Reproduction and plasticity within the reproductive polyphenism of an aphid, *Acyrthosiphon pisum*

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Submitted in accordance with the requirements for the degree of
Doctor of Philosophy

The University of Leeds
School of Biology

December 2023

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Acknowledgements

When thinking about writing my thesis (which for those who know how hastily I formatted it, it will probably come as some shock that I thought about it much at all), for the longest time I looked forward probably most of all to getting to write the acknowledgements, because producing this piece of work has been some effort for myself and a collective mammoth effort for many people who I have known, some for many years, and some I have come to know over the course of the PhD and hope to continue to know for many years to come, and acknowledging their efforts is important (and fun).

To start, I cannot begin to express how grateful I am to have had Liz as not only an incredible supervisor, but a brilliant mentor and role-model of what it is to be both a great scientist and an exceptionally kind and encouraging person. Without her enthusiasm and support, and the way she has built her lab to be such a great and nurturing environment, I have no doubt that producing this thesis would have been significantly more difficult, and I would have enjoyed the experience a whole lot less. Liz, thank you (not least of all for introducing me to the aphids, who I will miss deeply).

Thank you also to my co-supervisor Andrew Peel, and to all of the academics, postdocs, staff and students who I have got to know over the years – I feel lucky to have shared an office, lab space and floor with so many friendly and interesting people. I am particularly grateful to Ruth Hughes and Sally Boxall in the Bioimaging Facility for their assistance with imaging and cell sorting, and to Jeanine Williams in the School of Chemistry for her kindness and for facilitating our many attempts to run HPLC (Zoe, good luck!). I would also like to thank the fantastic technical staff, particularly Jenny, Lin, Marco and Fiona for all their advice, knowledge and time.

Over my years, Liz's lab group has had many students, all of which have been fantastic to get to know (even the ones who ask a million questions a day). In particular, I have shared in enthusiasm for the aphids with Steph, George, Sarah, Grace, Jenna, and Matthew and gotten to know many others over various other critters and techniques. I have also had the privilege of overlapping with four other PhD students and a postdoc. Jens, I ridiculed you so much for your habit of eating a whole bag of shredded cheese, but after writing the thesis, I get it, I should have never have questioned you; thanks for putting up with all my stupid questions way back in the day. Rosie, pineapples are funny. Apples are not. I think Rosie's and my friendship stems from the same place of silliness that makes this an obvious

fact. Rosie, thank you for all your kindness and silliness. Bananas? They're funny too. I could write a whole thesis on what makes bananas funny. I could write this whole acknowledgements about bananas, who's going to stop me. On the subject of bananas, Ant, we've eaten many bananas together over the years, and I think we have both kept each other sane (somehow?) over our shared appreciation for the many potential delicacies the lab has to offer, try not to eat all of the broad beans once I'm gone, they're for the aphids! Zoe, we haven't known each other properly for long, but it has been great getting to know you and I know you'll do great in the lab – I'm glad I at least got to see you become known as an insect serial killer like the rest of us in the lab, it's a rite of passage. James, thanks for all your teaching, advice and stories – I hope I've not contributed to too many more (though, Millport must rank highly).

I was incredibly lucky to make some great and undoubtedly, life-long friends in the early days of the PhD (who knew organised fun and cohort building activities could lead to such a thing). Some have been friends and housemates, Felix and Meg, we shared some dark times together in Headingley Rise and some better times in better places, Meg I'm sorry I burnt your non-microwaveable plate in the microwave, thanks for all of the Harry Potter viewing, quotable moments and cakes over the years, and Felix, expect the unexpected – you are by far my favourite yank, we've had some great laughs over the years and hopefully have many more to come. Claire and Utkarsh, we've lived together mostly through the deepest pits of thesis writing, Claire this thesis literally probably wouldn't be done without your help, thank you always (even if I still won't justify). Julia, you are truly an amazing person, thank you for (literally) saving me from drowning, for always being an incredible friend and for the many good times over the years, name a James Bond actor. Scott, I think without that PS2, or the cards, or our shared appreciation for goblins and gnomes, these PhD days would have been a whole lot less manageable, I hope we play that PS2 for many years until it only works at a very particular angle. Meg, I'll never forgive you for repeatedly putting rogue things in my food, peas in a cookie is not funny (try something else next time, like a pineapple or a banana), but thank you for putting up with the constant noise of games night and for being a great friend and temporary lab-collaborator (I think those were some of my favourite days in the lab). Faye, I will never forget coming down to lunch after you got back from Tenerife, I hope we have many more years of tennis club. Last, but by no means least, Betty, we've had a rocky road, we started

as day 1s, hit rock bottom (I'm not sorry I asked what's the point of conservation, it was character building), then came way back up again, here's to many more years at the casino covered in mocha, thanks for being my first friend (and enemy) in Leeds.

I also would like to give a shoutout (because I know this acknowledgements is getting way too long, anyone actually reading this thesis may begin to spot a pattern) to some other people who have been there along the way: Seb, Katie, Diego, Tara, Nok, Jake, Powelly, Lauren, Ben, Sam, Emily & Chad, thank you for being there, and sorry if I have been frequently AWOL, I hope you will at least maybe flick through this and partially understand why.

Thank you also to my family, as for most PhD students, they don't really get what I do, but they have supported me in various ways throughout and I wouldn't be here without them. Thank you mum, dad, Nam, Adam, Isabella, Amelia and Nanny.

Finally, thank you Emily. You have had to endure me at probably my most difficult points, and I am incredibly grateful for all of your support – thank you for putting up with my burping (I think it's a symptom of thesis writing), for feeding me, for your reassurance and for always reminding me to be silly.

Thank you also to the BBSRC and the White Rose DTP in Mechanistic Biology for funding and to all of the aphids that made this possible.

For Jonny,
who taught me everything I know about the North.

Abstract

Cyclical parthenogenesis is a key evolutionary novelty of the aphids and a striking example of phenotypic plasticity – a phenomena which is exemplified in the insects. Within cyclical parthenogenesis, aphids use both asexual and sexual reproduction, two fundamentally different approaches to producing offspring, with the asexual, viviparous mode being unusual among insects and animals. The primary aims of this thesis were to first, investigate how epigenetic factors may be involved in the asexual reproductive mode and potentially have contributed to its uptake as a strategy or underpin the ability to switch from it to sexual reproduction, and secondly, to improve our understanding more generally of the molecular underpinnings of cyclical parthenogenesis, primarily at the level of the ovaries of aphids that will produce asexual or sexual offspring, thus determining some of the potentially more proximate causes of effecting the switch and linking known and putative modulators of the switch to these putative proximate causes.

To this end, I explored the use of RNAi as a tool to investigate the functions of genes and discern their potential involvement in the reproductive switch, in order to causatively link suspected factors to it – however, in doing so I contributed to a growing body of evidence to suggest that RNAi susceptibility is highly variable, which I discussed, in chapter 3. The ovary-specific expression pattern and evolutionary history of two *A. pisum dnmt3* paralogs were explored in chapter 4, revealing an Aphidomorpha specific duplication and expression in germ cells and early embryos, and additionally, a role of DNA methyltransferases in asexual reproduction. Genes relating to known and putative modulators of the reproductive switch were then assayed at the level of the ovary, revealing differential expression of several insulin and juvenile hormone related genes, and the two *dnmt3* paralogs between ovaries containing embryonic aphids fated to produce asexual and sexual offspring, associating them with the reproductive switch, though few of them appeared directly responsive to artificial elevation of juvenile hormone signalling, which was able to redirect reproductive mode. Finally, a technique to target specific cell types was optimised and directed at the germ cells in order to characterise the expression profiles of these cells derived from aphids producing asexual and sexual offspring, the germ cells being a possible key place for the switch to be effected.

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Abbreviations

AU	Airy unit
CA	*Corpus allatum*/*corpora allata*
CDE	Clathrin-dependent endocytosis
ChIP-seq	Chromatin immunoprecipitation
DAPI	4',6-diamidino-2-phenylindole
DEG	Differentially expressed gene
DMSO	Dimethylsulfoxide
DNMT	DNA methyltransferase
dsRNA	Double-stranded RNA
EE	Extra-embryonic
ELISA	Enzyme linked immunosorbent assay
ETH	Ecdysis-triggering hormone
FACS	Fluorescence-activated cell sorting
GLM	Generalized linear model
GLMM	Generalized linear mixed effects model
GOI	Gene of interest
ILP	Inuslin-like peptide
IPC	Insulin producing cell
IR	Insulin receptor
IVT	*In vitro* transcription
JH	Juvenile hormone
LD	Long-day
LRT	Likelihood ratio test
MeDIP	Methylated DNA immunoprecipitation
NSC	Neurosecretory cell
PBS	Phosphate buffered saline
RNA-FISH HCR	RNA-fluorescent *in situ* hybridisation chain reaction
RNAi	RNA-interference
SD	Short-day
siRNA	Short interfering RNA
SNP	Single nucleotide polymorphism
TET	Ten-eleven translocation dioxygenase
TF	Transcription factor
TOR	Target of rapamycin
WGD	Whole-genome duplication

Chapter 1 General Introduction

1.1 Insects, their evolutionary success and plasticity

Insects represent a highly successful class of animals. They occupy an impressive diversity of ecological niches across almost all habitats, and have colonised every continent (Condamine, Clapham & Kergoat, 2016). They (along with other arthropods) account for half of the world's animal biomass (Bar-On, Phillips & Milo, 2018), and (alone) an incredibly large proportion of the biodiversity encompassed by multicellular organisms (May, 1986; Stork, 2018). Evolving ~ 480 million years ago (Misof et al., 2014), the evolutionary success of insects is due to several phenomena. One is the evolution of winged flight (which is thought to have evolved ~ 410 million years ago, insects being the first organisms to do so (Misof et al., 2014)), which has allowed insects to colonise otherwise unreachable niches (La Greca, 1980). Another arguable contributor to the evolutionary success of insects is their incredible adaptability mediated by plasticity (phenotypic plasticity can be found among animals, plants, fungi and prokaryotes (West-Eberhard, 1989; Slepecky & Starmer, 2009; Matuła et al., 2019), but insects stand out because they exhibit some of the most striking and well-studied examples (Simpson, Sword & Lo, 2011)), which amongst other things contributes to the array of forms exhibited among insects (it has been hypothesised that sequential development such as exhibited by complete metamorphosis may have resulted from assimilation of what were originally environmentally dependent morphs (Minelli & Fusco, 2010)), which can be highly specialised to different niches and lifestyles (Moczek, 2010; Simpson, Sword & Lo, 2011). Plasticity is the sensitivity that a biological process or phenotype has to the environment. It encompasses the changes that occur to an organism's or process' form that are not genetically determined. Phenotypic plasticity is in essence, the ability of a single genome to produce multiple phenotypes (West-Eberhard, 2003; Pigliucci, 2001). Phenotypic Plasticity has been argued to have adaptive significance (where responses are appropriate to the current/predicted environment, otherwise they can be maladaptive) as it allows organisms to respond to variation in their environment over relatively short time periods (Ghalambor et al., 2007).

To some extent, more direct, simple plasticity, where biochemical and biophysical processes are directly affected are more a product of how living systems work than evolved traits (Schlichting & Pigliucci, 1995; Nijhout, 2003), this kind of plasticity is a fundamental part of biological systems. For example the additional energy provided by higher temperature, control over interactions conferred by ionic concentration, or folding of proteins mediated by pH can all affect these processes and are (at some level) environmentally determined. In its more intricate form, however, plasticity (and degree of plasticity) is of course an evolved trait (Nijhout, 2003); this is the case for the developmental plasticity (phenotypic plasticity that arises during the development (embryonic or post-embryonic) of an organism, and due to the early stages at which it acts, is capable of facilitating relatively large differences between morphs (Lafuente & Beldade, 2019)), such as that explored in this thesis. Some organisms are more plastic than others, and this is underpinned by an organism's genetic architecture, which is dictated by evolutionary history (Fusco & Minelli, 2010). In addition to being a product of evolutionary forces, plasticity is also a source of diversity that facilitates novelty and can increase survivability under changing conditions and through this, provide substrate for evolutionary forces to act on (Moczek et al., 2011; Fusco & Minelli, 2010); though, it has also been argued that plasticity may reduce the need for speciation and thereby inhibit genetic diversification (Lalejini et al., 2021; Fox et al., 2019). Thus, plasticity is dictated by genetics but is also likely to contribute to evolutionary processes.

For many of the reasons I have discussed, phenotypic plasticity can be largely seen as having contributed to the great success of the insects. Exactly because of the incredible adaptive benefits plasticity can confer, the vast majority of organisms display some level of plasticity (Sommer, 2020). Insects, however appear to have developed an incredible capacity for plasticity. Some of the most striking examples of plasticity appear across the insects, where extremely distinct phenotypes are encoded in single genomes (Simpson, Sword & Lo, 2011). Where plasticity is extreme to the extent of distinct discrete phenotypes rather than continuous plasticity, this is known as polyphenism (Simpson, Sword & Lo, 2011; Nijhout, 2003).

1.2 Insect Polyphenism

Polyphenisms are extreme examples of phenotypic plasticity. They occur where, rather than a phenotypic trait increasing/decreasing incrementally with equivalent changes in intensity/exposure to a given environmental cue, thresholds in cue separate distinct phenotypes (typically two, rarely more) (figure 1.1) (Nijhout, 2003). As a result, the phenotypes displayed often encompass a greater capacity for diversity, because they are not incrementally different phenotypes in direct response to environmental cues, but uncoupled phenotypic responses arising from potentially substantially different inputs (or very similar inputs separated by a threshold), that arise from 'all or nothing'-type switches (Simpson, Sword & Lo, 2011). Generally, these examples, because they often involve drastically different developmental programs, occur during development (a period with great capacity for change relative to the rest of an insect's life) and are examples of what is termed developmental plasticity (Brisson & Davis, 2016). In insects, developmental plasticity encompasses embryonic development and post-embryonic development, the progression between developmental stages by moulting. Holometabolous insects are likely more capable of polyphenism post-embryonically than hemimetabolous insects (Yoon et al., 2023), because they undergo complete metamorphosis that encompasses distinct developmental stages that differ drastically from the adult form, as opposed to incomplete metamorphosis, exhibited by hemimetabolous insects which rather than having distinct post-embryonic developmental forms, produce nymphs that are for the most part, miniaturised versions of the adults (Truman & Riddiford, 2019). During development, brief critical periods of increased sensitivity to inductive stimuli/the pathways they stimulate exist, eventual phenotype being largely dictated during these periods, where typically, a switch from one phenotype to another does or does not begin to occur (in the case of polyphenism), or greater sensitivity in phenotypic plasticity, more generally, is experienced (Nijhout, 1999). In polyphenism, typically the inducing environment (the conditions that stimulate onset of phenotype switching) and the selective environment (the conditions in which the selected phenotype is adaptive) are separate (Lafuente & Beldade, 2019), thus the polyphenic organism must be able to make predictions, based on cues experienced in the inducing environment, of the conditions of the selective environment.

Polyphenism, especially, within phenotypic plasticity, therefore hinges on perception of reliable signals that are predictive of the future environment, as switches that are often irreversible and involve large degrees of difference do or do not occur, and therefore major commitment to a particular form, which if the signals are misleading may be maladaptive, occurs (Halali et al., 2021; Bonamour et al., 2019). The role the separation between inductive and selective environment plays is likely two-fold. Firstly, development or development of particular traits takes time and the time lag allows phenotypes to be assumed in time for when they are adaptive. Secondly, the polyphenism is made more robust (importantly, I must point out that by robustness here I mean the certainty that the selection of one morph over another is appropriate, rather than more resistant to environmental variance) because there is a longer period of exposure to any environmental stimuli, and multiple cues may be used, and more accurate predictions can therefore be made (Bonamour et al., 2019).

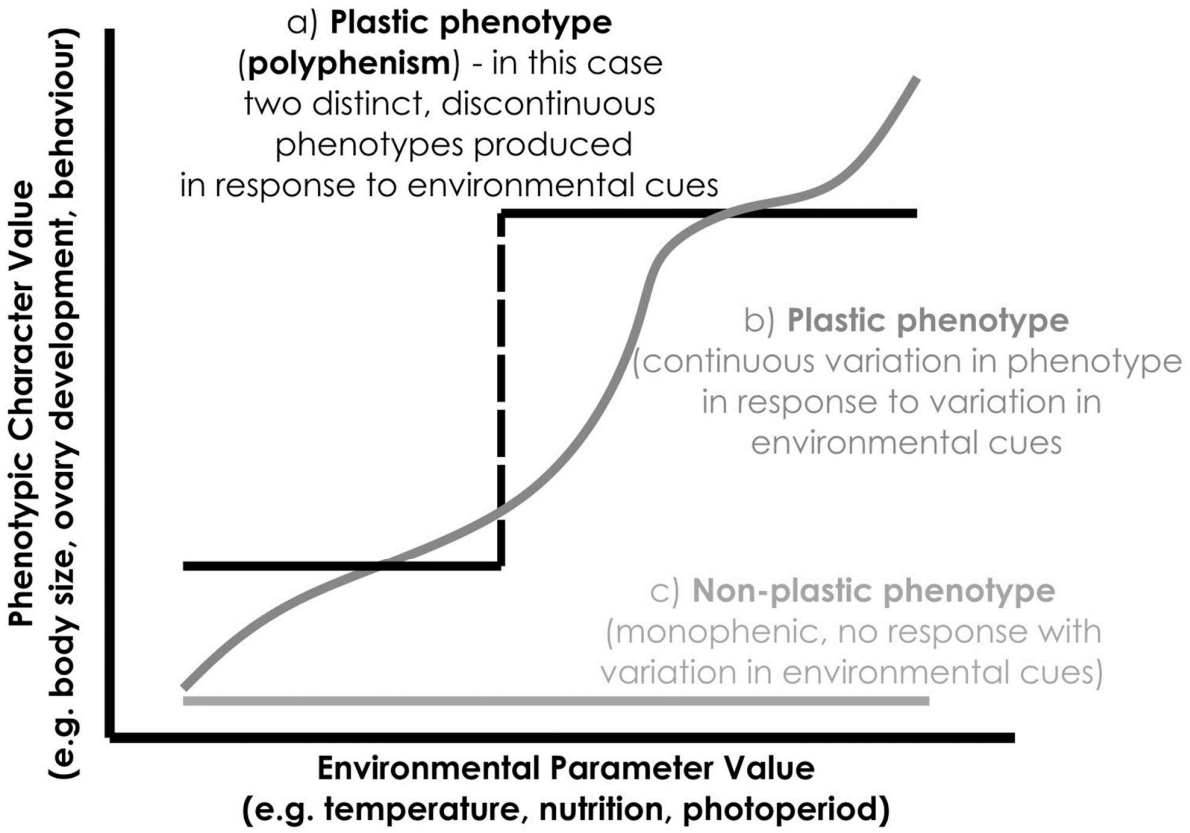


Figure 1.1. Graphical representation of the relationship between environmental cue(s) and phenotype (reaction norms), across different types of plasticity.
(a) an illustration of polyphenism, where discrete phenotypes are produced in response to continual variation in environmental cue. (b) while also an example of phenotypic plasticity, represents continuous plasticity whereby phenotype varies continuously with variation in environmental cue. (c) a representation of a non-plastic trait that does not vary in response to an environmental cue. Adapted from Yoon et al. 2023.

Polyphenisms in insects can be responsive to a vast array of types of cues, including being density-dependent (e.g. locust phase polyphenism), nutrition dependent or seasonal (often being temperature and/or photoperiod dependent) (Simpson, Sword & Lo, 2011). Key examples are the reproductive division of labour exhibited by social insects where worker and queen castes are specified during larval development, which in *Apis melifera* is primarily driven by differential nutrition of larvae leading to one form or the other (Barchuk et al., 2007; Slater, Yocum & Bowsher, 2020); the horn phenotype of polyphenic male horned-beetles which develop as either small morphs with reduced horns or large morphs with large horns, again, a primarily nutrition driven polyphenism, whereby sufficiently large male larvae ‘commit’ to developing large horns, but

insufficiently large male larvae exhibit reduced horns (Moczek, 1998; Emlen et al., 2005); the season dependent wing eye-spot pattern of some nymphalid butterflies, which, in *Bicylus anynana* is dependent on temperature during the wandering larval stage (Monteiro et al., 2015); and the wing and reproductive polyphenisms of aphids, which are primarily responsive to crowding (or cues associated with it) and photoperiod, respectively (Müller, Williams & Hardie, 2001; Ogawa & Miura, 2014; Yan, Wang & Shen, 2020). These examples span a diverse range of traits and inducing cues, though there are elements many of them have in common (as we will see).

Uncovering the underlying bases of these polyphenisms greatly expands our understanding of evolution, diversity and the capacity for responding to change in the environment. Beyond understanding, this presents practical importance with regards to, notably, climate change, given that the time-scale over which climate change is occurring will mean species that are not sufficiently plastic are more likely to be threatened than those displaying more plasticity, and pest control, as accounting for plasticity and/or using it as a tool (for example, by manipulating the morph taken on by a population toward a less problematic one) may enable more effective management (Fox et al., 2019; Richard et al., 2019; Rodrigues & Beldade, 2020).

1.2.1 The mechanics of a switch

1.2.1.1 Sensors, modulators and effectors

Insect phenotypic plasticity operates through three main components: sensors (and associated integrators), modulators and effectors (figure 1.2) (Yoon et al., 2023). In this way, the sensory aspect is the only part that need be truly sensitive to the environment, while the modulators and effectors do not need to be. This makes the system flexible, as different cues can be linked to the same modulators and/or effectors for a given plastic trait/polyphenism (which can also make the system more robust, as detecting more cues may improve predictive capacity (Dore et al., 2018)), and the same modulators can be responsive to different cues (associated with different plastic traits/polyphenisms), leading possibly to interaction with different effectors (figure 1.2). This essentially reduces the need for diversity in modulators.

Environmental cues are detected by sensors, which collectively across organisms are capable of capturing a huge diversity of environmental information. Sensors can be sensitive to generic cues and/or highly specialised cues. Generics cues are those which are detected by all insects, involving relatively basic environmental conditions such as temperature, photoperiod and nutrition – no specialised structures are required to detect them (Klein et al., 2015; Helfrich-Förster, 2020; Badisco, Van Wielendaele & Vanden Broeck, 2013). Specialised cues on the other hand are typically detected by specialised systems and are parts of more derived systems typically at the species or family level (Rajakumar et al., 2018; Mast et al., 2014). Combining generic and/or specialised cues may improve fidelity, and/or introduce redundancy and increase the accuracy at which plastic response occurs, though more work is required to discern how systems using multiple cues work (i.e. how is the information integrated and then passed on to (possibly shared) modulators, and how much importance is given to each cue and how might this be controlled) (Dore et al., 2018; Yoon et al., 2023). This is especially important given that in many contexts assuming a mismatched phenotype may be maladaptive (Schneider, 2022). Sensors are necessarily relatively diverse, as their diversity must encompass the extent of the diversity of possible cues (with some degree of overlap). Whether the cue is generic or specialised, the information encoded within it must be integrated and then passed to modulator systems if the intended effect occurs somewhere other than where detection occurs.

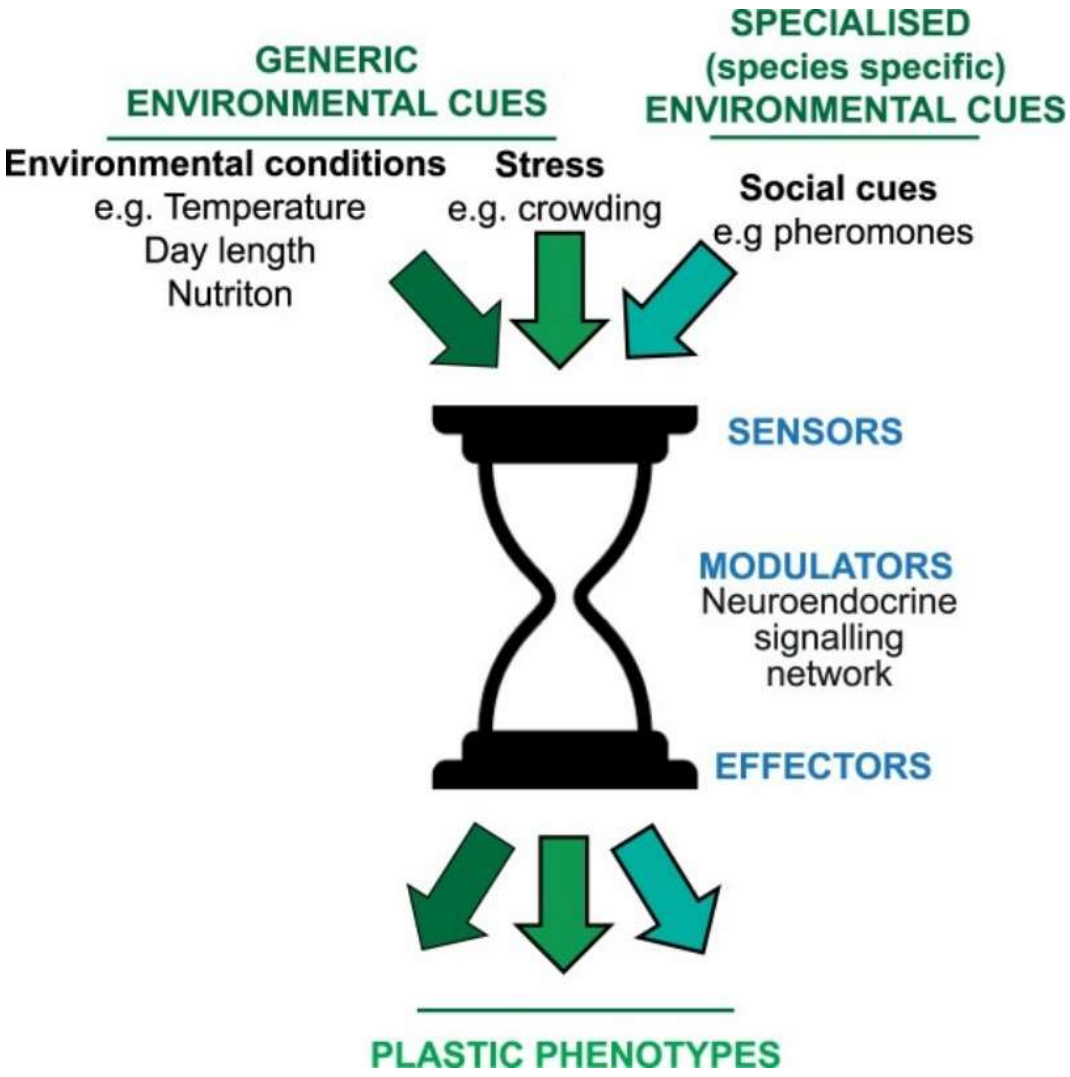


Figure 1.2 Requirements for developmental plasticity to function. Insects must first detect cues using specialised or non-specialised sensors (which are diverse in their nature within insects), then transmit information derived from detected cues to the tissue(s) where the downstream effect(s) will be realised using a relatively limited range of modulators, then gene expression in the affected tissue must change in response to bring about alternative developmental trajectories, encompassing plastic phenotypes, these downstream genes being effectors. Adapted from Yoon et al. 2023.

Modulators link sensory perception with the target tissues in which plastic responses will ultimately be effected. In insects, the neuroendocrine signalling system is principally responsible for modulation (figure 1.3) (Hartenstein, 2006). Beyond its role in plasticity, the insect neuroendocrine system is well conserved, and it is involved in a huge range of insect biological processes and life-history strategies/phenomena (Knapp et al., 2022; Libbrecht et al., 2013; Mirth et al., 2014; Orchard & Lange, 2024). It primarily encompasses juvenile

hormone, insulin signalling, ecdysteroids and biogenic amines (chiefly dopamine, octopamine and serotonin) (Orchard & Lange, 2024). Many examples of plasticity in insects are underpinned by these systems (Nijhout, 1999; Simpson, Sword & Lo, 2011). Modulation represents the most conserved part of the plastic response in insects. The same pathways transduce information associated with sensing of cues by a diverse range of sensors to diverse effectors to give rise to distinct and separate phenotypes both within individuals and across species (Yoon et al., 2023). How this conserved and limited set of modulators can influence such separate and distinct plastic responses, especially within a single individual (and especially where phenotypes are extreme, as in polyphenism), is not clear. Though, switches generally, involve variation in modulator secretion, timing of sensitive period(s) and/or the threshold of sensitivity (Nijhout, 1999) and it is likely compartmentalisation is also achieved through this.

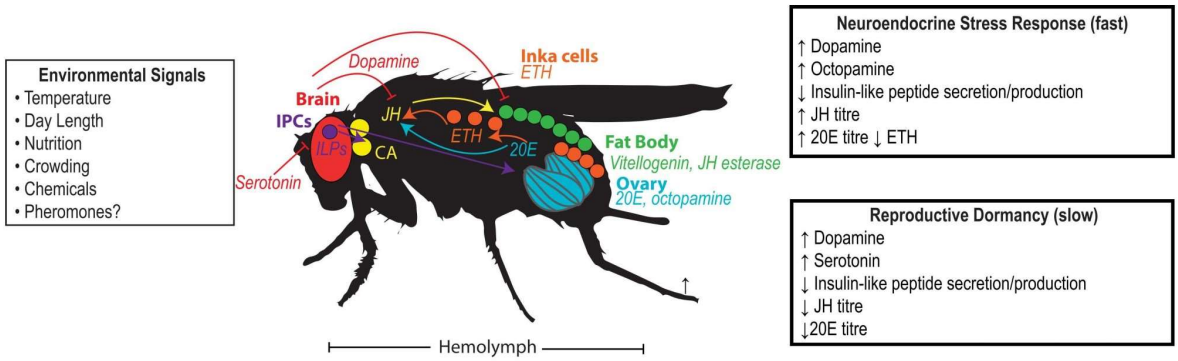


Figure 1.3 An example, using *D. melanogaster*, of modulatory systems (encompassing the major neuroendocrine signalling systems) that are responsive to environmental signals to modulate reproduction.
D. melanogaster are able to detect a variety of environmental cues, many of which are able to effect reproduction (either quickly, primarily associated with the neuroendocrine stress response, or in a more slow and persistent way, through reproductive dormancy). The major, conserved, insect neuroendocrine systems (biogenic amines, JH, 20E, ILPs) are known to be responsive to various environmental cues. CA, corpus allata, IPC, insulin producing cell, ILP insulin-like peptide, JH, juvenile hormone, ETH, ecdysis-triggering hormone, 20E, 20-hydroxyecdysone. Adapted from Knapp et al., 2022.

Ultimately, the plastic response is realised through effectors. These are the genes and their regulatory networks that give rise to differential plastic phenotypes (Cameron, Duncan & Dearden, 2013; Tian & Monteiro, 2022). For

example, by affecting patterning, cell growth, cell fate and cell death. As an example, during the final stages of *A. melifera* larval development, extensive cell death of germ cells occurs in worker-destined ovaries, while germ cell cluster formation (through cell division) occurs in queen-destined ovaries in response to nutrition, leading to ovaries containing relatively few ovarioles, or many ovarioles, respectively (Hartfelder & Steinbrück, 1997). Thus, the cell death or cluster formation of germ cells largely realises differences between castes in this example of polyphenism. Similarly to sensors, effectors, necessitated by the diversity of phenotypes, also appear to be incredibly diverse, even between relatively closely related species exhibiting functionally identical polyphenisms. That is to say, that while the end phenotype is grossly the same, the genes associated with one phenotype or another are different between species. For example, in locusts, transcriptomic responses (in the head and thorax) during the phase polyphenism of closely related species have been demonstrated to be species-specific (Foquet, Castellanos & Song, 2021), while functionally, the genes involved in the responses of different species are similar. Similarly, honeybees and bumblebees (which both display eusociality, though to different extents, advanced in honeybees and primitive in bumblebees (*Bombus terrestris*)) have been demonstrated to possess different caste-specific expression profiles after a critical period (Collins et al., 2021). Although, in both of these examples, there was a small degree of overlap - in the bee study by Collins and colleagues, 0.68 – 2.13 % of differentially expressed (between castes) orthologues, depending on larvae age, were overlapping between *A. melifera* and *B. terrestris*. Importantly, these studies do not necessarily discriminate between genes involved in sensory systems, modulatory systems, or effectors. And, for example, in Collins *et al. kr-h1* is identified as an overlapping gene, *kr-h1* being a response element of juvenile hormone signalling (Minakuchi, Namiki & Shinoda, 2009; Minakuchi, Zhou & Riddiford, 2008; Lozano & Belles, 2011), which is a commonly deployed modulator of insect polyphenism (Nijhout, 1999). Thus, while the overlap is small and does not necessarily exclude effectors, it is possible that the true overlap in effectors is lower than the percentages reported, though this is difficult to unpick. Taken together, these examples highlight that functionally identical polyphenisms can be underpinned by separate sets of genes (in terms

of identity, though functionally, genes may be similar (i.e. be involved in the same processes)).

1.3 Aphids and Polyphenism

1.3.1 The evolutionary novelty of viviparous parthenogenesis

Aphids are globally distributed, soft-bodied, sap-feeding hemimetabolous insects of the order Hemiptera (Dixon, 1977). Of the over 5000 described aphid species, approximately 250 are agricultural pests that inflict damage on plants directly through feeding and indirectly through the transmission of viral plant pathogens (Dixon, 1985; Emden & Harrington, 2007). Because of this, aphids are responsible for huge reductions in crop production and are therefore of great economic importance (though the difficulty in assessing the impact exactly, partially due to high between-year variation has been noted (Dedryver, Le Ralec & Fabre, 2010)). This has attracted a great deal of attention to their control and their biology. Among those aphids that are major pests, the pea aphid, *Acyrthosiphon pisum*, on which this thesis focuses, is one that is particularly economically problematic (Paudel et al., 2018; Sandhi & Reddy, 2020).

All aphids primarily (or solely) reproduce by viviparous parthenogenesis (Simon, Rispe & Sunnucks, 2002), without meiotic recombination or haploid gametes due to a modified meiosis (termed apomictic parthenogenesis) which produces only a single polar body through a single maturation division (Blackman, Minks & Harrewijn, 1987; Le Trionnaire et al., 2008). They typically exist as lineages in clonal colonies of genetically identical (except for variation arising from spontaneous mutation) viviparous parthenogenetic females (Dixon, 1977). In addition to (and conferred in part by) parthenogenetic reproduction, they also have very short generation times, and the oldest embryos developing within an adult mother will within it, typically contain its own embryos (in the early stages of development), meaning a reproductive adult aphid encompasses three generations (this is termed the telescoping of generations) (figure 1.4) (Dixon, 1985; Kindlmann & Dixon, 1989). Upon reaching adulthood, aphids almost immediately start reproducing. This reproductive strategy, which also forgoes the need for males and therefore avoids the two-fold cost of sex (Lehtonen, Jennions & Kokko, 2012), having a population that can all produce offspring,

and avoiding the need to seek mates, contributes to aphids having some of the fastest population growth rates of any insect (Kindlmann & Dixon, 1989; Dixon, 1987). Parthenogenesis has evolved independently and been secondarily lost across several groups of organisms (Parker et al., 2019; Fujita et al., 2020; Sperling et al., 2023), though it only evolved once in the aphid lineage (Carter, Simon & Nespolo, 2012), reflecting its successfulness as a reproductive strategy – one that contributes a great deal to the pest status of aphids.

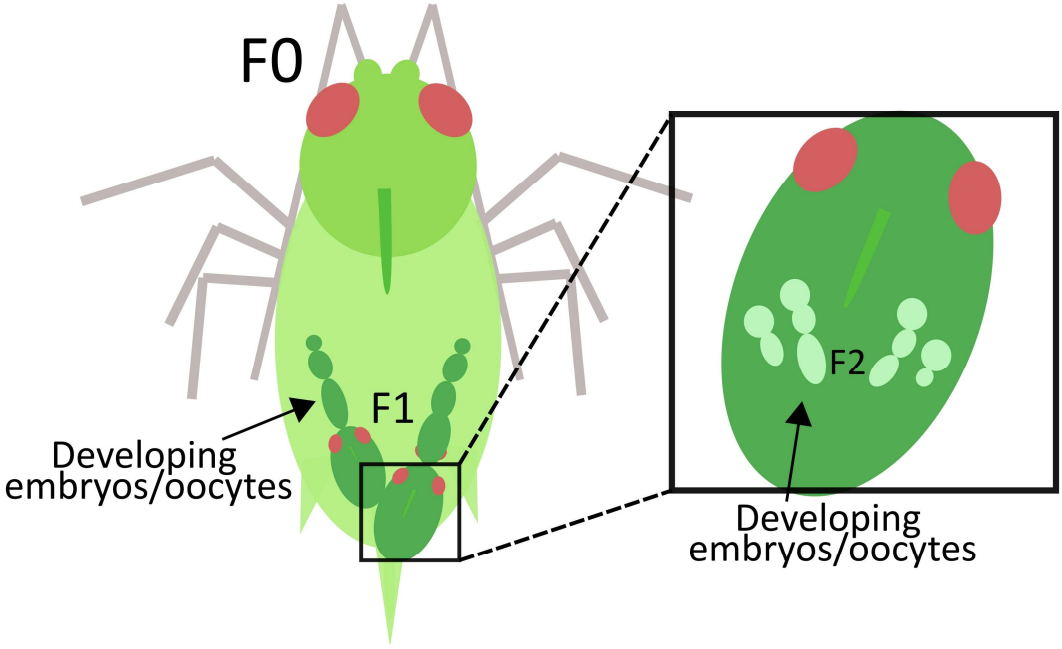


Figure 1.4 Schematic of the telescoping of generations.
Asexual viviparous aphids (F0) possess embryos (F1) at various stages of development in their ovaries, the more mature of these embryos in turn possess ovaries containing developing embryos (F2) of their own. Thus, a single aphid can represent three generations of individuals at a given time.

1.3.2 Novelty in Aphid Biology

Aphids have attracted interest for their unique biology and lifestyle, especially encompassing their displays of plasticity, symbiosis, and plant-insect interactions in developmental biology, evolutionary biology and ecology (Shigenobu & Yorimoto, 2022). The nature and role of their ancient obligate endosymbionts, *Buchnera aphidicola*, which provision essential amino acids otherwise not provided by their unbalanced diet of phloem (Shigenobu et al., 2000; Blow et al., 2020), have also been extensively studied (Chong & Moran, 2018; Byrne et al., 2022; Brinza et al., 2009; Feng et al., 2019). Other facultative heritable symbionts have also been described (Meseguer et al.,

2017; Heyworth, Smee & Ferrari, 2020). Aphids have evolved specialised cells called bacteriocytes in which they house *B. aphidicola* (figure 1.5) (Braendle et al., 2003). *A. pisum* is perhaps among the most widely used model aphid species owing to its ease of culturing and manipulating. Several aphid species, and close relatives have had their genomes sequenced, which has facilitated in-depth molecular and genetic investigations (Mathers et al., 2020; Biello et al., 2021; Byrne et al., 2022; Wei et al., 2022). *A. pisum* was the first aphid to have its genome sequenced, in 2010 (The International Aphid Genomics Consortium, 2010), and its genome assembly has since been greatly improved - there are currently two high-quality chromosome-level assemblies of two different strains (Li et al., 2019b; Mathers et al., 2021).

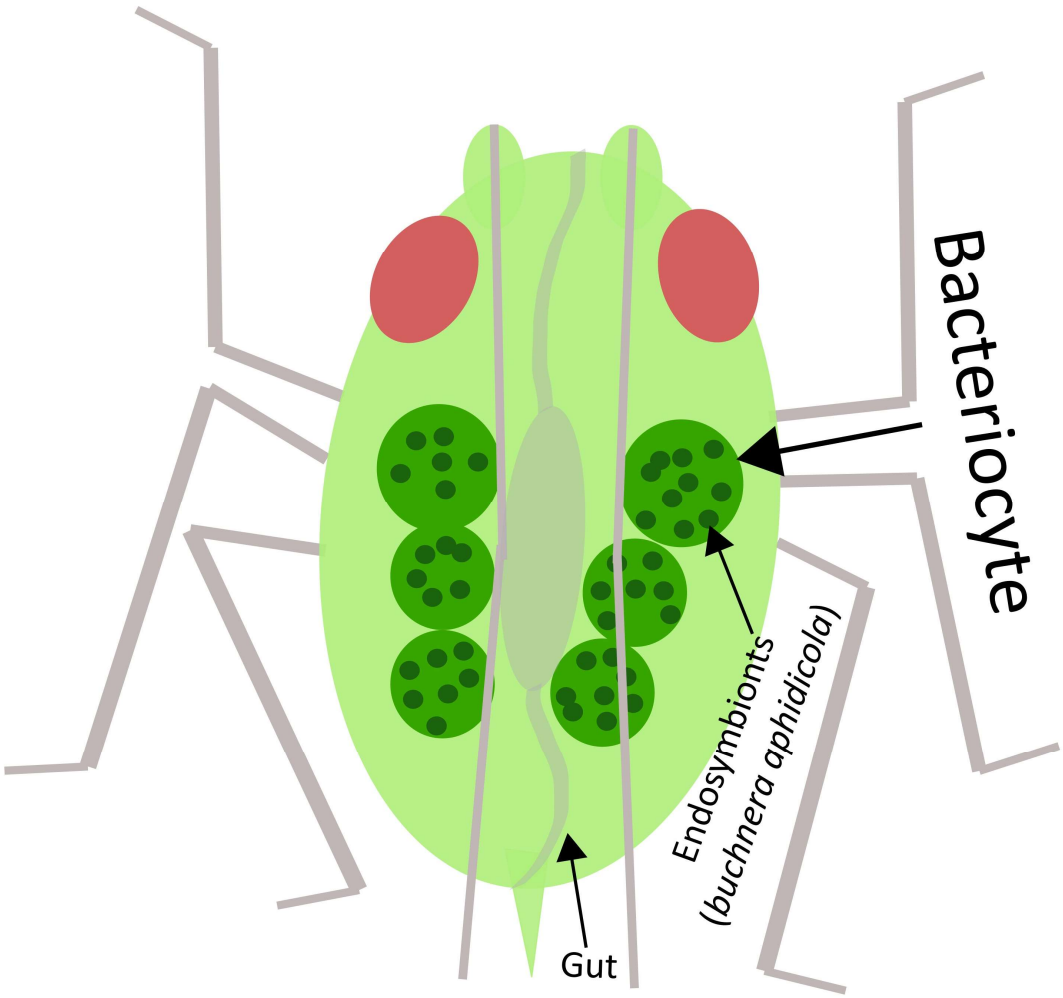


Figure 1.5 Schematic of the conserved aphid symbioses, in which aphids possess specialised cells, bacteriocytes, to host endosymbiotic bacteria (principally the obligate symbiont *B. aphidicola*).
Endosymbionts are inherited from the mother during embryogenesis and are contained within the cytoplasm of the bacteriocytes. Endosymbionts associate closely with the gut and provide nutritional support to aphids which rely otherwise on a relatively nutrient-poor diet of plant phloem sap.

Many of the noteworthy parts of aphid biology have been suggested to be linked, partially, to gene duplication (Shigenobu & Yorimoto, 2022). Gene duplication is a major source of genetic material on which evolutionary processes may act to generate novelty and innovation (Crow & Wagner, 2006). The aphid lineage displays an incredible amount of duplication (one study found that for inspected Aphidomorpha species, the percentage of genes with duplicates ranges from 54 to 81 % (Li et al., 2023b)), and both ancestral

duplications (in an ancestor of the Aphidomorpha or the Aphididae (Julca et al., 2020; Ribeiro Lopes et al., 2020)) and species-specific duplications have occurred. For instance, in *A. pisum*, 29 % of proteins have been shown to have in-paralogs (arising from species specific duplication) (Julca et al., 2020), and similar proportions were revealed in other aphid species, some of the species-specific duplications being parallel with those of other aphid species; similarly, the study that first reported the *A. pisum* genome (The International Aphid Genomics Consortium, 2010) reported 2459 gene families with aphid specific duplications). The pea aphid genome appears particularly enriched for species-specific duplications (Mathers et al., 2017). A large-scale duplication is estimated to have occurred before the diversification of the Aphidomorpha (it has been suggested that one whole-genome duplication (WGD) may have occurred during the evolutionary history of the aphids, which may be difficult to detect as a result of the propensity of aphid genomes for reshuffling, resulting in a general lack of synteny (Julca et al., 2020) (which is the traditional approach to identifying ancient WGDs), and many of the genes duplicated in ancestors of this lineage are involved in basic aspects of aphid biology: e.g. endosymbiosis and their plant feeding lifestyles (Julca et al., 2020). Aphid genomes more generally, have more taxonomically restricted genes than is commonly observed in other insects, again this likely plays a role in their very specific biology, and some of these taxonomically restricted genes are also duplicated (Shigenobu & Stern, 2013; Korgaonkar et al., 2021; Shigenobu & Yorimoto, 2022). Another quirk exhibited by aphid genomics is the extreme extent of chromosome reorganization that appears to have occurred even relatively recently (within 30 million years) in the aphid lineage (but seemingly, not in other Hemiptera), far more than is commonly observed in other insects (though, studies have mostly been restricted to Dipteran (Sved et al., 2016; Dudchenko et al., 2017) and Lepidopteran species (d'Alençon et al., 2010)) and hemipterans (Mathers et al., 2021; Li et al., 2023b). While this is true of the autosomes, the X chromosome is relatively conserved in terms of gene content in investigated aphids (Mathers et al., 2021). Although, the X chromosome is relatively fast evolving at the nucleotide and protein levels (nonsynonymous substitutions are enriched on the X compared to the autosomes in *A. pisum*, *Myzus persicae* and *A. gossypii*) (Jaquiéry et al., 2012, 2018; Li, Zhang & Moran, 2020). Their holocentric chromosomes may have contributed to their

general propensity for genetic rearrangement (Coghlan & Wolfe, 2002), as holocentric chromosomes have diffuse kinetochores and therefore associate diffusely with microtubules (rather than having localized centromeres) (Mandrioli & Manicardi, 2012), and because of this DNA fragments may remain attached and then be retained and inherited by cells (this can among other things, disperse members of paralog pairs) (Li et al., 2019b), as opposed to possessing fragments lacking centromeres which are lost (Schrader, 1947), though, the fact that other hemipterans which also possess holocentric chromosomes do not always show similar extents of rearrangement is noteworthy (Mathers et al., 2021). It is possible that these genetic dynamics and duplications, as sources of novelty themselves, may have contributed to some of the novelty displayed in aphid biology (Mandrioli et al., 2019; Fernández et al., 2020).

One evolutionary novelty of the aphids (specifically of the Apidoidea, which includes the true aphids, but not of the other members of the Aphidomorpha, the Phylloxeroidea and Adelgoidea) is viviparity (figure 1.6) (Davis, 2012). Viviparity is the development of embryos within the ovary and subsequent live birth. Viviparity is relatively rare (in terms of true viviparity, as opposed to ovoviviparity whereby eggs hatch to release larvae in the ovary) in insects, occurring ubiquitously in the true aphids, though it has been observed in some species of cockroach (Hagan, 1939; Roth & Willis, 1957), and two groups of earwig (Jaglarz et al., 2019) for example. Though, importantly, viviparity has independently evolved many times across many taxonomic groups of animals (Kalinka, 2015), and within the insects, highlighting its successfulness as a reproductive strategy. Typically, oviposition (or sperm where sexual reproduction occurs) is required to activate eggs which are otherwise arrested (Went, 1982). The evolution of viviparity therefore necessitated a bypass of cell cycle arrest, and encompassed a change in development – in aphids, viviparous parthenogenetic oocytes are much smaller than oviparous oocytes and are associated with smaller nurse cells with reduced connections, and in embryogenesis (which occurs over a much shorter timespan during viviparous development), cellularization occurs earlier (Davis, 2012; Couchman & King, 1979; Tsitsipis & Mittler, 1976). Viviparity allows aphids to have direct contact with their developing embryos from oocyte to embryonic maturity (though, the degree of connectivity reduces as the embryo develops its cuticle), and

therefore constant and exquisitely tight control over their developmental environment (Bermingham & Wilkinson, 2009; Ogawa & Miura, 2014). Oviparous insects deposit an egg and typically provision and convey information to it in the form of maternally-synthesized transmissible signals, primarily mRNA, up to and at the time of oviposition (Pokrywka & Stephenson, 1995; Li et al., 2020b; Hilker, Salem & Fatouros, 2023; Couchman & King, 1979). The period where the mother is able to direct the fate of the embryo therefore encompasses only the beginning of development, and control over developmental environment is only during this period. Viviparous aphids are able to constantly signal to their offspring throughout development while they are developing within their ovaries. This yields them a development that has the capacity for tight, rapid and continual responsiveness to the environment as perceived by the mother. Viviparity may therefore accommodate a greater and more flexible ability to respond to the environment (Ogawa & Miura, 2014).

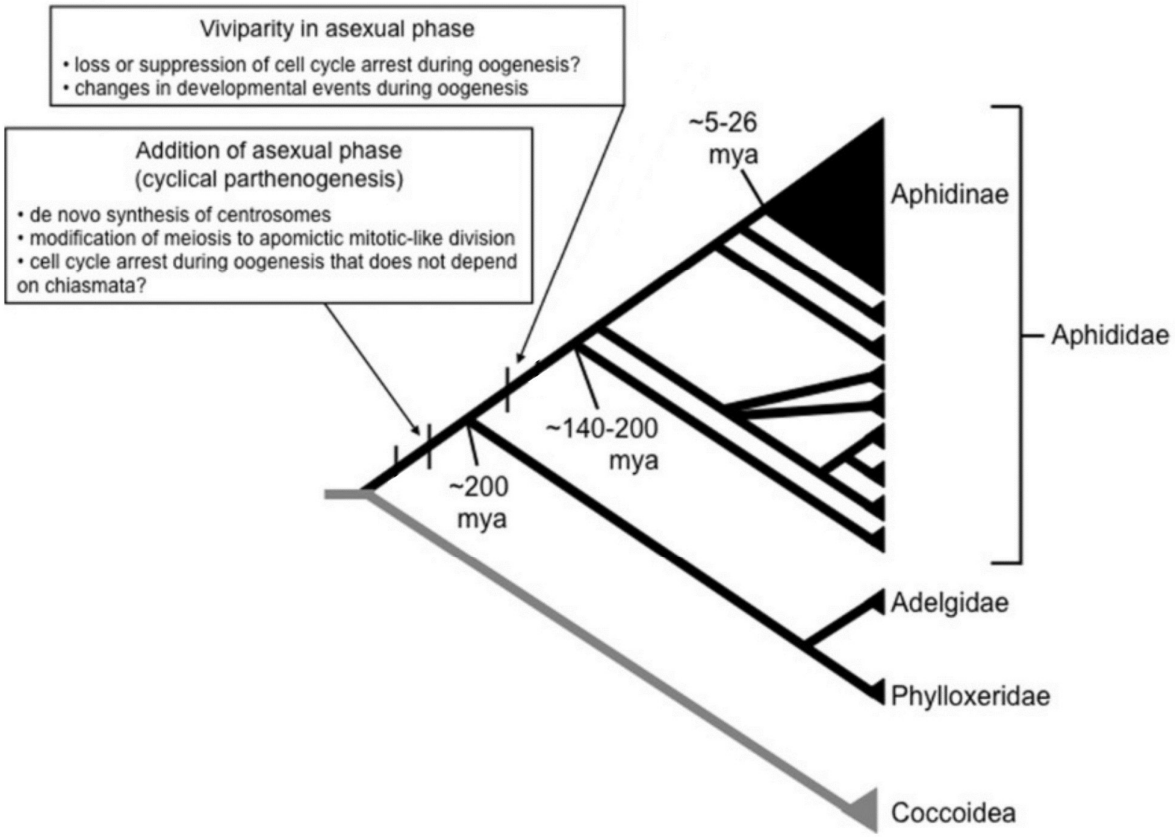


Figure 1.6 Simplified phylogeny of extant Aphidomorpha species, and evolutionary novelty associated with them.
The approximate emergence of two evolutionary novelties within the aphidomorpha (black lines, encompassing the true aphids, Aphididae, the Adelgids and the Phylloxeridae) group, cyclical parthenogenesis (common to all three groups) and viviparity associated with the asexual mode (specific to the Aphididae), alongside possible major steps required for their development. Coccoidea is included as the sister group to the Aphidomorpha. Adapted from Davis, 2012.

1.3.3 Oviparous sexual reproduction and cyclical parthenogenesis as a polyphenism

While parthenogenetic (asexual) reproduction allows aphids to thrive in fast establishing, high density populations of genetic clones, foregoing sex has its costs. For one, without genetic recombination conferred by mating (Hales et al., 2002b), a lack of genetic diversity may lead to reduced fitness, especially if an environmental condition changes (Bürger, 1999; Rice, 2002; Grapputo et al., 2005). For another, aphids are poikilotherms, which are not able to regulate their internal temperature and therefore are unable to thrive at low temperatures

(generally, aphid development has a lower limit of around 4 °C (Hullé et al., 2010; Kilian & Nielson, 1971). A reduction in temperature is an environmental change that typically accompanies the onset of autumn and winter, and this reduction is usually fairly drastic in the temperate regions that most aphid species inhabit (Dixon et al., 1987). Aphids, necessitated by the need to overcome harsh conditions are typically capable of exhibiting a reproductive polyphenism in response to environmental cues associated with the onset of autumn (figure 1.7) (Simon, Rispe & Sunnucks, 2002). Importantly, this polyphenism leads to the production of oviparous sexual females and males. This has the two-fold benefit of overcoming the drawbacks associated with parthenogenesis and also, by production of a cold-resistant diapausing egg (Strathdee, Howling & Bale, 1995) after mating, allows individuals to avoid the harsh conditions associated with winter while still ensuring the next generation (Simon, Rispe & Sunnucks, 2002). Reproductive mode is determined during embryonic development in response to maternal signals. This ability to switch from parthenogenesis to sexual reproduction is termed cyclical parthenogenesis and is exhibited by most extant aphids (Simon, Rispe & Sunnucks, 2002). Though, some species and even lineages have secondarily lost cyclical parthenogenesis and are obligately parthenogenetic (Moran, 1992; Simon, Rispe & Sunnucks, 2002; Peng, Qiao & Chen, 2017). These aphids are typically found outside of temperate zones (or in greenhouses) where winter conditions are not harsh enough to necessitate this reproductive strategy (Dixon et al., 1987; Vuong, Kim & Song, 2003; Nelson, Denlinger & Somers, 2010). Those that can respond to photoperiod within cyclical parthenogenesis are termed holocyclic, while those that cannot are termed anholocyclic.

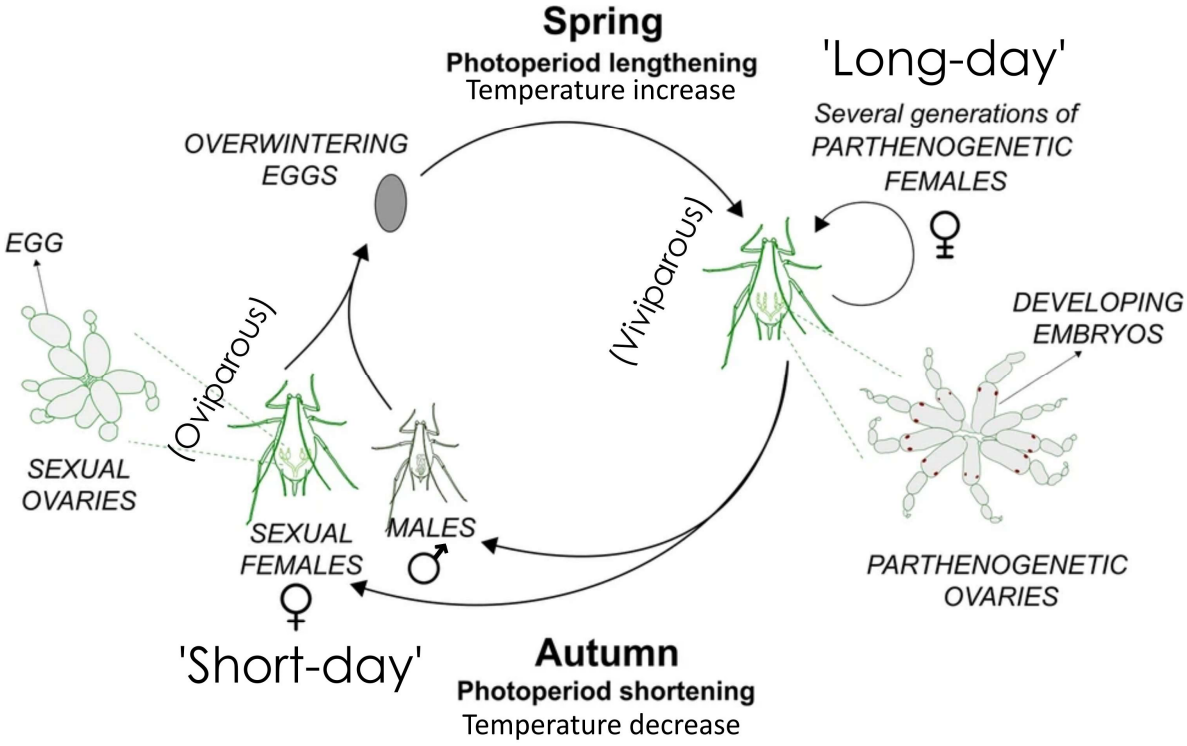


Figure 1.7 Cyclical parthenogenesis exhibited by aphids.
Most extant aphid species display cyclical parthenogenesis (the exception being species and strains that have secondarily lost the ability to reproduce sexually), in which many generations of asexually reproducing viviparous aphids are produced in the spring and summer, when photoperiod is sufficiently long and associated with higher temperatures, until, in response primarily to reduced photoperiod in autumn, sexual oviparous females and males are produced asexually, which mate to produce cold-resistant diapausing eggs. These eggs hatch in the spring, producing asexually reproducing viviparous females. The principal difference between asexual and sexual females is in their ovaries, the former possessing string of many embryos at various stages, and the latter possessing relatively few yolky eggs. Adapted from Colizzi, Martínez-Torres & Helfrich-Förster, 2023.

Cyclical parthenogenesis occurs in an annual cycle (Ogawa & Miura, 2014). Reproduction occurs through viviparous parthenogenesis for much of the year (typically including ten to thirty generations). At the onset of autumn, perception of environmental cues leads to the eventual reproductive switch (Ogawa & Miura, 2014). As discussed for cases of developmental plasticity more generally (Section 1.2), the inducing environment and the adaptive environment are separate (though highly associated), and there is a delay between perception of environmental cue and actualisation of the switch. This delay spans generations. The primary cue is a reduction in photoperiod, which is secondarily modulated by temperature (aphids at sufficiently high temperatures will not

switch between reproductive modes, regardless of photoperiod; for *A. pisum* and another aphid, *Megoura viciae* this temperature has been reported as 25 °C (Kenten, 1955; Lees, 1959)). Photoperiod is perceived initially by light penetrating the cuticle of the head (Hardie, Lees & Young, 1981), with specialised neurons (photoreceptors) not associated with the eyes responsible for detection. In response to the perception of reduced photoperiod, development is redirected, leading eventually, if the environmental cue persists, to the production of a single generation of sexually reproducing aphids (Ogawa & Miura, 2014). Sexual reproduction requires that females of the generation prior to the sexual one must produce, by viviparous parthenogenesis, sexual females but also sexual males.

The production of males is a product of the XO sex-determination system present in most aphids. Females are XX and identical to their mothers, while males are XO (hemizygous) and lose one X chromosome during the single maturation division (Blackman & Hales, 1986). Which of the X chromosomes is lost is thought to be random (Wilson, Sunnucks & Hales, 1997). During meiosis in males, viable spermatocytes are formed only where an X chromosome is present and which of the sets of autosomes associate with the X chromosome (the other set being lost) is also thought to be randomly determined (Hales et al., 2002a). In this way, the following generation are all female. Males and sexual females represent relatively rare morphs, which has implications for the selection pressures experienced by genes expressed specifically in them. Beyond the elimination of one X chromosome in males, sexual females and males are genetically identical to their mothers, though their reproductive phenotypes are fundamentally separate.

Haploid sperm and oocytes are produced by males and oviparous females respectively through reductive meiosis. After mating, females oviposit frost-resistant, overwintering eggs (Strathdee, Howling & Bale, 1995). Sexually produced embryos reach maturity much slower than viviparous parthenogenetically produced embryos, three months versus days and hatch in spring (whether development is continual but slowed, and/or halted appears to vary between aphid species (Shingleton, Sisk & Stern, 2003; Durak et al., 2020, 2023), surviving conditions that adults and nymphs would not necessarily be able to. From the eggs hatch viviparous parthenogenetic females called stem

mothers or fundatrices, which resume viviparous parthenogenetic reproduction. A seasonal timer exists to prevent these females, and the generations immediately following, from initiating the switch to sexual reproduction again (Matsuda et al., 2017). In *A. pisum*, aphids are unresponsive to reduced photoperiods for 70 to 90 days (depending on temperature) after their progenitor hatched from an egg (Matsuda et al., 2017). This is facilitated by substantial transcriptomic changes in heads (between aphids with operational and expired seasonal timers, and in particular, differentially expressed genes (DEGs) associated with epigenetic modification are enriched), where photoperiod is sensed and integrated (suggesting that this is the site of the seasonal timer) (Matsuda, Numata & Udaka, 2020).This is likely an adaptation to prevent losses in productivity associated with production of further diapausing eggs, and loss therefore of several generations of highly reproductive asexuals. This mechanism, which has been observed in other aphids, has been suggested to be a result of perception of the still relatively reduced photoperiods of early spring (Lees, 1959), which may be sufficiently short to, without a seasonal timer, initiate switching to oviparous reproduction without the need to do so (Matsuda et al., 2020). The timer may therefore act for long enough to allow enough generations that by the time it expires, the photoperiod is not sufficiently short for the switch to oviparous reproduction to occur. This is an example of an intergenerational reproductive polyphenism, whereby the signal is perceived by the parent (F0) and transmitted to the offspring (F1), and potentially the grand-offspring (F2), as a result of the telescoping of generations (the F0 can essentially encompass the F1 and F2 (Kindlmann & Dixon, 1989)). Although, while classical studies indicated that the critical area where light was able to redirect the polyphenism was the dorsal part of the protocerebrum of adults (Hardie, Lees & Young, 1981), whether embryos are able to directly perceive photoperiod through the cuticle of their mother is as yet, unknown. Aphids, and the two other groups constituting the Aphidomorpha: the Adelgidae and Phylloxeridae, evolved this reproductive polyphenism from sexual ancestors before their divergence ~ 150-200 million years ago (Davis, 2012; Ortiz-Rivas & Martínez-Torres, 2010; Ren et al., 2013). Cyclical parthenogenesis paired with viviparity represents an extreme example of phenotypic plasticity displayed by aphids. Each of the components that

constitute plastic systems, sensors, modulators and effectors play a role, therefore, in facilitating it.

1.4 Sense, modulation and effect in the reproductive polyphenism of aphids

1.4.1 Detection and integration of photoperiod

The primary sensors, the aspect that allows initiation of perception of the environmental cue, for the aphid reproductive polyphenism are thought to be the photoreceptors in the dorsal *pars lateralis* of the brain (Lees, 1964; Barberà, Collantes-Alegre & Martínez-Torres, 2022), which directly (rather than through the visual system) detect light through the cuticle of the head (Hardie, Lees & Young, 1981). These photoreceptors encompass a group of flavins (short wavelength sensitive) and opsins (sensitive to longer wavelengths) with photopigments responsive to wavelengths of light ranging from near UV (365 um) to red, but most sensitive to blue light (450-470 um) (reviewed in Colizzi, Marínez-Torres & Helfrich-Förster, 2023). The expression levels of seven opsin genes have been demonstrated to be lower under long day (LD; conditions under which only production of viviparous parthenogenetic individuals occurs) conditions than short day (SD; conditions under which the switch to sexual reproduction can be initiated) conditions in aphids of a holocyclic strain but not an anholocyclic strain of *A. pisum* (Collantes-Alegre et al., 2018), consistent with these genes being important for light cue detection specifically as part of the reproductive polyphenism. Given that higher expression of opsin genes, which would intuitively be linked to greater reception of light, is associated with exposure to SD, it may be that upregulation occurs to make the switch more robust. That is, if light receptivity is higher, increasing sensitivity, but the photoperiod is still perceived to be short, commitment toward the sexual mode is more likely to be appropriate. Along a similar vein, it has been demonstrated that expression of genes related to dopamine's role in sclerotization and melanisation of the cuticle (*aaNAT, black*, *ebony* and *yellow*) are similarly downregulated in the heads of SD exposed L2 and L4 stage (sexuparae – asexuals that will produce sexual offspring) aphids, and a similar pattern is observed in SD embryos (Le Trionnaire et al., 2022). This may also modulate light penetration and thereby increase accuracy in detection of photoperiod.

Light perception must then be integrated into photoperiodic information spanning many day-night cycles. After light detection by photoreceptors, information is passed to the photoperiodic timer, also putatively situated in the *pars lateralis* (Steel & Lees, 1977). The timer compares information from the receptors to an internal clock (which also receives information from the photoreceptors), in aphids the nature of which is thought to be a highly dampened circadian clock system (Colizzi, Martínez-Torres & Helfrich-Förster, 2023). This highly dampened circadian clock exhibits a single photosensitive period that occurs rhythmically (the circadian clock, rhythmic, element), but then dampens if the dark period does not come (the dampened element, if aphids are maintained in constant darkness after light exposure at the critical period, their ability to respond to darkness during the photosensitive period is abolished, thus the system also relies on light-dark cycles; the system can thus be dampened if the night length changes) (Hardie & Vaz Nunes, 2001; Colizzi, Martínez-Torres & Helfrich-Förster, 2023). *A. pisum* has been demonstrated to possess most of the circadian clock genes (the exception being *jetlag*) possessed by *D. melanogaster* (in which our knowledge of circadian clocks is extensive) (Cortés, Ortiz-Rivas & Martínez-Torres, 2010), and they are expectedly, rhythmically expressed in the head (Collantes-Alegre et al., 2018). Though, *A. pisum* circadian clock genes (notably, *tim*, *per* and *cry*) appear to have diverged from their counterparts in other species, with highly accelerated rates of evolution relative to the otherwise highly consistent insect orthologues (Cortés, Ortiz-Rivas & Martínez-Torres, 2010). A circadian clock in this context works to modulate the reproductive switch by light exposure during a light sensitive phase in the night. The advantages of a dampened clock are that they can phase-shift more easily and thereby adapt if the photoperiod changes, and that they are high amplitude under non-constant conditions (Bertolini et al., 2019; Colizzi, Martínez-Torres & Helfrich-Förster, 2023). By comparing the photoreceptor derived information to the internal (circadian) clock, the photoperiodic timer is able to determine if the experienced photoperiod is above or below a threshold.

If the photoperiod is perceived to be below a threshold (i.e. the photosensitive phase is not interacted with sufficiently by light, indicating a sufficiently long night) by the photoperiodic timer, the photoperiodic counter then counts the

number of days that this occurs for (Colizzi, Martínez-Torres & Helfrich-Förster, 2023). This integrated information then interacts with the modulatory systems.

1.4.2 Modulation by neurosecretion

Sufficient or insufficient integration of photoperiod by the photoperiodic counter likely leads to differential stimulation of a group of neurosecretory cells (NSCs) in the *pars intercerebralis* in the dorsoanterior region of the protocerebrum, to which the *pars lateralis* are situated laterally (Steel, 1977). Classical studies identified a bundle of axonal projections from ten NSC group I cells (five in each hemisphere of the *pars intercerebralis*; though, importantly this was in *M. viciae*, recent evidence points to *A. pisum* having four pairs of involved cells) along with two NSC group II cells (one in each hemisphere of the *pars intercerebralis*) extending from the brain to the vicinity of the ovaries (Steel, 1977; Cuti et al., 2021). Arborizations originating from eight NSC group I cells in *A. pisum* have been observed in the *pars lateralis,* perhaps allowing them to interface with the putative clock neurons (dorsal neurons expressing circadian genes, *period* and *timeless*) and photoreceptors (cells expressing non-visual opsin genes, and *cryptochrome1*) located there (Cuti et al., 2021). Indeed, PDF-positive and CRY-positive clock neurons overlap dendrites of these neurosecretory cells and extend to the *pars lateralis*, and *pdf* expression is higher under SD; PDF (pigment-dispersing factor) being an important circadian clock element (Colizzi, Martínez-Torres & Helfrich-Förster, 2023), adding to the suggestion that the *pars lateralis* is the site for detection and integration. Subsequently, by ablating the areas containing the group I NSCs and the *pars lateralis*, these cells have been demonstrated to be involved in promoting development of parthenogenetic embryos (under LD), by secreting what was termed by the authors virginoparin (ablating led to production of sexuals under LD conditions) (Steel & Lees, 1977). The virginoparin is a modulator, a substance that is involved in transducing the signal from where it is perceived in the brain to where it takes effect, in the ovary. There are multiple candidates for the identity of virginoparin, and its exact nature has not been conclusively shown. Although, it appears likely that multiple systems beyond just the virginoparin may have a modulatory role on the polyphenism, as multiple systems have been linked (though, in many cases not definitively) to the switch (as we will see, below). Gene expression differences between LD and SD holocyclic (but, importantly, where

tested, not anholocyclic, those aphids that do not switch reproductive mode) aphids have been demonstrated extensively, especially in heads where the integration and some modulation occurs, as well as whole bodies, which alone and by stimulating further study have implicated several key systems (Le Trionnaire et al., 2009; Ishikawa et al., 2012; Liu et al., 2014; Collantes-Alegre et al., 2018; Matsuda, Numata & Udaka, 2020; Le Trionnaire et al., 2022).

1.4.3 Insulin signalling

The insulin system is a conserved component of the insect toolkit and is a conserved feature of metazoans generally (Jin Chan & Steiner, 2000; De Meyts, 2004). It primarily functions as a nutrition responsive pathway, is stimulated by insulin-like proteins (ILPs) and regulates basic insect functions like growth (facilitating it when nutrition is high, and reducing it when nutrition is low), reproduction and longevity (Brogiolo et al., 2001; Ikeya et al., 2002; Puig et al., 2003; Badisco, Van Wielendaele & Vanden Broeck, 2013; Okamoto & Yamanaka, 2015; Nässel & Broeck, 2016a). It has recently been demonstrated that two of the seven ILPs, *ilp1* and *ilp4*, encoded by the *A. pisum* genome are upregulated in the heads of aphids exposed to LD conditions relative to SD, though only at particular zeitgeber times (Barberà, Cañas-Cañas & Martínez-Torres, 2019). This is suggestive of the involvement of ILPs in the switch from asexual to sexual reproduction, most likely being closely responsive to detection and integration of photoperiod, given the head specific expression pattern. The link to zeitgeber time is also suggestive of some link to photoperiod and the circadian clock. This expands on a previous study that has shown LD to be associated with upregulation of an insulin receptor and downregulation of an insulin-degrading enzyme in heads (Le Trionnaire et al., 2009). Taken together, these findings point towards high insulin signalling during LD and therefore associated with maintaining parthenogenetic reproduction, and low insulin signalling during SD, linked to a shift towards sexual reproduction. Furthermore, characterisation of the group I NSCs of the *pars intercerebralis* and their axonal projections (some of which extend to the vicinity of the ovaries) suggest that they may secrete both ILP1 and ILP4 (Barberà, Cañas-Cañas & Martínez-Torres, 2019; Cuti et al., 2021). This quite firmly implicates insulin signalling for a role in the reproductive polyphenism, and potentially as a key modulator, possibly the virginoparin. Although, any causative role of insulin signalling in

the switch has not yet been demonstrated functionally. Of the *A. pisum* ILPs, ILPs 1 to 4 are the most typical of insulin proteins released from peptidergic neurons or neurosecretory cells (Huybrechts et al., 2010) and they also appear to have arisen from several duplications, possibly specific to the aphids (Barberà, Cañas-Cañas & Martínez-Torres, 2019). *ilp1* and *ilp4* are the only two of these that appear to be appreciably expressed in heads of adult females (based on public head specific RNAseq libraries) (Barberà, Cañas-Cañas & Martínez-Torres, 2019). The axonal projections of the NSCs carrying (putatively) ILP1 and ILP4, two lateral and one medial, have also been characterised, and importantly, ILP4 appears to be synthesised and transported equally in holocyclic and anholocyclic strains (suggesting that if ILP4 is involved in affecting the reproductive switch, another aspect of the pathway may account for the inability of anholocyclic strains not to switch) (Cuti et al., 2021). These projections appear to originate from the branching of two bundles as they pass through the *corpora cardiaca* (Cuti et al., 2021), a neuroendocrine organ just outside of the brain (Bowers & Johnson, 1966; Johnson, 1963). The medial nerve extends almost to the end of the abdomen, while the lateral nerves, importantly, appear to terminate in the vicinity of the germaria (Cuti et al., 2021). Germaria may therefore be the site where the modulatory effects of insulin occur. Taking these results together, it is becoming increasingly clear that the insulin signalling pathway is linked to the reproductive polyphenism in some capacity.

The insect insulin signalling pathway is a complex system that is primarily understood to be activated in states of sufficient nutrition encompassing carbohydrate and amino acid availability (Badisco, Van Wielendaele & Vanden Broeck, 2013; Yoshinari et al., 2021; Ling & Raikhel, 2023). Nutrition stimulates release of ILPs from NSCs in the brain, which bind primarily to insulin receptors (IRs) (Smýkal et al., 2020; Viola et al., 2023). Binding activates the tyrosine kinase activity of the receptor leading to autophosphorylation (Lee & Pilch, 1994; Defferrari et al., 2018). Briefly, by direct IR activity and by employment by IRs of insulin receptor substrates (notably, Chico), activation of key intracellular pathways occur (figure 1.8) (Clancy et al., 2001; Slack et al., 2015; Valzania et al., 2019). Among these is the FoxO signalling cascade (Gui & Burgering, 2022). Activation and phosphorylation of a series of targets leads eventually to

recruitment of Akt. Akt phosphorylates forkhead box protein O (FoxO), causing it to translocate from the nucleus to the cytoplasm and thereby preventing it from acting on its target genes to increase their expression (Brunet et al., 1999, 2001; Puig & Tjian, 2005; Chen et al., 2023). Targets of FoxO include *inr*, *4e-bp* and *bmm*, among many other genes typically associated with growth and reproduction across a range of insects (Hansen et al., 2007; Roy & Raikhel, 2012; Kang et al., 2017; Zeng et al., 2017; Dong et al., 2021). The target of rapamycin (TOR) pathway also interacts with the insulin signalling pathway, sharing key regulators (principally Akt and FoxO) and each is able to regulate aspects of the other (Hay, 2011; Chowański et al., 2021); TOR can be activated by insulin signalling or independently by amino acids (Hansen et al., 2004).

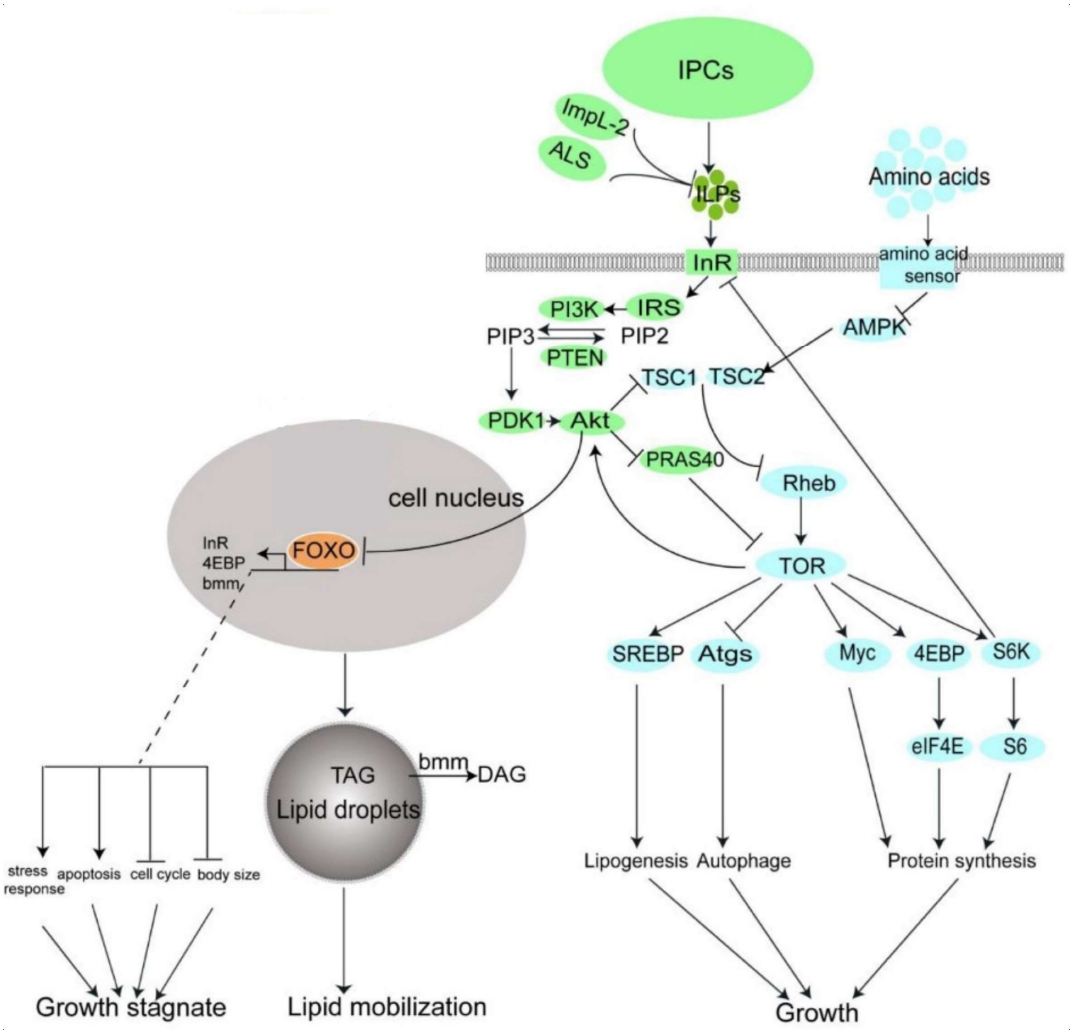


Figure 1.8 Simplified representation of the insulin signalling pathway in insects (based on studies in *D. melanogaster*), and its association with TOR signalling, another nutrition responsive element.
Briefly, IPCs (insulin-producing cells) in the brain release ILPs (insulin-like proteins) in response to various cues, which then bind to InR (insulin receptor). InR autophosphorylates, leading to a phosphorylation cascade that leads principally, to FOXO (a key node in the insulin signalling pathway) being outside of the cell nucleus (as a result of phosphorylation by Akt). Not being in the nucleus, FOXO is then unable to act as a transcriptional regulator of key downstream genes, involved primarily in regulating growth and mobilization of lipid reserves. Insulin signalling also interacts with TOR (target of rapamycin) signalling, to further regulate response to nutrition. Adapted from (Miao et al., 2022).

