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RESEARCH

Molecular and mitochondrial biology of eusocial bees: responses to stress and xenobiotics

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy (PhD)

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Author declaration

I declare that this thesis is a presentation of original work, and I am the sole author. All sources and work which is not my own are acknowledged as references. This work has not been previously submitted for an award of a degree at this, or any other, university.

Chloe Sargent

Abstract

Bees are one of the most important pollinators and provide substantial economic value via their pollination services. Over recent decades the decline in pollinator diversity and abundance has been of great concern. The cause of these declines has been attributed to a combination of factors including habitat loss, agriculture intensification and pesticide use, climate change, and disease. Hence, it is important to elucidate how these stressors may affect the physiology and molecular biology of bees and thereby help to establish potential stress-biomarkers to monitor and mitigate negative environmental effects. This thesis aims to investigate the effects that environmental stressors and xenobiotics have on mitochondrial function and epigenetic modifications in the eusocial bees, *Bombus terrestris* and *Apis mellifera*. Using high-resolution respirometry and tethered flight mills this thesis shows how mitochondrial function and flight performance are affected by chemical stressors. Furthermore, it is demonstrated that bumblebees can be used as effective model organisms for research and development of pharmaceutical drugs. To detect the epigenetic modification, N^6-methyladenine (6mA) methylation, in the DNA of bees, the use of techniques including Oxford Nanopore sequencing and a new approach termed the immuno-southern (iSouthern) are also demonstrated.

This thesis shows that mitochondrial function in response to the neonicotinoid insecticide, imidacloprid, is tissue-specific; this highlights the necessity to examine tissue-specific effects of insecticides on mitochondrial function. It is further demonstrated that xenobiotics which target neuronal function alter the mitochondrial function and flight velocity in bumblebees. Finally, it is concluded that 6mA methylation in DNA may be present in eusocial bees and is associated with the presence of an endosymbiont bacteria from the genus *Arsenophonus*.

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I would like to thank my secondary supervisor Emyr Davies for his support and Rothamsted for providing the flight mills which allowed a fundamental part of the third chapter to go ahead. Also, for bringing back past trauma from my MSc project of gluing tiny metal tags to bees. I would also like to thank Ian Hardy, my supervisor during my first year. Although short and sweet his continuous support throughout has been most appreciated. I would also like to thank Ian for his generosity in providing the funding which allowed me to attend the International Congress of Entomology in Helsinki which was one of the highlights of my PhD.

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To all my friends and family, I know most of you have no idea what I have been doing these past four years (except that I work with bees), but nevertheless, I hope you are proud and know that I couldn't have done it without you. To my parents, for your words of affirmation and making me believe I could achieve whatever I set my mind to, but mainly for the never-ending support you have both given me throughout the years. To my grandad, your words on the value on education and working hard will forever stay with me. I hope you are looking down with a smile. This journey was, and always has been, because of you.

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Covid Impact Statement

I began my PhD in October 2019, immediately prior to the beginning of the covid-19 pandemic. On 23rd March 2020 the government announced lockdown restrictions to which all lab work at the university was suspended. In the autumn of 2020, the university began a phased return to lab work with limited access to the labs and final year students taking priority. This meant unfortunately my research was severely hindered for the first year and half of my PhD. I was fortunate that the university was able to provide me with a six-month extension, however this was not able to make up for the total amount of time I had lost due to the pandemic and meant I was unable to conduct as much research as I would have liked. Besides the physical limitations of not having the facilities to conduct my lab work, the mental toll that the pandemic had on myself was also extremely difficult and challenging. In lockdown and separated from all my friends and family it was difficult to stay motivated and in a positive mindset. However, despite the challenges of the pandemic I would like to say the last few years of my PhD (post-covid) were some of the most enjoyable and I am incredibly grateful for all the amazing people I had the privilege of working alongside.

Abbreviations

ATP – adenosine triphosphate

ADP – adenosine diphosphate

DA – dopamine

DNA – deoxyribonucleic acid

DNMT – DNA methyltransferase

ECL – enhanced chemiluminescence

ETC – electron transport chain

ETS – electron transport system

FCR – flux control ratio

F1,6BP – fructose-1,6-bisphosphate

F6P – fructose-6-phosphate

GA3P – glyceraldehyde-3-phosphate

GPDH – glycerol-3-phosphate dehydrogenase

G3P – glycerol-3-phosphate

HK – hexokinase

HRP – horseradish peroxidase

IMD - imidacloprid

IMM – inner mitochondrial membrane

IMS – intermembrane space

MAS – malate aspartate shuttle

MBP – methyl-binding proteins

MTase – DNA methyltransferase

mtDNA – mitochondrial DNA

NA - noradrenaline

NAD^+ – nicotinamide adenine dinucleotide

NGS – next-generation sequencing

OXPHOS – oxidative phosphorylation

PFK-1 – phosphofructokinase-1

RNA – ribonucleic acid

ROS – reactive oxygen species

ROX – residual oxygen consumption

SRC – spare respiratory capacity

TCA cycle – tricarboxylic acid cycle

Tet – Ten-eleven translocase

TGS – third-generation sequencing

5-HT – serotonin

5mC – 5-methylcytosine

6mA – N^6-methyladenine

Publications

List of published manuscripts that I have authored or co-authored during my PhD. Publication 2 is not included in this thesis.

1. **Sargent, C**., Ebanks, B., Hardy, I. C. W., Davies, T. G. E., Chakrabarti, L., & Stöger, R. (2021). Acute Imidacloprid Exposure Alters Mitochondrial Function in Bumblebee Flight Muscle and Brain. Frontiers in Insect Science, 1(December), 1–8. https://doi.org/10.3389/finsc.2021.765179

2. Ebanks, B., Wang, Y., Katyal, G., **Sargent, C**., Ingram, T. L., Bowman, A., Moisoi, N., & Chakrabarti, L. (2021). Exercising D. melanogaster Modulates the Mitochondrial Proteome and Physiology. The Effect on Lifespan Depends upon Age and Sex. International Journal of Molecular Sciences, 22(21), 11606. https://doi.org/10.3390/ijms222111606

1. Introduction

1.1 Melittology (Bees)

1.1.1 The bee

If you are to look up the definition of a bee in the dictionary you would find it often described as a black and yellow insect that makes honey and can sting. These definitions, along with what most would perceive a bee to be, are tailored around the honeybee. Although not incorrect, the majority of bee species do not produce honey, are a variety of colours, and are solitary in nature. There is only one genus which makes honey, *Apis*, containing around nine species, out of the >20,000 species of bee found worldwide. [1-4] Despite arguably the most well-known bee species displaying eusociality, approximately only around one in ten of all bee species are eusocial and have large complex societies. [5,6]

There are seven families of bees belonging to the superfamily of Apiodea in the Hymenopteran order of insects. These families are: Melittidae, Apidae, Megachilidae, Andrenidae, Halictidae, Stenotritidae and Colletidae (**Figure 1.1**). Apidae is the largest family which contains three subfamilies, Nomadinae, Apinae and Xylocopinae. Nomadinae are commonly known as cuckoo bees; they are kleptoparasites, also known as brood parasites, who invade host species nests to lay their eggs.[5] Rather than building their own nests and resources the Nomdinae utilise food provisions provided for the host offspring.[5] Xylocopinae contain the bees commonly known as carpenter bees, whilst Apinae, the largest of the three subfamilies, contains the most widely known eusocial genera *Apis* and *Bombus*.

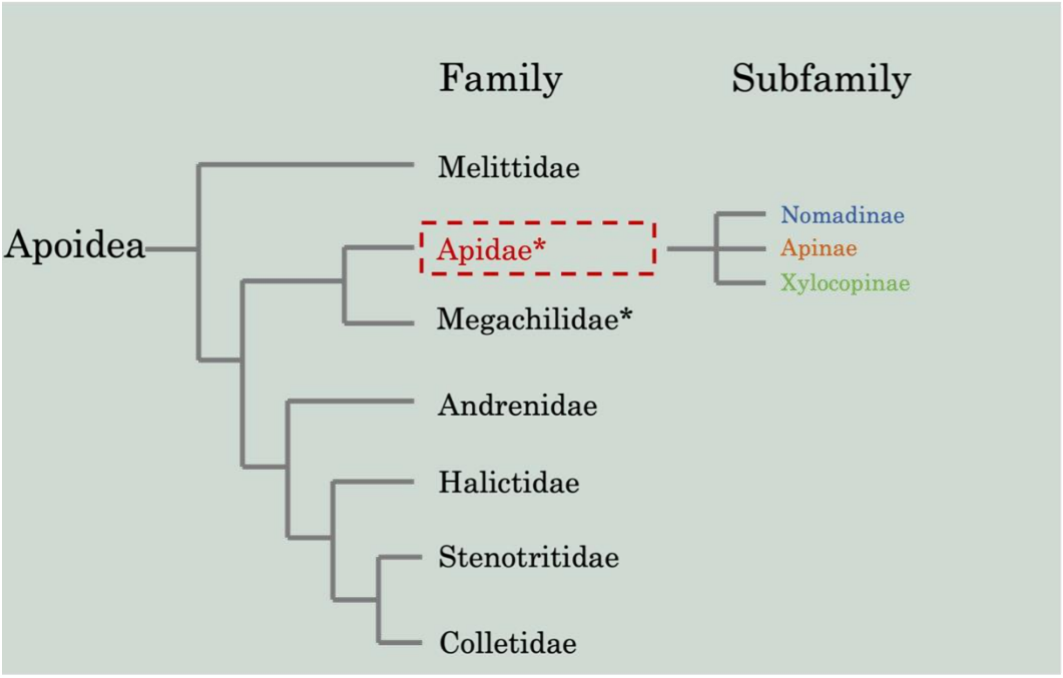

Figure 1.1: Tree diagram of the families belonging to the superfamily Apoidea contained within the Hymenopteran order of insects.

*long-tonged bees

Figure adapted from Hedtke *et al* [7]

The six other families of the Apoidea superfamily contain the bees commonly known as leafcutter and mason bees (Megachilidae), mining bees (Andrenidae), sweat bees (Halictidae), plasterer (or polyester) bees and yellow-faced bees (Colletidae).

The wide variety of bee species exhibit vast differences phenotypically, morphologically, and behaviourally making it difficult to generalise when using the term bee. In this thesis I have primarily focused on bees that display sociality (described in section 1.1.4), and in particular two species belonging to the *Apis* and *Bombus* genus, *Apis mellifera* (European honeybee) and *Bombus terrestris* (buff-tailed bumblebee).