Insulin signalling is a key regulator of insect reproduction (Leyria, Orchard & Lange, 2021; Chen et al., 2023; Yan et al., 2022; Wang et al., 2023d; Pan et al., 2022; Xu et al., 2021b). As one of the major energetic demands (perhaps, the major) of female insect life, reproduction is inherently hinged upon nutrition, to which insulin signalling is responsive (Rivero-Lynch & Godfray, 1997; Smykal & Raikhel, 2015). While ILPs are principally produced in the head and enter the haemolymph, their receptors in some cases are highly expressed in the ovaries (Silva-Oliveira et al., 2021; Smýkal et al., 2020). It has been demonstrated in a wide range of insects that ILPs stimulate ovary activity and disruption of insulin signalling leads to deficiencies in reproduction, including arrested ovaries, failures in oogenesis and vitellogenesis, and ultimately reduced ovaries and reproductive output (Brown et al., 2008; Sheng et al., 2011; Lu et al., 2018a; Fujinaga et al., 2019; Silva-Oliveira et al., 2021; Pan et al., 2022). Following from its clear role in reproduction, insulin signalling is involved in facilitating reproductive diapause (Chen et al., 2023; Schiesari et al., 2016; Sim & Denlinger, 2008). Reproductive diapause is often exhibited by insects to deal with exposure to harsh conditions, predominantly in response to cues that telegraph the oncoming winter and involves inactivation of ovaries and a cessation of reproduction (Tatar & Yin, 2001). In many cases, the phenotypes exhibited by insects where insulin signalling has been disrupted bear great similarity to those experienced during reproductive diapause (Xu et al., 2021b; Al Baki et al., 2019), for instance silencing of *ilp1*, *ilp2* and *ir* in *Diaphorina citri* led to inhibited ovarian development and reduced laying of eggs, and reduced the expression of a highly reproduction related gene, *vg* (Wang et al., 2023d), and similarly, in *Chrysopa pallens*, silencing of *inr2* reduced *vg* expression, ovarian development and fecundity (silencing of other insulin related genes had similar, adjacent effects) (Han et al., 2020), consistent with reproductive diapause phenotypes. Additionally, as reproductive diapause occurs in response to environmental cues (Saunders, Henrich & Gilbert, 1989), it is an example of plasticity. The inducing cue is frequently photoperiod. As the aphid reproductive polyphenism can be regarded as a type of diapause, it is noteworthy that these phenomena bear remarkable similarity, at least in their function.

Insulin signalling has been implicated in an impressive array of further polyphenisms, especially where differential growth/size is involved, a product perhaps of the system's most principal function (Nijhout & McKenna, 2018; Fawcett et al., 2018; Libbrecht et al., 2013). Perhaps the most striking insect example of polyphenism is the division of reproductive labour exhibited by eusocial insects. Honey bee larvae destined to be queens have greater expression levels of genes encoding ILPs and IRs than do worker destined larvae (Wheeler, Buck & Evans, 2006) and disruption of insulin signalling has demonstrated a similar relationship (Wolschin, Mutti & Amdam, 2010). In ants, where in a number of species, nutritional state drives caste determination, similar observations have been made (Okada et al., 2010; Chandra et al., 2018). ILPs also play a role in the wing polyphenism of *Nilaparvata lugens*, which exhibits long or short wings in response primarily to crowding, where two IR isoforms work antagonistically to the action of each other (Xu et al., 2015). Further examples of the involvement of insulin signalling in polyphenism are presented extensively in (Nijhout & McKenna, 2018). Wing polyphenism (which is one of the most commonly occurring insect polyphenisms, such as those explored by Nijhout & McKenna, 2018) appears to be frequently linked to insulin signalling, though it should be noted that wing polyphenisms that appear to not be mediated by insulin signalling also exist (Gudmunds et al., 2022).

Importantly, insulin is also involved in the other polyphenism displayed by aphids, which is a wing polyphenism. *ilp5* is more highly expressed in the heads of uncrowded pea aphids than crowded pea aphids fated to produce winged offspring (though, none of the other ILP encoding genes were differentially expressed – this may account for the separation in activating each of the polyphenisms, if ILP1 and ILP4 are involved in the reproductive polyphenism) (Yuan et al., 2023). Though, whether higher *ilp5* expression is merely correlated with reduced crowding (for instance, reduced crowding may increase nutrition, which increases insulin signalling) or is a cause (or even consequence) of the lack of crowding/production of wingless offspring has not been tested. However, differential expression of the two genes encoding IRs were not found to be differentially expressed in embryos at the stages critical to the polyphenism (late development, stage 18 and stage 20) (Ogawa & Miura, 2013), and curiously their inhibition did not affect the production of winged offspring in the same

study. Another study has demonstrated that *inr1* is more highly expressed in stage 18 wingless-fated embryos (Grantham et al., 2020). Importantly, knockdown of *FoxO* in uncrowded pea aphid adults was able to increase the production of winged offspring, further suggesting insulin, through FoxO exerts control over wing development genes (Yuan et al., 2023). This study provides the most concrete evidence for the involvement of insulin signalling in the wing polyphenism. The aphid wing polyphenism is one which presents a trade-off between dispersal and fecundity, as the winged females are able to translocate away from unfavourable conditions but suffer a reduction in reproductive output (Braendle et al., 2006). The principal inducing cues for induction of production of winged offspring (as for the reproductive polyphenism, wing morph is in many cases prenatally determined, though in others fate can be affected during the early nymphal stages) appear to be tactile stimulation encouraged by crowding, presence of predators or parasitoids, and degraded food resources (all of which may interact) (Chen et al., 2019; Müller, Williams & Hardie, 2001; Dixon & Glen, 1971). An important point to note is that the aphid reproductive polyphenism and wing polyphenism display a degree of mutual exclusivity, in that the sexual morph appears to preclude the winged morph (with few exceptions) (Hille Ris Lambers, 1966). The mechanism underpinning the preclusion by oviparous and also stem-mother identities of developing wings is unknown, though it is likely linked, in the case of the oviparous identity, to compartmentalisation in their utilisation of the same modulatory pathways (perhaps by tissue specificity or through expansions of key gene families, for instance, seven ILPs are encoded in the *A. pisum* genome (Huygens et al., 2022) and while *ilp1* and *ilp4* have been associated with the reproductive polyphenism (Barberà, Cañas-Cañas & Martínez-Torres, 2019; Cuti et al., 2021), only *ilp5* has been associated with the wing polyphenism (Yuan et al., 2023)). Additionally, whether or not it is possible to stimulate the production of winged viviparous or winged oviparous individuals in place of wingless oviparous individuals under SD conditions by sufficiently (by which I mean maximising the cue, e.g. by causing aphids to be extremely overly crowded) providing the inducing cue has not been explored. Similarly, no experiment has yet set out to induce, by direct manipulation of for example, insulin signalling, production of winged oviparae; doing so may help to determine the mechanism controlling the apparent mutual exclusivity, but will likely require high tissue specificity and greater understanding of how

compartmentalisation may be occurring. It is likely that control over these multiple polyphenisms occurs partially through the concurrent use of several systems, beyond insulin signalling.

1.4.4 Juvenile hormone

In addition to potential direct roles of ILPs at targets (in the ovary), insulin signalling is also known to modulate synthesis of juvenile hormone (JH) by the *corpus allatum* (an endocrine gland which in aphids and some other insects consists of the fused *corpora allata*), which sits posteriorly adjacent to the *corpora cardiaca*, and juvenile hormone degradation (Tu, Yin & Tatar, 2005; Tatar et al., 2001; Libbrecht et al., 2013; Rauschenbach et al., 2017; Pan et al., 2022). Juvenile hormone is a core insect neurohormone, and it is involved in most, if not all of the examples of polyphenism that I have explored in the sections previous, to some extent (Nijhout, 1999). Much like insulin signalling, it appears to have been co-opted into control of plastic responses repeatedly.

Juvenile hormones are a class of (mostly) insect specific non-peptidergic hormones synthesised by the *corpora allata* (or the functionally identical *corpus allatum*) (Cusson et al., 1991). They are responsible for mediating core processes during insect life, including reproduction, growth, development, and metamorphosis, which was the first function identified by Wigglesworth in the 1930s (Wigglesworth, 1936, 1940; Zhang, Li & Liu, 2022). Synthesis of JH is controlled by the brain, principally through neural connections and by neurohormones, primarily allatotropins (which exert an upregulatory effect) and allatostatins (which exert an inhibitory effect), and by negative feedback loops (Noriega, 2014). The canonical JH pathway, briefly, occurs when JH (principally JHIII, the most commonly occurring member of the family) binds to its receptor methoprene-tolerant (Met), a bHLH-PAS family transcription factor (figure 1.9) (Ashok, Turner & Wilson, 1998; Konopova & Jindra, 2007; Charles et al., 2011). JH binding reduces homodimerization of Met (and to paralogs of Met where they exist, as in the case of Gce in *D. melanogaster*), allowing Met to instead associate with Taiman (Tai) (also a bHLH-PAS family protein) (Charles et al., 2011; Zhang et al., 2011; Li, Mead & Zhu, 2011). The Met-Tai complex is then translocated to the nucleus where it is able to bind to JH-response motifs and induce expression (Kayukawa et al., 2012; Li et al., 2014). A key early responder of the JH-Met-Tai complex is *krüppel homolog 1* (*kr-h1*), which

encodes a conserved transcription factor that then acts on several further downstream targets (Li, Mead & Zhu, 2011; Kayukawa et al., 2012; Zou et al., 2013). In this way, the juvenile hormone signalling pathway is able to exert control over downstream processes.

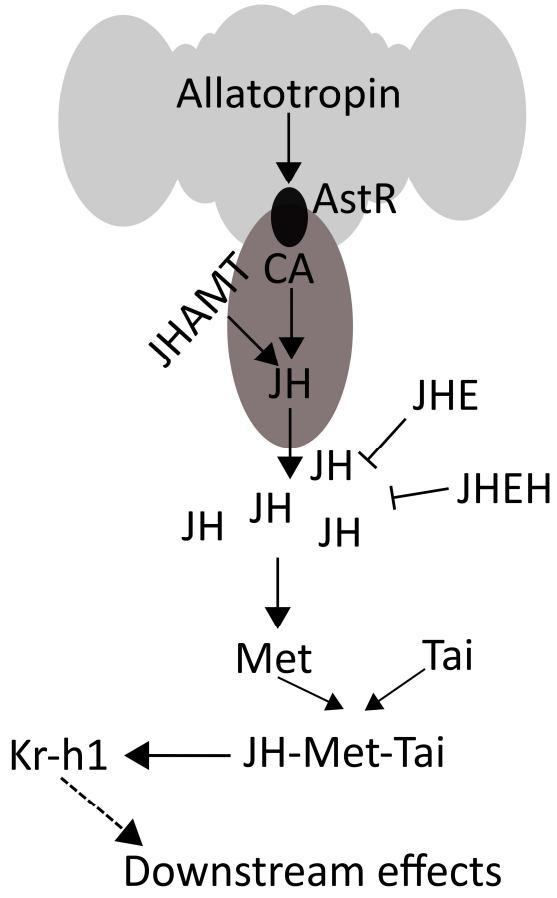


Figure 1.9 Simplified schematic of JH synthesis and activity.
Allatotrophin released from the brain interacts with its receptor, AstR, in the CA (corpora allata, the site of JH synthesis) to stimulate synthesis of JH (JHAMT is a key enzyme involved in the final steps of JH synthesis). JH is released from the CA into the body more generally (through the haemolymph), where it can be degraded by the activity of JH degrading enzymes (JHE and JHEH), and binds to its receptor Met. The JH-Met complex then associates with Tai, and the JH-Met-Tai complex can then associate with DNA, the key inducible elements of this complex being Kr-h1, a transcription factor with multiple downstream targets.

JH was an early-hypothesised identity for the virginoparin, the substance involved in the reproductive polyphenism of aphids shown to maintain the parthenogenetic offspring fate (Steel & Lees, 1977). Clear involvement of JH signalling in the aphid reproductive switch has since been demonstrated. JHIII titre is higher in LD exposed pea aphids producing parthenogenetic offspring

than SD exposed pea aphids producing sexual offspring (and this is also associated with differential expression of JH related genes) (Ishikawa et al., 2012) and the same has been observed in *M. viciae* (Hardie et al., 1985). This relationship may be driven by differential expression of genes encoding juvenile hormone esterase (JHE), primarily *jhe1*, the products of which degrade JH, which has been demonstrated to be upregulated at certain stages in SD exposed *A. pisum*, though exactly how active the product of this gene is in degrading JH is unknown (Ishikawa et al., 2012). Furthermore, classic studies demonstrated that through application of JH and its analogs (principally, kinoprene), it is possible to redirect aphids exposed to SD from production of sexual offspring (oviparae) toward production of asexual offspring (viviparae) (or, intriguingly, offspring with mixed ovaries containing embryos and vitillogenic eggs) (Hardie & Lees, 1985; Mittler, Nassar & Staal, 1976; Corbitt & Hardie, 1985).

Roles for JH in reproduction more generally, where it stimulates oogenesis and vitellogenesis (Hardie, 1987a; Gujar & Palli, 2016; Zhang et al., 2018; Smykal et al., 2014; Leyria, Orchard & Lange, 2022), and controls ovarian development (Marchal et al., 2014; Lin, Yao & Wang, 2015; Yue et al., 2018; Hernández-Martínez et al., 2019; Tang et al., 2020; Wynants et al., 2023), along with controlling reproductive diapause (Ma et al., 2021; Tian et al., 2021; Gao et al., 2022; Li et al., 2022c), have been extensively investigated across a wide range of species. Thus, JH signalling is a system that is fundamentally important to many aspects of insect life, and one that appears to have been deployed in many contexts, some of which are arguably, derived forms of what JH is typically known to regulate, such as phenotypic plasticity and polyphenisms involving size and reproduction (Nijhout, 1999; Jongepier et al., 2018). In addition to direct activity of JH to control downstream effectors, JH signalling is also known to interact with and regulate insulin signalling in some insects (Sheng et al., 2011; Mirth et al., 2014; Hatem et al., 2015).

The links between insulin signalling and juvenile hormone are clearly demonstrated by the systems that they co-regulate (JH is also implicated in the aphid wing polyphenism (Hardie, 1980; Ishikawa et al., 2013), for example) but also in the more direct interactions between them. Insulin and juvenile hormone are able to regulate the activity of each other (ecdysteroids, the other major

hormone ubiquitous to the insect toolkit, primarily 20-hydroxyecdysone (20E), also interact (Leyria et al., 2022; Mirth et al., 2014; Keshan, Thounaojam & Kh., 2017), but because 20E does not appear to be particularly implicated in the aphid reproductive switch is mostly beyond the scope of this thesis, though it is important to note that it is heavily involved in the wing polyphenism (Vellichirammal, Madayiputhiya & Brisson, 2016a; Vellichirammal et al., 2017; Deem et al., 2024)). Met and Kr-h1 interact with *ilp* genes (binding sites for Kr-h1 appear to occur far more frequently than for Met) and Kr-h1 activates some *ilp*s while repressing others in *Aedes aegypti* ((Ling & Raikhel, 2021)). Similarly, knockdown of *inr* resulted in JHIII titre reduction and reduced Met expression in *Spodoptera litura* (Pan et al., 2022).

Because insulin signalling and JH signalling are both elevated in aphids in LD conditions producing viviparous offspring relative to aphids in SD conditions producing oviparous offspring, and given that they co-regulate each other, both regulate the wing polyphenism, and axonal projections carrying ILPs appear to project close to the *corpus allatum* where JH is synthesized, in addition to their shared roles in controlling reproduction, it is plausible that they are arranged in a complex system to control the reproductive polyphenism.

To briefly introduce ecdysone, this is another developmental hormone that is linked primarily to moulting, where it is involved in coordinating activities at moulting and metamorphosis (Truman & Riddiford, 2002). It acts principally by binding to its nuclear receptor, which then in association with ecdysone is able to regulate transcription. Like the other major modulators of insect development, it has similarly been implicated in a range of insect examples of phenotypic plasticity and polyphenism (Nijhout, 1999; Simpson, Sword & Lo, 2011). Among these is the aphid wing polyphenism, which in *A. pisum* has been linked to differential expression of several genes related to ecdysone (genes associated with greater ecdysone signalling were higher in aphids producing wingless offspring) (Vellichirammal, Madayiputhiya & Brisson, 2016a; Vellichirammal et al., 2017). Meanwhile, a link between ecdysone signalling and the aphid reproductive polyphenism has not been demonstrated (though, the expression levels of some key genes relating to ecdysone, such as *broad*, an early ecdysone inducible gene, and *EcR*, the ecdysone receptor, have been investigated in LD and SD (Ishikawa et al., 2013).

1.4.5 Biogenic amines

An aspect of neuromodulation that has been largely ignored with regards to the aphid reproductive polyphenism is the role of biogenic amines. In insects, biogenic amines (principally, dopamine, octopamine and serotonin) act directly on target tissues as neurohormones and neurotransmitters (Blenau & Baumann, 2001; Farooqui, 2007). They are responsive to the environment and are involved in reproduction, likely mediated through their modulatory effects on insulin and JH (Fuchs et al., 2014; Li et al., 2020a; Knapp et al., 2022). They have also been implicated in insect examples of polyphenism (e.g. locust phase polyphenism (Wang & Kang, 2014)). Receptors for serotonin, dopamine and octopamine are found in the *Drosophila* insulin producing cells (IPCs) (Luo et al., 2012; Andreatta et al., 2018; Crocker et al., 2010), which appear analogous to the aphid group I NSCs. Elevation of serotonin and dopamine levels are associated with reduced production and/or release of ILP from IPCs (Andreatta et al., 2018), similarly, knockdown of an octopamine receptor (OAMB) and a serotonin receptor (5-HT_{1A}) in *D. melanogaster* increased expression of *ilp3*, and *ilp2 and ilp5* respectively in the brain, suggesting that levels of insulin-like peptides may be responsive to signalling by these biogenic amines, which may act directly on the IPCs (Luo et al., 2014). Octopamine receptors have been identified in the bird cherry-oat aphid, *Rhopalosiphum padi*, and their expression has been demonstrated to be responsive to starvation conditions (Wang et al., 2022), revealing a possible link between octopamine and insulin signalling in this species. Recently, dopamine has been shown pharmaceutically to mediate the wing polyphenism in the pea aphid (which also show reduced titres of dopamine, serotonin and octopamine when crowded) (Liu & Brisson, 2023; Vellichirammal, Madayiputhiya & Brisson, 2016a). Octopaminergic signalling has also been demonstrated to be involved in the wing polyphenism in the pea aphid, where reduction in signalling led to a decline in production of winged individuals (Wang et al., 2016). A role for dopamine has been identified in the reproductive polyphenism of *A. pisum*, though it appears to be in modifying the extent of sclerotization and melanisation of the cuticle rather than in signalling (Le Trionnaire et al., 2022). This may affect the ability of light to stimulate the photoreceptors and may therefore modulate responsiveness to photoperiod, perhaps to enhance robustness. In addition to dopamine, octopamine and

serotonin, histamine may also play a role in the aphid polyphenisms. Histamine in insects is a solely photoreceptor associated neurotransmitter (Nässel, 1999). Given that photoreceptors mediate the aphid reproductive polyphenism, it is likely that histamine is involved.

Exactly what role, if any, these biogenic amines play in the aphid reproductive polyphenism are not entirely known. Owing to their environmentally responsive nature, demonstrable role in the wing polyphenism, and interactions with known/proposed polyphenism-mediating systems – JH and insulin signalling, it is possible that they are involved in the reproductive polyphenism.

1.5 Epigenetic mechanisms and plasticity

Epigenetic mechanisms represent potential effectors which may underpin changes at the level of the tissue where polyphenism is ultimately realised (Duncan, Gluckman & Dearden, 2014; Richard, Jaquiéry & Le Trionnaire, 2021; Budd et al., 2022). These are heritable, non-sequence modifications of DNA, encompassing: non-coding RNAs, histone modifications, chromosome remodelling, and DNA methylation (Duncan, Gluckman & Dearden, 2014; Lezcano et al., 2020; Cavalieri, 2021). Importantly, epigenetic modifications (of a genome, collectively, the epigenome) are environmentally responsive. DNA methylation has perhaps historically been linked, among epigenetic marks, most strongly to phenotypic plasticity (Duncan, Cunningham & Dearden, 2022; Kucharski et al., 2008). It primarily occurs at cytosines in CpG dinucleotides and in mammals is confined mostly to gene regulatory regions and is associated with repression of gene expression (Ehrlich et al., 1982; Suzuki & Bird, 2008). In insects, sparse (but variable) DNA methylation is observed in the epigenomes of many insects (though not all, notably it appears to be missing in the Diptera) (Bewick et al., 2017; Provataris et al., 2018), where it is confined primarily to the gene bodies rather than regulatory regions, and is associated with genes that are highly conserved and generally, stably expressed (i.e. housekeeping genes) (Foret et al., 2009; Elango et al., 2009; Glastad, Hunt & Goodisman, 2014). The evidence for a link between DNA methylation and differential gene expression in insects is not strong, but DNA methylation has been proposed to be involved in differential splicing (Duncan, Cunningham & Dearden, 2022).

The core DNA methylation toolkit consists of DNA methyltransferases 1 (Dnmt1) and 3 (Dnmt3), which are primarily responsible for maintenance and *de novo* methylation respectively (Bogdanović & Veenstra, 2009; Bewick et al., 2017) and Ten-eleven translocation dioxygenase (TET) which is involved in demethylation (Pastor, Aravind & Rao, 2013; Wojciechowski et al., 2014; Crawford et al., 2016). Across insects, genes encoding Dnmt1 occur frequently, while genes encoding Dnmt3 have been commonly lost (Bewick et al., 2017; Provataris et al., 2018). Dnmt1 has been suggested to have, in some cases, *de novo* methylation capabilities, which may explain how insects lacking Dnmt3 still often display DNA methylation (Fatemi et al., 2001; Mitsudome et al., 2015; Haggerty et al., 2021; Bewick et al., 2017).

The link between DNA methylation (or parts of the DNA methylation machinery) and plasticity in insects is not incredibly clear (Duncan, Cunningham & Dearden, 2022), though there are hints at a relationship between them in some species. In *S. gregaria*, differential expression of genes involved in DNA methylation, and differences in DNA methylation profiles between the two dispersal morphs, along with a putative role for Dnmt3 have been demonstrated (Robinson et al., 2016; Hou et al., 2020). Similarly, in *Nasonia vitripennis*, which demonstrates a photoperiod responsive embryonic diapause (Saunders, 1966), different photoperiods have been associated with differential methylation, and disruption of the DNA methylation machinery disrupted the effect of photoperiod on diapause (Pegoraro et al., 2016). Aphids possess a complete suite of DNA methylation genes and the *A. pisum* genome shows global low levels of methylation (Walsh et al., 2010; Clément et al., 2021). The potential role of DNA methylation and the DNA methylation machinery in the photoperiod responsive reproductive polyphenism is as yet unexplored.

Altogether, the aphid reproductive polyphenism encompasses a highly complex system of sensors, modulators and effectors which act together to facilitate assumption of one morph or the other. While there is strong evidence for involvement of the ‘usual suspects’, JH and insulin signalling (Ishikawa et al., 2012; Barberà, Cañas-Cañas & Martínez-Torres, 2019; Cuti et al., 2021), in depth analyses of individual components, especially separating tissues and isolating systems are lacking. Here, I present an exploration of changes associated with the reproductive polyphenism primarily at the level of the ovary

of aphids in which it is realised (as their ovaries bear future oviparae - sexual aphids, or virginoparae - asexual aphids). A diagram presenting the basis and main line of investigation for this thesis is presented in figure 1.10.

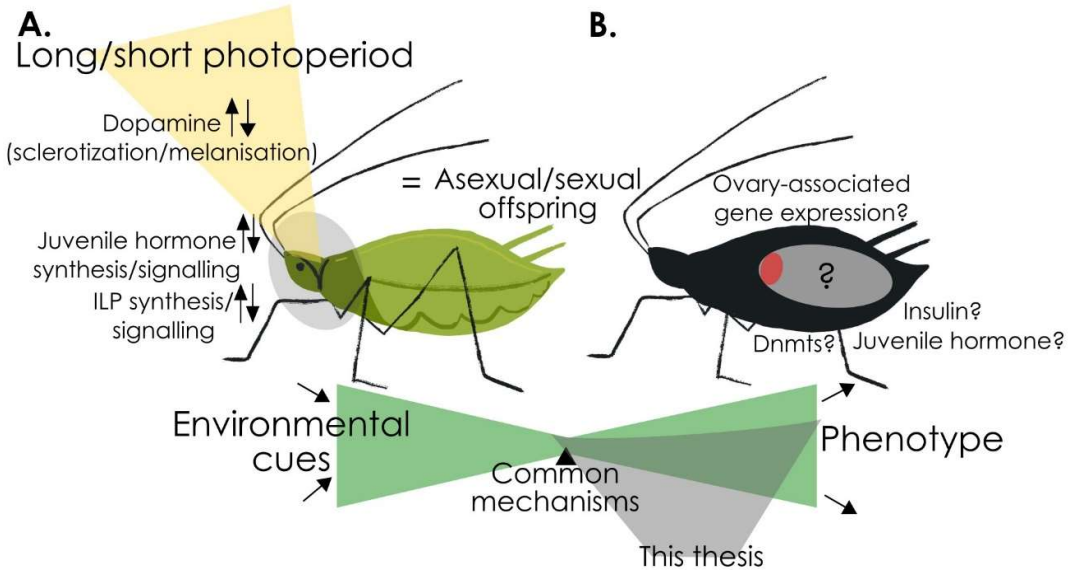


Figure 1.10 Key known components of the aphid reproductive switch, and unknown components focused upon in this thesis.
While differences in detection of environmental cues and mother-associated transcriptomic profiles (especially in heads) between aphids exposed to long and short photoperiods are fairly well understood (A), the changes that occur in the ovaries of aphids of the same rank (being the aphids that produce asexual or sexual offspring) are not (B). Beyond this, the ovaries can be looked at in even higher resolution, e.g. investigating the germaria (in red) specifically to further increase our understanding of the reproductive switch. Generally insect plasticity is responsive to a wide array of environmental cues to give a wide range of phenotypes, though this is often achieved through common mechanisms, some of which are investigated in this thesis.

1.6 Outline of thesis

The overarching aim of this thesis was to investigate how the reproductive polyphenism of aphids may be controlled at the level of the ovary, in response to putative modulatory systems, and relating to mechanisms that may be common to a broader range of insects and examples of plasticity. To do so, I use *A. pisum*, a model aphid species that exhibits polyphenism, was the first aphid to have its genome sequenced (with significant improvements on the original assembly having been made, since), has been demonstrated to be amenable to functional investigation (both RNAi and CRISPR-Cas9) and is an

important agricultural pest to explore some of the genes involved in facilitating a reproductive polyphenic switch.

Chapter 3 investigates the use of the N116 strain of pea aphids in RNAi based functional analysis and explores potential reasons for the apparent inconsistency of its efficacy in aphids (and other groups of insects), generally, as a tool to knockdown the expression of genes to study their function. I focus here on the several barriers that can inhibit efficient RNAi and how they might be mitigated, along with implications for the application of RNAi based pesticides.

Chapter 4 explores the role of two paralogs of a key gene in the DNA methylation toolkit, *dnmt3*, in reproduction by profiling expression in the ovaries of viviparous aphids and exploring their evolutionary history, revealing potential involvement in the reproductive polyphenism. And, following from the results of chapter 3 with regard to the efficacy of RNAi, this chapter uses pharmacological manipulation to inhibit the activity of the DNA methylation machinery to inspect its role in reproduction.

Chapter 5 builds on evidence for JH's involvement in the switch, in addition to recent studies in aphids that have implicated the insulin signalling pathway as a potential identity for the virginoparin, which maintains production of parthenogenetic offspring. In addition to investigating potential involvement of the two *dnmt3* paralogues explored in chapter 4 in the reproductive switch. I used RT-qPCR to explore the expression levels of key genes of these systems in the ovaries of virginoparae (producing parthenogenetic offspring only) and sexuparae (producing sexual offspring). I also investigated the changes in gene expression that occur downstream of JH signalling, and isolated from differential expression directly caused by sensory/modulatory aspects associated with varying photoperiod, by treating aphids kept under SD conditions with a JH analog.

In chapter 6, I aimed to profile differences in gene expression of germ cells from virginopara and sexupara aphids by RNA-seq. Because the germaria present a major avenue for aphid mothers to continually interact with their oocytes and early developing embryos, facilitated by viviparity, because of the proximity of the germaria to the terminations of projections carrying the putative virginoparin, and following on from expression patterns observed in chapter 4 and chapter 5,

the germ cells represent a possible point for the modulators to interact with effectors of the aphid reproductive switch. To investigate germ cell specific differential expression of genes associated with the reproductive polyphenism, I optimised a technique to combine ACME-dissociation, RNA-FISH HCR and FACS isolation of cells, toward Probe-Seq, in order to sequence RNA from cells positive for a germ cell specific marker.

Chapter 7 discusses the general findings of this thesis and the relationship of these findings to each other and the wider context. I discuss the links between *dnmt3* paralogues as possible effectors of the switch to JH and insulin signalling, synthesising the findings across the data chapters. I suggest future work that will further our understanding of the aphid reproductive polyphenism, other polyphenisms like it, and plasticity in general, and discuss considerations for functional studies in aphids going forward.

Chapter 2 General Methods

2.1 Aphid culture and maintenance

All laboratory experimental data presented in this thesis was performed using the N116 strain of *A. pisum*, which has been maintained by our laboratory for several years, encompassing many viviparous generations (at least twenty). The primary colony (from which individuals or other populations for use in experiments were derived) is maintained under long-day conditions – 16 light:8 dark, 20 °C,70 % humidity in a climate controlled growth chamber. Aphids taken from this colony were always viviparae. To generate sexuparae and/or oviparae, aphids were reared under short-day conditions – 12L:12D, 15 °C, 70 % humidity in a climate controlled growth chamber. Aphids were reared on *Vicia faba* seedlings which were regularly replaced to avoid deterioration of the plants as a food resource and by the growth of mould, and the population was regularly thinned for the same reason and to reduce the production of winged morphs.

2.2 Dissections

All dissections were performed in ice-cold PBS under a dissecting microscope (GXMXTL3T), using fine forceps. Dissections were performed on live aphids and they were performed quickly to minimise effects on gene expression and RNA degradation from the act of dissection. Ovaries were always removed first (where dissections of multiple body parts were taken), by using one pair of forceps to gently hold the aphid in place at the thorax, and using the other to make an incision at the anterior of the ventral/dorsal abdomen and then dragging towards the posterior and pulling the ovary out, as this quickly killed the aphid. For ovaries where parts of or whole ovarioles broke loose (and the aim was to collect the ovaries in their entirety), these were collected with a P1000 pipette equipped with a P1000 tip which was cut with a razorblade to increase the size of the opening. Tissue was collected into a 1.5 ml eppendorf tube containing PBS, on ice. Dissections of single tissues originating from single aphids, for RT-qPCR, were immediately frozen in liquid nitrogen, then stored at

-80 C. Where multiple aphids were included in a sample, dissections were performed for no longer than thirty minutes before processing.

2.2.1 Ovary fixing

To prepare ovaries for staining, they were fixed immediately after the dissection period by nutating at RT for one hour in a 1:1 mix of 4% formaldehyde: heptane in PBS (phosphate buffered saline). After, the lower phase was removed and 500 µl ice-cold methanol was added, and tubes were inverted gently several times. Ovaries were then washed briefly twice in ice-cold methanol, and finally stored at – 20 °C in ice-cold methanol.

2.2.2 Total RNA extraction

Total RNA was extracted (unless stated otherwise) from single tissues using the Zymo Direct-zol RNA Microprep Kit (Zymo), with the on-column DNase step. RNA was eluted in nuclease-free water, and, unless stated otherwise, quantified and its quality checked using a Nanodrop 2000 Spectrophotometer and stored at – 80 C.

2.2.3 cDNA synthesis

cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific), according to the manufacturer's instructions, and where indicated, cDNA synthesis was performed immediately following an additional dsDNase (Thermo Scientific) treatment (including dsDNase inhibition with 10 mM DTT (Thermo Scientific)). cDNA was synthesised from 1 ug total RNA, unless otherwise stated, and the conversion of RNA to cDNA is assumed to be 1:1. cDNA was stored at – 80 C.

2.2.4 RT-qPCR

Primers against target genes were designed by first identifying mRNA and genomic DNA (gDNA) sequences in NCBI or AphidBase (Legeai et al., 2010), where necessary, by BLASTing known *Drosophila melanogaster* sequences from FlyBase (Gramates et al., 2022). cDNA sequences were aligned to gDNA sequences using Splign (Kapustin et al., 2008) to identify intron-exon boundaries. Primer design was targeted, where possible, at intron-exon boundaries. Primers design was then performed using Primer3 plus

(Untergasser et al., 2012), and primers with product sizes ~ 100 bp, 50 % GC content and differing in Tm by at most 1 (and ~ 60 C) were preferentially selected. The deltaG of primer pair self and cross dimerisation was assessed using Beacon Designer (Premier Biosoft), primer pairs with deltaGs below 3 were preferentially used. The specificity of primer pairs was assessed using Primer-BLAST (NCBI) using the *A. pisum* databases as reference. Primers were synthesized by Integrated DNA technologies (IDT).

RT-qPCR was performed using a BioRad CFX RT-PCR detection system. Each RT-qPCR reaction consisted of (unless otherwise stated), 7.5 µl SsoAdvanced Universal SYBR Green Supermix (BioRad), 1.6 µl nuclease-free water, 0.45 µl forward primer (10 uM), 0.45 µl reverse primer (10 uM) and 5 µl cDNA (unless otherwise stated, containing 25 ng cDNA). Two (or three, where indicated) technical replicates were run for each biological replicate, and no-template and no-RT control reactions were included. Unless otherwise stated, reactions for a given target gene were run for all samples and replicates together on a single plate. The RT-qPCR program was: 95 °C for thirty seconds, followed by forty cycles of 95 °C for five seconds and 60 °C for thirty seconds, with plate reading at the end of each cycle. Following the forty cycles, a melt curve analysis was performed from 65 °C to 95 °C in increments of 0.5 °C, each for five seconds.

Prior to performing RT-qPCR for any target gene, the efficiency of the primers used to amplify it were determined by performing RT-qPCR of serially diluted (1, 1:10, 1:100, 1:1000, 1:10000 dilutions) cDNA generated from viviparous *A. pisum* individuals in triplicate (and with a no-template control), following MIQE guidelines (Bustin et al., 2009). The Cq values were plotted against log10 starting quantity, and the slope, efficiency and r^2 determined by the BioRad CFX software. Primers with efficiencies between 90 and 110 % were used preferentially.

After performing quantification, melt curves were checked (to assess for non-specificity) and the deviation between technical replicates was assessed (only biological replicates with two technical replicates within 0.5 Cq of each other were used going forward. Expression of Genes of interest (GOIs) were normalised to the geometric mean of the relative quantities of (unless stated otherwise) two reference genes – which are specified in chapter specific methods. Unless otherwise stated, reference genes were validated using the

geNorm2(), measureM() and pairwiseV() functions of the *ctrlGene* package in R (R Core Team, 2021) version 4.1.2 (2021-11-01). Relative expression ratios were determined using the Pfaffl method (Pfaffl, 2001), accounting for exact primer efficiencies. Relative expression ratio was calculated using the geometric mean of the control group sample ΔCts.

2.3 Agar-leaf plates for maintaining aphids

Where aphids needed to be maintained and isolated in small groups, they were often kept on agar-leaf plates (this is specified in chapter specific methods where it is the case). Agar-leaf plates were prepared by first pouring 2 % agar, supplemented with 10 % final volume Miracle-Gro solution (10 X Miracle-Gro all purpose soluble plant food (Miracle-Gro) solution, 1.25 ml Miracle-Gro in 100 ml total volume dH_2O) and 0.3 % final volume antifungal (10 % nippagin solution), into petri dishes (55 x 15 mm, Sarstedt), approximately 5 ml agar per dish. While the agar was still molten, but had cooled slightly, *V. faba* leaves freshly cut from plants (maintained ambiently) at the petiole and close to the leaf blade using a 70 % ethanol sterilised razor blade, were placed underside up into the agar, cut end first. Leaves of similar sizes (or multiple leaves constituting the size of one 'standard' leaf) were used. In order for the agar to set in only part of the petri dish, leaving bare surface for the aphids to walk on and for them to access the backside of leaves, agar was cooled, prior to insertion of leaves at a shallow angle – this meant only the parts of the leaf closest to the cut end were submerged in agar. After being allowed to solidify and cool, the leaf-agar plates were ready for use in maintaining aphids, with the lid-on to enclose them.

While maintaining aphids on agar-leaf plates, no more than ten aphids were allowed to be on a plate at a time (plates were checked frequently, and aphids were moved where necessary). This was done to reduce crowding effects which are known to induce production of winged morphs. Where one aphid produced more than ten offspring, these were split across multiple leaf agar plates but treated as one sample. Leaf quality and aphid mortality were inspected at least every three days, and live aphids were transferred to new agar-leaf plates at least every three days (more regularly if leaves showed signs of deterioration) by lightly brushing aphids with a paintbrush to cause them to withdraw their stylet from the leaf, and then picked up gently and transferred with the paintbrush once they were free.

2.4 HCR RNA-FISH

Third-generation HCR RNA-FISH (Choi et al., 2018) was carried out on fixed ovaries using a protocol adapted from the HCR RNA-FISH *D. melanogaster* protocol available online at (https://www.molecularinstruments.com/) and the aphid ISH protocol described in (Duncan, Leask & Dearden, 2013; Duncan, Benton & Dearden, 2013). All solutions, probes and hairpins were supplied by Molecular Instruments. Briefly, in all cases except for in chapter 6, fixed ovaries were rehydrated through a methanol/0.3 % PTw series (0.3 % Tween-20 in PBS), washed three times in 0.3 % PTw for ten minutes each, re-fixed for twenty mintues in 4 % formaldehyde (in 0.3 % PTw), washed as before three times, and then incubated for fourty-five minutes in detergent solution (1 % SDS, 0.5 % Tween-20, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0), 150 mM NaCl). Ovaries were then rinsed six times in 0.3 % PTw. Following this, pre-hybridisation and hybridisation were carried out as specified in the HCR RNA-FISH *D. melanogaster* protocol, except for the use of 1 pmol of each probe mixture. After overnight incubation at 37 °C, probe was removed as in the RNA-FISH HCR *D. melanogaster* protocol, using preheated wash buffer. Then, ovaries were pre-amplified in 100 µl amplification buffer for thirty minutes at RT. Concurrently, 6 pmol in 2 µl (or 3 pmol in 1 µl, where specified) hairpin h1 and equal quantities of hairpin h2 were prepared by heating at 95 °C for ninety seconds and allowing to cool to RT in the dark for thirty minutes. The pre-amplification solution was removed and hairpin solutions were added, and samples were incubated overnight at RT in the dark. Excess hairpin was then removed as specified in the HCR RNA-FISH *D melanogaster* protocol, protecting samples from the light, except for the inclusion of 1 µl DAPI (5 mg/ml, 4′,6-diamidino-2-phenylindole, Invitrogen) during the first thirty minute wash, to stain nuclei. Ovaries were then stored in 1 ml 70 % ultrapure glycerol and stored at 4 °C protected from light, prior to microscopy.

2.4.1 Mounting ovaries

Ovary samples were transferred to staining dishes and/or glass slides to select and disconnect individual ovarioles (or parts of ovarioles) and embryos from the ovaries, using fine forceps and syringe needles (1 ml 25GA x 5/8in Luer, BD Plastipak) equipped with tungsten needles, under a dissecting microscope (GXMXTL3T). Ovarioles/embryos were then transferred to new glass slides with

minimal transfer of glycerol. A small amount of SlowFade Diamond AntiFade Mountant (Invitrogen) was added, and samples were covered with a coverslip (where preparations were of larger embryos, bridge-mounting was used) and then sealed with nail polish. Mounted samples were stored in the dark at 4 °C prior to imaging.

2.4.2 Confocal microscopy and imaging

Confocal microscopy was performed using an LSM880 Upright Confocal Microscope (Zeiss), situated in the Bioimaging and Flow Cytometry facility at the University of Leeds, and the Zen Black software (Zeiss). Imaging was always carried out within a week of sample preparation. Lasers at wavelengths of 405 nm (diode, for DAPI), 561 nm (DPSS) and 633 nm (HeNe) were used to excite fluorophores. 1 AU was used along with EC Plan-Neofluor 10x/0.3, Plan-Apochromat 20x/0.8 and Plan-Apochromat 63x/1.4 Oil DIC objectives.

2.5 Data analysis

All data analysis was performed and graphical presentations of data built using R (R Core Team, 2021) version 4.1.2 (2021-11-01), specifics of analyses are reported in chapter specific methods. Data figures were modified (for clarity) and diagrams were built and/or adapted using InkScape (Inkscape Project, 2020).

Chapter 3 Testing and optimising RNAi in the N116 *Acrythosiphon pisum* clone

3.1 Introduction

RNA interference (RNAi) allows sequence specific knockdown of gene expression. It is considered a gold standard technique for studying the function of genes. By knocking down gene expression, investigators can, through assessment of effects on phenotype or aspects of molecular biology, study how the expression of particular genes leads to particular biological phenomena (Mocellin & Provenzano, 2004; Bellés, 2010). RNAi presents a powerful tool for studies in developmental and evolutionary biology (for a review of functional analysis in hemiptera, see (Jain et al., 2020)) especially when paired with RT-qPCR, another gold-standard technique for assessing the expression levels of genes, and (fluorescence) *in situ* hybridization techniques, such as RNA-FISH (Choi et al., 2018), that allow investigators to visualize the expression of developmental genes which may be responsive to RNAi-mediated knockdown of other genes.

Beyond research, RNAi has also been leveraged for its potential for use as the basis of highly specific insecticides (see (Vogel et al., 2019; Christiaens et al., 2020) for reviews of this area). RNAi relies on the synthesis of highly specific dsRNA against a specific gene in a target organism and subsequent delivery of this dsRNA into target organisms (either as embryos, as larvae or directly into adults). dsRNA can be designed in such a way to target only a single species or strain (in fact, the requirement for very high sequence specificity necessitates that this is often the case), or even particular life-history stages or morphs (because expression of some genes is highly dependent on these aspects, for instance, one gene may be expressed appreciably in adults but not larvae, or in one morph within a plastic phenotype, but not another), by careful targeting of genes (Neumeier & Meister, 2021; Shang et al., 2016). Targeting of genes is also influenced by our understanding of developmental biology, and enhanced by understanding across a wide range of organisms, that is, if we know what genes are important when and where, we can more efficiently select genes for particular purposes (e.g. affecting reproduction but not lifespan). It is preferable

that RNAi based management of pests reduces population sizes without inducing significant mortality (i.e. by affecting reproduction) (Shelby et al., 2020), one reason being that this minimizes the effects on other species (for instance, organisms that predate a pest or have some other relationship with it may also perish if the pest population is exterminated). Thus, target gene selection must be highly considered. Knowledge of developmental biology feeds into the careful selection of target genes for RNAi. Thus, RNAi is an integral tool for studying developmental and evolutionary biology; and developmental and evolutionary biology form the basis for effective and considered use of RNAi as a potential tool in the management of pests.

Aphids are a case where RNAi presents an incredibly powerful tool, being a group possessing several species which are emerging (or emerged, such as *A. pisum*) model organisms (Shigenobu & Yorimoto, 2022; Srinivasan & Brisson, 2012; The International Aphid Genomics Consortium, 2010; Wenger et al., 2020), and possessing several species that are prevalent pests (some of these species are also the ones that we have the best understanding of), responsible for huge (though, difficult to exactly quantify) damage to crops, which has economical implications and threatens food security (Dedryver, Le Ralec & Fabre, 2010). The voracity of aphids as pests is owed mostly to their extreme productivity, which is facilitated in part by their possession of viviparous parthenogenetic development, a reproductive strategy which allows quick reproduction of clonal populations (Dedryver, Le Ralec & Fabre, 2010; Emden & Harrington, 2017). Viviparous parthenogenesis is commonly encompassed by a polyphenism in aphids, whereby switching from parthenogenetic to sexual reproduction occurs (Srinivasan & Brisson, 2012). Viviparous parthenogenetic development itself, and cyclical parthenogenesis (the term for the reproductive switch) are evolutionary novelties which have undoubtedly contributed to the great success of the aphids (Davis, 2012). Aphids, generally speaking, display two polyphenisms, extreme examples of phenotypic plasticity, and thus present useful organisms for investigating the molecular basis of plasticity, along with, notably, plant-pest interactions, viviparity, parthenogenesis and endosymbiosis (Chapter 1.3; (Srinivasan & Brisson, 2012; Shigenobu & Yorimoto, 2022; Shih, Sugio & Simon, 2023)). Thus, development and application of efficient RNAi in aphids has and will continue to benefit multiple areas of interest.

Among insects, RNAi efficiency is highly variable between species and even between strains (and even between tissues or life stages) (Cooper et al., 2019). While in some groups, such as Coleoptera, RNAi sensitivity has been demonstrated to typically be high (though, only a tiny amount of the diversity of the Coleoptera has been assessed) (Baum et al., 2007; Mehlhorn et al., 2020; Willow & Veromann, 2021), in others such as Hemiptera and Lepidoptera (where sensitivity is low except for in embryonic stages), it is far more variable (Christiaens & Smagghe, 2014; Jain et al., 2021; Terenius et al., 2011; Xu et al., 2022a). This is despite general conservation in the (nature of) core RNAi machinery between insect groups; though, core RNAi gene copy number variation between groups has been noted (Dowling et al., 2016). Possessing more copies of core RNAi genes may be linked to higher RNAi efficiency (notably, several Coleoptera groups have two copies of *R2D2*, which encodes a dsRNA binding protein; *T. castaneum* additionally possesses three *sil* genes (encoding proteins involved in dsRNA uptake) and two *Ago2* genes (encoding proteins that associate with siRNA to target mRNA for degradation) (Tomoyasu et al., 2008). Successful (and unsuccessful attempts at) RNAi has been reported in several aphid species, including the pea aphid, by several means of delivery of dsRNA, including spraying (Yan et al., 2020; Linyu et al., 2021), ingestion through artificial diets (Mao & Zeng, 2012; Jacques et al., 2020) or through transgenic plants (host-mediated RNAi) (Dong et al., 2022; Zhang et al., 2023), transgenic symbionts (making use of the presence in aphids of an ancient obligate symbiont, *Buchnera aphidicola*, and a range of other symbionts) or bacteria (reviewed in (Li et al., 2022b)), and injection (Jaubert-Possamai et al., 2007; Mao, Liu & Zeng, 2013; Ye et al., 2019a, 2019b). The range of modes of delivery highlights the scope of the need to develop efficient and useable systems. In laboratory settings, injection into the body cavity is considered the simplest and most direct method of delivery, and it is a commonly deployed method (the other basic method being feeding, which is less preferable due to the instability of dsRNA in the insect digestive tract) (reviewed in (Zhu & Palli, 2020; Jain et al., 2020)), which may allow more systemic spreading of dsRNA to the various tissues of the insect body (Sapountzis et al., 2014).

dsRNA faces a number of barriers that have been suggested to influence the efficiency of RNAi (explored partially in figure 3.1). Increasingly, in insects, the presence of non-specific endonucleases in the body and fluids (primarily in the haemolymph, gut and salivary glands) that degrade dsRNA has been demonstrated. Not only are non-specific endonucleases detected widely, it has also been demonstrated in *Hyphantria cunea* that expression of the genes encoding them (in this case, for two out of the four encoded nucleases) increase in response to dsRNA exposure (Zhang et al., 2022a). These insect endonucleases have been suggested to not be order-specific, and cluster in groups rooted in their nucleic acid substrate, that is, particular non-specific endonuclease orthologues may have different targets. It has been demonstrated that knockdown of non-specific endonucleases can lead to improved silencing of target genes in a range of insect species, including the pea aphid (Chung et al., 2018; Peng et al., 2020; Li et al., 2022a; Zhang et al., 2022a). Furthermore, it has been demonstrated in several insects that by increasing the expression of genes encoding core RNAi machinery enzymes, chiefly *Dicer2*, *Argonaute2* and *R2d2*, the susceptibility to RNAi can be improved (Li et al., 2015; You et al., 2020; Ye et al., 2019a); several methods of upregulating the core RNAi machinery have been demonstrated. You and colleagues over-expressed *Dicer2* (the product of which cleaves long dsRNA into small interference RNAs) and *Argonaute2* (which encodes a protein that digests target mRNA), two core RNAi machinery genes in *Bombyx mori*, a Lepidopteran (which is a group that has been particularly recalcitrant to RNAi (Terenius et al., 2011; Shukla et al., 2016)), leading to enhanced gene silencing; after increasing *Dicer2* expression 46-fold, *EGFP* relative expression was reduced to 15 % compared to for control individuals, after twenty-four hours, and in individuals co-expressing *Dicer2* and *Argonaute2*, relative expression was reduced to 8 % (You et al., 2020). Ye and colleagues, similarly, demonstrated that by pre-exposing pea aphids to generic dsRNA (*gfp*), subsequent exposure to dsRNA against a target gene, *hb*, at a relatively low dose (60 ng, a dose at which, without pre-exposure, no silencing was achieved) led to efficient silencing; pre-exposure was associated with increased expression of *Dicer2*, *Argonaute*, and *R2d2* (Ye et al., 2019a). By injecting *A. pisum* individuals with 60 ng ds*gfp* and then 600 ng ds*hb* seventy-two hours later, Ye and colleagues silenced *hb* by 33 % thirty-six hours post second

injection, and when the first injection was 600 ng ds*gfp*, and the second was 60 ng ds*hb* this increased to 81 % silencing.

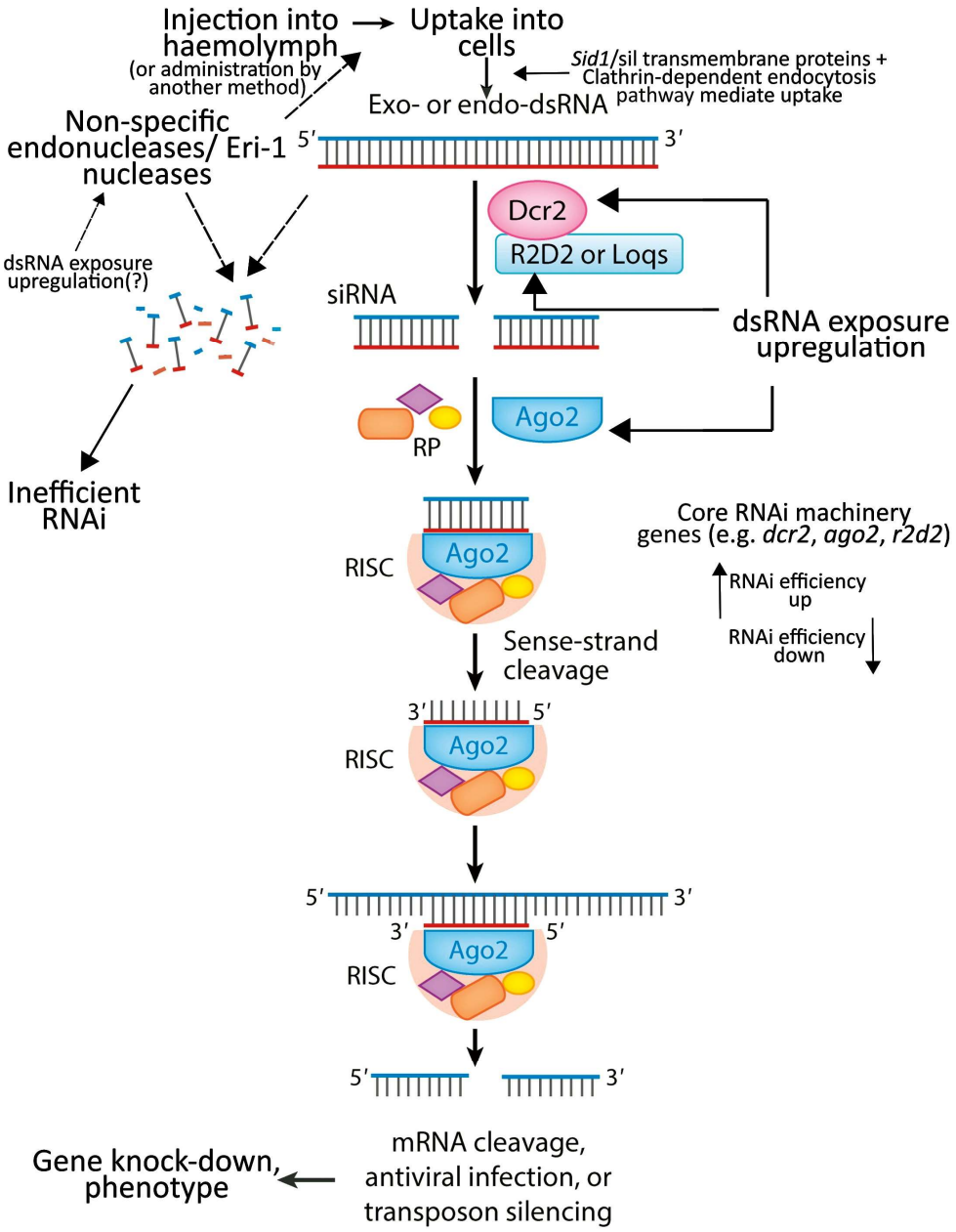


Figure 3.1 Schematic of the siRNA pathway, with known modulators of RNAi efficiency.
Briefly, the siRNA pathway, which is primarily an anti-viral mechanism, operates in insect RNAi first by uptake of long dsRNA. dsRNA can be degraded by non-specific endonucleases before entry into cells, or intracellular nucleases once inside cells, which can lead to poor RNAi efficiency. Active uptake of dsRNA into cells by two mechanisms, Sid1/Sil transmembrane proteins and clathrin-dependent endocytosis can facilitate greater uptake and thereby greater RNAi efficiency. Once inside cells, dsRNA is processed by several components to generate an antisense strand which guides degradation of its complementary mRNA. If efficient, RNAi-mediated gene silencing can be achieved. The expression levels of core RNAi machinery genes (*ago2*, *r2d2*, *dcr2*) can also affect RNAi efficiency, and their expression can be responsive to dsRNA exposure. Adapted from (Zhu & Palli, 2020).

In an attempt to improve the efficiency of pea aphid RNAi in our strain, I used a three-injection RNAi protocol, an RNAi machinery primed double RNAi (RNAi of a primed RNAi). First, injecting aphids with ds*gfp* in the hopes of upregulating core RNAi machinery genes, then injecting with a cocktail of dsRNAs targeting two non-specific endonucleases (that I show to be expressed widely in the aphid body, and targeted based on personal communications with Amol Ghodke and Charles Robin, University of Melbourne) that putatively target dsRNA for degradation, then finally injecting with dsRNA targeting the ultimate gene of interest. I hypothesized that, by exposing aphids to generic dsRNA and subsequently treating with dsRNA against two non-specific endonucleases, their knockdown would be greater than without pre-exposure to generic dsRNA, and that together these would improve the efficacy of RNAi of target genes, leading to knockdown. The aim ultimately being to deploy this optimised RNAi injection regime to research questions explored within this thesis, first and foremost, the functions of *dnmt3a* and *dnmt3x* in the ovary in reproduction and the reproductive switch.

3.2 Methods

3.2.1 Aphid stock

All aphids used in this chapter are viviparae derived from the LD stock population of N116 strain *A. pisum* described in the general methods. Only wingless adults or wingless destined nymphs, where specified, were used. Aphids were reared pre- and post- injection in LD conditions.

3.2.2 Approach to optimising RNAi in *A. pisum*

Based on personal communications with Amol Ghodke and Charles Robin, University of Melbourne, I decided to take two approaches towards improving the efficiency of RNAi in *A. pisum*. The first was attempting to increase the activity of the core RNAi machinery by stimulating upregulation of the genes encoding core RNAi enzymes, non-directly by injecting dsGFP (non-specific dsRNA). The second approach was, before attempting to knock down target genes, knocking down the expression of non-specific endonucleases to reduce the degradation of target gene dsRNA.

3.2.2.1 Identification of *A. pisum* non-specific endonuclease genes

To identify non-specific endonucleases in *A. pisum*, I used the sequences of non-specific endonucleases from similar studies in *B. mori* (Arimatsu et al., 2007) and *B. tabaci* (Luo et al., 2017) as bait for BLAST searches against the *A. pisum* genomic database curated by NCBI. I used an E-value cut-off of e-30 to identify homologs.

I used the identified *A. pisum* homolog sequences, together with *B. mori*, *B. tabaci*, and identified *Schistocerca gregaria* (the sequence which was used to root the tree) sequences to perform a phylogenetic analysis and construct a phylogenetic tree. 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 .fa-gb file was then realigned using Clustal Omega. 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 1 million iterations, mixed models and default priors was run using MrBayes (Huelsenbeck & Ronquist, 2001). The consensus tree was visualised using Figtree (Rambaut, 2010), and edited for clarity using InkScape (Inkscape Project, 2020). I used the clustering structure of the tree to identify which of the *A. pisum* sequences corresponded most closely to which *B. mori*/*B. tabaci* sequences.

3.2.2.2 Qualitative assessment of expression of non-specific endonucleases

To qualitatively assess the presence of expression of nucleases in various *A. pisum* tissues, RNA was extracted from: heads (twenty per sample), ovaries (twenty per sample), guts (twenty per sample), whole bodies (five per sample) and carcasses (the body parts remaining after removal of head, thorax, ovaries and guts, but encompassing the fat body, twenty per sample) of adult viviparous aphids, (derived from the long-day (LD) stock population, detailed in the general methods). cDNA was generated from RNA (as detailed in the general methods, except for in the case of gut samples, where 500 ng RNA was used, otherwise 1 µg template was used), and PCR (using 100 ng cDNA template and HS taq (NeoBiotech) (94 °C 3 mins, 40x [94 °C 30 secs, 60 °C 30 secs, 72 °C 1 min], 72 °C 5 mins) using primers designed as specified in the general methods

(appendix table A.1) was used to amplify target genes. Genomic DNA was isolated from whole adult aphids using a PureLink Genomic DNA mini kit, according to the manufacturer's instructions. PCR products were then run on 1.5 % agarose in SB gels containing 0.5 µg/ml ethidium bromide, in SB buffer (10 mM sodium hydroxide, pH adjusted to 8.5 with boric acid) (Brody & Kern, 2004) at 150 V, alongside 1kb+ ladder (Invitrogen), no-template controls, no-RT controls, genomic DNA samples and positive controls (using *tubulin* primers). Loading dye (Invitrogen) was added to samples in a ratio of 1:5 loading dye:sample. Visualisation and imaging of DNA was achieved through a Syngene InGenius Gel Doc System and the GeneSnap program. Gel images were prepared using InkScape.

3.2.3 RNAi

3.2.3.1 Plasmid preparation

pEasy-T3 cloning kit Trans1-T1 phage resistant competent *Escherichia coli* cells were transformed to introduce LITMUS28i plasmids containing dsRNA template for *eGFP* and *Aphb* respectively, which were previously generated by Elizabeth Duncan. Cells were transformed using the Peasy-T3 Cloning Kit (TransGen Biotech), according to the manufacturer's instructions in SOC (10 µl 1M MgCl2, 10 µl 1M MgSO4, 1000 µl 2M glucose, 8980 µl SOB). After overnight incubation of cells spread on selective plates (150 µl 100 mg/ml ampicillin per 150 ml media), the following morning, plates were placed at 4 °C until the evening. Then, a single colony was picked and grown in 3 ml LB media inoculated with ampicillin (150 µl 100 mg/ml ampicillin per 150 ml media), overnight at 37 °C,220 rpm, in an orbital shaker. Plasmids were isolated using a QIAGEN Plasmid Midi Kit, according to the manufacturer's instructions. Plasmid yield was then assessed using a NanoDrop 2000 Spectrophotometer. Plasmid insert sequences are presented in supplementary table A.2.

3.2.3.2 dsRNA template generation

To generate dsRNA template for *nuc1* and *nuc2*, RNA was extracted from individual viviparous aphids and assessed as laid out in the general methods. cDNA was generated as laid out in the general methods, without the additional dsDNAse step.

To generate template for *in vitro* transcription, I used forward and reverse primers that had T7 promoter sequences appended on to their 5' ends (appendix table A.1) in PCR (Q5 High-Fidelity DNA polymerase (NEB), 100 ng template, 98 °C 1 min, 30x [98 °C 10 secs, 60 °C 30 secs, 72 °C 30 secs], 72 °C 2 mins) to amplify from cDNA generated from RNA from whole viviparous adult aphids, for *nuc1* and *nuc2*, or I used M13 forward and reverse primers to amplify template from isolated plasmids (for *gfp* and *hb*) (Q5 High-Fidelity DNA polymerase (NEB), 1 ng template, 98 °C 1 min, 30x [98 °C 10 secs, 55 °C 30 secs, 72 °C 60 secs], 72 °C 2 mins). PCR products were purified using the Monarch PCR & DNA Cleanup Kit (NEB). Template size was verified by gel electrophoresis (as detailed below, appendix figure A.2). The approximate location of the region that was amplified to produce template dsRNA is visualised within the context of each relevant mRNA in appendix figure A.4.

3.2.3.3 dsRNA synthesis

dsRNA was synthesised from template (described above) using a MEGAScript T7 transcription kit (Invitrogen) according to the manufacturer's instructions, including the turboDNAse step to degrade template. To increase yield of dsRNA, the reaction volumes were doubled. After synthesis, dsRNA was purified and concentrated using an RNeasy Mini-Kit (Qiagen), according to the manufacturer's instructions. dsRNA was eluted in 15 µl nuclease-free water, then the 15 µl elutant was used to re-elute from the column a second time, and then a 1/10 dilution was quantified and its quality assessed using a NanoDrop 2000 spectrophotometer. dsRNA was further assessed by running a sample of 1/10 diluted dsRNA on a 1 % agarose in SB gel containing 0.5 µg/ml ethidium bromide in SB buffer at 150 V, with 1kb+ ladder (DNA rather than RNA ladder) (appendix figure A.3). dsRNA gels were run briefly (for less than fifteen minutes) to minimise RNA degradation. 1 µl aliquots of dsRNA were stored at – 80 C.

3.2.3.4 RNAi injection regimes

dsRNA in nuclease-free water, or nuclease-free water alone was injected into vivipara aphids at various stages of development, with the point of needle insertion being either directed posteriorly in the mid dorsal abdomen or the upper abdomen, or directed posteriorly, medially and laterally between the mid and hind legs. Injection needles were either prepared from glass capillary tubes

(1.14 mm, 3.5”, WPI), pulled using a Narishige PD-5 micropipette puller (Narishige) set to ‘heater’ = 9, ‘magnet’ = 9, ‘main magnet’ = 9, with or without manual sharpening against the edge of a coverslip, and with or without subsequent needle sharpening using a Narishige EG-45 microgrinder (Narishige), or using pre-prepared needles (TIP10XV119, WPI). Needles were backfilled with mineral oil (M3516, Sigma-Aldrich) and attached to a Nanolitre 2000 microinjector (WPI), then front filled with either dsRNA in nuclease-free water or nuclease-free water alone.

To inject, aphids collected from the stock LD population were immobilised on 2 % agar plates in which a small gap was made with a razor blade (figure 3.2, a). Aphids were gently placed into the gap by manipulating them with a paintbrush, while using forceps to gently part the two sides of agar. Two approaches were used for immobilizing aphids during the optimisation process. The first was to place aphids upright into the agar, so that most of their abdomen was embedded, exposing the upper abdomen, thorax and head. The second approach was to place the aphids laterally into the agar, with about a fifth of the aphid’s width not below the surface of the agar, so that the lateral surface was exposed. Aphids were injected with either 46 nl or 120 nl injectant, under a dissecting microscope (GXMXTL3T), using an MM33 micromanipulator (Drummond Scientific) to manipulate the microinjector which was set to ‘slow’. After injecting, aphids were transferred to insect rearing bags (15 x 8 cm, BugDorm Store) or agar-leaf plates (described in the general methods) at low densities (between one and ten individuals), and placed into a humidity controlled incubator set to 16L:8D, 20 °C,50 % or 70 % humidity. Aphids that were sampled for downstream expression quantification were sampled thirty-six hours after injecting. Where aphids were injected multiple times, they were injected thirty-six hours after the previous injection (figure 3.2, b). Thirty-six hours was taken as the interval for sampling and injecting based on observations by Ye and colleagues that showed maximal *hb* knockdown at this time (Ye et al., 2019b).

nuc1 and *nuc2* RNAi efficiency was examined by RT-qPCR of aphids injected once with ds*gfp* (150 ng, as L3 nymphs), then a second time with a cocktail containing ds*nuc1*+ds*nuc2* (100 ng of each) to equal aphids injected twice with

ds*gfp* (equal amounts). Five ds*gfp* and four ds*nuc1*+ds*nuc2* were sampled and processed (as described above) for RT-qPCR.

hb silencing was examined by RT-qPCR of aphids injected once with ds*gfp* (150 ng, as L3 nymphs), then a second time with a cocktail containing ds*nuc1*+ds*nuc2* (100 ng of each), and finally with ds*hb* (150 ng) and equal aphids injected once with ds*gfp* (150 ng, as L3 nymphs), then a second time with a cocktail containing ds*nuc1*+ds*nuc2* (100 ng of each), then with ds*gfp* (150 ng) for the third injection. Three aphids injected for the third time with *dsgfp* and five aphids injected for the third time with *dshb* were sampled and processed (as described above) for RT-qPCR.

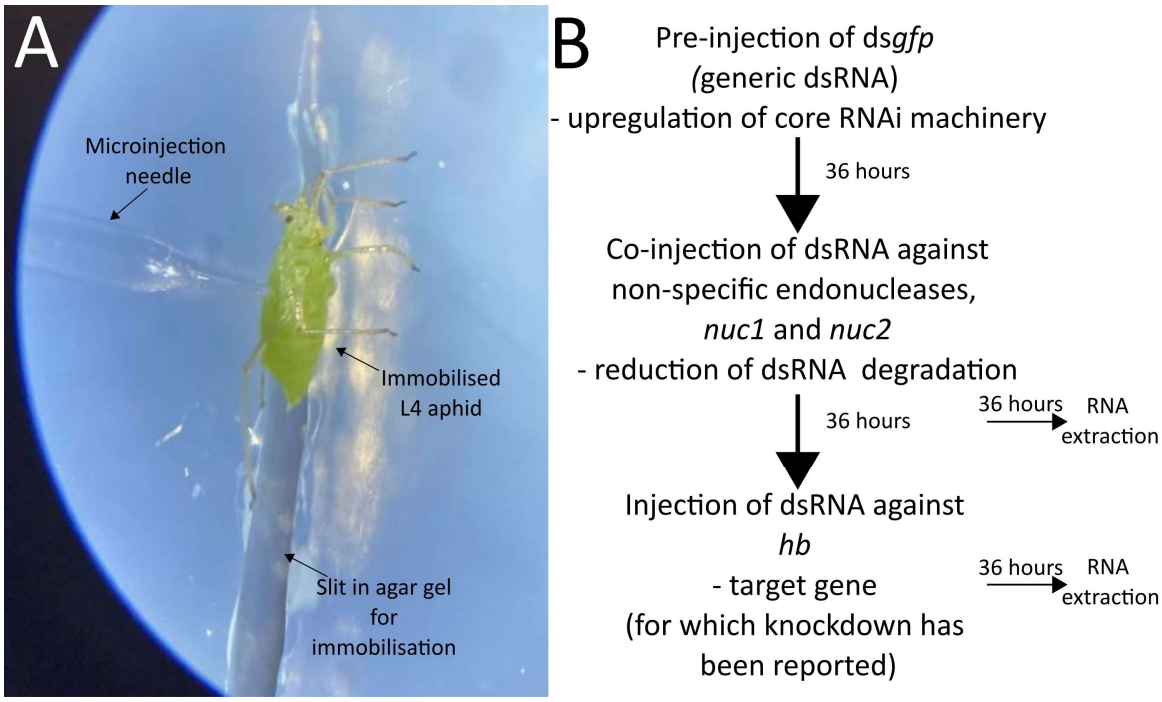


Figure 3.2 Optimised RNAi injection regime to assess efficiency.
A viviparous parthenogenetic Acyrthosiphon pisum L4 immobilised in a slit in a 2 % agarose gel for administration of solution (water or dsRNA in water) via a microinjection needle, which is directed in this case, toward lateral insertion between the mid and hind legs (A). Three injection protocol for A. pisum, combining pre-injection with generic dsgfp to elicit an increase in activation of the RNAi machinery, injection of dsnuc1 and dsnuc2 (two non-specific endonucleases), to reduce degradation of dsRNA within the aphid body, concurrently, and finally injection of dshb, a gap gene with a well understood function that has been targeted and reported to be silenced in several studies in A. pisum, with all injections separated by thirty-six hours (an amount of time at which it has been shown that hb silencing is maximal), with RNA samples taken thirty-six hours after the second and third injections for downstream expression analysis (B).

3.2.3.5 Sample preparation and RT-qPCR

dsRNA injected aphids were collected individually and frozen at -80 °C, thirty-six hours post terminal injection (the nature of which depended on the particular injection regime each aphid was exposed to). RNA was extracted as specified in the general methods, using an on-column DNAse step, from individual whole aphids for assessment of expression of nucs, or from ovaries for assessment of *hb* expression. cDNA was generated as specified in the general methods. Amplification for each set of RT-qPCR primers was confirmed by PCR (98 °C 1 min, 30x [98 °C 10 secs, 60 °C 30 secs, 72 °C 30 secs], 72 °C 2 mins) and 1.5 % agarose gel electrophoresis in SB buffer (as specified above) (appendix table A.1).

qPCR reactions were set-up and run as laid out in the general methods, using 5 ng cDNA. *tub* was used as a reference gene. NoRT and NT controls were run, and primers were designed and their efficiencies were validated (appendix table A.1) as specified in the general methods. Melt curves were checked for specificity of amplification. Approximate locations of qPCR primer targeting within the context of each mRNA are visualised in appendix figure A.4; primers used for qPCR and IVT template generation were non-overlapping.

3.2.4 Data analysis

RT-qPCR data was analysed by the Pfaffl method (Pfaffl, 2001), taking into account the primer efficiencies for each gene, and taking the control group averages (geometric mean) as reference. Statistical analysis was performed by running GLMs (either with an inverse Gaussian distribution and $1/mu^2$ link function (*nuc1*) or a gamma distribution with inverse link function (*hb*, *nuc2*)) performed using base R, after data exploration and assessment using the *fitdistrplus* package, *ggplot2* package and base R, followed by likelihood ratio tests comparing full and null models (*lmtest* package). Boxplots were built using ggplot2. All figures were assembled using InkScape.

3.3 Results

3.3.1 Approaches to optimise *A. pisum RNAi*

3.3.1.1 Non-specific endonucleases in *A. pisum*

By using *B. mori* and *B. tabaci* non-specific endonuclease protein sequences from similar studies as bait, I identified three potential orthologues in *A. pisum*. A phylogenetic tree of nucs is presented in figure 3.3. The first, XP_003242652.1, is consistent with *nuc1* identified by Chung and colleagues (Chung et al., 2018), and so from herein I refer to it as *nuc1*. *nuc1* appears to possess only a single transcript variant, and a NUC superfamily domain encompassing an active site, Mg^{2+} binding site and substrate binding site, predicted by PFAM. It branches closely to *B. tabaci dsRNAse1*. Another identified orthologue, XP_029342033.1, branched most closely with Nuc1 and appeared to possess the same domains and a single transcript variant. I termed the gene encoding this protein *nuc3*. This protein corresponds to ACYPI30083 in the study by Chung and colleagues. The long branch length of Nuc3 may suggest divergence from Nuc1, though it may be that, given it has been noted that branching patterns of nucs from various species may relate to substrate (Zhang et al., 2022a), and that Nuc1 and Nuc3 branch together, divergence may be in spatial or temporal expression. A third orthologue, which appears to have four isoforms was identified, also appearing to possess the full NUC domain, though its corresponding proteins branched away from the two other *A. pisum* proteins, the *B. mori* protein, and one of the *B. tabaci* proteins. However, these isoforms branched most closely with a second *B. tabaci* protein. I termed this protein, given its grouping with what has been termed *dsRNAse2* in *B. tabaci*, *nuc2*. Because it has been noted that nucleases may cluster based on substrate specificity (while non-specific endonucleases hydrolyse nucleic acids without specificity, they do possess some sequence preference (Wang et al., 2007)), I decided to pursue all three *nuc*s as potential targets for RNAi knockdown.

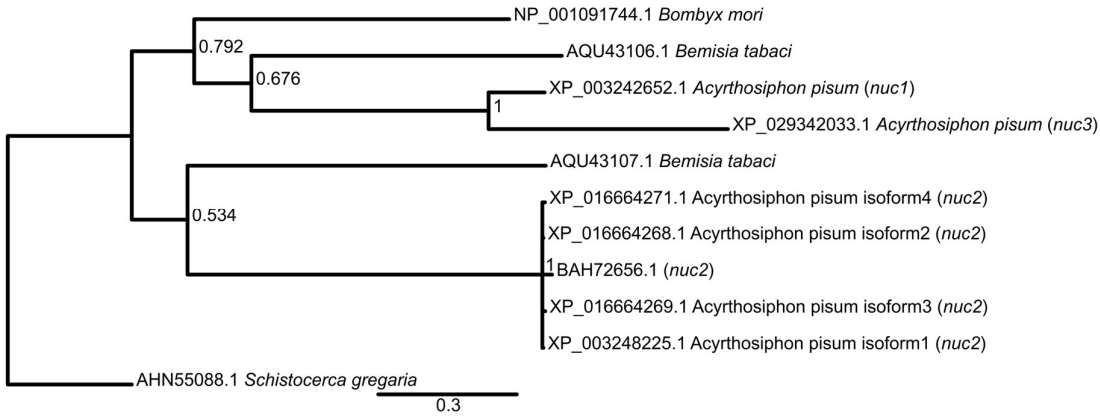


Figure 3.3 Consensus tree of non-specific endonuclease protein orthologue sequences from *A. pisum*, *B. tabaci* (which along with *A. pisum*, belongs to the hemiptera), *B. mori* (a lepidopteran, which, similarly to *A. pisum* has been reported to be relatively recalcitrant to RNAi), and *S. gregaria* (the sequence for which is used to root the tree).
BAH72656.1 (the middle *nuc2* branch) is presumed to originate from the same locus as each of the four *nuc2* isoforms due to sequence similarity and branch pattern. Scale bar corresponds to substitutions per site.

3.3.1.2 *nuc1* and *nuc2* show widespread expression across the tissues of viviparae N116 *A. pisum* adults

Having identified three potential non-specific endonucleases, I sought to assess expression across various tissues in adult viviparae by qualitative RT-PCR. While both *nuc1* and *nuc2* (based on primers targeting regions conserved between all four *nuc2* isoforms) expression signal was detected in gut, head, ovary and fat body (abdomen with gut and ovaries removed) samples (all tissue types assessed), *nuc3* did not appear to be appreciably expressed in any individual tissue or whole body samples (figure 3.4, appendix figure A.1). On the basis of this, and the limit on dsRNA injection volume imposed by the size and survivability of aphids, I synthesised dsRNA against *nuc1* and *nuc2*.

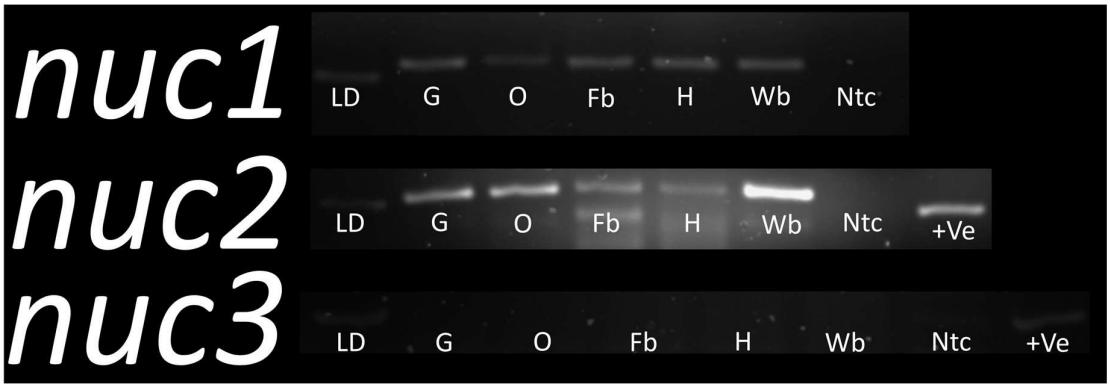


Figure 3.4 Tissue specificity assay for each of three non-specific endonucleases identified in *Acyrthosiphon pisum*.
nuc1 is XP_003242652.1, nuc2 encompasses four isoforms (XP_003248225.1, XP_016664268.1, XP_016664269.1 and XP_016664271.1) and nuc3 is XP_029342033.1. Each nuc possesses typical non-specific DNA/RNA endonuclease domains (active site, substrate binding site and Mg2+ binding site) and therefore may be capable of degrading dsRNA. Ladder, LD, gut, G, ovaries, O, fat body, Fb, head, H, whole body, Wb, no-template control, Ntc, tubulin positive control band, +Ve. LD band is 100 bp. Expression of both nuc1 and nuc2 expression were both apparent across all tissues, while nuc3 expression appeared absent in all cases.

3.3.1.3 Injection optimisation and GFP pre-injection

Before injecting dsRNA against *nuc1* and *nuc2* in hopes of knocking down their expression, I optimised the injection protocol to maximise survival from injection generally and from a pre-RNAi GFP injection, in hopes of upregulating the RNAi machinery. I hypothesized that by using a 'primed RNAi of RNAi' dsRNA injection protocol, priming aphids for efficient RNAi by upregulating the RNAi

machinery, then downregulating the expression of nucs, and finally injecting dsRNA against targets, RNAi efficiency would be improved.

A summary of iterative attempts at optimising the RNAi injection protocol is presented in table 3.1. Generally, survival after injection (which was mostly comparable between water, ds*gfp* and dsRNA against target treatments) was variable between attempts, but improved with various approaches toward injections and aphid maintenance post- and pre- injection (a representative set of attempts under various approaches are presented in table 3.1). The most successful approach toward optimising injection survival was to use a pre-pulled needle (WPI) to inject aphids ventrally between the mid and hind leg after thorough sterilisation of tools and equipment with 70 % ethanol, then rearing aphids at low density on leaf-agar plates. Thus, these parameters were used for RNAi.

Table 3.1 Iterative approaches to improving survivability of microinjection of asexual *A. pisum* individuals,
encompassing various injection conditions, injection volumes and injectant identities ('injection'), consensus age at injection ('stage at injection'), and number of injections separated by thirty-six hours (i.e. 1 = entry based on aphids that were injected once, 2 = entry based on aphids that were injected once and then injected a second time thirty-six hours after, values being associated with the second injection, 3 = entry based on aphids that were injected once, followed up with a second injection thirty-six hours later, and then a third injection after a further thirty-six hours, values being associated with the third injection; sequential entries with progressive numbers are typically associated) ('injection number'). n represents the number of individuals that were alive while assessing survivability immediately after the final aphid in an entry was injected (occasionally, aphids died from or immediately following injection (or appeared to be dying) and were therefore, not counted), % survival corresponds to the % of n alive thirty-six hours after injection. Injection conditions identifiers: 1, injecting dorsally into abdomen, 2, injecting ventrally between mid and hind leg, a, leaf agar plate, b, needle sharpened under coverslip, c, individuals maintained (post-injection) in insect rearing bags, d, injected using pre-pulled needle, e, needle filed down with fine forceps to increase sharpness, f, needle sharpened using needle microgrinder, g, humidity reduced from 70 to 50 %, h, individuals maintained (post injection) at low density (less than three individuals per leaf-agar plate), i, large (for stage) aphids injected, j, increased sterility (70 % ethanol used to sterilise tools). Injections were performed using the 'slow' setting of a Nanolitre 2000 microinjector.

Injection Conditions	Injection	Stage at injection	n	% survival	Injection number
a,b,1	46nl H2O	L3	35	31.4	1
b,c,1	46nl H2O	L3	17	5.9	1
			5	0	1
a,d,1	46nl H2O	L3	33	27	1
			45	40	1
a,d,2	46nl H2O	L3	23	30.4	1
a,b,e,2	46nl H2O	L3	43	69.8	1
	46nl H2O	L3	20	50	1
	46nl H2O	L4	10	40	2
	46nl H2O	Adult	4	0	3
a,d,2	125ng ds*gfp* in 46 nl	L3	20	0	1
		Adult	10	0	1
		L3	16	0	1
		Adult	20	0	1
a,b,f,g,h,2	125ng ds*gfp* in 46 nl	L3	42	85.7	1
	125ng ds*gfp* in 46 nl	L3	20	20	1
	50 ng ds*nuc1* + 50 ng ds*nuc2* in 46 nl, or 100 ng ds*gfp* in 46 nl	L4	21	4	2
a,b,f,g,h,j,2	125ng ds*gfp* in 46 nl	L3	31	45.2	1
	125ng ds*gfp* in 46 nl	L3	20	0	1
	50 ng ds*nuc1* + 50 ng ds*nuc2* in 46 nl, or 100 ng ds*gfp* in 46 nl	L4	14	0	2

Injection Conditions	Injection	Stage at injection	n	% survival	Injection number
a,d,g,h,j,2	150 ng ds*gfp* in 46 nl	L3	13	92.3	1
		Adult	15	86.7	1
		L3	30	53.3	1
	100 ng ds*nuc1* + 100 ng ds*nuc2* in 46 nl, or 200 ng ds*gfp* in 46 nl	L4	11	81.8	2
		L4	16	NA	2
	150 ng ds*gfp* in 46 nl	L3	42	30.96	1
		L3	52	28.8	1
		L3	31	45.2	1
	100 ng ds*nuc1* + 100 ng ds*nuc2* in 46 nl, or 200 ng ds*gfp* in 46 nl	L4	12	NA	2
		L4	14	78.6	2
a,d,g,h,j,2	150 ng dsgfp in 46 nl	L3	11	72.7	1
	100 ng ds*nuc1* + 100 ng ds*nuc2* in 46 nl, or 200 ng ds*gfp* in 46 nl	L4	8	54.5	2
	150 ng ds*hb* in 46 nl, or 150 ng ds*gfp* in 46 nl	Adult	6	33.3	3
a,d,g,h,j,2	150 ng ds*gfp* in 46 nl	L3	46	60.9	1
	100 ng ds*nuc1* + 100 ng ds*nuc2* in 46 nl	L4	20	60	2
	150 ng ds*hb* in 46 nl, or 150 ng ds*gfp* in 46 nl	Adult	12	75	3
a,d,g,h,j,2	150 ng ds*gfp* in 46 nl	L3	65	67.7	1
	100 ng ds*nuc1* + 100 ng ds*nuc2* in 46 nl	L4	30	76.7	2
	200 ng dsgfp in 46 nl	L4	11	36.4	2
	150 ng ds*hb* in 46 nl	Adult	12	58.3	3
	150 ng dsgfp in 46 nl	Adult	3	100	3
a,d,g,h,j,2	120nl H2O	L3	19	42.1	1
	300 ng ds*gfp* in 120 nl	L3	19	21.1	1
a,d,g,h,j,i,2	120 nl H2O	L3	12	83.3	1
		L4	10	80	2
		Adult	8	87.5	3
a,d,g,h,j,2	300 ng ds*gfp* in 120 nl	L3	22	50	1
	180 ng ds*nuc1* + 180 ng ds*nuc2* in 120 nl, or 360 ng dsgfp in 120 nl	L4	11	72.7	2

3.3.2 Knockdown of *nuc1* and *nuc2*

Expression of *nuc1* and *nuc2* after pre-injecting ds*gfp* and then injecting ds*nuc1*+ds*nuc2*, or pre-injecting ds*gfp* and then injecting ds*gfp* was assessed by RT-qPCR. There were no significant differences in either *nuc1* (GLM, LRT (df = 1), $\chi^2 = 0.5828$, p = 0.4452, figure 3.5) or *nuc2* (GLM, LRT (df = 1), $\chi^2 = 1.2284$, p = 0.2677, figure 3.6) expression levels between conditions, indicating no knockdown.

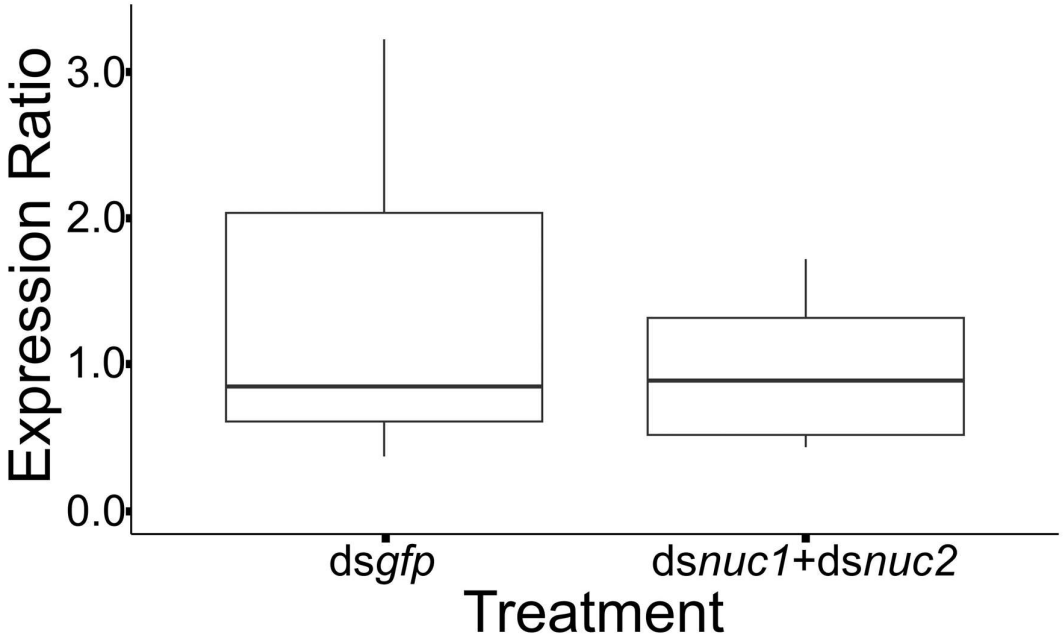


Figure 3.5 Relative expression of *nuc1* after RNAi.
Relative expression of *nuc1* in parthenogenetic viviparous *A. pisum* individuals injected 36 hours prior to freezing for quantification, with either ds*gfp* (non-specific dsRNA) or ds*nuc1*+ds*nuc2* (dsRNA against two non-specific endonucleases, which putatively degrade dsRNA) injection, after injection in both cases of ds*gfp* (in the hopes of upregulating the core RNAi machinery to improve silencing), 36 hours prior. No statistical significance was detected by GLM.

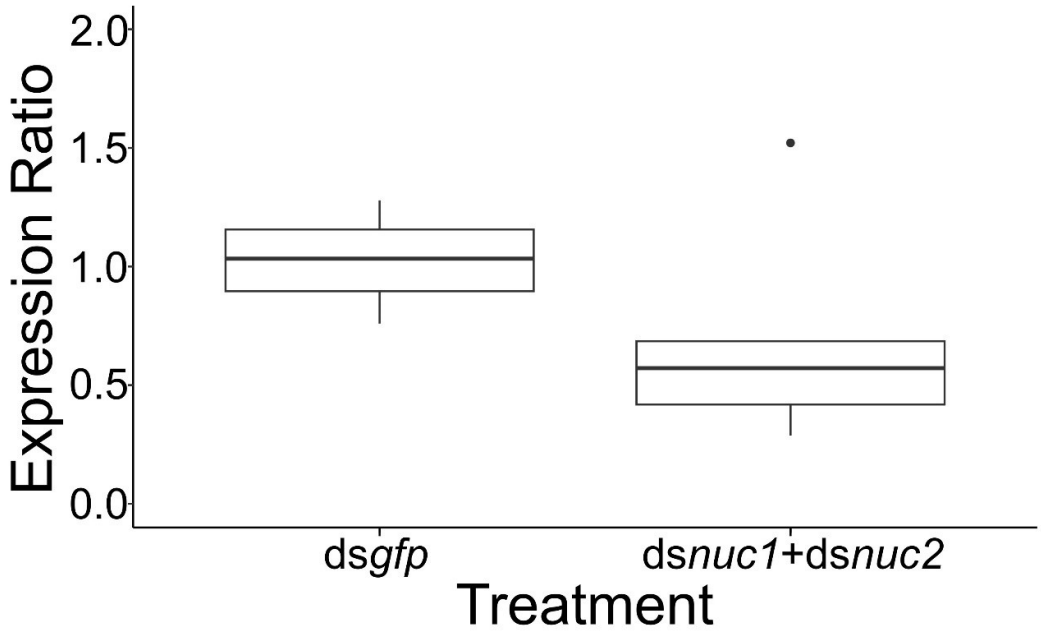


Figure 3.6 Relative expression of *nuc2* after RNAi
Relative expression of *nuc2* in parthenogenetic viviparous *A. pisum* individuals injected 36 hours prior to freezing for quantification, with either ds*gfp* (non-specific dsRNA) or ds*nuc1*+ds*nuc2* (dsRNA against two non-specific endonucleases, which putatively degrade dsRNA) injection, after injection in both cases of ds*gfp* (in the hopes of upregulating the core RNAi machinery to improve silencing), 36 hours prior. No statistical significance was detected by GLM.

3.3.3 **Knockdown of *hb***

I selected *hb*, a conserved and key regulator of insect anteroposterior patterning (Schröder, 2003; Liu & Kaufman, 2004; Wilson & Dearden, 2011), as the ultimate target of RNAi during optimisation, because the role of *hb* in development is well understood and distinct phenotypes would be expected from its knockdown, constituting loss of segments or thorax/gnathal segments taking on abdominal identities in embryos, as in *T. castaneum* (Marques-Souza, Aranda & Tautz, 2008) and *O. fasciatus* (Liu & Kaufman, 2004), and/or enhanced mortality. Modest RNAi knockdown of *hb* has been demonstrated in *A. pisum* through feeding (Mao & Zeng, 2012) and injection (Ye et al., 2019a) of dsRNA. *hb* was thus used as the terminal target in a primed double RNAi protocol, to assess possible improved efficiency (though no statistically significant effect from ds*nuc1*+ds*nuc2* injection was detected, a small decrease in *nuc1*/*nuc2* expression, even if undetected, may have had biological relevance

for *hb* knockdown). Aphids injected with ds*gfp*, then ds*nuc1*+ds*nuc2*, and then ds*hb* showed equal expression of *hb* compared to aphids injected with ds*gfp*, then ds*nuc1*+ds*nuc2*, then ds*gfp* (GLM, LRT (df=1), $\chi^2 = 0.2091$, $p = 0.6475$, figure 3.7). In the present protocol, therefore, injection of ds*hb* did not efficiently silence *hb*. Additionally, injection of dsRNA against *nuc1* and *nuc2*, following pre-injection with ds*gfp* was therefore not able to elicit efficient RNAi of *hb* in this case.

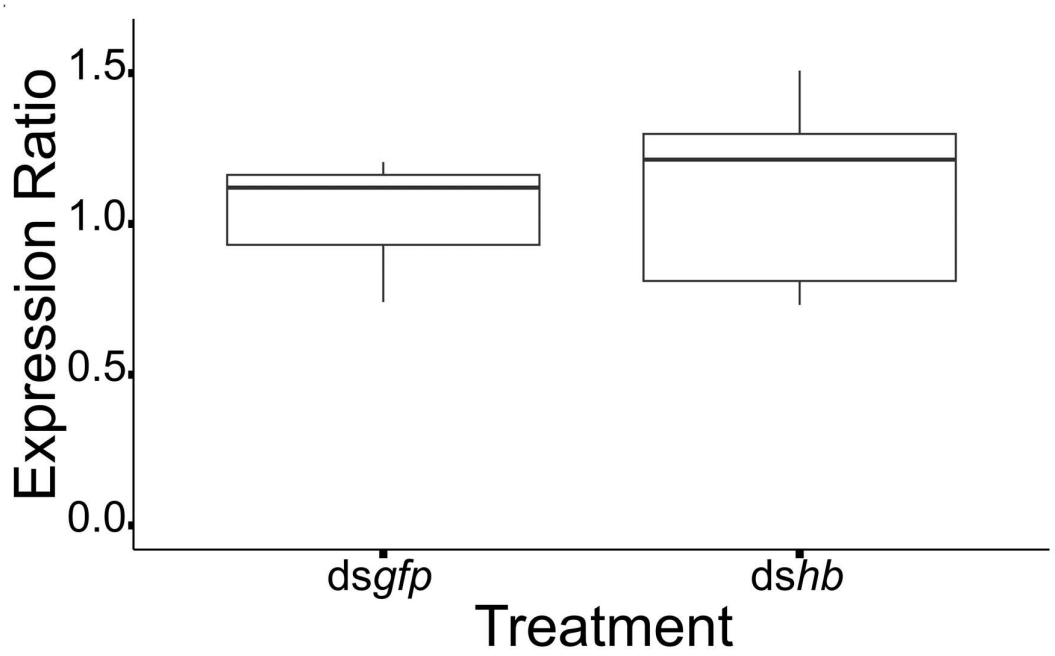


Figure 3.7 Relative expression of *hb* after RNAi
Relative expression of *hb* in the ovaries of parthenogenetic viviparous *A. pisum* individuals injected 36 hours prior to freezing for quantification, with either ds*gfp* (non-specific dsRNA) or ds*hb* (dsRNA against *hb*, a gap gene that is involved in anteroposterior patterning in aphid embryos) injection, after injection in both cases of ds*gfp* and then ds*nuc1*+ds*nuc2* (two genes encoding non-specific endonucleases that are expressed widely in the tissues of adult *A. pisum* virginoparae and have been demonstrated to reduce RNAi efficiency), each separated by 36 hours. No significance was detected by GLM.

3.4 Discussion

Under the parameters presented here, sequential injection of ds*gfp*, dsRNA against *nuc1*+*nuc2*, then dsRNA against a target, does not appear to be a reliable approach to RNAi in the presently used strain of aphids. Here, I demonstrated that while three non-specific endonucleases are encoded in the genome of the pea aphid, only two were expressed at detectable levels in vivipara adults. By attempting to optimise RNAi in pea aphids of the N116 strain by combining two approaches that have, independently of one another, previously been demonstrated to improve RNAi mediated silencing, but not being able to enhance silencing of a target gene, *hb* (a target for which RNAi mediated silencing in *A. pisum* has previously been reported), I demonstrate poor efficiency of RNAi in this strain using the presented protocol.

Detection of mRNA of two non-specific endonucleases, *nuc1* and *nuc2* widely across tissues and at broadly (though, not assessed quantitatively) comparable levels in adult pea aphids, differs slightly from what has been previously demonstrated. Chung et al. (Chung et al., 2018), in the pea aphid, explored expression of *nuc1*, and while, in agreement with the data presented here, they reported that, though it was primarily expressed in the gut, it showed a lower level of expression elsewhere, they also noted that *nuc2* was not expressed at appreciable levels, on the basis of publically available transcript abundance data (Jing et al., 2015), and so did not pursue *nuc2*. While the assessment of expression here was based on qualitative rather than quantitative or semi-quantitative RT-PCR, and it is therefore not possible to comment confidently on expression level, that *nuc1* and *nuc2* are qualitatively similar is suggestive of comparable expression levels. *nuc2* expression in the presently used population of aphids may therefore represent an artefact of the method used (Chung and colleagues note that in the data of Jing et al. (Jing et al., 2015), *nuc2* is not appreciable expressed, though this is based on single replicates of pooled three-day old pea aphids (Harris clone), derived from whole-body and gut RNA, whereas the results here are based on adult aphids and many PCR cycles), or it could be real biological variation (as I will discuss below). Regardless, the apparent expression of both *nuc1* and *nuc2* was the rationale for targeting both *nuc1* and *nuc2*. This expression pattern likely corresponds, partially, also to enrichment in the haemolymph as observed or suggested (by degradation) of

nucleases in several insect species, including aphids (Christiaens, Swevers & Smagghe, 2014; Peng et al., 2020; Zhang et al., 2022a). Though, importantly, while I did not detect *nuc3* expression, only adult aphids under one condition were investigated. Feasibly, *nuc3* might be upregulated in response to exposure to dsRNA, but its relative expression was not assayed. Similarly, the first of three injections occurred in L3 nymphs, and it is feasible that *nuc3* is expressed at pre-adult stages. Importantly, mRNA and protein will necessarily be separated temporally, to some degree, and so the apparent lack of expression of *nuc3* in adults may not correspond to a lack of Nuc3 protein in adults (Liu, Beyer & Aebersold, 2016). Thus, future studies should further assess under what conditions (if any) *nuc3*, which branched closely with *nuc1*, is expressed, along with possible divergence from *nuc1* and its possible function. Similarly, while pre-injection of ds*gfp* has been demonstrated previously to upregulate core RNAi machinery components, and subsequently improve RNAi efficiency (Ye et al., 2019a), it may also cause an upregulation of other response elements, such as non-specific endonucleases (as was demonstrated for two nucs in the lepidopteran, *H. cunea* (Zhang et al., 2022a)), including *nuc1* and *nuc2* investigated here, and other nucleases that may reduce RNAi efficiency. Intracellular nucleases, like Eri-1/3'Exo nuclease (Christiaens, Swevers & Smagghe, 2014) may also play a role in pea aphid RNAi response, and while one study reported no upregulation of pea aphid *eri-1* in response to dsRNA exposure (Christiaens, Swevers & Smagghe, 2014), it may be generally expressed at a high level in some cases and also, therefore, reduce RNAi effectiveness. In Lepidoptera, a group for which, similarly to aphids, insensitivity to RNAi has been frequently reported, an order specific gene encoding an RNAi efficiency-related nuclease (REase) has been identified, and similarly, upregulation of this gene has been reported in response to dsRNA exposure (Guan et al., 2018). A similar phenomena may be occurring in the pea aphid, involving the non-specific endonucleases pursued here, other nucleases, or other elements of the RNAi machinery. Future studies that characterise and assess any roles of additional nucleases that may degrade dsRNA in aphids may lead to further improvements in RNAi efficiency.

Another approach to optimisation of RNAi may involve enhancement of endocytosis mediated dsRNA uptake by cells. It has been demonstrated in *A.*

pisum that key genes involved in the clathrin-dependent endocytosis (CDE) pathway (one of the two systems involved in invertebrate dsRNA uptake by cells, the other being systemic RNA interference deficient 1 transmembrane channel proteins (SID-1) or like-proteins (Sil), which is a faster uptake pathway) are important factors in RNAi susceptibility (Ye et al., 2021). The importance of dsRNA uptake to efficient RNAi has been demonstrated in several insects (Wynant et al., 2014; Xiao et al., 2015; Pinheiro et al., 2018). While CDE has been investigated in *A. pisum*, the role of SID-1/Sil has not, partially because SID-1 is not present in Dipterans (where RNAi is possible) or several other insects, in addition to being present but not appearing to be important to RNAi efficiency in other species (Tomoyasu et al., 2008; Ye et al., 2021). Thus, the potential contribution of SID-1/Sil to RNAi efficiency in aphids (which have been shown, in the cases of *A. pisum*, *Aphis glycines* and *Aphis gossypii*, to possess *sid-1* genes (Bansal & Michel, 2013)) is unresolved. Investigating any involvement of SID-1 will make more complete our understanding of factors influencing RNAi inefficiency in aphids, and possibly present better approaches to improving efficiency. Protective vectors, for instance, proteins, lipids and polymer nanoparticles that encapsulate/bind to otherwise naked RNA, such as those explored by Pugsley and colleagues (Pugsley et al., 2023) can improve uptake (in addition to reducing degradation), and may therefore present an option for improving efficiency (Taning et al., 2016; Gillet et al., 2017; Christiaens et al., 2018) – though the investment required in their synthesis/verification may make them difficult to apply.

One barrier faced in the present study was the difficulty in establishing efficient delivery of dsRNA and getting consistently low mortality, through injection, which in pea aphids (small and soft-bodied) is time consuming and difficult to perform. Especially given the three injection protocol, which placed a relatively high amount of stress on the aphids. Topical application (which has been demonstrated to be an effective RNAi approach in *A. pisum* by Niu and colleagues for *hb* (Niu et al., 2019); and other targets in another aphid species, *D. citri* by Killiny et al. (Killiny et al., 2014), these studies demonstrate that efficient delivery is possible without carriers, as explored above) may present a simpler approach to dsRNA delivery. Niu and colleagues saw significant knockdown of *hb* with as little as 60 ng of ds*hb* applied topically in *A. pisum*,

and Killiny et al. saw knockdown of five CYP4 genes from topical application of ds*cytochrome-P$_{450}$* at amounts as little as 10 ng, with downregulation as extreme as > 80 % at 20 ng (though, in *Diaphorina citri*, another aphid species). Nanocarriers, such as those designed and explored by Shen and colleagues (Zheng et al., 2019; Yan et al., 2020; Zhang et al., 2022b) have been demonstrated to improve delivery in a range of aphid species (Linyu et al., 2021; Guo et al., 2022a), including in a greenhouse trial administering dsRNA through spraying (Ma et al., 2023); though, in the greenhouse aspect of this study, they used only mortality as a readout for efficiency and did not use a non-specific (e.g. ds*gfp*) dsRNA control. While nanocarriers present an enticing avenue for improving the efficiency of RNAi (both protecting the dsRNA and improving its uptake), the expertise, resources and cost associated with their production meant they were not used in the work presented here (though, (relatively) simpler and cost reduced methods may increasingly be developed (Li et al., 2019a).

The results here suggesting poor RNAi efficiency despite attempts at optimisation are consistent with some studies in pea aphids, but contrary to others. For example, Ye and colleagues used a similar approach (pre-injection and ds*hb*, though a substantially higher amount of ds*gfp* was injected (600 ng)) and saw a silencing effect (Ye et al., 2019a), and Mao and Zeng, who fed ds*hb* similarly reported reduced expression of *hb* (Mao & Zeng, 2012). Though, interestingly, neither of these studies demonstrated phenotypes in the subsequent generation, thus whether the observed silencing effect translates to realised biologically relevant effects (as would be necessary for application to commercial RNAi) is unclear. Mao and Zeng reported increased mortality in the focal aphids, but given the nature of *hb* as a gap gene involved in anteroposterior patterning, a mis-pattering phenotype in the offspring of the focal *dshb* aphids would be more convincing of effective knockdown. Mortality is often the reported phenotype in response to RNAi, though it has been commented that this is a particularly vague phenotype that may arise from several factors, and more specific phenotypes (as I would expect to arise from significant silencing of *hb*) are preferable when assessing RNAi effectiveness (Mehlhorn et al., 2021). Sapountzis et al. demonstrated specific phenotypes in response to RNAi of *cathepsin-L* (Sapountzis et al., 2014). Niu et al. reported

efficient silencing of *hb* by topical application of just 60 ng dsRNA (Niu et al., 2019). Conversely, Christaens, Swevers and Smagghe (Christiaens, Swevers & Smagghe, 2014) did not detect upregulation of *DcR2*, *ago2* or *r2d2* after injection of dsRNA (though, they used a relatively low dose of 50 ng). The *hb* sequence used here as template for dsRNA synthesis was partially overlapping the *hb* sequence used by Ye and colleagues (Ye et al., 2019a), for which they were able to demonstrate efficient knockdown. While their fragment spanned, in XM_008184541.3, nucleotides 1383 to 1811 and was therefore 428 bp, our fragment spanned nucleotides 834 to 1913 and was 1070 bp. Both sizes are within the range of lengths commonly used in RNAi and shown to be able to elicit silencing, thus, taken together with the overlap shared between our and their sequences, the discrepancy in RNAi efficiency is likely not due to design of dsRNA fragments. Additionally, the *nuc1* dsRNA fragment was 328 nt long and is identical to the fragment against *nuc1* used by Chung and colleagues (Chung et al., 2018), for which they demonstrated significant knockdown (though, through feeding). The *nuc2* dsRNA fragment was similarly, 330 nt. Thus, all dsRNA fragments designed against targets were appropriate sizes, and would be expected to be able to elicit a silencing response.

Recently, Elston et al. targeted *nuc1* in addition to *C002* (another gene for which successful RNAi has been reported in the pea aphid) in *A. pisum* and found, similarly to the results here, no improvement in silencing from *nuc* knockdown (Elston et al., 2023). While the method used here was injection, Elston et al. fed their ds*nuc1* via artificial diet over eighteen hours, at a concentration of 100 ng/1 µl in 100 µl total volume; though it is difficult to compare actual dsRNA exposure between prolonged feeding of low concentration dsRNA and one-time exposure of high concentration dsRNA through injection, the same result across two methods of delivery is noteworthy. Though, in the same study, they were able to elicit a phenotypic response from injection of ~200 ng dsRNA against *EcR*. This is a comparable amount to that of ds*nuc1*, ds*nuc2* and *hb* injected in the present study. While the amount of dsRNA necessary to elicit a silencing effect is dependent on the initial expression level of the gene (Chen et al., 2021), the dsRNA doses used here are comparable to (or higher than) what has been published in the pea aphid as being able to elicit silencing (Ye et al., 2019a; Jaubert-Possamai et al., 2007;

Sapountzis et al., 2014; Niu et al., 2019) (yet, relatively high compared to what has been reported to yield successful RNAi in some other hemipterans (Wang et al., 2018; Castellanos et al., 2019)). Thus, the lack of a silencing effect is suggestive of some other factor.

A total lack of or partial activation of the core RNAi machinery has been reported in *A. pisum*. Here, the expression levels of core RNAi machinery genes in response to injection of dsRNA were not assessed. While I would anticipate some amount of upregulation from either or both of ds*gfp* injection or injection of dsRNA against *nuc1* and *nuc2*, it is possible that insufficient activation of the machinery occurred. Though, this is less likely to be related to dose, as a study by Yang and colleagues (Yang et al., 2020) demonstrated that with as little as 6 or 60 ng (both doses considerably lower than those used here) dsRNA (in this case, against *hb* and *gfp*), multiple components of the core RNAi machinery (beyond just *dicer*, *Argonaute* and *R2D2*) were differentially expressed in the pea aphid. More direct upregulation of core RNAi machinery, for instance by introduction of transgenes (as in (Li et al., 2015; You et al., 2020)) may be preferable, though this is a greater investment than indirect upregulation through ds*gfp*, where it is known to work, and far less amenable to commercial applications, as generating sufficiently transgenic organisms/strains requires delivery to cells/organisms at early developmental stages (as in (Li et al., 2015; You et al., 2020)) and has obvious implications relating to genetic modifications outside of laboratory contexts. A lack of upregulation could be due to the amounts of dsRNA injected, or a result of some other factor. Alternatively, the lack of silencing of *nuc1*, *nuc2* and/or *hb* observed here may have arisen from discrepancies between the sequences of these genes recorded in NCBI and the actual sequences encoded in the genomes of the aphids used, as was observed by Mao and Zeng (Mao & Zeng, 2012) who observed low RNAi efficiency in *A. pisum* when they generated ds*hb* based on mRNA sequences in GenBank (from *A. pisum*), but high efficiency when they based ds*hb* synthesis on their own sequencing. This has relatively severe implications for RNAi as a pesticide, as sequence homology within species even is not necessarily high, though careful selection of highly conserved (at the nucleotide level) targets could help to mitigate its effects. Though, here, I did not observe even a hint of

knockdown of *hb*, suggesting this is not the major contributor to the lack of silencing.

One possible factor for a lack of silencing effect, despite combination of multiple approaches that have been demonstrated in some cases to enhance silencing, is strain, along with potential differences between lab populations. To my knowledge, RNAi (successful or unsuccessful) has not yet been reported in the N116 strain used here. Yoon and colleagues (Yoon et al., 2020) assessed 83 pea aphid genotypes and found RNAi efficiencies across a spectrum encompassing no effect to complete mortality, demonstrating high intraspecific variation. Among the genes that they found to be significantly correlated with RNAi response (though, in this case with relation to RNAi of a single gene, *AQP1*, which encodes an aquaporin (water channel) in the gut and salivary glands), GO terms relating to RNA/DNA metabolic processes and transcription were, not unexpectedly (given the correlation with RNAi efficiency) enriched. When they assessed the RNAi responsiveness of strains that were highly or lowly susceptible to RNAi against *AQP1*, to another gene, *snakeskin* (which while functionally distinct, is still enriched in the gut), they found only partial consistency in response for the highly susceptible to *AQP1* group (two out of four of the tested genotypes showing significant susceptibility to *snakeskin*), though, high consistency in the lowly susceptible to *AQP1* group. These results are suggestive of genotype specific RNAi susceptibility, but also suggest that this may be gene-specific and rely on multiple factors.

Variation in RNAi efficiency within and between populations has also been demonstrated in *Locusta migratoria* (Sugahara et al., 2017). Not only do different labs often work on different pea aphid strains (possibly being a major reason for differences in RNAi efficiencies), but even for a given strain, differences between various lab cultured populations, which are often maintained for many generations over many years, may also contribute to inconsistencies in reports of RNAi. As the RNAi system functions principally in response to exposure to viruses, it is possible that variation in viral load may contribute to inconsistencies between studies, even within strains (as in (Elston et al., 2023), where they deliberately targeted four strains that previously showed large RNAi responses, but did not themselves see a significant effect). If one strain in one laboratory has a higher viral load, this may lead to greater

baseline expression of the core RNAi machinery, or if it is a strain that in ecological conditions has had high exposure, historically, to viruses, then selection may have led to a greater capacity for the RNAi system to activate. Likewise, high viral loads may lead to high activity of the RNAi system, but mean that its ability to react to exogenous dsRNA is dampened due to competition for machinery. Feasibly, attempts to upregulate the machinery in some systems may not work if the insects tested are already experiencing a high viral load leading to the system operating at or near capacity. Additionally, some viruses have been demonstrated to be able to suppress the RNAi response of their host (Mierlo et al., 2012; van Cleef et al., 2014; Weinheimer et al., 2015; van Mierlo et al., 2014; Samuel et al., 2016; Bonning & Saleh, 2021). Taken together, viral infection likely affects sensitivity to dsRNA, and may explain variation between populations. An approach to test if viral load is likely to account for (at least some of the) variation between laboratory populations is to take an RNAi susceptible strain, and gradually increase its viral load (RNA viruses that are not pathogenic to aphids may be preferable, to avoid effects on survivability which might affect RNAi susceptibility by non-direct mechanisms, aphid-borne viruses are detailed in (Gadhave et al., 2020; Guo et al., 2022b); feeding on plants known to be infected with an aphid-borne virus may present a means of doing this) and assess the effects on gene silencing efficiency. The viral load of RNAi susceptible and RNAi recalcitrant strains could also be assessed to less directly assess the association between viral load and RNAi susceptibility.

Additionally, it is feasible that differences in efficiency between experimenters and strains may be explained by differences in expression by default of target genes between the aphids used. I.e. if in one study, expression of a given gene in their strain of aphid is relatively high, and in another it is relatively low, RNAi effectiveness is likely to differ. Though, it is not likely that this would account for such wide discrepancies in knockdown observed between studies targeting the same gene.

Yoon et al. (Yoon et al., 2020) identified several genes which appeared to be related to RNAi susceptibility, with various SNPs associated with greater or lesser susceptibility. Feasibly, for lab studies at least, expanding on and then implementing (in terms of selection of strain) understanding of genes associated

with greater susceptibility may be the best approach toward consistent and effective RNAi. While this is a viable solution in a research lab setting, RNAi is increasingly being developed with commercial application in control of pests, including control of aphids, in mind. If some strains (of pea aphids, other aphids, or other insects) are not susceptible to RNAi, then RNAi may quickly become a less-viable option for control of pests, especially given that as susceptibility is clearly driven, at least partially, by genetic variation, selection of RNAi insusceptible strains would be likely to occur, leading potentially to RNAi resistance. Importantly, resistance would likely be partially dsRNA non-specific. Resistance of aphids to conventional insecticides is already an issue (Mota-Sanchez & Wise, 2023; Bass & Nauen, 2023), and resistance to dsRNA by a population of a prolific pest, the western corn rootworm, generated by artificial selection, has already been demonstrated (Khajuria et al., 2018). Interestingly, concerning western corn rootworm dsRNA resistance, the authors suggest (based on qualitative assessment of dsRNA degradation by gut extracts, and differences in dsRNA localisation in midgut cells between susceptible and resistant individuals) this was related to uptake of dsRNA but not dsRNA degradation. This further suggests uptake mechanisms as good targets for improving efficiency (but again, highlights the breadth and variability of cases, as degradation is clearly a driver in other studies/systems, as discussed above), and possibly in addressing potential dsRNA resistance. Although, further studies in this area may identify ways to mitigate strain-specific variation in susceptibility to RNAi, for example by exploring concurrent upregulation of multiple regulators (including core RNAi machinery genes), downregulation of multiple others (e.g. non-specific endonucleases, intracellular nucleases, etc.) and prolonged exposure to high concentrations of, possibly, protected (e.g. by nanoparticles as reviewed in (Pugsley et al., 2021)) dsRNA, most feasibly through transgenic expression in plants (or perhaps, spraying, although the issue of degradation, which is one of the perceived benefits of dsRNA as a pesticide, may make this less feasible, unless dsRNA is protected). Additionally, synthesis of sufficient amounts of dsRNA has historically been expensive and getting highly concentrated RNA can be an issue – production of dsRNA in microbial systems such as *E. coli* may become increasingly preferable to dsRNA synthesis by IVT (Verdonckt & Vanden Broeck, 2022). Taken together, while it is tempting to suggest that for lab experiments, careful selection of strain

and/or genetic screening may be the most appropriate approach for studies using RNAi as a tool to assess gene function, it will be fundamentally important to further elucidate the genetic and putatively environmental underpinnings of variation in RNAi susceptibility, for RNAi to be an effective and sustainable method of pest control.

As an alternative to RNAi, where strains do not appear to be susceptible to it (as is the case in the work presented here), CRISPR-Cas9 offers an alternative approach to studying gene function by knocking-out (rather than knocking-down) gene expression. Although, knock-down may in many cases be preferable, due to it being less likely to elicit significant mortality, and because knock-out by CRISPR-Cas9 often generates highly mosaic organisms (that is, not all cells in an organism will have the modification that is induced by CRISPR-Cas9). Additionally, RNAi being transient means that a reversal of the effect can be observed, increasing confidence in the link between knock-down and phenotype. CRISPR-Cas9 is also a relatively newly explored technique in the pea aphid, and it has not yet been widely adopted or optimised (Le Trionnaire et al., 2019, 2022; Takenaka, Konno & Kikuta, 2023); successful attempts so far have relied on microinjection into sexually produced eggs which develop over three months, and the efficiency has been low, with significant mortality, making it also a significant task to achieve. Delivery into viviparous individuals has been less successful (Jamison, Thairu & Hansen, 2018). Optimised delivery of Cas9 ribonucleoprotein, through microinjection in adults and subsequent transport to the ovaries to affect the germline, for example by using a system similar to the Receptor-Mediated Ovary Transduction of Cargo (ReMOT) technology (which would require identification of suitable delivery ligands for *A. pisum*, which is not helped in the viviparous mode by the fact that the oocytes do not undergo vitellogenesis), or the recently demonstrated to be a workable system in *A. pisum*, using cell-penetrating peptides (CPPs) to target tissues from the haemolymph (Takenaka, Konno & Kikuta, 2023), may allow more efficient injection, and targeting of adult asexual individuals.

In summary, I was unable, after attempting to optimise RNAi by pre-injecting non-specific dsRNA and dsRNA targeting two non-specific nucleases which I demonstrated to be widely expressed, to silence a target gene by RNAi, in *A. pisum* vivipara of a lab-maintained strain (N116). The lack of effective RNAi

may stem from strain (e.g. SNPs in core RNAi or RNAi efficiency associated genes) or population (e.g. viral load) specific differences between the aphid population used here, and populations used by other investigators. Given the fact that the doses used here were comparable to those reported in studies that report significant silencing, even of the same target (*hb*), it seems likely that the observed lack of effect stems from differential RNAi susceptibility. Thus, this chapter builds on an increasing, and concerning body of research that indicates some level of diversity in RNAi susceptibility, even within species, which may present severe implications for the deployment of RNAi as a pesticide. Though, several avenues to potentially circumvent this by addressing points in the RNAi system where differential RNAi susceptibility can be realised, are increasingly being identified and explored, as I discuss here. Going forward, more in-depth analyses of genetic (genotyping and screening for RNAi susceptibility-related genes across a wider range of strains) and environmental differences (e.g. assessment and manipulation of viral load in relation to RNAi susceptability) between strains of pea aphids used for RNAi in which RNAi susceptibility differ, and development of understanding of how these factors interact with different target genes or groups of genes (owing to the fact that there appears to be some difference between genes in their response) will greatly increase our understanding of the underpinnings of differential RNAi susceptibility.

Chapter 4 Exploring the role of the DNA methylation machinery in aphid parthenogenetic reproduction and profiling the expression pattern, spatially in the pea aphid ovary along with the evolution across the diversity of insects, of two paralogs of the *de novo* methyltransferase gene *dnmt3*

4.1 Introduction

Aphids present a group of insects with a distinct biology. They, except in some species and strain specific cases, exhibit cyclical parthenogenesis, which, itself an evolutionary innovation, the inter-generational ability to switch from production of asexual to sexual offspring, encompasses also viviparity, a further innovation, in the asexual mode (Srinivasan & Brisson, 2012; Davis, 2012). The ability to switch reproductive mode is naturally, genetically determined and over relatively short time periods (within species), genetic changes that result in the loss of this ability can occur (Blackman, Minks & Harrewijn, 1987; Srinivasan & Brisson, 2012). The viviparous mode of reproduction encompasses chains of developing embryos and oocytes, terminating at the anterior end with germaria, in the ovarioles of the telotrophic meroistic ovaries of aphids (Büning, 1985; Michalik et al., 2013). The mechanisms involved in development in the parthenogenetic mode generally, and that may facilitate the polyphenic switch between developmental modes as effectors, are not well understood in aphids.

DNA methylation is an epigenetic mechanism, a heritable change to DNA that does not involve a change in sequence, and is carried out by DNA methyltransferases (DNMT1 being responsible primarily for maintenance methylation, and DNMT3 for *de novo* methylation) (Goll & Bestor, 2005). Primarily, DNA methylation involves the addition of a methyl group to cytosines in CpG dinucleotides (figure 4.1), and while in mammals its function is relatively well understood (methylation marks being confined mainly to gene regulatory regions and associated mainly with repression of expression), in insects its role is unclear (Glastad, Hunt & Goodisman, 2014; Duncan, Cunningham & Dearden, 2022). This is particularly true of hemimetabolous insects, as much of the research effort has settled on holometabolous groups, especially Hymenoptera (Kucharski et al., 2008; Wojciechowski et al., 2014; Pegoraro et al., 2016; Lonsdale et al., 2017; Marshall et al., 2020; Marshall, Lonsdale & Mallon, 2019; Pozo et al., 2021; Renard, Gueydan & Aron, 2022; Hunt et al., 2023). Partly, this focus on Hymenopteran species, principally eusocial bees and ants, is related to DNA methylation having been demonstrated to be

environmentally responsive or differential DNA methylation states being associates with particular morphs in these species (Shi et al., 2011; Bonasio et al., 2012; Alvarado et al., 2015). Several studies have attempted to link DNA methylation to differential gene expression, akin to what is seen in mammals, though, conclusive evidence for a link is lacking, and also, have attempted to link DNA methylation to phenotypic plasticity; in particular, the extreme example of phenotypic plasticity observed in, for example, reproductive castes of eusocial insects, which have drawn major attention (Duncan, Cunningham & Dearden, 2022). In insects, a link between DNA methylation and differential splicing has been suggested (enrichment of methylation at genes that are alternatively spliced genes has been described in some insects, the proposed mechanism being adjustment of DNA accessibility (Bonasio et al., 2012; Lyko et al., 2010)), however, similarly to for an association between DNA methylation and gene expression, the results are inconsistent (Duncan, Cunningham & Dearden, 2022); several insect whole-genome dataset analyses have demonstrated a lack of consistency in the relationship between DNA methylation and differential splicing (that is, DNA methylation may not be enriched in genes that may be alternatively spliced) (Marshall, Lonsdale & Mallon, 2019; Lewis et al., 2020).

Although, some studies investigating differences in DNA methylation between organisms in different states within phenotypically plastic phenotypes, for instance in worker and queen destined larvae of honeybees and similarly, between castes in other eusocial insects, have suggested that in some cases, DNA methylation can be higher in some modes relative to others; though, as I have already pointed out, the findings of these studies are not always consistent (reviewed in (Oldroyd & Yagound, 2021)). Several studies have failed to identify a link between DNA methylation and castes, such as (Libbrecht et al., 2016), which is an example where sufficient biological replication was included and (Herb et al., 2012) (in Herb et al., 2012, differences were detected between worker subcastes but not between workers and queens). Though, importantly, these studies often look at the whole-body level or rely on pooled samples, which introduces noise, especially because it is reasonably expected that different tissues will respond differently (Song et al., 2009; Xu et al., 2018, 2021a; Hunt et al., 2023) and so might individuals, or suffer from poor biological replication ((Oldroyd & Yagound, 2021); and as commented on by (Libbrecht et al., 2016)). Early studies suggested links between honey bee caste determination and DNA methylation, but they suffered from poor sample sizes, and subsequently poor replication, or sampled in periods where large scale differences in gene expression/methylation state would be expected, or

investigated few loci; all of which have made interpretation of their results difficult. The critical paper that suggested DNA methylation to be the principal mechanism for worker-queen caste determination in honeybees was by Kucharski and colleagues, who used RNAi against *dnmt3* in larvae, leading to subsequent development of the queen phenotype (Kucharski et al., 2008). Subsequent studies have frequently (though, again, inconsistently) found an unconvincing link between methylation state and differential expression (Harris et al., 2019). The exact role, if there is one, of DNA methylation in differential expression and phenotypic plasticity is therefore still heavily obscured.

Interestingly, methylation independent roles of Dnmt1, one of the major (especially given that in several groups, Dnmt1 is the only present DNA methyltransferase type) facilitators of DNA methylation have recently been identified in reproduction in the hemipteran, *Oncopeltus fasciatus* (hemimetabolous) (Bewick et al., 2019; Washington et al., 2021) and the beetle, *T. castaneum* (holometabolous) (Schulz et al., 2018), which, together with the observation of *dnmt1* in the genomes of some insects where DNA methylation is not thought to be present (Bewick et al., 2017; Duncan, Cunningham & Dearden, 2022) hints at potentially conserved functions of DNMTs that extend beyond DNA methylation. Embryogenesis related functions of Dnmt1 have also been reported in *Blatella germanica* (Ventós-Alfonso et al., 2020) and *B. mori* (Xu et al., 2022b), though these do appear to be associated with DNA methylation (in the former, through reduced representation bisulfite sequencing and the latter whole-genome bisulphite sequencing).

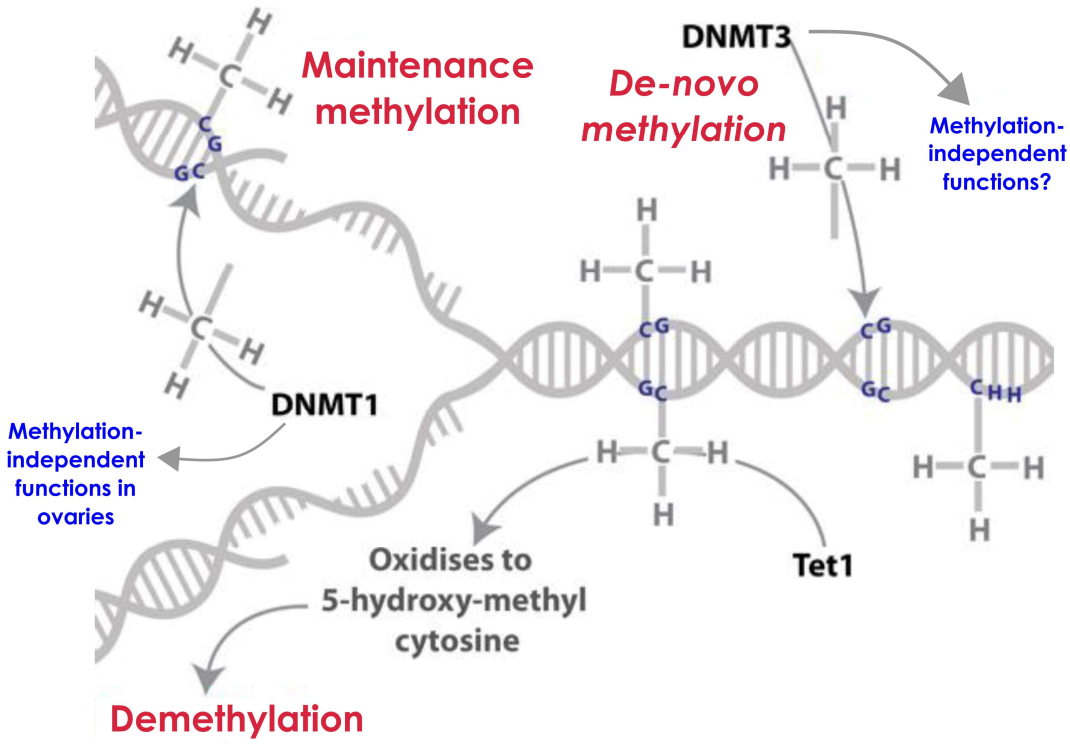


Figure 4.1 DNA methylation and demethylation mechanisms.
DNA methylation is a reversible epigenetic mark, the addition of a methyl group primarily to cytosine residues. DNA methyltransferases are responsible for catalysing the addition of this residue to DNA. Dnmt3 is associated with catalysing *de novo* methylation, that is applying methylation marks to unmethylated DNA, whereas Dnmt1 is associated with catalysing maintenance methylation, to maintain methylation signatures on replicated DNA. DNA demethylation occurs by oxidation of methylated cytosine to 5-hydroxymethylcytosine, catalysed by TET proteins. Additionally, DNA methylation independent roles of DNMT1 have been demonstrated in the ovaries of several insects (a beetle, a hemipteran bug, and an ant, explored in text); whether there are methylation independent functions for DNMT3 proteins is unresolved. Adapted from (Duncan, Cunningham & Dearden, 2022).

Further, a study in *Ooceraea biroi*, an ant that shows some plasticity in reproduction, revealed a potential role for *dnmt1* (the protein and mRNA of which both appear to be maternally provisioned) in early oogenesis and maturation (Ivasyk et al., 2023) by knockout, but treatment did not affect the plastic mode assumed. Though, this study suffered from incredibly low sample sizes, and the use of G0 CRISPR-Cas9 mutagenized individuals, which may have been mosaics. While this does make interpretation of its results tenuous, the consistency between the observed effect and those observed in studies of the ovaries of *O. fasciatus* and *T. castaneum* after similar manipulations (using RNAi, rather than CRISPR-Cas9) make it more convincing. Together, these results pose the possibility of roles for the DNA methylation machinery in further

insect species, in the ovary and in reproduction. Especially given the degree of evolutionary separation between the groups to which these examples belong, given that Hemiptera (*O. fasciatus*) and Holometabola (*T. castaneum*, *O. biroi*) are thought to have diverged at least ~ 330 million years ago (Misof et al., 2014).

Given the relative scarcity of *dnmt3* in insects, compared to *dnmt1*, which is demonstrably capable of assuming the role that *dnmt3* classically is associated with (*de novo* methylation) (figure 4.2) (Bewick et al., 2017), it is interesting that *dnmt1* (which is presumably under selection to maintain DNA methyltransferase activity, in species that have intact DNA methylation systems – importantly, some organisms (e.g. *T. castaneum*) that possess *dnmt1* are not thought to have methylated DNA (Schulz et al., 2018)) potentially has conserved roles in oogenesis and reproduction, and raises the possibility that *dnmt3*, as an apparently more dispensable gene (Bewick et al., 2017; Duncan, Cunningham & Dearden, 2022), may also have roles beyond DNA methylation. After all, if *dnmt3* can be readily dispensed of, cases where it is present (by maintenance from the ancestral system) raise the questions of why it is being maintained in specific genomes, and what functions it may carry out (which may be independent of DNA methylation). Many insects possess highly simplified DNA methylation toolkits (encompassing Dnmt1 only, or single copies of the present DNA methylation orthologues) (figure 4.2) (Bewick et al., 2017), resultantly, where more sophisticated systems exist, this may be an indicator that the expanded toolkit is linked to some novelty in the organism's biology, because maintenance of additional components (e.g. *dnmt3*) that are not essential to DNA methylation may be under relaxed selection, and because duplication (when it is maintained) often leads to novelty through sub- or neo-functionalization, if duplicates do not become pseudogenes (Flagel & Wendel, 2009; Edger et al., 2015; Birchler & Yang, 2022). In addition to this, because of increasing evidence for functions of Dnmt1 independent of DNA methylation in the ovaries of insects (and in other animals, e.g. *Xenopus laevis*, where it is a direct repressor of transcription during embryogenesis (Dunican et al., 2008)), suggesting some amount of conservation, if Dnmt3 is active in the ovary, then its role may have diversified beyond DNA methylation (as for Dnmt1), but feasibly not be related to oocyte maturation and general reproduction (at least in relation to the aspects that Dnmt1 appears to be involved in). This further raises the possibility for more specialised roles for Dnmt3 in the ovary and in reproduction, such as in mediating plasticity. For instance, relating to reproduction (Nguyen et al., 2021) or, feasibly, the reproductive polyphenism exhibited by aphids.

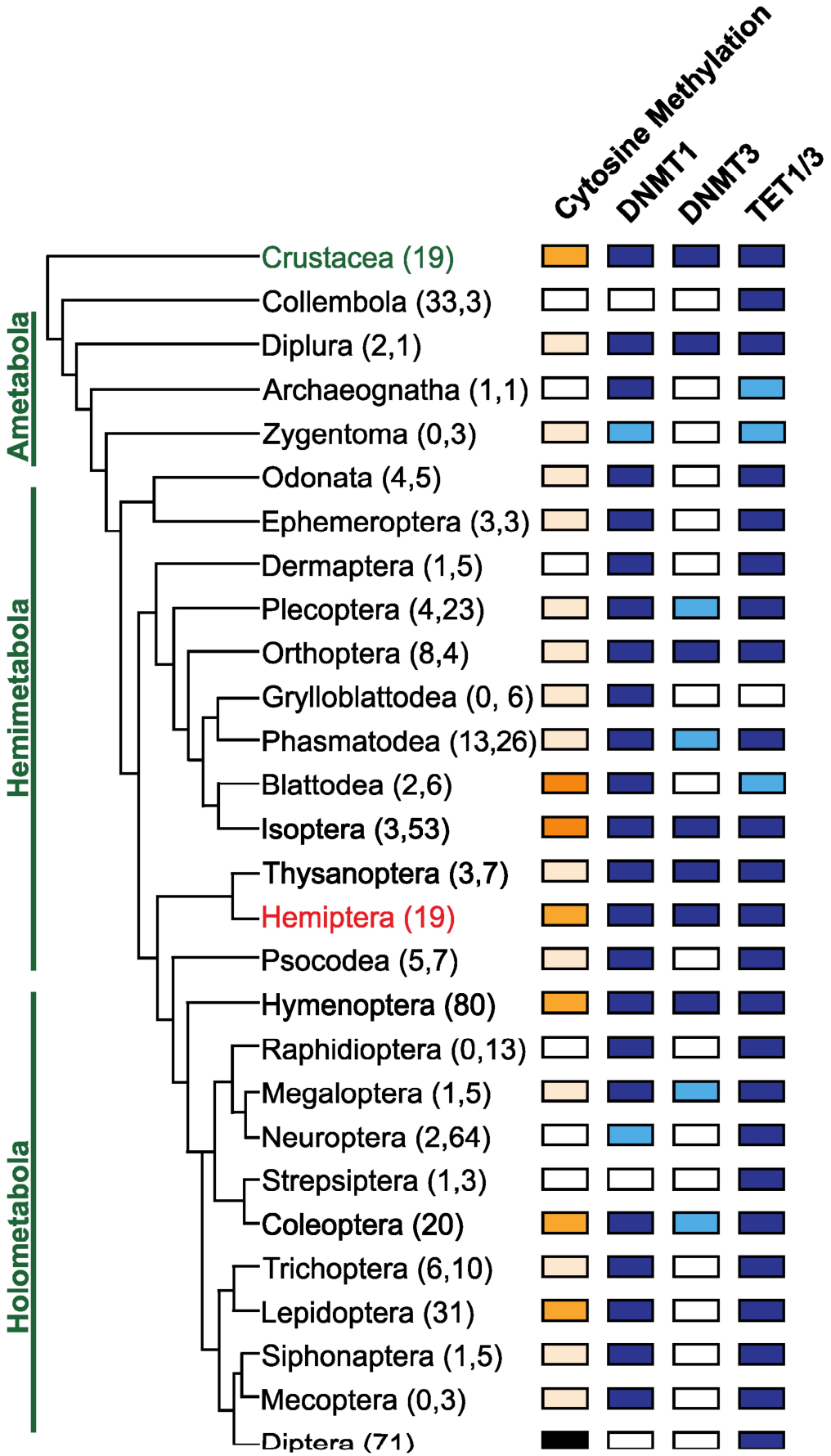

Cytosine Methylation
DNMT1
DNMT3
TET1/3
Ametabola
Hemimetabola
Holometabola
Crustacea (19)
Collembola (33,3)
Diplura (2,1)
Archaeognatha (1,1)
Zygentoma (0,3)
Odonata (4,5)
Ephemeroptera (3,3)
Dermaptera (1,5)
Plecoptera (4,23)
Orthoptera (8,4)
Grylloblattodea (0, 6)
Phasmatodea (13,26)
Blattodea (2,6)
Isoptera (3,53)
Thysanoptera (3,7)
Hemiptera (19)
Psocodea (5,7)
Hymenoptera (80)
Raphidioptera (0,13)
Megaloptera (1,5)
Neuroptera (2,64)
Strepsiptera (1,3)
Coleoptera (20)
Trichoptera (6,10)
Lepidoptera (31)
Siphonaptera (1,5)
Mecoptera (0,3)
Diptera (71)

Figure 4.2 Appearance of DNA methylation and DNA methylation machinery genes across the diversity of insects.
Phylogenetic relationships between insect orders are based on (Misof et al., 2014). Numbers in parentheses indicate the number of genomes (first) and transcriptomes (second) that were interrogated to determine presence/absence for each DNA methylation related gene (right); DNA methylation presence/absence is based on published data. For cytosine methylation, dark orange corresponds to experimentally confirmed DNA methylation, apricot corresponds to inferred presence of DNA methylation by CpG observed/expected, white corresponds to absence of DNA methylation based on CpG observed/expected, and black corresponds to experimental confirmation of absent DNA methylation. For DNA methylation components, dark blue indicates greater than 50 % of assessed species having identifiable homologs (by BLAST searching) lighter blue indicates less than 50 % of assessed species having identifiable homologs, and white indicates complete absence. Modified from (Duncan, Cunningham & Dearden, 2022).

The pea aphid genome presents an especially interesting point from which to explore this, as its genome, which exhibits a complete suite of DNA methylation genes, possesses two paralogs of *dnmt3* (Walsh et al., 2010). Based on predictions, one, termed *dnmt3a*, appears to possess domains necessary for function as a DNA methyltransferase, the other, termed *dnmt3x*, putatively, does not. Though, the functionality of each of these paralogs has not yet been validated. If these paralogs appear in further aphid species, this may suggest they are related to the unusual biology of aphids in some way. I have already discussed, in the general introduction, how duplications, which appear frequently in the genomes of aphids (Fernández et al., 2020; Julca et al., 2020), may have facilitated some of the novelty exemplified by aphid biology.

The pea aphid genome shows low (relative to mammals and plants) cytosine methylation (Walsh et al., 2010). One study found that when taking the whole genome, ~3 % of cytosines were methylated, increasing to ~ 9 % when just taking coding regions. The same study, using bisulphite and RNA sequencing, suggested a general correlation between methylation and expression in *A. pisum* (Clément et al., 2021), though suffering from the low sample sizes typical of experiments using these techniques. While the level of methylation in the pea aphid is low relative to mammals and plants, it is relatively high compared to many insects (insect DNA methylation is generally low, but variable), for instance, in *Apis melifera* it is thought ~1 % of cytosines are methylated (Provataris et al., 2018; Glastad et al., 2011).

In *A. pisum*, three genes related to juvenile hormone regulation (juvenile hormone being heavily implicated in the reproductive polyphenism of aphids), a JH-esterase binding protein (which is noteworthy because degradation of

juvenile hormone has been suggested to be a major driver of differential JH titre during the reproductive switch (Ishikawa et al., 2013)), a JH epoxide hydrolase and a predicted cytoplasmic JH binding protein have been shown to be methylated in the pea aphid, while four other JH related genes did not appear to be methylated (Walsh et al., 2010). Although, the link between methylation of these JH related genes and their expression levels was not assessed. This hints at a possible relationship between DNA methylation and the reproductive polyphenism, though differential methylation of genes between aphids exposed to long day (LD) or short day (LD) conditions, which lead to the continual production of parthenogenetic offspring or the eventual production of sexual offspring, respectively, has not been demonstrated.

Given the developmental determination of the reproductive mode in response to photoperiodic cues perceived by the mother and transduced to her future offspring, it is useful to characterise the expression patterns of genes involved in DNA methylation, as possible downstream components of the reproductive switch, in the ovary. The duplication of *dnmt3* in the *A. pisum* genome presents an interesting case of potential investment in an increased use of *dnmt3* beyond what is typical, potentially indicating it is involved in facilitating some novelty in the aphids. In this chapter, to explore this, I first identified *dnmt3a* and *dnmt3x* orthologues across the aphids and insects more generally to follow their evolutionary history. I then explored the genetic contexts of these genes to make inferences about their relation to each other. Following this, I used RNA-FISH HCR to profile their expression in the ovaries of virginopara (parthenogenetic aphids producing parthenogenetic offspring) pea aphids. Because of distinct expression patterns of these genes in the ovary, I pharmacologically inhibited the DNA methylation machinery more generally and assessed effects in the ovary and on reproduction.

4.2 Methods

4.2.1 Phylogenetic analysis and genome organisation of *dnmt3a* and *dnmt3x*

To detect conserved domains in the *A. pisum dnmt3a* and *dnmt3x* genes (table 4.1), the NCBI conserved domain database was used with default parameters (Wang et al., 2023a).

Pea aphid *dnmt3a* and *dnmt3x* sequences were then used to search for homologs in other insect species (a subset of homolog protein sequences are presented in appendix tables B.2, B.3 and B.4), using BLASTn and BLASTx to

databases in NCBI's nucleotide collection, and available on AphidBase (Legeai et al., 2010), in collaboration with Elizabeth Duncan. To identify positive hits, an E-value cut-off of 1e-20 was used, and results were examined. The protein sequences corresponding to the extracted positive hits were aligned using Clustal Omega (Sievers et al., 2011) with default parameters. 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 .fa-gb file was then realigned using Clustal Omega; sequence alignment figures were prepared using Jalview (Waterhouse et al., 2009). 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).

Table 4.1 Nucleotide sequences of *A. pisum dnmt3* paralogs.

Gene	Nucleotide Sequence
dnmt3a LOC100568466	TGATTGCTGCACTTGTTTCAAAAGCCCAAAAAAGATTATCATCTTTCTGCAGCGCTTGCATTTTGTTTTTAAATAGTAAAAACAAGAAAAATTTTTTTAGGCAATCGTTTCGATCAAAAAATTT ATCTTTATCGTAATTCTCCCTGTTTGGATAATATTGGACGACTAATTTTGCATTACTCTTATTATTGTCCTGATTATTATCATTTAAATAACTAGTTTCCAGAATCTTGAACGGAAATCCTAGA GTTTGTAAAACTTTTGGTACATTGTTTTGAATATCTTTTAAATTCAGGACATGAATCTCTTTTATTTTATACTGTTCCTTGGCACAGTCTTTTTTAGCGTTAGCTGGATACAACATATAGATAT GTCTTAGTTGTTCTTTGGTACTCCTCGGAACTAGATTACTGATCTTTAAAGTAGTGGAACTGCATGCAATGCATGCCCATGCATTGTCTATAAGAATTTTTTCCAAAGCTTTTTGTCCACAGT AAAATTCTAAGCATTCATTGCAATAAGCTCTTATACAAAAACCGCACAATACCAA
dnmt3x LOC100572675	atcttcgtcaagagcaaaaacctctaaattaccacgaataacatcttcatcaaatccaaaaccATCTAAATTACCaCGACTAAGAGTACTGTCTCTCTTTGACGGAATTGGCACAGGGTATTATGCATTGTTGA AATTAGGGTTTGATATTGAAGTAATTTATGCAAGTGAAATAGATAAAGATGCGTTAATGGTAACTAAATATCATTTTTCCGACAACATTAAACAATTGGGTTCGgTTACTGAAATAACCACAA AAATGCTGGACCAAATCGCACCTATAAATCTGCTTTTtGGTGGTTCACCTTGCTCCGACCTATCTGGTGTCAATTATCGAAAAAAAGGACTTTTCGATCCAAAAGGAACAGGTATATTATTCT ATGATTATTACCGTATATGGAACTATCTAAGTGTAAAAGCTGCTAGAGAGAATACTCCTTTTtATTGGTTATATGAAAATGTGGCAAGCATGGAAATTAAAAATAAAGACACCATTTCAAAAT TTTTTGAATGTCAACCAATTGTTCTTGATTCATTACACTTCAGCCCACAGCGACGTAAAAGATATTTCTGGTCGAGCCTCCCAGGGATAACAATGTTGCCTCATATTCAGAAGACGAATTAT GCTACTAATGCACCAAAAaCTAGAAGATTAtTTAGAAAAGAATTTAGACAGACAGGCTAATGTAGAAATAgTTGGAACCATCACTTCAAAAAGAAGTTGTTTACAAGATAGCAAATCTAGAAA TCCTGTATGTCAAGACGGACAATACACAGGCTTATTTATAACAGAGATTgAAGCAATATTTGGTTTGCCACCACATTTTACTGATGTAGgTGATttgtcaatatcaagtcgccaaaaactttgggaagag cgtggagtgt

The genetic arrangement and synteny of *dnmt3a* and *dnmt3x* between aphid species and another hemipteran bug, *Bemisia tabaci*, were assessed in order to investigate the likely nature of the duplication (e.g. large or small duplication) and assess conservation in the genes surrounding each copy, more generally, to detect conserved blocks of genes. To do this, a chromosome level assembly of the pea aphid genome (pea_aphid_22Mar2018_4r6ur_v2 (GCF_005508785.2)) was used to explore the genetic contexts of each of the paralogs. The identities of the flanking genes (two or four most immediately flanking) on each side of each orthologue in the genomes of *A. pisum* and another aphid species, *Myzus persicae* (which groups with *A.* pisum in the Macrosiphini tribe) and an Aleyrodidae species, *B. tabaci*, were recovered from NCBI. Orthologues of non-*A. pisum* flanking gene in the *A. pisum* genome were identified, using the same approach laid out above (taking the top BLAST hit), and their positioning in the *A. pisum* chromosome assembly identified.

4.2.2 Expression pattern of *dnmt3a* and *dnmt3x* in long-day exposed parthenogenetic viviparous pea aphids

Virginopara pea aphids, mostly adults but also L3 and L4 stage nymphs to yield younger embryos, maintained in LD conditions were collected from the stock population (detailed in the general methods) and dissected to yield ovaries, which were then fixed (as described in the general methods). RNA-FISH HCR of ovaries was carried out as described in the general methods, after tissue (stored in methanol at – 20 C) was hydrated through a methanol:PTw (PBS, 0.3 % Tween-20) series by nutation at RT for ten minutes each at ratios of 3:1, 1:1, then 1:3. Ovaries were stained using either *Ap-vasa* and *Ap-dnmt3a* or *Ap-vasa* and *Ap-dnmt3x* probes and B4-647, B2-546, or B3-546 hairpins for *Ap-vasa*, *Ap-dnmt3a*, and *Ap-dnmt3x* respectively. 6 pmol of each of hairpin 1 and hairpin 2 in 2 µl hairpin storage buffer each were prepared and used for *Ap-dnmt3a* and *Ap-dnmt3x*, but because of the abundance of *Ap-vasa* mRNA in the ovary (and associated strong signal), 3 pmol of hairpin 1 and hairpin 2 in 1 µl hairpin storage buffer each were prepared and used for *Ap-vasa*. Concurrently, control samples were prepared by omitting the probe, but otherwise following the same protocol. After staining, clearing and mounting on slides, samples were imaged within five days, and imaging and processing were carried out as detailed in the general methods. Staging of oocytes and embryos was performed based on (Miura et al., 2003).

4.2.3 Chemical inhibition of DNA methyltransferases

Fourth instar parthenogenetic pea aphids (viviparae) were collected from the stock population and reared on an established artificial diet (the recipe for the preparation of which is documented in appendix B.1, table B.1) supplemented with either 50 µm final concentration 5-azacytidine in DMSO or DMSO as a control. After being activated and metabolically converted to its active form, 5-azacytidine's active nucleotide is incorporated into DNA in place of cytosine, and upon association, DNA methyltransferases are bound to the site of incorporation irreversibly, functionally reducing their ability to perform their role and resulting in a reduced pool of free DNA methyltransferases, and subsequently, a reduction in DNA methylation (Stresemann & Lyko, 2008).

To prepare treatment supplemented artificial diet, single-use aliquots of diet stored at -20 °C were supplemented with 5-azacytidine in DMSO to yield a final 5-azacytidine concentration of 50 μM, or DMSO only (herein referred to as 'control treatment') immediately prior to feeding, each day; the final concentration of DMSO was 0.5 % volume/volume. I determined this concentration of 5-azacytidine through preliminary experiments, where mortality was consistent between feeding on diet supplemented with 50 μm 5-azacytidine and DMSO and supplemented diet, and because it is a comparable concentration to what has been used in similar studies in insects (Cook et al., 2015; Claudio-Piedras et al., 2020).

To supply aphids with supplemented diet, aphids were reared individually in the wells of a sterile, transparent 96-well standard, flat based cell culture plate. To feed them, first, sterile flat PCR strip tube caps were filled with 10 μl (per cap) of artificial diet (supplemented with either 50 μM 5-azacytidine in DMSO or DMSO). Then, the strips of caps were gently inserted, open face first, into 70%-ethanol-sterilised parafilm stretched over a 70%-ethanol-sterilised 96-well PCR tube holder. The parafilm was stretched to allow aphids access to the diet, being careful to put the parafilm under enough tension for their stylets to be able to penetrate it. The caps were inserted in such a way as to not penetrate the parafilm. This created a system akin to the leaves and stems of plants that aphids pierce to feed on phloem sap. On the first day of feeding, aphids were transferred to wells using forceps and cap-parafilm complexes were placed over the wells, enclosing the aphids. Aphids were maintained in this way, under LD conditions (16L:8D, 20 °C,70 % RH) for the duration of the feeding period, and fresh diet (in new cap-parafilm complexes) was provided each day. To replace the diet, aphids, which were often attached to parafilm, feeding, were first dislodged by gentle brushing with a paintbrush. This allowed them to withdraw their stylet and subsequently be placed in or drop to the bottom of the well. Aphids were fed in this way for six days, at which point all focal aphids were adult and reproductive; nymphs and cuticles were removed daily while replacing the diet, and the general state of nymphs were recorded.

After six days of treatment, focal aphids were assayed in two ways: RNA-FISH HCR and phalloidin staining of ovaries to assess effects in the ovary and on

early development, and allowing adults to reproduce to assess their reproductive phenotype and the phenotype of their offspring.

4.2.3.1 Ovarian and developmental defects

A sample of aphids were dissected on day six and processed as detailed in the general methods to yield fixed ovaries for RNA-FISH HCR. RNA-FISH HCR was carried out as detailed in the general methods, using *Ap-vasa* (using 3 pmol of hairpin, rather than 6, as in 4.2.2, above) and *Ap-wg* probes to generate stained and mounted ovarioles and embryos. Additionally, a sample of aphids were dissected on day six, and another on day two, to produce ovaries that were processed immediately after dissection (dissection was carried out as specified in the general methods) for staining with phalloidin, which binds to actin and allows visualisation of morphology. For phalloidin staining, ovaries were fixed in 1 ml of a mix of PBS, heptane and 37 % formaldehyde in a ratio of 5:4:1 by nutating for forty-five minutes. After fixing, the solution was removed and ovaries were washed for five minutes in PTw (0.3 % Tween-20) by nutating, five times, then incubated in PTw for one hour. Following, ovaries were washed as before, once, then a solution containing 10 µl Alexa fluor 488 phalloidin (Molecular Probes) in 200 µl total volume PTw was applied. Ovaries were nutated, covered from light, for thirty minutes. After which, 800 µl PTw and 1 µl DAPI (5 mg/ml; 4'-6-diamidino-2-phenylindole) were added to the ovaries in solution and they were nutated, covered from light for a further thirty minutes. Ovaries were then washed three times as before, and then a final time for five minutes in PBS. Control ovaries processed in the same way, except for the inclusion of phalloidin were concurrently prepared. Processed ovaries were then stored in 70 % glycerol before mounting on slides in 70 % glycerol and a small amount of SlowFade Diamond AntiFade Mountant (Invitrogen). Both RNA-FISH HCR and phalloidin stained samples were imaged within two days, and images were processed as specified in the general methods.

4.2.3.2 Reproductive output assay

Aphids were maintained following exposure to supplemented diet for six days and their survival and productivity, in addition to the survival and phenotype of their offspring were assessed every day, encompassing, including the six days of treatment, twenty days total. To do this, aphids were maintained on leaf-agar

plates (prepared as described in the general methods), one adult per plate. Because at this stage aphids were reproductive, plates therefore contained a single adult and some of its offspring, or its offspring alone. To prevent over-crowding, no more than ten aphids were maintained on a single plate at one time. Offspring were spread across multiple plates where necessary and aphids were moved by first brushing them gently with a paintbrush, then using forceps to pick them up by a leg. Plates were replaced every three days.

To assay the reproductive phenotype of the offspring of the focal adults, these aphids were raised to adults and pooled per focal adult. The phenotype of their offspring was assayed (for the presence of disturbed offspring) for ten days following the first of these offspring reaching adulthood.

4.2.4 Data analysis

A Cox proportional hazard model was run to assess differences in lifespan between 5-azacytidine and DMSO control focal aphids. Differences between 5-azacytidine and DMSO control aphids in their daily reproductive output was assessed by zero-inflated poisson GLMM fitted with a log link function, and with aphid ID as a random effect and treatment as the fixed effect. This was followed by performing a likelihood ratio test comparing the full model to a null model with the fixed effect dropped (using the *lmtest* package); data was truncated to remove entries prior to initiation of reproduction by a given aphid (all only being able to be 0). 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.

4.3 Results

4.3.1 *dnmt3* gene evolution and maintenance

4.3.1.1 *dnmt3x* arose from an Aphidomorpha specific duplication of the ancestral copy, *dnmt3a*

I first set out to establish the distribution of orthologues amongst aphids and then insects more widely of the two *dnmt3* paralogs known to be present in the genome of the pea aphid: *dnmt3a* and *dnmt3x*, in order to determine whether the gene duplication giving rise to them was a pea aphid specific event, occurred early in the lineage giving rise to the aphids, or occurred in a more distant ancestor shared between more disparate groups of insects, as understanding the evolutionary history of this duplication may inform if it is likely to be involved in aphid specific novelties or not. As paralogs (the genes are taken to be paralogs resulting from duplication based on homology between them) of the *de novo* methyltransferase gene, it is possible that one or both of *dnmt3a* and *dnmt3x* are important to reproduction in *A. pisum* (as elevated expression of *dnmt3* has been associated with gametogenesis and embryogenesis (or reproductive females), in a range of insects (Zhang et al., 2015; Kay, Skowronski & Hunt, 2016; Zwier et al., 2012), and functions of Dnmt1 relating to reproduction have been demonstrated (Ventós-Alfonso et al., 2020; Xu et al., 2022b), as explored in 4.1) and possibly the reproductive switch, as Dnmt3s canonically facilitate DNA methylation, which may be involved in the reproductive polyphenism exhibited by aphids (as has been explored, though, with inconsistent reports for several other examples of polyphenism in insects, see 1.5).

dnmt3a is thought to encode a protein functional as a DNA methyltransferase. I reconfirmed this here, as *dnmt3a* appears to possess a methyltransferase domain, encompassing DNA, substrate and cofactor binding sites. *dnmt3x* on the other hand has been suggested to be missing many of the key residues thought to be necessary for activity as a Dnmt3 (Walsh et al., 2010). In accordance with this, I did not detect a methyltransferase (catalytic) domain in *dnmt3x* (figure 4.3). Interestingly, *dnmt3x* appears to possess a PWWP and ADD domain (figure 4.3), both of which are thought to be involved in directing Dnmts to histones with particular methylated residues (Kucharski et al., 2023;

Otani et al., 2009), thus, while likely not being able to directly carry out DNA methylation, *dnmt3x* is likely functional in the sense of being able to associate with histones, which may mean it is involved in establishing epigenetic marks on histones (e.g. methylation or acetylation) or at least reading histone marks to direct other proteins. *dnmt1* was not predicted to possess these domains. The ancestral *dnmt3* is likely to encompass the methyltransferase domain, PWWP domain, and ADD domain – as the closely related non-aphid, *B. tabaci dnmt3* is predicted to possess all three of these domains (figure 4.3).

The PWWP domain typically recognises H3K36me3/me2 (Rona, Eleutherio & Pinheiro, 2016), and is thus a reader of these histone marks (though diversification of PWWP domain target towards methylation of another H3 lysine residue, H3K27, has been reported in the hymenoptera, in which two PWWP domains have been observed in *dnmt3* (Kucharski et al., 2023)). Conversely, the ADD domain is known to target unmethylated H3K4, and is thus a reader seeking non-methylated sites; when this domain is not associated with unmethylated H3K4, it is able to block the associated catalytic domain of the methyltransferase, thus preventing DNA methylation (though this is based on mammalian proteins) (Otani et al., 2009). DNA methylation and histone methylation are thus clearly tightly linked.

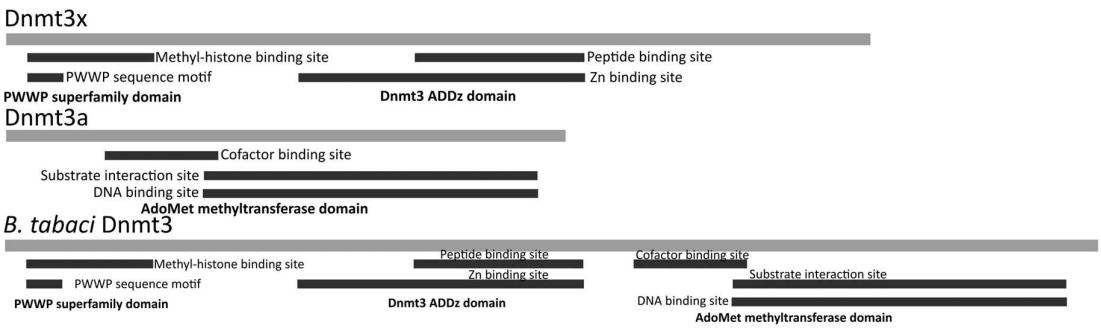


Figure 4.3 Conserved domains present in *A. pisum* Dnmt3 paralogs (Dnmt3x and Dnmt3a) and the singular *B. tabaci* Dnmt3.
Conserved domains were identified using the NCBI conserved domain database.The domains of the two *A. pisum* paralogs together reconsistute the domains in the *B. tabaci* Dnmt3. While Dnmt3x is missing the methyltransferase domains, and is likely therefore to be unable to catalyse DNA methylation, it possesses domains associated with histone binding. While Dnmt3a is missing these histone binding domains, it does possess the methyltransferase domain and therefore may be functional as a DNA methyltransferase. *B. tabaci* may represent the ancestral form, while the two paralog configuration of *A. pisum* is likely to be derived.