1.1.2 Environmental and economic importance of bees

Bees are thought to have emerged between 140-110 million years ago; coincidently around the same time as flowering plants began to diverge.[8] They are fundamental for both ecosystem stability and food production; more than 85% of angiosperms and 75% of crops produced for human consumption require insect pollination, of which around 80% is contributed by bees.[9-18] Furthermore, insect pollination services have been estimated to provide a global economic value of more than €150 billion a year.[18-20]

The European honeybee, *Apis mellifera*, has often been labelled as one of the most important insect pollinators for agriculture.[20,21] The use of domestic (or managed) honeybees for pollination services in agriculture has been on the rise, however, with reports of annual colony losses at ~40% it poses a challenge to meet the current agricultural demands.[21,22] Colony collapse disorder (CCD) is a term used to describe such losses and has been associated with a range of causes including pesticide use, habitat loss and climate change, as well as diseases and parasites such as the *Varroa destructor* mite.[19,22,23] In addition to honeybees, there has also been a rise in the use of bumblebees as commercial pollinators, particularly for the pollination of greenhouse crops such as tomatoes.[20,24] In 2005 it was reported that annually ~15,000 colonies were used on soft fruit farms around the UK, while more recent estimates from 2016 by the Bumblebee Conservation Trust predicted 65,000 colonies were imported to the UK each year.[24,25]

The growing use of commercialised bees in agriculture also brings environmental concerns. Use of large-scale bees can resort in competition with native species and subsequently reduce floral resource.[24] Furthermore, the spread of disease from commercially reared to wild bees may cause native populations to become vulnerable and further exacerbate the current declines in wild bees that we are reporting.[24,26,27]

Solitary bees make up ~85% of bee species and so it is important to also grasp the impact that these bees are having both environmentally and

economically.[2,28] Wild plants and crops can be more efficiently pollinated by native solitary species than that of honeybees, and thus the importation and commercialisation of honeybees may not be the best course of action to increase crop yields.[2] Therefore, it is important to spread awareness to farmers and growers that they can benefit from increasing the diversity of bee species.[2]

1.1.3 Bee population declines

Declines in managed honeybees as well as in wild bee populations has been reported over recent decades, with a particular focus in Europe and North America, although it is predicted to be a global issue.[4,20,26,29] The cause of these declines has been attributed to a combination of factors including habitat loss, agriculture intensification and pesticide use, climate change, and disease.[4,18,19,26]

Given the economic importance of bees, declines in populations can have detrimental effects on the pollination services they provide and subsequently food production. Furthermore, pollinator-dependent crops are large sources of essential vitamins and nutrients in the human diet and so any reduction in the production of these crops may elicit an increase in nutritional deficiencies, particularly in developing countries.[18]

Overall, by improving our understanding of how anthropogenic activity is affecting bees we can take appropriate measures to help mitigate the effects and protect species biodiversity as well as the agricultural output.

1.1.4 Sociality in bees

Eusociality is the highest level of sociality in animals and is defined by the overlap of generations, reproductive division of labour, and cooperative brood care.[30-32] Eusocial bees have a distinct caste structure in which the queen is the sole egg layer and female workers are sterile, except under certain circumstances such as the absence of a queen.[33,34] Furthermore, sex in social insects is determined by haplodiploidy in which diploid females have twice the number of genes compared to haploid males (**Figure 1.2**).

Distinct morphological and phenotypic differences between castes is also seen as queens have much longer lifespans and are larger in size than workers (**Figure 1.3**).[35,36] In honeybees, queens can live for up to five years, whilst bumblebee queens live for ~ 11 months.[37,38] In contrast, worker lifespan ranges from four to six weeks in the summer for both bumblebees and honeybees.[39,40] However, unlike bumblebees, honeybee colonies overwinter. During the winter months worker lifespan increases to ~20 weeks, suggesting honeybee workers exhibit greater plasticity in regard to lifespan than bumblebee workers.[39-42]

Division of labour in eusocial bees sees workers being responsible for foraging, colony defence, nest cleaning and brood care.[6] Male drone bees are thought to have little purpose besides mating with a queen, after which they soon die.[38] In honeybees, female workers show distinct age-based division of labour which is less prominent in bumblebees. Workers begin life as nurse bees where they remain in the hive and perform tasks such as cleaning, building wax cells and brood care.[39,40,43] They then later switch to become foragers where they leave the hive in search of nectar and pollen to feed developing larvae and themselves.[29,39]

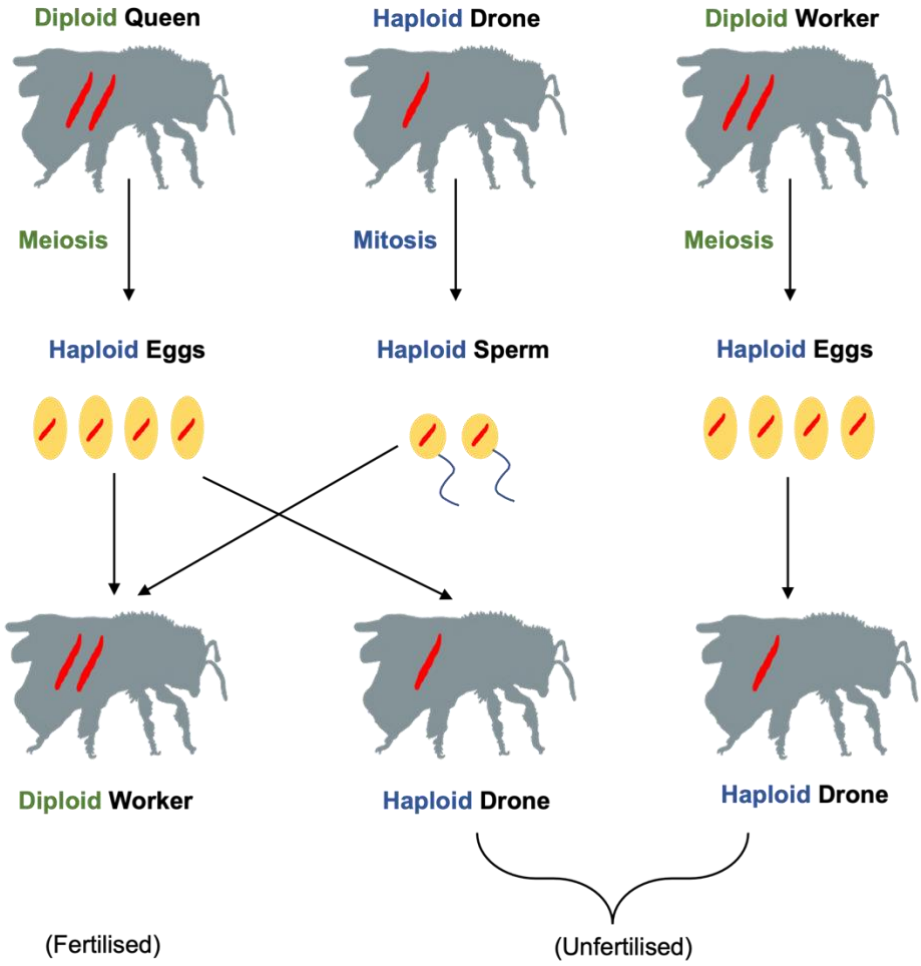

Figure 1.2: Diagram showing reproduction in eusocial bees displaying haplodiploidy. Diploid females have twice the number of genes compared to haploid males.

1.1.4.1 Life cycle of a honeybee hive

For the most part, honeybee colonies are monogynous and therefore contain a singular queen.[44] However, there are periods where multiple queens are reared from workers, such as when the current queen is old or the hive is preparing to swarm.[44,45] If multiple gynes (virgin queens) are reared, they will either leave in an afterswarm or the first to eclose from her cell will attempt to destroy any sister queen cells.[44] In the case that multiple gynes eclose simultaneously then they will fight until one queen remains and claims the hive.[44]

Swarming, or colony fission, is often considered as a form of reproduction and occurs when the colony has grown too large for the nest and therefore decides to split into two separate hives.[46] The primary swarm is when the original queen and approximately half the workers leave the hive to establish and new separate colony.[46] Multiple gynes along with the remaining workers are left in the hive. It is also common that an afterswarm may occur in which a new gyne will leave the hive with a group of workers to establish another colony.[46]

When honeybee gynes take their maiden flight, they can mate with multiple males; studies have reported queens mating with up to 45 drones.[47] This display of polyandry increases the genetic diversity between castes within the colony and is thought to contribute to an increase in colony fitness.[47]

Drone populations tend to be highest in the late spring to early summer months when gynes are most abundant and there is the highest chance of swarming.[48] Drone production is thought to be controlled by both the queen and workers. Workers construct wax combs in which the queen deposits eggs, and as drone cells are made larger than worker cells this enables drones to reach full size.[49] Therefore, the number of drone cells produced by workers can limit the maximum number of drones that can be reared.[49] Workers are also responsible for tending to the developing drone larvae and are therefore able to control the level of investment they put towards drone production and ultimately decide whether they make adulthood.[49] The queen can control the production of workers or drones by deciding whether to fertilise the egg; females are fertilised diploid eggs whilst drones are unfertilised and haploid (**Figure 1.2**).[48,50] Towards the end of the summer drone numbers decline and are normally non-existent during overwintering before the beginning of spring when the cycle continues.[48]

Unlike other bee species, honeybees are the only species that make honey. This is to provide them with sufficient food to last throughout the winter.[41] Workers produce honey from the nectar collected during foraging. Foragers transfer nectar via trophallaxis to receiver bees in the hive which then by a series of behaviours concentrate the nectar via evaporation before storing in cells.[51]

1.1.4.2 Life cycle of a bumblebee colony

In contrast to honeybees, the life cycle of a bumblebee colony is annual with gynes being produced in late summer to autumn.[38] The virgin queens leave the colony on their maiden flight to mate with a single male before hibernating over the winter months.[38] In spring the queen will then scout out a suitable site before establishing her colony and producing her first diploid eggs. These eggs develop into workers which take over brood care and foraging activities whilst the queen remains in the colony and predominantly focuses on egg laying.[31] Once the colony size has reached a few hundred workers the queen will switch to produce unfertilised haploid male eggs.[38,52] The queen will also produce a last batch of diploid eggs that develop into gynes.[38] The drones and gynes leave the colony to find a suitable mate which then begins the cycle over.

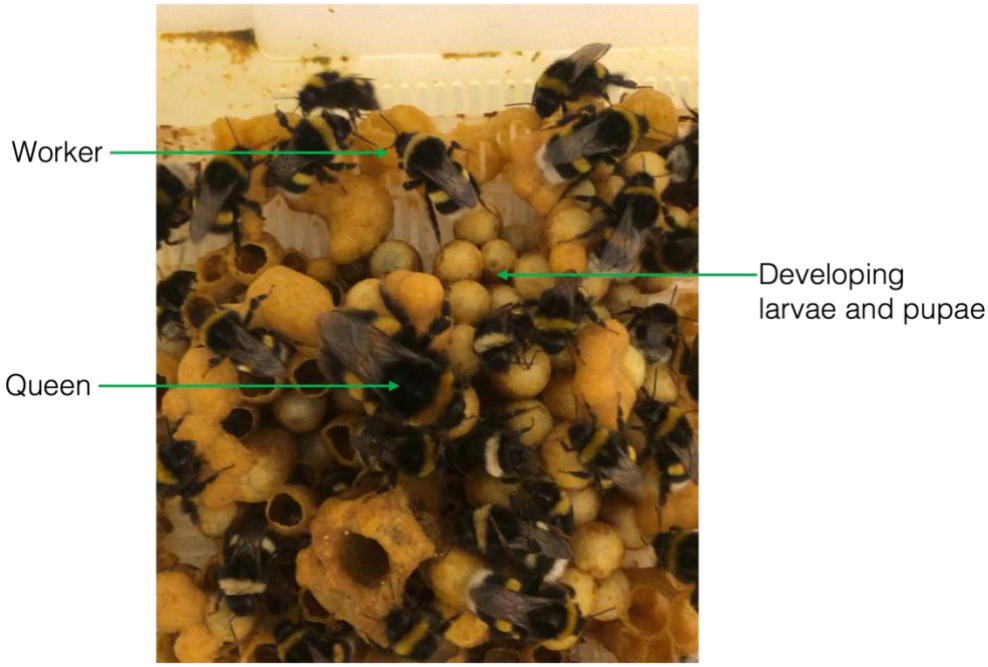

Figure 1.3: Image of a young *B. terrestris* colony. Morphological differences between female castes with queens being much larger in size.