Homologs of *dnmt3a* and *dnmt3x* appeared frequently in representative species across the Macrosiphini and Aphidini (which, together, make up the Aphidinae, the largest subfamily of the true aphids, the Aphididae) (figure 4.4, and appendix table B.3, B.4), suggesting the duplication event giving rise to the derived copy occurred at least before the divergence of these groups ~ 62 million years ago.

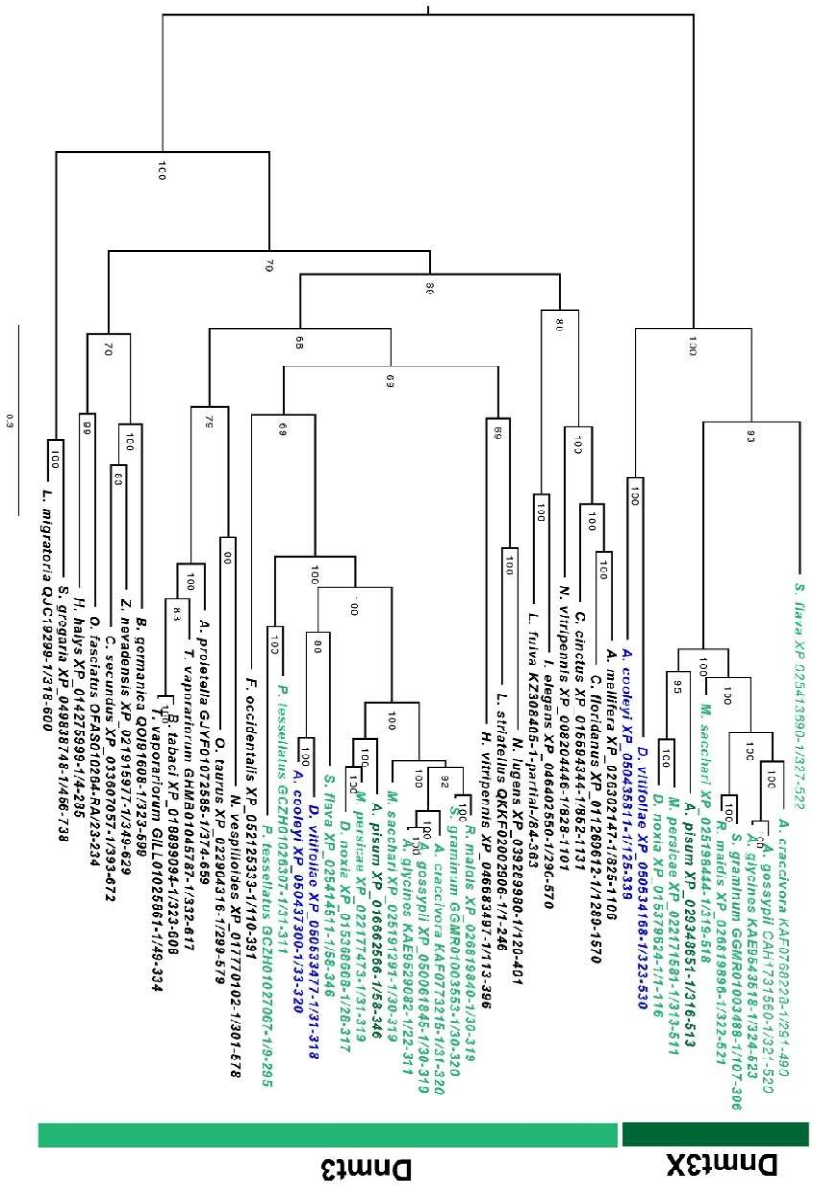


Figure 4.4 Phylogenetic tree of homologs of *A. pisum* Dnmt3x and Dnmt3a.

Homologs of Dnmt3x and Dnmt3a were identified by BLAST searching against public databases housed by NCBI. Numbers at nodes represent posterior probabilities. Scale bar is rate of substitutions. Most insects possess a homolog of Dnmt3 only, whereas Aphidomorpha species (alone) possess Dnmt3a and Dnmt3x. Aphidomorpha Dnmt3a clusters with the rest of the insect Dnmt3s, suggesting it is more similar to the ancestral identity, while Dnmt3x appears to be an Aphidomorpha specific diverged copy. Taken together, an aphidomorpha specific duplication is likely to have occurred. For identities,green indicates aphid sequences (dark green is *A. pisum*) and blue indicates non-aphid Aphidomorpha sequences. Built as a collaborative effort between Kane Yoon and Elizabeth Duncan.

Beyond the Aphididae, orthologs to both *dnmt3a* and *dnmt3x* were detected in representative genomes from the Phylloxera, *Daktulosphaira vitifoliae*, the grape phylloxera, and from the Adelgids, *Adelges cooleyi* (figure 4.4). This extends the likely time of duplication to before the divergence of these groups from the Aphididae, which are thought to have last shared a common ancestor ~ 150-200 million years ago (Ren et al., 2013; Ortiz-Rivas & Martínez-Torres, 2010; Davis, 2012). Homology between Dnmt3a sequences was better than for Dnmt3x sequences (appendix tables B.3, B.4).

Beyond this, while *dnmt3a* was detected (and had high protein homology), *dnmt3x* is convincingly absent from the genome of other insects, including the closely related to aphids, fellow Sternorrhyncha suborder Aleyrodidae species, *Bemisia tabaci* (a species for which there are high-quality genome assemblies (Chen et al., 2016; Xie et al., 2017)). This, in conjunction with the ubiquitous absence of orthologues of *dnmt3x* in the genomes of all insects inspected outside of the Aphidomorpha, suggests that *dnmt3x* is the diverged paralog and it arose from an Aphidomorpha specific duplication. Phylogenetic analysis provides support for this observation (associated sequence alignments are presented in appendix figures B.6, B.7, B.8 and B.9), as protein sequences corresponding to *dnmt3a* homologs group with the majority of Dnmt3 proteins that were analysed, while the protein sequences corresponding to *dnmt3x* homologs form a distinct group with very long branch lengths.

4.3.1.2 *dnmt3a* and *dnmt3x* have been maintained in the genomes of Aphidomorpha species for a vast amount of evolutionary time

Following the observation of *dnmt3x* and *dnmt3a* being ubiquitously spread amongst the aphids, I used a chromosome level assembly of the pea aphid genome (pea_aphid_22Mar2018_4r6ur_v2 (GCF_005508785.2)) to explore the two paralogs in greater detail, including their arrangement and their wider genetic contexts as tools to further assess the nature of the duplication i.e. whether they are arranged in close proximity, which may suggest they share regulatory features or function (Gherman, Wang & Avramopoulos, 2009) or if they share flanking genes for similar reasons or because they arose from a large-scale duplication. I also explored the conservation of synteny between species for the same reasons.

A. pisum dnmt3a and *dnmt3x* are both found on the X chromosome (Genbank. XP_016662566, NC_042493.1; XP_029348651, NC_042493.1). *dnmt3a* consists of seven exons, and is found between 132292988 and 132291722 bp, spanning 1266 bp. *dnmt3x* consists of fifteen exons, and is found between 126095319 and 126070929 bp, spanning 24390 bp. Divergence in exon-intron structure between duplicate genes, in many cases, leads to diversification of function (Xu et al., 2012). *dnmt3a* and *dnmt3x* are separated by only 6,196,403 bp, making them relatively close duplicates within the context of the X chromosome which is the second largest of the four chromosomes possessed by pea aphids at 132,544,852 bp. Both *dnmt3a* and *dnmt3x* are therefore situated at the extreme end, *dnmt3a* being less than 1 % of the total bp away from the end, and *dnmt3x* less than 5 % away. The two genes immediately flanking each of the pea aphid *dnmt3* paralogs shared no identity with each other (appendix figure B.2). Additionally, the flanking genes were not shared between each of the orthologous within-genome paralogs, or between each of the *Ap-dnmt3* paralogs and their orthologue in *M. persicae* or *B. tabaci* (in the case of *B. tabaci*, only for *dnmt3a* orthologues owing to a lack of *dnmt3x* orthologue) (appendix figure B.3). Although, while pea aphid orthologues of the two immediate flanking genes of the *B. tabaci dnmt3a* are located on the pea aphid A1 chromosome, and those of the genes flanking the *M. persicae dnmt3a* orthologue are located on the *A. pisum* A2 and at distance on the X chromosome (at ~ 66 Mb), the *M. persicae dnmt3x* flanking gene orthologues are both located on the *A, pisum* X chromosome. Taken together, these results support the conclusion drawn from the phylogenetic analysis, that the duplication of the ancestral *dnmt3* occurred deep in evolutionary history. Additional chromosome level genome assemblies will allow investigation of if the appearance of *dnmt3a* and *dnmt3x* on the *A. pisum* X chromosome is consistent in further aphid species.

4.3.2 *dnmt3a*, *dnmt3x* and *vasa* mRNA colocalize in the germaria, oocytes, and early embryos, and the germ cells only of developing embryos once they have been specified

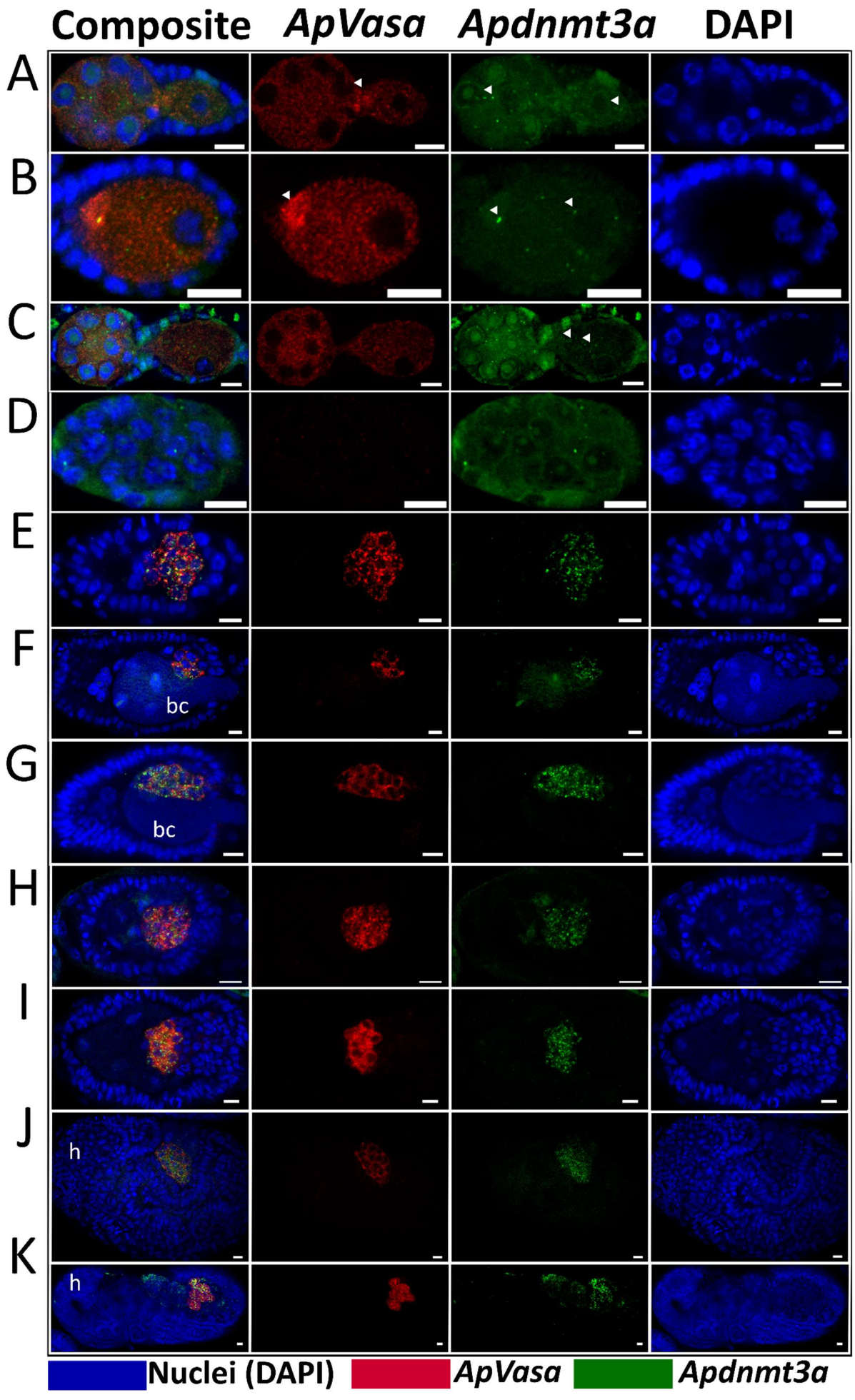
To examine potential roles for Dnmt3a and Dnmt3x in the ovary, I explored mRNA localisation using RNA-FISH HCR. Expression of both *dnmt3a* and *dnmt3x* consistently localise to the germ cells (figure 4.5, figure 4.6), which are identified by expression of the conserved germ cell marker, *vasa* (Chang et al., 2006, 2007). In the germaria, *vasa* mRNA is ubiquitously present and highly concentrated in the cytoplasm of nurse cells and prospective oocytes, and in the trophic core and cord (figure 4.5, a, b, c, figure 4.6, a, b). It is also markedly present in the early oocytes that have not yet separated from the germarium (stage 0, previtellogenic) and those that have just become pinched off from the germarium by follicle cells (but are still connected to the germarium by trophic cords, stage 1) (figure 4.5 a, b, figure 4.6, b); particularly, *vasa* mRNA is concentrated at the anterior of the oocyte (figure 4.5 a, b, figure 4.6, b, arrowheads). *dnmt3a* and *dnmt3x* mRNA is also detected, though weakly relative to *vasa*, in the germaria and in oocytes (figure 4.5 a, b, c, figure 4.6 a, b, c, arrowheads point to examples of punctate signal). The colocalisation of *vasa*, *dnmt3a* and *dnmt3x* in the germaria and oocytes can only be maternal provisioning.

vasa, *dnmt3a* and *dnmt3x* mRNAs are not strongly detected at stage 2 of development (during which the singular maturation division occurs), likely due to clearance of maternal mRNA. *vasa* mRNA is not detected again until stage 5 (by which point, the oocyte has developed into a syncytial embryo), where it is localised to the cytoplasm surrounding nuclei associated with the periphery (figure 4.5 d, figure 4.6 d, e). *dnmt3x* mRNA also localises to the cytoplasm of these nuclei, but is most strongly localised to the posterior end, where the germ cells will be specified. While an image for *dnmt3a* mRNA at stage 4 is missing, given the otherwise consistent pattern of expression between *dnmt3a* and *dnmt3x*, it is likely that *dnmt3a* is also localised to these nuclei. mRNA localisation to nuclei at the periphery may suggest maternal provisioning through the ovariole sheath (a structure which facilitates transfer of metabolites from the haemolymph to the embryos) (Bermingham & Wilkinson, 2009) and the more intense signal at the posterior end of both *vasa* and *dnmt3x* may be part of the maternally provisioned germ plasm. Two nuclei in particular, among the presumptive germ cells, appear to be slightly more enriched for *vasa* and *dnmt3x* (figure 4.6, d).

At stage 6, the germ cells, after cellularization during stage 5, are specified toward the posterior end of the embryo and separate the embryo into a central syncytium and posterior syncytium after they invaginate to the embryo's centre (figure 4.5, e, figure 4.6, f). From this stage onwards, through all embryonic stages (the bacterial mass invading, figure 4.5, f, g, germ band invagination, figure 4.5, h, i, and mid-late embryogenesis, figure 4.5, j, k), *vasa*, *dnmt3a* and *dnmt3x* mRNAs are restricted to the germ cells, and the intensity of *dnmt3a* and *dnmt3x* signal increases almost but not quite reaching the intensity of *vasa*. Thus, while *dnmt3a* and *dnmt3x* expression appears weak relative to *vasa* (which is highly expressed) in oocytes and embryos prior to specification of germ cells, once germ cells are specified expression of these genes increases.

The observed expression pattern therefore represents, in virginopara ovaries, persistent temporal and spatial colocalization of *dnmt3a* and *dnmt3x* with *vasa* to the germ cells of developing embryos, the germaria and early oocytes, and the cytoplasm surrounding nuclei of embryos just before germ cell specification at stage 6. This suggests a possible role for *dnmt3a* and *dnmt3x* in germ cell specification and/or function and oogenesis, facilitated by maternal provisioning (presumably, this is partially why expression appears to be so high in the germ cells of late-stage developing embryos, in which the germ cells will eventually form or have formed clusters, their germaria) and zygotic expression. The association between the two paralogs also suggests they are, at least in virginoparae, likely tightly linked in their function, or may exhibit functional redundancy. A linked function is consistent with the above genomic analysis, which demonstrated relatively close proximity of *dnmt3a* and *dnmt3x* on the *A. pisum* X chromosome, and conservation of these genes over vast evolutionary history.

To summarise, the linked expression of *dnmt3x* and *dnmt3a* in the ovary comes on early and stays on, encompassing essentially the entirety of a virginopara pea aphid's development and reproductive period. It is therefore likely that they are important to reproduction in the pea aphid, at least in the parthenogenetic mode in virginoparae.


Composite
ApVasa
Apdnmt3a
DAPI
A
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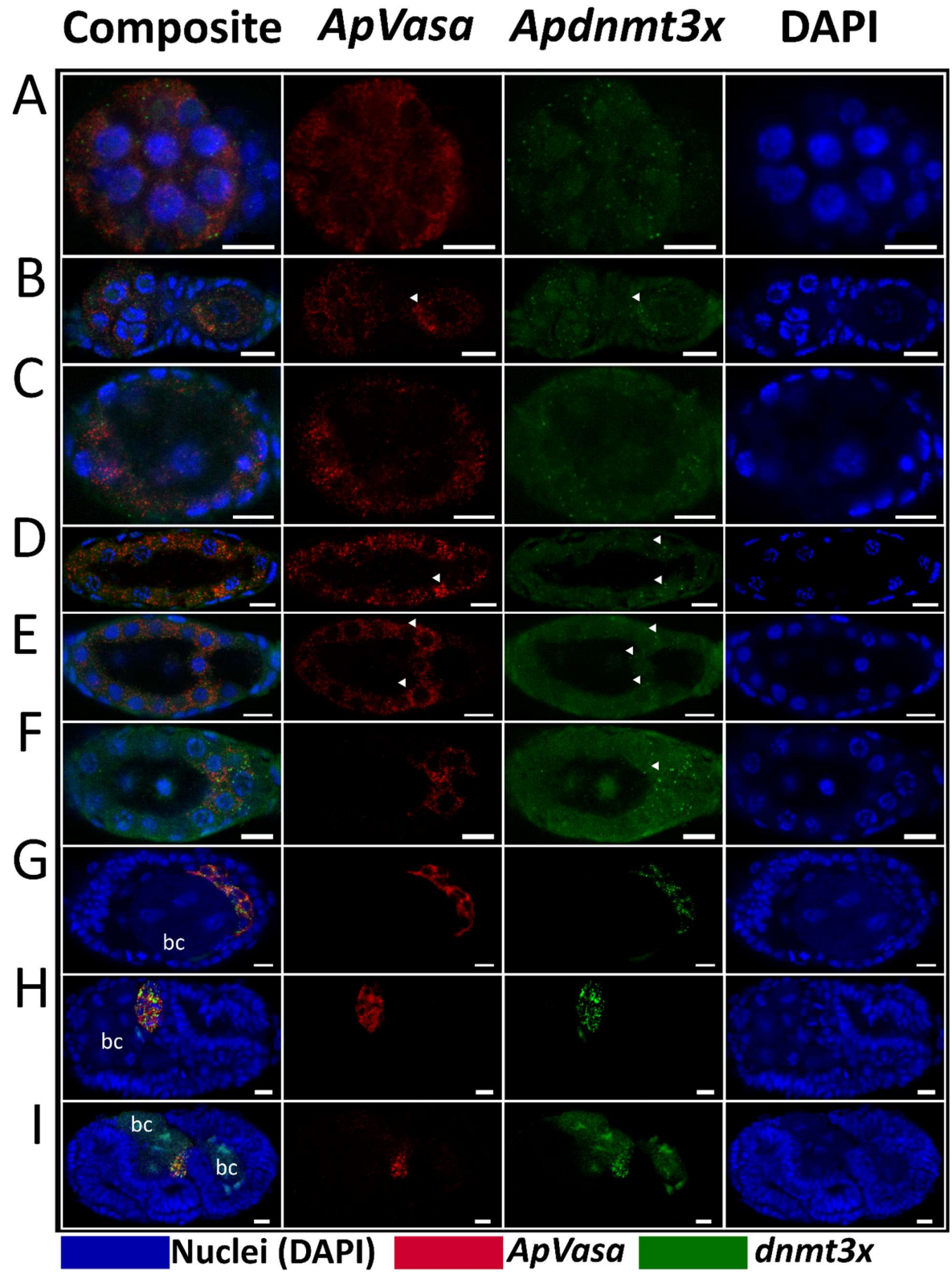
H
I
J
K
bc
bc
h
h
Nuclei (DAPI)
ApVasa
Apdnmt3a

Figure 4.5 Expression patterns of *vasa* and *dnmt3a*, generated by RNA-FISH HCR, and DAPI to visualise nuclei in parthenogenetic viviparous, long-day exposed *A. pisum* ovaries.
Images were taken using a Zeiss LSM880 confocal microscope in a single plane. Various stages of development, encompassing oogenesis (A-C) and early to late embryogenesis (D-K) are presented. Scale bars represent 10 μm. Staging (and order) based on Miura et al., 2003. A, germarium and late st0 oocyte; B, st1; C, germarium and st1; D, st3; E, st6; F, early st7; G, st7; H, st8; I, st9; J, st12, K, st17. *vasa* distinctly marks the germ cells only, beyond oogenesis and early embryogenesis (before the germ cells are specified), *dnmt3a* follows a similar pattern of expression. Individual channels are presented alongside a composite of all channels. Arrowheads are provided for emphasis of punctate signal in earlier stages, bc = bacteriocytes, h = head.


Composite
ApVasa
Apdnmt3x
DAPI
A
B
C
D
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F
G
H
I
bc
bc
bc
bc
Nuclei (DAPI)
ApVasa
dnmt3x

Figure 4.6 Expression patterns of *vasa* and *dnmt3x*, generated by RNA-FISH HCR, and DAPI to visualise nuclei in parthenogenetic viviparous, long-day exposed *A. pisum* ovaries.
Images were taken using a Zeiss LSM880 confocal microscope in a single plane. Various stages of development, encompassing oogenesis (A-B) and early to late embryogenesis (C-I) are presented. Scale bars represent 10 μm. Staging (and order) based on Miura et al., 2003. A, germarium; B, germarium and st1 oocyte; C, st2 oocyte; D, early st5; E, late st5; F, st6; G, st8; H, st10; I, st12. *Vasa* distinctly marks the germ cells only, beyond oogenesis and early embryogenesis (before the germ cells are specified), *dnmt3x* follows a similar pattern of expression. Individual channels are presented alongside a composite of all channels. Arrowheads are provided for emphasis of punctate signal in earlier stages, bc = bacteriocytes, h = head.

4.3.3 The DNA methylation machinery is important to reproduction in virginoparae

Following on from the clear expression patterns of *dnmt3a* and *dnmt3x,* I attempted to disrupt the DNA methylation machinery and assay effects in the ovary and on reproduction generally. To do so, adult aphids were exposed to 5-azacytidine, an inhibitor of DNA methylation that causes DNMTs to become irreversibly bound and degraded (Stresemann & Lyko, 2008).

4.3.3.1 Disruption of DNA methyltransferases causes defects in morphology and the organisation of germaria, oocytes and early, but not late embryos

After six days exposure to 5-azacytidine, based on visualisation of HCR prepared and phalloidin stained ovaries, germaria and early stage embryos and oocytes showed gross morphological deformities (figure 4.7, figure 4.8). In the germaria, the morphology was generally disturbed and the cells within them appeared somewhat disorganised (figure 4.7, a, a'). Ectopic tissue which stained positive for phalloidin (which reveals cortical actin) and *Ap-vasa* appeared in some cases, with few oddly located nuclei (indicated by DAPI staining) attached to germaria or embryos (figure 4.7, c, figure 4.8). The phalloidin signal suggests this may be cortical actin, though the structures are clearly not oocytes or embryos. Occasionally, a mass of nuclei associated with intense phalloidin signal was also observed (figure 4.7, figure 4.8), similarly to the ectopic membrane structure, but containing many nuclei. Additionally, an increased number of presumptive oocytes (which are distinct from the nurse cells owing to their much smaller nuclei) at the posterior end of the germaria were occasionally observed (figure 4.8). Arrested oocytes at the posterior end, seemingly failing to progress to stage 0 (follicle cells surrounding the oocyte) may account for some of the oddity in the appearance of the germaria.

Additionally, the disconnection between some adjacent early oocytes (stage 0, stage 1) appeared reduced, with follicle cells not pinching them off from each other or the germaria. In some cases, attached to the germaria appeared to be an uncharacteristically long series of multiple oocytes in what appeared to be a continuous follicle, with consistent follicle cells at the periphery. Together, these results are suggestive of 5-azacytdine (and thus, disruption of the DNA methylation machinery) disrupting oogenesis and early embryogenesis. Specification of oocytes and their subsequent separation from one another and the germarium that they arose from thus appears aberrant. Clearly, disruption of the DNA methylation machinery (though, broadly) leads to severe reproductive defects.

Early stage embryos (and/or oocytes of stage 2) were disturbed to the extent that, generally, confidently staging them was not possible. This was owing to mismatches between gross morphology, expected size and number and positioning of nuclei, and expression patterns of *vasa* and *wg* (a segment polarity gene, involved therefore in patterning, and for which a distinct expression pattern would be expected) (figure 4.7). For instance, *vasa* mRNA appeared intensely in embryos that were disorganised and substantially shorter than would be expected for an embryo with specified germ cells, and not following the expression patterns of earlier embryos/oocytes described in the previous section; additionally, intense *vasa* signal surrounding a few nuclei (presumed to be germ cells, situated posteriorly) occurs alongside disorganised signal in other parts of the embryo (figure 4.7, b, arrowheads). *Wg* expression was equally aberrant, seemingly occurring earlier than is typical and in a disorganised pattern; *wg* signal is visible throughout what appear to be relatively early embryos (figure 4.6, a', a''), when it would be expected to be tightly confined to the posterior and only in relatively larger and later stage embryos. These results taken together suggest disorganisation of cell specification (given the odd expression pattern of *vasa*) and possibly segmentation (given the disruption to *wg*, a segment polarity gene) and odd morphology. Many embryos had an unusually high number of nuclei for their size and shape, suggesting a disruption to cell division, or morphological defects. The disorganisation of *vasa* signal, especially, suggests disorganisation of germ cell fate specification. This, taken together with the expression patterns observed in the previous section, is consistent with a potential role for *dnmt3a*/*dnmt3x* in proper specification of the germ cells. The general disorganisation of embryos and apparent disruption of oogenesis further suggests possible integral roles in oogenesis and embryogenesis for *dnmt3a* and/or *dnmt3x*, and/or possibly *dnmt1*.

While early embryos, oocytes and germaria were distinctly and extensively disturbed, embryos in the later stages of development (from stage 7, at which point the germ cells are specified and the bacterial mass invades from the mother's body cavity, onwards) appeared typical, with *vasa* expression, morphology, size and arrangement appearing consistent with expectations and control samples, and germ cells forming clusters (figure 4.7 d). The same phenotype of disruption of early development was also observed after two days of treatment with 5-azacytidine. This suggests that the effects of disruption are quick acting and further implicates DNMTs as being important for oogenesis and early embryogenesis.

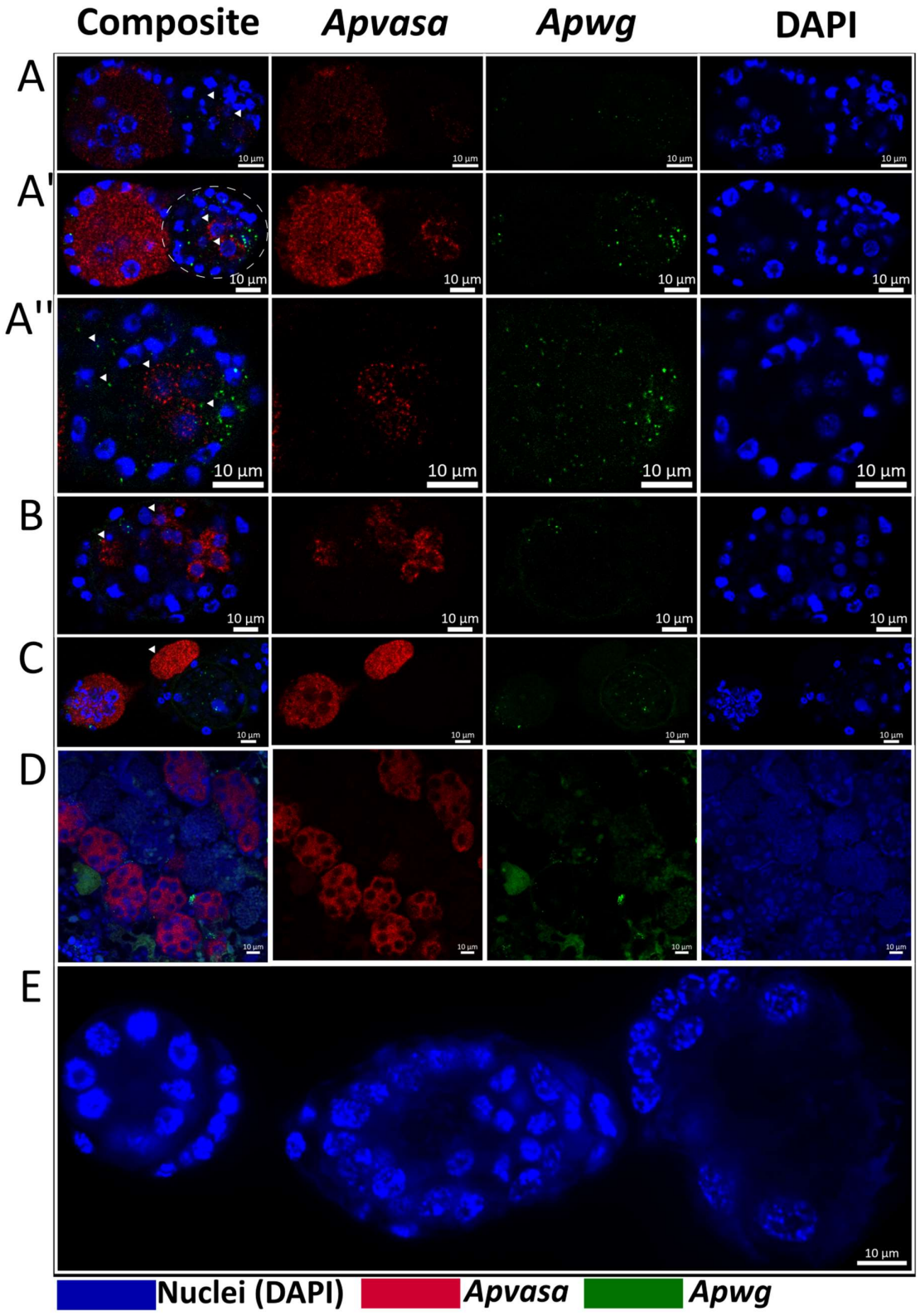
Composite
Apvasa
Apwg
DAPI
A
A'
A''
B
C
D
E
10 µm
Nuclei (DAPI)
Apvasa
Apwg

Figure 4.7 Representative images of various developmental stages from ovaries of *A. pisum* viviparae treated with 5-azacytidine, an inhibitor of DNA methyltransferases.
Each row of images represents a single plane of focus, divided into a composite image followed by three individual channels, which are HCR derived *vasa* signal, HCR derived *wg* signal, and DAPI, which stains nuclei. 5-azacytidine treatment led to severe defects of morphology and disruption of expression pattern. A, A' and A'' show early defects associated with oogenesis/early embryogenesis, as the morphology and size of the embryo does not match the expected staining of *vasa* or *wg*, and the number of nuclei is disrupted for the size. B similarly reveals decoupling of morphology, nuclei and staining patterns. C indicates a case consistent with A and B, and additionally presenting an example of extra regions of staining that appear to be beyond the bounds of the embryo. D is a close image of a late-stage embryo, with focus on the germ cell clusters which appear typical and have formed rosettes; late-stage embryos were not disturbed. E shows slightly odd morphology of a mid-stage embryo at the posterior end (right), but otherwise typical ovaries. Scale bars represent 10 μm. Arrowheads indicate disrupted elements.

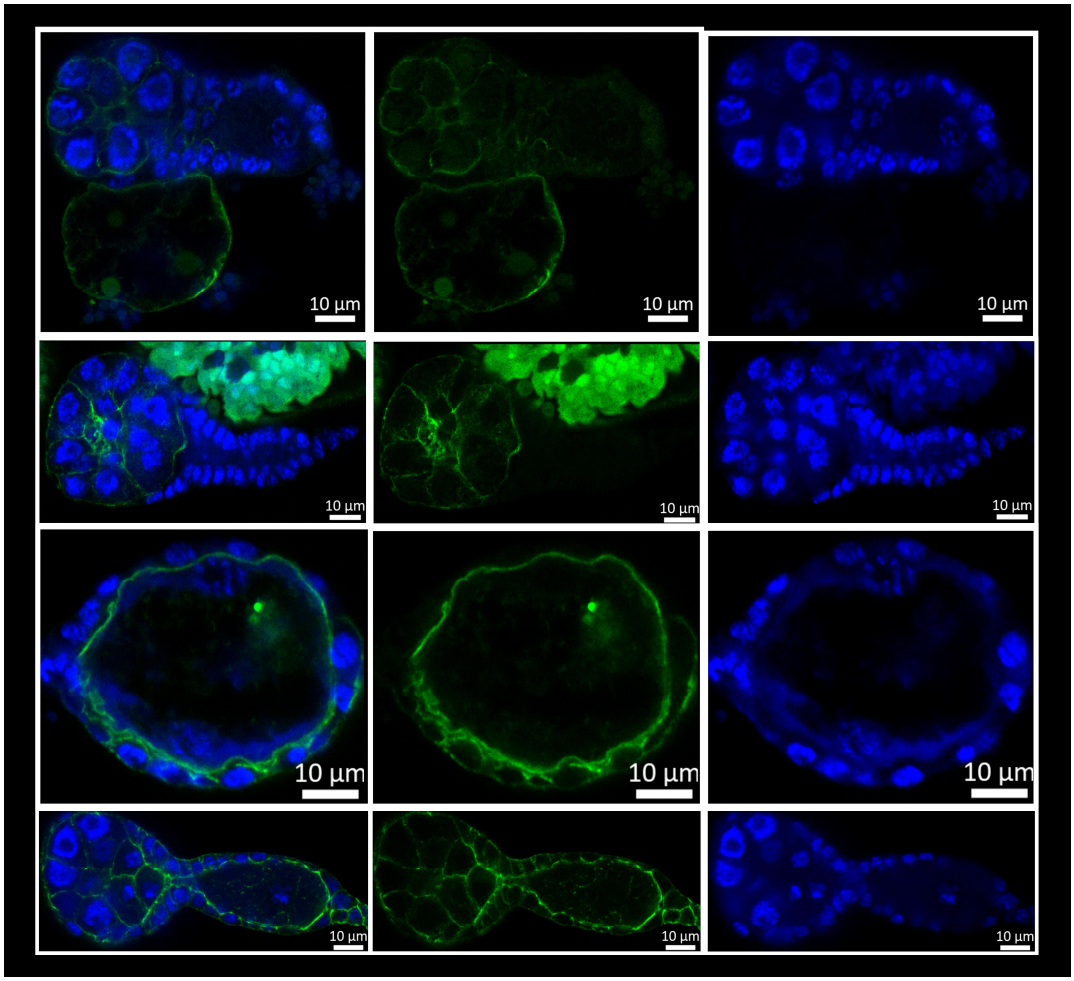

Figure 4.8 Representative images of *A. pisum* ovary samples stained with phalloidin (stains actin, green, middle) and DAPI (stains nuclei, blue, right) after aphids were treated with 5-azacytidine, a disruptor of DNA methyltransferases.
Images are single plane of focus per row, and individual channels are presented alongside composite (left). The top row shows an unusual structure, positive for actin and appearing ectopic to the germarium and oocyte, possibly a mass of cortical actin. The second row shows two adjacent oocytes and the germarium, the oocytes appear odd in that they do not appear to have separated from eachother, and form a long continuous chain. The third row shows an embryo with an odd size and morphology for the number of nuclei. The bottom row shows an oocyte/embryo adjacent to the germarium, though its morphology and nuclei number do not match expectations. Scale bars represent 10 µm.

4.3.3.2 Disruption of DNA methyltransferases affects reproduction and offspring phenotype

Disruption of the DNA methylation machinery with 5-azacytidine caused disturbance in the ovaries and developing embryos of virginopara aphids, but it was not clear exactly the effect this might have on reproductive output and the status of the nymphs produced from these affected ovaries. To investigate the

effect on reproduction, aphids exposed to 5-azacytidine or control treatments for six days were assayed for reproduction and lifespan.

While having equal lifespans (Cox proportional hazards model, $p = 0.7$, appendix figure B.1), 5-azacytidine treated aphids produced significantly fewer nymphs per day, on average, once reproductively active, than control aphids over a twenty day period (GLMM, LRT: $\chi 2 = 8.7787$, $p = 0.003048$, $df = 1$; treatment: $z = 3.828$, $p = 0.000129$; figure 4.9, b). The time from L4 to reproduction was approximately consistent between treatments (5-azacytidine mean first reproductive day = 3.71, control mean first reproductive day = 4.2, Wilcoxon rank sum test: $W = 25.5$, $p = 0.3531$, figure 4.9, a); as this delay accounted for progression from the L4 nymph stage to adulthood, the delay to adulthood is inferred to also be consistent. The greatest difference in nymph production was observed between days twelve and sixteen after initiation of treatment (therefore, given the ~ four day delay from initiation of treatment, which lasted for 6 days to onset of reproduction, this period occurred approximately between eight and twelve days after the onset of reproduction). On day sixteen in particular, 5-azacytidine treated aphids collectively produced zero offspring, while control aphids continually reproduced. This period of particularly reduced reproductive output partially coincided with the production, by 5-azacytidine treated aphids only, of nymphs that were stillborn or died shortly after birth (figure 4.10) (azacytidine treated aphids produced significantly more of these nymphs than did DMSO-control aphids, Wilcoxon rank sum test, $W = 3995$, $p = 0.001441$; figure 4.10, a). These nymphs were produced during days twelve to fourteen (spanning three days), but mostly on day twelve (six days after the cessation of treatment) (figure 4.10, b). Control treatment aphids produced only typical nymphs, and either maintained a roughly equal rate of production or an increased one, during the same period. Whether they were born dead or died shortly after, these disturbed nymphs failed to extend their legs away from their bodies (new-born nymphs, ordinarily do this immediately). The failure to extend their legs may indicate a failure of an extraembryonic membrane to rupture, possibly associated with incomplete/absent eversion during katatrepsis (the embryo flipping undergone by embryos of hemimetabolous insects) (Panfilio, 2008), although, births were not directly observed, so whether or not some embryos did emerge un-flipped I am unable to say. 71 % of all 5-azacytidine treated adults (that survived to reproduce during the period in which disturbed nymphs were produced, ($n = 7$)) produced at least one disturbed nymph, with each producing up to four in total. Outside of these disturbed nymphs, the offspring of 5-azacytidine treated aphids appeared outwardly typical.

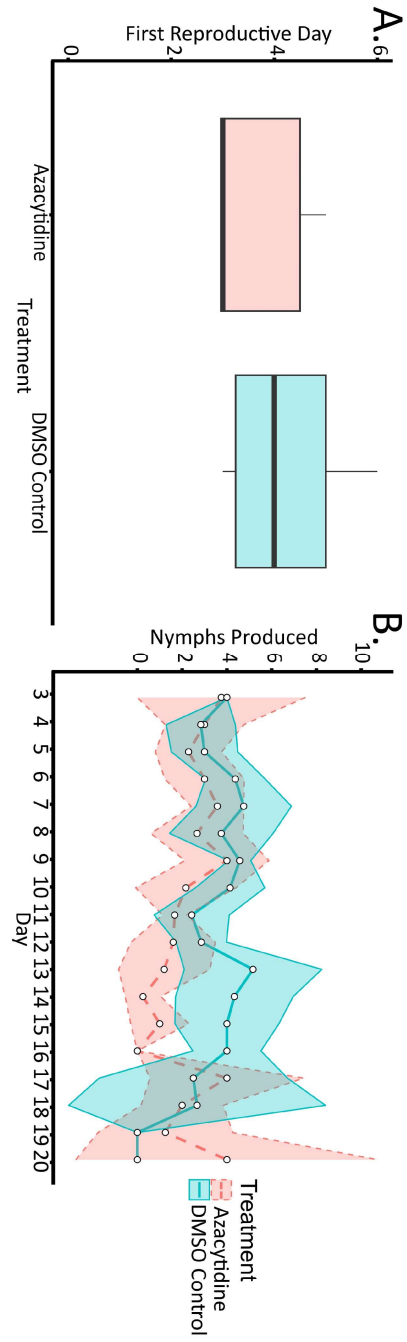

Figure 4.9 Reproductive phenotypes in response to treatment of *A. pisum* with 5-azacytidine (50 um).
Treatment started on day 0 with L4 nymphs, and continued until day 6. A. Boxplots representing statistics for the first day reproduction was observed (with daily monitoring) for 5-azacytidine and DMSO control exposed groups. 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, several individuals had died, durastically reducing the sample size). Points represent mean nymphs produced by all individuals of a treatment, and ribbons indicate 95 % confidence intervals.

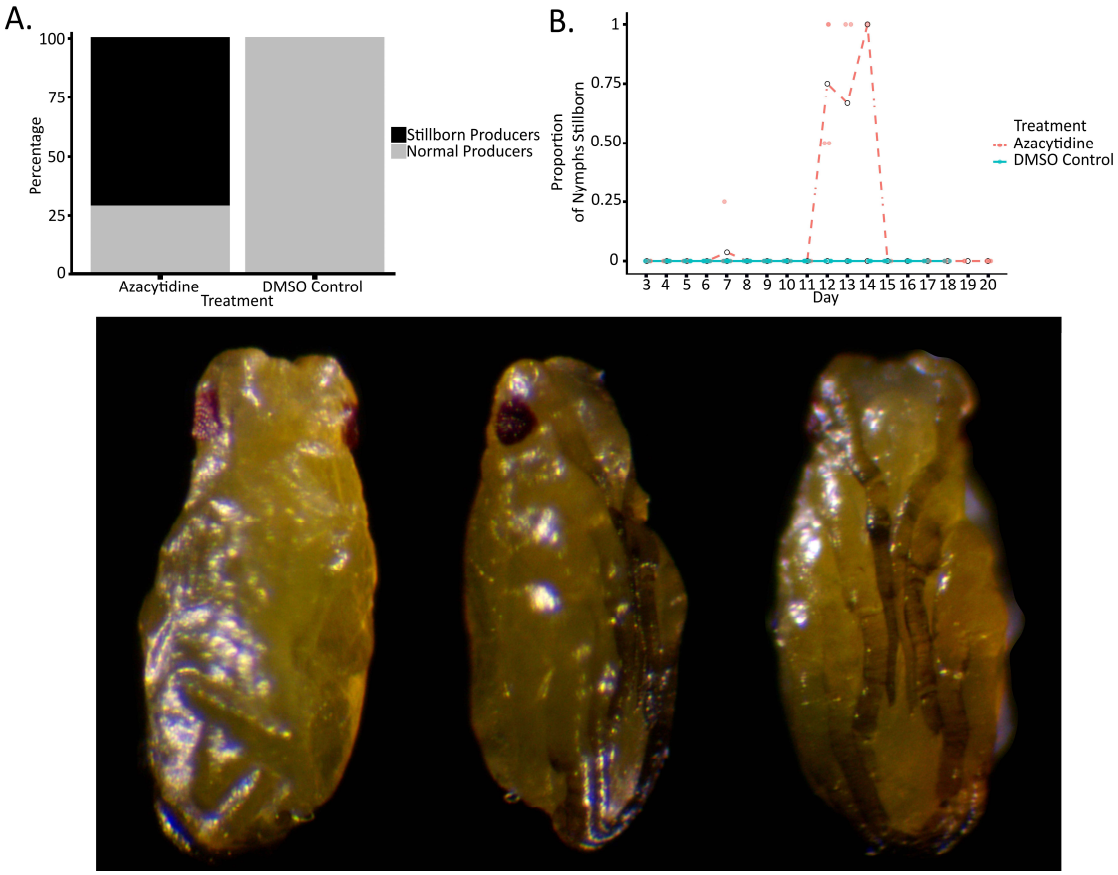

Figure 4.10 Late reproductive phenotypes of 5-azacytidine treated *A. pisum*.
A. Percentage of all 5-azacytidine (50 um) treated and control (DMSO) aphids that produced 'stillborn' (highly disturbed, dead at birth or shortly after birth, though morphogically typical) aphids after treatment from day 0 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. The bottom panel is an image of a stillborn nymph, the time between birth and photographing is not clear, but is less than 24 h. The legs are close to the body and have failed to be extended.

It is important to note that, while reproduction slowed down during the period where disturbed nymphs were produced, it generally did not stop completely (figure 4.9). And, where more than one disturbed nymph was produced by any single adult, they were produced in chains uninterrupted by typical nymphs (except for one disturbed nymph produced by a single aphid on day seven, the only disturbed nymph to occur outside of days twelve to fourteen). The uninterrupted nature of the production of disturbed nymphs is suggestive of a specific stage or stages where this phenotype is realised, rather than it being a result of general disruption to developmental processes throughout development, occurring somewhat randomly. Following the period of supressed

reproduction, and reproductive inactivity (on day sixteen), reproductive output rebounded, at day seventeen, to an average level comparable to those before the onset of repressed reproduction, and comparable to that of control treated aphids (figure 4.9). That reproductive output was able to recover to normal levels suggests that the effects of 5-azacytidine are not permanent and likely therefore do not involve major damage to the germaria. It is also important to note that while treatment occurred for six days, disturbed nymphs appeared over only three days. A timeline of phenotypes is presented in figure 4.11.

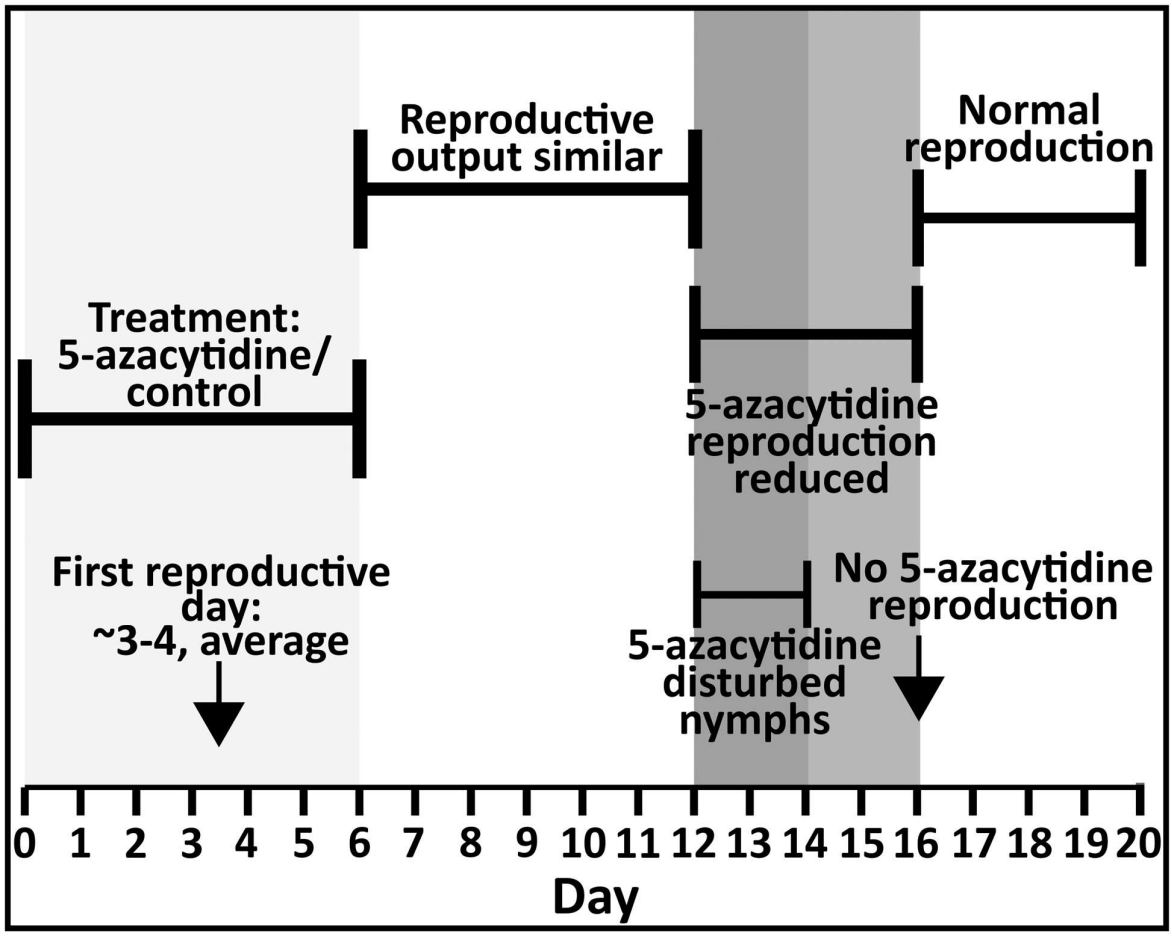

Figure 4.11 Schematic of the effects of 5-azacytidine treatment (50 um) on *A. pisum* reproduction.
Age-matched aphids were treated from day 0 with either 50 μm 5-azacytidine or DMSO as a control, from the L4 nymph stage, by ingestion through artificial diet, and were fed daily until day 6. Reproductive output was assessed over twenty days.

To inspect for effects on otherwise healthy appearing nymphs, representing transgenerational effects, the survivability of nymphs produced by 5-azacytidine treated and nymphs produced by control treated aphids were compared. There was no significant difference between the overall survival of each group (proportion of total nymphs dead, Wilcoxon, $W = 49$, $p = 0.1874$). After rearing these nymphs to adulthood and allowing them, as pooled groups of sisters, to reproduce, the reproductive capacity of the offspring (aside from those produced during the feeding period, days three/four to six) of treated aphids was assessed, though, on a very coarse scale as the pools consisted of individuals produced pre-reduced-reproduction-period, mid-reduced-reproduction-period and post-reduced-reproduction-period. Though, given that the earliest adults were likely exposed to 5-azacytidine in the ovaries of their mother from mid to late development, while they were not obviously affected themselves, this likely would have (owing to the telescoping of generations) affected their ovaries in the same way as it did their mothers, as the oocytes and embryos contained within them would be at the early stages of development. This encompasses a good proportion of the individuals (produced between days seven to fifteen). Importantly, and somewhat surprisingly, disturbed nymphs were not observed among the offspring of these aphids, in either treatment.

Collectively, these results suggest important roles in reproduction for DNMTs, the disturbance of which leads to a reduction in reproductive output to the extent of ceased reproduction (albeit for a short period), along with a role in development, in particular, mid to late embryogenesis, as indicated by the presence of developed but stillborn/disturbed embryos. Additionally, the observation of a period of typical reproduction, followed by a period of repressed reproduction and production of disturbed nymphs, is consistent with observations made of 5-azacytidine treated ovaries (section previous), in which oogenesis and early embryogenesis, but not late stage embryos appeared disturbed.

4.4 Discussion

The role of DNA methylation and the machinery that underpins it in parthenogenetic viviparous reproduction, which is exhibited by all aphids, and its potential role in cyclical parthenogenesis, an example of polyphenism, are as yet unknown. Here, I investigated the distribution of *dnmt3* homologs amongst the aphids and insects generally, revealing an ancient duplication of *dnmt3* in an ancestor of the Aphidomorpha, leading to two copies which are co-

expressed spatially and temporally in the ovary. I also provide evidence for a role of the DNA methylation machinery in viviparous reproduction in *A. pisum*.

The presence of orthologs to both *dnmt3a* and *dnmt3x* across the diversity of the aphids, and in the genome of *D. vitifoliae*, a Phylloxera (one of two sister groups to Aphididae), and in the genome of *A. cooleyi*, an Adelgid (the other sister group of the Phylloxera and Aphididae) suggests that the duplication of the ancestral *dnmt3* occurred before the divergence of these groups, thought to have occurred at least ~ 150-200 million years ago, and is thus specific to the Aphidomorpha (Ortiz-Rivas & Martínez-Torres, 2010; Ren et al., 2013). While orthologs of *dnmt3a* were present across some of the diversity of insects (though, having also been secondarily lost fairly frequently, as has been reported (Bewick et al., 2017; Duncan, Cunningham & Dearden, 2022). The notable absence of a *dnmt3x* orthologue in the genome of *B. tabaci* (the most close relative of the Aphidomorpha assessed here, another member of the Sternorrhyncha suborder) provides support for confinement of *dnmt3x* to the Aphidormopha. It is also apparent that *dnmt3a* is more consistent with the ancestral copy, and *dnmt3x* is the more derived duplicate copy (figure 4.3).

Cyclical parthenogenesis is thought to have evolved in the Aphidomorpha, before divergence of the three sister groups over ~ 150-200 million years ago (Davis, 2012; Ortiz-Rivas & Martínez-Torres, 2010; Ren et al., 2013), and apart from where it has been secondarily lost, is the reproductive strategy deployed by all known aphids. Viviparity in the asexual phase, however, arose in the Aphididae lineage (true aphids). Phylloxera and Adelgids reproduce exclusively by oviparous cyclical parthenogenesis (Davis, 2012). Because the duplication of *dnmt3* appears to have occurred in an ancestor of these three groups, while the duplication may not have facilitated uptake of viviparity in the parthenogenetic phase, it may be involved in facilitating cyclical parthenogenesis. As an evolutionary novelty of the Aphidomorpha, cyclical parthenogenesis being associated with a gene duplication would not be surprising – duplication being a key mechanism by which novelty can occur (Long & Langley, 1993), and one that has been suggested to have played a major role in the unusual biology of aphids (Shigenobu & Yorimoto, 2022), a group that is seemingly enriched for gene duplication events (Julca et al., 2020).

Not only do *dnmt3a* and *dnmt3x* appear to have been maintained together in the genomes of Aphidomorpha species over vast evolutionary time (no cases of just one or the other copy were detected, convincingly, in the Aphidomorpha), spanning over ~ 150-200 million years, they are also maintained in *A. pisum* on the X chromosome (the chromosome locations of these genes in the genomes

of a greater diversity of aphid species will be important to determining if their being on the X chromosome is consistent). The pea aphid X chromosome is particularly mutable, though generally chromosome arrangement and gene content is consistent, and it appears to be incredibly duplication prone relative to the autosomes (Li, Zhang & Moran, 2020; Mathers et al., 2021). Among the four chromosomes, the X harbours the most within chromosome paralogs and a large frequency of ancestral duplications. The mutability of the X chromosome may be partially due to relaxed purifying selection, as a result of its being masculinized (Jaquiéry et al., 2022) and therefore many of the genes being rarely expressed (as the males, along with the oviparous females, appear for only a very brief period each year) and thus shielded from selective pressures. Generally, *A. pisum* X-linked genes are expressed at low levels, which may again be partially because of masculinization of the X chromosome (Jaquiéry et al., 2022). Potentially tied to the low expression levels, X-linked genes generally show faster evolution of proteins compared to autosomal genes, because if the genes are not expressed at appreciable levels or are rarely expressed, they may be under relaxed selection, allowing mutation to be maintained more frequently and thus leading to protein evolution (Li, Zhang & Moran, 2020; Jaquiéry et al., 2018). This is consistent with diversification of *dnmt3a* and *dnmt3x* from each other, but the protein sequence homology between *dnmt3a* orthologues with other *dnmt3a* orthologues, and *dnmt3x* orthologues with other *dnmt3x* orthologues across aphid species was generally high (appendix table B.3, B.4). If the observations made of the *A. pisum* X chromosome, that they are more mutable, are true of aphids more generally (and *dnmt3a* and *dnmt3x* are located on the X chromosome of other aphids), then this is suggestive of increased pressure toward conserved amino acid sequences of *dnmt3a* orthologues and of *dnmt3x* orthologues among aphid species. Maintenance of *dnmt3a* and *dnmt3x* and conservation of their proteins over evolutionary time as vast as this is indicative of likely important roles for both paralogs, which appear to be diverged in their domain structure. The expression of *dnmt3a* and *dnmt3x* on the X chromosome also suggests, given the general pattern of the X, it being enriched for genes relating to the sexual morphs, that these genes may be involved in the switch to production of sexuals, or important to the identity of the sexuals.

Additionally, duplications are, generally, enriched on the *A. pisum* X chromosome in the region between 90 and 130 Mb, where a high frequency of transposable elements have also been reported (Li, Zhang & Moran, 2020; Mathers et al., 2021). Perhaps it is not surprising then that *dnmt3a* and *dnmt3x* are both located in this region, towards the far end of the chromosome. They are separated from each other by only ~6 Mb. Given the age of the duplication, this close proximity is noteworthy as it is likely an indication of pressure to maintain them close together. There are several possibilities for why they may have been maintained close together, including being functionally related (Weber & Hurst, 2011; Cera et al., 2019) or being co-regulated by shared transcription factor(s) at shared gene regulatory regions (Ibn-Salem, Muro & Andrade-Navarro, 2017). The close proximity of *dnmt3a* and *dnmt3x* taken together with the temporal and spatial colocalization of their mRNAs in the virginopara *A. pisum* ovary, makes one or more of these possibilities likely. Although, the 6 Mbs separating *dnmt3a* and *dnmt3x* makes shared regulation less likely; among the most well characterised long-range enhancers, is an enhancer for the mouse gene, *Shh*, the regulatory region of which spawns ~ 1 Mb (Kane et al., 2022). Gene duplication typically leads to production of pseudogenes or redundant genes, which are removed from the genome or silenced, over time (Birchler & Yang, 2022). The vast evolutionary time over which the ancestral copy (*dnmt3a*) and the derived copy (*dnmt3x*) have been maintained, especially given their positioning on the mutable X chromosome, therefore suggests that both copies are likely important and functional, and that (taking into account the divergence in domain structure) sub- or neo-functionalization is likely to have occurred.

Generally speaking, in insects, *dnmt3* genes appear to be highly dispensable. Many studied insects rely solely on *dnmt1* for DNA methylation, and while the presence of DNA methylation in insects is correlated with the presence of *dnmt1*, it is not correlated with *dnmt3*. Many species exhibit expanded *dnmt1* gene repertoires, but *dnmt3* has been recorded relatively rarely to be duplicated (Bewick et al., 2017; Duncan, Cunningham & Dearden, 2022). This is likely, in small part, due to the fact that *dnmt3* is less likely to be present at all. For example, Kucharski and colleagues (Kucharski et al., 2023) analysed 107 Hymenopteran genomes, and found that most species had between one and

three *dnmt1*s, but that *dnmt3* was single copy except in a few cases where it was lost (e.g. some paper wasps and some Braconid wasps), and a single family of parasitoid wasps, the *Dryinidae*, where it was duplicated. This raises two interesting points. The first, because Dnmt3 is clearly more dispensable in terms of DNA methylation (possibly indicating some conserved feature of Dnmt1 which means Dnmt3 is not integral to DNA methylation), this may have led to a relaxation of selection pressure on any methylation functionality of Dnmt3. This relaxation may have facilitated the acquisition of novel functions, for example, roles in oogenesis and embryogenesis, as explored here. The second, highly related point, in cases where *dnmt3* has been maintained, what selective pressures are there for this to happen, especially where duplications have occurred and then also been maintained, such as in the case of the Aphidomorpha. Duplication has likely released *dnmt3x* from selective pressure towards its ancestral function, and it may have developed a new function either through neofunctionalization or subfunctionalization with *dnmt3a* (to divide the ancestral function), or a combination of both (Mehlferber et al., 2017; Birchler & Yang, 2022). The apparent separation of the catalytic methyltransferase domain from the PWWP and ADDz domains into *A. pisum* Dnmt3a and Dnmt3x respectively, is suggestive of some amount of subfunctionalization; though, as I have already discussed, this may be of functions of the ancestral Dnmt3 that are not related to DNA methylation (or DNA methylation only).The Aphidomorpha are exemplary cases of evolutionary innovation, and duplications have been suggested to be linked before to the novelties observed in aphid biology (Davis, 2012), thus, taking everything together, there is a possibility that duplication of *dnmt3* relates to cyclical parthenogenesis or some other atypical aspect of aphid biology.

While Dnmt3a is presumed to be functional as a DNA methyltransferase, and indeed, is predicted to possess the catalytic methyltransferase domain, *dnmt3x* has been suggested to be non-functional as a DNA methyltransferase (Walsh et al., 2010), and my analysis confirmed that it is not predicted to possess the catalytic domain necessary for it to be. The PWWP domain, which is typical of DNA methyltransferases and facilitates interactions with histones (typically, binding preferentially to particular methylated histone lycine residues, depending on species) and DNA and the ADD domain (which, similarly, binds to

unmethylated H3K4) appears absent from *dnmt3a* (Otani et al., 2009; Dhayalan et al., 2010; Kibe et al., 2021; Uehara et al., 2023). *dnmt3x* on the other hand, does appear to possess both a PWWP and an ADD domain. The appearance of the PWWP and ADD domains in *dnmt3x* is noteworthy because this suggests Dnmt3x, while not functional as a DNA methyltransferase, may still be able to interact with DNA and with histones (and thus interact with an epigenetic mark beyond DNA methylation), and mediate interactions by other proteins (Zhang et al., 2010). As histone methylation marks are known to guide DNA methylation by DNA methyltransferases, facilitated by the PWWP and ADD domains, this may mean that Dnmt3x is able to direct the activity of Dnmt3a. Direction by Dnmt3x of Dnmt3a could be to modulate DNA methylation, as is true (in some cases, recent evidence pointing towards in oocyte methylation in some groups of rodents (Behluli et al., 2023)) for the mammalian Dnmt3L (though Dnmt3L has an ADD domain, but not a PWWP domain), which is also catalytically inactive but necessary for DNA methylation, meaning a possible non-direct role in DNA methylation for Dnmt3x (Bourc'his et al., 2001; Kaneda et al., 2004). To this end, subfunctionalization of the ancestral Dnmt3 function across Dnmt3a and Dnmt3x may have occurred, consistent with the conserved domains of the ancestral Dnmt3 appearing to be split across these two paralogs. Alternatively, Dnmt3x might direct Dnmt3a to DNA to carry out some function not-related to DNA methylation, or, Dnmt3x itself might associate with DNA independently of Dnmt3a to carry out another role. For instance, Dnmt3x might act as a regulator of transcription by binding to sites, guided by PWWP and ADD, and thereby blocking access by transcription factors or epigenetic modification enzymes. An example of a DNA methyltransferase acting to regulate transcription, independently of catalytic activity, has been demonstrated in Dnmt1 in *X. laevis* embryos (Dunican et al., 2008). One or multiple of these possibilities may explain the role of Dnmt3x.

Additionally, it has recently been observed that in Hymenoptera, Dnmt3s ubiquitously possess a duplicate of the PWWP domain, and that the PWWPs in these cases have diversified to be able to bind to H3K27 (Kucharski et al., 2023). This novelty in Hymenoptera may be linked to evolutionary novelties observed in this group, such as those facilitating aspects of eusociality, and importantly, presents a further example of an unusual *dnmt3* system, in a group

that presents a textbook example of phenotypic plasticity (Simpson, Sword & Lo, 2011; Yang & Andrew Pospisilik, 2019), especially considering that *dnmt3* has been suggested to play a role in caste determination in hymenopterans, e.g. *A. melifera* (Kucharski et al., 2008). Characterising the aphid *dnmt3x* PWWP domain will further elucidate if PWWP is facilitating some evolutionary innovation in either case, and likewise for the ADD domain. Based on conserved domains, *dnmt3a* may be fully capable of carrying out DNA methylation on its own, while *dnmt3x* certainly would not; although, this does need to be functionally determined. Taken together, these observations further raise the question on the nature of the functions of proteins encoded by *dnmt3a* and *dnmt3x*. And of the functions of Dnmt3s in insects more generally.

Interestingly, in the crustacean, *Daphnia magna*, two *dnmt3* paralogues have also been identified (Nguyen et al., 2020). The proteins of one of which, Dnmt3.2, appears to have a functional methyltransferase domain and an ADD domain, but no PWWP domain. While the protein of the other, Dnmt3.1, possesses a diverged methyltransferase (catalytic) domain, which appears to be missing catalytic functionality, but does possess a PWWP domain and ADD domain. The similarities between this system and that of Aphidomorpha are obvious, with *Dapma*Dnmt3.1 being similar to *dnmt3x* and *Dapma*Dnmt3.2 to *dnmt3a* in terms of structural domains. *Daphnia* and the Aphidomorpha are separated evolutionarily by ~ 500 million years (Misof et al., 2014) and many insects possessing sometimes one, or frequently no orthologues of *dnmt3* (Provataris et al., 2018). Therefore, the duplications of *dnmt3* in these groups undoubtedly represent a case of convergent evolution, though whether they are functionally linked I cannot say within this chapter.

dnmt3a and *dnmt3x* mRNA localize temporally and spatially in the virginopara *A. pisum* ovary. The presence of their mRNAs in the germaria, trophic cords, oocytes and germ cells of embryos suggests they are maternally provisioned, and they may therefore be integral to early development. This is supported by the marked disruption of oogenesis and early embryogenesis seen in 5-azacytidine treated aphids, whereby oocytes appeared to be stalled (leading to backed up chains of oocytes) and early embryos were disturbed morphologically and developmentally to the extent of not being able to confidently stage them. While 5-azacytidine is non-specific in which DNA

methyltransferases it interacts with, affecting Dnmt1s and Dnmt3s due to its mechanism of action being activation and incorporation into DNA in place of cytosine (and thus becoming the target substrate for DNA methyltransferases) (Stresemann & Lyko, 2008), the overlap between the expression pattern of *dnmt3*s in the ovaries, and the defects observed resulting from 5-azacytidine treatment leads me to suggest that the observed defects are at least partially due to disruption of Dnmt3a and/or Dnmt3x. In order to truly test the function of each *dnmt3* paralog, it will be necessary to isolate them from each other and from *dnmt1*, and to test for any interaction between them (by also targeting *dnmt3a* and *dnmt3x* together), by using RNAi or CRISPR-Cas9 (both of which have been demonstrated to be useable tools in aphids and the pea aphid specifically, though they are not always tractable (see chapter 3, (Le Trionnaire et al., 2019, 2022)) to target them specifically. For reasons I discussed in chapter 3, it was not possible to use RNAi here to assess more specifically, the functions of *dnmt3a* and *dnmt3x*, though doing so will likely be integral to understanding the functions of these genes, and revealing their putative roles in reproduction and possible roles in cyclical parthenogenesis; perhaps use of a more RNAi-conducive strain will facilitate functional analysis (as explored in chapter 3). It will also be useful to confirm if the two Dnmt3 paralogues are able to interact with cytosine (as this is the mode of action of 5-azacytidine, and thus, for this treatment to directly affect Dnmt3a and Dnmt3x, it is necessary that they target cytosine), and if they alone (likely only in the case of Dnmt3a) or together (Dnmt3a and Dnmt3x, together reconstructing the ancestral Dnmt3 in terms of domains) are able to carry out DNA methylation; DNA methylation assays (e.g. ELISA) after transgenic expression and purification of Dnmt3a and Dnmt3x, or assessment of methylation levels, by MeDIP or Nanopore sequencing after knockdown of either and both of *dnmt3a* and *dnmt3x* present means of assessing for these traits. In addition to expression in the germaria, oocytes and early embryos, more concentrated *dnmt3a* and *dnmt3x* mRNA (along with *vasa* mRNA) signal was observed in a few cells at the posterior end of the stage 5 embryo, where the germ cells are specified in the subsequent stage. This suggests that they may be provisioned as part of the germ plasm, which is involved in specifying the germ cells, and that they might play a role in germ cell specification (at least, they are associated with germ cell identity).

Whether or not the roles of *dnmt3a* and/or *dnmt3x* in the ovary are facilitated by methylation is not clear. Importantly, clear roles for DNA methyltransferases beyond methylation of DNA are emerging in insects. Roles for Dnmt1 in oogenesis, spermatogenesis, embryogenesis, and subsequently, reproduction have been reported in the large milkweed bug (*O. fasciatus*), independently of DNA methylation (Bewick et al., 2019; Washington et al., 2021; Cunningham et al., 2023). In *T. casataneum*, where very low or absent levels of methylation are reported (Zemach et al., 2010), *dnmt1* is upregulated in the ovaries (Schulz et al., 2018). Maternal knockdown of *dnmt1* in this system leads to early arrest of development. Similarly, in *B. tabaci*, *dnmt1* RNAi led to reduced fecundity and egg viability, disturbed follicular cells (which support oogenesis) and lack of localisation of Dnmt1 to oocyte nuclei, in the ovary (Shelby et al., 2023). Again, global DNA methylation levels were not different between *dnmt1* RNAi samples and controls. Similarly, in a study looking at the effects of 5-azacytidine on the crustacean, *D. magna*, the authors observed reduced fecundity (though, cumulative) after seven days of exposure, linked to a reduction in expression of *vitellogenin* (which is a key factor in providing nutrition to developing embryos, by loading the yolk), though this was associated with a global reduction in methylation (they did not inspect methylation in the ovary specifically) (Lindeman et al., 2019). This example is noteworthy because it expands the link between disturbed DNA methylation machinery and effects on reproduction to beyond the insects (though, still relatively closely related, all falling within the pancrustacea) and explores the use of 5-azacytidine in adults which are parthenogenetic and in a species that exhibits cyclical parthenogenesis (Huylmans et al., 2016). Together the phenotypes observed in these examples, after knockdown of *dnmt1*, bear striking resemblance to the phenotype observed here in aphids in which DNA methyltransferases were inhibited by 5-azacytidine, chiefly, disruption of oogenesis and early embryogenesis, and reduced reproductive output. While it is possible that these phenotypes occurred because of disruption of Dnmt1, the expression patterns of *dnmt3a* and *dnmt3x* lead me to believe that it is likely that they occurred because of disruption of one or both of these genes, too. Arguably, because *dnmt3x* is predicted to lack the methyltransferase domain necessary to methylate cytosine, it may not interact with the activated and incorporated 5-azacytidine, and therefore be unaffected by it. Again, the ability of each of these paralogs to

interact with DNA should be confirmed to determine the effects 5-azacytidine is likely to have on them. Additionally, it cannot be ruled out completely that the observed effects in the ovary are as a result of disturbance by 5-azacytidine upstream of the ovaries. For example, 5-azacytidine could feasibly disrupt DNA methylation (or non-methylation related functions of Dnmt3a/Dnmt3x) in the *corpus allatum* leading to dysregulation of JH titre, or in the *pars intercerebralis* leading to dysregulation of insulin signalling, which might account for disruption in the ovary (the expression patterns of *dnmt3a* and *dnmt3x* were not inspected in any tissue other than the ovaries). Though, again, the expression of *dnmt3a* and *dnmt3x* in the ovaries make disruption there the more parsimonious explanation. Expression of *dnmt3a* and *dnmt3x* in individual tissues of *A. pisum*, beyond the ovaries, was not assessed here.

5-azacytidine treatment had a number of effects on reproductive output. The most apparent of the effects, the production of disturbed nymphs that were born stillborn or died shortly after birth, occurred only in a relatively short window, lasting less days than the number of days that aphids were treated for (though, the days of treatment and presence of the phenotype were separate). This is indicative of a relatively small window in development in which 5-azacytidine is able to disrupt development in this way. The fact that stillborn nymphs were birthed at all indicates that while treatment disturbed a process important to proper development, this stage was not so critical, or so early (or was not perceived to be, by the mother) that with regards to this phenotype, embryos passed successfully through several other stages of development and were birthed, or there is a stage at which development of a particular embryo is committed and will be birthed regardless of its status. Morphologically, except for the legs-close-to-body phenotype, these disturbed, ‘stillborn’ nymphs appeared outwardly normal. They may correspond to the mid-stage embryos visualised by HCR that were not observed to be disturbed, but may have subsequently failed to pass successfully through an as yet undefined stage of development. The halt in/severely-reduced reproduction of 5-azacytidine treated aphids around the period where severely disturbed nymphs were produced, taken together with the extent of disruption of oocytes and early embryos (visualised by HCR and phalloidin staining) is suggestive of resorption of embryos and oocytes that were sufficiently disturbed; certainly, the very early

embryos that appeared disturbed after visualisation would be unlikely to take on the proper form, and thus must not have corresponded to the disturbed birthed nymphs. One approach to confirm that resorption is taking place would be to allow 5-azacytidine and control treated aphids to reproduce until they become post reproductive (in the lab, asexual *A. pisum* individuals commonly reach post-reproductive age, and identifying post-reproductive *A. pisum* individuals can be done visually, or by dissection (Saberski et al., 2016)), with monitoring of the number of nymphs produced. If 5-azacytidine aphids survive past their reproductive period (which relies on their lifespan not being reduced), the total number of offspring produced can be compared. As asexual aphids possess a set number of germ cells that become nurse cells, and that eventually become oocytes (thus, being capable of 'running out' of their stock of oocytes; reported to be approximately 20 nurse cells and 11 oocytes in *A. pisum* (Miura et al., 2003)), with germaria being specified before they are born (Büning, 1985; Blackman, Minks & Harrewijn, 1987), if 5-azacytidine treated aphids produce less offspring before becoming post-reproductive, it is likely that resorption is occurring. Resorption would explain the reduction in nymph production, as it would leave gaps in the ovarioles, and the time between nymphs maturing would be increased. Resorption, typically of oocytes (oosorption), is a reproductive strategy of female insects that usually occurs in response to unfavourable conditions in order to recover resources that might otherwise be wasted, and delay reproduction until more favourable conditions arise (Moore & Attisano, 2011). Here, it may be occurring in response to nonviability or severe developmental defects of disturbed oocytes and/or embryos, which would be wasted investments, given the high level of disturbance observed. Aphid ovaries are structured such that there is a slight stagger in the ages of oocytes and embryos contained within and between each ovariole, that mature in sequence (Büning, 1985; Brough & Dixon, 1990a). Because there are several ovarioles in each of the paired ovaries (six or seven per ovary), typically, there are many embryos and oocytes within an aphid that are very close in age, and some that are at the same developmental stage (though, at most only one per ovariole) (Büning, 1985).

The appearance of relatively few disturbed nymphs (ranging from one to four, where present) is again suggestive of an exquisitely small developmental

window where 5-azacytidine produces this phenotype (embryos still birthed but disturbed). The observation of seemingly typical nymphs preceding and following disturbed nymphs further suggests this, as they would likely be close in age to disturbed nymphs. Though, the typical nymphs that came after may actually have originally been more separated from the disturbed nymphs in terms of sequence in the ovary, but because of resorption, the sequence is shortened. Given that aphids were treated for six days, the appearance of disturbed nymphs over a window of just three days further suggests resorption, as without resorption (or a stop in embryos advancing through the ovariole) some aphids younger than the critical period would develop, over this time, to reach the critical period during the time when exposure to 5-azacytidine was still high, leading to the disturbed phenotype. One possible factor affecting the birth of disturbed nymphs is a potential increase in the degree of separation between the mother and her embryos introduced by the increasing level of sclerotization (Stadler, 1995), though this has not been explored substantially. As embryonic development progresses through the mid-stages, cuticle is deposited and hardens and the embryo may gain some independence from the control of the mother; this has been suggested as a possible mechanism for control of the diverging prioritisation of resources by the mother and the offspring during periods of nutritional stress, as it has been noted that more developed embryos are less susceptible to resorption (Stadler, 1995). The increasing cuticle may make resorption less possible, and as a result, birth still occurs.

Additionally, the apparent reinitiation of typical reproduction on day seventeen after reduced reproductive output and production of disturbed nymphs is also consistent with resorption occurring. Day seventeen is separated by eleven days from the final day of 5-azacytidine treatment, and while the exact rate of development of aphids is difficult to determine, Kindlmann and Dixon (Kindlmann & Dixon, 1989) suggest for *M. viciae* at 20 °C, that embryonic development takes ~ ten or eleven days (to my knowledge, there is no estimate for *A. pisum*). These timings are consistent with the delay observed in the present study (which was also run at 20 °C), which suggest that the nymphs being born on day seventeen were probably specified as oocytes shortly after the treatment period ended, and thus were the first of the embryos/oocytes not to be exposed to treatment.