1.1.4.3 Nutrition and caste determination

In honeybees, queen and worker caste determination is influenced by nutrition and in particular royal jelly. Royal jelly is secreted by the hypopharyngeal and mandibular glands of young workers and fed to all developing larvae.[36,40,53-55] However, after the third larval instar developing workers are fed a mix of royal jelly, pollen and nectar whilst queens are fed an exclusive diet of royal jelly and will continually feed on it throughout adulthood until they die.[55-58] Trying to elucidate how royal jelly can influence the longevity and morphological differences between queens and workers has led to an increase in research,[55,58] particularly regarding the role of epigenetic modifications[36], however as of yet no general consensus has been reached. In bumblebees however, the development of queens and workers is thought to be associated with the quantity of food provided during the larval stage rather than any nutritional differences.[35,59] This may suggest nutritional quantity can also influence caste differentiation in honeybees and that a combination of factors determines caste fate rather than royal jelly alone.

1.2 Neonicotinoids

1.2.1 History and development of the neonicotinoids

Agricultural intensification and pesticide use has been heavily associated as a contributing factor in the decline of bee populations and other insects.[19,18,26] Agriculture depends heavily on the use of synthetic pesticides (which includes herbicides, fungicides and insecticides).

The organochlorines, including dichloro-diphenyl-trichloroethane (DDT), were brought about after WWII in the 1940s and were the first insecticides to show high efficacy with low toxicity to vertebrates.[60] However, as written in 1962 by Rachel Carson in her powerful book Silent Spring[61], they were later found to be present throughout the food chain and bioaccumulate in ecosystems.[60,61] Consequently, this led to the ban of organochlorines in the 1970s-1980s which initiated the use of alternative insecticides including the organophosphates, carbamates and pyrethroids throughout the late 20th century.[60] However, the rise of insecticide-resistant pests sparked the need to develop novel synthetic insecticides. This brought about the era of the neonicotinoids.

1.2.2 Mode of Action

Neonicotinoids are a class of neuroactive insecticides which were first introduced in the 1990s and are highly toxic to insect pests with low toxicity to vertebrates.[11,60] They are agonists which target and bind to nicotinic acetylcholine receptors (nAChRs) located in the brain of insects causing over-stimulation of nerve cells, mitochondrial depolarisation, and hyperactivity.[9,10,62-64] This can result in paralysis, convulsions, and eventually death.[10,11,65] There are seven neonicotinoids approved for use: imidacloprid, thiacloprid, thiamethoxam, clothianidin, acetamiprid, nitenpyram and dinotefuran.[60]

1.2.3 Neonicotinoids and bee health

Neonicotinoids are often used as systemic insecticides via seed coating which are readily absorbed by plants and found ubiquitously throughout all

tissues.[9,11,29,66,67] This allows neonicotinoids to target herbivorous insects, however the systemic nature results in the entire plant being a potential vector to off-target species, with a particular threat to pollinators.[60]

Trace residues of neonicotinoids have been found in the pollen and nectar of plants; this can cause exposure to foraging bees which land on flowers, as well as the colony via contaminated food sources.[9,11,66] Sublethal doses of these trace amounts found in pollen and nectar have been associated with impairments in learning and memory, foraging efficiency, navigation and homing ability, and immune response.[9,11,16,66,68,69]

It is important to analyse the effects of insecticides on bees in a caste-specific approach. Differences in behaviour, physiology and lifespan between castes may influence their response and sensitivity to pesticide exposure.[11] Neonicotinoids have been shown to alter gene expression, however, the types of genes that are differentially expressed can differ between castes.[11,70] Furthermore, pesticides may also influence caste determination during development. Chlorpyrifos, an organophosphate pesticide, has been shown reduce the number of larvae which develop into queens.[71] Whether this is directly associated with the pesticide itself or rather by a decrease in the amount of nutrients larvae consume requires further research. Nevertheless, this is detrimental to colony reproduction as female caste differentiation is, in part, determined by the quantity of nutrients received during development.[55-59]

1.2.3.1 Neonicotinoid exposure and flight activity

Current research suggests that neonicotinoids impact flight activity in honeybees and bumblebees. Hyperactivity caused by over stimulation of nAChRs by neonicotinoids can alter the velocity, duration, and total distance flown in bees.[16,72] Acute and chronic neonicotinoid exposure affect flight activity differently; chronic exposure does not appear to elicit the same hyperactive state seen with acute exposure and has significant negative effects on the distance and duration flown in honeybees.[72] Acute exposure appears to have mixed results on flight performance between different bee species and

neonicotinoids.[16,72] In bumblebees, the acute effects of imidacloprid hinder flight duration as it increases velocity which is thought to cause muscle fatigue, premature exhaustion and early flight termination.[16] In contrast, acute thiamethoxam exposure in honeybees appears to benefit their flight performance by increasing flight duration and distance.[72]

The effect of neonicotinoids and other pesticides which hinder flight activity will not only contribute to a decline bee health but also decrease their pollination services by reducing the area in which they can forage. This will increase economic costs by putting a greater reliance on commercial bees to meet the demand for crop pollination.

1.2.4 Current use and legislation

Following the discovery of the detrimental effects of neonicotinoids on pollinators, in 2013 the European Union banned the agricultural use (outside of commercial greenhouses) of the three main neonicotinoids, thiamethoxam, imidacloprid, and clothianidin.[16,73,74] However, countries are able to apply for emergency authorisation for their use if a pest cannot be controlled via any other means, and they still remain some of the most widely used insecticides globally.[16,73] In January of this year (2024) the UK government approved the emergency use of thiamethoxam on sugar beet for the fourth consecutive year, further proving that despite legislation the use of neonicotinoids is still prevalent.[75]

1.2.5 Mitigation strategies for insecticide use

With substantial evidence regarding the harmful effects of neonicotinoids on pollinator health it would seem rational to elicit a global ban. However, this may not be the best course of action to protect pollinators if this initiates the production of novel pesticides that have limited research surrounding their effects on non-target species. The development of novel pesticides to replace banned ones needs to be evaluated on an evidence-based approach which analyses both acute and chronic exposure, and lethal and sub-lethal doses on all non-target species.[76]

Mitigation methods have been proposed as a more appropriate course of action to reduce pollinator declines, particularly in vulnerable areas that are exposed to high levels of chemicals.[77] These approaches can include prohibiting foliar spray application on certain crops during flowering periods as applications nearer the time of flowering causes higher concentrations of insecticide in the pollen and nectar.[76,78] Pesticide application methods may also have a significant role in effectiveness. Insecticides applied by drip irrigation have been shown to have higher efficacy against pests than conventional foliar spray methods as the initial high concentration sprayed is often short-term and declines rapidly.[76,78] Drip application also reduces any direct pesticide exposure and has shown to reduce the adverse-effects of thiamethoxam in bumblebees.[78] Hence, by restricting pesticide application to certain methods this can further limit the risk of exposure to bees. Currently, however, there is very little or absent product recommendations which inform users on the best application approaches to protect pollinators.[76]

Prohibiting the combined use of pesticides can also help reduce the negative impact to bees as they can often interact and have synergistic effects. For instance, in solitary bees when clothianidin was combined with the fungicide, propiconazole, there was a decrease in ovary maturation, feeding, and overall survival.[79] Insecticide toxicity can also be enhanced by certain fungicides; those which inhibit ergosterol biosynthesis, a critical sterol found in plasma and mitochondrial membranes of fungi, have minimal toxicity alone yet significantly increase the toxicity of neonicotinoids.[80,81] Therefore, the mixing of pesticides should not be permitted until there is sufficient evidence that there is no synergism between them.

The use of multiple mitigation methods can improve the likelihood of reducing the impact of pesticides on pollinators. However, governments need to provide greater incentives and engage farmers in integrated pest management practices which will decrease insecticide use whilst still producing high crop yields.[77]

1.3 Cellular respiration in bees

1.3.1 Glycolysis and ATP production

ATP production can occur via oxidative phosphorylation (OXPHOS) in the mitochondria or via glycolytic pathways. Glycolysis occurs in the cytosol and converts glucose into two pyruvate molecules via a series of enzymes (**Figure 1.4**). Unlike OXPHOS, glycolysis produces ATP without the need for oxygen, however it yields much fewer molecules making it less efficient. During OXPHOS approximately 30-32 molecules of ATP are generated from the ETS, whereas glycolysis yields two ATP molecules and two NADH molecules from one molecule of glucose.[82,83] One advantage of glycolysis however it that it is much quicker than OXPHOS, with estimates of it being around 100 times faster.[84] This may be important in insects that need quick ATP availability such as when initiating flight. The pyruvate molecules produced from glycolysis can be converted into acetyl- CoA and subsequently fed into the tricarboxylic acid (TCA) cycle.[85]

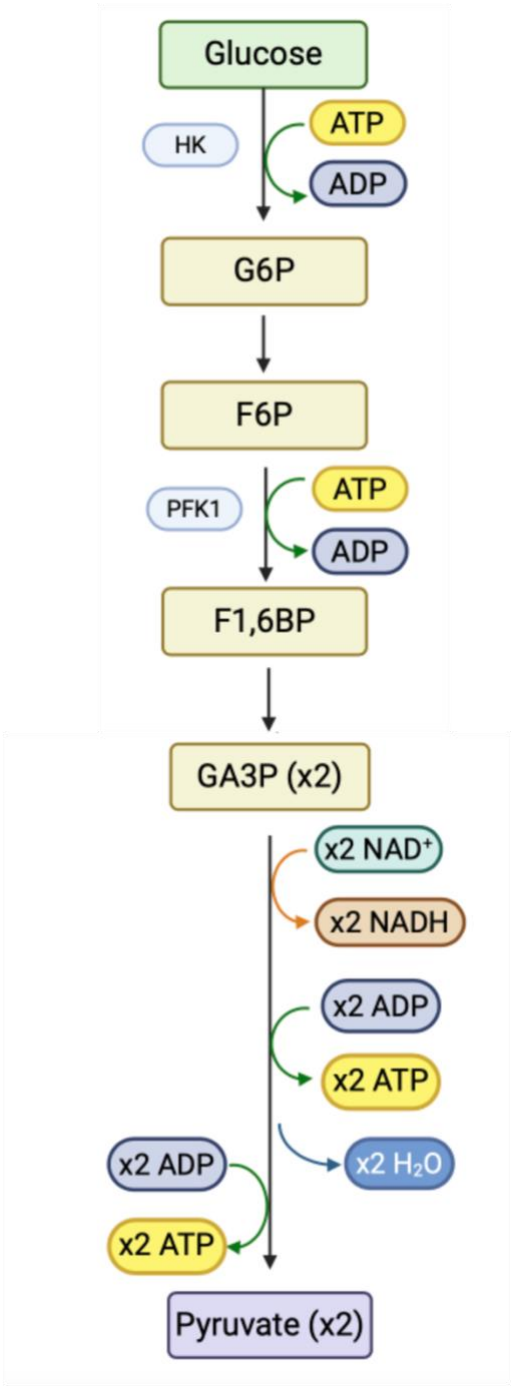

Figure 1.4: Simplified diagram showing the glycolytic pathway. Glucose is the initial substrate and is first converted into G6P via HK which hydrolyses one molecule of ATP. G6P is then converted into F6P which is subsequently converted into F1,6BP via PFK-1 which hydrolyses another ATP molecule. F1,6BP is then converted into two molecules of GA3P. A series of enzymatic reactions then convert two GA3P molecules into two pyruvate molecules along with four ATP

molecules, providing a net gain of two ATP per one glucose molecule. Two NADH molecules are also produced.
Abbreviations: G6P: glucose-6-phosphate; HK:Hexokinase; F6P: fructose-6-phosphate; F1,6BP: fructose-1,6-bisphosphate; PFK-1: phosphofructokinase-1; GA3P: glyceraldehyde-3-phosphate. Image created with BioRender.com