The exact stage of development at which the developmental failure leading to disturbed nymphs occurred is not completely clear. Though, I would speculate that, given the legs-close-to-body-phenotype, which may have been a by-product of the cause of death, a failure of the serosa to rupture and withdraw over the body completely may have occurred, leaving the nymphs encapsulated. The serosa is an extraembryonic (EE) tissue that, jointly with the second insect extraembryonic tissue, the amnion, encloses the embryo (Panfilio, 2008). Generally speaking, while the amnion lies at the ventral side of the embryo and encloses the amniotic cavity, the serosa, as the outer membrane, surrounds the embryo and the amnion (Panfilio, 2008). While we do not know how either of the EE tissues/membranes work in *A. pisum*, we do have a good understanding of these structures in the closely related *O. fasciatus* (Panfilio & Roth, 2010). Being extraembryonic, the EE membranes do not contribute directly to the development of the body (Panfilio, 2008), which is consistent with the observed disturbed nymphs appearing morphologically normal. In *T. castaneum* both EE tissues rupture and withdraw following germband retraction during late development (Hilbrant et al., 2016; Koelzer, Kölsch & Panfilio, 2014). In *O. fasciatus*, another hemipteran, rupture of the EE tissues occurs at the initiation of katatrepsis, the flipping of the embryo (in hemimetaolous insects, typically, prior to katatrepsis embryos are upside down and back to front compared to their final axes and the axes of the egg) (Panfilio, 2008), and katatrepsis is driven in this case by the serosa and its rupture (Panfilio & Roth, 2010). In *A. pisum*, katatrepsis (stage 15) occurs at approximately 50 % completion during parthenogenetic embryonic development (Miura et al., 2003), and is independent of germband retraction. Furthermore, the serosa in the ovaries of the parthenogenetic (but not that of the oviparous, which is more similar to that of *O. fasciatus*, both being oviparous) pea aphid has been suggested to be reduced and its function is unknown and has been suggested to be diverged from that of the oviparous pea aphid (in which the serosa encompasses the yolk and forms a serosal cuticle) and other oviparous insects (Miura et al., 2003); asexually developing *A. pisum* embryos do not produce a serosal cuticle. Although, in depth exploration of the nature and functions of the extraembryonic membranes of aphids have not yet been carried out. Despite being relatively closely related, the parthenogenetic development occurring in the ovaries of asexual aphids fundamentally differs from the more

typical oviparous development exhibited by *O. fasciatus* (Büning, 1985; Michalik et al., 2013). While the exact function and mechanisms by which the serosa may be involved in katatrepsis in aphids has not yet been investigated, it is possible that because of the atypical viviparous development of asexual aphids, the serosa of these aphids may differ from what is observed in other insects, even other hemipterans. Katatrepsis is a short-lived, high activity, late development stage of great change that, given many manipulations disturb it, has been suggested to be quite sensitive, reviewed in (Panfilio, 2008). Thus, it may be at this stage (and possibly associated with EE membrane rupturing) that the highly disrupted ('stillborn') phenotype arises. More careful and frequent assessment to monitor expected stage and polarity of mid-to-late stage embryos from whole ovaries/ovarioles of 5-azacytidine treated individuals would help to determine if defects in katatrepsis may be occurring, as (partial or whole) inversion may be expected if this were the case; given the relatively small window for production of these disrupted offspring, it is likely that if this were the case, it would be easily missed in stained ovary samples, and partial katatrepsis may be difficult to observe. Juvenile hormone analogs applied to some insect species (*Locusta migratoria*, *Acheta domesticus*) have been shown to disturb katatrepsis, while they often do not appear to disturb the rest of embryonic development (Erezyilmaz, Riddiford & Truman, 2004; Kidokoro et al., 2006; Panfilio, 2008); if the effect of 5-azacytidine inhibition of DNA methyltransferases here is partially through disturbing katatrepsis, this (tentatively) links DNA methyltransferases to a known modulator of the reproductive polyphenism, juvenile hormone.

Reproduction halted (a complete drop in reproductive output) for between one and three days, for 5-azacytidine treated aphids. Given that otherwise, mean daily nymph production by individual 5-azacytidine treated aphids was between two and four, this again suggests a relatively small window in development is affected to produce this phenotype, possibly representing zero to two embryos 'lost' per ovariole (considering that each of the paired *A. pisum* ovaries consists of six or seven ovarioles). Resorption may also explain the general reduction in productivity of 5-azacytidine compared to control aphids for the majority of the experiment, which otherwise may be explained by a slowing or halting of either development or progression along the ovarioles or embryos being held prior to

birthing, that is, if the embryos are not resorbed, the parsimonious explanation for the differences in reproductive output is that advancement along the ovarioles (which can be thought of as conveyor belts) is slowed or is stopping (this is logically, the only alternative explanation, at least that is clear to me). A further possible explanation for potential resorption, aberrant early embryo morphology and arrest of oocytes is disturbance of insulin signalling. Dnmt1 has been demonstrated to affect the expression of IR genes in mice (which, importantly, appear to use methylation marks differently to insects) through the activity of NT5C2 (Chen et al., 2020); overexpression of *dnmt1* in this study led to inhibition of an IR gene. Similarly, one of the two *D. magna* (a crustacean) *dnmt3* paralogs (the copy bearing no DNA methyltransferase domain, discussed above) has been linked to nutritional status, along with reproduction and lifespan (Nguyen et al., 2020, 2021) – all elements that are highly linked in insects, to insulin signalling (Leyria et al., 2022). Additionally, knockout of *dnmt3.1* in *D. magna* led to differential expression of a gene that appears to encode an insulin-like peptide and *tobi*, which is an insulin responsive gene (Nguyen et al., 2021). Resorption in several species of insects, including the hemipteran *O. fasciatus* and several species of aphid is frequently associated with nutritional stress (in addition to other environmental factors (Moore & Attisano, 2011; Xu et al., 2019) (reviewed in (Bell & Bohm, 1975)), which is heavily related to insulin signalling. Resorption has been well documented in aphids, including the pea aphid (Brough & Dixon, 1990b; Ward & Dixon, 1982). Insulin signalling is also known in insects (though, principally, based on studies in *Diptera*) to regulate apoptosis (Bikopoulos et al., 2004), cell proliferation (Zhao et al., 2022:p.2) and growth (Nässel & Broeck, 2016b; McKenna, Tao & Nijhout, 2019), along with oocyte maturation, pre-vitellogenic development and vitellogenesis (and thus, reproduction generally) (Richard et al., 2005; Shim, Gururaja-Rao & Banerjee, 2013; Al Baki et al., 2019; Han et al., 2020) and reproductive diapause (Sim & Denlinger, 2008; Chen et al., 2023); defects in some of these processes, principally oocyte maturation, pre-vitellogeneic development and cell proliferation appear consistent with the results of 5-azacytidine treatment on the ovary, and defects in reproduction consistent with the observed phenotypes. Knockdown of genes encoding ILPs in *N. lugens* led to a reduction in *dnmt3* expression (Lu et al., 2018a). It is also noteworthy that knockdown of *dnmt3* in *A. melifera* led to a phenotype mimicking that

associated with consumption of royal jelly (development of queen-like ovaries), also suggesting a possible link between *dnmt3* and nutrition/insulin (Kucharski et al., 2008). Similarly, a recent study found that RNAi against *dnmt1* in *B. germanica* led to activation of nutrition-related pathways (based on KEGG analysis), including insulin secretion and insulin signalling (Zhao et al., 2023). If DNA methylation or DNMTs are involved in the aphid reproductive polyphenism, then it is possible their involvement is through regulation or response to insulin signalling in the mother. Disruption of insulin signalling in other insects (importantly, this is known only in oviparous insects, while aphids exhibit viviparous and oviparous reproductive modes) is associated with disrupted ovarian development (Sim & Denlinger, 2008; Chen et al., 2023), and associated disruption of the genes underpinning ovarian development and egg production (Roy, Hansen & Raikhel, 2007). Thus, aberrant activation/inactivation of insulin signalling or downstream processes as a result of inhibited action of DNA methyltransferases may have contributed to the observed disturbed phenotypes. More in-depth inspection of the links between DNMTs and insulin signalling is required to assess if this may be the case. Investigating the effects of disrupted activity of DNMTs (*dnmt3a* and *dnmt3x* especially) on gene expression (paired with DNA methylation assessment) at the level of the ovary will help to determine if these systems are linked. It is feasible that perturbation of DNA methyltransferases in other tissues than the ovaries, for example the head (where *dnmt1* and *dnmt3* expression have been demonstrated in several insects, though in many cases higher expression is associated with ovaries/reproduction (Kay, Skowronski & Hunt, 2016; Robinson et al., 2016; Zhang et al., 2015)), may have contributed to the observed phenotypes – perhaps by leading to knock-on effects on expression of insulin signalling related genes, as an example.

Importantly, however, effects on reproductive mode (viviparous or oviparous reproduction) were not assessed here. Though, the phenotypes demonstrated in the presented examples fundamentally differ from those experienced during the reproductive switch, chiefly from asexual to sexual reproduction. If DNA methyltransferases are involved in these examples, through the insulin signalling system, and are equally involved in the insulin signalling pathway's

mediation of the reproductive polyphenism, this is suggestive of fine control and thresholds during typical switching (for functional reproduction to be occurring).

The non-disturbed aphids from the same generation as the disturbed nymphs described above, developed normally, and they produced no stillborn nymphs themselves. While it is unsurprising that the older nymphs developed normally, owing to their presumably having aged past some critical period at the onset of treatment (and/or there being a dilution effect because of their advanced development (already possessing a large number of cells, and perhaps linked to *dnmt3a* and *dnmt3x* expression being confined to the germ cell clusters only at late stages (results, this chapter)) and the mechanism of action of 5-azacytidine, which must be incorporated, typically, during DNA replication/repair leading to a dilution effect) (Stresemann & Lyko, 2008). And it is likewise unsurprising that the younger nymphs developed normally, given that they likely would have similarly avoided treatment at critical periods. It is noteworthy that there was no obvious transgenerational effect, and, especially, an absence of production of disturbed nymphs by the older individuals. A transgenerational effect might be expected, based on the telescoping of generations, that is, that late stage embryos (f1) possess early stage embryos (f0) of their own developing inside of them (Kindlmann & Dixon, 1989), and the expectation that 5-azacytidine would be activated and incorporated in the DNA of the replicating cells of these embryos within embryos (as the pool of substrate to build DNA is necessarily shared between the mother, her embryos, and her grand-embryos). Thus, while the f1 may have not been obviously affected by 5-azacytidine, their embryos would be expected to have received a roughly equivalent treatment to the f1. If the stages of the f2, at the time of treatment, were the same as the stages of the f1 that were disturbed, then we might expect to see the same disturbed phenotype in the f2. Although, given the coarseness of the assessment of the offspring of the f1, which pooled f1 adults together into groups of sisters, I was unable to finely assess differences in reproductive output which might have indicated resorption. The absence of disturbed nymphs in this generation, therefore, suggests that the critical stage at which the severe disturbance occurs and resorption cannot occur, is beyond the developmental stages that occur within the ovaries of developing embryos.

In order to conduct these experiments more conclusively with regards to developmental stages, it will be useful to know the embryonic development rates of the used strain and species of aphid under a given set of conditions, principally temperature. This will allow rough estimations of the stages that we would expect to be exposed to treatment, and assess the progression of phenotypes based on this. To this end, EdU staining presents a powerful tool, that, similarly to the action of 5-azacytidine, works by incorporation into DNA when it is replicated (Flomerfelt & Gress, 2016). While in the present study, after trialling this method, I was not able to successfully stain *A. pisum* ovaries with EdU, this was likely a product of the method of application, injection, which has since been demonstrated to be an ineffective way of administering the chemical in a range of insects (Anton et al., 2023). Optimisation of such techniques in aphids will be necessary to assess *in vitro*, the rates of development and inspect more closely the stages at which 5-azacytidine is interacting; feasibly, coadministration of both 5-azacytidine and EdU could be used, although this would need to be validated. As part of characterisation of the two Dnmt3 paralogs, it will also be useful to assess if they are both able to interact with cytosine, and therefore be affected by 5-azacytidine upon its incorporation into DNA in the place of cytosine.

4.4.1 Future work

Building on the findings of this chapter, several experimental approaches will greatly increase our understanding of the putative roles of the two aphid *dnmt3* paralogs, *dnmt3a* and *dnmt3x*. Most obviously, functional characterisation of each of these genes by knocking them down (by RNAi) independently and together, and assessing effects on reproduction and reproductive mode (both in LD, where asexual aphids are expected and SD conditions, where the eventual production of sexual aphids is expected) will allow us to understand one, if the effects of 5-azacytidine observed here are due (or partially due) to the activity of either or both Dnmt3s, and 2, if Dnmt3a and/or Dnmt3x are involved in the reproductive switch, either by effecting the switch to sexual development or by maintaining the asexual mode. By examining both *dnmt3a* and *dnmt3x* singularly and together, the possibility of subfunctionalization, and the need for proteins of both for full functionality can be inspected. Doing so in conjunct with methylation analysis, by bisulfite, MeDIP or nanopore sequencing (with the

latter two being preferable), both at the whole-body and just the level of the ovary, and across LD and SD conditions will help to ascertain how these genes relate to DNA methylation, if they have ovary-specific functions that are or are not related to DNA methylation, and if differential DNA methylation, carried out by *dnmt3a* and/or *dnmt3x* is related to photoperiod and to the aphid reproductive polyphenism. Similarly, investigating the activity of *dnmt3a* and *dnmt3x* together and independently, through an ELISA against methylated DNA, by expressing and purifying their proteins will also help to determine their functionality as DNA methyltransferases and the likelihood of their being affected by 5-azacytidine treatment. Functional analysis of Dnmt1 in *A. pisum* by the same means, and comparison of the effects of *dnmt1* knockdown to the observed effects of 5-azacytidine treatment, observed here, will help to understand how the disturbances generated by 5-azacytidine treatment may have occurred, and allow for inspection of consistency between insect species for DNA methylation independent roles of Dnmt1.

Additionally, analysis of the regulatory regions of *dnmt3a* and *dnmt3x* by ChIP-seq will allow searching for domains associated with particular TFs and other DNA binding proteins that are linked to these genes, and possibly reveal interactions with other key modulatory systems, like the JH and insulin signalling pathways. An inspection of a greater number of chromosome-level aphid genome assemblies for the chromosomal localisation of *dnmt3a* and *dnmt3x*, will help us to understand the general genome organisation of these genes (beyond the context of just *A. pisum*) and assess if their appearance on the X chromosome in *A. pisum* is a species-specific quirk, or if, by occurring on the X frequently, their appearance there is likely linked to some unusual aspect of aphid biology.

4.4.2 Concluding remarks

To summarise, it is likely that a duplication event took place in an ancestor of the Aphidomorpha to give rise to *dnmt3a* and *dnmt3x*, the latter being the more diverged copy which is specific to the Aphidomorpha lineages. Presence of a single copy of *dnmt3* is noteworthy, that there are two is unusual. Due to cyclical parthenogenesis being an evolutionary novelty that is shared by members of the Aphidomorpha, it is possible that the duplication of *dnmt3* contributed to the development of this example of reproductive polyphenism, or

some other novelty of aphid biology. Expression in the ovary of *dnmt3a* and *dnmt3x* appears to overlap spatially and temporally, being detected in the germaria, oocytes, early embryos, and in germ cells of embryos once they were specified, in viviparous pea aphids producing viviparous offspring. Inhibition of DNA methyltransferases produced defects in oogenesis (arrest) and early embryos (disorganisation), but not later stage embryos. Additionally, inhibition reduced the production of offspring for several days, suggesting resorption of disturbed oocytes and early embryos, and resulted in the production of highly disturbed offspring that were still birthed for a small window of time. The roles of the two *dnmt3* paralogs in the virginopara ovary are unclear, though given the increasing recognition of Dnmt1 as having methylation independent roles, it is possible that the action of one or both *dnmt3* paralogs may be methylation-independent also.

Chapter 5 Exploring the molecular underpinnings of the reproductive switch in the sexupara aphid Ovary

5.1 Introduction

5.1.1 Aphid reproductive polyphenism

The aphid reproductive polyphenism, cyclical parthenogenesis, encompasses a switch from viviparous parthenogenetic reproduction, the typically occurring mode, to oviparous sexual reproduction, a relatively rare form seen for a single generation, as discussed in chapter 1 (Srinivasan & Brisson, 2012; Blackman, Minks & Harrewijn, 1987). The sexual reproductive mode is the ancestral means of reproducing, while viviparity arose in a common ancestor of extant aphids (Davis, 2012). Cyclical parthenogenesis is exhibited by most, but not all aphid lineages. Where it is not present, it has been secondarily lost. Loss has been observed in whole species but also strains within species (Blackman, Minks & Harrewijn, 1987; Srinivasan & Brisson, 2012). As an adaptation to primarily overcome the decrease in temperature associated with the onset of winter, these losses are typically found in groups located outside of temperate zones (Dixon et al., 1987). Both viviparity and cyclical parthenogenesis are evolutionary innovations of the aphids, and just cylical parthenogenesis of the aphidomorpha (Davis, 2012). Each reproductive mode deploys its own developmental program (Duncan, Leask & Dearden, 2013), and individuals employing the two distinct modes, each being birthed themselves parthenogenetically, arise from the distinct signalling and gene expression patterns of, primarily, their mother upon exposure to varying photoperiods, after detection and integration of the environmental signal. Briefly, photoperiod is detected in the head of the mother and then, if the photoperiod is sufficiently long, a substance termed virginoparin is released, likely from neurosecretory cells in the *pars intercerebralis* or arising from the effects of released signals from these neurosecretory cells, reviewed in (Colizzi, Martínez-Torres & Helfrich-Förster, 2023). This substance then transduces the signal from the head of the mother to her ovaries and the fate of the embryos developing within them is maintained as asexual. If the photoperiod is sufficiently short, virginoparin secretion is reduced and this leads, eventually, to the production of

sexual offspring. Asexual aphids that produce asexual offspring only are termed virginopara, while those asexual aphids that will produce some amount of sexual offspring are termed sexupara, and sexual females have been termed ovipara. The exact mechanisms underpinning the switch, a key example of insect polyphenism, are unknown (Yan, Wang & Shen, 2020). Though, much research effort has been directed at uncovering them, revealing convincing roles for JH signalling (Hardie, 1987a; Hardie & Lees, 1985; Ishikawa et al., 2012), and possible roles for insulin signalling (Le Trionnaire et al., 2009; Barberà, Cañas-Cañas & Martínez-Torres, 2019; Cuti et al., 2021).

5.1.2 Detection across generations

A long-standing open question in the reproductive polyphenism of aphids is exactly how many generations are truly required for the switch to occur (Le Trionnaire et al., 2009). This is an issue that is particularly difficult to dissect as a result of the telescoping of generations. Telescoping of generations refers to the fact that adult asexual aphids contain, within their ovaries, mature embryos that possess developed ovaries of their own, and oocytes and early stage embryos developing within them (Büning, 1985). This means that three generations can be represented within a single aphid, mother, daughter (embryo) and granddaughter (embryo's embryo/oocyte). This presents the possibility that the daughter embryo may be able to detect the photoperiod signal, independently of the mother, and transduce this signal to the granddaughter embryo/oocyte. Assessment of known and putative modulators of the reproductive switch in ovaries and the embryos contained within them is useful, therefore, to determine what if any signalling the embryos might be using to convey information directly to their own embryos/oocytes.

5.1.3 Insulin signalling and co-option

Insulin signalling is a key and conserved pathway across several groups of animals, including insects. It is, canonically, deployed in the sensing of and responding to nutritional status (Leyria et al., 2022). It has been implicated in several examples of insect polyphenism, such as caste determination in honeybees (where it enhances JH signalling), horn development in some species of scarab and stag beetle (where nutrition is tightly linked to horn size), and the wing morph determination of aphids, the brown planthopper and the

soapberry bug (Wheeler, Buck & Evans, 2006; Wolschin, Mutti & Amdam, 2010; Nijhout & McKenna, 2018; Fawcett et al., 2018). Thus, it appears to be a commonly deployed environmentally responsive system, which is not incredibly surprising given its high responsiveness to nutrition. Relating to nutrition, insulin signalling appears especially linked to plasticity involving body size (Casasa & Moczek, 2018; McKenna, Tao & Nijhout, 2019) and female reproduction (diapause, as an example (Chen et al., 2023)), female reproduction being perhaps the most energetically (and therefore nutritionally) demanding part of insect life (Smykal & Raikhel, 2015). Examples include the aphid wing (which involves a reproductive component, as wingless aphids tend to possess larger ovaries than their winged sisters, and differ slightly in their reproductive strategies) (Ishikawa & Miura, 2009; Grantham et al., 2020; Yuan et al., 2023) and reproductive polyphenisms such as reproductive diapause in *Culex pipiens* (Sim & Denlinger, 2008) and *Coccinella septempunctata* (Chen et al., 2023), where it may act independently or by additionally inducing the secretion of juvenile hormone (Zeng et al., 2017; Jiang et al., 2022), another major player common to many examples of insect polyphenism. The application of insulin signalling toward control over reproduction is a generally observed phenomena in insects, where reproduction is, necessarily, exquisitely sensitive to nutrition (Smykal & Raikhel, 2015). Thus, while nutritional sensitivity is likely an ancestral feature, examples where plasticity involves extreme differences (as in the case of polyphenism) represent extreme utilisation of the system in novel ways, possibly to the extent of co-option.

Putative insulin producing cells and several insulin-like peptides (ILPs) have been characterized in aphids (Barberà, Cañas-Cañas & Martínez-Torres, 2019; Cuti et al., 2021). Seven ILPs have been identified to be encoded in the pea aphid genome, of which, ILP1 to ILP4 (which form a monophyletic group, seemingly owing to aphid specific duplication events) are considered classical insulin molecules (Huygens et al., 2022). They have characteristics of insulin molecules secreted by endocrine cells or peptidergic neurons. While previous experiments have demonstrated very low expression of ILP2 or ILP3 generally. Both ILP1 and ILP4 have been shown to be more highly expressed under long days than short (Barberà, Cañas-Cañas & Martínez-Torres, 2019; Cuti et al., 2021), indicating association between these ILPs and the switch, though no

study to date has functionally examined this (or a link between insulin signalling and the reproductive polyphenism more generally) by manipulating insulin signalling. ILP4 and ILP1 have been shown to be secreted by the two groups of four type I neurosecretory cells (NSCs) of the *pars intercerebralis* (Barberà, Cañas-Cañas & Martínez-Torres, 2019). These cells and the *pars intercerebralis* generally, along with the *pars lateralis*, which is located medially adjacent on each side of it, were demonstrated to be important to the switch by classical studies (Steel & Lees, 1977). Ablation of these structures led to switching, under LD, to the production of sexual offspring, which is usually experienced under SD conditions. The authors proposed a substance which they termed virginoparin to be secreted from the NSCs and be responsible for maintaining parthenogenetic reproduction (Steel & Lees, 1977). Recent evidence suggests this substance may be ILP1 and/or ILP4 (Barberà, Cañas-Cañas & Martínez-Torres, 2019). ILP1 and ILP4 are produced in the NSCs, and ILP4 has been shown to be transported to the *corpora cardiaca*, and then on to the abdomen via three projections, two lateral and one medial (Cuti et al., 2021) (ILP1 localisation is currently unknown) (figure 5.1). Importantly, the lateral projections appear to terminate in the vicinity of the germaria (though not in direct contact with the oocytes or embryos) of the ovaries (Cuti et al., 2021). Taken together, insulin signalling appears linked to the reproductive polyphenism, and may be the identity of the virginoparin, though, further work (primarily functional, i.e. knockdown/inhibition) will be required to assess if insulin signalling is cause or consequence of the switch.

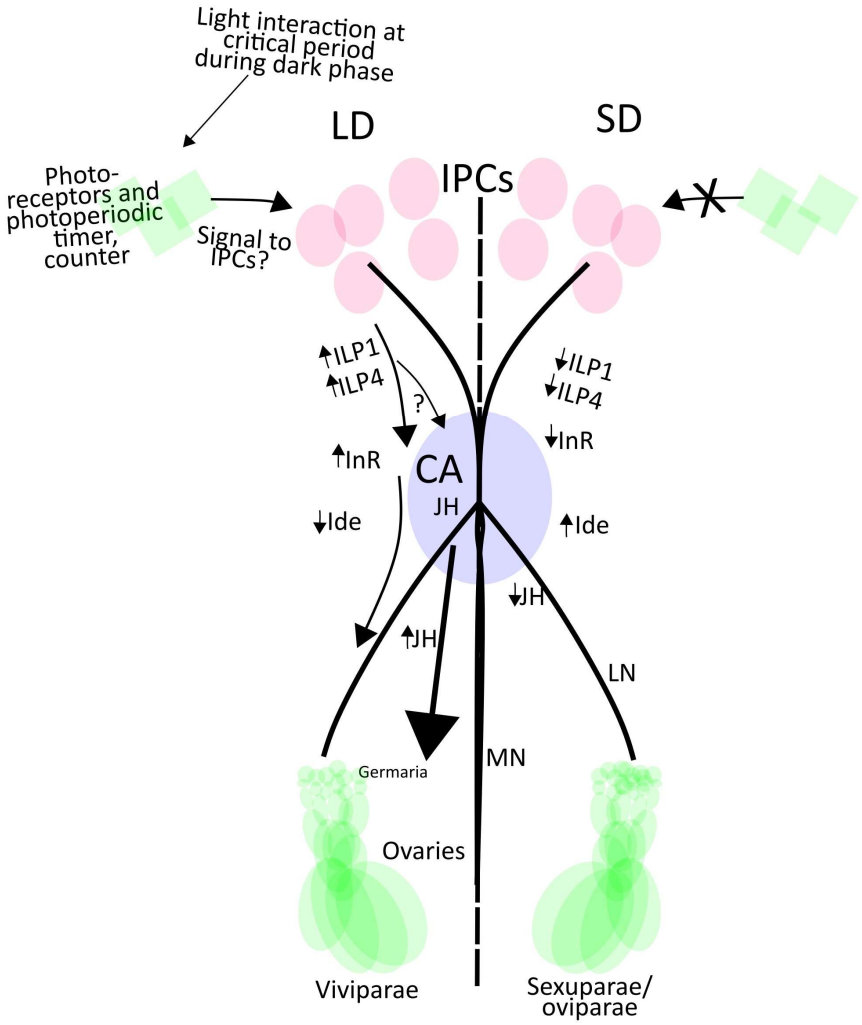

Figure 5.1 Schematic diagram of proposed elements of the photoperiod responsive reproductive polyphenism of aphids, cyclical parthenogenesis, principally concerning insulin signalling and juvenile hormone signalling.
In response to detection of light by photoreceptors in the protocerebrum of aphids during a critical stage in the 'dark phase', information about photoperiod is thought to be integrated by elements of the photoperiodic response. After integration, signalling to IPCs is likely to occur (though, the mechanism by which this occurs is not resolved). These neurosecretory cells produce ILPs (insulin-like peptides). Projections from the IPCs extend past the CA (corpora allata), the site of JH (juvenile hormone) synthesis, where they may be capable of affecting its synthesis/release, and the LNs (lateral nerves) terminate in the vicinity of the ovaries. Differential expression of two other insulin signalling related genes, *inr*, encoding an insulin-like peptide receptor, and *ide*, encoding an insulin-like peptide degrading enzyme, has been detected in the heads of aphids exposed to LD or SD (short-day) conditions. Juvenile hormone titre is also increased under LD conditions (associated with transcriptomic differences in JH related genes between LD and SD), and JH has been functionally determined to be able to modulate the reproductive switch.

5.1.4 Juvenile hormone signalling

Juvenile hormone and 20-hydroxyecdysone (the active form of ecdysone) are two major insect endocrine hormones (Orchard & Lange, 2024). The juvenile hormone pathway is a ubiquitous insect system responsible for controlling many basic functions, including metamorphosis, development, reproduction, diapause and plasticity (Wigglesworth, 1940; Ling & Raikhel, 2021). It often acts in conjunct with or antagonistically to the activity of ecdysone. It is a system that plays a myriad of roles both fundamental to the basic biology of, being a basic component of the insect molecular toolkit, and also more diverged phenotypic phenomena of a huge diversity of insects. It has been co-opted into and deployed as part of an array of polyphenisms and phenotypic plasticities, where it acts as a modulator (Nijhout, 1999; Simpson, Sword & Lo, 2011). Many of the examples presented in chapter 1 are facilitated at least in part by juvenile hormone. It is typically associated with having a juvenilising effect, which is to say that one of its first identified primary roles was in maintaining insects in juvenile states, controlling progression from larval, in holometabolous insects, or through nymphal stages, in hemimetabolous insects, eventually to mature adults (Wigglesworth, 1936, 1940). The parthenogenetic and wingless aphid morphs can be seen as sort of juvenile-like states, as individuals lack wings and are not capable of sexual reproduction, therefore lacking traits typical of most adult insects (Blackman, Minks & Harrewijn, 1987; Srinivasan & Brisson, 2012). Given the juvenile-like states, it is relatively unsurprising that JH appears to be functioning in both polyphenisms (Hardie, 1987a; Ishikawa et al., 2013). Curiously, however, the sexual mode appears to frequently preclude the development of wings (Hille Ris Lambers, 1966). The mechanism underpinning this is not clear.

Juvenile hormone is typically produced in the corpora allata (Tobe & Stay, 1985), though in aphids this is fused to give the *corpus allatum* (Hardie, 1987b). Our understanding of juvenile hormone signalling comes primarily from studies in the model system, *D. melanogaster*. In *D. melanogaster*, JH (JHIII being the principal JH in insects) binds to its receptor Methoprene-tolerant (Met) and activates downstream signalling through recruitment of Taiman (Tai) (Ashok, Turner & Wilson, 1998; Charles et al., 2011; Abdou et al., 2011) (figure 5.2). Kr-h1, a transcription factor, is the main transducer of JH signalling, after its

expression is upregulated by binding of the JH/Met/Tai complex at E-box-like motifs (Minakuchi, Zhou & Riddiford, 2008). Juvenile hormone exerts a degree of control over reproduction in many insects (figure 5.2). In many orders it is absolutely necessary for vitellogenesis (Wu et al., 2021), in others it is additionally essential for ovulation (Luo et al., 2021) and therefore is integral for reproduction to occur at all. Following on from classical experiments by Steel and Lees (1977) and Hardie and Lees (1985), juvenile hormone was suggested to be the virginoparin, the substance which is most important in determining reproductive morph. Though, the nature of the virginoparin has not yet been conclusively validated.

JH analogs or application of JH extracts have previously been demonstrated to be capable of redirecting the developmental trajectory of aphids in SD conditions towards a LD condition-like fate; although (Steel & Lees, 1977; Hardie & Lees, 1985). The observation of JH and its analogs diverting reproductive fate, along with the observation of elevated JH III titres (while there are many insect JHs, JH III is the only one present in *A. pisum* and the JH most commonly deployed in insects generally) in aphids reared in LD conditions relative to SD conditions (Ishikawa et al., 2012) provides some of the basis for the hypothesis of juvenile hormone in part underpinning the reproductive switch. In insects more generally, juvenile hormone is considered the master endocrine regulator of reproductive diapause (Denlinger, Yocum & Rinehart, 2012; Ma et al., 2021; Li et al., 2022c; Zhou et al., 2022), which typically involves arrest of ovarian development to overcome harsh winter conditions (Koštál, 2006). The oviparous morph within aphid cyclical parthenogenesis can be considered as analogous to reproductive diapause, with the production of overwintering eggs representing a form of diapause over winter (overwintering eggs themselves also encompass embryonic diapause of the embryos within them (Shingleton, Sisk & Stern, 2003), but at the population level, a type of reproductive diapause is ocuring) (Srinivasan & Brisson, 2012). Though, importantly, the mechanisms of typical reproductive diapause, in which ovaries are arrested and reproductivity reduced as a result of cessation of oogenesis and vitellogenesis (Kubrak et al., 2014; Gao et al., 2022), and the oviparous morph, encompassing egg development, with nutrition and resources still being supplied to maturing oocytes, are fundamentally different (Shingleton, Sisk & Stern, 2003; Michalik et

al., 2013). While reproductive diapause for the majority of insects represents a reduced allocation of resources to offspring, arguably, production of sexual eggs is energetically more demanding, at least more intense given the need to provide the putative embryo with all the nutrition it requires to grow at time of laying, than viviparous development (Ogawa & Miura, 2014; Bermingham & Wilkinson, 2009). But, the parthenogenetic mode is comparable to diapause in some ways – the reproductive output is not reduced, but there is a cessation of production of yolky, vitellogenic eggs (though oogenesis and the production of embryos are not reduced) (Michalik et al., 2013). The links between JH, reproductive diapause, and the reproductive switch are clear.

Pea aphids possess a complete complement of JH related genes, and differences in JHIII titre observed between aphids in LD and in SD conditions are thought to arise partially from differential levels of juvenile hormone esterases, the enzymes responsible for degrading juvenile hormone. Ishikawa and colleagues (Ishikawa et al., 2012) found *jhe1* upregulation, but only in their first SD generation (producing males, but also producing asexual females, during the discrete periods of production of each), and *jhe2* upregulation in their second generation, before the production of sexual, but not asexual females. Other genes they investigated, related to JH titre regulation did not present as clear patterns. They also reported strong negative correlation between *jhe1* expression and JHIII titre, but no correlation between JHIII titre and the expression of *jhe2*, *jheh*, *allatotropin* (a stimulator of JH synthesis), or *AstR* (encoding the receptor of a JH synthesis inhibitor, allatostatin). However, as they based gene expression on whole body samples, the importance of other genes in the JH pathway, such as those involved in synthesis (which will be most highly concentrated in the head, owing to the site of synthesis being the *corpus allatum*), in controlling JHIII titre may have been obscured, as tissue-specific and antagonistic relationships may exist; this may also have been exacerbated by the pooling of individuals in samples. Additionally, functional determination of this relationship has not yet been attempted, and so the causative ability of JHEs to degrade JH and through this affect the aphid reproductive switch is as yet unresolved.

In classical studies, topical applications of kinoprene (a JH analog) and of JH by several investigators in the pea aphid and other aphid species demonstrated

the ability of JH to redirect reproductive fate away from the oviparous morph (Mittler, Nassar & Staal, 1976; Mittler et al., 1979; Hardie & Lees, 1985; Corbitt & Hardie, 1985). Interestingly, it was noted that within an ovary, and even within individual ovarioles, asexual embryos and sexual eggs sometimes developed together, which is contrary to the either unanimously asexually developing offspring or unanimous sexual eggs seen typically in asexual and sexual females, respectively (Michalik et al., 2013). Why these partial phenotypes occurred is not clear, though it might be related to critical periods or quick clearing of JH/JH-analog (owing to upregulation of juvenile hormone esterase, for example), which may have meant that only some developing aphids (oocytes and/or embryos) were exposed sufficiently to the chemical, or doses being close to thresholds. Studies using JH and its analogues to divert aphids from production of sexual to asexual offspring, importantly, have only treated from the fourth instar onwards in the sexupara generation only, and have used relatively low doses over infrequent and few applications. Because late-stage virginopara embryos already contain within them specified germ cells, oocytes and even developing embryos of their own, their treatments may have missed critical stages. Furthermore, the causative effects of elevated JH on gene expression in aphids has not been assessed.

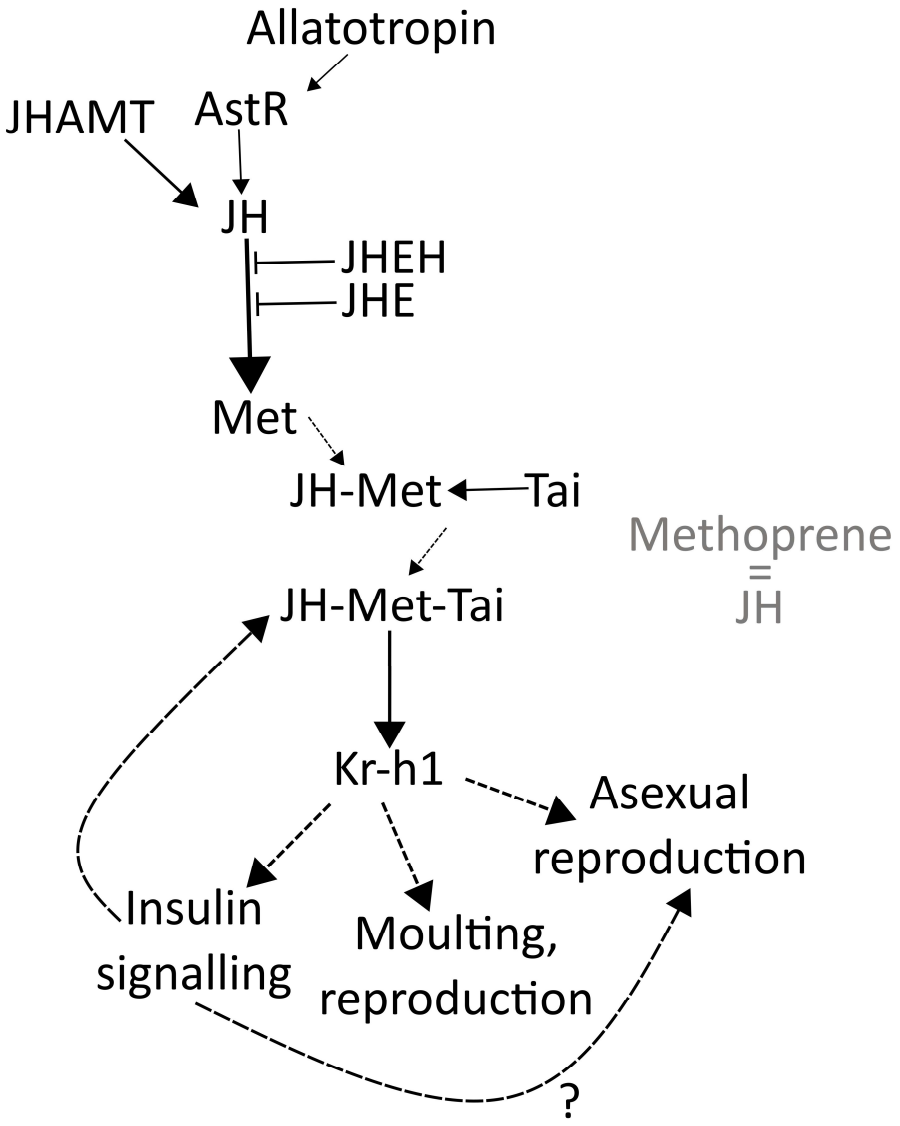

Figure 5.2Figure 5.2. Simplified diagram for the juvenile hormone pathway.
Allatotrophin produced by the brain has a stimulatory effect on the corpus allata/corpora allata, by binding to its receptor AstR, to produce JH. JHAMT contributes to JH biosynthesis by conversion of inactive precursors of JH to active forms. JH degrading enzymes, JHE and JHEH, act antagonistically to reduce JH levels. JH binds to its receptor Met, and they then form a complex together with Tai. The JH-Met-Tai complex is then able to bind to DNA and principally exerts downstream effects on gene expression through the activity of the primary JH inducible element Kr-h1. JH signalling then, primarily through the activity of Kr-h1, regulates moulting and reproduction, as well as regulating insulin signalling, and also plays a role in the reproductive polyphenism of aphids, high JH titre being associated with the asexual reproductive mode (as opposed to the sexual reproductive mode). Additionally, insulin signalling is known to regulate JH signalling in some insects, and is associated (though, not yet causatively) with the aphid reproductive polyphenism.

5.1.5 Joining the dots

There is strong evidence to suggest that juvenile hormone and suggestive evidence (though, only correlational) that insulin signalling both may play a role in the transduction of signal that gives rise to the reproductive polyphenism of aphids. Juvenile hormone and ILPs are both key endocrine signalling molecules that are nutritionally sensitive, linked to reproduction, and linked to each other (Libbrecht et al., 2013; Rauschenbach et al., 2017; Leyria et al., 2022; Pan et al., 2022). They have also been linked to photoperiodism (the system on which the reproductive switch hinges primarily) in other insects (Miki et al., 2020; Mano & Goto, 2022; Li et al., 2023a). While the interlink between these systems is, in general, well understood (though, relying mostly on studies in *D. melanogaster*) (Zhang, Li & Liu, 2022; Leyria et al., 2022), their interaction in the reproductive polyphenism is relatively little understood. If insulin signalling is involved in the reproductive switch, whether ILPs acts directly on the ovary to stimulate a switch in phenotype of offspring, or they do so through JH is unclear, likewise for JH signalling and whether it acts directly on the ovary or by regulating insulin signalling, perhaps in addition to direct signalling by JH itself. ILPs exert some control over the biosynthesis and release of lipophilic hormones like JH (and ecdysteroids) (Pan et al., 2022; Leyria et al., 2022). Likewise, JH activity can modulate insulin signalling (Mirth et al., 2014; Sheng et al., 2011; Ling & Raikhel, 2021). As such, the relationships between these two systems and reproduction and the reproductive polyphenism are likely complicated. Whether both act on each other in a feedback loop, and/or both work at the level of the ovary together is unclear.

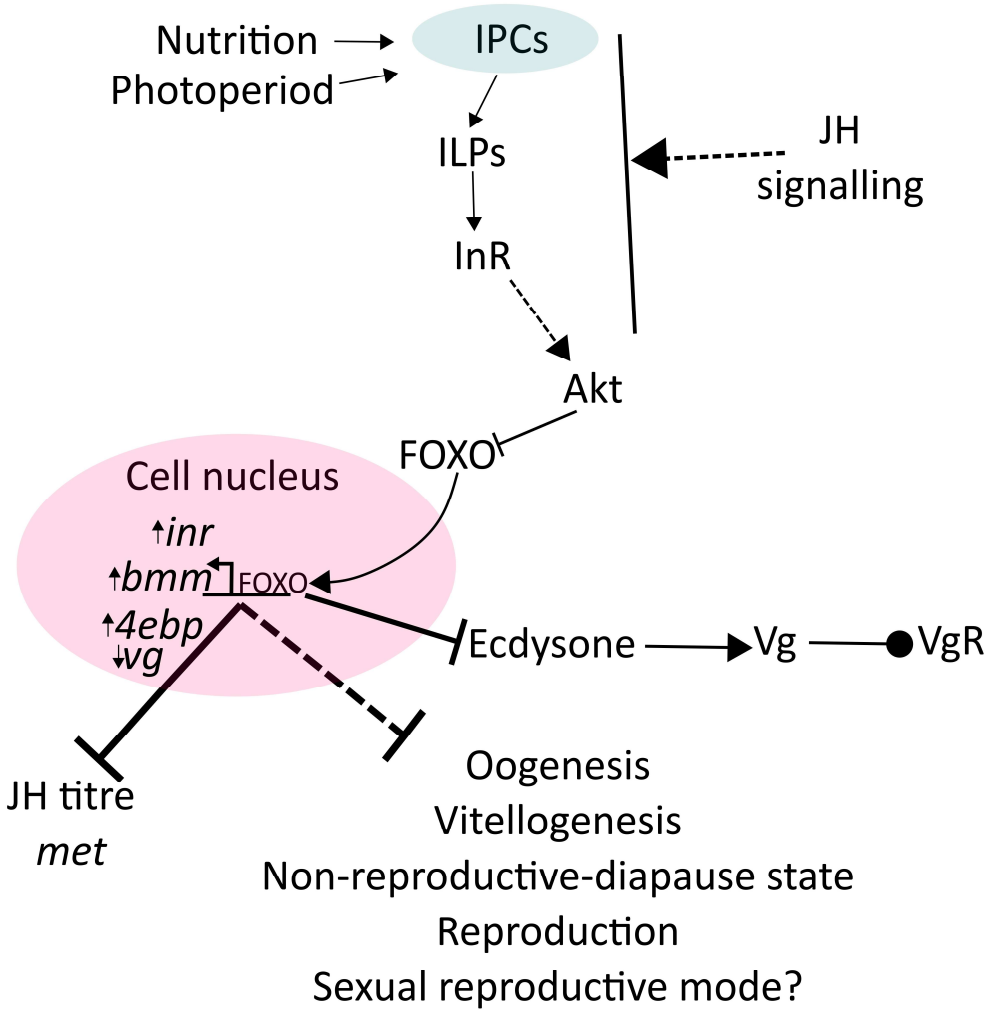

Figure 5.3 Simplified schematic of insulin signalling in insects (based primarily on studies in *D. melanogaster*).
Insulin signalling is principally responsive to nutrition, but in insects is also known to respond to other environmental cues, including photoperiod. IPCs (insulin-producing cells, as they are described in *D. melanogaster* and other insects, and NSCs, neurosecretory cells in aphids) produce ILPs, insulin-like peptides in response to nutrition and photoperiod. ILPs bind to the insulin receptor (InR), which then through a phosphorylation cascade activates Akt. Akt phosphorylates FOXO, a key node in the insulin signalling pathway, causing it to localise outside of the nucleus, where it would otherwise act to regulate the expression of insulin-responsive genes by binding to DNA. The activity of FOXO, if localised to the nucleus, includes regulation of egg production and maturation (oo- and vitello- genesis), and thereby reproductive diapause and reproduction generally, as well as enhancing the expression of *inr* (increasing sensitivity to ILPs), and regulation of growth (through, for example, *4ebp*) and juvenile hormone signalling. Differential insulin signalling may also be involved in the reproductive polyphenism of aphids, as enhanced expression of positive insulin signalling genes has been detected in the heads of asexual offspring producing *A. pisum*. Juvenile hormone signalling is also known to be able to modulate insulin signalling.

5.1.6 Sensors, modulators and effectors

Classically, approaches have aimed to identify molecular pathways and genes involved in the reproductive polyphenism of aphids (Le Trionnaire et al., 2009; Ishikawa et al., 2012). These approaches pull out important components, but whether or not these elements represent sensors (aspects involved in the detection of the signal), modulators (those aspects that act in transducing a signal from where it is perceived to where the effect occurs), or effectors (the genes that more directly exert an effect on phenotype) – essentially which elements are proximately or ultimately responsible for the polyphenism, and which signal between them – can be hard to dissect. This is especially difficult in the case of aphids, where multiple generations exist at once for a single individual, with each generation potentially having the capacity to exhibit all three categories (Kindlmann & Dixon, 1989). As such, an experimental design that is able to isolate each of sensors, modulators and effectors from each other, and specific putative mechanisms for each (e.g. insulin signalling and juvenile hormone signalling) is important to gain greater understanding of this complicated polyphenism. Manipulation of known elements of the pathway, while keeping constant others, presents a means to this end, allowing isolation and study of mechanisms in detail.

By bypassing the sensory aspect by artificially manipulating the modulatory system, effectors and modulators closer to where the phenotype is effected can be identified. This allows separation that would otherwise not be easy to achieve within standard gene expression approaches, especially given the telescoping of generations, and the possibility of similar modulatory systems as are active in the mother being active also in developing embryos. Classical approaches in aphids have bypassed the sensory aspect by applying JH and its analogs (as discussed above), though they have not sought to then assess effects on gene expression to establish cause and effect of the action of JH on other aspects of the switch. Juvenile hormone mimics present a powerful tool therefore, to dissect the aphid reproductive polyphenism.

Studies investigating the molecular basis of the switch have thus far mainly focused on adult heads (Le Trionnaire et al., 2009; Ishikawa et al., 2012; Liu et al., 2014; Matsuda, Numata & Udaka, 2020; Le Trionnaire et al., 2022), where

the photoperiod is detected and integrated (Colizzi, Martínez-Torres & Helfrich-Förster, 2023), and whole bodies, but little focus has fallen on the ovaries or the germ cells where the effect occurs. It is likely that several points of control exist at the level of the ovary (Ishikawa et al., 2012; Cuti et al., 2021), relating to modulation and sensitivity to signalling, in addition to downstream effects which contribute to taking on one phenotype or another, all of which may therefore have been obscured or missed; if the reproductive polyphenism relies on juvenile hormone and insulin signalling, and both are released at the ovary, then it is likely that they interact in some way at this tissue The effects of juvenile hormone and insulin, or of other yet unknown modulators, at the level of the ovary during the switch are a little studied, but important part of the system.

5.1.7 Aims and hypotheses

In this chapter, I aimed to investigate how the expression of genes relating primarily to insulin signalling and juvenile hormone differ between the ovaries of sexupara and virginopara aphids, containing future oviparous and viviparous offspring respectively. I hypothesized that aphids that will give birth to asexual offspring would show higher expression in genes associated with greater levels of both insulin and juvenile hormone signalling than those that would give birth to sexual offspring (sexuparae), reflecting differential utilisation of these pathways towards alternate reproductive modes, potentially highlighting some amount of direct modulation of signal by embryos, and uncovering downstream factors involved in the aphid reproductive polyphenism. While differential expression of insulin signalling related genes and various genes involved in juvenile hormone signalling have been detected between heads and whole bodies of sexuparae and virginoparae, effects specifically at the level of the ovary, where the switch is ultimately realised, have not yet been examined in detail.

I also hypothesized that the regular application of a juvenile hormone analog, methoprene, at a higher than naturally occurring level will prevent aphids from being able to produce sexual offspring, partially consistent with published data, but expanding on previous studies by completely diverting fate toward production of sexuals only, rather than generating mixed offspring. This, I hypothesized, will affect the expression of insulin signalling and juvenile hormone signalling relevant genes towards the virginopara pattern, and

additionally allow isolation of the effect from the sensory aspect of the switch by bypassing it.

5.2 Materials and Methods

5.2.1 Aphid Stocks

Aphids for these experiments were established from the stock population (described in the general methods) by rearing in relatively low densities for the preceding generations under LD conditions. L4 aphids were targeted initially for approximate age matching.

5.2.2 Switch experiment

5.2.2.1 Switch induction and maintenance of aphids

Wingless, parthenogenetic L4 aphids derived from the general parthenogenetic stock were isolated and reared in LD conditions (16:8 L:D, 15 °C,70 % humidity) in groups of ten in insect rearing bags (NHBS) on *V. faba* seedlings to adulthood. After two days, at which point all aphids had emerged as adults, aphids were placed singularly on leaf-agar plates (preparation of leaf-agar plates and maintenance of aphids on them is detailed in the general methods) in LD (identical conditions to in the prior step) or SD (10:14 L:D, 15 °C,70 % humidity) conditions. Once they started to reproduce, they were allowed to continue to do so for three days. No more than ten offspring were reared on a single plate to avoid crowding and reduce the production of winged morphs as well as leaf degradation and mortality. All aphids used in the subsequent steps were wingless. When an aphid mother produced more than ten offspring, these were split across multiple leaf-agar plates (but treated as one sample).

Aphids maintained on leaf-agar plates were monitored daily (to check for mortality, reproduction and leaf quality) and moved to new leaf-agar plates at least every three days (they were moved more often if leaves began to show visible signs of declining quality). Aphids were transferred by gentle brushing with a paintbrush to cause them to remove their stylet from the leaf (and avoid damaging it) and then picked up gently with a paintbrush once they had withdrawn it.

Aphids were reared in this way to produce a series of generations of individuals. To produce each subsequent generation (after F0), a subset of individuals (one per mother) from the previous generation reared to adulthood on leaf-agar plates were placed singularly in insect rearing bags onto *V. faba* seedlings (one bag and one mother aphid per seedling). Mothers were placed onto plants once reproduction had started and within twenty-four hours of the onset of reproduction. Mothers were allowed to reproduce on the seedling for three days, at which point nymphs were collected from insect rearing bags by gently removing the bags and transferring (as detailed above) to the necessary number of leaf-agar plates.

5.2.2.2 Phenotyping and ovary sampling

Once adults, ovaries were dissected (as described in the general methods) from the remaining individuals in each generation (that weren't isolated to produce the subsequent generation). Sampling occurred before the onset of reproduction during the mid photophase. Sampling was carried out before reproduction started as under SD conditions, pea aphids produce a series of progeny encompassing typically, sexual females, then males, and finally parthenogenetic females in sequence. By sampling adults relatively early, before the onset of reproduction, ovaries are at their maximal load of developing sexuals and sexual fated embryos are actively developing. Adult aphids were assessed for parthenogenetic reproduction by the presence of compound eyes visible through the cuticle of the abdomen, if they weren't sampled, or by inspection of ovaries for developing embryos after dissection, under a dissecting microscope (GXMXTL3T). All dissections were performed as laid out in the general methods, and samples were flash frozen in liquid nitrogen before being stored at – 80 °C prior to processing for RT-qPCR (below), or fixed, as described in the general methods, for RNA-FISH HCR (below).

5.2.2.3 RNA-FISH HCR

RNA-FISH HCR of sexupara ovaries and subsequent imaging were carried out as specified in the general methods. Images were processed and enhanced to maximise brightness and the signal to noise ratio for clarity using Zen Blue.

5.2.3 Using methoprene to isolate the role of JH

In an effort to investigate effects of photoperiod downstream of juvenile hormone signaling, without upstream effects, for example, the direct response to differential light perception, or effects arising from other mechanisms (insulin signaling particularly, ILPs being a proposed identity for the virginoparin) methoprene, a juvenile hormone analog, was used to treat aphids raised in SD, oviparous producing conditions. This presents an opportunity to analyse the role of JH directly in the reproductive switch.

5.2.3.1 Methoprene treatment and maintenance of aphids

L4 viviparous aphids, raised in LD conditions (16:8 L:D, 20 C) for many generations (over twenty) were collected and raised together in groups of ten individuals to adulthood, in insect rearing bags on Sutton variety *V. faba* seedlings under SD conditions (12:12 L:D, 15 C). After two days, at which point all aphids had emerged as adults, aphids were placed in groups of three or two onto agar leaf plates. Plates were randomly assigned as methoprene or acetone control treatment groups. 100 nl of either 0.125 mg/ml methoprene diluted in acetone, 0.25 mg/ml methoprene diluted in acetone, or acetone alone were applied to the dorsal abdomen of each aphid, in two applications of 50 nl delivered using the high speed setting of a Nanolitre 2000 microinjector (WPI) through pulled-glass borosilicate capillary tubes (1.14 mm, 3.5”, WPI) pulled using a Narishige PD-5 micropipette puller (Narishige) set to ‘heater’ = 9, ‘magnet’ = 9, ‘main magnet’ = 9 (Narishige). Needles were broken against a cover slide to create a relatively blunt edge, such that aphids were not penetrated by the needle when applying solutions. The solution, a small drop of liquid relative to the size of an adult aphid, quickly dissolved across the cuticle. Where necessary, aphids were held still with a pair of fine forceps to prevent them from dispersing in response to the needle, application of solution or to the alarm pheromone released by other aphids (Vandermoten et al., 2012). After one day, adult aphids were separated onto individual plates and after two days, aphids had generally started reproducing. Aphids were treated with methoprene or acetone every two days. Nymphs produced by these adults were treated with 50 nl of solution in the same way as adults, until reaching L4, as 100 nl completely covered young nymphs. Adult aphids were allowed to reproduce for three days, after which they were removed and their offspring were retained.

Leaf-agar plates contained no more than ten offspring and were replaced every three days to prevent degradation of the leaf as a food source and the growth of mold, as this would reduce survivability and encourage the production of winged morphs.

5.2.3.2 Phenotyping and ovary sampling

Upon reaching adulthood, the wing phenotype of each aphid was recorded, and a subset of unwinged aphids were randomly selected for sampling. Only unwinged aphids were used for consistency, and because the production of winged offspring is dependent on an additional polyphenism in aphids and winged aphids are incapable of producing winged offspring (which could feasibly be associated with differential gene expression). Aphids were dissected before they started reproducing and within one day of emerging as an adult as described in the general methods. Ovaries were immediately snap frozen in liquid nitrogen, then stored at -80 C.

The remaining aphids were isolated singularly on leaf-agar plates (described in the general methods) and allowed to reproduce for three days; adults were treated with methoprene or acetone up until the end of this period, as before, but their offspring were not. Offspring were monitored daily and observations of phenotype recorded. Offspring were isolated on leaf-agar plates in groups of no more than ten individuals and allowed to reach adulthood, at which point their wing phenotype was determined by external morphology using a dissecting microscope (GXMXTL3T) and ovary phenotype was determined by dissection, also using a dissecting microscope (GXMXTL3T). It is important to note that the individuals from which samples were collected were not the same as those that produced these offspring, as sampling occurred before the onset of reproduction. Though, they occupied the same leaf-agar plates prior to sampling. A layout of the experimental procedure is presented in figure 5.4.

Images of wing phenotype are representative and were acquired using a GXCAM-U3 Series camera (GT Vision) attached to a dissecting microscope (GXMXTL3T), and the GX Capture software (GT Vision). Offspring phenotype images were captured using the same equipment and software.

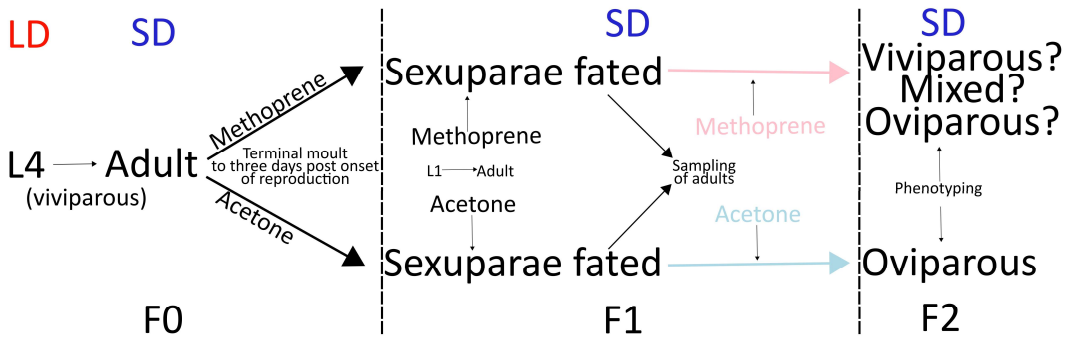

Figure 5.4 Experimental layout for methoprene induced redirection of reproductive fate of SD (short-day) condition *A. pisum*.
Methoprene is a juvenile hormone (JH) analog, and thus, is expected to be able to prevent the eventual production of sexual morphs (which are associated with reduced JH). Aphids raised from L4 in LD (long-day) conditions until reaching adulthooh, and then allowed to reproduce in SD conditions typically produce sexupara offspring (asexual aphids that will produce sexual offspring), but treatment with methoprene is able to prevent this (resulting in offspring consistent with what would be produced in LD).

5.2.4 RT-qPCR

Ovary samples were processed for RT-qPCR analysis. RT-qPCR was performed after RNA isolation and cDNA extraction (with the additional dsDNAse step) from single pairs of ovaries as detailed in the general methods with the exception that, for the methoprene experiment, due to variation in phenotype data, twenty biological replicates for methoprene treatment and ten biological replicates for acetone treatment were used. Seven (LD) and six (SD) biological replicates were used for RT-qPCR in the differential photoperiod reproductive switch experiment, because extra samples were added for each target gene (to account for an outlier, removed) on additional plates. To account for inter-plate variation, the additional replicates were run alongside a calibrator sample from the original RT-qPCR plate and GOI RT-qPCRs were run alongside reference gene RT-qPCRs on the same plate to allow quantification of relative gene expression to then calibrate gene expression values back to the original plates using this inter-plate calibrator. Otherwise, all RT-qPCR plates were run using a sample maximization approach. Two technical replicates were included per sample. *rpl7* and *ef1α* were selected as appropriate reference genes for the methoprene experiment using geNorm and the ctrlGene package in R (appendix table C.4). *tub* and *ef1α* were similarly selected as the most

appropriate reference genes for the switch experiment (appendix table C.3). RT-qPCR reactions were run as specified in the general methods.

5.2.5 Gene identification and targeting

GOI sequences were identified by comparing protein sequences of targeted genes from FlyBase and AphidBase against *A. pisum* sequences in the nr NCBI database using BLASTx, as detailed in the general methods. Primers were designed as specified in the general methods, and where multiple isoforms were present, primers were designed to span shared regions such that all were detected in a single RT-qPCR reaction to give a single product. Target genes and associated primers are presented in appendix table C.2.

5.2.6 Methoprene gene expression clustering

Due to the mosaic of phenotypes observed across the ovaries of methoprene treated aphids, and following from gene-expression analyses based on methoprene treatment group, I sought to group samples into clusters more representative of seemingly more methoprene-responsive (asexual producers), intermediately methoprene-responsive (dual asexual and sexual producers, and producers of aphids with mixed ovaries), and methoprene-unresponsive phenotypes (sexual producers). I selected *lsd* and *cyclinJ* as sexual and asexual markers respectively. *lsd* encodes a protein associated with lipid storage droplets, and its expression has been demonstrated to be highly specific to sexual oocytes and germaria in the pea aphid ovary, likely linked to yolk accumulation in sexual oocytes ((Gallot et al., 2012; Beller et al., 2010); Duncan, unpublished data). Similarly, *cyclinJ* has been demonstrated to be expressed specifically in the oocytes and germaria of asexual pea aphid ovaries (Gallot et al., 2012). It encodes a protein involved in oogenesis and early embryonic divisions in *D. melanogaster* (Kolonin & Finley, 2000). I clustered samples using the expression ratios of these genes using the k-means++ clustering method with a Euclidean distance matrix, having determined the appropriate number of clusters (k = 3) by the elbow method. I assessed the quality of clustering by silhouette analysis.

5.2.7 Data analysis

Relative gene expression was calculated as specified in the general methods, normalizing to reference genes (*rpl7* and *ef1α* for the methoprene experiment, and *tub* and ef1α for the LD/SD switch experiment) and using the geometric mean of acetone/LD (control) treatment sample values for normalisation. The optimum number and identity of reference genes was determined via the *ctrlGene* package, using the geNorm2(), measureM() and pairwiseV() functions.

To analyse difference in gene expression, GLMs were built using the glm() function in base R, fitted with quasipoisson distributions (to account for under-dispersion) and log link functions, after visual inspection of frequency histograms, Cullen and Frey plots, diagnostic plots and by inspecting dispersion. Null models, produced by dropping treatment/cluster as the fixed term, were compared to the full model using the lrtest() function in the *lmtest* package and pair-wise comparisons were performed where appropriate using the emmeans package, which uses the Tukey method to correct for multiple-testing. Inspections of correlation in gene expression were performed using the cor.test() function in base R supplied with method = “kendall”.

K-means++ clustering was carried out using the cluster and *factoextra* packages, using set.seed(123) and the kmeans() function supplied with nstart=10000 and nclusters=3 using a 2D matrix consisting of *lsd* and *cyclinJ* expression ratios. Preceding clustering, the Elbow Method for Optimal Number of Clusters was performed using base R and the ggplot2 package, and following clustering, silhouette analysis was performed using the silhouette() function in the *cluster* package to confirm cluster number.

In the methoprene experiment, to assess survival between groups, Cox proportional hazard models were run using the *survival* package and then pairwise comparisons were computed using the *emmeans* package. For assessment of wing production phenotype proportion, a binomial GLM fitted with a logit link function was run using the *glmmTMB* package, and the full model was then compared to the null model, with the fixed effect of treatment dropped, using the lrtest() function in the *lmtest* package. To assess the ability of methoprene treatment to redirect reproductive mode of treated aphids, a Fisher’s exact test was used to compare proportion of sexual-only producers to

combined asexual-only and mixed producers, in the three days following onset of reproduction.

Graphs were constructed using ggplot2 or base R (silhouette analysis), and formatted using InkScape.

5.3 Results

5.3.1 Switch experiment

5.3.1.1 The switch can occur across few generations

I first set out to determine the number of generations that would be necessary to induce production of oviparous morphs under the specified experimental design. After exposing the f0 to SD upon maturing to adults, but before the onset of reproduction, the f1 produced in the first three days after the onset of reproduction, and reared under the same conditions produced oviparous offspring upon reaching adulthood themselves. These SD f1 therefore represent sexuparae, and their offspring produced in the first three days of the onset of reproduction were invariably oviparous, bearing ovaries containing sexual eggs. All SD f1 individuals produced on the first, second and third day by f0 individuals produced sexuparae. Equal aphids exposed to LD conditions produced viviparous offspring only in all generations. Because of this, the f1 were selected as the focal generation of the following experiments, as their ovaries contained developing future oviparae embryos, oviparae destined oocytes, and because the f1 is the generation where the switch is likely primarily detected and integrated in the brain, and effected in the ovary. This is especially clear given the relatively small amount of time the f0 were exposed to SD conditions for.

5.3.1.2 Distinct gene expression profiles between SD and LD aphids

Having identified the f1 as the sexuparae, I next quantified the expression of several genes putatively involved in the reproductive switch. These genes were selected, mostly, for their involvement in the JH and insulin signaling pathways, two highly inter-linked pathways, which are known to be responsive to photoperiod (SD/LD) and involved in the switch. I targeted these genes in the

ovary to assess potential differential expression downstream of adult reception/integration, specifically targeting ovary- and embryo-level changes.

I began by searching for homologues of key insulin and juvenile hormone signaling related genes in the pea aphid. I identified *A. pisum* homologues of *bmm*, *foxo*, two copies of *inr*, *ilp1*, *ilp4*, *ide*, three copies of *idel*, *e75*, *kr-h1*, *ddc*, *dnmt3x*, *brc*, *jhe1*, *jhe2*, two copies of *met*, *vg* and *vgr*. Of the three copies of *idel*, I only targeted one, which I termed *idel1* (LOC100163575, which was the most appreciably expressed of the three, based on NCBI RNAseq data), due to difficulties in designing sufficiently efficient primers targeting the other two identified *idel* genes. *foxo* (principally), *ilp1*, *ilp4*, *inr*, *ide* and *idel* can essentially be seen as readouts for insulin signalling, while *kr-h1* (principally), *jhe1*, *jhe2* and *met* are readouts for juvenile hormone signalling, and *brc* and *e75* are readouts for ecdysone signalling. *4-ebp* is a key part of the insulin signaling pathway, but curiously, I could not identify it in the *A. pisum* genome. I did, however, identify homologues of it in several other insect species, including representative of the hemiptera. I quantified the expression of the identified homologues, in addition to *dnmt3a*, *dnmt3x* and *vasa*, three genes for which I explored the ovary specific expression patterns of in chapter four, revealing *dnmt3a* and *dnmt3x* expression to be specific and colocalised in the germ cells, oocytes and early embryos.

In SD relative to LD ovaries, of the assessed genes, eight showed significant differential expression. *bmm*, *dnmt3x*, *dnmt3a* (the two DNA methyltransferase 3 paralogues explored in chapter four which arose from an Aphidomorpha-specific duplication), *met*, *vgr*, *krh1*, and *inr1* were upregulated in SD conditions relative to LD, while *idel1* was downregulated in SD conditions (figure 5.5, figure 5.6) (comparisons for each gene are presented in appendix table C.5). While differences in relative expression were mostly modest (being typically < 2 for upregulation in SD relative to LD), *bmm*, *dnmt3x* and *vgr* were more differentially expressed between conditions (figure 5.5), with *vgr* showing the greatest level of difference, with the SD average relative expression value being 6 <. Taken together, the expression profile of SD ovaries is characterized by upregulated insulin signaling and upregulated juvenile hormone signaling, and associated upregulation of genes putatively downstream of these systems (*bmm*, *vgr*), and by an upregulation of DNMTs.

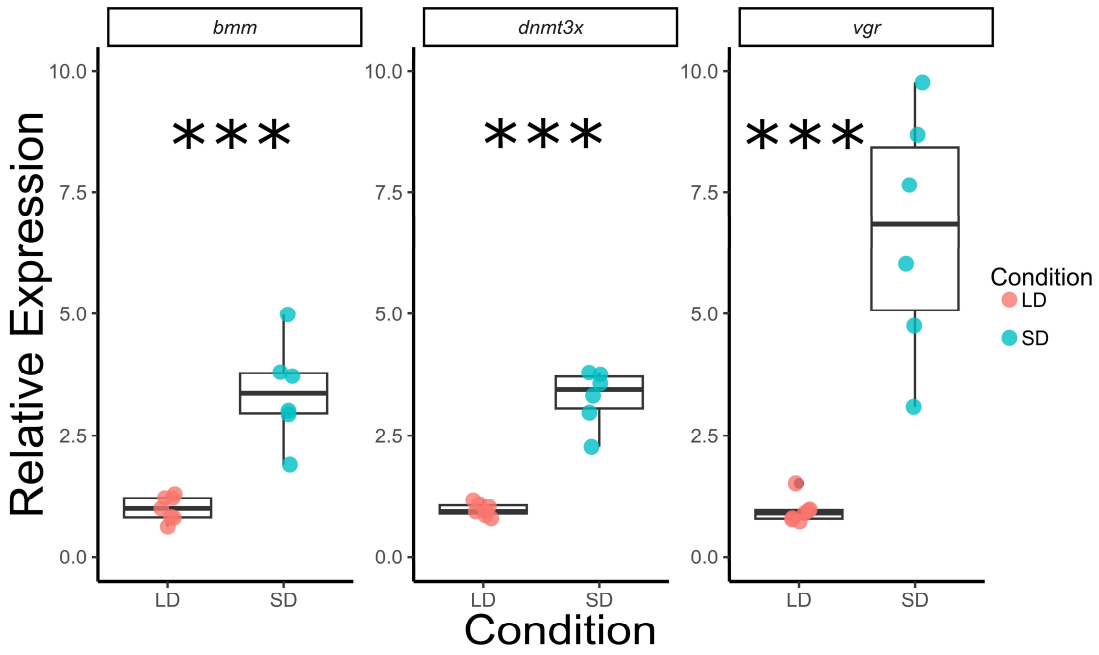

Figure 5.5 *bmm*, *dnmt3x* and *vgr* relative expression in ovaries of *A. pisum* in LD (long-day) and SD (short-day) conditions.
Relative expression was measured by performing the Pfaffl method after RT-qPCR (see methods). Relative expression of *bmm*, *dnmt3x* and *vgr* was revealed (by GLMs) to be significantly higher under SD conditions than LD conditions. *** indicates p-values <0.001.

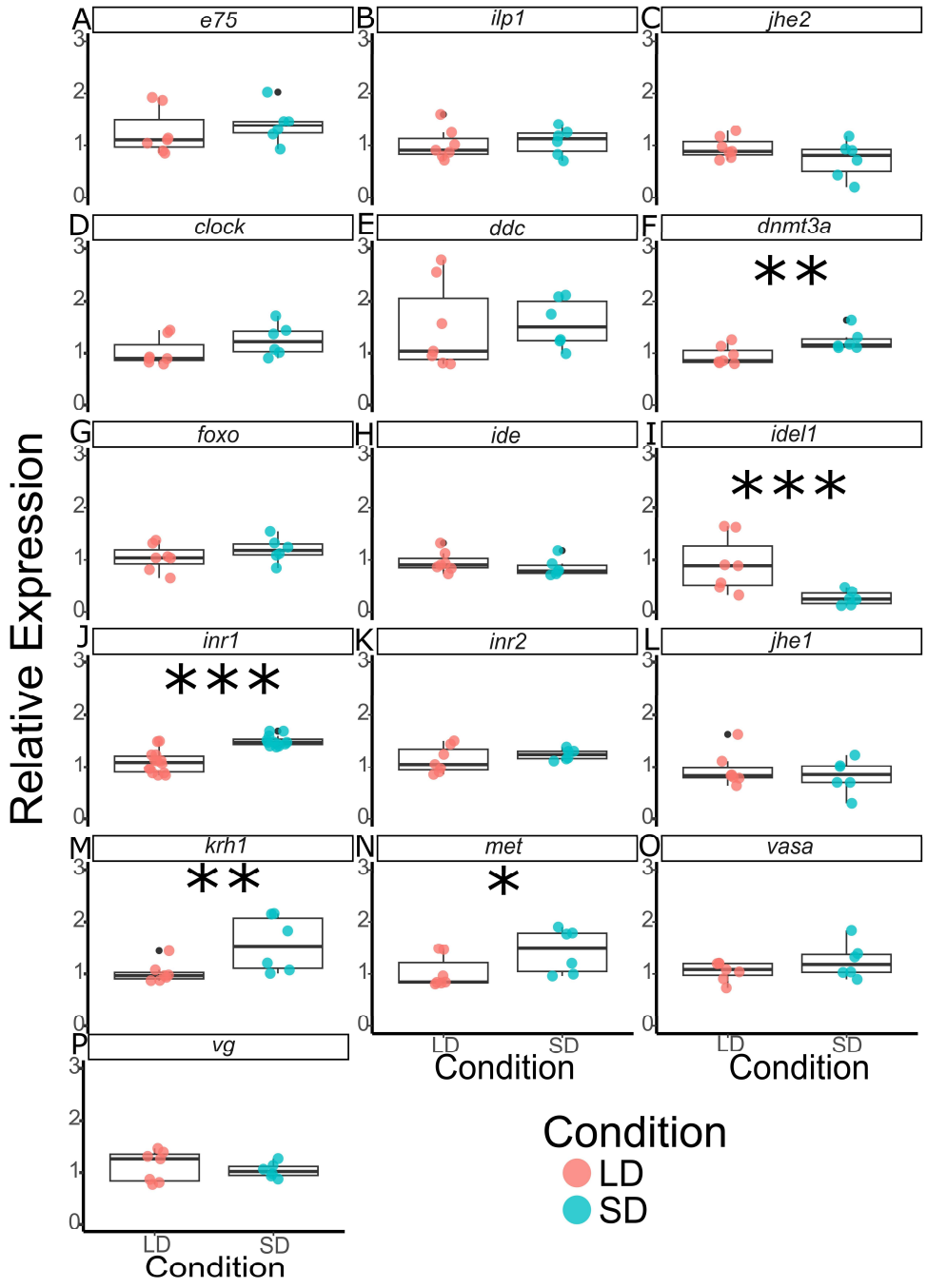

Figure 5.6 Relative expression of several genes, relating primarily to juvenile hormone and insulin signaling, related and downstream factors, and genes demonstrated to be expressed in ovaries, in ovaries of *A. pisum* individuals in LD (long-day) or SD (short-day) conditions. Relative expression was measured by performing the Pfaffl method after RT-qPCR (see methods). GLMs revealed significant differences between conditions for F. *dnmt3a*, I. *idel1*, J *inr1*, M *krh1* and N, *met*. In each of these cases, except for in the case of I, *idel1*, relative expression was significantly higher in SD relative to LD conditions. Statistical significant comparisons are denoted on plots, *, p = < 0.05, **, p = < 0.01, ***, p = < 0.001.

5.3.1.3 dnmt3x exhibits an expanded expression pattern in sexuparae ovaries

Because *dnmt3x* expression was relatively highly upregulated (especially compared to the upregulation seen for many of the other genes investigated) in the ovaries of SD relative to LD condition aphids, I investigated its expression pattern in SD ovaries using RNA-FISH HCR in the same way as laid out in chapter 4. Preliminary visualization of *dnmt3x* mRNA in the ovary suggests an expression pattern partially consistent with that observed in LD conditions (see chapter 4), in that expression is enriched in germ cell clusters, germaria and early embryos (figure 5.7, a, b, c, d, e, f; ellipses/circles highlight germ cell signal in embryos). Additionally, and beyond the expression pattern observed in the ovaries of aphids reared in LD conditions, sexupara embryos appear to have a slightly expanded expression profile of *dnmt3x* (figure 5.7, g, h, i, j). Punctate, uniformly distributed *dnmt3x* signal can be observed in the germband (from stage 11 onwards, figure 5.7, g, h, i, j, arrowheads provide emphasis of some areas of signal). Although, data from more developmental stages will be necessary to confirm if the pattern is consistent during development. Regardless, *dnmt3x* expression shows an expanded range, coincident with upregulation of this gene detected by RT-qPCR in sexuparae. The *vasa* signal was, as expected, confined to the same regions as demonstrated in LD ovaries (see chapter 4), in accordance with the observation of consistent expression levels between LD and SD ovaries.

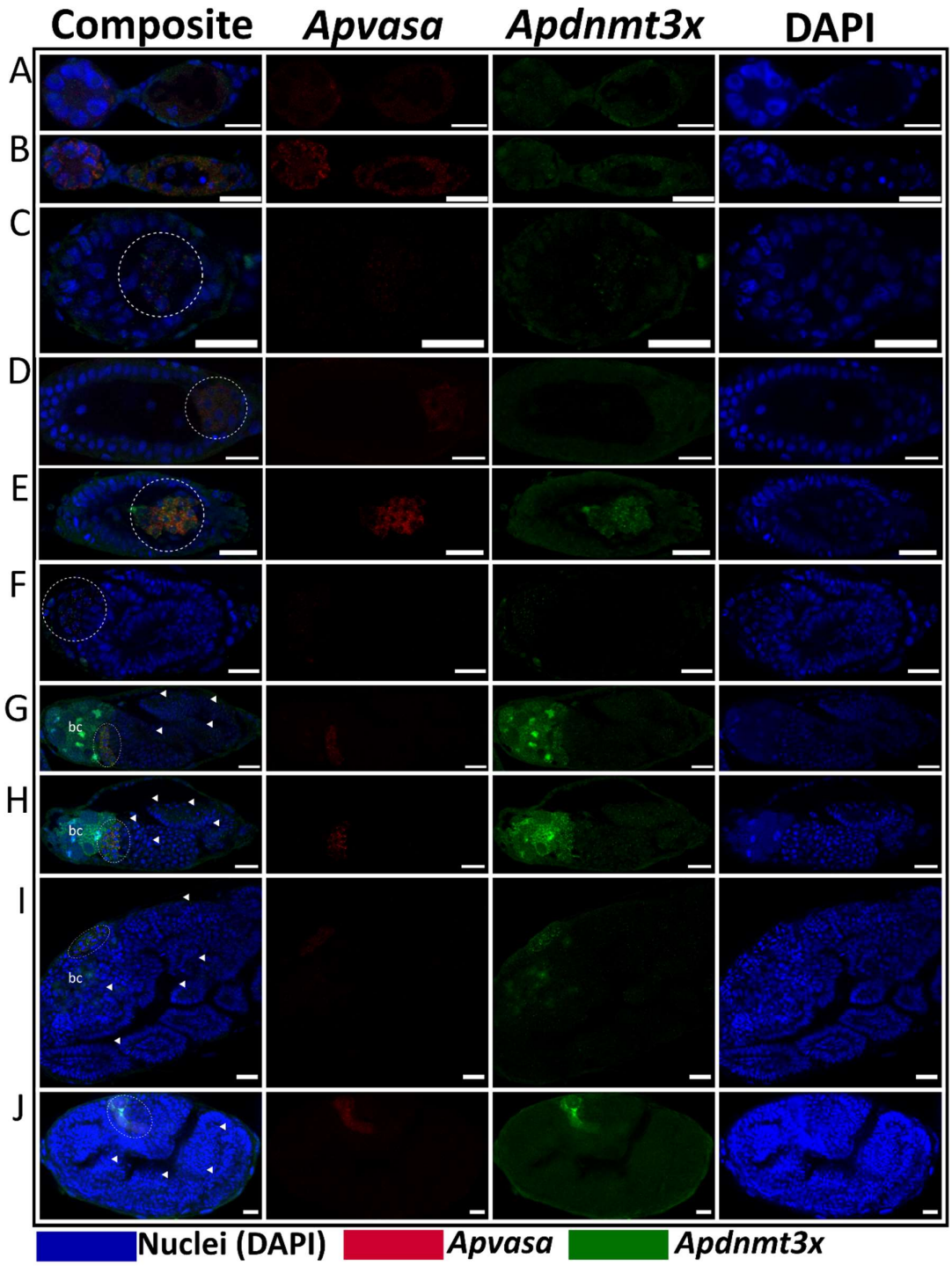
Composite
Apvasa
Apdnmt3x
DAPI
A
B
C
D
E
F
G
H
I
J
bc
bc
bc
Nuclei (DAPI)
Apvasa
Apdnmt3x

Figure 5.7 Expression pattern of *vasa* and *dnmt3x* in *A. pisum* sexupara ovaries.
Germaria (A, B, left), oocytes (A, right) and embryos (B, right to J) from ovaries of *A. pisum* sexuparae, generated by confocal microscopy of RNA-FISH HCR processed ovaries targeting mRNA of *vasa* and *dnmt3x*, and counterstained with DAPI (to stain nuclei). Composite images are presented alongside individual channels. Panels are presented in descending order from least to greatest level of development. Rows A to F show *dnmt3x* expression patterns consistent with virginoparae, being confined to germaria, very early stage embryos and germ cells (which are made apparent by *vasa* signal, highlighted in relevant composite images by dashed ellipses/circles), while rows G to J show *dnmt3x* expression expanded to and diffuse within the germ band (examples of this signal are emphasised in composite images by arrowheads), indicating an expanded expression pattern. bc = bacteriocytes.