1.3.2 Mitochondria – the powerhouse of the cell

Flight is an aerobic activity primarily driven by the use of adenosine triphosphate (ATP) produced by oxidative phosphorylation (OXPHOS) in the mitochondria of eukaryotic cells.[15,23] During aerobic respiration the mitochondria take up oxygen and substrates and convert them into energy as ATP.[23,86]

OXPHOS consists of the electron transport system (ETS) located in the inner mitochondrial membrane (IMM) which transports electrons via a series of electron carriers.[87,88] During the ETS, NADH:ubiquinone oxidoreductase (complex I) oxidises NADH releasing two electrons which are transferred to ubiquinone whilst four protons are translocated across the membrane and into the intermembrane space (IMS) (**Figure 1.5**).[86,88,89] Ubiquinone, an electron carrier, transfers the electrons throughout the ETS via a series of redox reactions.[88] Ubiquinone is first reduced to ubiquinol by the addition of electrons from complex I and II.[88] Succinate dehydrogenase (complex II), gains electrons from $FADH_2$ which passes them to ubiquinone, however as no protons are translocated across the membrane, less ATP can be synthesised by this complex.[86,88] Ubiquinol cytochrome *c* oxidoreductase (complex III) has an iron core which is reduced and oxidised as electrons pass through.[88] Ubiquinol is oxidised back into ubiquinone in complex III via cytochrome b and passes the electrons to cytochrome c.[88] The electrons are then shuttled to complex IV whilst protons are pumped across the IMM.[88] Cytochrome c oxidase (complex IV) transfers the electrons to oxygen, the final electron acceptor.[88] The release of energy enables protons to be shuttled into the intermembrane space.[86] This translocation of H^+ by the ETS causes a difference in pH between the

intermembrane space and matrix, with a higher pH in the latter.[87] Furthermore, a voltage gradient, or membrane potential, is formed across the IMM with a more negative voltage in the matrix.[87] Both the pH and voltage gradients across the IMM constitute an electrochemical proton gradient which drives the production of ATP via ATP synthase.[86,87,89,90] ATP synthase is made up of the subunits, F0 and F1, which act as a rotational motor system.[86,88,90] F0 is embedded in the IMM and the repeated protonation and deprotonation causes its rotation which catalyses the reaction of adenosine diphosphate (ADP) with an inorganic phosphate (Pi) to form ATP.[88,90] The soluble F1 subunit is able to reverse and hydrolyse ATP to pump protons back across the IMM against the electrochemical gradient into the intermembrane space.[87,88,90]

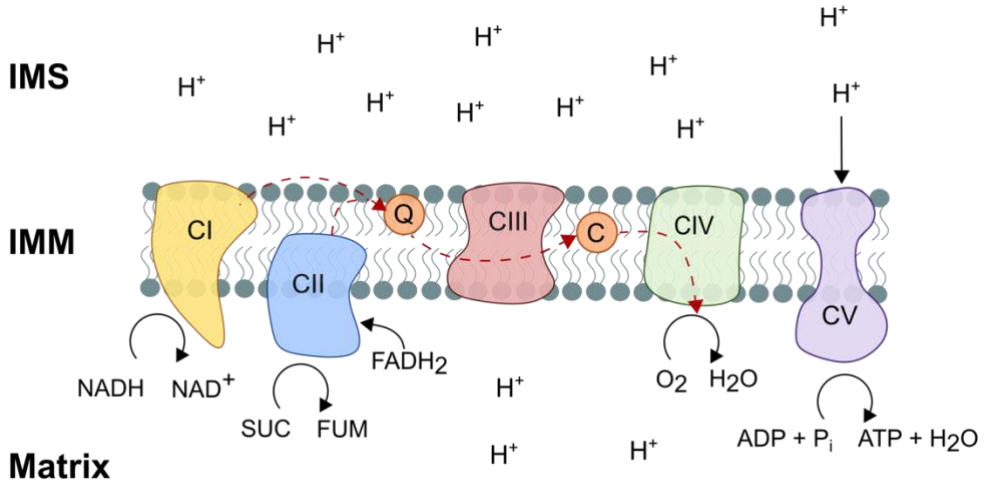

Figure 1.5: Electron transport system (ETS) located in the inner mitochondrial membrane (IMM).

Electrons donated from NADH enter at CI whilst the lower energy electrons donated from $FADH_2$ enter at CII. CI and CII pass the electrons to Q which is reduced to ubiquinol and transfers the electrons to CIII. Electrons pass through CIII, translocating protons across the IMM, before being passed to cytochrome c which carries them to CIV. CIV passes the electrons to oxygen, the final electron acceptor, whilst more protons are pumped into the intermembrane space. Protons then flow down the electrochemical gradient from the intermembrane space into the matrix via ATP synthase (CV) producing ATP.

Abbreviations: Q: Ubiquinone; C: Cytochrome c; SUC: succinate; FUM: fumarate; IMS: intermembrane space; IMM: inner mitochondrial membrane

Image created with BioRender.com

The tricarboxylic acid (TCA) cycle (or Krebs cycle), occurs in the mitochondrial matrix and provides a source of electrons to the ETS (**Figure 1.5; Figure 1.6)**.[85] During the TCA cycle, acetyl-CoA, a product of glycolysis, β-oxidation of fatty acids, and the catabolism of certain amino acids, first enters the cycle and reacts with oxaloacetate to form citrate.[85,91] TCA intermediates are then oxidised by dehydrogenase enzymes leading to the reduction of the cofactors, nicotinamide adenine dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD) to NADH and $FADH_2$, respectively.[23] In addition to acetyl-CoA, other substrates such as proline can supply intermediates into the TCA cycle. Proline can be oxidised to glutamate which subsequently enters the TCA cycle as α-ketoglutarate (**Figure 1.6**). Completion of the TCA cycle generates three molecules of NADH and one molecule of $FADH_2$.[85] Electrons from NADH are supplied to complex I of the ETS, whilst those supplied from $FADH_2$ enter at complex II.[86,85]

The TCA cycle is coupled with OXHPOS; the oxidation of NADH and $FADH_2$ at complexes I and II are required to maintain TCA cycle functioning.[86,85] Furthermore, complex II of the ETS catalyses the oxidation of succinate to fumarate. Hence, any dysfunction in the cycle may result in a decline in OXPHOS efficiency leading to decreased ATP synthesis.[87,92]

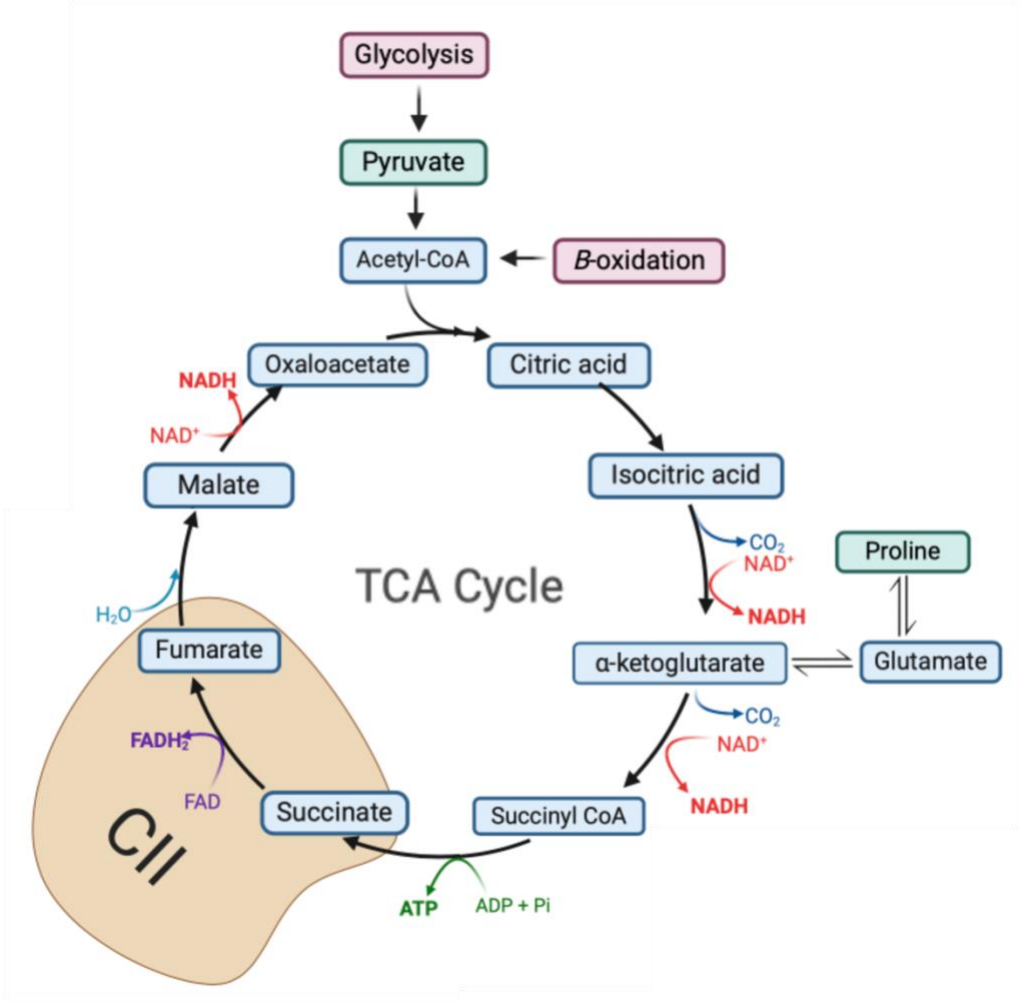

Figure 1.6: Diagram of the TCA cycle located in the mitochondrial matrix. During the TCA cycle acetyl-CoA reacts with oxaloacetate to form citrate. The TCA intermediates are then oxidised via dehydrogenase enzymes and reduce the cofactors NAD^+ and FAD to NADH and $FADH_2$, respectively. Per cycle three molecules of NADH and one molecule of $FADH_2$ are produced. Electrons from NADH and FADH are then supplied to the ETS for the production of ATP.