5.3.2 Methoprene experiment

Following the switch experiment, I sought to further examine the reproductive switch at the level of the ovary by isolating juvenile hormone responsive and unresponsive elements, and targeting gene expression differences downstream of sensory/modulatory systems by bypassing the induction of juvenile hormone titre increases associated with exposure to LD.

5.3.2.1 Methoprene phenotypes

First, I characterised the general effects of methoprene on survival and phenotype. In addition to a role in the reproductive polyphenism, juvenile hormone and methoprene are known to exert effects on the wing polyphenism (Ishikawa et al., 2013; Bai et al., 2022). To assess comparability between treatments, I assessed the wing-mode of focal aphids upon reaching adulthood. There was no significant difference in the appearance of winged individuals relative to wingless individuals in this generation and the general occurrence was low, ranging from 2 to 9.3 % (GLM, LRT, df = 2, 3 χ^2 = 3.5026, p = 0.1736, figure 5.8). Although there was a trend towards a reduced proportion of winged individuals in the 0.25 mg/ml methoprene treatment relative to 0.125 mg/ml methoprene and acetone treatments. Where winged morphs did occur in 0.125 mg/ml and 0.25 mg/ml methoprene treatments (but not acetone), wings always appeared crumpled, over-inflated or reduced, and/or unilateral and always appeared non-functional (figure 5.9); where present, these phenotypes persisted for the entire study period, which is to say that non-functional wings did not later become functional. Additionally, the wing muscles appeared

reduced. No winged individuals were observed in the subsequent generation for any treatment.

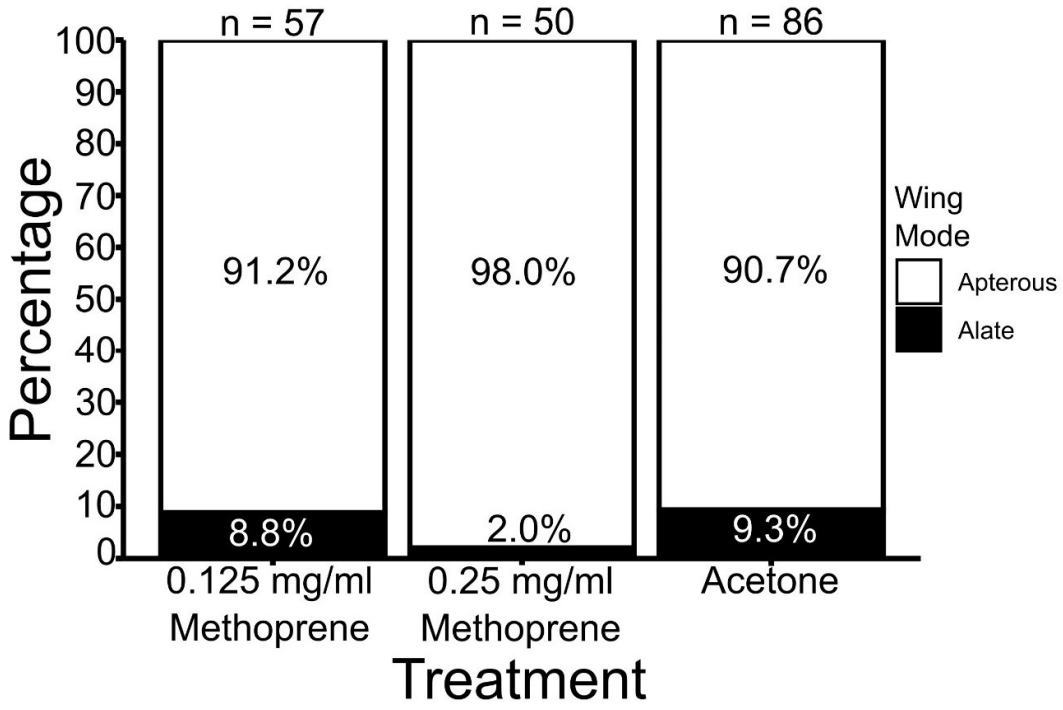

Figure 5.8 Methoprene effect on wing production mode.
Percentage of SD (short-day) condition *A. pisum* individuals produced by adults treated with 100 nl, and themselves treated with 50 nl or 100 nl upon reaching the L4 nymph stage, methoprene at concentrations of 0.125 mg/ml or 0.25 mg/ml in acetone, or acetone as a control every two days from birth to adulthood that were alate (winged) or apterous (wingless) as a proportion of the total number of individuals per treatment, upon reaching adulthood.

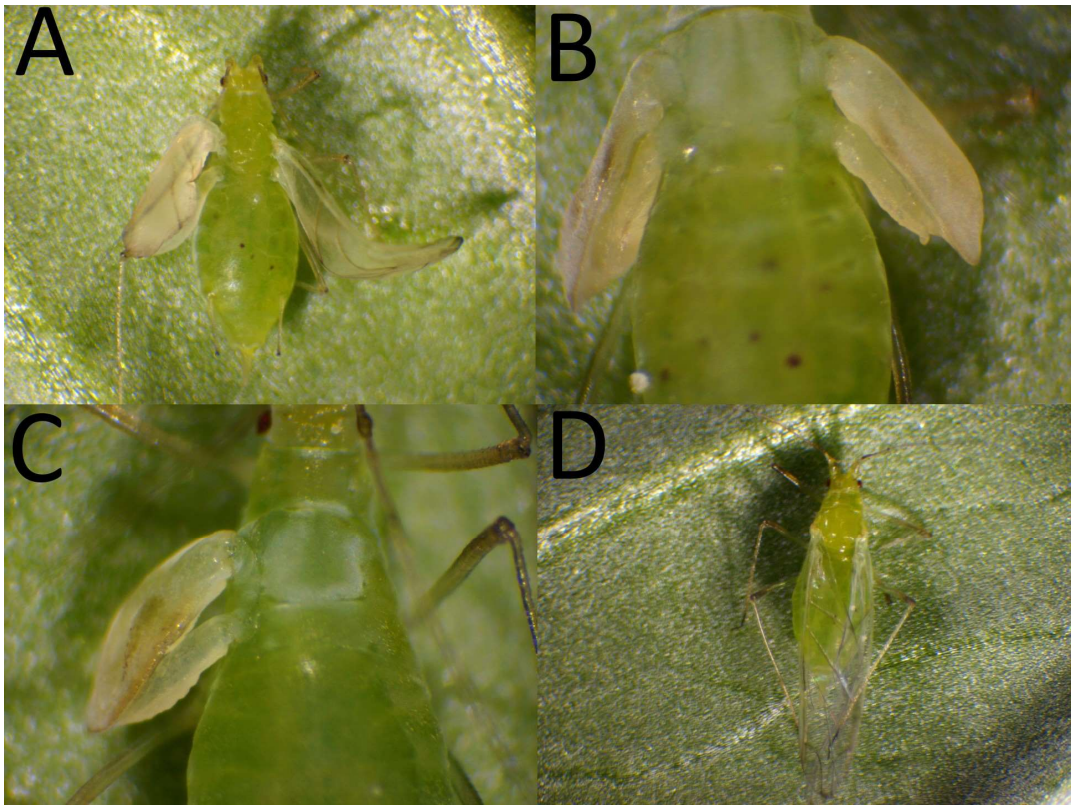

Figure 5.9 Methoprene treatment effects on wings.
Representative images of disturbed wing phenotypes of parthenogenetic *A. pisum* individuals treated with 0.125 mg/ml or 0.25 mg/ml methoprene, including wings that were crumpled (A), over-inflated and reduced (B, C), and unilateral (C), and reduced wing muscles alongside an acetone treated *A. pisum* individual displaying the typical wing form and muscularization of the thorax. Individuals bearing these phenotypes did not then go on to develop typical, functional wings. Individuals were treated indirectly while embryos by treatment of their parthenogenetic mothers from the L4 nymphal stage to reproduction, and then every two days following their birth.

Aphids were treated with methoprene or acetone from birth until they were sampled or removed (aphids were removed after they had reproduced sufficiently for subsequent investigation). Focal 0.25 mg/ml and 0.125 mg/ml methoprene treated aphids had equal survival between birth and day fifteen, at which time a large proportion of adults were sampled, though their lifespans were significantly lower than for acetone treated aphids (Cox proportional hazard, p = <0.001, figure 5.10, appendix table C.1). A subset of aphids were not sampled and were left in order to assess the reproductive phenotype of the next generation. However, 0.25 mg/ml methoprene treated aphids often died before reproducing at all or at least at sufficient levels (comparable to the

production of 0.125 mg/ml methoprene treated or acetone treated individuals). This effect was a combination of delay in emerging as adults, dying during the moulting process from L4 to adult, and a slightly (though, not statistically significant (Cox proportional hazard, p = 0.128), figure 5.11) increased mortality rate (over 0.125 mg/ml individuals). As a result few offspring of 0.25 mg/ml methoprene treated individuals were available for assessment of ovary phenotype. As such, all analyses following are based on acetone and 0.125 mg/ml methoprene (referred to simply as methoprene in the following) treated aphids.

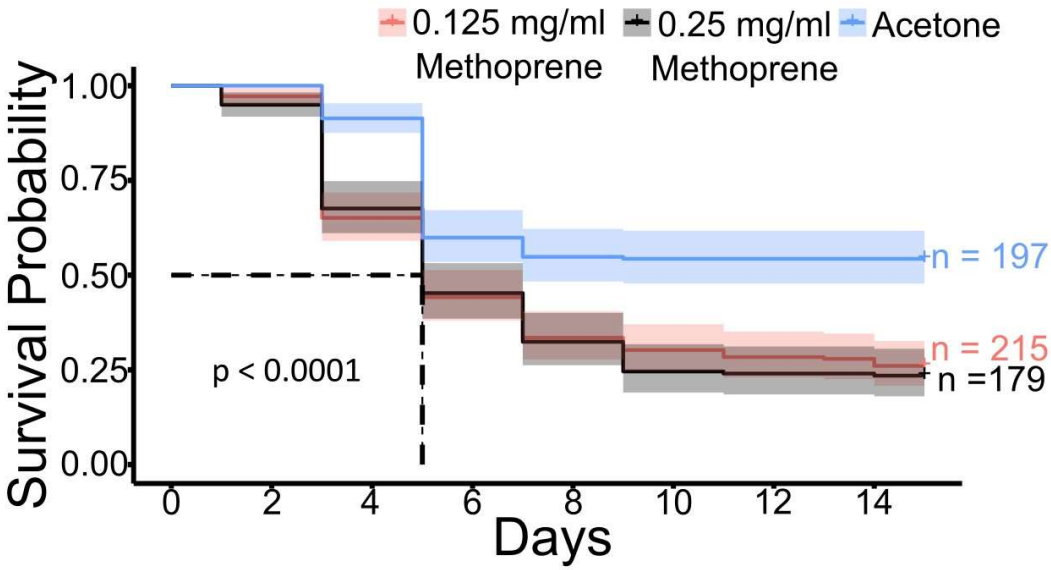

Figure 5.10 Survival of aphids in the methoprene experiment prior to sampling period.
Kaplan-Meier survival curve of *A. pisum* individuals produced by adults treated with 100 nl, and themselves treated with 50 nl or 100 nl upon reaching the L4 nymph stage, methoprene at concentrations of 0.125 mg/ml or 0.25 mg/ml in acetone, or acetone as a control from birth to adulthood or death. Day 0 is specified as the third day after the onset of reproduction. No individuals were removed for sampling (for RT-qPCR experiments) during the days represented here, except on day 15.

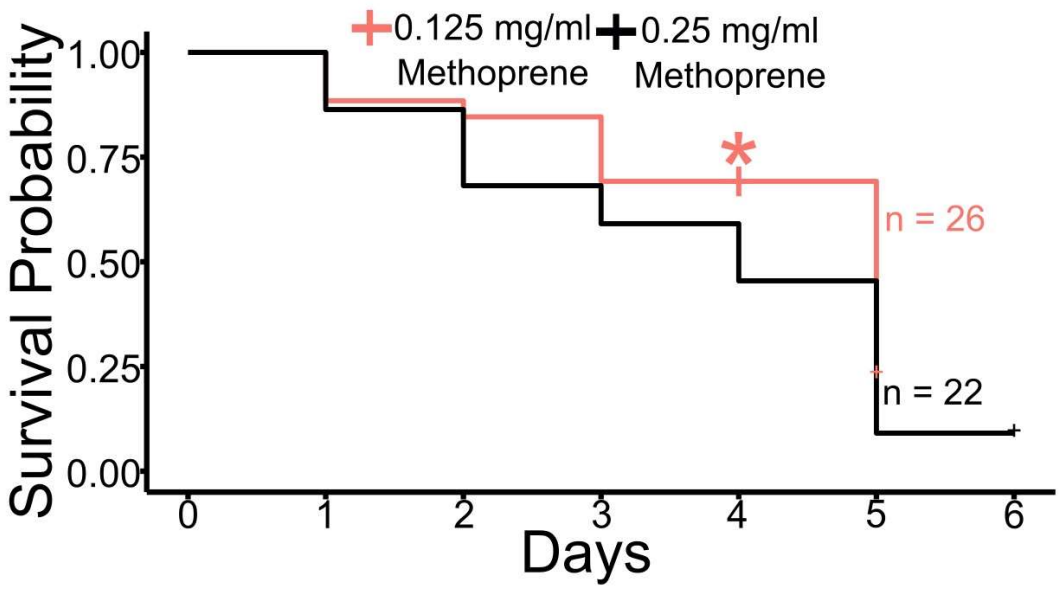

Figure 5.11 Survival of aphids in methoprene experiment after the main sampling period.
Kaplan-Meier survival curve of A. pisum individuals produced by adults treated with 100 nl, and themselves treated with 50 nl or 100 nl upon reaching the L4 nymph stage, methoprene at concentrations of 0.125 mg/ml or 0.25 mg/ml in acetone from birth to adulthood or death. Day 0 is specified as the first day of sampling of aphids that reached adulthood. Acetone survival is omitted from the figure due to large amount of sampling that occurred prior to this period drastically reducing sample size. The red asterix represents the day at which a large number of 0.125 mg/ml individuals were censored due to having reproduced sufficiently for further study (expression analysis by RT-qPCR), and this largely explains the perceived drop in survival from day 4 to day 5. No such censorship was performed for 0.25 mg/ml individuals due primarily to insufficient/no reproduction having occured.

Methoprene treatment yielded a mosaic of phenotypes. Ordinarily, in SD conditions, all offspring produced by aphids at the level of the focal adults used here are sexual (see results, above (5.3.1.1)). This was observed for acetone control treated aphids. However, of the aphids treated with methoprene, 15/22 produced at least some offspring with (at least partially) asexual ovaries in the three days following the onset of reproduction, while 7/22 produced only offspring with sexual ovaries. Altogether, methoprene treatment resulted in significantly more non-sexual only reproductive modes being observed (Fisher's exact test, $p = <0.001$, figure 5.12). Some aphids produced offspring possessing mixed ovaries, containing both developing sexual eggs and asexually produced embryos.

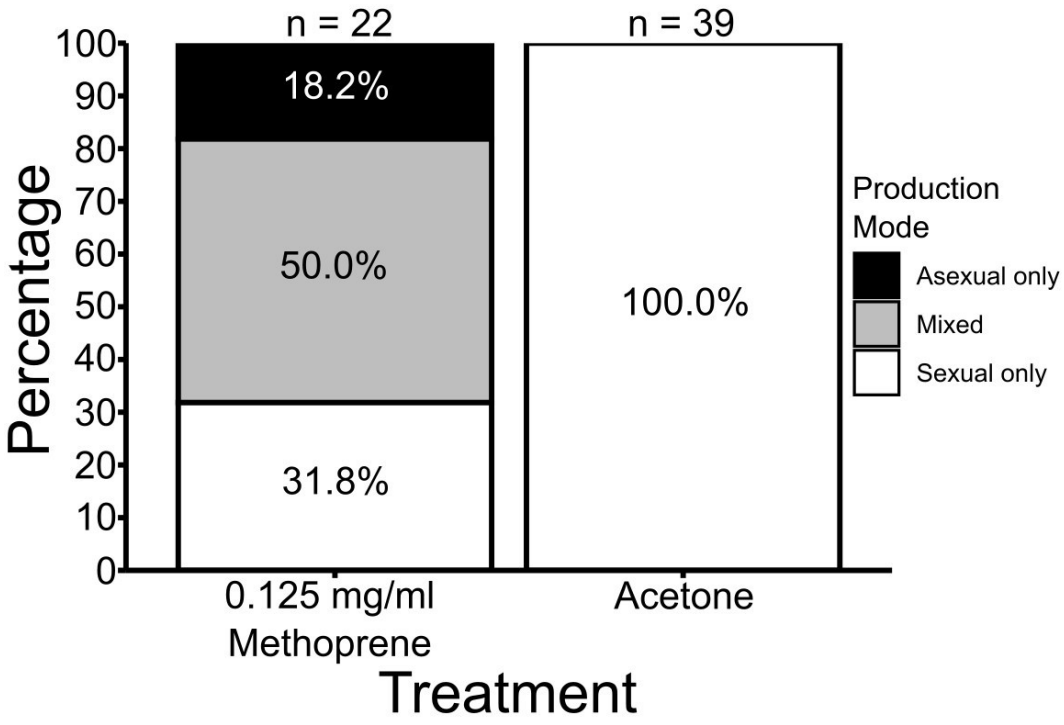

Figure 5.12 Effects of methoprene on reproductive nature of offspring. Percentage of *A. pisum* individuals produced by adults treated with 100 nl, and themselves treated with 50 nl or 100 nl upon reaching the L4 nymph stage, 0.125 mg/ml methoprene in acetone or acetone as a control from birth to adulthood that possessed ovaries containing future asexual individuals only, future sexual individuals only, or both, under SD conditions, assessed by the phenotype of their offspring produced over the first 3 days of reproduction upon reaching adulthood. Acetone treatment produced sexual offspring only while 0.125 mg/ml methoprene produced a mosaic of phenotypes, dominated by mixed and asexual only production.

In addition, some methoprene treated individuals produced a small proportion of disturbed offspring, which were either born dead or died before extending their legs away from their body after being birthed (figure 5.13). Though, they were relatively rare. These disturbed offspring were not observed to be produced by acetone treated aphids.

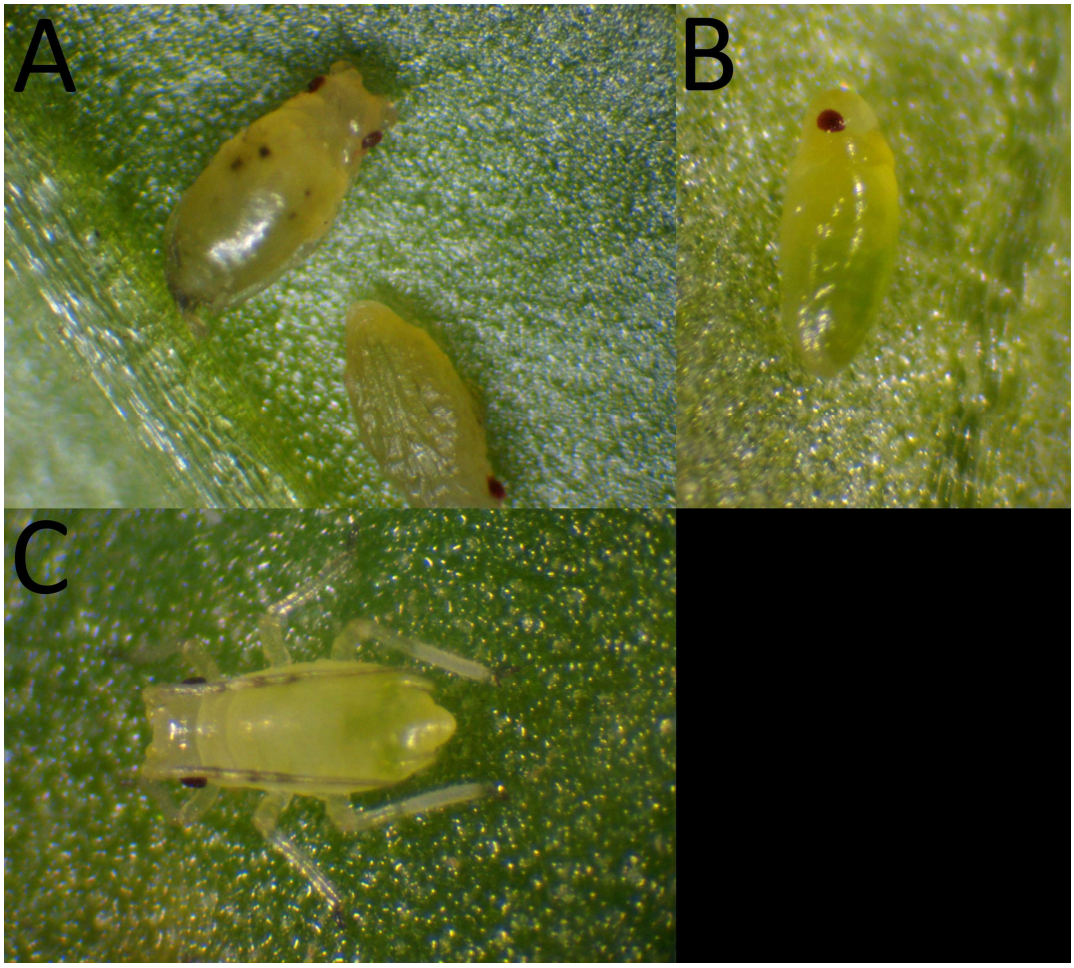

Figure 5.13 Rare disturbed phenotype resulting from methoprene treatment.
Representative images of disturbed offspring produced by *A. pisum* individuals treated with 0.125 mg/ml or 0.25 mg/ml methoprene, representing a low proportion of the total offspring (A, B). Disturbed nymphs did not unfurl their legs or develop further and were either born dead or died shortly after being birthed. Disturbed nymphs are presented alongside a typical nymph (C), representative of most nymphs produced by methoprene treated individuals and all acetone treated individuals. The parthenogenetic mothers of these nymphs were treated pre-natally by treatment of their parthenogenetic mothers from the L4 nymphal stage to reproduction, and then every two days following their birth until reproduction.

5.3.2.2 Gene Expression based on Methoprene Treatment Condition

Having confirmed, phenotypically, that regular application of 50/100 nl (depending on developmental stage) methoprene at 0.125 mg/ml was capable of diverting reproductive fate of offspring, I then investigated gene expression in the ovaries of methoprene and acetone treated aphids. I targeted genes I demonstrated to be differentially expressed between SD and LD ovaries in the switch experiment, and putatively therefore associated with the reproductive switch, to assess if they are responsive to JH, JH being a clear mediator of said switch, or if they are likely responsive to another modulator. By assessing gene expression under pseudo-JH-elevated-conditions, I hoped to unpick exactly how JH, mechanistically, contributes to the switch.

Following from the observation of a mosaic of phenotypes across methoprene treated ovaries, and the fact that the sampling method rendered it impossible to directly assess individual ovaries (aphids were sampled before the onset of reproduction, but offspring were required to assess phenotype), twenty methoprene ovary biological replicates and ten acetone ovary biological replicates were included for RT-qPCR for better representation of each phenotype.

Of the genes shown to be differentially expressed between LD and SD ovaries, I targeted *kr-h1*, *dnmt3x*, *idel1*, *bmm*, *met*, *vgr* and *inr1* (figure 5.14, figure 5.16). The relative expression of these genes did not differ significantly between methoprene and control treated ovaries, except in the case of *idel1* (figure 5.16, c) (comparisons for each gene are presented in appendix table C.6) which was upregulated in methoprene treated ovaries relative to acetone treated ovaries, suggestive it is responsive in the ovary to juvenile hormone signalling. Though, the observation of a positive correlation between *kr-h1* and *dnmt3x* expression (Kendall rank correlation, tau = 0.3977011, p = 0.001723, figure 5.15) suggests a connection between juvenile hormone signalling, which is facilitated in part by a signalling cascade leading to *kr-h1* upregulation, and *dnmt3x*, one of the two *dnmt3* paralogs (the derived, diverged copy) investigated and shown to have expression specific to the germ cells in chapter 3. Together, these results suggest that many of the genes activated in the ovary by SD conditions (and putatively associated with the switch to oviparous reproduction) are not responsive to JH, at least within a simple relationship.

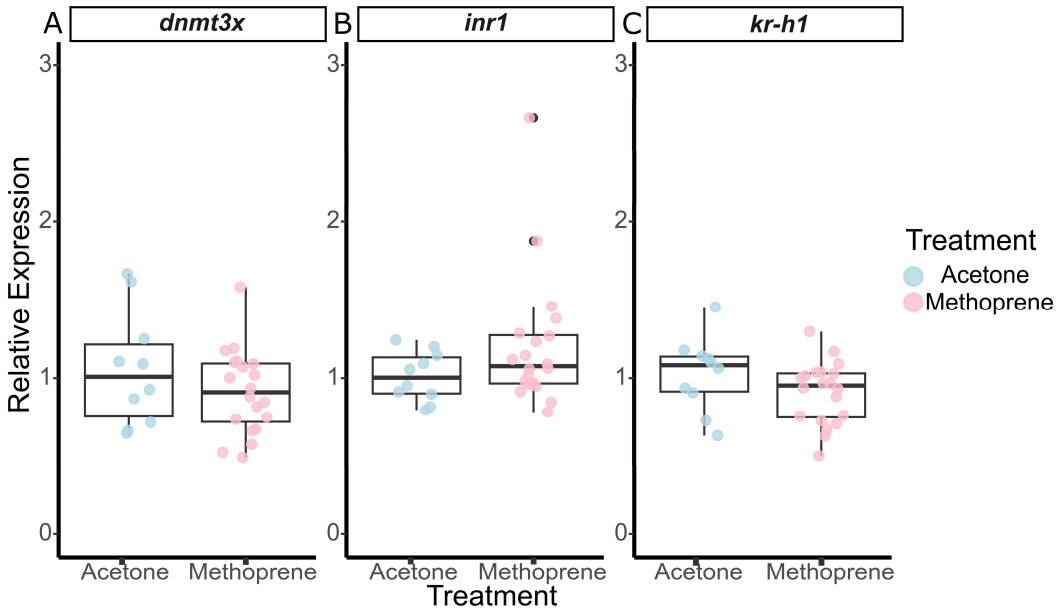

Figure 5.14 Relative expression of *dnmt3x*, *inr1* and *kr-h1* in acetone and 0.125 mg/ml methoprene treated SD (short-day) condition *A. pisum* ovary samples (by treatment).
Due to variation in phenotypic data (the methoprene treatment group encompassing individuals that produced asexual, sexual or mixed reproductive offspring), twenty methoprene biological replicates and ten acetone biological replicates are included. No statistically significant difference was detected (by GLM) between treatments for any gene.

Because of the partial penetrance of methoprene in affecting reproductive fate, and because *kr-h1*, a primary responder to juvenile hormone signalling, was not elevated by methoprene exposure (but was elevated in SD relative to LD samples), I quantified the expression of four juvenile hormone related genes, which may have explained how methoprene activity could be dampened: *met*, *clock*, *jhe1* and *jhe2* (two putative receptors and two putative degrading enzymes of JH). None of these genes showed significant differential expression between conditions, suggesting that these genes are not responsible for a lack of effect of methoprene, and that they may not be responsive to JH signalling, again, at least in a simple relationship.

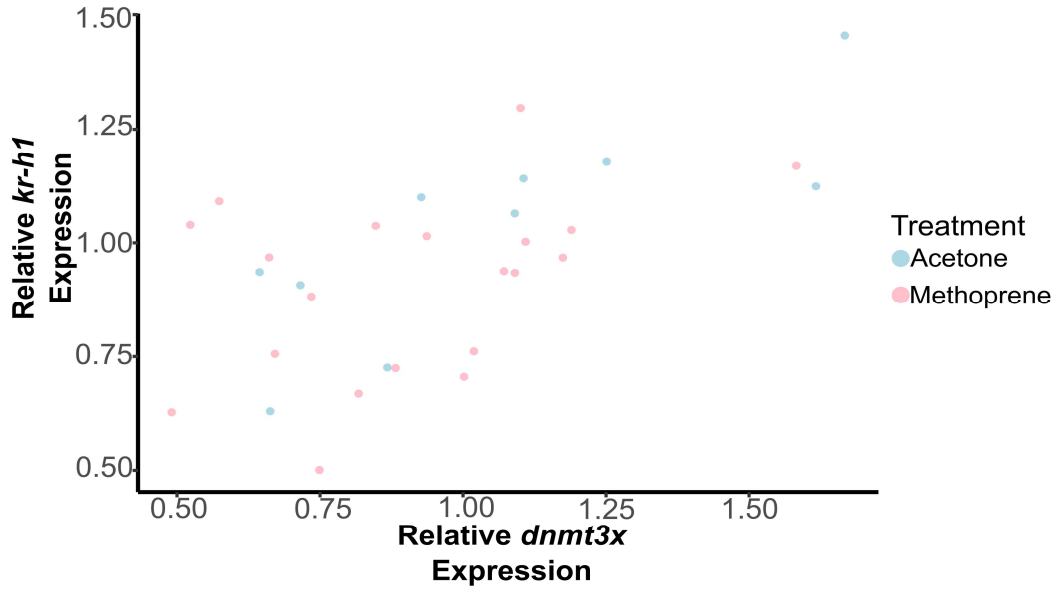

Figure 5.15 Correlation between *kr-h1* and *dnmt3x* expression in SD condition *A. pisum* ovary samples.
kr-h1 relative gene expression plotted against *dnmt3x* relative gene expression of acetone and 0.125 mg/ml methoprene treated *A. pisum* ovary samples. Due to variation in phenotypic data, twenty methoprene biological replicates and ten acetone biological replicates are included (the methoprene treatment group encompassing individuals that produced asexual, sexual or mixed reproductive offspring). There is a strong positive correlation between *kr-h1* and *dnmt3x* expression (kendall rank correlation, tau = 0.3977011, p = 0.001723).

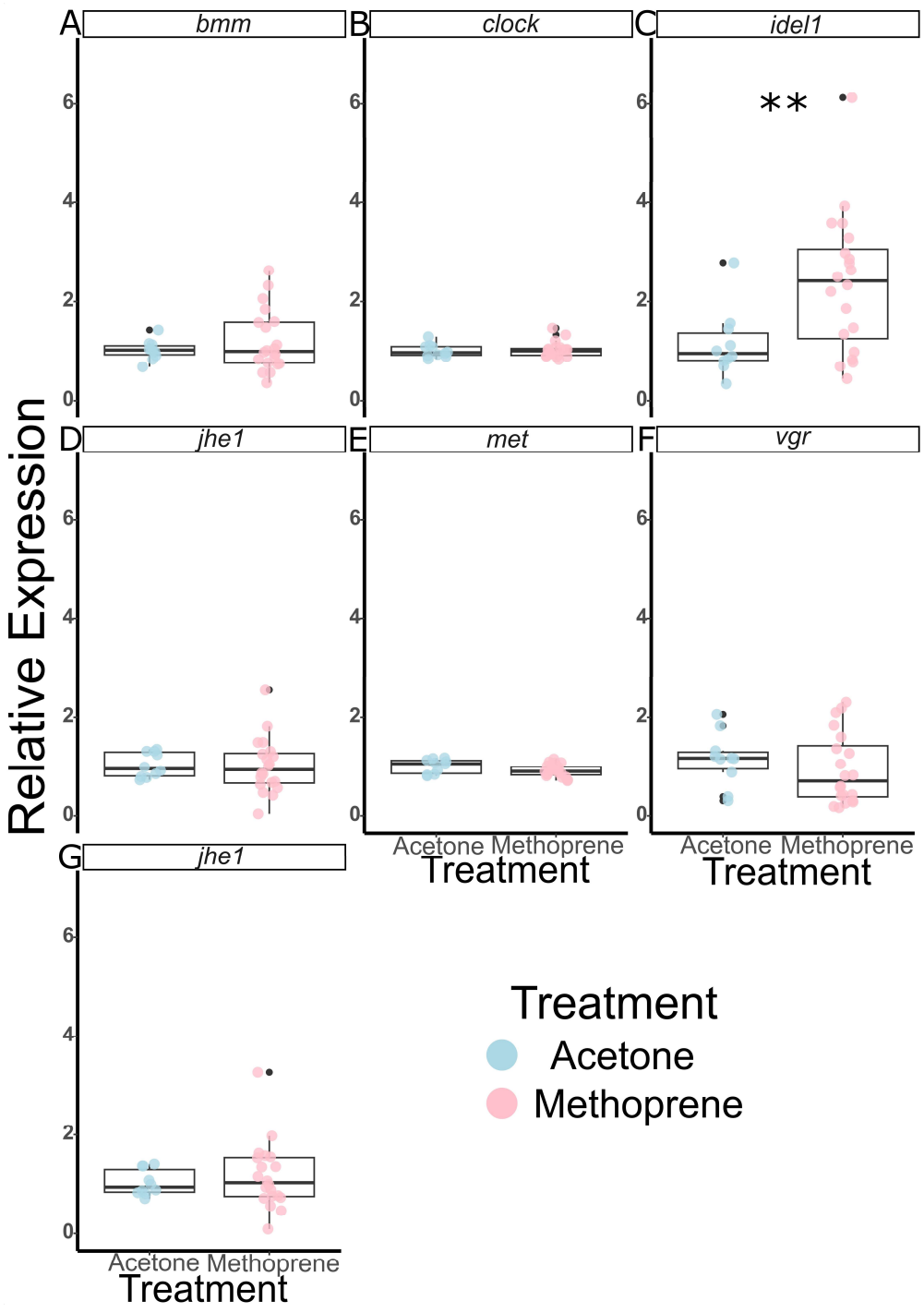

Figure 5.16 Relative expression of *bmm*, *vgr*, *idel1* and four genes more directly related to juvenile hormone (*met*, *clock* (a homolog of *met*), *jhe1* and *jhe2*) in acetone and 0.125 mg/ml methoprene treated SD (short-day) condition *A. pisum* ovary samples.
Due to variation in phenotypic data (the methoprene treatment group encompassing individuals that produced asexual, sexual or mixed reproductive offspring), twenty methoprene biological replicates and ten acetone biological replicates are included. No statistically significant difference was detected (by GLM) between treatments for any gene except *idel1*.

5.3.2.3 Gene Expression Based on Asexual- and Sexual- Specific Gene Clustering

Following the observation of partial penetrance of methoprene in affecting the reproductive fate of offspring (apparent by the mosaic of phenotypes observed in the ovaries of offspring), I assessed the relative expression of a sexual-reproduction specific gene, *lsd* and of an asexual-reproduction specific gene, *cyclinJ*, in an attempt to cluster samples based on phenotype (presumed asexual producers vs presumed sexual producers) rather than treatment. Visual inspection of *lsd* expression ratio plotted against *cyclinJ* expression ratio indicated separation of samples into three prospective clusters with distinct expression profiles for these two genes. *lsd* expression was negatively correlated with *cyclinJ* expression when all data was considered (Kendall rank correlation, tau = -0.3563218, p = 0.005297, figure 5.17), consistent with these genes being markers of sexual (*lsd*) and asexual (*cyclinJ*) oocytes and germaria, as previously reported, but not when acetone treated samples were taken alone (Kendall rank correlation, tau = -0.4222222, p = 0.1083) (likely arising from the little variation associated with the fully sexual nature of acetone treated aphids – high *lsd*, low *cyclinJ*). This further indicated the appropriateness of these two genes as proxies for sexual and asexual development. K-means++ clustering of total gene-expression ratio data using two dimensions (*lsd* and *cyclinJ*), a K of three (indicated as an appropriate number of clusters by the elbow method) and a Euclidean distance matrix confirmed the occurrence of three distinct clusters (figure 5.17, b) characterised by: high *cyclinJ* expression and low *lsd* expression (relative to acetone samples), this cluster included only methoprene treated samples and likely contains samples derived from aphids who were highly affected by methoprene; baseline *cyclinJ* and baseline *lsd* expression (relative to acetone samples), this cluster represents sexupara acetone treated aphids (all acetone treated aphids belonged to this cluster) and seemingly unaffected-by-methoprene (in terms of sexual mode of offspring) methoprene treated aphids; and, surprisingly, a third cluster characterised by high *lsd* expression, and baseline *cyclinJ* expression (relative to acetone samples), composed of methoprene treated samples only. Cluster robustness was confirmed by silhouette analysis (average silhouette widths greater than 0.5 for each cluster, appendix figure C.2) and the elbow

method (appendix figure C.1). Differences in relative expression of *lsd* (GLM, LRT, df = 2, 28, χ^2 = 8.6196 p = <0.001) and *cyclinJ* (GLM, LRT, df = 2, 28, χ^2 = 7.9924, p = <0.001) between clusters were confirmed statistically. For *lsd*, all clusters showed high statistically significant dissimilarity from each other, importantly, both clusters containing methoprene-treated samples only were significantly lower (cluster 3), or higher (cluster 1) for *lsd* expression than the acetone-like cluster (figure 5.18, appendix table C.9). For *cyclinJ*, while cluster 3 has significantly higher expression than cluster 1 and cluster 2, cluster 1 and cluster 2 were not significantly different suggesting approximately equal proportions of late-stage viviparous embryos (zero) (figure 5.18, appendix table C.9).

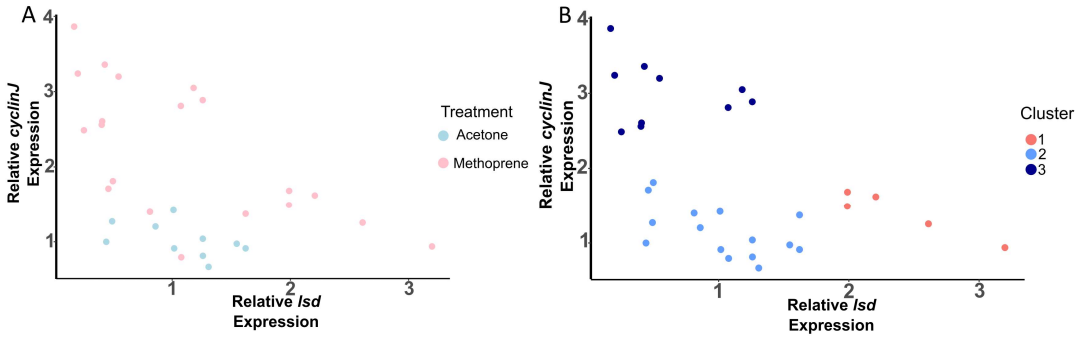

Figure 5.17 Expression of *lsd*, a sexual *A. pisum* specific marker plotted against expression of *cyclinJ*, an asexual *A. pisum* specific marker, in acetone and 0.125 mg/ml methoprene treatment *A.pisum* ovary samples. Samples are separated by treatment (A), and by k++ means clusters formed based on *lsd* and *cyclinJ* expression profiles (B). Clustering reveals three distinct populations within the grouped twenty methoprene treatment and ten acetone treatment samples, representing one acetone/methoprene mixed cluster and two distinct methoprene clusters.

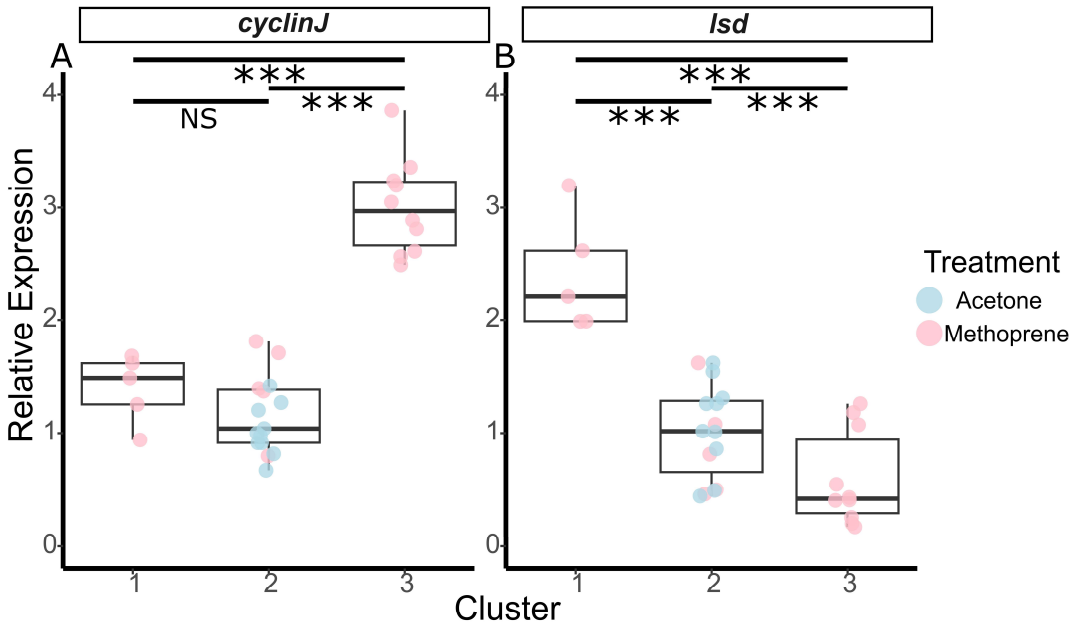

Figure 5.18 Relative expression of *cyclinJ*, an asexual specific marker, and *lsd*, a sexual specific marker, in three clusters determined by *cyclinJ* and *lsd* expression profiles and k++ means clustering of *A. pisum* ovary samples.
Samples encompass acetone and 0.125 mg/ml methoprene treated samples. Clusters one and three contain methoprene treatment samples only while cluster two represents acetone and methoprene treatment samples. *cyclinJ* is elevated in cluster 3 relative to clusters 1 and 2, only, and *lsd* is elevated in cluster 1 relative to clusters 2 and 3, and in cluster 2 relative to cluster 3. *** represents significant p-values <0.001 determined by GLM.

I then compared gene expression between these three clusters for the same genes as were analysed using methoprene/acetone treatment to group samples (*kr-h1*, *dnmt3x*, *bmm*, *clock*, *met*, *idel1*, *jhe2*, *jhe1*, *vgr* and *inr1*) (figure 5.19, figure 5.20) (GLM contrasts for each gene and pairwise comparisons, where appropriate, are presented in appendix tables C.7 and C.8). *idel1* expression was significantly higher in cluster 1 (high *lsd* methoprene) and cluster 3 (low *lsd* methoprene) relative to cluster 3 (acetone and acetone-like methoprene), but clusters 1 and 3 did not differ significantly, suggesting that this gene is responsive, in the ovary, to JH signalling, but not necessarily in the capacity of the reproductive switch (as clusters 1 and 3 are interpreted to be associated with sexual fate and asexual fate respectively), or not in a simple relationship (i.e. both sides of the switch may rely on JH to control *idel1* expression in different contexts/ways). *bmm* was significantly elevated in cluster 1 relative to clusters 2 and 3, while clusters 2 and 3 showed no difference, which again

demonstrates a complicated relationship, seemingly not being associated with the difference between production of asexual and sexual offspring, but suggesting it is, under particular circumstances, responsive to JH signalling. *vgr* was significantly elevated in cluster 1 relative to clusters 2 and 3, and in cluster 2 relative to cluster 3, suggesting that this gene is possibly correlated with degree of sexual egg production (being lowest in presumed asexual producers, then in normal sexual producers, then highest in sexual producers with an enhanced *lsd* expression). No significant differences in gene expression were found between any pair of the three clusters for any of the other genes inspected, consistent with the results comparing between acetone and methoprene treatment and thus suggesting again that these genes may not be responsive, in sexupara ovaries, to JH signalling, despite differential expression being associated with LD and SD conditions.

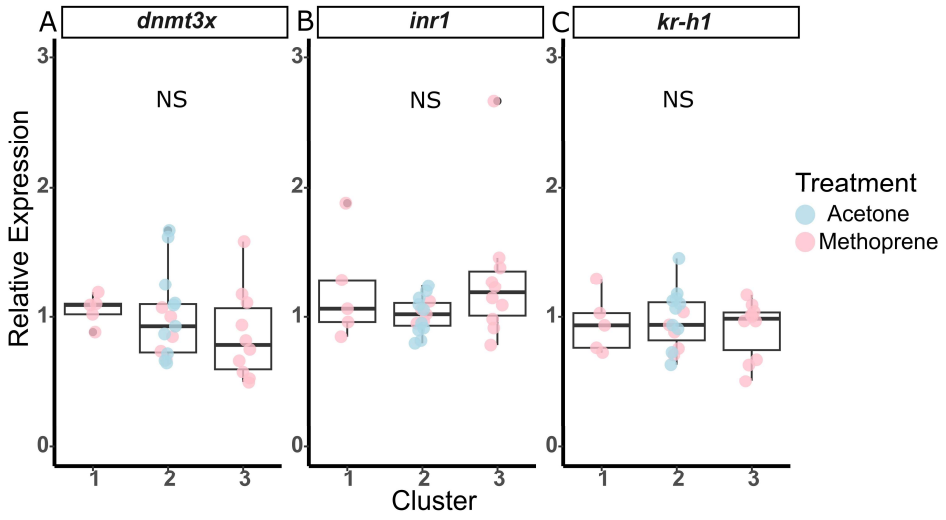

Figure 5.19 Relative expression of *dnmt3x*, *inr1* and *kr-h1* in acetone and 0.125 mg/ml methoprene treated SD (short-day) condition *A. pisum* ovary samples, grouped and compared by cluster (determined by expression of an asexual specific marker, *cyclinJ* and a sexual specific marker, *lsd*). Clusters one and three contain methoprene treatment samples only while cluster two represents acetone and methoprene treatment samples. Cluster 2 is characterised by high *lsd* and low *cyclinJ* expression, and thus is inferred to represent eventual production of sexual offspring, while cluster 3 is characterised by low *lsd* and high *cyclinJ* expression, and thus is inferred to represent eventual production of asexual offspring. Cluster 1 was consistent with cluster 2, except for having elevated *lsd* expression, inferred to be associated with enhancement of the sexual fate. No statistically significant difference was detected (by GLM) between clusters for any gene.

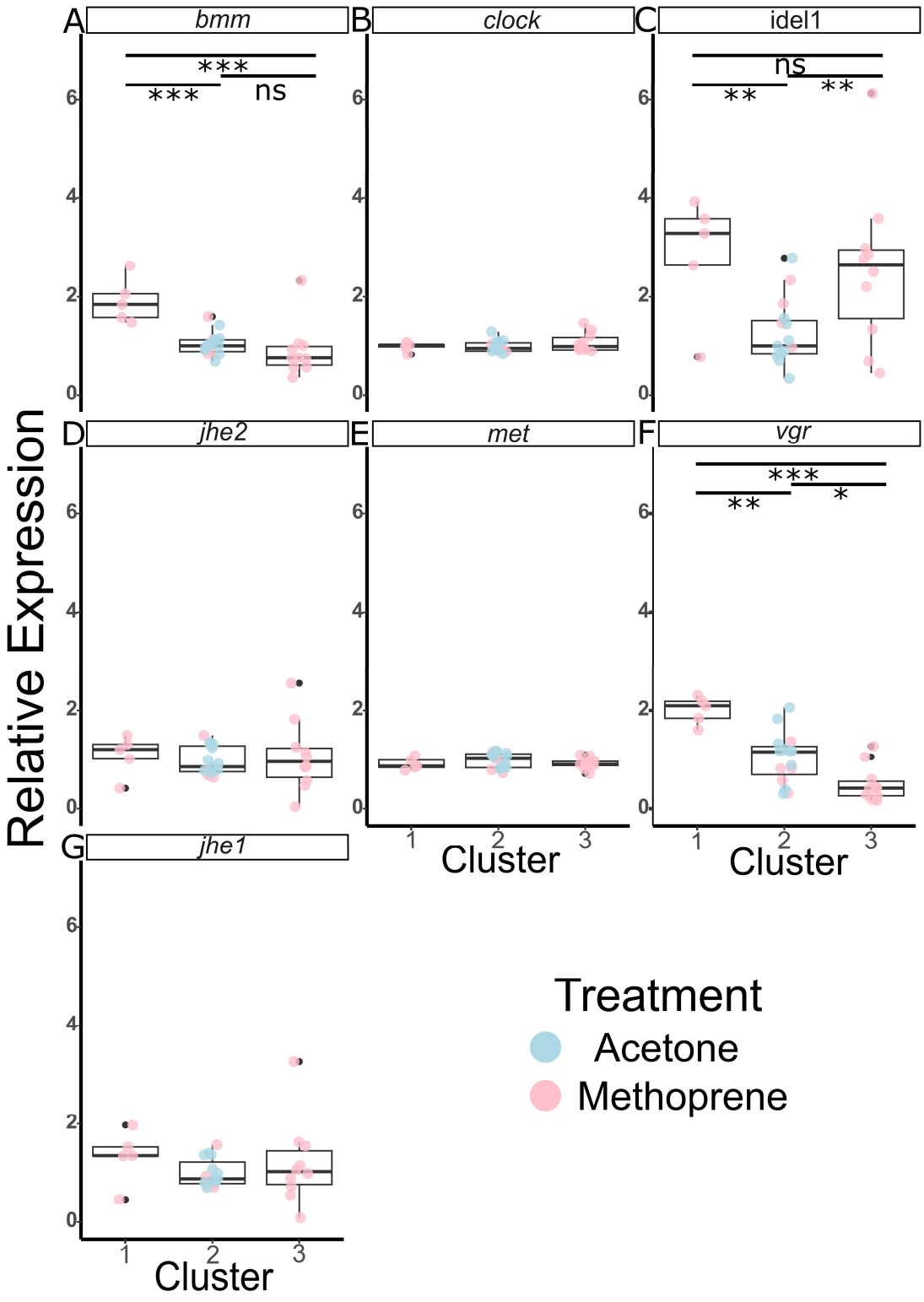
A
bmm
B
clock
C
idel1
D
jhe2
E
met
F
vgr
G
jhe1
***
***
ns
ns
**
**
***
**
*
Relative Expression
0
2
4
6
1
2
3
Cluster
Treatment
Acetone
Methoprene

Figure 5.20 Relative expression of *bmm*, *vgr*, *idel1* and four genes more directly related to juvenile hormone (*met*, *clock* (a homolog of *met*), *jhe1* and *jhe2*) in acetone and 0.125 mg/ml methoprene treated SD (short-day) condition *A. pisum* ovary samples, grouped and compared by cluster (determined by expression of an asexual specific marker, *cyclinJ* and a sexual specific marker, *lsd*).
Clusters one and three contain methoprene treatment samples only while cluster two represents acetone and methoprene treatment samples. Cluster 2 is characterised by high *lsd* and low *cyclinJ* expression, and thus is inferred to represent eventual production of sexual offspring, while cluster 3 is characterised by low *lsd* and high *cyclinJ* expression, and thus is inferred to represent eventual production of asexual offspring. Cluster 1 was consistent with cluster 2, except for having elevated *lsd* expression, inferred to be associated with enhancement of the sexual fate. Differential expression was detected (by GLM with post-hoc pairwise comparisons) for *bmm*, *idel1* and *vgr*, but no other genes. *bmm* was elevated in cluster 1 relative to clusters 2 and 3 only, while *idel1* was elevated in clusters 1 and 3 (both clusters including only methoprene treated samples) relative t ocluster 2, only, and *vgr* was higher in cluster 1 than 2, and higher in cluster 2 than 3. Statistical significance is denoted, where pairwise comparisons were made, on plots, ns, not significant *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

5.4 Discussion

The two doses of methoprene used here (0.125 mg/ml and 0.25 mg/ml, at 50 nl as nymphs and 100 nl as adults, corresponding to 6.25 ng and 12.5 ng as nymphs and 12.5 ng and 25 ng as adults) were at far higher concentrations than have previously been demonstrated to be typically circulating in the haemolymph of pea aphids (~1.5 to 2 ng/aphid in viviparae, and in sexuparae, <1 ng/aphid (Ishikawa et al., 2012)). High doses were selected because degradation of juvenile hormone has been suggested to be a critical step in the determination of JHIII titre during the reproductive switch (Ishikawa et al., 2012), thus, the increase in degradation associated with SD may result in rapid reduction in the amount of circulating methoprene that is then able to act on JH receptors (though, it is important to note that while methoprene is similar to JHIII, the ability of the *A. pisum* JH degradation enzymes (JHE1, JHE2, JHEH) to degrade methoprene has not been validated, nor has the ability of these enzymes to degrade JH). It is also possible that the expression levels of genes encoding juvenile hormone degrading enzymes may increase in the body of the adult in response to exposure to JH or its analogues (especially when there is a mismatch between the photoperiod and the JH titre, as in this case, though again, this has not been tested) (Stay et al., 1980; Noriega, 2014). Degradation also formed the rationale for treating as frequently as every two days (in

preliminary experiments, treating with either higher doses or daily exposure led to increased mortality (data not shown)). Additionally, treatment was carried out over a much longer period than has been documented before (for application of JH or its analogs), encompassing two generations (adults prior to reproduction, and their offspring until pre-reproductive adults themselves). While previous studies demonstrated that short-term treatment of aphids in SD conditions with JH or kinoprene was able to elicit production of parthenogenetic offspring in place of sexual offspring, the effect was short-lived and often, the progeny sequence included individuals with mixed ovaries (Mittler, Nassar & Staal, 1976; Hardie & Lees, 1985). By treating earlier and for longer, I hoped to demonstrate more complete disruption of the switch, proportional to the exposure to methoprene. All together, the presented dosages and timings for treatment with methoprene were selected in order to maximise the chance of eliciting a strong phenotypic response, and because SD conditions are known to upregulate the expression of JHEs, which degrade JH, in adults, and JHE expression (or expression of other JH signalling relevant genes) may also be affected by JH/JH-analog titre through feedback loops. The higher dose, 0.25 mg/ml, resulted in increased mortality, increased delay to adulthood and failed moulting at the terminal moult (which led to death). These phenotypes are not surprising given the known roles of JH in these processes. The lower dose, 0.125 mg/ml, while still increasing mortality (relative to control samples), did not inflict these additional defects but was sufficient to, in the majority of cases, redirect the reproductive phenotype of some developing embryos.

While not statistically significant, treatment with 0.25mg/ml methoprene relative to 0.125 mg/ml methoprene and acetone displayed a trend towards reduced production of winged morphs. Additionally, under both methoprene treatments, all winged morphs produced displayed disturbed phenotypes encompassing (presumably non-functional): fluid-inflated, crumpled, uni-lateral and/or reduced wings and seemingly degenerated wing muscles (figure 5.13). It has previously been demonstrated that methoprene treatment can facilitate wing-muscle degeneration in the pea aphid and affect wing morph (Bai et al., 2022; Mittler, Nassar & Staal, 1976). Also of note was that while a large proportion of offspring of methoprene treated individuals were redirected from sexual to asexual fates, none of the individuals of that generation were winged; while this

may be related to the low production of winged offspring generally (as seen in the previous generation), that none of the asexual offspring were winged is suggestive of the mechanism that dictates asexual/sexual fate being slightly removed from the mechanism that appears to prevent wing development during the sexual mode – in that, it is likely that this mechanism does not rely on JH, at least JH alone. Ordinarily, sexuparae (generally) produce no winged daughters, as the oviparous morph appears to preclude the winged morph (Hille Ris Lambers, 1966), likely due to both phenotypes being energetically demanding and the need to produce overwintering eggs being more important than dispersal – after all, what good is it to disperse if the environment will be uninhabitable either way. While the lack of wings may be due to a lack of cues to stimulate their production (primarily crowding), the generation previous exhibited a small but present proportion of winged morphs (8.8 %) under the same conditions. If the observed lack of wings in viviparous producers was real, then this suggests that while juvenile hormone being elevated is capable of removing the effect on reproductive mode of SDs, it cannot reverse the preclusion of winged morphs associated with SD and oviparae. Because elevated JH is associated with virginoparae, which ordinarily can produce winged morphs, and because high JH is associated also with wingless morphs, but both are determined during embryonic development in the pea aphid (and other aphids), this emphasises the highly structured and complex arrangements that this system operates in to control co-occurring phenotypes arising from seemingly antagonistic processes. This likely involves tissue-specific control. Otherwise, another system stimulated by SD/LD reception, which was still affected by SD conditions in the asexual phenotype aphids, may modulate the inability to produce winged morphs under SD conditions. Given that the wing polyphenism is also sensitive to insulin signalling (discussed below, (Grantham et al., 2020; Yuan et al., 2023)), ILPs may also exert some control over this phenomena (which may offer an explanation for methoprene treated, SD exposed asexual aphids putatively not being able to possess wings, as typical insulin signalling might be expected), though, given the link between insulin signalling and the reproductive polyphenism also (discussed in 1.4.3 and discussion, below), it is likely that it plays an equally complicated role. Further research will be needed to confirm the consistent preclusion of winged morphs after treatment with JH analogs under SD conditions, and the potential role of

insulin signalling in this system, perhaps by inhibiting or increasing insulin signalling (independently from and in combination with manipulation of JH signalling). To my knowledge, no study has attempted to examine in any detail the interplay between the reproductive and wing polyphenism thus far.

Based on *cyclinJ* (asexual specific (Gallot et al., 2012)) and *lsd* (sexual specific (Gallot et al., 2012)) expression, methoprene and acetone treated samples were separated into three clusters. Two expected clusters, one represented by high *cyclinJ* and low *lsd* expression (methoprene treated individuals where reproductive fate of developing embryos were affected), and the other represented by base-line, low *cyclinJ* and base-line to (relatively) high *lsd* expression (acetone and methoprene treated individuals where oviparous reproductive fated embryos were developing, as is typical) occurred. Surprisingly, a third cluster was also present, characterised by base-line *cyclinJ* expression but significantly higher *lsd* expression than the other two clusters. Members of this cluster were exclusively methoprene treated. This cluster is suggestive of an effect of methoprene toward enriched oviparous egg production, or greater loading of oviparous eggs with yolk/lipid (perhaps representing more mature eggs (based on the association of *lsd* expression with sexual eggs/germaria (Gallot et al., 2012) and the known role of Lsd in *D. melanogaster* being to load eggs (Cermelli et al., 2006; Beller et al., 2010)), though the pattern of Lsd production (or that of other sexual egg specific proteins) in sexual aphid eggs of varying maturity has not been explored), and may indicate that there is a threshold in juvenile hormone signalling. If JH/JH-analog exposure is sufficiently high, reproductive fate can be altered, but if the JH threshold is not reached (but JH titre is still effectively higher than is typical) a feedback system may work to reduce it, reducing it beyond what is typically experienced and resulting in increased *lsd* expression, possibly. One possible explanation for this would be an upregulation of JHE genes, as has been observed in *Coccinella septempunctata* exposed to methoprene (though, it is likely that upregulation of genes encoding juvenile hormone degrading enzymes would occur most strongly in the body of the adult more generally, rather than in the ovary) (Li et al., 2022c), another could be a downregulation of *met*, as has been observed in methoprene treated *Tuta absoluta* (Wang et al., 2023c), though I did not detect differential expression of either of two genes encoding

Met-like JH receptors in the ovary (where we would likely expect JH to interact) between any pairs of the three clusters. Alternatively, JH may play further roles in oviparous development and the inverse of the relationship in the mother (which is characterised by low JH) may be present to ensure proper provisioning of sexual eggs, the production of which are associated with JH in other (sexual, oviparous) insects. Elevated JH is associated with greater ovarian development and reproductive output in a range of insect species (Gijbels et al., 2019; Al Baki et al., 2019), which again, suggests that this phenotype may be related to a threshold not being met. The observation of the first cluster, containing putative enhanced-oviparous methoprene treated samples, and the second cluster, which included all presumed oviparous acetone-treated and methoprene-treated samples, reveals the intricate likely inter-play of signalling pathways involved in control of the reproductive polyphenism.

Evidently, JH activity alone is capable of preventing/reversing the SD induced oviparous fate, and it is clearly an important regulator of the reproductive switch. Though, the fact that affected individuals were more often than not observed to produce asexual and sexual progeny, regardless of the extended period of treatment, suggests that JH is either not the major (or only) component, or that it does not work in a simple on/off binary relationship; either way, this is suggestive of other important factors. It is highly likely that the insulin-like peptide pathway is interacting with the juvenile hormone pathway. Evidence is increasingly implicating ILPs as possible contributors to both aphid polyphenisms (Barberà, Cañas-Cañas & Martínez-Torres, 2019; Cuti et al., 2021; Yuan et al., 2023). Juvenile hormone may act by modulating the expression of insulin-like peptide related genes, in cases where the phenotype was acetone-like, modulation of expression may not have occurred sufficiently. Similarly, reduced ILP signalling as a result of the SD conditions may dampen the ability of JH to redirect reproductive fate, in some cases sufficiently, in others insufficiently; as an example, insulin signalling may sensitize the ovary to the effects of JH, perhaps by increasing expression of genes encoding JH receptors, and thereby enhance the intensity of JH signalling, or it may regulate degradation (as has been observed in *B. mori* (Zeng et al., 2017)). This may present an explanation for the inconsistent effects of methoprene on

reproductive fate. An intricate interaction between juvenile hormone and insulin signalling pathways in the reproductive polyphenism would mimic the general insect relationship they exhibit in reproductive regulation more generally. Further studies manipulating insulin signalling will help to determine this relationship. Additionally, assessment of insulin signalling related genes in the heads and bodies of aphids in response to manipulation of JH signalling (by treatment with analogs or by RNAi, for instance) will be useful to further characterising links between these systems.

Of the genes I assessed the relative expression levels of, *vgr*, *bmm*, *dnmt3x*, *dnmt3a*, *inr1*, *idel1*, *met* and *kr-h1* were differentially expressed in SD reared aphids relative to LD reared aphids. These genes are therefore implicated in the reproductive switch, though it is not possible to say with certainty which of them, if any, are directly linked to the switch (as ovary level response elements), rather than arising from some independent correlate (e.g. photoperiod may have other effects on the ovary that are independent of the reproductive switch). With the exception of *dnmt3x* and *dnmt3a*, these genes have clear known roles in the insulin and juvenile hormone signalling pathways. *inr2*, *vg*, *clock* (a homologue of *met*), *jhe1*, *jhe2*, *vasa*, *ide*, *foxo*, *ddc*, *e75*, *ilp1*, and *ilp4* did not show differential expression between conditions. *jhe1*, *jhe2*, *ilp1* and *ilp4* showing consistent expression between ovaries of LD exposed (virginoparae) and SD exposed (sexuparae) aphids is not an overly surprising result, given that these genes would not be expected to be overly expressed in the ovaries, aside from perhaps being expressed in the more mature embryos. *ilp1* and *ilp4* have both been shown to be more highly expressed in the heads of virginoparae *A. pisum* relative to sexuparae, though importantly only at particular zeitgeiber times (Barberà, Cañas-Cañas & Martínez-Torres, 2019). That they are not differentially expressed here, suggests that if they are involved in the reproductive switch, then the mature embryos are likely not differentially signalling through ILPs, and affecting the development of their ovaries themselves. Or if they are, they may be doing so through other ILPs (remembering that the *A. pisum* genome encode seven and ILP2 and ILP3 are also similar to classical insulin proteins (Huygens et al., 2022)).

Vitellogenin, encoded by *vg*, is a protein produced primarily by female insects and incorporated, after processing to vitellin, into the yolk as the major yolk

protein. In this capacity, it is deployed to a far greater extent by oviparous pea aphids than by viviparous pea aphids due to the fundamental differences in how these modes supply nourishment to their developing embryos. Viviparous aphid oocytes contain very little to no yolk, due to the enhanced and continual contact between developing oocytes and embryos and the mother. Vitellogenin level has been shown to be regulated by JH in *Locusta migratoria*, *Blatella germanica*, *Zeuogodacus cucurbitae*, *Propylea japonica*, *Liposcelis entomophila* and *Tribolium castaneum* (Dhadialla & Wyatt, 1983; Comas, Piulachs & Bellés, 2001; Sheng et al., 2011; HuangFu et al., 2021; Chen et al., 2022; Yang et al., 2023). Vg is transported into oocytes during vitellogenesis by receptor mediated endocytosis facilitated by the vitellogenin receptor, Vgr (Schonbaum, Perrino & Mahowald, 2000). Insulin-like peptides can cause an increase in uptake of vg through activation of the insulin-signalling pathway, whereby ILPs cause phosphorylation of FOXO and prevent it from translocating to the nucleus and binding to *vgr*, where it would otherwise repress expression. Sheng and colleagues (Sheng et al., 2011) showed that *T. castaneum* ILP2 and ILP3 RNAi reduced expression of VgR, and FOXO RNAi increased expression. The link between insulin signalling and vitellogenesis has been demonstrated in several insects (Richard et al., 2005; Roy, Hansen & Raikhel, 2007; Corona et al., 2007). Vitellogenesis is extremely underpinned by nutrition, it being one of the major energetic demands of insect life. Sheng et al. 2011 suggest that JH's effect on *vg* is mediated through the insulin-signalling pathway, where JH increases the expression of key ILPs, as injection of insulin and knockdown of FOXO independently rescued *vg* expression in JHAMT and Met RNAi beetles.

While *vg* was not differentially expressed between LD and SD conditions, *vgr* was upregulated under SD. The elevated *vgr* expression of SD aphids relative to LD can be viewed through two lenses. The first, considering that juvenile hormone levels are expected to be reduced in SD exposed aphids (Ishikawa et al., 2012) makes this observation surprising, as reduced juvenile hormone would be expected to cause reduced *vg* expression based on results from other insects. The second, considering the unusual reproductive biology of aphids encompassed by their viviparous development and the reduced production of yolk, would lead to opposite expectations. Importantly, while *vgr* expression is expected in the ovaries, *vg* expression is associated primarily with the fat body

(Shang et al., 2018; Hu et al., 2019; Han et al., 2022). One possibility is that the elevated *vgr* expression is associated with the most mature, sexual-fated embryos, which will have partially developed ovaries of their own and be specifying sexual oocytes. However, given the relatively small proportion of the ovaries of SD aphids represented by embryos developed sufficiently to possess relatively developed ovaries, I don't expect that the effect from oocytes of oviparous embryos would be significant. Additionally, if *vgr* upregulation is associated with these embryos, we might expect upregulation of *vg* to also be present, as these embryos would possess ovaries and fat body tissue, and *vg* and *vgr* expression has been demonstrated to be temporally consistent in *S. frugiperda* and *S. furcifera* (a hemipteran) (Hu et al., 2019; Han et al., 2022) (unless *vg* is expressed by the mother and is then transported to the ovaries of her developing embryos, though, this does not seem parsimonious). One alternative is that expression of *vg* and *vgr* are uncoupled, temporally, in *A. pisum*. There are two additional possibilities, neither of which I am able to rule out. The first, that vitellogenin is deployed beyond the context of yolk production for nourishment, as has been demonstrated in several insects, principally hymenopteran insects relating to different castes, e.g. in *A. melifera* workers and queens, but also possibly in *N. lugens* (Morandin, Hietala & Helanterä, 2019; Corona et al., 2013; Morandin et al., 2014; Shen et al., 2019; Tufail & Takeda, 2008), possibly facillitated by VgR for involvement in the reproductive switch. And/or, that while juvenile hormone levels are reduced (owing likely to upregulation of JHEs, as suggested by (Ishikawa et al., 2012)) in whole-bodies of viviparous aphids exposed sufficiently to SD conditions, the relationship between photoperiod and JH is not consistent in ovaries. That methoprene treatment did not increase ovary expression of the TF *kr-h1*, the primary response gene (and an early one) of JH signalling, despite causing (at least partially) diversion away from oviparous offspring fates adds to the plausibility of this, especially given that upregulated JH signalling is associated with asexual fate, and the LD, asexual fate was shown to be associated with lower expression of many of the JH responsive genes investigated in ovaries.

Silencing of *met*, the receptor of JH, in *P. japonica* and *Diploptera punctata* led to downregulation of *kr-h1* and *vg* (HuangFu et al., 2021; Marchal et al., 2014). Similarly, knockdown of *jhe* and *jheh*, and topical application of methoprene led

to increased *kr-h1* and *vg* expression (in whole females) in diapause destined (the reproductive diapause observed in several insect species is analogous, in a way, to the SD induced oviparous morph of cyclical parthenogenesis in aphids) *C. septempunctata*, reversing the diapause phenotype (Li et al., 2022c). Methoprene treatment in *T. absoluta* larvae led to upregulation of *kr-h1*, while knockdown of *kr-h1* led to reduced *vg* and *met* expression (Wang et al., 2023c). It would be reasonable to expect, then, that methoprene would upregulate *kr-h1* and *vg*, especially given that *vgr* expression was affected by methoprene treatment. Exactly how elevated JH in the haemolymph of an adult and reduced JH in the same adult's ovary may coexist but are apparently compartmentalised, especially given the close degree of contact between viviparous aphid mothers and their embryos, is not clear. A lack of differential expression of *jhe1* and *jhe2* in the ovary between LD and SD conditions, and no effect on *kr-h1* after methoprene treatment suggests the mechanism is not ovary specific control of JH titre by degradation (as has been suggested to be the main driver of JH titre control in whole bodies during the reproductive switch (Ishikawa et al., 2012)). Regardless, *vgr* and *kr-h1* upregulation in conditions that reduce JH titre in the adult (SD) are both surprising and unsurprising findings.

Functions of *vg* in a broad range of processes have been demonstrated in the viviparous morph of *Aphis citricidus* (the brown citrus aphid), where it has been observed that AcVg is missing a domain (the DUF1943 domain, for which the exact function is unknown, though it contains 2 β-sheets that contribute to the lipid binding cavity) common in other insect Vgs (Shang et al., 2018). More generally, it has been demonstrated that aphid Vgs are diverged from the Vgs of several other groups of insects (including other hemiptera, and all other groups surveyed by Shang et al.), with high conservation outside of the aphids, while aphid *vgr*s were consistent with those of other insects. Additionally, the *A. pisum vg* has been described as *vg*-like B, a group that differs from the conventional *vg*s (Morandin, Hietala & Helanterä, 2019). In *A. citricidus*, roles for *vg* in delay to reproduction, length of reproductive period, survival, embryonic development and nymphal development were identified (Shang et al., 2018), and a role in fecundity was observed in viviparous *Aphis gossypii* (Wang et al., 2023b). Though, there is little characterisation of *vg* and *vgr* in

aphids beyond these cited papers. The divergence of the aphid *vg* genes may be related to their unusual biology that makes the use of Vg to develop yolk (at least, large quantities of yolk) a relatively rare phenomenon, given that the oviparous morphs that would do so appear typically as a single generation in a year (Blackman, Minks & Harrewijn, 1987), leading perhaps to relaxed selection (as has been suggested for similarly rarely expressed genes, associated with sexual females and males (Li, Zhang & Moran, 2020; Jaquiéry et al., 2022)). This divergence may encompass novel functions. While I did not explore *vg* expression in the fat body/whole bodies of virginoparae and sexuparae, it is additionally possible that differential *vgr* expression in the ovary may facilitate alternative functions of Vg. Alternatively, *vgr* expression may be associated with mature oviparous embryos, and *vg* may not be being differentially expressed at the sampling period due to *vgr* being upregulated in advance of *vg* expression. In order to assess where the differential expression of *vgr* (and other genes implicated here, for relation to the reproductive switch) is occurring, RNA-FISH, and quantitative RNA-FISH HCR (Schwarzkopf et al., 2021) will be useful tools.

Upregulation of *bmm* (*brummer*), the product of which is responsible, in other insects, primarily for mobilization of lipid reserves which provide nourishment for developing embryos, but also has a role in vitellogenesis (Lu et al., 2018b), in SD compared to LD conditions is similarly not unexpected. Especially given that *kr-h1* was upregulated and expression of *kr-h1*, *met*, and *tai* are known to be upregulated by Bmm in *N. lugens*, along with *vg* and *vgr* expression (Lu et al., 2018b). *bmm* expression in *N. lugens* is enriched in the fat body and the ovaries (Zhou et al., 2018), the expression in the fat body raises the possibility of expression being associated with mature embryos. But again, curiously, *vg* was not observed to be differentially expressed here. Bmm's primary function is to break down lipids (the primary energy source of insect embryos, typically), in part to supply energy and metabolic intermediates to developing embryos (Zhou et al., 2018). *vgr* and *bmm* upregulation without upregulation of *vg* is therefore unusual.

Additionally, *vgr* and *bmm* were also differentially expressed as a result of methoprene treatment. While differential expression was not revealed by comparing treatments, separating samples based on clustering unveiled subtle differences. *bmm* elevation in the cluster of methoprene samples that had

elevated *lsd* expression (inferred to have enhanced sexual egg production) relative to control/control-like samples and high *cyclinJ* samples (inferred to be producing asexuals), these latter two clusters being similar, is an observation that difficult to rationalise. JH signalling increasing *bmm* expression is not completely surprising, given the known role of JH in modulating insulin signalling, which can then affect *bmm* expression (in *D. melanogaster*) (Luong et al., 2006; Grönke et al., 2005). However, that *bmm* is not differentially expressed between the control group and the switched (to producing asexuals) group is unexpected, given the significant upregulation of *bmm* in SD relative to LD ovaries. Additionally, in *D. melanogaster* exposed to starvation, *lsd1* is downregulated, and *bmm* is upregulated (in whole flies) (Grönke et al., 2005), which makes sense given the antagonistic natures of the functions of these genes – but again, this is inconsistent with (opposite to, even) the results observed here. The relationship for *vgr* is more consistent with expectations, with the asexual group having downregulated *vgr* expression relative to the other two clusters (which were also significantly different from each other). While both *bmm* and *vgr* are clearly responsive to JH signalling in the *A. pisum* ovary, the relationship for *bmm* appears more complex and will require further inspection. Characterisation of both of these genes, and *vg* in *A. pisum* will help to determine if their functions in aphids are consistent with other insects, or if the unusual biology of aphids means they are used in novel ways.

Importantly, *met*, which encodes a putative (based on homology with a *D. melanogaster* gene of the same name) JH receptor, was expressed more highly under SD, relative to LD, conditions. Again, at first glance this appears counterintuitive to what has been observed in whole-body and head samples across the same comparisons. High JH signalling is associated with LD conditions and the production of asexuals (Ishikawa et al., 2012), although, importantly, Ishikawa and colleagues did not detect differential *met* expression (while I identified *met* and its homolog, which I have described as *clock*, they only assessed expression for *met*) between LD and SD, likely owing to their use of whole-bodies. Feasibly, *met* expression in the ovary may increase under SD conditions to increase sensitivity to JH, thereby increasing the certainty of SD conditions and well-informed switching to sexual reproduction. That *met* expression did not change in response to methoprene treatment (neither based

on treatment or clustering) suggests that the differential *met* expression seen between LD and SD ovaries is not driven by JH signalling, but likely some other modulator that is responsive to photoperiod – again, it is easy to suggest that this is insulin signalling (based on recent studies implicating insulin signalling in the reproductive switch (Barberà, Cañas-Cañas & Martínez-Torres, 2019; Cuti et al., 2021)). Assessment of *met* expression after manipulation of insulin signalling in *A. pisum* will help to verify if insulin-signalling and JH signalling are interacting by this means. While *met* and *clock* shared homology with each other and the *D. melanogaster* orthologue, and possess the same conserved domains, only *met* was differentially expressed. Possibly, *met* being differentially expressed in the ovary, while *clock* is not, may be linked to the separation of JH signalling in the ovary (which appears, based on the data presented here, to be characterised, in SD, by high signalling) and in the body of the mother more generally (which appears to be characterised, in SD, by low signalling). To my knowledge, the expression of *clock* has not been assessed between LD and SD conditions beyond the presented study, and the function and character of this homolog should be assessed more closely.

Similarly to how positive JH signalling related genes being upregulated in the ovary appears initially to be counterintuitive to what we know about the reproductive polyphenism from whole bodies and heads, two insulin related genes, *inr1* and *idel1*, were differentially expressed between ovaries from LD and SD conditions. *inr1*, which has previously been demonstrated to be upregulated under LD conditions (Le Trionnaire et al., 2009; Barberà, Cañas-Cañas & Martínez-Torres, 2019) in heads, was found here to be downregulated under LD conditions in ovaries. Similarly *ide* has been shown to be upregulated under SD conditions in heads (Le Trionnaire et al., 2009). In the present study, *ide* was not differentially expressed, though another gene, which I termed *idel1*, bearing homology to *ide*, and possessing an insulinase domain was downregulated in ovaries under SD conditions; as *idel1* appears to have an insulinase domain, it is reasonably expected that downregulation in SD conditions would be associated with greater signalling by ILPs – though importantly, which if any of the seven ILPs encoded in the *A. pisum* genome it degrades must be functionally determined. This is again, contrary to expectations, though in keeping with the rest of the presented results, points to

fine-tuning and tissue specific expression of putative modulators of the switch – the environment of the mother, speaking generally, being separate from the environment of the ovaries. Despite differential expression of *inr1* between LD and SD conditions, *inr1* expression was not affected by methoprene treatment, suggesting that expression of this gene is not responsive to JH signalling. *idel1*, on the other hand, was upregulated by methoprene treatment, regardless of whether samples were in the cluster characterised by high expression of *cyclinJ* and therefore inferred to be producing asexuals (cluster 3), or the cluster characterised by elevated expression of *lsd* and therefore inferred to have enhanced production of sexuals (relative to acetone and non-affected methoprene treated samples). That both of these clusters, which are inferred to be at opposite ends of the spectrum, had elevated *idel1* expression, suggests that *idel1* is responsive to juvenile hormone, perhaps beyond the context of just being involved in the reproductive switch (as aphids presumed to have not switched, from cluster 1, also had elevated expression). That JH increases *idel1* in the ovary is consistent with the expression pattern between LD and SD conditions, as elevated JH in the body of the mother is expected under LD, where *idel1* was also elevated. Thus, the differential *idel1* expression in the ovary under different photoperiods is likely underpinned by JH signalling.