1.3.3 The flight of the bee – fuelling flight

Insect flight muscle is characterised by high dependence on the aerobic oxidation of cytosolic NADH through the malate aspartate shuttle (MAS) and glycerol-3-phosphate dehydrogenase (GPDH) shuttle, or GP shuttle, during OXPHOS.[93,94] Cytosolic NADH is produced during glycolysis and cannot

enter the mitochondrial matrix for use in the ETS, yet NAD^+ needs to be regenerated to sustain glycolysis.[95] The MAS and GP shuttles allow the reducing powers of cytosolic NADH to be utilised in the ETS whilst simultaneously oxidising NADH back into NAD^+.[95]

In comparison to pathways such as glycolysis and the GP shuttle, MAS is a much slower and complex pathway which uses cytosolic NADH to convert oxaloacetate into malate whilst producing NAD^+ for glycolysis (**Figure 1.7**). Malate can then be diffused across the IMM via a decarboxylate carrier.[95] Once inside the mitochondrial matrix malate is oxidised to oxaloacetate during the TCA cycle and reduces NAD^+ to NADH which can then supply electrons to complex I of the ETS.[93,95] Oxaloacetate is converted to aspartate before being transported back into the cytosol.[95]

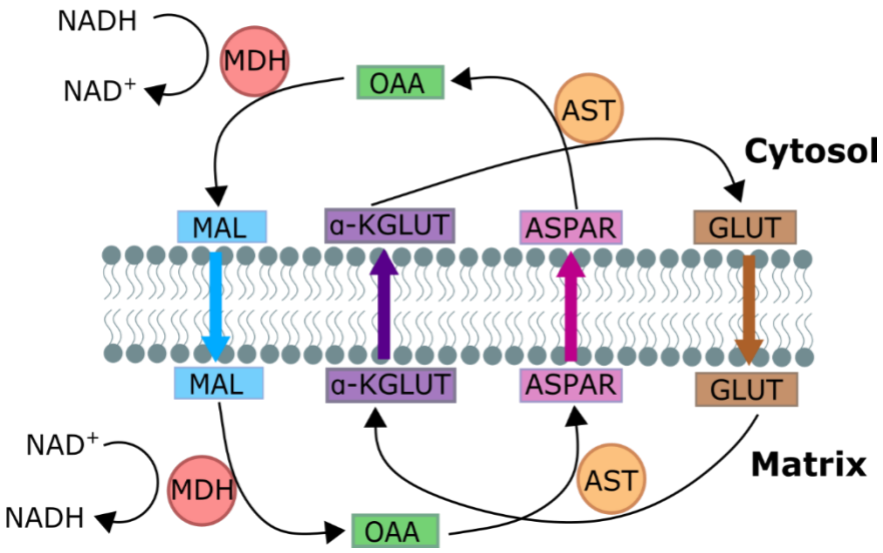

Figure 1.7: Diagram showing the MAS pathway. MDH converts OAA into MAL whilst production NAD^+ from cytosolic NADH. MAL can then be diffuse across the IMM via a decarboxylate carrier, into the mitochondrial matrix. MAL is then oxidised back into OAA during the TCA cycle and reduces NAD^+ to NADH which subsequently supplies electrons to complex I of the ETS. OAA is converted back into ASPAR, via AST, before being transported back to the cytosol.

Abbreviations: OAA: oxaloacetate; MAL: malate; MDH: malate dehydrogenase; ASPAR: aspartate; AST: aspartate amino transferase; α-KGLUT: α-ketoglutarate; GLUT: glutamate
Image created with BioRender.com

The GP shuttle is faster than the MAS shuttle at oxidising cytosolic NADH but less efficient in ATP synthesis as it transforms the reducing power of cytosolic NADH into $FADH_2$.[93,95] Firstly, dihydroxyacetone phosphate (DHAP) is reduced by GPDH to glycerol-3-phospahte (G3P) whilst oxidising cytosolic NADH to NAD^+.[93,95] Mitochondrial GPDH (mGPDH), located on the IMM, oxidises G3P back to DHAP and reduces FAD into $FADH_2$ which can then supply electrons to complex II (**Figure 1.8**). [93,95]

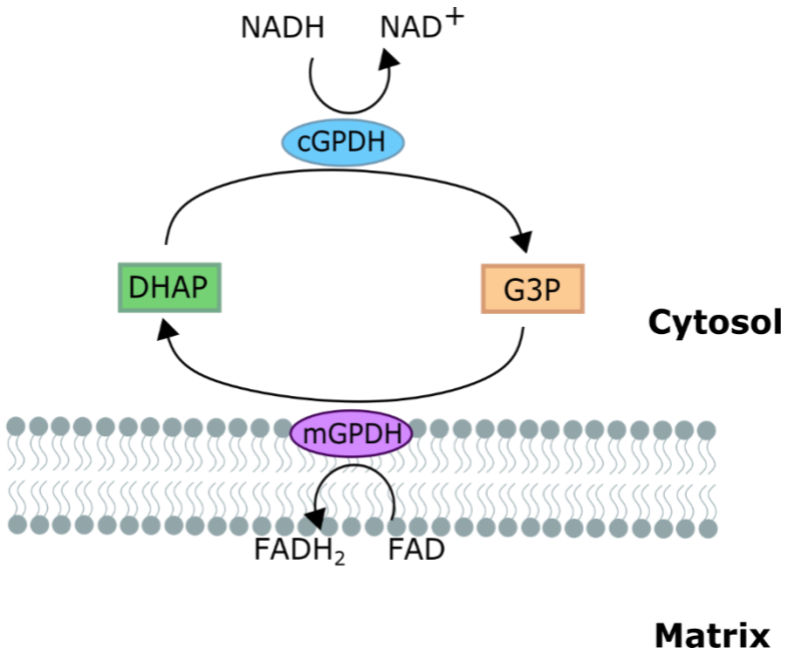

Figure 1.8: Diagram of the glycerol phosphate shuttle. DHAP is reduced by cGPDH to G3P whilst oxidising cytosolic NADH to NAD^+. mGPDH, located on the IMM, oxidises G3P back to DHAP and reduces FAD into $FADH_2$ which can then supply electrons to complex II of the ETS.

Abbreviations: DHAP: dihydroxyacetone phosphate; G3P: glycerol-3-phosphate; cGPDH: cytosolic glycerol-3-phosphate dehydrogenase; mGPDH: mitochondrial glycerol-3-phosphate.
Image created with BioRender.com

Bumblebees have been shown to have a greater OXPHOS capacity with mGPDH compared to honeybees despite the lower levels of metabolic efficiency and large contribution to reactive oxygen species (ROS) production.[93] This suggests there is an alternative advantage for the use of the GP shuttle in bumblebees that may include a greater stability of cytosolic ATP production without the accumulation of by-products such as lactic acid, thus allowing flight muscle to contract without acidification when glycogen is present.[94] Furthermore, mGPDH is also thought to contribute to pre-flight thermogenesis in bumblebees as the energy loss caused by the conversion of NADH to $FADH_2$ may be utilised as heat.[96] However, in both honeybees and bumblebees, complex I is thought to be the largest contributor to the ETS and provides approximately 40% of the proton motive force which drives the movement of protons down the electrochemical gradient.[86,93] Hence any dysfunction in complex I will have significant impact in energetically demanding tissues, such as the flight muscle of insects.[86,97]

1.3.3.1 Flight activity and ROS production

In bees it has been suggested that foraging can damage carbohydrate metabolism as high reactive oxygen species (ROS) production associated with increased flight activity has been shown to damage enzymes involved in the glycogen synthesis pathway.[97,98] Proton leakage, when protons flow back into mitochondrial matrix independent of ATP synthase, along with the ETS, control the mitochondrial membrane potential.[89,99] This in turn controls respiratory rate, ATP synthesis, and ROS production.[99] A decline in mitochondrial energy utilisation may therefore reflect an accumulation in ROS leading to oxidative damage of biomolecules.[99,100] Differences in ROS production may also occur between bee species as higher levels were found in

honeybees compared to bumblebees.[101] This may be due to the higher metabolic rate seen in honeybees putting a greater demand on the ETS and generating a large difference in membrane potential across the IMM leading to an increase in free radicals.[101]

1.3.4 Muscle contractions and flight

The flight muscle of bees, and other Hymenopteran species, have some of the highest mass-specific metabolic rates among all animals and is near the maximum oxidative capacity for power generating muscle.[15,80,93,101,102] More than 40% of the flight muscle volume is occupied by mitochondria, thus allowing the high ATP turnover required for flight muscle contraction.[15,23,93] Furthermore, sustained aerobic respiration during flight requires the mitochondria to be distributed in a structured lattice arrangement which promotes the production of ATP.[93]

Bees contract flight muscles at 100-300 Hz and are asynchronously stimulated contracting up to ten times from a single nerve impulse.[93,103] Asynchronous muscle is unique to certain insect taxa.[104] Unlike synchronous stimulated muscle that has a 1:1 ratio of stimuli to contraction, asynchronous is much more energy efficient.[93,103,105] In asynchronous muscle there is no direct communication between individual contractions and action potentials; the motor neurons sustain oscillatory contraction and the frequency is dependent on wings and thorax rather than neuronal firing rate.[104,106] This decrease in neuronal input reduces Ca^{2+} cycling and thus the surface area of the sarcoplasmic reticulum is reduced in cells.[104] This allows for a greater volume of myofibrils in the muscle which increases the mechanical power output and reduces ATP demand per contraction.[104]

In bumblebees, thoracic muscle contraction is also important for ‘buzz’ pollination and pre-flight thermogenesis. ‘Buzz’ pollination is unique to bumblebee species and comprises the strong shaking of flowers by intense flight muscle contractions.[107] Flight muscle contractions also contribute to pre-flight thermogenesis enabling bumblebees to fly in low temperatures.[16]

1.3.5 Bioenergetics in the brain

Energy metabolism is not only important for flight but crucial for neuronal signalling in the brain.[108] Neurons contain vast quantities of mitochondria to fulfil their high energy demands, hence any decline in mitochondrial respiration can lead to neuronal pathology and cognitive impairment.[108] Cognitive function is critical for navigation, homing senses, memory, and learning in bees.[109] Therefore, in bees it is important to maintain mitochondria function in the brain to prevent negative effects on foraging and pollination capacity.[109] Furthermore, variations in brain mitochondrial respiration may also explain differences in behaviour.[108,110] In honeybees, differences in aggressive behaviour was found to be associated with changes in brain mitochondrial respiration.[108,110]

In humans, during fatiguing exercise neural changes can occur, such as changes in the concentration of serotonin (5-HT), dopamine (DA) and noradrenaline (NA), which are key in the signal transduction between neurons.[111] These changes have been associated with fatigue arising from the central nervous system. [111] This fatigue, termed 'central fatigue', is thought to influence the perception of effort and hence the feeling of fatigue.[111] Therefore, it could be proposed that flight activity may affect neuronal signalling in bees and contribute to muscle fatigue during foraging. However, more research is required to understand the impacts of flight on the brain bioenergetics in bees.

1.3.6 Influencing factors on bioenergetics

1.3.6.1 Substrates

Hymenopteran species do not oxidise lipids but rely primarily on carbohydrates to fuel flight.[97,99,107,112,113] Bumblebees and honeybees store nutrients as non-polymerised sugars such as glucose, fructose and trehalose, which are dissolved in the haemolymph and flight muscles.[107,112,113] The level of substrates in the haemolymph need to be high enough to sustain flight as

limited amounts of glycogen are stored in the flight muscle, and only small quantities of amino acids are used as fuel.[112]

The major products of oxidation of these sugars are G3P, a glycolytic intermediate, and pyruvate, a final product of glycolysis.[107] OXPHOS and respiration rates vary with different substrates; pyruvate and G3P have been shown to produce the highest rates of respiration in bumblebees and honeybees.[101,107] As previously mentioned, complex I substrates are the largest contributors to OXPHOS in bees, which may suggest bees have adapted to favour complex I substrates such as pyruvate to facilitate the high energy demands required for flight.[93] Despite G3P being an FAD-linked substrate and bypassing complex I, the additional advantages of using this substrate, such as the reoxidation of cytosolic NADH may explain the high levels of respiration.[94]

In mammalian high-resolution respirometry (HRR) studies the most commonly used TCA substrates are pyruvate plus malate, glutamate plus malate, or succinate.[107] However, in insect flight muscle mitochondria the presence of these substrate transporter proteins does not seem necessary for high OXPHOS capacity.[107] It has been proposed that insect flight muscle cannot oxidise exogenous malate, glutamate and succinate due to the absence of transporters.[107,114]

The current research of succinate has controversial findings regarding its oxidation efficiency in flying insects.[102,107,115] Previous studies in bumblebees and honeybees have reported an absence or low number of the dicarboxylate transporter required for succinate oxidation, thus explaining the low rates observed in insect mitochondria.[101,107,114] Unlike succinate, G3P does not require a transporter as the mGPDH enzyme is located on the cytosolic side of the IMM which may explain the high efficiency of G3P.[107]

The high carbohydrate diet and high energy expenditure seen in bees could have driven the evolution of flight muscle mitochondria toward an optimisation of OXPHOS with glycolytic products rather than that of the TCA

cycle.[107] This could explain a decrease in the activity of the transmembrane carriers required for TCA intermediates and the high respiration rates seen with pyruvate and G3P substrates.[107]

In contrast to mammalian, bumblebee mitochondria have been shown to maintain a high membrane potential in the absence of exogenous substrates suggesting that there are substantial quantities of oxidizable substrates in the matrix.[107] This could have evolved to compensate for the low activity of the corresponding substrate transporters.[107]

The amino acid, proline, is a OXPHOS substrate with much uncertainty surrounding its use in bees.[102,107] Pollen is proline rich and could therefore provide a source of amino acids for bees.[102] However, it has been reported that honeybees have no capacity to oxidise proline alone, or in combination with carbohydrate, yet proline oxidation in the bumblebee, *B. impatiens*, doubled the mitochondrial oxygen consumption when compared to carbohydrate metabolism alone.[102,107] However, in a different bumblebee species, *B. terrestris*, proline oxidation was found to be slow with low rates of respiration suggesting it is not a major fuel for flight.[107] Thus, it appears proline oxidation is taxonomically labile and underscores the importance of studying multiple bee species and genera, as individual species cannot be assumed to be representative for all Hymenopterans.[102]

Although proline oxidation can occur alone supplying electrons directly to the ETS, the highest rate seen in some bees occurs when combined with anaplerotic substrates that increase TCA cycle intermediates.[102,107,113] Insect mitochondria have been shown to be impermeable to many TCA intermediates; the role of proline may be to replenish and increase these intermediates as well as maintain the potential for pyruvate oxidation.[102] However, more research regarding the potential role of proline as a substrate in eusocial bees is required.

1.3.6.2 Age

It has been widely acknowledged that energy metabolism and ATP production decline with age in mammals, and has also been shown in the flight muscle of *Drosophila melanogaster*.[10,92,116] During development honeybees exhibit temporal polyethism; worker bees go through a series of behavioural and functional life stages in an age-dependent manner.[103] This would suggest that during the latter stages of life when workers transition to foragers they would have lower ATP production compared to younger nurse bees. However, oxidative capacity of honeybee flight muscle has been previously shown to increase with age; older bees have greater density of mitochondria and increased activity of glycolytic and oxidative enzymes.[80] These age-dependent biochemical changes correlate with the transition from nurses to foragers and would appear to meet the increase in metabolic rate and energy demand required for flight.[80] It has, however, been suggested that the increase in mitochondrial density with age could reflect a lower mitochondrial turnover leading to mitochondrial dysfunction and decreased efficiency.[99] Hence, the increase in mitochondrial density may compensate for a decrease in function.[99] Furthermore, ATP levels were found to decrease with age in honeybee fat cells which may suggest younger bees can utilise energy more efficiently. [99]

These contradictory findings regarding ATP production with age and polyethism may be explained by tissue-specific OXPHOS capacities. Tissues taken from the head and abdomen of nurse bees had higher OXPHOS capacity compared with foragers, whereas in the thorax this was the opposite.[117] As foragers require higher ATP in the flight muscle for efficient flight performance this may explain why OXPHOS increases in the thoracic muscle with age but decreases in other tissues.

It should also be noted that further changes occur when bees transition from nurses to foragers including a decrease in body mass and increases in thoracic glycogen stores and metabolic rate.[98] Furthermore, the age of transition can be variable, thus the physiological changes which occur after foraging onset must be associated with the changes in behaviour and increased flight activity rather than with ageing itself. [98]

1.3.6.3 Temperature

Mitochondrial respiration rate is temperature dependent.[101] High metabolic rates support increased thoracic temperatures during flight, and high thorax temperatures enable flight muscle to produce maximum power.[80] In addition, the respiration rates differ between bee species; the maximum respiration rate for mitochondria is lower in the flight muscle of *B. terrestris* than that of *A. mellifera*.[101] Furthermore, mitochondria in honeybees are adapted to higher temperatures than that of bumblebees.[101] This may be associated with the evolutionary adaptation to protect against hornet attacks as honeybees can survive at internal body temperatures between 48-60^{o}C whilst the lethal temperature to a hornet is 44-46^{o}C.[101]

Bumblebees can survive at sub-zero temperatures allowing some to pollinate during the winter months.[101] The relatively high dependence of mGPDH seen in bees and other insects may also be partially explained by the low sensitivity to temperature changes seen in mGPDH enzymes, thus allowing flight to occur within a larger range of environmental temperatures.[101] Hence, under lower temperatures mGPDH and NAD-linked enzymes may function better in bumblebees compared to honeybees.[101]

Bumblebee flight can only occur when the flight muscles reach a minimum of 30^{o}C.[107] To achieve this, bees undergo pre-flight thermogenesis.[96] The FAD-linked respiration substrate, G3P, is associated with low ETS energy production and hence 'uncoupling' nature which releases heat, warming of the flight muscles to reach the minimum temperature required for flight.[101]

1.3.7 Caste-specific differences in bioenergetics

Facultative polyphenism, in which adult phenotypes depend on and are influenced by environmental factors during development, are seen in social bee castes.[98,117,118] An example of this in honeybees is the differential feeding of larvae (described in section 1.1.4.3) to produce either queens or workers. These nutritional differences also influence reproductivity and lifespan as

workers are facultatively sterile, smaller in size, and have much shorter lifespans compared to the larger, highly reproductive queens.[118]

As previously mentioned, OXPHOS capacity can change with age in honeybees to enhance thoracic ATP production in foragers.[117] Foraging bees have a higher metabolic rate in the flight muscle compared to non-foraging bees with rates reported to reach 100-120 ml O_2 g^{-1} h^{-1} in honeybees, which is 10-100 x higher than seen in non-foraging bee behaviour.[97,99] Furthermore, mitochondrial densities and cristae surface areas are thought to be near the upper limit of locomotory muscles, to maximise OXPHOS capacity.[113] This high rate subsequently increases the production of ROS such as superoxide anions, hydroxyl radicals and hydrogen peroxide.[97] In honeybees, it was found that intense flight increases oxidative damage and when restricting flight longevity can increase up to eight-fold.[40,97] In addition, after two weeks of foraging oxidative damage can begin to accumulate in the brain and contribute to cognitive decline.[97]

The high demand for oxygen, particularly during flight may suggest that oxygen supply as well as metabolite substrates could be a limiting factor for sustained flight activity. Fountain *et al* demonstrated that both oxygen and substrate availability were essential for flight in the Glanville fritillary butterfly, *Melitaea cinxia*.[119] Low levels of trehalose were a factor in reducing flight in butterflies that flew under normoxic conditions, whilst there was no relationship in hypoxic conditions.[119] Furthermore, flight was still reduced under hypoxic conditions despite sufficient trehalose.

Oxygen availability has also been shown to affect thoracic temperature and abdominal pumping in bumblebees.[120] However, bumblebees were able to maintain metabolic rates at 15 and 10 kPa O_2, and meet the energetic demands of endothermy at 15 kPa O_2 .[120] The effect of oxygen availability of respiration and flight is likely to vary between species. For instance, certain bumblebee species, such as *Bombus impetuosus,* inhabit higher altitudes with lower oxygen levels, however, it has been shown there is some plasticity with bees being capable of tolerating higher altitudes than that of their natural habitat.[121]

Overall, it has been shown that both oxygen and substrate availability affect mitochondrial respiration in bees. However, under normoxia, despite the high oxygen demands of flight, it is likely that the cause of premature termination during foraging in bees is associated with a scarcity of food resources.

It's been suggested that mitochondrial metabolism may have a role in caste differentiation; one study found oxygen consumption and ATP production was much higher in queen larvae compared to worker larvae, although mitochondria efficiency was similar for both female castes.[118] The quantity of mitochondria was also shown to be higher in honeybee queens, but the maximum mitochondrial capacity was lower compared to workers.[118] This higher demand for oxygen by queen mitochondria may be due to the availability of more energy rich substrates and quantity of royal jelly received during development which may increase energy metabolism.[118] Furthermore, the higher concentration of lipids present in royal jelly may be associated with a higher quantity of mitochondria and OXPHOS levels in queen fat cells.[118] However, it should be considered that this area of research is limited and there is little supporting literature or additional research to corroborate these findings and thus further research is required.