Taken together, these results suggest that while low insulin signalling in the mother may be a factor in specification of oviparous offspring (Barberà, Cañas-Cañas & Martínez-Torres, 2019; Cuti et al., 2021), high insulin signalling is associated with actually ultimately generating the oviparous fate, and that this is controlled in these individuals not by elevating ILPs, but by reducing degradation and increasing sensitivity. Barberà and colleagues (Barberà, Cañas-Cañas & Martínez-Torres, 2019) suggest that elevated *ilp1* and *ilp4* expression in *A. pisum* heads is consistent with the observation of elevated expression of ILP encoding genes and not entering diapause exhibited by other insects (Sim & Denlinger, 2008; Chen et al., 2023), though because of the fundamental differences between aphid ‘reproductive diapause’ (the reproductive switch) and typical reproductive diapause (the first encompassing switching to sexual reproduction with commitment of a great deal of resources to its germ (Blackman, Minks & Harrewijn, 1987; Büning, 1985), and the second representing, typically, a complete redirection of resources away from the ovary

(Karp, 2021)) they are not directly comparable. While the reproductive switch is a form of diapause (functionally, reproduction slows to the extent of stopping over winter at the level of the population (Srinivasan & Brisson, 2012)), it is fundamentally opposite to the reproductive diapause experienced more typically by insects. The switch to oviparous sexual reproduction encompasses a reversal in relation to vitellogenesis (Büning, 1985; Miura et al., 2003; Couchman & King, 1979; Brough & Dixon, 1990a) and, as observed here primarily through the upregulation of *vgr* and other positive insulin signalling and JH related genes in SD relative to LD conditions, the switch to oviparous reproduction is associated with enhanced expression of genes that would ordinarily be supressed during diapause (Liu et al., 2019; Jiang et al., 2020; Chen et al., 2023).The reproductive switch of aphids may therefore represent a co-option of the reproductive diapause response, deploying it instead in the opposite direction of what is typical (short photoperiod eliciting the opposite response).

Manipulation of *idel1* levels, perhaps by RNAi, will help to discern what, if any, function *idel1* has in the reproductive switch. Furthermore, the effects of methoprene on *idel1* expression (and the expression of other genes targeted here that showed differential expression) should be assessed in LD conditions, to investigate if the effect of methoprene (and therefore juvenile hormone signalling) on these genes is specific to SD conditions, perhaps as a result of modulatory mechanisms that are activated differentially between LD and SD. Similarly, the effects of methoprene treatment on gene expression (of some of the genes targeted here, most obviously, *jhe1*, *jhe2*, *inr*, *ide*, *idel1*, *met*, *clock*, *ilp1* and *ilp4*) should be assessed in heads and in bodies with ovaries removed from individuals in SD conditions to confirm if these genes are JH responsive elsewhere.

The fact that *kr-h1*, *inr1*, *met* and *dnmt3x* were upregulated in ovaries in SD conditions relative to LD conditions, but were not downregulated in SD conditions in methoprene treated relative to control aphids suggests that while these genes may be involved in the switch, the ovary being the place where effectors of the switch must exist to execute the switch (which is known to be responsive to juvenile hormone, and has been linked to insulin signalling), JH cannot act alone to modify their expression or they are not responsive to JH at

all. Given that methoprene was able to reverse the switch, these genes are either not major reproductive polyphenism components in the ovary (that is to say, the switch is able to occur, at least partially, without differential expression of these genes occurring) and they are responsive to some other modulator (possibly insulin signalling), or JH acts downstream of them to affect reproductive mode (although, this is perhaps unlikely, given that JH is synthesized in the *corpus allatum* of the mother (Hardie, 1987b)); feasibly, JH operates upstream (as a modulator in the mother) and downstream (as a modulator in the embryo) in opposite directions. If these genes are not major contributors to the ability to switch reproductive fate, but are part of the reproductive switch, given that the effects of methoprene treatment were partial, it is possible that they are involved in making the switch more consistent or permanent. It may be that, given the upregulation of *inr1* in SD vs LD ovaries but not in the methoprene experiment, JH enhances insulin levels in the adult, but some other mechanism upregulates *inr1* in the ovary, increasing sensitivity, and both are necessary for robust maintenance of the viviparous form. Additionally, because in the methoprene experiment, none of *kr-h1*, *inr1*, *met* or *dnmt3x* were differentially expressed between viviparous and oviparous embryos (between acetone exposed ovaries, methoprene exposed ovaries bearing some viviparous offspring and methoprene exposed ovaries bearing only oviparous offspring), the expression pattern of these genes observed between LD and SD ovaries in the switch experiment must not be driven by the downstream phenotype, which is to say it is not a product of realised oviparous vs viviparous fates, and is instead a cause of it. Under natural conditions, the switch from viviparous to sexual offspring production is irreversible. Though it encompasses often, a series of progeny phenotypes, once the photoperiod cue is sufficiently detected and integrated, sexual offspring inevitably follow (Blackman, Minks & Harrewijn, 1987). Given the seemingly partial effect demonstrated in the mixed ovaries of methoprene treated individuals, it may be that some genes not controlled by JH are involved in canalising the effect and rendering it irreversible. *dnmt3x* and *inr1* may represent such canalising components.

In chapter 4, I showed that *dnmt3x* and *dnmt3a* expression in the ovaries was highly specific to the nurse cells, oocytes and germ cells (in addition to

expression being present in early embryos before germ cell specification). Its expression in the nurse cells and subsequent provisioning to early developmental stages is particularly interesting given that the germaria are in close proximity to terminations of the lateral nerves shown to carry a putative virginoparin (ILP4) from the *pars intercerebralis*. The virginoparin has been suggested to be one of or both of ILP1 or ILP4. If juvenile hormone was the virginoparin, we might expect *dnmt3x* to be differentially expressed in the germaria of methoprene treated ovaries as primary points of contact, given the pattern of *dnmt3x* expression observed between LD/SD conditions. That *dnmt3x* expression did not appear to respond to methoprene, and that *dnmt3x* and *inr1* were both upregulated in response to SD (in ovaries) further suggest insulin may be the identity of the virginoparin (though, this is not to exclude the role of JH, which is likely also being transported from the CA to the ovary, and clearly can effect reproductive fate). The appearance of a phenotype in some methoprene samples may result from JH bypassing this part of the pathway. Given that one of the phenotypic observations of methoprene treatment presented here, and of azacytidine (an inhibitor of DNA methylation machinery, including *dnmt3x*) treatment (chapter 4) were shared (production of disturbed nymphs) and that *dnmt3x* and *kr-h1* expression were correlated in the switch experiment, in addition to the observations of no differential expression in the methoprene experiment, it is reasonable to suggest that in the ovary JH signalling is downstream of *dnmt3x*. Additionally, in *D. magna* a cyclically parthenogenetic species of crustacean, treatment with 5-azacytidine led to reduced fecundity of the focal generation (as observed in chapter 4 for parthenogenetic *A. pisum*) and interestingly, an increase in expression of *met* in the f1 generation (though, not to the extent of statistical significance, and using pooled whole body individuals as samples) (Lindeman et al., 2019). This provides a suggestive (though by no means conclusive) link between DNMTs and juvenile hormone signalling, Met being the receptor of JH. Several genes involved in the JH pathway have been shown to be methylated in *A. pisum* (Walsh et al., 2010), but whether or not these genes are differentially methylated between reproductive morphs (virginoparae, sexuparae or oviparae) is unclear; furthermore, as discussed in the general introduction, there is not a strong link between differential methylation and differential expression in insects. Though, as I discussed in chapter 4, *dnmt3x*'s role in DNA methylation,

if it has one, is unclear. Interestingly, *dnmt3a* which appears to be the copy possessing methylation capabilities was also differentially expressed between LD and SD, but to a lesser extent than was *dnmt3x*. The rational conclusion is that differential methylation between future oviparous and viviparous oocytes or embryos may be occurring, perhaps facilitated by *de novo* methylation, perhaps with Dnmt3x acting in a capacity similar to the mammalian Dnmt3-L (Bourc'his et al., 2001) to modulate the activity of Dnmt3a. Though, that *dnmt3x* expression was seemingly more upregulated by SD conditions than *dnmt3a* is suggestive that something else may be occurring, perhaps involving novel functions for Dnmt3x (as explored in chapter 4). Undoubtedly, these genes are relevant to the reproductive switch.

The distribution of *dnmt3x* mRNA in sexupara ovaries appeared expanded beyond that observed in vivipara, where it is confined to the germ cells in embryos later than stage six (at which point the germ cells are specified, chapter 4). Convincing *dnmt3x* mRNA signal was observed in the germ band (the embryo proper) fairly uniformly, in sexupara ovaries. Expanded expression of *dnmt3x* is observed therefore in early embryonic development of oviparae, which links it quite firmly to the reproductive switch. The expansion of expression into the embryo proper may relate to establishing new methylation marks by *de novo* methylation, though, that the expression pattern appears to persist throughout the mid-late embryonic stages observed, suggests *dnmt3x* may be performing some other function. In depth inspection of methylation levels in sexupara and virginopara ovaries will help in determining if differential *de novo* methylation is occurring.

Differential expression of *dnmt3x* beyond what was observed for *dnmt3a*, in addition to the differential expression pattern of *dnmt3x*, between sexupara and virginopara ovaries, reveals a possible decoupling of expression of *dnmt3x* from expression of *dnmt3a*, though this will need to be validated by inspecting the expression pattern of *dnmt3a* in sexupara ovaries. Therefore, expanding on the exploration presented in chapter 4, it is likely that *dnmt3x* and *dnmt3a* are involved in the reproductive polyphenism of aphids. To further confirm this, functional characterisation of *dnmt3x* and *dnmt3a* is necessary. Targeted silencing or knockout of *dnmt3x* and *dnmt3a*, independently and together (to reveal if the Dnmt3x interacts with Dnmt3a), perhaps in conjunct with

assessment of methylation state (bisulphite sequencing, MeDIP or nanopore sequencing (Harris et al., 2010; Liu et al., 2021), to determine any methylation related functions of Dnmt3a and/or Dnmt3x), offspring phenotyping (primarily of aphids in SD conditions, under which the expression of *dnmt3a* and *dnmt3x* is upregulated, to assess the ability of these genes to contribute to/control the switch) and/or expression analysis will provide an invaluable tool toward functional characterization. Taken together, *dnmt3a* and *dnmt3x* appear to be good candidates for downstream effectors of the reproductive switch in aphids.

Interestingly, in the crustacean, *D. magna*, which possesses two *dnmt3* paralogues, the proteins of one of which, Dnmt3.1, possesses a diverged methyltransferase (catalytic) domain, appearing to be missing catalytic functionality, while possessing a PWWP domain and ADD domain, the gene encoding which is upregulated in response to starvation (Nguyen et al., 2020). This protein is putatively similar to *dnmt3x*, which also appears to possess a PWWP domain and an ADD domain, but appears to lack the methyltransferase domain ((Walsh et al., 2010),chapter four). *D. magna* is also reported to possess an additional Dnmt3, Dnmt3.2, which has the conserved methyltransferase domain and an ADD domain, but no PWWP domain (Nguyen et al., 2020); thus being tentatively similar to *dnmt3a* in *A. pisum*. Both *Dapmadnmt3.1* and *Dapmadnmt3.2* are expressed during embryogenesis (Nguyen et al., 2020), again, consistent with *A. pisum dnmt3a* and *dnmt3x* (chapter 4). Importantly, the authors identified a candidate FOXO-binding site in the promoter of *dnmt3.1*, both in *D. magna* and the closely related *Daphnia pulex*. Inspecting the promoters of *A. pisum dnmt3a* and *dnmt3x* for FOXO-binding sites may further link these genes to the aphid reproductive switch, through the insulin signalling pathway (in which FOXO is a key node), but will rely on ChIP-seq data to accurately identify promoters (and may also directly indicate interactions between FOXO and the promoters of these genes). Furthermore, in a subsequent study, the same authors used CRISPR-Cas9 to silence *Dapmadnmt3.1* (Nguyen et al., 2021). Under nutrient rich conditions (which would be expected to elevate insulin signalling), growth and fecundity were not affected by mutation. When starved, WT individuals presented a trade-off between growth and reproduction in favour of growth in the growth and reproduction phase (individuals produced less eggs), but in the same phase,

starved mutant individuals had significantly higher growth, and produced even less eggs than WT. This relationship was confined to the growth and reproduction phase, but did not occur in the previous growth phase, or the subsequent reproduction phase. Additionally, mutation reduced survival under starvation, but not nutrient rich conditions (Nguyen et al., 2021). Their RNA-seq analysis reported, interestingly, starvation associated downregulation, which was exacerbated in the mutants, of a vitellogenin gene and several other lipid transport related genes, in addition to downregulation in starved mutants of several genes involved in carbohydrate metabolism, including *target of brain insulin tobi*. They also reported that *vgr* was downregulated in mutants relative to WT, but not between starved and fed WTs. And, importantly, *ftz-f1*, which is known to mediate intracellular JH signalling (being upregulated by JH) (Dubrovsky et al., 2011), and has been shown to be part of a positive feedback loop with JH and Vg in *A. melifera* (Mello et al., 2019), was also downregulated by starvation in WT, and more so in starved mutants. These observations further tether *dnmt3.1* to insulin signalling, and possibly to JH signalling, to which the genes detailed above are known to be responsive to. Furthermore, the authors suggest that in the starvation response, Dnmt3.1 may not be important in methylation. Additionally, in the same organism, disruption of the DNA methylation machinery by treatment with 5-azacytidine decreased *vg* expression (and affected *met* expression, as discussed above), further tethering DNMTs, insulin signalling, reproduction and JH signalling together (Lindeman et al., 2019).

Not only is this system of Dnmt3s reminiscent of that observed in the pea aphid, but that the *D. magna* paralog bearing similarity to *dnmt3x* is responsive to starvation, appears to have a FOXO binding site in its promoter, and that its silencing leads to differential expression of genes downstream of insulin signalling (Nguyen et al., 2020, 2021) suggests a strong link between Dnmt3.1 and insulin signalling. This further implicates *dnmt3x* for a role in response to photoperiod encompassed in the aphid reproductive polyphenism, possibly independent of DNA methylation, and likely in response to insulin signalling (consistent with *A. pisum dnmt3x* expression not being affected by methoprene treatment, but being differentially expressed between LD and SD conditions). Importantly, *D. magna* is a species that exhibits cyclical parthenogenesis. In the

parthenogenetic mode of cyclically parthenogenetic *Daphnia*, embryogenesis occurs within the brood chamber of the mother, somewhat analogous to viviparity in the parthenogenetic reproductive mode of aphids, and in the sexual phase, by laying fertilised eggs in an egg case that will diapause to avoid unfavourable conditions (Hebert, 1978; Ebert, 2022) – analogous to oviparity in the sexual reproductive mode of aphids. Thus, the similarities between these systems, principally their unusual maintenance and duplication of *dnmt3* (Walsh et al., 2010; Lindeman et al., 2019) and the shared use of cyclical parthenogenesis with viviparity (or something akin to viviparity) in the asexual phase, and diapausing laid eggs in the sexual phase (Blackman, Minks & Harrewijn, 1987; Hebert, 1978), are striking and suggestive of the duplication of *dnmt3* bearing importance to the reproductive polyphenism of aphids (and *Daphnia*), as explored in chapter 4 and consistent with the results of this chapter. Though, it will be important to determine whether or not the expression of *dnmt3x* (and *dnmt3a*) is greater or not in the germaria of SD exposed aphids than in the germaria of LD condition pea aphids, specifically (thus implicating it as being directly responsive to ILPs perhaps as the virginoparin), rather than just the germbands (inferred from expanded expression profile), to assess in more detail the putative importance of *dnmt3x* in the switch; quantitative RNA-FISH HCR or expression profiling of isolated germaria are two approaches that would help determine if this is the case.

Ultimately, aphid cyclical parthenogenesis appears to be an example of an increasingly apparent phenomena in insects of co-option of the insulin signalling pathway toward polyphenism, and more specifically to control over reproductive phenotype. Nutrition sensing mediated by the insulin signalling pathway being a major hinge on which reproduction ordinarily hangs (being a highly conserved and deeply rooted system, across vast evolutionary time (Jin Chan & Steiner, 2000)), it may be an easy point of access for other environmental factors to affect reproductive phenotypes. Whether examples are underpinned by shared ancestry or convergent evolution is unclear.

5.4.1 Future study

Being released from specific regions of the brain in response to environmental cues, the link between ILPs and juvenile hormone and biogenic amines, which act as neuromodulators and neurotransmitters in the brain is clear (reviewed in

(Knapp et al., 2022)), yet biogenic amines are a little studied area of the switch. Studies have implicated dopamine for a role in the switch, where genes involved in its synthesis are differentially expressed between LD and SD (in heads) (Le Trionnaire et al., 2022), but primarily in facilitating differential melanisation and sclerotization of the cuticle (perhaps affecting the ability of light to penetrate the head capsule and stimulate the photoreceptors responsible for perceiving light) rather than acting differentially as a signalling molecule. Beyond this, studies of biogenic amines in aphids have, to my knowledge, only explored their levels in relation to the wing polyphenism (Liu & Brisson, 2023). Dopamine, octopamine and serotonin are important bioamines that have been linked to polyphenism, nutrition, reproduction and insulin signalling in insects (as explored in chapter 1, (Monastirioti, Linn, Jr. & White, 1996; Gruntenko et al., 2007; Ling & Raikhel, 2018; Sasaki et al., 2012; Wang et al., 2022; Knapp, 2022; Knapp et al., 2022)). Regarding polyphenisms, dopamine, octopamine and serotonin all play roles in the phase polyphenism displayed by *Locusta migratoria* (Ma et al., 2011; Guo, Ma & Kang, 2013; Ma et al., 2015; Wang & Kang, 2014). Additionally, there is mounting evidence for involvement of dopamine (most strongly), octopamine and serotonin in the wing polyphenism of aphids where higher titres are associated with crowded mothers (associated with production of winged daughters), and artificial manipulation of dopamine has been shown to affect morph (Wang et al., 2016; Vellichirammal, Madayiputhiya & Brisson, 2016b; Liu & Brisson, 2023). It is as yet, unclear what role these biogenic amines play in the aphid reproductive switch, and determining any interactions they may have with insulin and JH signalling may greatly improve our understanding of this polyphenism. Determination of biogenic amine titres in aphid heads, bodies and ovaries should therefore be pursued by HPLC techniques, and in-depth quantitative analysis of expression of genes closely related to biogenic amines should be carried out.

One natural progression of this study is to investigate how methoprene treatment affects gene expression in the body of the mother (heads and bodies separately, preferably), without the contribution of the ovaries. Some of the genes investigated here, principally *jhe1*, *ilp1* and *ilp4*, while not being differentially expressed in the present study targeting ovaries, have been shown to be differentially expressed in heads of SD and LD exposed aphids (Ishikawa

et al., 2012; Barberà, Cañas-Cañas & Martínez-Torres, 2019). Though, it is important to note that when comparing the primers designed against *jhe1* in the present study to those used by Ishikawa and colleagues (in a study in which they reported differential expression of *jhe1*), their primers appear to be able to amplify two additional similarly sized products (according to NCBI primer BLAST against *A. pisum* databases), which may mean their quantification was not accurate. Assessing whether or not these genes are responsive to JH signalling by treating with methoprene, or manipulating JH signalling more finely (RNAi against *met, jhamt*, *jhe* etc.) will further our understanding of how they might contribute to the reproductive switch. This may also help to disentangle the relationship between juvenile hormone and insulin signalling.

Because there is a suggestion of a role for ILPs in the switch (Barberà, Cañas-Cañas & Martínez-Torres, 2019; Cuti et al., 2021), but juvenile hormone is capable of redirecting it, more in depth investigation of the role ILPs are playing are necessary. In a similar way to how JH signalling was isolated here, manipulation of insulin signalling by artificially elevating ILP titres, increasing the expression of receptors, or inhibiting ILP breakdown in SD conditions, or doing the inverse in LD conditions may similarly help elucidating its exact role. This could be achieved through RNAi, CRISPR-Cas9, application of exogenous insulin or chemical inhibition (e.g. (Brown et al., 2008; Haqshenas et al., 2019; Chen et al., 2023)). Although, it is important to note that this may not be easy to disentangle, given that the expression patterns appear to be tissue-specific. For instance, inhibiting InR by injecting dsRNA against *inr* into the body cavity may lead to silencing and thus reduced insulin signalling, which might encourage sexual development. On the other hand, the results presented here suggest that reduced insulin signalling in the ovary is associated with asexual development. Likely, partial penetrance consistent with that observed after treatment with JH and its analogs (this chapter, (Hardie & Lees, 1985; Corbitt & Hardie, 1985)) would occur, dependent on dose. Feasibly, experiments attempting to functionally characterise insulin signalling in these ways (along with greater characterisation of the gene expression effects of JH) may further elucidate how insulin signalling and juvenile hormone interact, and if either one of them is more important to control of the switch.

Additionally, greater characterisation of *dnmt3a* and *dnmt3x*, functionally – both in terms of knockdown and assessment on the ability of the reproductive switch to occur, and with regards to DNA methyltransferase functionality – and by greater analysis of their promoters and interaction with TFs, along with expression more specifically (i.e. is *dnmt3x* and *dnmt3a* upregulation under SD conditions associated with the germ cells, or just in the embryo proper), will greatly aid our understanding of how these genes may, through DNA methylation or otherwise, be involved in the reproductive switch as ovary level effectors. MeDIP/nanopore sequencing, ChIP-seq, and single-cell/probe-seq-like sequencing (Park, 2009; Amamoto et al., 2019; Liu et al., 2021) will all be important tools for tackling this characterisation, and for understanding the molecular bases of the reproductive switch more generally.

5.4.2 Concluding remarks

Here, I elucidated the expression patterns of some of the genes involved in key pathways known to be important to the ability of aphids to switch reproductive morph during cyclical parthenogenesis. This study expands on an increasing base of evidence for the involvement of insulin signalling in the switch and explores the delicate interaction between insulin signalling and juvenile hormone signalling in the ovary of sexupara aphids for the first time. By isolating the effects of juvenile hormone away from other modulatory components and perception and integration I explored how the polyphenism is controlled by this mechanism only. Further studies isolating insulin signalling and targeting the ovaries in greater detail, along with determining of the role of biogenic amines, if there are any, will greatly improve our understanding of the aphid reproductive polyphenism, and in turn enhance our understanding of control of plasticity (especially considering the apparent recurrent co-option of conserved insect systems), and open potential avenues for more effective and aphid-specific pest control.

Chapter 6 Germ cell specific probe-seq

6.1 Introduction

6.1.1 Aphid polyphenism

The aphid reproductive polyphenism (cyclical parthenogenesis) is an example of extreme developmental plasticity (Simpson, Sword & Lo, 2011). It encompasses, in response primarily to varying photoperiod, either asexual (parthenogenetic viviparous) or sexual (oviparous) reproduction. Reproductive mode is determined during embryonic development (Srinivasan & Brisson, 2012).

The reproductive polyphenism occurs across generations. Over which, continued exposure to reduced photoperiods (at sufficiently low temperatures) leads eventually to the production of sexual females and males, while sufficiently long photoperiods maintain aphids in the more commonly occurring parthenogenetic viviparous mode (Blackman, Minks & Harrewijn, 1987). Light is detected directly through the cuticle of the head by photoreceptors in the *pars lateralis* (Hardie, Lees & Young, 1981; Colizzi et al., 2023). The cells involved here in the perception of light and the determination of photoperiod interact with eight/ten (eight in *A. pisum*, ten in *M. viciae*) group I neurosecretory cells (NSCs) and two group II NSCs in the *pars intercerebralis* (Hardie, Lees & Young, 1981; Barberà, Cañas-Cañas & Martínez-Torres, 2019). These cells produce what has been termed virginoparin, a substance responsible for maintaining production of parthenogenetic offspring. These cells have projections which may deliver this substance from the head to the vicinity of the ovaries where it acts in some unknown way to promote the production of parthenogenetic embryos; reduced exposure of embryos to virginoparin leads to production of sexuals (Corbitt & Hardie, 1985; Blackman, Minks & Harrewijn, 1987). While the detection, determination and integration of photoperiod are becoming fairly well understood (Blackman, Minks & Harrewijn, 1987; Colizzi et al., 2023), reviewed in (Colizzi, Martínez-Torres & Helfrich-Förster, 2023), the exact mechanism by which the virginoparin affects the development of embryos

is unclear. The ovaries, being the tissue where a putative identity for the virginoparin, ILP4, appears to be transported to (Cuti et al., 2021), and being the site of oogenic and embryonic development, the site at which the polyphenism is rooted, present a natural point of investigation.

6.1.2 Aphid ovary structure

Viviparous parthenogenetic aphids have paired telotrophic meroistic ovaries, in which nurse cells situated in the single germarium at the terminal end of each ovariole provide nourishment to diploid oocytes and early embryos; sexual female ovaries are consistent in structure, with nurse cells nourishing only haploid oocytes instead (figure 6.1) (Büning, 1985; Miura et al., 2003; Brough & Dixon, 1990a; Couchman & King, 1979). Each ovary consists of several ovarioles (the number varies between species and between aphids of different reproductive and wing phenotypes, with fundatrices, those parthenogenetic aphids which arise from eggs typically possessing the most, and winged aphids the least) (Michalik et al., 2013) which are joined at their most anterior end in the upper abdomen by their terminal filaments. Connected, in parthenogenetic ovaries, on the other side of each germarium is a series of developing oocytes (typically a single matured/maturing oocyte and several arrested prospective oocytes at the posterior of the germarium) and embryos of progressively later developmental stages (or one or few yolk filled eggs in sexual ovaries), in the vitellarium (Büning, 1985; Miura et al., 2003; Brough & Dixon, 1990b; Couchman & King, 1979). In addition to the nurse cells, the germaria (of aphid ovarioles with the capacity to release more oocytes) contain resting, prospective oocytes and typically, a single previtellogenic oocyte. The nurse cells, which are polyploid and connected to one another by a common trophic core, nourish oocytes and early embryos (encompassed by the first and second ovarian follicles, and only uncellularized embryos) through the trophic cord which extends into their cytoplasm (Büning, 1985; Brough & Dixon, 1990a; Couchman & King, 1979). The trophic cord is a structure of extensively arranged microtubules that is enriched in free ribosomes and mitochondria (Bermingham & Wilkinson, 2009). I have previously shown in Chapter 3 that *ApVasa*, *Apdnmt3a* and *Apdnmt3x* appear to be provisioned to oocytes by the nurse cells.

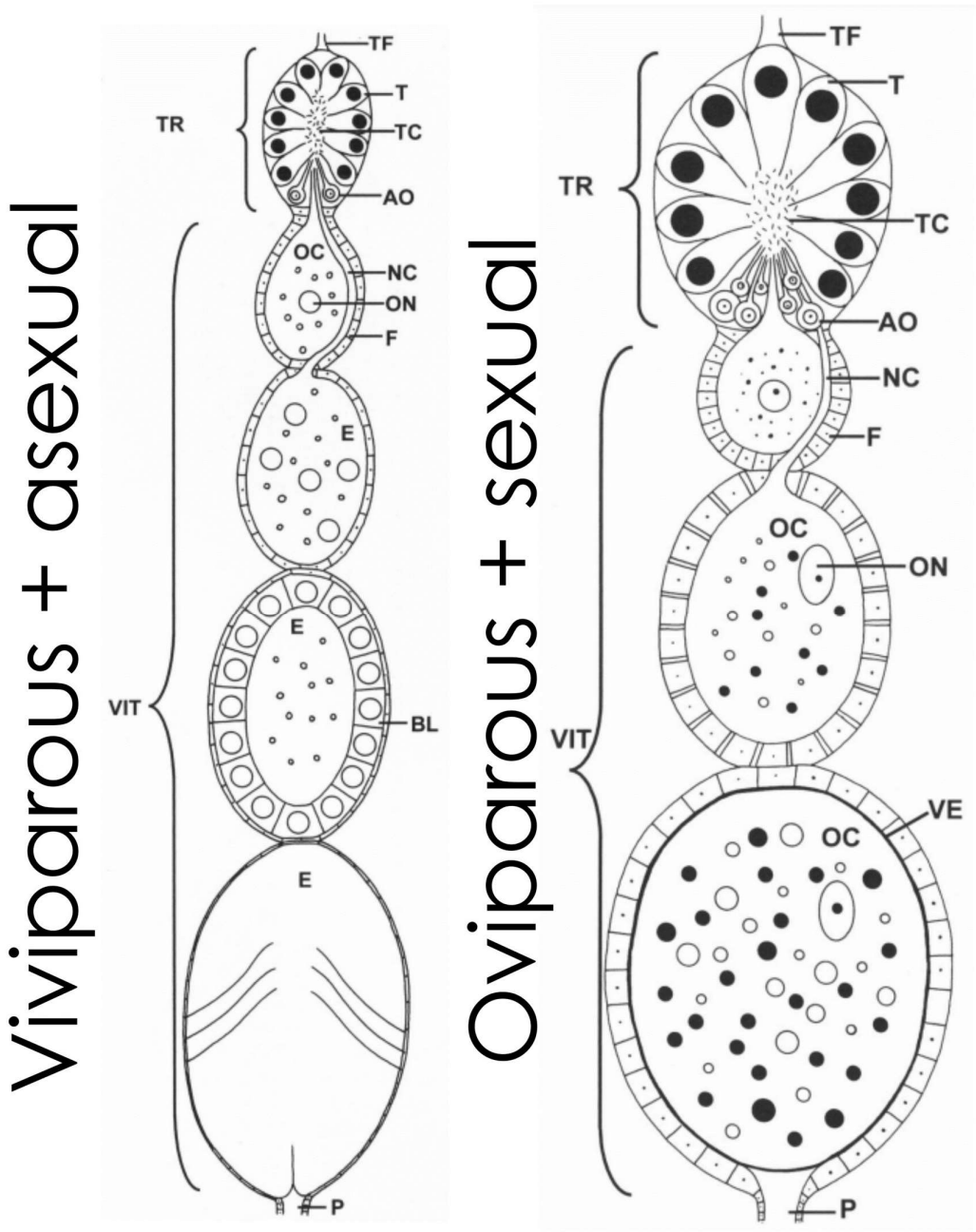

Figure 6.1 Schematic of typical aphid ovarioles of viviparous, asexual individuals (left) and oviparous, sexual individuals (right).
At the anterior end (top), the terminal filament (TF) anchors ovarioles together, and attaches to the germarium (consisting primarily of trophocytes (T, nurse cells) and prospective and arrested oocytes (AO)). The trophoctes nourish oocytes and early embryos through nutritive cords (NC, trophic cords), to which they attach through the trophic core (TC). Sexual females possess enlarged trophocytes relative to asexual females, and develop larger oocytes (O), which maintain a longer association with the follicular epithelium (F). ON, oocyte nucleus, E, embryo, BL, blastoderm, VE, egg envelope, VIT, vittelarium, P, pedicel. Adapted from Michalik et al., 2013.

In the pea aphid, nurse cells and oocytes are both derived from the thirty-two cell germ cell cluster (itself arising from synchronous oogonial divisions of a single stem cell) (Blackman, 1978), with some portion becoming nurse cells (typically in aphids, reported as ~ 24 (Michalik et al., 2013), and in *A. pisum* approximately twenty (Miura et al., 2003)) and the remainder presumptive oocytes. Being maternal in nature and through provisioning, the nurse cells provide a direct and close avenue of communication between the mother and her eventual offspring, from the earliest stages of development (Bermingham & Wilkinson, 2009; Winata & Korzh, 2018). Not just by adult aphids, but also by late stage embryos which already have their germ cells specified and arranged into germaria, and contain within them oocytes and early stage embryos of their own (Dixon, 1985; Brough & Dixon, 1990a), presenting the possibility of mature embryos being able to affect the trajectory of their own germ. The alternate avenues for a mother to interact with her germ is through the follicle cells and ovariole sheath. The follicle cells surround oocytes while they are in the follicle envelope, resultantly, while the association is continual and long in oviparous ovaries, in parthenogenetic ovaries it is comparatively short-lived (Bermingham & Wilkinson, 2009). The ovariole sheath on the other hand is a unicellular layer, enriched in mitochondria, ribosomes, endoplasmic reticula and Golgi apparatus, which encompasses the developing embryos (surrounding the ovariole) and appears to support embryos from the blastula stage onwards by facilitating transfer of nutrients from the haemolymph to embryos (Bermingham & Wilkinson, 2009; Couchman & King, 1979; Brough & Dixon, 1990a). The high degree of continued contact between a mother and her developing offspring afforded by viviparity, a mode of development in insects that is exemplified in aphids (in the true sense of viviparity), allows exquisite control over development (Bermingham & Wilkinson, 2009; Ogawa & Miura, 2013). This, likely, affords greater developmental plasticity than oviparous reproduction, and may partially facilitate fine control over the two polyphenisms (reproductive mode and wing morph) demonstrated in aphids; though, it is important to note that cyclical parthenogenesis appeared earlier than viviparity in the asexual phase, with viviparity being specific to the *Aphididae*, and cyclical parthenogenesis appearing across the *Aphidomorpha* (Davis, 2012). The fact that the projections thought to be carrying the virginoparin (associating with the NSCs known to exert control over the switch (Hardie et al., 1985; Cuti et al.,

2021)) terminate in the proximity of the germaria, rather than the developing embryos, suggests that the virginoparin's effect may be facilitated by the trophocytes of the germaria (or indeed, by directly interacting with the oocytes), rather than the ovariole sheath or follicle cells.

In the pea aphid, shorter photoperiods experienced over generations result in the eventual production of sexual aphids (Corbitt & Hardie, 1985; Le Trionnaire et al., 2009; Matsuda et al., 2017) (I have previously demonstrated in our strain that this can be over two generations, with the offspring of the second being sexual (see chapter 5)). This is accompanied by a suite of changes in gene expression in the sexupara generation ((Le Trionnaire et al., 2009; Ishikawa et al., 2012; Barberà, Cañas-Cañas & Martínez-Torres, 2019; Matsuda, Numata & Udaka, 2020) and as explored in chapter 5). Recent investigations of the mechanisms of the reproductive switch have increasingly implicated the insulin signalling pathway as a potential player. In aphid mothers, ILP4 appears to be transported from the *pars intercerebralis* of the protocerebrum, where it is thought to be synthesised in the NSC I, through a medial nerve and two lateral nerves which travel through the corpus cardiaca, with the lateral nerves terminating in the vicinity of the ovaries (Cuti et al., 2021). As *ilp1* and *ilp4* expression have been shown to be higher in the brains of aphids in LD conditions (Barberà, Cañas-Cañas & Martínez-Torres, 2019), and *inr* expression has been shown to be higher, and *ide* expression lower, also in LD heads (Le Trionnaire et al., 2009), and I revealed several insulin-related genes to be differentially expressed in the ovaries of LD and SD condition aphids (chapter 5), a putative physical link between a putative modulator of the reproductive switch and the germaria is revealed. Through this system, reception of environmental cue in the brain of the mother may be being conveyed to the ovary where the effect on reproductive mode is effected.

The germ cells are thus a strong candidate for having differential gene expression in response to the different photoperiods which result in the production of asexual or sexual aphids, likely, at least partially in response to differential exposure to virginoparin (and other modulators). Additionally, germ cell specification occurs relatively early in development, in the pea aphid, at stage six of twenty (Miura et al., 2003). The germ cells migrate with the

elongating germ band (embryo proper) then eventually form distinct clusters corresponding to germaria following katatrepsis (stage 15, which occurs at approximately 50 % of total embryonic development), that are situated bilaterally in the dorsal abdomen region of the embryo (Miura et al., 2003). The germ cells of viviparous and oviparous aphids are specified through the inheritance mode, in which maternally provided germ plasm accumulates in the embryo (Chang et al., 2006; Lin et al., 2014). This mode of germ cell specification is thought to be derived compared to the ancestral induction mode, in which signal induction occurs independently of maternal input (Extavour & Akam, 2003; Whittle & Extavour, 2019), and is unconventional for hemipterans (it is not known to occur in any other hemipteran, e.g. *Oncopeltus fasciatus* (Ewen-Campen, Jones & Extavour, 2013)), only occurring in aphids, where it is employed in viviparously and oviparously produced embryos. This mode of germ cell specification appears, therefore, to have independently evolved in aphids and may have been gained following or leading up to the emergence of cyclical parthenogenesis. Thus, germ cells present an early stage of development (in terms of specification) that may be interacted with by the environment to facilitate the reproductive polyphenism.

The products of several genes show germline specific localisation in the viviparous pea aphid (in the asexual and sexual phase, some genes being differentially expressed between these modes (Duncan, Leask & Dearden, 2013)). Among them is *Ap-vasa1*, one of the four *vasa* homologs, all of which encode DEAD-box protein family ATP-dependent RNA helicases, *Ap-vasa1* being the only one specific to the germline (Chang et al., 2006, 2007; Lin et al., 2014). In metazoans generally, *vasa* is considered amongst the most conserved germ-cell specific genes (Gustafson & Wessel, 2010; Lin et al., 2014).

To date, studies aimed at the determination of differential expression profiles between parthenogenetic aphids exposed to LD and SD conditions (or attempting to assess expression differences as part of the wing polyphenism) have made use of whole bodies, heads (the two tissues types most commonly used), ovaries, carcasses (typically the body without the ovary, to remove the influence of embryos) and embryos (especially, later stage embryos) (Le Trionnaire et al., 2007, 2009; Huybrechts et al., 2010; Ishikawa et al., 2012;

Vellichirammal, Madayiputhiya & Brisson, 2016b; Barberà, Cañas-Cañas & Martínez-Torres, 2019; Matsuda, Numata & Udaka, 2020; Le Trionnaire et al., 2022). In order to explore potential differential gene expression profiles between germ cells, which are a relatively rare cell type within the total population of ovary cells (and thus, the differences in expression profiles of which are likely usually swamped by the effects of RNA from the rest of the tissue), of pea aphids in LD (producing virginopara aphids) and SD (producing sexupara aphids) conditions, I used ACME dissociation (García-Castro et al., 2021) in a novel protocol modifying RNA-FISH HCR (Choi et al., 2018) toward Probe-Seq (Amamoto et al., 2019) to isolate germ cells from ovaries using a germ cell specific marker, *Ap-vasa*. ACME is a dual dissociation and fixation media. Combining dissociation and fixation prior to RNA-seq minimises changes in gene expression that typically occur when isolating dissociation of live cells from fixation (with fixation typically occurring after lengthy protocols, for instance after several wash, incubation, staining, centrifugation, and sorting steps, as I use below); use of nuclei extracted from frozen tissue has been proposed, but this eliminates the mature mRNAs concentrated in the cytoplasm (García-Castro et al., 2021). Thus, ACME, which has been demonstrated to produce fixed cells with high-integrity RNAs (García-Castro et al., 2021), presents a powerful tool for various RNA-seq applications. Its use has been demonstrated in a range of species by García-Castro and colleagues, but not yet in a hemimetabolous insect.

After optimisation of each aspect of the method, the protocol was successfully used to isolate presumptive germline cells from aphid ovaries for RNA-seq. To my knowledge, this is the first study using an approach like Probe-Seq or ACME dissociation in a hemipteran bug, and the first to target sequencing at germ cells in aphids at all.

6.2 Methods

6.2.1 Aphids

Aphids were derived from a viviparous asexual colony reared long-term in LD conditions, 16L:8D, 20 °C, 70% RH. Aphids were isolated from this colony and reared in low density under LD conditions for two generations to avoid crowding effects which are known to be transgenerational. All aphids used in the following procedure were wingless. Upon reaching adulthood, an age-matched (within three days) population from the second generation of aphids was isolated and allowed to reproduce in low density LD conditions. The earliest L4 aphids from this population were isolated and represent the F0 of this study. At this point, L4 aphids were split into LD and SD (12L:12D, 15 °C, 70% humidity) condition populations. Aphids were reared to adulthood and allowed to produce the focal generation of aphids of this study. Owing to different temperatures, the time to reach adulthood, the time to onset of reproduction, and reproductive rate differed between LD and SD aphids. This resulted in desynchronization temporally of aphids in the two conditions. I have previously demonstrated (data not shown) that SD conditions applied at these stages cause a ~ six day delay in the onset of reproduction in SD aphids compared to LD aphids; this desynchronization is expanded the longer the conditions are experienced. To account for this, samples of aphids in SD conditions were taken one week after samples of aphids in LD conditions were taken. L3 and L4 aphids were isolated from the LD and SD groups when appropriate, with younger nymphs and the F0 adults being removed. These aphids were seeded onto fresh plants at a density of approximately five aphids per broad bean seedling. These aphids were reared to adulthood and sampled, with SD aphids being sampled nine days after LD aphids. At the time of sampling, aphids were reproductive, and to avoid crowding effects prior to sampling, nymphs were routinely removed following the onset of reproduction. Due to differences in productivity, nymphs had to be removed from the LD condition more regularly, and in greater number than from the SD condition. The sampled SD aphids were confirmed as sexuparae by dissection of a sample of the offspring produced by their generation, which revealed oviparous ovaries. A schematic of the experimental procedure is presented in figure 6.2.

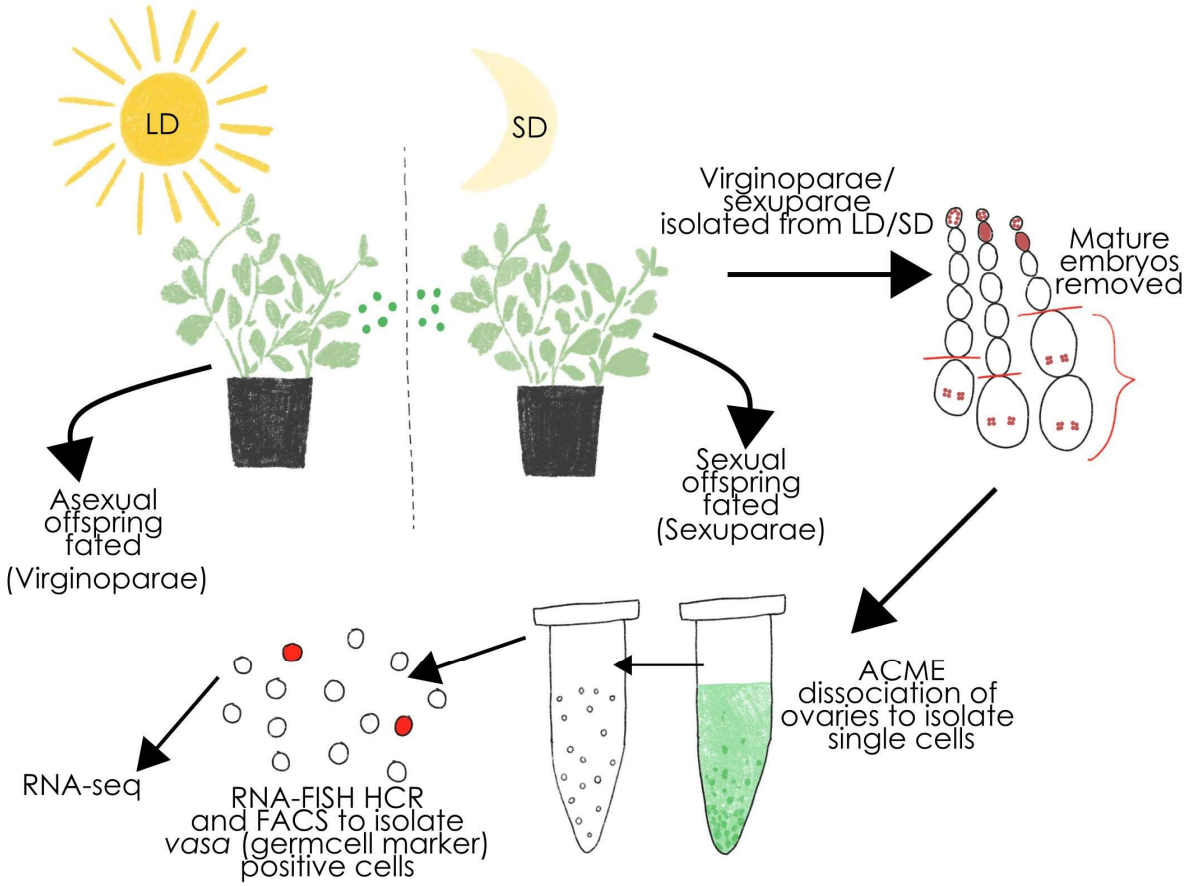

Figure 6.2 Schematic of experimental approach for RNA-seq of FACS sorted RNA-FISH HCR stained ACME dissociated single cells derived from pooled ovaries from virginoparae (asexual aphids in LD (long-day) conditions that will produce asexual offspring) or sexuparae (asexual aphids in SD (short-day) conditions that will produce sexual offspring) *A. pisum*.
Ovaries were dissected from adults, and the most mature embryos were removed (to avoid possible differential expression associated with late-stage sexual embryos specifying and developing sexual eggs), tissue was then dissociated into single cells, then cells positive for the germ cell marker, *vasa* were stained with RNA-FISH HCR and cells were sorted using FACS. RNA was then extracted from *vasa* positive cell populations for RNA-seq.

6.2.2 Cell dissociation

On the day of sampling for both LD and SD conditions, adult aphids were collected within the first two hours of lights-on. For each condition, three biological replicates and a probeless negative control were prepared sequentially, with aphids being collected using forceps, dissected and then processed into ACME solution (described below) before collection of the next set. Ovaries were dissected out in cold PBS using a dissecting microscope

(GXMXTL3T) and 70 % ethanol-sterilised fine forceps. Bits of gut, cuticle, and cauda belonging to the mother were often attached to ovaries and were discarded. Embryos at approximate stages 18-20, corresponding (generally) to the most mature one to four embryos, were removed from ovaries and the remaining tissue consisting of strings of developing embryos and oocytes, and germaria and loose ovary parts (recovered using a P1000 pipette) were placed into a microcentrifuge tube on ice, with minimal transfer of PBS. The late-stage embryos were identified primarily by morphology and size (these stages see significant growth relative to the rest of embryogenesis), and were removed because they possess ovaries that contain sexual eggs (in the case of SD aphids) or relatively developed embryos (in the case of LD aphids) which might affect interpretation, in addition to possessing a large percentage of untargeted cells. After no more than thirty minutes of dissection, PBS was aspirated and ovaries were placed in ACME solution. The following describes a modified version of the ACME cell dissociation method described in (García-Castro et al., 2021). ACME solution, a cell dissociation and fixation media (that also partially permeabilises cells), was made fresh and consisted of water:methanol:glycerol:glacial acetic acid in a ratio of 13:3:2:2. 1 ml of ACME solution was applied to each tube of ovaries (30 ovaries per) and tubes were gently rocked on a nutator for (depending on replicate) five to six hours at RT. Roughly every 15 minutes, tube contents were triturated by pipetting up and down several times using, initially, a P1000 pipette, then a P200 once partial dissociation had occurred, and finally with a P20 towards the end, to aid dissociation. After the final trituration the solution was a mostly homogenous liquid with no large clumps visible to the eye. To remove ACME solution, samples were centrifuged at 1,000 g for five minutes at 4 °C and the supernatant was removed. The resultant pellet of dissociated cells was resuspended in PTw (0.05 % Tween-20, a lower concentration than used for whole tissue HCRs due to the lytic effect of higher detergent concentrations on single cells; 0.05 % BSA (Thermo Scientific) to reduce clumping). Cell suspensions were kept on ice when not nutated until pre-hybridisation.

As a confirmatory step in preliminary experiments, a subset of cell suspension was stained with DAPI (1 µl, 5 mg/ml, 4′,6-diamidino-2-phenylindole, Invitrogen) after the final wash, after washing with and then resuspension in 500 µl PBS

instead of PTw. Cells were visualised on a Leica M165FC Fluorescence Stereo Microscope with a Q Imaging Retiga EXI colour cooled fluorescence camera and Q Capture Pro 7 software.

6.2.3 Dissociated cell RNA-FISH HCR

Following, cell suspensions were processed through a modified version of the RNA-FISH HCR protocol described in the general methods, optimised for minimal damage to cells, minimal cell loss during washes, and maximal probe and hairpin penetrance. Precautions were taken to minimise the presence of RNAses. Cell suspensions were centrifuged at 1000 x g for five minutes at 4 °C, then the supernatant was removed and pellets were gently partially resuspended in 500 µl PTw (0.05 % Tween-20, 0.05 % BSA) and washed for ten minutes on a nutator at RT, for a total of three times. Suspensions were then centrifuged at 1000 x G for five minutes at 4 °C and the supernatant was removed, then 500 µl PTw (0.05 % Tween-20, 0.05 % BSA) was applied, the pellet was gently partially resuspended, and the samples were nutated at RT for thirty minutes. Cells were centrifuged again (as above), the supernatant was removed, and the pellet was resuspended in 200 µl pre-warmed HCR probe hybridization buffer (Molecular Instruments) at 37 °C for thirty minutes. 2 µl (6 pmol) *ApVasa* probe (Molecular Instruments) was added to each of the biological replicate samples, but not to the probeless negative control, and then samples were pipetted up and down gently with a P200 pipette to mix. Samples were incubated at 37 °C O/N (~ thirteen hours).

500 µl pre-warmed HCR wash buffer was added to each sample and centrifuged at 2000 x G for five minutes at RT to form a more compact pellet (while in HCR probe hybridization buffer, a pellet does not form very well and so aspiration of supernatant was carried out very carefully, leaving some liquid). All centrifuges following, until FACS, were 2000 x G for five minutes at RT. To minimise changes in temperature, samples were quickly transferred from the hybridisation chamber at 37 °C to the centrifuge or vice versa, and samples and HCR wash buffer were placed back in the hybridisation chamber after each centrifuge and taken out and replaced for washes one at a time. Samples were washed by applying 500 µl pre-warmed HCR wash buffer and gently disturbing the pellet from the side of the tube, but not resuspending, then centrifuging and

the supernatant removed (more completely with each wash) as described for a total of four times. 500 µl 5 x SSCT (5 x SSC, 0.1% Tween-20) was added to the pellets, then they were disturbed away from the side of the tube, but not resuspended, and washed for five minutes, then centrifuged and the supernatant was removed, repeated a total of two times. Samples were gently partially resuspended in 200 µl amplification buffer equilibrated to RT to pre-amplify for thirty minutes at RT. At the same time, HCR hairpins B4-594 h1 and B4-594 h2 (separate, in PCR-tubes, and in hairpin storage buffer) (Molecular Instruments), 4 µl of each per sample, were prepared by heating to 95 °C for 90 seconds in a thermal cycler, then allowed to cool to RT protected from the light for thirty minutes. After, 4 µl of each hairpin solution and a further 200 µl RT amplification buffer were added to each sample. Samples were gently pipetted up and down to mix and partially resuspend, then incubated at RT protected from the light until the following day (~20 hours). Samples were protected from light as much as possible until after FACS.

Samples were centrifuged as described and amplification buffer was carefully removed, leaving some liquid. 500 µl 5 x SSCT (5 x SSC, 0.1% Tween-20) was added to samples and pellets were gently disturbed from the side of the tube. Pellets were washed in 5 x SSCT (5 x SSC, 0.1 % Tween-20) on a nutator at RT, centrifuged and aspirated of supernatant, five times in total, the first two and the fifth washes being 5 minutes long, the third and fourth being thirty minutes (with 2 µl DAPI (5 mg/ml; 4′,6-diamidino-2-phenylindole; Invitrogen) added during the first). After the final centrifugation, pellets were washed briefly in 500 µl PBS (0.1 % BSA) at RT, centrifuged and washed again in 500 µl PBS (0.1% BSA), centrifuged and aspirated, then thoroughly resuspended in 500 µl PBS (0.1 % BSA) using a P1000 pipette and tips pre-wetted with PBS (0.1 % BSA), then placed on ice. Cell suspensions were filtered through sterile single-use 70 µm nylon mesh cell strainers (Fisher Scientific) pre-wetted with PBS (0.1 % BSA) into 50 ml conical tubes pre-wetted with 500 µl PBS (0.1 % BSA), then the filtrate was transferred to a pre-wetted with PBS (0.1 % BSA) microcentrifuge tube, protected from light and put on ice until FACS.

As a confirmatory step, in preliminary experiments, diluted samples of cells were then visualised by confocal microscopy (as described in the general

methods). Additionally, a sample of cells was used for RT-PCR of two housekeeping genes (*ef1α* and *rpl7*) to confirm RNA isolation (following 2.2.2 and 2.2.3 in the general methods, with the additional dsDNAse step), after RNA assessment using a Nanodrop 2000 spectrophotometer. PCR conditions were (HS taq (NeoBiotech), 1 µg template, 94 °C 3 mins, 40x [94 °C 30 secs, 60 °C 30 secs, 72 °C 1 min], 72 °C 5 mins), run with a NTC, No-RT, gDNA and positive control (cDNA from RNA derived from whole ovary). PCR products were then run on 1.5 % agarose in SB gels containing 0.5 µg/ml ethidium bromide, in SB buffer (10 mM sodium hydroxide, pH adjusted to 8.5 with boric acid) (Brody & Kern, 2004) at 150 V, alongside 1kb+ ladder (Invitrogen). Loading dye (Invitrogen) was added to samples in a ratio of 1:5 loading dye:sample. Visualisation and imaging of DNA was achieved through a Syngene InGenius Gel Doc System and the GeneSnap program.

6.2.4 FACS and RNA Isolation

Fluorescent Activated Cell Sorting (FACS) of RNA-FISH HCR processed cell suspensions was carried out on a BD FACSMelody Cell Sorter (BD Biosciences) in a class II recirculating cabinet by Dr Sally Boxall and Dr Ruth Hughes at the Bio-imaging and Flow Cytometry facility in the Faculty of Biological Sciences at the University of Leeds. Prior to running, the instrument was thoroughly decontaminated by flushing with bleach followed by detergent, ethanol, and then sterile PBS, and pre-cooled to 4 °C. Samples were kept on ice and transferred to 5 ml FACS tubes just prior to running. To set gating parameters and inspect the cell populations from each replicate and the negative control, 10,000 event runs of each sample were carried out prior to each being sorted. Cells were first gated using SSC (side scatter) and FSC (forward scatter), to remove debris, then SSC-W (SSC width) and SSC-H (SSC height), to remove clumps, and FSC-W (FSC width) and FSC-H (FSC height), to specify single cells. The probeless negative control was used, by comparison to each of the positive samples, to gate cells positive for fluorescence (relative to control). The same gating parameters were applied to each sample. In addition to fluorescence positive cells, a small sample of cells derived from a low-fluorescence area of each of the positive samples, corresponding to *ApVasa* negative cells were concurrently sorted. Sorting was carried out under

cooled conditions (4 °C sitting temperature, 8 °C running temperature) into microcentrifuge tubes containing 500 µl PBS (0.1 % BSA) which were then placed on ice. Due to timings, for LD and SD conditions, the first two samples were run, and then centrifuged at 17000 x G for five minutes, aspirated of PBS and then the pellet was resuspended in 300 µl TRI Reagent (Zymo), cell lysis was aided by pipetting up and down with a P1000, and lysates were placed on ice. Concurrently the third sample was run, and then immediately processed in the same way, leaving it in TRI Reagent for five minutes prior to RNA extraction. As a result, the first two samples were processed into TRI Reagent (Zymo) approximately thirty minutes before the third. Though, given the RNA protective and RNAse inhibitory properties of TRI Reagent, I do not expect this to have had an effect on the RNA integrity or quantity, or, subsequently, on the RNA-seq output or interpretation. Following this, RNA extraction was carried out as described in the general methods, without manual homogenisation, on the *ApVasa* positive and negative samples.

Initial quantification of RNA was carried out on a Qubit 4.0 fluorometer using the RNA HS Assay Kit (Invitrogen) and 1 µl of RNA per sample. RNA was then flash frozen in liquid nitrogen and stored at -80 °C prior to RNA-seq. RNA for SD samples was extracted and stored ~ one week after RNA for LD samples, due to the experimental setup.

6.2.5 Sequencing

Samples were sent for sequencing by Novogene , following their Plant and Animal Eukaryotic mRNA-seq workflow (using an Illumina NovaSeq 6000 sequencing system (150 bp paired-end)) in November 2023.

6.3 Results

6.3.1 ACME-RNA-FISH-HCR optimisation

I first attempted to optimise ACME dissociation of aphid ovaries. While in *Schmitea mediterranea* (the focal species of the original ACME paper (García-Castro et al., 2021)), dissociation took place to yield sufficiently dissociated tissue after ~ one hour, pea aphid ovaries of around a similar volume reached sufficient dissociation after upwards of four hours (sufficient dissociation was assessed by the presence of relatively few clumps of debris, or debris that no longer appeared to be dissociating), with regular tituration (though, the volume of ACME used was significantly lower, 1 ml here vs 10 ml in *S. mediterranea*). The acidic properties of ACME solution should preserve the integrity of RNA during dissociation.

6.3.1.1 Cell disassociation

In order to assess the efficiency of ACME for cell dissociation and permeabilisation, cells were stained and visualised using DAPI, which stains DNA to reveal nuclei. Visual inspection of nuclei of cells that underwent the ACME cell disassociation protocol showed mostly dispersed cells, with some clusters of multiple nuclei appearing (figure 6.3 a, a', b, c) (corresponding to undissociated cell aggregates, which occur in all dissociation methods, but are later removed through the downstream cytometry step, where they are excluded based on FSC, described below), though seemingly far less frequently than seemingly single cells, suggesting the protocol is appropriate and efficient for dissociating aphid ovary cells.

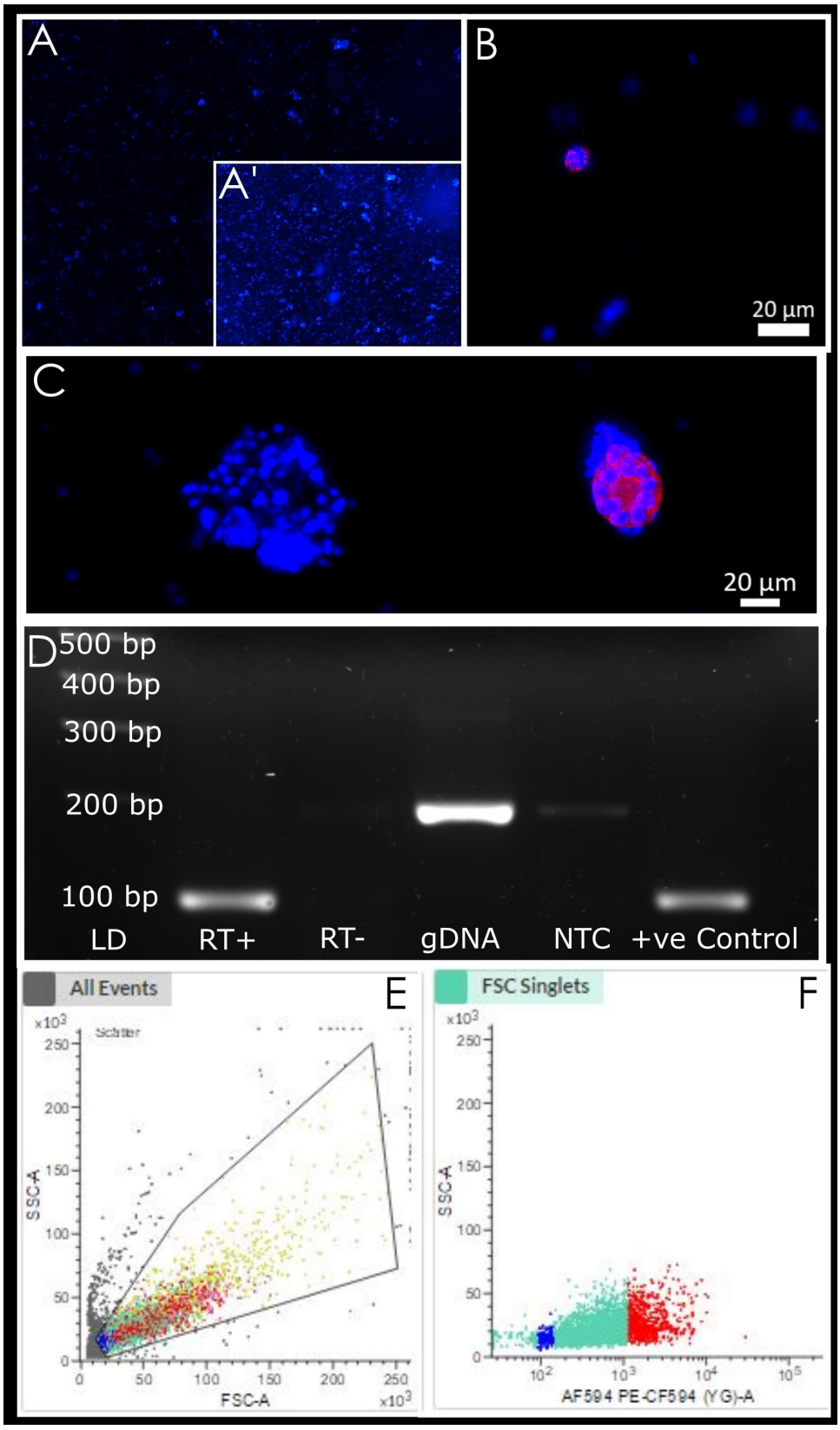
A
A'
B
20 µm
C
20 µm
D
500 bp
400 bp
300 bp
200 bp
100 bp
LD
RT+
RT-
gDNA
NTC
+ve Control
All Events
E
Scatter
SSC-A
FSC-A
FSC Singlets
F
SSC-A
AF594 PE-CF594 (YG)-A

Figure 6.3 Workflow checkpoints for RNA-FISH HCR FACs dissociated cell RNA-seq protocol, prior to sequencing.
Briefly, ACME cell dissociation was performed to fix and isolate single cells from pooled *A. pisum* ovary samples. Dissociated cells are visualised (by staining with DAPI, blue) in A and A', cells appear mostly dispersed, though some clusters are visible. Dissociated cell samples were then run through a modified RNA-FISH HCR protocol to stain for *vasa* (red), a highly specific marker of germcells in *A. pisum* ovaries. B. a single *vasa* positive cell and several *vasa* negative nuclei. C. two clusters of cells that have not been completely dissociated, one negative for *vasa*, and the other (which is a germarium, a cluster of germ cells – nurse cells and presumptive oocytes that exist at the terminal end of each aphid ovariole) enriched for *vasa*. After RNA-FISH HCR, the ability to extract sufficiently undegraded RNA from samples was assessed by performing RT-PCR on cDNA produced from extracted RNA. *tub*, a housekeeping gene, was used in this case to assess integrity. Ladder, LD, reverse transcriptase + cDNA synthesis, RT+, reverse transcriptase – cDNA synthesis, RT-, genomic DNA control sample, gDNA, no-template control sample, NTC, positive sample (whole aphid) control, +ve. After RNA-FISH HCR, FACS was used to isolate *vasa* positive single cells from debris and *vasa* negative cells. Examples of gating strategies are presented in panel E (gating out debris) and panel F (gating based on *vasa* associated fluorescence).

6.3.1.2 RNA-FISH HCR

Following efficient cell dissociation, I attempted to stain germline cells by using a modified version of RNA-FISH HCR, using *Ap-Vasa* as a target-specific probe, with more cell-friendly solutions (the inclusion of BSA and reduced detergent concentration) and handling, then visualising by confocal microscopy. Dissociated cells positive for *Ap-Vasa* were identifiable in cell suspensions, though these cells were very rare (figure 6.3, b, c). This rarity corresponds to the relative abundance of *Ap-Vasa* positive cells, the germ cells of developing embryos, the trophocytes in the germaria, and the early oocytes, within the ovaries of viviparous pea aphids (see chapter 4).

6.3.1.3 RNA assessment

Having successfully stained target dissociated cells, I endeavoured to assess the availability and integrity of total RNA within cell suspensions exposed to the ACME dissociation and RNA-FISH HCR protocols. RNA extraction of and subsequent PCR of cDNA generated using the extracted RNA from, RNA-FISH HCR cell suspensions produced and revealed RNA of useable quality (for RT-PCR)), and relatively high yield, as assessed by spectrophotometry (though, of the total cell suspension population rather than the comparatively rare *Ap-Vasa*

stained population, total RNA of which represents a small percentage of the total pool), demonstrating the optimised protocol's suitability for downstream applications, which are dependent on the recovery of sufficient amounts of high integrity RNA. PCR of cDNA produced from the total RNA amplified two targeted housekeeping genes (housekeeping genes were targeted for their relatively high expression), *Ap-ef1α* and *Ap-rpl7*, which were visualised by gel electrophoresis, further demonstrating RNA integrity (figure 6.3, d).

6.3.1.4 FACS and Germline cell RNA assessment

To confirm the isolation of cells advanced through the optimised ACME-RNA-FISH HCR protocol, and approximate expected RNA yields from them, I then performed FACS (operated by Sally Boxall and Ruth Hughes at the Bioimaging and Cytometry Facility in the Faculty of Biological Sciences at the University of Leeds) on cell suspensions generated from whole ovaries and ovaries with the most mature embryos (corresponding to approximate embryonic stages 18-20) removed. A higher concentration of BSA (0.1 %) was found to improve recovery of fluorescent single cells. FACS analysis further confirmed the efficiency of dissociation, by inspection of scatter plots, and confirmed positive *Ap-vasa* associated fluorescence in a small proportion of the total population of isolated cells (figure 6.3, e, f). The positive populations of cells were collected and pelleted, though, the pellet was non-visible (or in the case of LD cells documented below, barely visible). Total RNA isolated from these populations was quantified and revealed low (but useable) amounts of RNA.

6.3.2 ACME RNA-FISH HCR of LD and SD aphid vvaries

LD cell populations averaged a mean input of 7.77 million events (corresponding roughly to number of cells) (n = 3), while SD cell populations had a mean of 3.69 million events (n = 3). For LD samples, a mean of 144,660 cells (n = 3) were gated as positive for A594 fluorescence, corresponding to presumptive *Ap-Vasa* positive cells, and were collected for RNA isolation, while the mean number of cells of the same type from SD samples was 42,164. As the number of aphids dissected for each replicate and between conditions was consistent (thirty ovaries per), the discrepancy between LD and SD samples in terms of events and *ApVasa* positive cells is noteworthy. I believe that the difference in number of events is due to batch specific differences in the loss of

material over the three-day long protocol (which requires many careful washes and aspirations of supernatants from solutions containing weakly compacted pellets), rather than differences in the amount of *vasa* positive tissue in SD and LD ovaries (as explored in chapter 5, *vasa* expression was equal between ovaries from LD and from SD aphids – equal to the samples used here). I do not expect this to have had an effect on the interpretation of the RNA-seq data, as cell loss through washes would be unbiased. Differences in recovery of positive cells stems primarily from this reduced input, but also initial gating based on SSC-A and FSC-A and a slightly reduced percentage of gated single cells being gated as *ApVasa* positive.

6.3.3 Sequencing

Samples (three biological replicates each of *Ap-Vasa* positive LD and SD cells, and of *Ap-Vasa* negative LD and SD cells) were sent for sequencing in November 2023.

6.4 Discussion

I have demonstrated that ACME dissociation is an efficient cell fixation and dissociation solution for use in the ovary of the pea aphid for downstream RNA-related applications, such as Probe-Seq, and feasibly scRNA-seq. It is likely appropriate for use in other tissues, in other aphids, and in other hemimetabolous insects. I have also demonstrated the capacity of RNA-FISH HCR to be pivoted into Probe-Seq sequencing of targeted cell types/populations through use of HCR-probes and HCR-hairpins, using probes complimentary to mRNA of genes that we know the expression pattern for (in our tissue of interest).

The presented protocol offers a powerful tool for isolation of distinct cell identities/populations. It is a useful technique for studies such as this, where total RNA can be extracted from bulk cells representing a distinct cell population, with specific markers to reveal putative generalised patterns between different morphs, and across conditions. Additionally, scRNA-Seq where further discrimination between individual cells allows greater resolution of differences in expression profiles, in conjunct with probe-seq will allow more exact assessments of expression profiling. Studies that implement scRNA-Seq

can also act as springboards for probe-seq investigations, where cell-type specific gene expression is revealed in single cell data, allowing well informed selection of specific targets, and targeting of subpopulations of particular cell types.

6.4.1 Concluding remarks and future work

This study presents for the first time, an attempt to target RNA-sequencing specifically at the germ cells, by combining (principally) two techniques, ACME cell dissociation and RNA-FISH HCR, showing that they can be combined in aphids to target specific cell types. In doing so, I hoped to unveil different transcriptomic profiles in the germ cells of virginopara and sexupara *A. pisum* aphids, to further our understanding of the reproductive switch at fine detail and characterise the expression profiles of these cells, generally.

Further studies utilising ACME-RNA-FISH-HCR-Probe-Seq or comparable approaches to isolate and profile germline cells in a greater range of morphs/conditions will greatly enhance our understanding of the mechanisms facilitating and involved in polyphenisms, such as in crowded/non-crowded viviparous aphids that will produce winged offspring or wingless offspring (as part of the second aphid polyphenism), and in crowded/non-crowded sexuparae, as they are unable to produce winged females, to inspect how this exclusion may occur. Studies using the ovaries of aphids exposed to LD/SD conditions for variable lengths of time may identify gradual changes or switches in gene expression profiles in response to accumulation of exposure to reduced photoperiod.

Additionally, ACME-RNA-FISH-HCR-Probe-Seq aimed at other cell types within the ovary, and other tissues, in aphids and other organisms, will further elucidate control of polyphenism and other developmental biology relevant questions. For instance, applying this technique to follicular cells, by identifying genes with follicle cell specific expression among cells in the ovary to use as probes (through homolog searching and RNA-FISH HCR visualisation of mRNA, or scRNA-Seq, for example) may further reveal how the mother's perceived environment influences the developmental trajectory of her offspring; if the message is not conferred through the germ cells alone. Although, it is

important to note that ACME solution does not penetrate chorions or vitelline membranes (which, given the nature of viviparous parthenogenetic development, were not present in this study), or hard shells, it is instead suitable for soft tissue, and is thus unsuitable in its unmodified form for some applications without careful consideration (see(García-Castro et al., 2021)).

Chapter 7 General Discussion

In this thesis, I have explored the reproductive biology of an aphid, *Acyrthosiphon pisum*, with regards, at the most general level, to the parthenogenetic viviparous reproductive mode and the polyphenism that encompasses it, cyclical parthenogenesis, as an extreme case of phenotypic plasticity (a summary diagram of the key findings of this thesis are presented at the end of this chapter, in figure 7.1). In chapter 3, I explored possible improvements in the efficiency of RNAi, and was unable, despite attempts to increase RNAi efficiency to demonstrate gene knockdown. This contributes to a growing body of evidence, which highlights the variability between species and indeed between strains of insect, in their responsiveness to RNAi (Sugahara et al., 2017; Tomoyasu et al., 2008; Luo et al., 2017; Elston et al., 2023; Guan et al., 2018). I make recommendations for other approaches to potentially alleviate the lack of responsiveness (3.4), but the picture that this work contributes to is one with implications for RNAi's applicability to use as pesticide, and concerns over pesticide resistance . Further work to assess inter-strain variability and investigate commonalities in mechanisms that make groups seemingly recalcitrant to RNAi (that is, is there one or a few mechanisms that are commonly and strongly associated with lack of RNAi responsiveness that can be mitigated, or is the variability so high and the elements so numerous that mitigation must be tailored very widely and/or specifically) will greatly benefit researchers looking to perform functional knockdown of genes to study their functions (by allowing genetic screening, perhaps, to allow selection of strains in which investigators can elucidate function by RNAi, or though improvement of approaches to increase RNAi efficiency such that any strain or species might be useable) and allow more considered advancement of RNAi based pesticide (without good understanding of, and sufficient ways to mitigate potential resistance, RNAi based pesticide must be looked at very tentatively). Regardless, as RNAi did not present a viable option to study gene function

under the presented method, I took another approach to studying the functions of two DNA methyltransferase 3 genes which are duplicated in the genome of *A. pisum*.

In chapter 4, I showed that both *dnmt3a* and *dnmt3x* likely arose from a duplication event in an ancestor of the Aphidomorpha, with *dnmt3x* being the likely derived copy, specific to the Aphidomorpha. Both *dnmt3a* and *dnmt3x* have been conserved across the Aphidomorpha, and have diverged from one another, with Dnmt3a appearing to possess methyltransferase activity, and Dnmt3x appearing not to possess methyltransferase activity, instead having domains associated with histone interactions (PWWP and ADD) , which Dnmt3a is lacking, suggesting some amount of subfunctionalization and/or neofunctionalization. Furthermore, both copies are expressed in the germ cells and early embryos within the ovaries of parthenogenetic aphids raised in LD conditions, and once specified, the germ cells of their embryos, in complete overlap, temporally and spatially with each other. By inhibiting the activity of the *A. pisum* DNA methylation machinery more generally (Dnmt1 and Dnmt3s), I revealed roles for this system in reproduction. Namely, inhibition led to reduced fecundity, disturbed embryogenesis/oogenesis, and the production of highly disturbed, seemingly stillborn offspring. Given the divergence of *dnmt3x* from *dnmt3a*, especially in the presence/absence of DNA methyltransferase domain, I discussed how novel DNA methylation independent activity of *dnmt3x* may be associated with this reproductive phenotype.

To better understand the mechanisms involved in the switch from asexual to sexual reproduction, I investigated, in chapter 5, differential gene expression between the ovaries of viviparae (asexual aphids that will produce asexual offspring only) and sexuparae (asexual aphids that will produce sexual offspring). In doing so, I revealed higher expression of several genes related to insulin signalling and juvenile hormone signalling in the ovaries of sexual producers (and lower expression of one that putatively works antagonistically to insulin signalling, *idel1*), along with elevated expression of *dnmt3x* and to a lesser, but present, extent, of *dnmt3a*. Curiously, many of the differentially expressed genes did not appear to be downstream of JH signalling (inferred by lack of differential expression when the fate of SD condition exposed aphids was diverted away from sexuparae to virginoparae by treatment with the JH

analog, methoprene), further suggesting other systems, possibly and principally insulin signalling as a major modulator of the reproductive switch, as has been suggested (Barberà, Cañas-Cañas & Martínez-Torres, 2019; Cuti et al., 2021). The lack of differential expression of the same genes that were observed to be differentially expressed between comparable LD and SD condition aphids was surprising, given that treatment with methoprene was able to reverse the switch, at least partially, from sexual to asexual reproduction. The one exception was *idel1*, the expression of which was elevated, consistent with the upregulation of this gene in LD compared to SD conditions. This is suggestive of *idel1* being both JH responsive, and associated with the switch. Taken together, the results presented in chapter 5 call most strongly for further inspection of the role that insulin signalling may be playing in the aphid reproductive polyphenism, which may be part of a growing number of examples of reproduction being controlled by insulin signalling in diverse and specialised ways. The results of chapter 5, generally, expand our understanding of the genetic underpinning of this polyphenic switch, especially given that many of the genes that saw an increase in their expression in sexual relative to asexual producers in the ovary, might be expected to have the opposite relationship in the adult otherwise (based on head/whole-body gene expression profiles in LD and SD *A. pisum*) (Ishikawa et al., 2013; Barberà, Cañas-Cañas & Martínez-Torres, 2019). The differential expression of *dnmt3x* and dnmt3a, expanding on the expression profiling in chapter 4, further implicates a role for these duplicate genes in reproduction, but also associates them with this example of plasticity – it is noteworthy that *dnmt3x* is specific to the Aphidomorpha, amongst insects, and cyclical parthenogenesis also arose in an ancestor of this group (Davis, 2012), and that a maintained duplication of *dnmt3* (a relatively unusual feature, at least in insects) is also present in *Daphnia* species, another group that exhibits cyclical parthenogenesis (Nguyen et al., 2020; Ebert, 2022). Following this, I demonstrated that accompanying the increased expression of *dnmt3x* in the ovary of sexuparae may be an expansion of its expression pattern, with more widespread signal rather than confinement to the germ cells in developing embryos (as seen in chapter 4). More work is warranted to determine the exact function of *dnmt3x* (and indeed *dnmt3a*) with regards to DNA methylation, and independently of DNA methylation. Further work should also establish more clearly the link between JH signalling and insulin signalling in the switch, and

target insulin-signalling to assess its control over the switch, independently from JH, along with its potential modulation of JH signalling.

In the final data chapter, chapter 6, I attempted to extend assessment of differential expression in the ovaries of virginoparae and sexuparae, by probe mediated isolation of single cells, to assess differences between germ cells derived from virginoparae and sexuparae (and the non-germ cells, as bulk, for comparison), as these represent the earliest point of development, and are situated primarily in the germaria, close contact points for the terminations of the projections thought to carry virginoparin from the brain; in addition to being the major place of expression of *dnmt3a* and *dnmt3x* (in virginoparae). This technique will be a useful tool, and perhaps a relatively tractable one for labs already performing RNA-FISH HCR, for inspecting differential expression more specifically in targeted tissues and populations of cells, beyond the capacity of bulk single-cell techniques.

Thus, taken together, the results presented in this thesis contribute principally to our understanding of the control of an extreme example of plasticity, the reproductive polyphenism of aphids. As an integral and unusual aspect of aphid biology, and a likely major contributor to their success as a group (Blackman, Minks & Harrewijn, 1987; Srinivasan & Brisson, 2012), this contributes to our understanding of a group that includes several important pest species (Emden & Harrington, 2017; Paudel et al., 2018). Thus, this work contributes to knowledge that may be leveraged for more efficient pest control, perhaps most obviously, control strategies that take advantage of the reproductive polyphenism by encouraging production of oviparous individuals, which represent a more slow mode of population growth (Shingleton, Sisk & Stern, 2003; Durak et al., 2023) and therefore reduced damage inflicted upon plants, without completely reducing populations which may lead to knock on effects on other (beneficial or neutral) organisms (Shelby et al., 2020) . The work in chapter 4, specifically the phenotypic effects (disrupted reproduction) of 5-azacytidine treatment, highlight the possibility of targeting the DNA methylation machinery (perhaps by knockdown of specific DNMTs – though further characterisation of exactly what each of *dnmt1*, *dnmt3a* and *dnmt3x* do will be necessary for this to be most effective) as a means of controlling aphid populations, given that 5-azacytidine treatment led to reduced fecundity, which

was undone with some delay after treatment ended. Research that inspects, functionally, the involvement of some of the genes found to be differentially expressed between LD and SD conditions in ovaries here, may identify further gene targets, the silencing of which may lead to controlling the reproductive switch, to reduce aphid population growth. Although, the work in chapter 3 highlights, by contributing to an increasing body of evidence suggesting high variation between populations of aphids in their sensitivity to RNAi, that approaches attempting to advance/apply RNAi based pesticide should do so in a considered and careful manner, and attempts should be made to characterise the origin of this variation and optimise RNAi to make sensitivity roughly consistent.