It can be assumed that there is no difference in environmental oxygen during rearing of queen and worker honeybee larvae as they are situated in open cells adjacent to one another.[118] However, worker fat cells were found to have lower rates of oxygen consumption compared with queens yet they have a higher physiological demand.[118] One possible explanation which has been suggested is the Warburg effect.[118] This is seen in tumour cells and is when aerobic glycolysis occurs and glucose is converted to lactate even in the presence of adequate oxygen.[118,122] Compared with OXPHOS, aerobic glycolysis is less efficient at ATP synthesis.[122] As seen with worker and queen fat cells, tumour cells are more energetically demanding than healthy cells, therefore the switch to aerobic glycolysis results in a more rapid production of ATP to compensate for the lower ATP yield.[123] Furthermore, the Warburg effect may also explain an over-expression of hypoxia genes encoding for glycolytic enzymes and their

increased activity, as well as the lower number of mitochondrial functional units and OXPHOS seen in the fat cells of worker larvae.[118]

In summary, mitochondrial bioenergetics varies amongst castes and even between individual workers at different life-stages. Previous studies looking at mitochondrial respiration and function, particularly during flight, tend to focus on a singular caste despite the evidence that mitochondrial function and morphology is caste specific.[107] Therefore, it is important to research mitochondrial bioenergetics not only between individual bee species but also within each caste.

1.3.8 Effects of insecticides on bioenergetics

As previously described, bees can be directly exposed to pesticides whilst visiting flowers, or indirectly by contaminated nectar and pollen, thus exposing the rest of the colony and larvae.[23,79,124] Many insecticides have been shown to compromise energy production.[23] Therefore, understanding how bees process these xenobiotics will help to elucidate how insecticides alone, or in synergy with other stressors, may lead to declines in foraging activity and overall bee health.[66]

1.3.8.1 Neonicotinoids and bioenergetics in the brain

Neonicotinoids have been associated with negative effects on energy metabolism in bees. Imidacloprid directly acts on the electron transport system (ETS) and has been shown to inhibit state-3 respiration (oxygen consumption in the presence of ADP and substrates) and reduce ATP synthesis by the inhibition of complexes I and II in Africanised honeybees.[23] Furthermore, clothianidin has been associated with changes in energy use in bumblebee heads via the enrichment of genes linked to carbohydrate and lipid metabolism.[11]

The head is the site of the nervous system and contains vast quantities of the neurons known as Kenyon cells, which neonicotinoids predominately target in eusocial bees.[11] More than 40% of cells in bee brains are Kenyon cells and are

primarily located in the mushroom body; the structure responsible for sensory integration, learning, and memory in insects.[9,64,125]

Functional mitochondrial ATP production is fundamental for energetically demanding neurons. Hence, it is important to understand whether excessive excitatory stimulation via acetylcholine (ACh) or the synthetic agonists, neonicotinoids, affect mitochondrial function in bee neurons.[9] Field realistic concentrations (1-10 ppb) of clothianidin and imidacloprid have been shown to affect Kenyon cells cultured from bumblebee brains and cause mitochondrial depolarisation leading to impaired ATP synthesis.[9,125] This inability to generate sufficient energy for neuronal function has been found to disrupt neuronal homeostasis and plasticity, learning, and behaviour in mammals.[9,11,125] Mitochondrial dysfunction in Kenyon cells may therefore explain the deterioration in learning, memory and navigation ability seen in bees exposed to neonicotinoids.[9,11,68]

1.3.8.2 Insecticide detoxification mechanisms and bioenergetics

Mechanisms to avoid detrimental effects from pesticides and other synthetic toxins in honeybees are thought to be associated with CYP450 monooxygenases, glutathione transferases, and carboxylesterases; all of which are involved in metabolism and detoxification.[64,66] Although the exact processes of these mechanisms remain unknown, it is well established that metabolic detoxification mechanisms are energetically expensive.[64,66] Hence, if pesticides impair ATP synthesis this may subsequently hinder detoxification mechanisms and potentially increase their toxicity as they cannot be metabolised effectively.

Nicotine, which has a similar mode of action as neonicotinoids, was shown to be detoxified via CYP450-mediated pathways in honeybees.[64,66] Nicotine up-regulated proteins involved in complexes I, II, IV and ATP synthase of the OXPHOS pathway which is likely caused by the activation of detoxification and defence mechanisms, and indicates an increase in energy demand.[66] Increases in OXPHOS by-products such as ROS are associated with the

energetically demanding P450-mediated detoxification processes.[66] To compensate this increase, antioxidant enzymes were also up-regulated in nicotine exposed honeybees.[66]

1.3.8.3 Neonicotinoids and mitochondrial function during flight

The effects of imidacloprid on flight performance in bumblebee workers has also been tested in a laboratory-controlled environment with the use of a tethered flight mill.[16] Imidacloprid treated bees flew significantly shorter distances compared to non-treated bees (660 m and 1834 m, respectively), although interestingly, the average velocity was 33% higher during the initial stages of flight in insecticide treated bees compared to the control bees.[16] This decline in flight endurance seen in imidacloprid treated bees may be associated with premature physical exhaustion due to reduced mitochondrial functioning and ATP production in the flight muscles. Imidacloprid has previously been shown to reduce mitochondrial activity and impair respiratory processes, causing mitochondrial depolarisation in bumblebee neurons.[16] In addition, as neonicotinoids are agonists of nAChRs and increase neuronal activity this may explain the higher velocity seen during the early stages of flight.[16] Consequently, this hyperactivity may increase energy expenditure leading to faster muscle fatigue and energy depletion resulting in flight termination after a shorter duration.[16] Hence, reduced flight distances caused by neonicotinoids will lead to a reduction in the foraging area accessible to a colony, thus hindering their pollination services and colony survival.

The sublethal acute and chronic effects of thiamethoxam on flight activity in honeybees has also been tested under similar flight mill conditions.[72] Honeybees exposed to acute doses of thiamethoxam flew approximately 80% longer and 70% further than when they were not exposed.[72] However, when the bees were chronically exposed to thiamethoxam the flight duration, distance, and average velocity significantly reduced compared to non-exposed control bees.[72] Although the acute results differ somewhat from that seen in bumblebees exposed to imidacloprid, it does however show that thiamethoxam has an excitatory response in the short term which deteriorates with chronic exposure in honeybees.[72]

These tests on flight activity in both bumblebees and honeybees show that neonicotinoids have an acute excitatory effect which elicit different responses on the flight activity between bee species. This could be partially explained by differences in the receptor structure between bee species thus influencing the sensitivity to neonicotinoids.[125] Furthermore, small differences in the structure between neonicotinoids may have a significant impact on affinity and receptor selectivity thus affecting acute and chronic toxicities in bees.[125]

1.3.9 Effect of insecticides on caste-specific bioenergetics

Phenotypic effects and susceptibility to pesticide exposure may differ between bee castes.[11] A study analysing gene expression in the heads of neonicotinoid treated bumblebees found workers and queens differed in their response to pesticide exposure.[11] In queens, the differentially expressed genes were related to lifespan, lipid metabolism and ion transport. Whilst in workers, genes were related to growth regulation, neuron projection and the Notch signalling pathway which is important for cell proliferation, differentiation and death.[11,126]

Biological reasons for a variation in response to pesticide exposure between castes could be associated with differences in behaviour and physiology.[11] As discussed, colony role, lifespan, and feeding behaviour all differ significantly between castes; workers forage, care for the brood and defend the colony whilst the queen lays eggs.[11] These differences may in turn influence caste-specific response thresholds to external stressors including insecticides.[11]

In the neotropical social bee species, *Plebeia droryana*, the organophosphate pesticide, chlorpyrifos, increased larval mortality and the likelihood of surviving larvae to become workers instead of queens.[71] This is likely to be related to a decrease in nutrients that the larvae consumed due to pesticide exposure.[71] This is particularly important in both stingless bees and bumblebees as unlike in honeybees neither receive royal jelly during larval

development, but rather female castes are determined by differences in larval nourishment.[71]

Overall, it is important to consider the biological and behavioural differences between bee castes when analysing the effects that different insecticides and xenobiotics may have on a species. The effect of an insecticide on a particular bee caste cannot be extrapolated to represent the whole colony, and thus, each caste should be analysed individually to truly elucidate the overall effects on a species.

1.4 DNA Methylation

1.4.1 Epigenetics and DNA methylation overview

The definition of 'epigenetics' is the stable and heritable change in gene expression, or phenotype, without alterations to the DNA sequence.[70,127,128] In essence, almost all cells within an organism contain the same DNA, yet the expression of genes varies between cell and tissue-type which is mediated by epigenetic mechanisms.[129] The most common processes involved in this type of genome regulation are modifications of histones and DNA methylation.

1.4.1.1 Histone modifications

Histones are a fundamental part of the formation of nucleosomes which make up the basis for chromatin which then further package the DNA into higher-order chromatin, seen for example in metaphase chromosomes.[130,131] Histone proteins organise the DNA into nucleosomes by wrapping this double-stranded nucleic acid around the octameric core of histone proteins (comprised of two H3-H4 histone dimers and two H2A-H2B dimers).[132,133] Chromatin is made up of nuclear DNA along with specific nuclear proteins and provides a dynamic structure for regulating gene transcription and DNA metabolism.[130,132] Gene expression requires transcription factors to bind to promotors which recruit RNA polymerase and initiate transcription.[131] Nucleosome spacing determines the chromatin structure and access to transcription factors; if densely packed it is categorised as transcriptionally inactive heterochromatin, and if it is less dense it is categorised as transcriptionally active euchromatin.[132] Chromatin

structure and its role in gene expression is largely regulated by post-translational modifications to the histones.[130,132]

On each of the core histones the N-terminal chain of amino acids, also known as the 'tail', extends out of the core of the histone protein.[131] The 'tails' are subject to an array of reversible covalent modifications which can alter the packaging of nucleosomes and regulate the accessibility for transcriptional regulators and RNA polymerases.[131] Histone modifications including acetylation, methylation, phosphorylation and ubiquitination, can therefore alter the chromatin structure resulting in changes to gene expression.[132,133] Histone modifications occur at a set of distinct amino acids, mainly lysine and arginine residues with different impacts to gene expression, depending on the combination of modifications.[130] For example, histone acetylation and deacetylation have been linked to increases and decreases in gene expression respectively.[130,134]

1.4.1.2 DNA methylation

DNA methylation is one of the most studied mechanisms of epigenetics.[135] It is the addition of a methyl ($-CH_3$) group to a nucleotide base in the DNA via DNA methyltransferases (DNMTs)(**Figure 1.9**).[136,137] DNA methylation can alter chromatin structure and regulate gene expression, and is also involved in various biological processes such as mammalian genomic imprinting and X-chromosome inactivation.[137,138] Diploid cells contain two copies of a gene, a maternal and paternal allele; generally, both these alleles are either active or inactive in a similar controlled way. However, in X-chromosomal inactivation and genomic imprinting one of these alleles is silenced.[136,139,140] DNA methylation marks the genes undergoing genomic imprinting and alters gene expression resulting in the imprinted copy of the allele being turned off and the non-imprinted allele turned on.[139]

In eukaryotes, the most common type of DNA methylation is 5-methylcytosine which contains a methyl group on the pyrimidine ring of cytosine at the 5th

position (**Figure 1.9 a**).[127,136-135,141] Other types of DNA methylation include, *i*) 5-hydroxymethylcytosine (5hmC), which has the addition of a hydroxymethyl group on the 5th position of cytosine; *ii*) N^4-methlcytosine (4mC), where cytosine is methylated on the N-4 position; and *iii*) N^6-methyloxyadenosine (6mA), where a methyl group is added on to the 6th position of the purine ring of adenine (**Figure 1.9 b**).[137,142,143] The latter two are considered to be found predominately in prokaryotes.[137,141,142,144]