As a key example of plasticity in insects (Nijhout, 1999; Simpson, Sword & Lo, 2011; Yang & Andrew Pospisilik, 2019), advancing understanding of the aphid reproductive polyphenism expands our knowledge of plasticity in general. This may be especially true given that the work presented in this thesis further characterises elements involved in modulation of a plastic response, which as discussed in chapter 1, is the aspect of insect plasticity that appears (and is necessarily) most conserved between examples across several insect polyphenisms and examples of phenotypic plasticity, compared to sensors (which are highly specific to the nature of the phenotypes involved in the polyphenism, and therefore diverse) and effectors (which are similarly tightly linked to phenotype, as they more directly dictate them, being genes that exert an effect on the realisation of one phenotype or another) (Yoon et al., 2023). Thus, the modulatory mechanisms inspected and discussed in this thesis may reveal aspects of a system that are shared between this and other insect polyphenisms. For example, the suggested interplay between juvenile hormone and insulin signalling, which must be further explored (perhaps most importantly, by manipulating insulin signalling to functionally confirm its role in contributing to modulation), is likely shared by other examples of plasticity and polyphenisms where insulin signalling and juvenile hormone are both thought to be involved – for example, the caste polyphenism of honey bees (Wheeler, Buck & Evans, 2006; Kucharski et al., 2008; Sim & Denlinger, 2008; Wolschin, Mutti & Amdam, 2010; Nijhout & McKenna, 2018; Chen et al., 2023). Insect polyphenisms may commonly deploy these two systems and the interaction

between them, which would suggest some conservation and perhaps ancestral utilisation of these system. If this is demonstrated to be true (or inferred further, by the exploration of additional insect polyphenisms), then a major unifier of examples of plasticity in insects will have been revealed, and the molecular underpinning of said plasticity will be better understood. Better understanding plasticity and its underpinnings will contribute to our ability to assess the potential for plasticity of individual or groups of organisms, which will likely be increasingly important as climate change advances (Bonamour et al., 2019). Understanding the capacity of particular species or groups (either directly or by inference based on shared evolutionary history/traits) may aid prediction of abundance increases in organisms that might pose threats to, for example, food security; i.e. species that display a large capacity for plasticity may be favoured under climate change, some of which may be problematic species or their increase may be problematic, and outperform other beneficial species, leading to exacerbation of the problem (Skendžić et al., 2021; Schneider, Rebetez & Rasmann, 2022). Aphids are, as a group, clearly highly plastic organisms (Srinivasan & Brisson, 2012; Yan, Wang & Shen, 2020), which may mean they will be 'winners' under climate change scenarios. This might be exacerbated by the fact that the aphid reproductive polyphenism, and aphid development generally is temperature dependent (Lees, 1959; Kilian & Nielson, 1971; Dixon, 1987). Thus, significant increases in temperature may increase the prevalence of the asexual mode (outside of its current temporal range in holocyclic groups), leading to greater and longer (within year) pressure by aphids on plants and food systems. Novel pesticides that are able to manipulate reproductive mode will therefore be of great value, though further work will be required to deduce the exact mechanism by which temperature modulates the polyphenism, for this approach to work.

In addition to elucidation of the modulatory effect of temperature on the reproductive polyphenism, other lines of investigation that would present natural follow-ons from this work relate mainly to insulin signalling and characterisation of the nature and function of the two *dnmt3* paralogs. If insulin is the virginoparin, as has been suggested (Barberà, Cañas-Cañas & Martínez-Torres, 2019; Cuti et al., 2021), then it follows that manipulation of insulin signalling (either by increasing the stimulation of the pathway, i.e. by applying

insulin, upregulating its synthesis, or blocking its degradation, or blocking the pathway, i.e. by inhibiting insulin receptors, reducing synthesis, or increasing degradation (Pan et al., 2022; Brown et al., 2008; Sim & Denlinger, 2008)) may be able to divert aphids exposed to either LD or SD conditions, to phenotypes associated with the other, as I explored here for juvenile hormone signalling (using the JH analogue, methoprene). That application of methoprene alone is able to cause what would ordinarily be oviparous aphids to be viviparous instead, is suggestive of (if both insulin and JH signalling are ordinarily involved in the switch) either JH signalling being downstream of insulin signalling (such that exogenous JH bypasses the need for insulin signalling) or upstream (such that exogenous JH increases insulin signalling, perhaps JH affects insulin degradation in the haemolymph) (Leyria et al., 2022; Ling & Raikhel, 2021; Rauschenbach et al., 2017; Al Baki et al., 2019; Pan et al., 2022; Tu, Yin & Tatar, 2005). Alternatively, insulin signalling may not be contributing to the switch (Nässel, 1999), but instead be associated with it (no causal link between insulin signalling and the reproductive switch has been demonstrated), or the methoprene exposure was high enough that the activity of insulin was not needed, bypassing the need for signalling by both systems. One possible mechanism by which JH and insulin signalling work together, consistent with methoprene causing switching to viviparous production only some of the time, is that JH signalling is the major determinant at the ovaries, but insulin signalling modifies the sensitivity to its effect – though how this might occur is as yet unknown. Experiments that manipulate insulin signalling and assess effects on reproductive mode, and on expression of genes, principally JH related genes in the body of the mother generally and specifically, in her ovary, will contribute to this area. Furthermore, assessment of insulin signalling genes in the non-ovary tissues (and haemolymph) of aphids exposed to methoprene will paint a clearer picture of any effects on insulin signalling that JH signalling may have. And, as *dnmt3x* expression was elevated under SD conditions, but not affected by methoprene treatment, assessing *dnmt3x* as a candidate response element to insulin signalling will also be neccessary.

In addition to greater characterisation of some of the factors I have mentioned, further research should attempt to establish whether there is a link between signalling by biogenic amines (primarily dopamine, octopamine and serotonin)

and the reproductive polyphenism of aphids, as is known for other examples of plasticity in insects (Sasaki et al., 2012; Guo, Ma & Kang, 2013; Ma et al., 2015; Liu & Brisson, 2023), and taking into account that insulin synthesis in *D. melanogaster* is also affected by biogenic amines (Luo et al., 2012, 2014). Quantification of titres of biogenic amines in the heads (primarily, as the site of detection and integration of photoperiod and the start point for transduction of signal to effect the reproductive switch (Colizzi, Martínez-Torres & Helfrich-Förster, 2023)), bodies and ovaries (to assess for downstream involvement of biogenic amines (Knapp et al., 2022)), and of possible photoperiod responsive signalling by biogenic amines carried out by mature embryos; quantification of histamine, as a photoreceptor associated biogenic amine (Nässel, 1999), may be useful for determining the ability to embryos to directly perceive light through the cuticle of their mother) of LD and SD exposed aphids by HPLC (high performance liquid chromatography) or derived techniques (perhaps ultra-HPLC) will provide a powerful tool in determining the association between specific biogenic amines and the reproductive switch. Similarly, expression analysis and knockdown of genes related to particular biogenic amines (their synthesis or signalling) across the same tissues, e.g. receptors, enzymes, will be a further means of assessing involvement.

Following from chapter sixth, in which I used ACME dissociation and RNA-FISH HCR in a novel probe-seq protocol to isolate germ cells from the ovaries of sexuparae and virginoparae, isolation and expression profiling of further cell types in the ovary will greatly contribute to our understanding of the aphid reproductive polyphenism, likely more so, given the increased depth afforded by targeting specific cell populations, than single cell sequencing of whole ovaries. Though, single cell sequencing of isolated germ cells would provide greater resolution, as the population of germ cells explored in chapter 6 would have represented germ cells from embryos of different ages (Büning, 1985; Miura et al., 2003; Michalik et al., 2013), and would have themselves been slightly different in nature – nurse cells and prospective oocytes for example. Other cells in the ovary that may be relevant to the reproductive polyphenism are the follicular cells, which provide an alternate (to by means of the nurse cells and trophic cord) means of providing signal to developing embryos (Bermingham & Wilkinson, 2009). Thus, they may play important roles in mediating the

reproductive switch, being a point of potential information transfer from the mother to her embryos, and a later and longer lasting one than the contact with the nurse cells (Büning, 1985). Exploration of possible follicle cell marker genes, perhaps by targeting homologues of *D. melanogaster* follicle cell specific genes (Dong et al., 2023), by RNA-FISH HCR, or exploration by bulk single cell sequencing of the *A. pisum* ovary, would be necessary for targeting of these cells.

In summary, this work expands on our understanding of the molecular underpinnings of the aphid reproductive switch at the level of the ovary, primarily by highlighting the association between insulin signalling and the reproductive switch, and between insulin signalling and juvenile hormone, and revealing possible links between the activity of the DNA methylation machinery (which exists in an unusual configuration in the aphidomorpha) to this switch and reproduction in aphids generally.

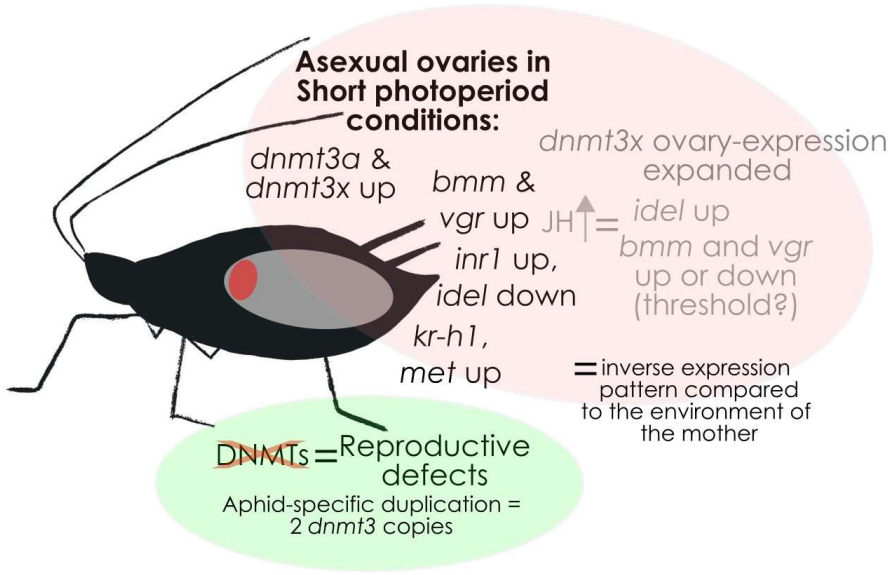

Figure 7.1 Key findings of this thesis.
In this thesis, in addition to exploration of two techniques (RNAi and a novel probe-seq method), the key findings were produced in chapters 4 (green overlay) and 5 (red overlay), in which I revealed important roles for DNMTs in reproduction, explored the Aphidomorpha specific duplication of *dnmt3*, and identified the expression pattern of both *dnmt3* paralogs in the ovaries; and, identified several genes upregulated (or downregulated in the case of an insulin degrading enzyme) in the ovaries of asexual aphids that would go on to produce sexual offspring (relating to insulin and JH and downstream genes, as well as the two *dnmt3* paralogs), representing expression patterns opposite to what is expected to occur in the adults bearing these ovaries, I also linked the expression of some of these genes to JH signalling.

Appendix A

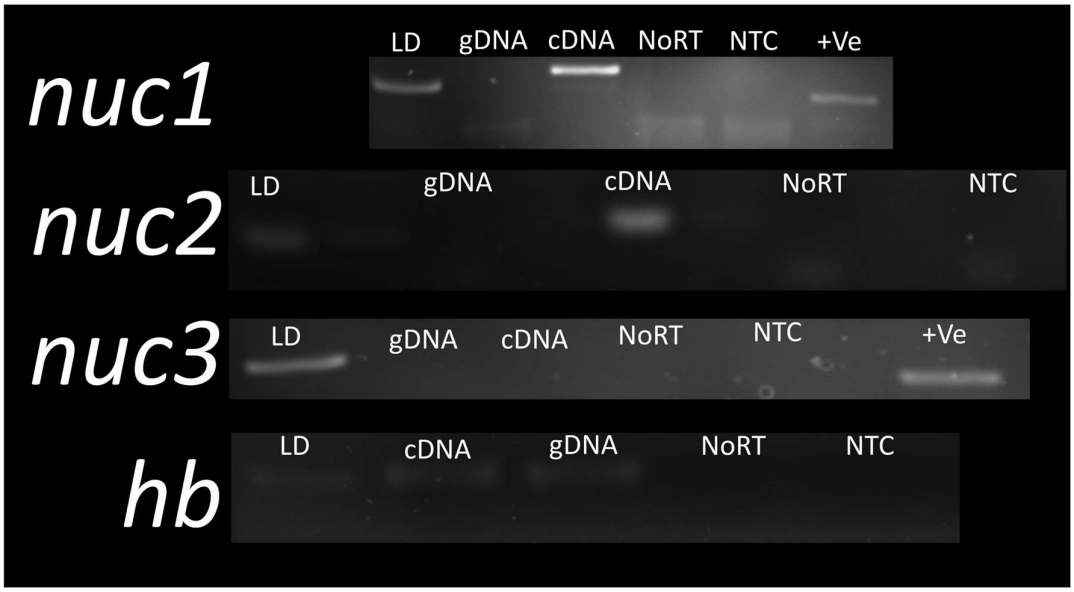

Figure A.1. Confirmation PCR of cDNA synthesis and RT-qPCR primer design of three non-specific endonucleases (*nuc1*, *nuc2*, *nuc3*) and a terminal RNAi target gene, *hunchback* (*hb*) in *Acyrthosiphon pisum* whole body samples.

LD = DNA ladder, 100 bp band; gDNA = genomic DNA sample with target specific primers; cDNA = cDNA sample with target specific primers; NoRT = no reverse transcriptase cDNA synthesis reaction sample; NTC = no template, negative, control sample; +Ve = cDNA sample with primers against *tubulin*, a positive control gene.

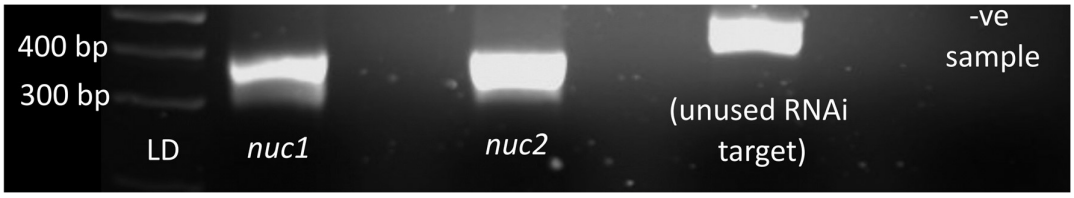

Figure A.2. Template primer product for T7 dsRNA synthesis. PCR reactions using cDNA generated from total RNA of *A. pisum* virginoparae and primers specific to targets, with T7 promoters appended on to their 5' ends, were used to generate template for synthesis of ds*nuc1* and ds*nuc2*. The -ve sample functions as a no-template control.

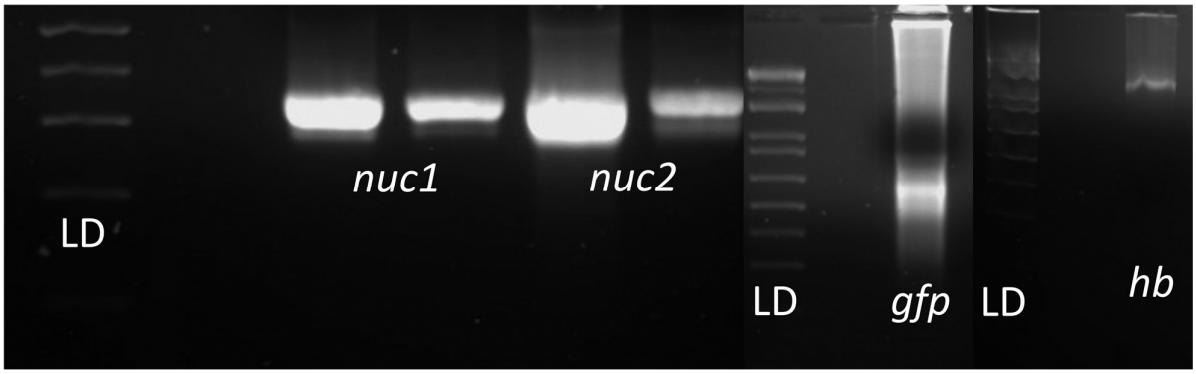

Figure A.3. dsRNA (1/10 dilutions) post clean-up, corresponding to *nuc1* and *nuc2*, *gfp*, and *hb*. DNA ladder (1kb+, Invitrogen), LD, is included for reference. Varying degrees of smearing appear as a result of degradation and/or secondary structure, but strong bands corresponding to the target sequence (roughly sized based on DNA ladder) are present.

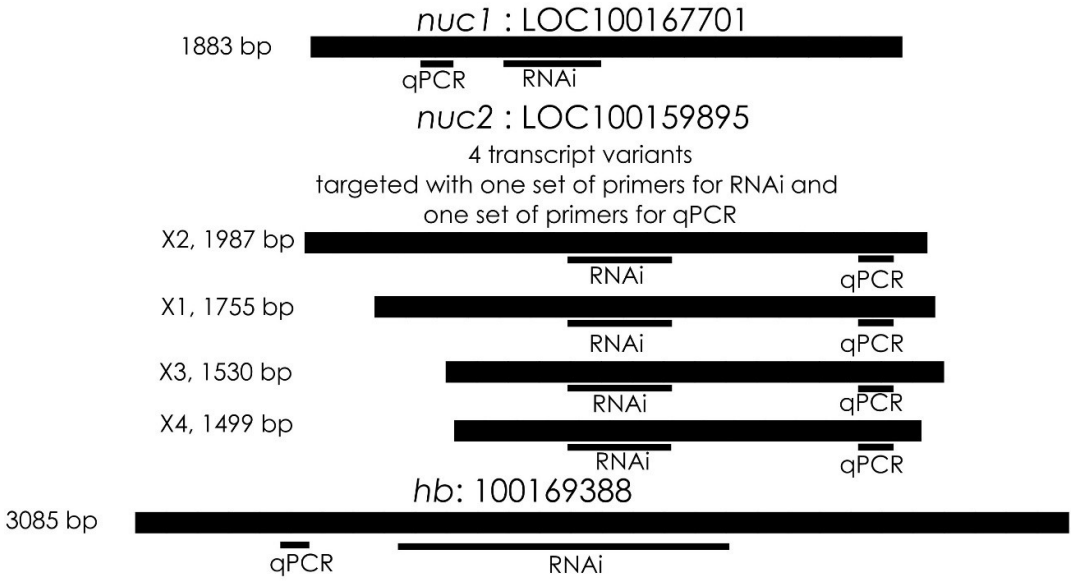

Figure A.4. Visualisation of targets of RNAi. Locations of qPCR primer- and RNAi primer- based amplification are approximately visualised within the context of targeted mRNAs. *nuc2* corresponds to the four transcription variants, each targeted by the same pair of RNAi primers and qPCR primers.

Table A.1. Primers used for RT-qPCR (and RT-PCR) and to generate template for dsRNA synthesis by IVT. *, *nuc1* and *nuc2* RNAi template forward and reverse primers had T7 promoter sequences (TAATACGACTCACTATAGGG) appended on to their 5' ends, [a], *nuc2* RT-qPCR and RNAi template primers were designed to target regions conserved between the four transcript variants, [b], *nuc1* RNAi template primers were taken from Chung et al., 2018, [c], length does not include T7 sequence. Efficiency is based on RT-qPCR of serially diluted cDNA (1, 1:10, 1:100, 1:1000, 1:10000).

Function	Target	Forward	Reverse	Length (bp) [c]	Identifier	Efficiency
RT-qPCR	*nuc1*	TCATTTGTTCGTG TCTTCTTCC	CATTTTGGGCTC GTCTTTTT	113	LOC100167701	99.2
RT-qPCR	*nuc2*[a]	CATAACGCCTCAA TGGGAAT	GCTTCCAGCTTC TTGTCGT	99	LOC100159895	108.4
RT-qPCR	*nuc3*	ACCGCCTATGGG AGAGATTT	CCGAGATAGCGA GTGTTGGT	88	LOC107882808	na
RT-qPCR	*hb*	CGGCTCGACCTA CAAATGAC	ATTGGCTGGTGT TCGTTTTC	96	XM_008184541.3	100.3
RNAi template*	*nuc1*[b]	ACCTCCGAAGTGT TGGTCAC	TGTTGCCGTACA GCTCTTTG	328	LOC100167701	na
RNAi template*	*nuc2*[a]	CCGAAGGACATTT AGCAAGC	TTTTAAACGGGT CGTTGGAG	330	LOC100159895	na
RNAi template	Plasmid (M13)	GTAAAACGACGG CCAGT	CAGTATCGACAA AGGAC	na	Plasmid	na

Table A.2. Sequences of dsRNA template for ds*hb* and ds*egfp* and their plasmid of origination. Template was amplified from plasmids using M13 primers, and then used in IVT to synthesise dsRNA for RNAi.

Plasmid	Sequence	bp
ApHb_pLit28i	aaaagcacaagtgcaaacactgtgggctggagtgcacagaaaaggtgcagtactggaagcacattcgcactcacataaaacct gaacagttgctggagtgcccaaactgtgagttcgccaccgacctgaaacaccactacgaataccacctgctgaaccacacgggtg ccaagccgttcacgtgcccggactgcgactacaagtgcgtgagcaagtcgatgctccaatcacacctcaagtcgcattcgaacgtg ttccagttccagtgttacgactgtggttacgcatctaagtacatgcacagcctcaagcagcacctgaagaagcgcgaccaccggcc ggccacgccgctcaacccagacggcacgccgaacccggacatcgtcatcgacgtggtgggcaaccgacgcgggccgcggca gaacaagaaacaaaaccggcacaacccgtatcagcaacagcaccagcagcaacagctccagcggtctatgacggtgtgttcgt cgcaggacgacggcggcagcagtagcggcggccacccatcaccgtactcgatgcaacagctgttgcagatgcctGCTTCCG GATGCGCCCAAGTTACGTATCCGACCTCGGCCGAGTCGTTTATGTATTCGATGATCACTA AACGCTCCATGGAGATGGTTGATTACCAGGCCGCCGAATACATGGCCATCAAGAACAACA ACAATAATAACAACTCCTCGTCGGTCGATGATAAGATGGAAGTGCAACAGCACCACCAAC AATCACTACAACACCACCACAACAATCATCACCATAAACGCGGCCATCAGTACCATTCAGA TGACGTGTTGAAAGTGGAATTGGCTGGGCGTAGCGGTAGTGGCggtgaactgacgcccgaaccac atgtacctgtgttggcggtcgccgatccttcgcccccgaccgtcgttttggccatcgagacgggaccgttgaacttaagcagagactc agtggctcctcgcgccgcgggcagcagccggcgtaaggggatcgcgtgcaaactcgagcgaccggcaaccgaatcgcagcct aagtcggtgtaatcgaattcctgcagg	1088
eGFP_pLit28i	gtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagctggacggcgacgtaaacggccacaagttcagc gtgtctggcgagggcgagggcgatgccacctacggcaagctgaccctgaagttcatctgcaccaccggcaagctgcccgtgccct ggcccaccctcgtgaccaccctgacctacggcgtgcagtgcttcagccgctaccccgaccacatgaagcagcacgacttcttcaag tccgccatgcccgaaggctacgtccaggagcgcaccatcttcttcaaggacgacggcaactacaagacccgcgccgaggtgaa gttcgagggcgacaccctggtgaaccgcatcgagctgaagggcatcgacttcaaggaggacggcaacatcctggggcacaagc tggagtacaactacaacagccacaacgtctatatcatggccgacaagcagaagaacggcatcaaggcgaacttcaagatccgc cacaacatcgaggacggcagcgtgcagctcgccgaccactaccagcagaacaccccatcggcgacggccccgtgctgctgcc cgacaaccactacctgagcacccagtccgccctgagcaaagaccccaacgagaagcgcgatcacatggtcctgctggagttcgt gaccgccgccgggatcactctcggcatggacgagctgtacaagtaa	716

Appendix B

B.1. Artificial diet recipe. Aphid artificial diet was made by first combining 5 ml amino acid (see appendix B, table B.1 for composition), 0.1 ml mineral (11 mg FeCl3.6H2O, 2 mg CuCl2.4H2O, 4 mg MnCl2.6H2O, and 17 mg ZnSO4 dissolved in 10 ml ddH2O), and 0.5 ml vitamin (0.1 mg biotin, 5 mg pantothenate, 2 mg folic acid, 10 mg nicotinic acid, 2.5 mg pyridoxine, 2.5 mg thiamine, 50 mg choline, and 50 mg myo-inositol dissolved in 5 ml ddH20) sterile stock solutions. Then 3 ml sucrose stock solution (10 mg ascorbic acid, 1 mg citric acid, 1.7 g sucrose, and 20 mg MgSO4.7H2O dissolved in 3 ml total volume ddH2O) was added, followed by 115 mg K2PO4 dissolved in 1 ml ddH2O, and then the solution was made up to 10 ml with ddH2O. The pH was confirmed as being between 7.0 and 7.5, then solution was filter-sterilised stored at – 20 °C in single-use aliquots.

Table B.1. Amino acid composition for preparing aphid artificial diet.

Amino Acid	mg to give final amino acid concentration when dissolved in 50 ml ddH_2O
Alanine	50.38
Asparagine	213.9
Aspartate/Aspartic Acid	189.7
Cysteine	42.5
Glutamic Acid	123.6
Glutamine	241.1
Glycine	9.0
Proline	65.6
Serine	59.9
Tyrosine	10.9
Arginine	300.2
Histidine	182.4
Isoleucine	114.1
Leucine	114.1
Lysine	158.9
Methionine	42.5
Phenylalanine	47.1
Threonine	103.6
Tryptophan	58.2
Valine	101.9

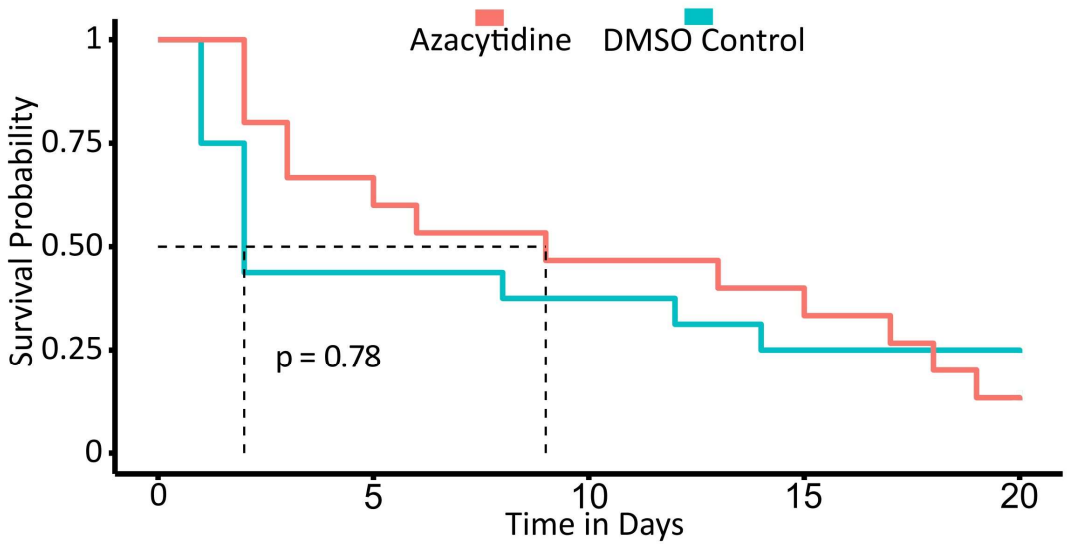

Figure B.1. Kaplan meier survival of LD maintained *A. pisum* individuals fed on an artificial diet containing either 50 µm 5-azacytidine in DMSO or an equal volume of DMSO as a control over twenty days, being fed for 6 days (fresh supplemented diet being provided on days 0 to 5), day 0 being the first day on which aphids were exposed to artificial diet. At this concentration, cox proportional hazards test indicated no significant difference in survival between azactydidine and DMSO treated groups.

Table B.2. Amino acid sequences of two paralogs of Dnmt3, Dnmt3a and Dnmt3x encoded in the *A. pisum* genome.

Protein	Amino Acid Sequence
Dnmt3a XP_016662566.1 DNA (cytosine-5)-methyltransferase 3C-like [Acyrthosiphon pisum]	MADNQSSDMEVDGNESTTVYSSKARTSELPWLRSSSRAKTSKLPRITSSSNPKPSKLPRLRVLSLFDGIGTGYYALLKLGFDIEVIYASEIDKDALMVTKYHFSDNIKQLGSVTEITTKMLDQIAPINLLF GGSPCSDLSGVNYRKKGLFDPKGTGILFYDYYRIWNYLSVKAARENTPFYWLYENVASMEIKNKDTISKFFECQPIVLDSLHFSPQRRKRYFWSSLPGITMLPHIQKTNYATNAPKLEDYLEKNLDR QANVEIVGTITSKRSCLQDSKSRNPVCQDGQYTGLFITEIEAIFGLPPHFTDVGDLSISSRQKLLGRAWSVQVIIDILDMLSGLFAKK
Dnmt3x XP_029348651.1 DNA (cytosine-5)-methyltransferase 3-like [Acyrthosiphon pisum]	MFIDAEKQQGQVVWAKMGKKSRLWAAIILSAKEMDLVYFEDKPGKTCVWWLGDDTMSQLQNDLIFEFRSNLKKGIADLIYARKDNFEQAIYILSLQFNFEFPKHGLVKWALVNLPIIMKGKNSR KEEFIIPDQFLKKITMVNNRIKRNIEENKVKNVESSPHGEKMKIDVSQISKMENKYCIACRFPSDELKIKHPLFKGLICNDCFINGKDIILSIAKDKSNDGCCVCLTQKDTLVLCGFCIRAYCNECLEFYC GQKALEKILIDNAWACIACSSTTLKISNLVPRSTKEQLRNIYMLYPANAKKDCAKEQYKIKEIHVLNLKDIQNNVPKVLQTLGFPFKILETSYLNDNNQDNNKSNAKLVVQYYPNRENYDKDKFFDR NDCLKKFFLFLLFKNKMQALQKDDNLFWAFETSAAIKVQEQKIISRFLEIEPIIIGMDTDDVQQRSRFIWTNITILNKDIRKFTNQNIYLHEIPKSIGRKSKKDKRNIDMPWCYTSIYIILSSFINNDIMF

Table B.3. Identities of proteins that were identified, by BLAST, to be homologous to *A. pisum* Dnmt3a (a subset of the proteins identified and included in phylogenetic analysis). Homologs with good % identity matches were present across a large diversity of the aphids, aphidomorpha (*D. vitifoliae*, e.g.), other hemipteran bugs (*B. tabaci*) and other insects (not shown).

Species	Protein ID	Homology to Dnmt3a	E-value
Myzus persicae	NCBI: XP_022177473.1 DNA (cytosine-5)-methyltransferase 3C-like [Myzus persicae]	230/274 (84% identity)	2.00E-175
Rhopalosiphum maidis	AphidBase: annotation_ogs1.0_gcf_003676215.2_asm367621v3_protein XP_026819840.1 DNA (cytosine-5)-methyltransferase 3C-like [Rhopalosiphum maidis]	230/314 (73% identity)	1.00E-174
Diurpahis noxia	NCBI: XP_015379624.1 PREDICTED: DNA (cytosine-5)-methyltransferase 3A-like [Diuraphis noxia]	232/275 (84% identity)	6.00E-178
Aphis gossypii	XP_027849764.1 DNA (cytosine-5)-methyltransferase 3C-like, partial [Aphis gossypii]	223/296 (75% identity	1.00E-167
Melanaphis sacchari	NCBI: XP_025191291.1 DNA (cytosine-5)-methyltransferase 3C-like [Melanaphis sacchari]	225/296 (76% identity)	8.00E-170
Myzus cerasi	AphidBase: annotation_v1.1_m.cerasi.v1.protein No good match		
Aphis glycines	NCBI: KAE9529082.1 hypothetical protein AGLY_012036 [Aphis glycines]	220/284 (77% identity)	6.00E-169
Rhopalosiphum padi	NCBI: XP_026819840.1 DNA (cytosine-5)-methyltransferase 3C-like [Rhopalosiphum maidis]	221/296 (75% identity)	2.00E-168
Cavariella salicola	No match	0	NA
Pterocomma pilosum	No match	0	NA
Aphis craccivora	NCBI: KAF0773215.1 DNA (cytosine-5)-methyltransferase 3C-like [Aphis craccivora]	215/274 (78% identity)	2.00E-166
Bemisia tabaci	**NCBI: ATN96644.2 DNA (cytosine-5)-methyltransferase 3 [Bemisia tabaci] (XP_018899094.1, XP_018899093.1, CAH0389569.1, XP_018899088.1 (all the same))**	**124/284 (44% identity)**	**9.00E-73**
Daktulosphaira vitifoliae	NCBI: XP_050533477.1 DNA (cytosine-5)-methyltransferase 3C-like [Daktulosphaira vitifoliae]	187/317 (59% identity)	8.00E-134

Table B.4. Identities of proteins that were identified, by BLAST, to be homologous to *A. pisum* Dnmt3x (a subset of the proteins identified and included in phylogenetic analysis). Homologs with good % identity matches were present across a large diversity of the aphids and aphidomorpha (*D. vitifoliae*, e.g.), but no detectable homolog was identified in the genomes of other hemiptera (for example, *B. tabaci*, a close relative of aphids) or other groups of insects (not shown).

Species	Protein ID	Homology to Dnmt3x	E-value
Myzus persicae	NCBI: XP_022171581.1 DNA (cytosine-5)-methyltransferase 3B-like isoform X1 [Myzus persicae] (isoforms X2, X3, X4 similarly good matches)	363/514 (71% identity)	0.00E+00
Rhopalosiphum padi	AphidBase: annotation_v2.0_rpadi_v2_pep g23824.t1	333/518 (64% identity)	0.00E+00
Aphis glycines	AphidBase: annotation_v1.0_ag_bt4_ogs1.0_20151030_proteins AG002968-PA unnamed protein product	340/517 (66% identity)	0.00E+00
Myzus cerasi	AphidBase: annotation_v1.1_m.cerasi.v1.protein Mca17618.t1-protein	73/105 (70% identity)	3.00E-37
Aphis gossypii	AphidBase: annotation_ogs1.0_gcf_004010815.1_asm401081v1_protein XP_027850238.1 uncharacterized protein LOC114129637 [Aphis gossypii]	153/221 (69% identity)	4.00E-100
Rhopalosiphum maidis	AphidBase: annotation_ogs1.0_gcf_003676215.2_asm367621v3_protein XP_026819896.1 DNA (cytosine-5)-methyltransferase 3C-like [Rhopalosiphum maidis]	343/517 (66% identity)	0.00E+00
Diuraphis noxia	NCBI: XP_015379612.1 PREDICTED: DNA (cytosine-5)-methyltransferase 3B-like [Diuraphis noxia]	41/60 (68.33% identity)	1.00E-23
Melanaphis sacchari	NCBI: XP_025198444.1 DNA (cytosine-5)-methyltransferase 3-like [Melanaphis sacchari]	134/184 (73% identity)	1.00E-74
Cavariella salicola	No match	0	NA
Pterocomma pilosum	No match	0	NA
Bemisia tabaci	**No match**	**5 low quality matches. The same accessions as for *dnmt3a***	**NA**
Daktulosphaira vitifoliae	**NCBI: XP_050534171.1 DNA (cytosine-5)-methyltransferase 3B-like isoform X1 [Daktulosphaira vitifoliae] (XP_050534172.1, XP_050534173.1 similar)**	**198/535 (37% identity)**	**1.00E-92**

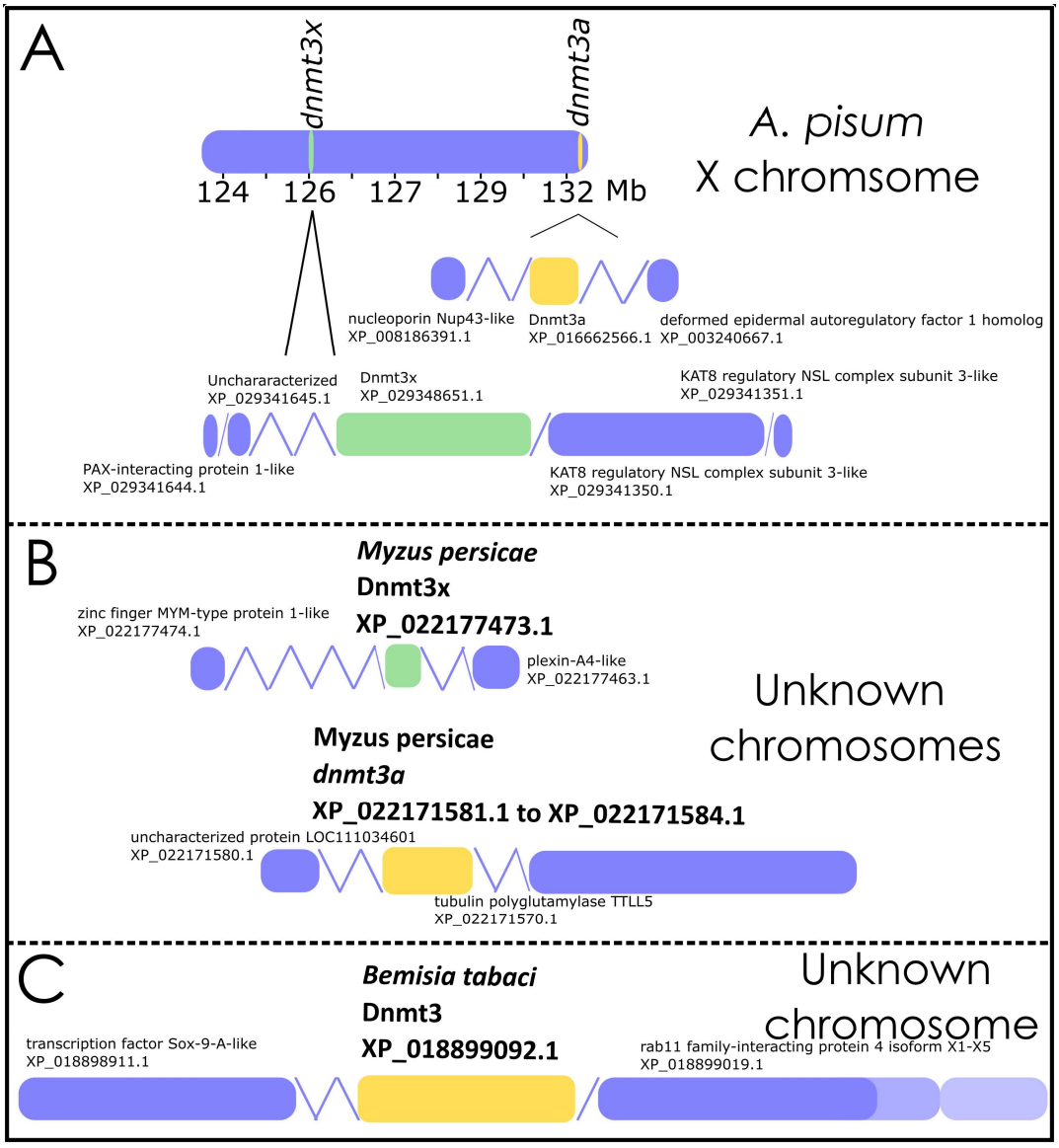

Figure B.2. Schematic representation of chromosomal placement and genetic context of Dnmt3 homologs, based on *A. pisum dnmt3a* and *dnmt3x*, in *A. pisum*, *M. persicae* and *B. tabaci*. A chromosome-level genome assembly for *A. pisum* reveals that *dnmt3a* and *dnmt3x* are positioned close together at the end of the X chromosome (which is just over 132 Mb) (A). The genes flanking each *A. pisum* duplicate are not conserved between them, nor are the flanking genes conserved between each *A. pisum* duplicate and its identified (by BLAST, with assessment of flanking genes using NCBI genome browser) orthologue in *M. persicae* (B) or *B. tabaci* (C). Notably *B. tabaci* has only a single copy of Dnmt3, bearing more homology to *A. pisum* Dnmt3a than *A. pisum* Dnmt3x. Chromosome-level genome assemblies of *M. persicae* and *B. tabaci* were not available to localise the orthologues to particular chromosomes.

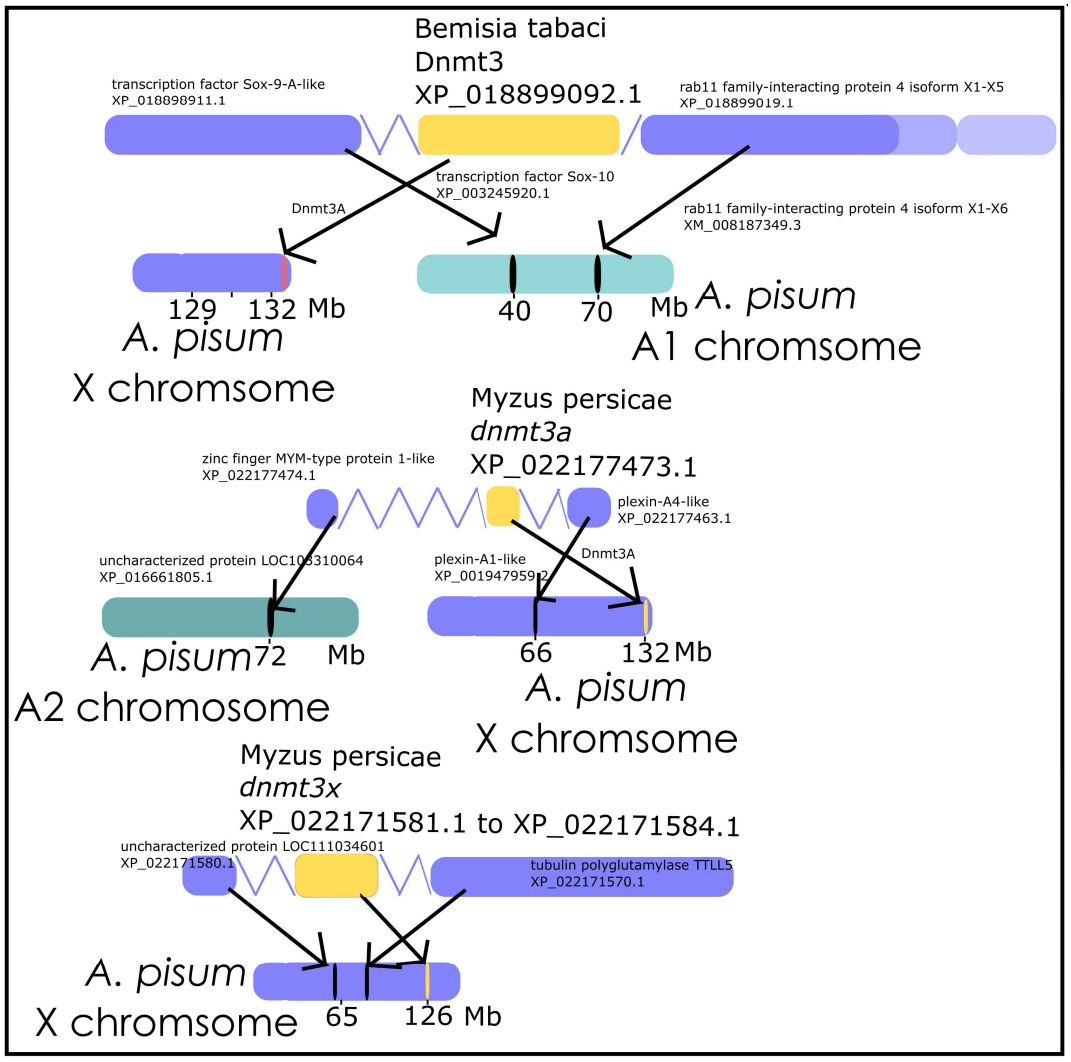

Figure B.3. Schematic representation of synteny based on *A. pisum dnmt3a* and *dnmt3x* and flanking genes, with mapping of orthologues of *A. pisum* Dnmt3x and/or Dnmt3a endoing genes and the genes directly flanking them (identified using NCBI genome browser) from *B. tabaci*, a non-aphid hemipteran 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.

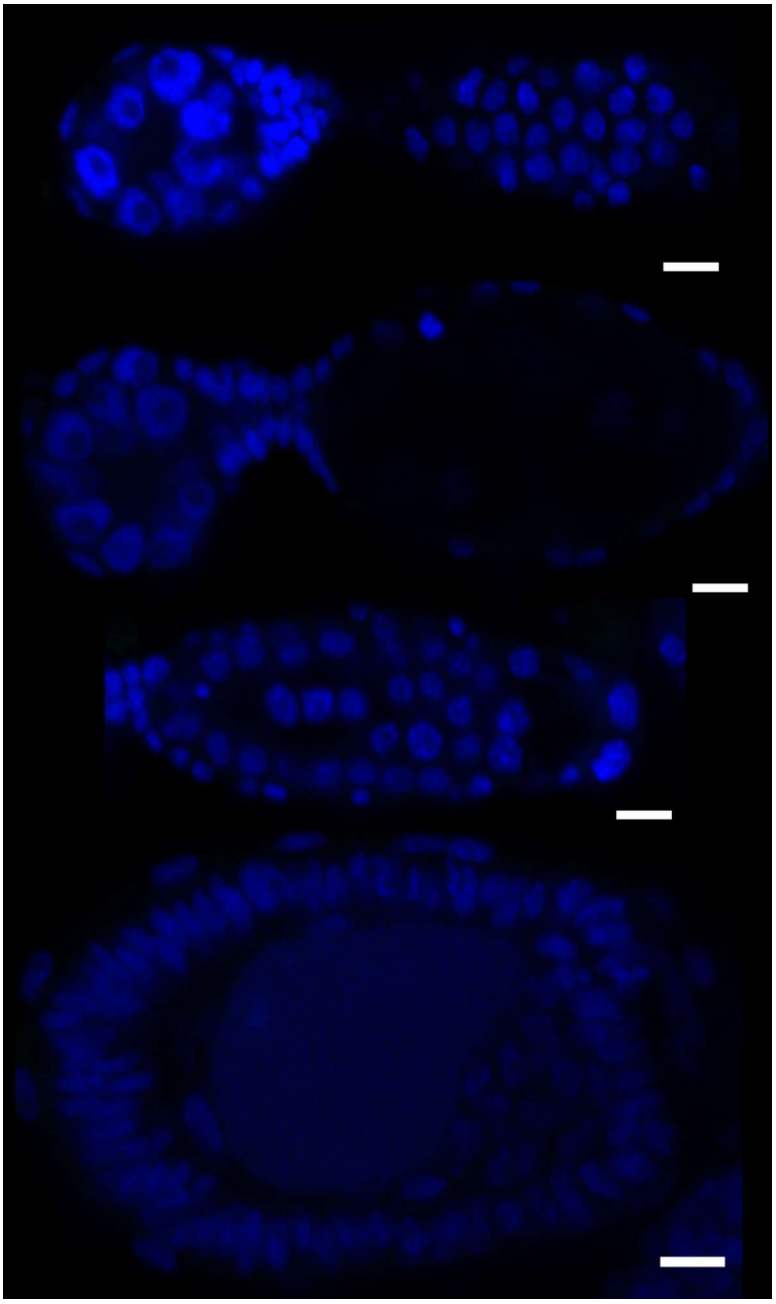

Figure B.4. *dnmt3a*/*dnmt3x* expression pattern RNA-FISH HCR probeless negative control images. Compound image of three channels (nuclei, DAPI, blue; *dnmt3a*/*dnmt3x*, green; *vasa*, red). Scale bars represent 10 μm.

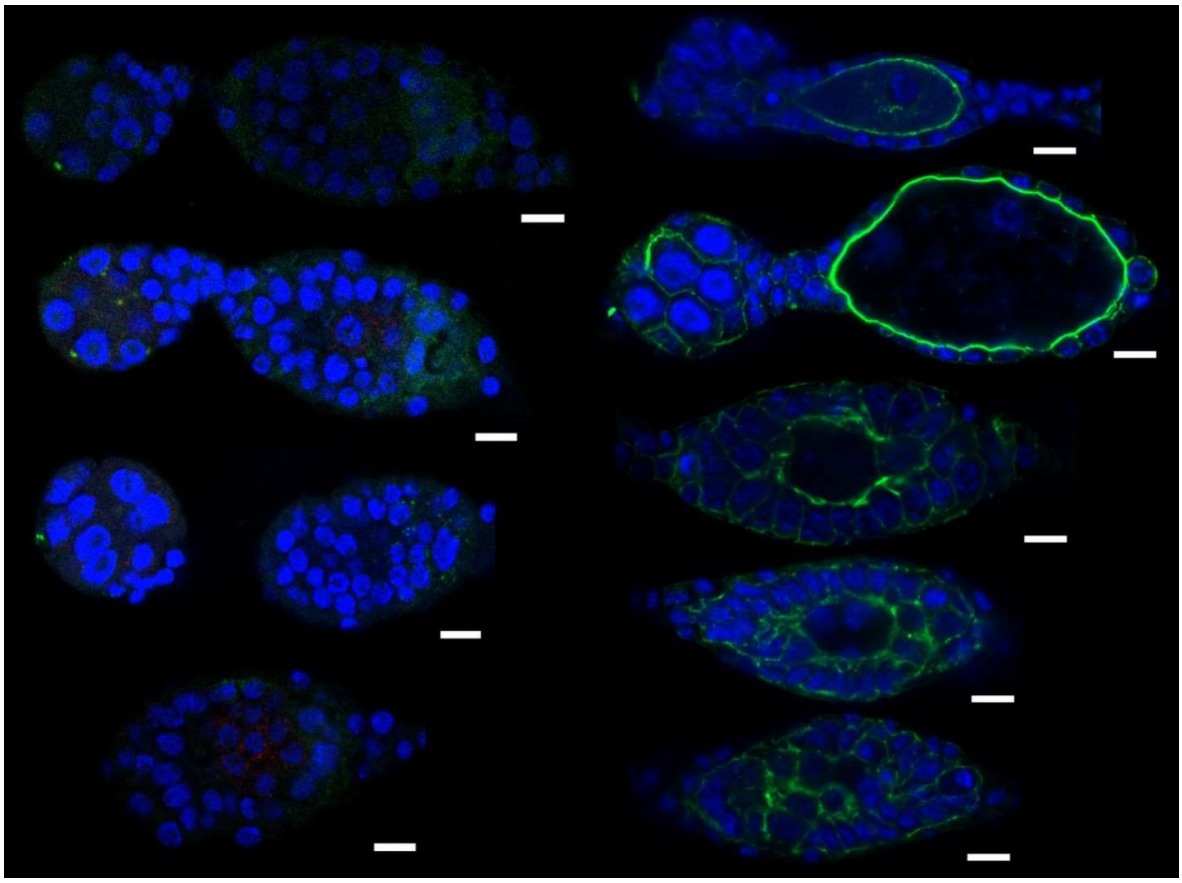

Figure B.5. 5-azacytidine experiment negative control images. Embryos, oocytes and germaria from DMSO control treated aphids. Left, RNA-FISH HCR visualisation of *vasa* (red) and *wg* (green), right, phalloidin (green) visualisation. Nuclei are also stained with DAPI (blue). Scale bars represent 10 μm.

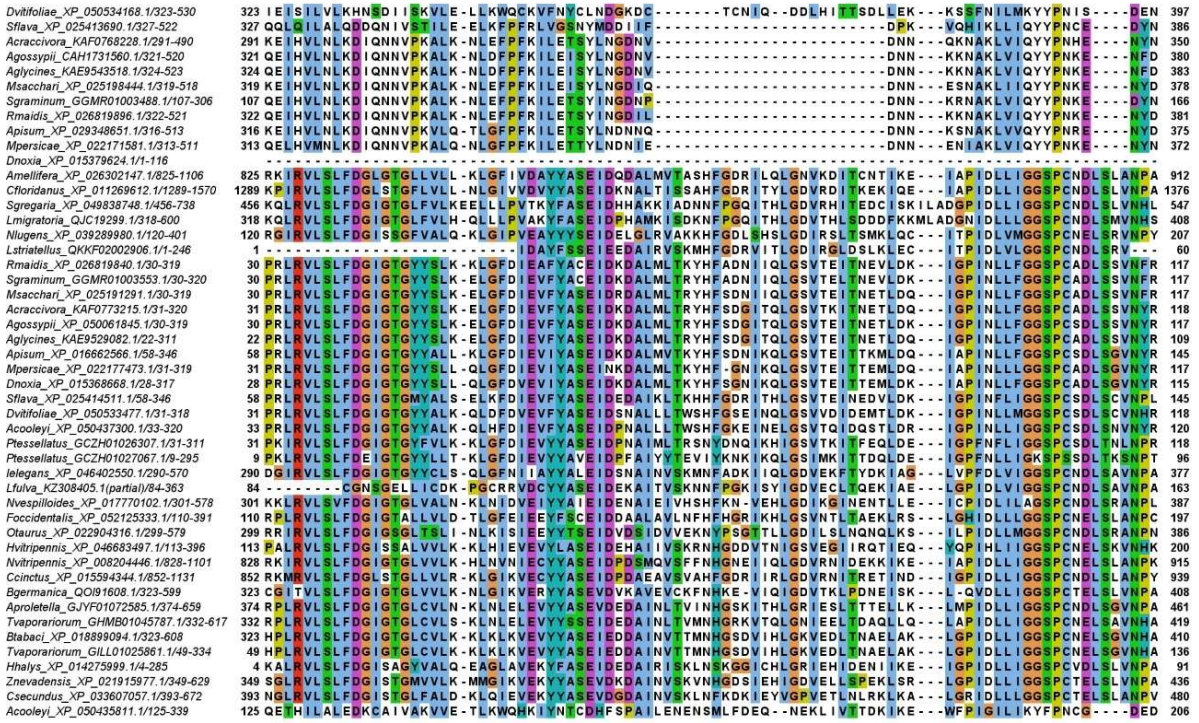

Figure B.6. Sequence alignment of inspected insect Dnmt3 proteins (1/4). Two major groups occur, encompassing proteins with homology to aphid *dnmt3a* (more ubiquitously), and proteins with homology to aphid *dnmt3x* (restricted).

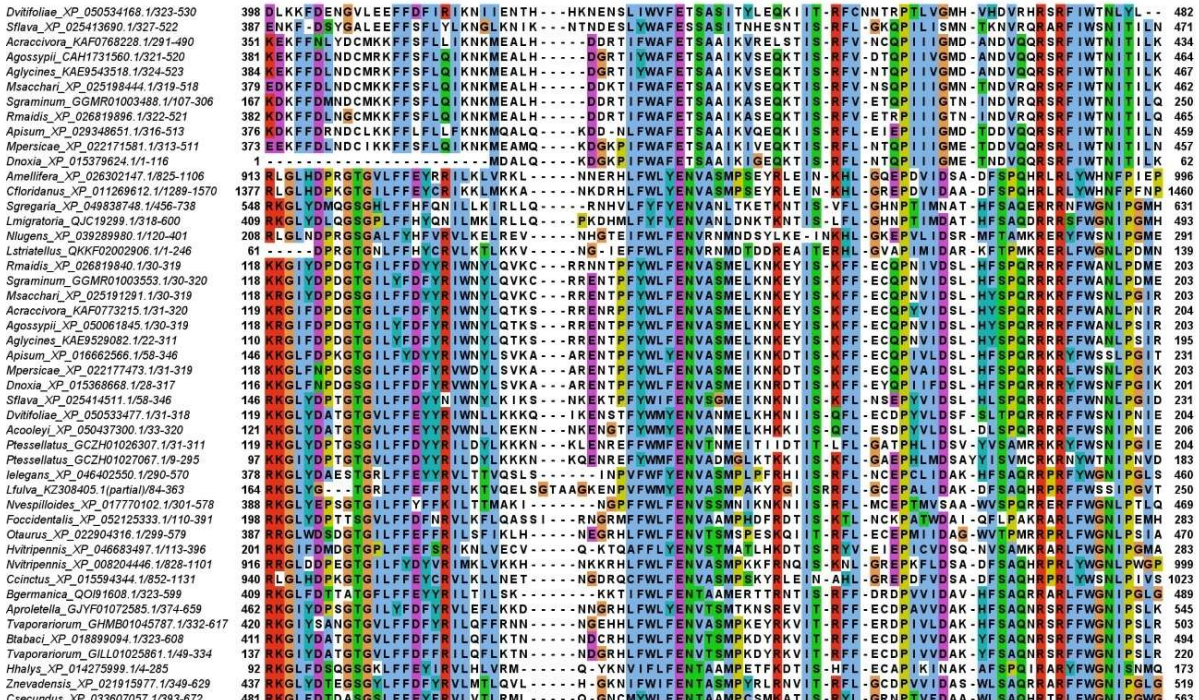

Figure B.7. Sequence alignment of inspected insect Dnmt3 proteins (2/4). Two major groups occur, encompassing proteins with homology to aphid *dnmt3a* (more ubiquitously), and proteins with homology to aphid *dnmt3x* (restricted).

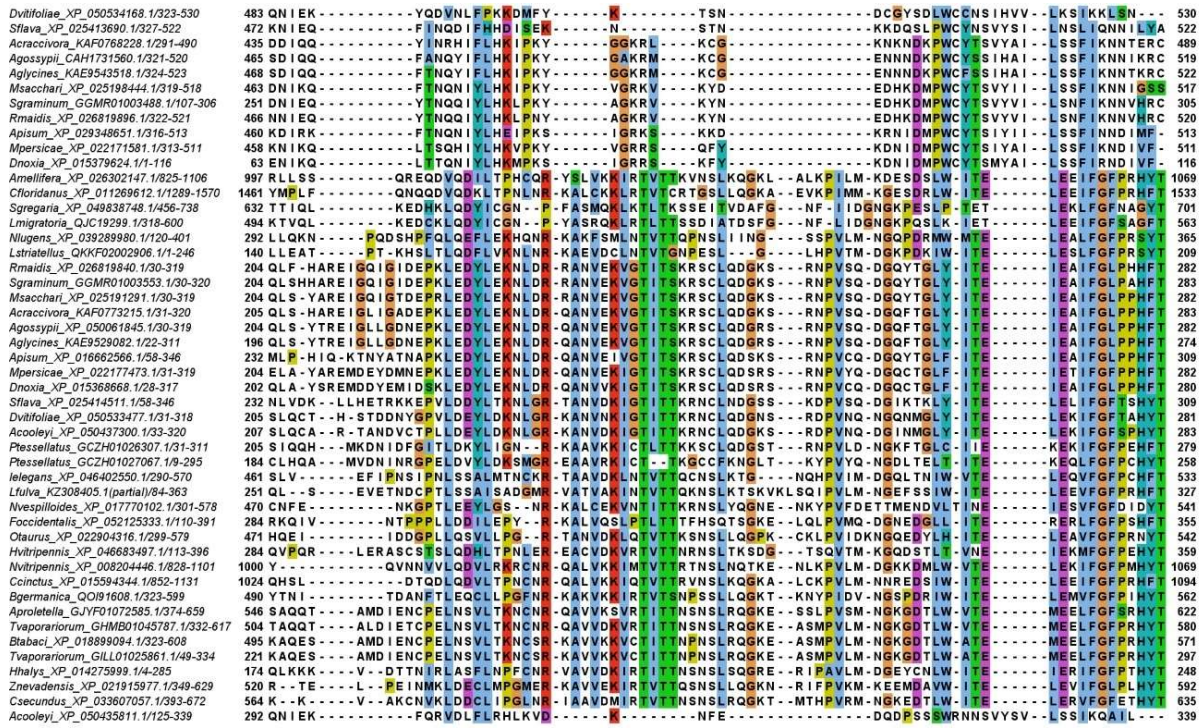

Figure B.8. Sequence alignment of inspected insect Dnmt3 proteins (3/4). Two major groups occur, encompassing proteins with homology to aphid *dnmt3a* (more ubiquitously), and proteins with homology to aphid *dnmt3x* (restricted).

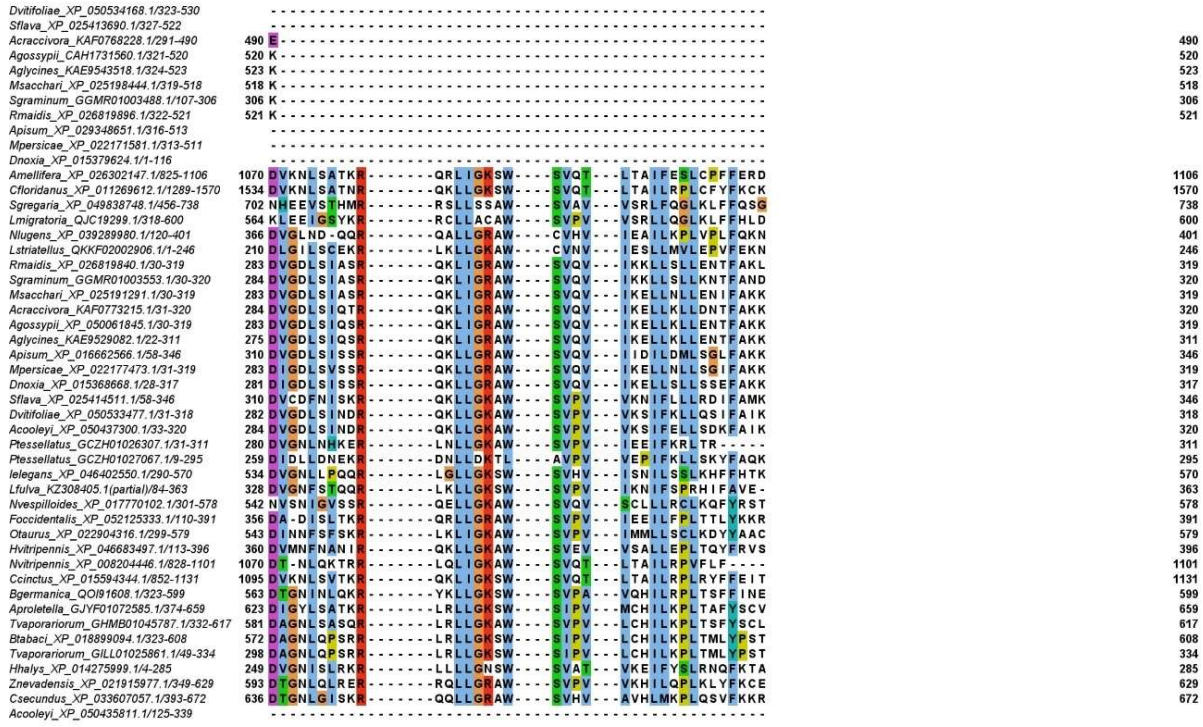

Figure B.9. Sequence alignment of inspected insect Dnmt3 proteins (4/4). Two major groups occur, encompassing proteins with homology to aphid *dnmt3a* (more ubiquitously), and proteins with homology to aphid *dnmt3x* (restricted).

Appendix C

Table C.1 Pairwise comparisons of survival, prior to sampling, in methoprene experiment.

Comparison	Estimate	SE	df	Z ratio	P value
Acetone-0.125 mg/ml methoprene	0.7771	0.132	Inf	5.881	<0.0001
Acetone – 0.25 mg/ml methoprene	0.8088	0.136	Inf	5.949	<0.0001
0.125 mg/ml methoprene – 0.25 mg/ml methoprene	0.0317	0.117	inf	0.272	0.96

Table C.2 Primers and associated targeted *A. pisum* genes used for RT-qPCR experiments comparing long-day and short-day condition aphid ovaries or methoprene and control treated aphid ovaries. Efficiencies were determined by RT-qPCR of serially diluted generic cDNA and computed using CFX manager.

Target	forward	reverse	efficiency	product size	target
foxo	GTCCGTCAGTCACGACCAAT	TTTCCCATAGAACACAGACATTTG	100.7	89	LOC100168097
inr1	AAGGATGAACAACAAATCCAA	TTGGTGAAGTATCACTTTTAGGG	100.0	97	LOC100168659
inr2	GCTTGACCAACATCACACGA	TATCGTTGCTCACTCCGTTG	104.4	108	LOC100169464
bmm	TTCTAGCAAGGAAGAAGTGATACAG	CAATCAAAGGTGGCACAAAT	111.1	83	LOC100162729
ilp1	ACCACCTTTCAACGAACCAC	TGATGACTAATGTCAAGAGGAGTGA	106.2	103	LOC100568938
ide	CCTCGTCAAATGGTTAGGTCA	AACACATGAACTGCTGTGATGA	99.1	96	LOC100167928
idel1	TGTATCCAGTGCCAAAGTCG	AAAATTCGTTTCTGCGTCGT	110.4	117	LOC100163575
dnmt3a	CTCCAGAGTCCAGATTTGTATTG	CTCATTGCCGTCTACTTCCA	106.3	106	LOC100572675
dnmt3x	AAAAAGACTGTGCCAAGGAA	TCTTGAACGGAAATCCTAGAG	95.5	114	LOC100568466
krh1	GCATTGACTACGCTCATCCC	CGATAGCCGTCTTTTGGTGC	94.4	109	LOC100165279
e75	TGCGAATCAACAGGAACAGG	CGAGCCTTTTCCCGTTTGG	92.6	112	LOC100166941
ddc	CACTTTTACCAACCGAGGCG	CCAATGAGTAACCCCTGGCA	84.6	95	LOC100168964
vasa1	AGACACGCACCCTTTCACG	GGCAACGCAAACAAACGGAA	99.2	117	LOC100144776
vg	ACTGCCAAGATGAGGGAAAGT	CGAATAGATGCCCAGCAAAGC	101.3	119	LOC100573020

vgr	ACTGTCATCCAAGTAATTGACCAGA	GACGATTTCACTTCCACAAGCA	99.4	120	LOC100163453
jhe1	ACTTTGTCAATGCAAATATTAATCCCA	CGCACCAAATACGAAACCGA	104.7	103	LOC103308679
jhe2	TCAATTAATGGGTATTGATTGGTCTT	CGCCAAATACAAACCCGAAA	104.1	114	LOC100166921
ilp4	ACGATCATCAGTTTACCACAATTCT	CCAAGAGGATTACACTCAAATACATAC	98.2	114	LOC100161832
brc	ACAATCACCGTCGTCCATACC	GCCGCCTGAAGATGGGTT	95.0	100	LOC100167818
met1 (met)	CACACAACACCGATGAACACAATA	TCTGGTCCCATGAACAAAGAGT	93.1	112	LOC100573780
met2 (clock)	CCTCCATGCATCAGAAACTGT	AACGAATGATCTGCTTCATGAATT	91.2	106	ACYPI004812
lsd1	CGTGGATCATGTGGTCGGAT	TGCGGCGTTCTAGTCAACA	111.5	120	LOC100574423
cyclinj	TGGTGGGAAACAGAGTACAGTG	AACAACACGGGTCGCAAATG	94.8	116	LOC100165266
tub	GTCAGTGCGGAAACCAAATC	GTAGGCTCCAGTTGGGTCAA	105.4	80	ACYPI008874
ef1a	CGTTGCTGTTGGTGTCATTA	CGCCTATTCATTTCTTCTTCTG	104.2	101	LOC100163340
rpl7	GCACGGAAGATTTAGTTCATTG	TTACGCCAGCCTCCAGTT	108.6	109	ACYPI010200

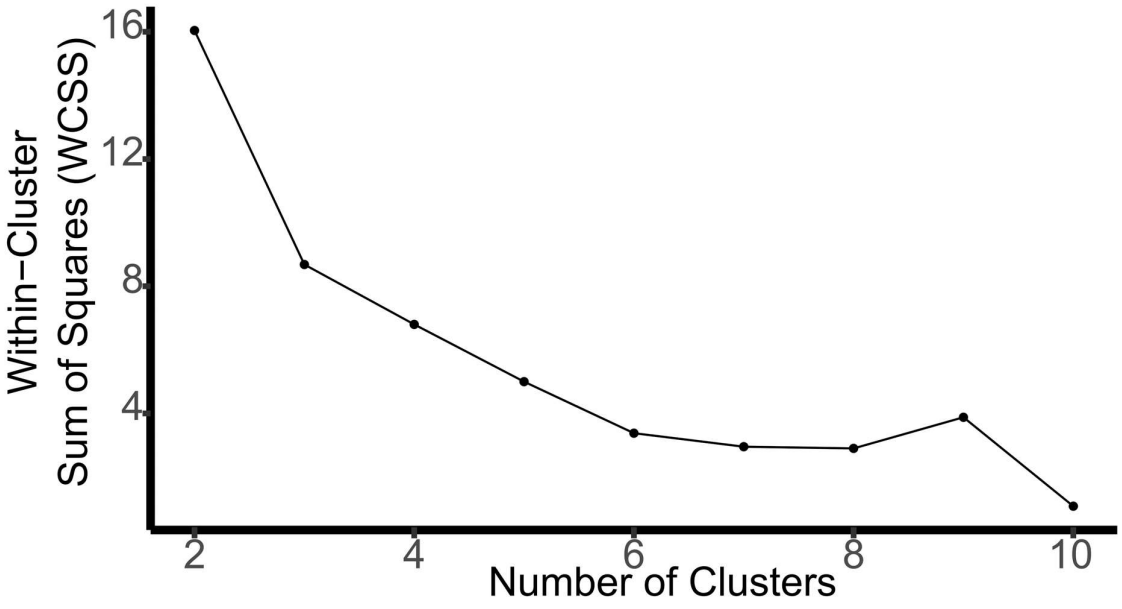

Figure C.1. Elbow plot for determination of clusters required to represent the variation in the data most effectively, of clusters produced by K++ means cluster analysis of methoprene treated (0.125 mg/ml) and acetone control treated *A. pisum* ovaries. 3 clusters, at which point the within-cluster sum of squares begins to show a less steep decline (forming an elbow in the plot), presents the optimal number of clusters. 3 clusters was thus used for further analysis of methoprene and acetone treated samples.

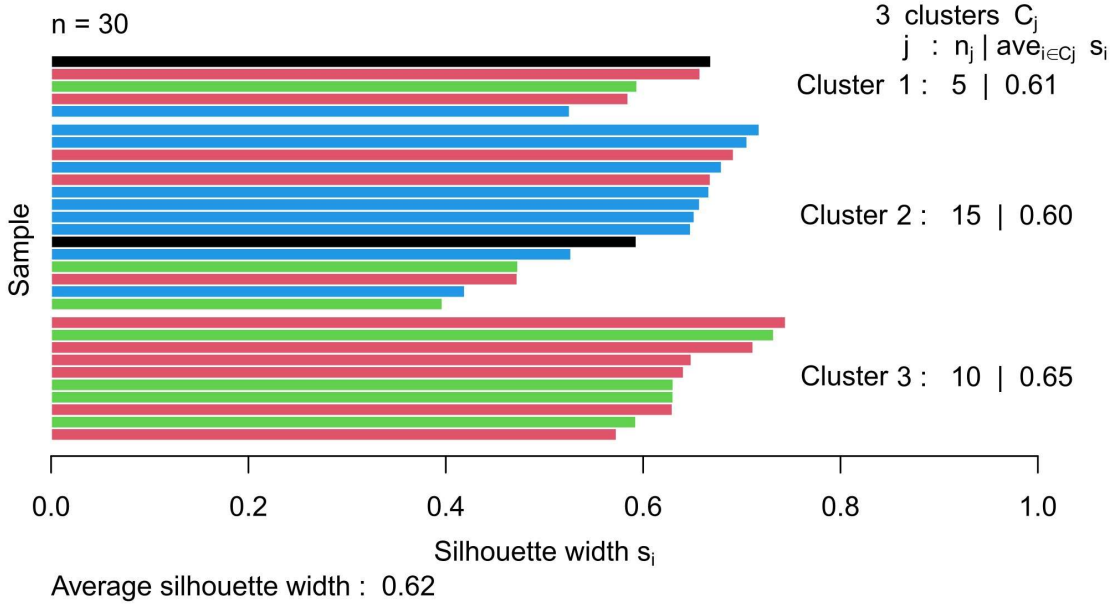

Figure C.2. Silhouette analysis of clusters formed by K++ means clustering of ovary samples from methoprene (0.125 mg/ml) and acetone treated *A. pisum* maintained in SD conditions. The elbow method identified 3 clusters as being appropriate, and the silhouete widths (which, on average for each cluster is 60=<) confirm this.

Table C.3. GeNorm2 determined stability of reference genes and validation of reference gene number for LD/SD switch experiment relative expression.

Gene	M
rpl7	0.2864934
ef1α	0.1963943
tub	0.1751604
Pairwise V2/V3 value	
0.006555372	

Table C.4. GeNorm2 determined stability of reference genes and validation of reference gene number for methoprene experiment relative expression.

Gene	M
rpl7	0.6892112
ef1α	0.4889308
tub	0.4259546
Pairwise V2/V3 value	
0.01559554	

Table C.5. LD/SD experiment relative expression contrasts. Summary table of results of contrasts (LRT) of GLMs fitted with quasipoisson distributions and log link functions, comparing relative expression for the indicated GOI based on condition (LD, long-day or SD, short-day) for *A. pisum* ovaries, with the full models being compared to their null models.

Goi	DF1	Df2	Chi2 (deviance)	p
dnmt3x	1	11	8.6215	2.2e-16
krh1	1	11	0.76691	0.006768
inr1	1	11	0.8453	2.757e-08
bmm	1	11	9.0318	1.39e-12
idel1	1	11	2.416	0.000621
jhe1	1	11	0.052764	0.5159
jhe2	1	11	0.19495	0.167
vgr	1	11	31.783	2.2e-16
clock	1	11	0.14286	0.1588
met	1	11	0.43039	0.04642
ddc	1	11	0.010537	0.8567
dnmt3a	1	11	0.2466	0.00601

foxo	1	11	0.0669	0.2746
ide	1	11	0.039576	0.3151
inr2	1	11	0.029887	0.3657
vasa	1	11	0.11231	0.1683
vg	1	11	0.019906	0.5296
e75	1	11	0.047845	0.5432
ilp1	1	11	0.0082459	0.7482

Table C.6. Methoprene experiment relative expression contrasts by treatment. Summary table of results of contrasts (LRT) of GLMs fitted with quasipoisson distributions and log link functions, comparing relative expression for the indicated GOI based on treatment (0.125 mg/ml methoprene or acetone) for *A. pisum* ovaries, with the full models being compared to their null models.

Goi	DF1	Df2	Chi2 (deviance)	p
dnmt3x	1	28	0.1411	0.2244
krh1	1	28	0.10104	0.1435
inr1	1	28	0.21997	0.1558
bmm	1	28	0.20181	0.3527
met	1	28	0.061101	0.07109
clock	1	28	0.0013506	0.8059
idel1	1	28	5.4761	0.004696
ihe1	1	28	0.11237	0.5328
jhe2	1	28	0.00080126	0.9529
vgr	1	28	0.25996	0.4497

Table C.7. Methoprene experiment relative expression contrasts by cluster. Summary table of results of contrasts (LRT) of GLMs fitted with quasipoisson distributions and log link functions, comparing relative expression for the indicated GOI based on cluster for *A. pisum* ovaries, with the full models being compared to their null models. Samples forming clusters included either or both of methoprene (0.125 mg/ml) and acetone treated individual ovary samples. Clusters were formed by k++ means clustering based on relative expression of an asexual specific marker, *cyclinJ*, and a sexual specific marker, *lsd*, resulting in 3 clusters that were characterised by different inferred reproductive outputs.

Goi	Df1	Df2	Chi2 (deviance)	P
dnmt3x	1	27	0.1664	0.444
krh1	1	27	0.028671	0.759
inr1	1	27	0.4257	0.1205
bmm	1	27	2.8725	8.306e-05
met	1	27	0.037056	0.4002
clock	1	27	0.043103	0.3626
idel1	1	27	7.61	0.001833
jhe1	1	27	0.42533	0.4706
jhe2	1	27	0.075918	0.8471
vgr	1	27	6.8751	4.003e-07

Table C.8. Methoprene experiment pairwise relative expression comparisons by cluster. Summary table of results of pairwise comparisons of relative expression for the indicated GOI between each of three clusters of methoprene (0.125 mg/ml) and acetone treated ovary samples, where statistical significance was detected previously by GLM. Clusters were formed by k++ means clustering based on relative expression of an asexual specific marker, *cyclinJ*, and a sexual specific marker, *lsd*, resulting in 3 clusters that were characterised by different inferred reproductive outputs. Cluster 2 was characterised by low *cyclinJ* and high *lsd* expression, and contained acetone and methoprene treated samples, and is inferred to represent sexual offspring producers. Cluster 3 was characterised by high *cyclinJ* and low *lsd* expression, and contained methoprene treated samples only, being inferred to represent asexual offspring producers. While cluster 1 was characterised by low *cyclinJ* expression and expression of *lsd* that was higher than observed for cluster 2 and included only methoprene samples, inferred to represent enhanced sexual production.

Goi	Pairwise	Estimate	SE	Z ratio	P
vgr	1-2	0.671	0.196	3.421	0.0018
vgr	1-3	1.375	0.263	5.224	<0.0001
vgr	2-3	0.704	0.247	2.848	0.0122
bmm	1-2	0.614	0.16	3.827	0.0004
bmm	1-3	0.753	0.181	4.157	0.0001
bmm	2-3	0.139	0.163	0.852	0.6704
idel1	1-2	0.81	0.273	2.969	0.0084
idel1	1-3	0.107	0.257	0.416	0.9090
idel1	2-3	0.703	0.236	2.982	0.0081

Table C.9 Methoprene experiment pairwise relative expression comparisons by cluster. *lsd* and *cyclinj* relative expression compared between three clusters formed based on K++ means clustering based on expression of the same genes.

Goi	Pairwise	Estimate	SE	Z value	P
lsd	2-1	0.8561	0.1741	-4.916	<0.001
lsd	3-1	1.398	0.2266	-6.168	<0.001
lsd	3-2	0.5419	0.2184	-2.481	0.0343
cyclinj	2-1	-0.19113	0.12762	-1.498	0.286
cyclinj	3-1	0.11958	0.11958	6.414	<0.0001
cyclinj	3-2	0.08589	0.08589	11.156	<0.0001

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