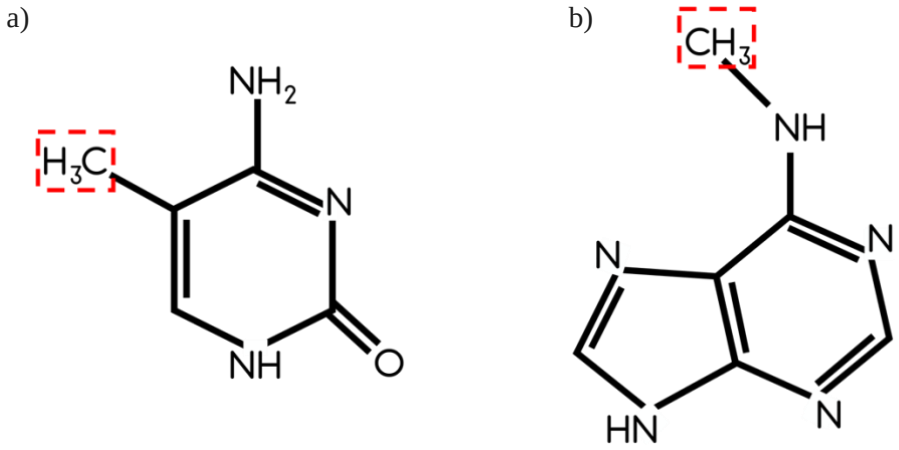

Figure 1.9: Skeletal formula of 5mC and 6mA. a) 5-methylcytosine (5mC); b) N^6-methyloxyadenosine (6mA). Red box indicates the added methyl group.

5mC methylation occurs on cytosines in CG dinucleotides (CpG) in humans and other mammals, as well as insects including honeybees.[144,145] CpG dinucleotide methylation allows the methylation on both strands of the DNA to be maintained during replication, making it key for epigenetic regulation.[144] CpGs are underrepresented throughout the mammalian genome except for 'CpG islands' which show increased rates in ~0.3-3kb stretches of DNA and are generally prevalent at promotor regions.[136] This high 5mC methylation at the promotor regions has been shown to generally supress gene transcription in mammals.[129,146] This suppression can be due to the prevention of DNA binding proteins which recruit the transcriptional activators, or the recruitment of methyl-binding proteins (MBPs) which initiate transcriptional corepressor complexes.[131,146]

DNA methyltransferases (MTases) are the group of enzymes responsible for the addition of a methyl group to a nucleotide base either at the C5 or N4 position of cytosine, or the N6 position of adenine.[147-149] They are responsible for establishing and maintaining DNA methylation in the genome.[148,149] The distinct catalytic motifs in DNA methyltransferases are used to generate the different types of methylations such as 5mC, 4mC or 6mA.[144] In eukaryotes MTases (also referred to as DNMTs) are grouped into a family of five members which are associated with the methylation of cytosine, DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L (**Table 1.1**).[129,136,148,150] In animals, DNMT1 maintains DNA methylation patterns during cell replication and methylates hemimethylated DNA whilst *de novo* methylation is mediated by DNMT3A and DNMT3B.[129,136,143,144,148,150-152] DNMT2 shares similarities to prokaryotic MTases and is an RNA methyltransferase which methylates tRNAs.[143,144,148] DNMT3L does not have a catalytic domain and thus cannot methylate DNA, however, it is thought to interact with DNMT3A and DNMT3B and improve their catalytic activity.[143,144] Different subfamilies of these eukaryotic MTases have also been identified in algae, fungi and vertebrates such as zebrafish.[147,153] N6-adenine methyltransferases (N6-adenine MTases, or N6AMTs) methylate the amino group of the C-6 position of the adenine ring.[143]

DNA methyltransferases catalyse the addition a methyl group from the donor, *S*-adenosylmethionine (SAM), to the nucleotide.[137,144,151,154] For example, during 5mC methylation, the target base (cytosine) is rotated into the catalytic pocket of the DNMT and the reaction is initiated via a conserved cytosine residue (catalytic cysteine thiolate) in motif IV of the DNMT which mediates a nucleophilic reaction on C6 of the cytosine.[137,144] A methyl group is then transferred from *S*-adenosylmethionine to C5 to from 5mC.[137,144]

DNA methylation is a reversible process and the modification can be lost by either passive or active demethylation.[155] Passive demethylation of 5mC in humans is associated with the failure of DNMT1, thereby reducing methylation during successive replication.[155] Active demethylation is catalysed

by the Ten-eleven translocase (Tet) family of enzymes which comprise of Tet1, Tet2 and Tet3 (**Table 1.1**).[155,156] Oxidation of 5mC is mediated via Tet enzymes and results in the conversion of 5mC to 5hmC, 5-formylcytosine (5fC), and 5-carboxycytosine (5caC).[155,156] Unlike 5mC, these oxidised products are not suitable substrates for DNMT1 and therefore actively promote the replication-dependent loss of the methyl group.[155,156]

Table 1.1: List of methyltransferases and demethylases and their target base (5mC or 6mA).

Protein	Family	Methyltransferase /demethylase	Methylation target	Organism
DNMT1 DNMT2 DMNT3A DNMT3B DNMT3L	DNMT	DNA methyltransferase	C5	*H. sapiens*
METTL3 METTL14	MT-A70	RNA methyltransferase	N6	*H. sapiens*
DAMT-1	MT-A70	DNA methyltransferase	N6	*C. elegans*
NMAD-1	AlkB	DNA demethylase	N6	*C. elegans*
DMAD	Tet	DNA demethylase	N6	*D. melanogaster*
Tet1 Tet2 Tet3	Tet	DNA demethylase (C5)	C5	*H. sapiens*

1.4.2 6mA methylation in eukaryotic DNA

Unlike in eukaryotes 6mA DNA methylation is the most prevalent epigenetic mark in prokaryotes.[138,157,158] In bacteria, methylation of adenine is known to have defensive properties by allowing the differentiation between methylated 'self' DNA and unmethylated DNA of invading bacteriophages.[136,138,142,157,158]

In addition, 6mA has also been shown to regulate mismatch repair, DNA replication and transcription.[141-143,159]

Previously, 6mA was thought to be only present in prokaryotes, however, in recent years there has been growing evidence of the presence of 6mA in eukaryotic DNA.[138,141,158,160] In eukaryotic DNA, 6mA was first thought to be detected in such small amounts that it was similar to the levels associated with DNA base damage, if detectable at all, and the low abundance initially cast doubt on its presence and functional relevance.[157] However, more recently 6mA in DNA has been reported in fungi, plants, *Drosophila*, *Caenorhabditis elegans*, as well as vertebrates such as mice, with claims of greater biological significance and potential epigenetic functions.[138,143,157,158] The level of 6mA currently reported in genomes of these eukaryotes varies; in early-diverging fungi up to 2.8% of adenines are methylated.[158] Whilst in *C. elegans* and *D. melanogaster* there are reports of 6mA in their genomes at levels of 0.01-0.4% and 0.001-0.07%, respectively.[138,143,161] In the green algae, *Chlamydomonas reinhardtii*, 6mA modifications are found to be present in >14,000 (84%) genes.[158,162] In these species 6mA was found to often occur at ApT dinucleotides and clustered around transcription start sites (TSSs) of expressed gene.[157,158,163] Furthermore, in *D. melanogaster*, 6mA was found in the gene bodies of transposons and the removal of 6mA during embryonic development resulted in transposon suppression suggesting it's vital in transposon activation.[158,163] 6mA also showed a negative association with nucleosome positioning together indicating that 6mA may have a role in eukaryotic gene expression regulation.[158]

6mA methylation may also be a transient DNA modification during development; during pig and zebrafish embryogenesis 6mA levels were shown to increase up to 0.1-02% of adenines, which then decrease to background levels (p.p.m, part per million) with developmental progression.[157,158] This is similar with 6mA levels reported in *D. melanogaster* which were found to be greater in early-stage embryos, further suggesting developmental dynamics of 6mA methylation in DNA.[143]

1.4.2.1 Eukaryotic 6mA methyltransferases and demethylases

In contrary to DNA, 6mA (also termed m6A in RNA) is the most prevalent modification in mRNA and is widely conserved in eukaryotes.[138] The family of enzymes which contain a MT-A70 domain are thought to have evolved from bacterial 6mA methyltransferases and include the human mRNA methyltransferases, methyltransferase-like protein 3 (METTL3) and METTL14 (**Table 1.1**).[138] The MT-A70 domain is the SAM binding subunit of the methyltransferase enzyme which catalyses the methylation of mRNA in humans.[138] In *C. elegans* a member of the MT-A70-like family of proteins, DAMT-1, has been proposed to be a DNA 6mA methyltransferase; the knockout of *damt-1* in *C. elegans* has been shown to decrease 6mA levels in genomic DNA.[138,149] The human RNA methyltransferases, METTL3 and METTL14, do not possess methylation activity on DNA; however, METTL4 is thought to be the closest ortholog to DAMT-1 in humans and has been proposed as a candidate DNA 6mA methyltransferase.[138,162]

6mA demethylases include the AlkB family which are responsible for the demethylation of mRNA in eukaryotes.[138] *C. elegans* have been found to have five AlkB members; one member, *nmad-1*(N^6-methyl adenine demethylase 1), when deleted, caused elevated 6mA levels *in vivo* suggesting it is a 6mA demethylase in *C. elegans*.[138,158] In *D. melanogaster*, DNMT1 and DNMT3 were evolutionarily lost, however, a homologue of the 5mC demethylase Tet family protein, DMAD (DNA 6mA demethylase), has been associated with the demethylation of 6mA.[138,143,151] The genomic DNA of *D. melanogaster* brains of *dmad* mutants showed 6mA levels that are ~100-fold greater than wild-type flies.[138,158] One study showed that the absence of 6mA from transposon regions by DMAD was associated with transposon suppression, yet another study found DMAD interacts with the Trithorax-related complex protein, Wds, and maintains active transcription by demethylating intragenic 6mA associated with neurodevelopment and function.[158,164] This suggests that 6mA may have different transcriptional outcomes depending upon tissue type and genes.[158,164]

1.4.2.2 Association between 6mA and histone methylation

DAMT-1 and 6mA have also been associated with histone methylation in *C. elegans*.[160,161] Deletion of the histone H3K4me1/me2 demethylase, SPR-5, in mutant worms increased 6mA levels.[161] Furthermore, the knockdown of the H3K9me binding protein, *eap-1*, which reduces H3K4me2 levels, also reduced 6mA levels.[161] This corresponds with Ma *et al* which found that transgenerational stress adaptation and 6mA induction lasted longer in SPR-5-null *C. elegans*.[160]

There is also evidence of interactions between 6mA methylation and histone modifications in *D. melanogaster* as DMAD-null flies had a reduction in Wds and the H3K4me3 marker.[164] Overall, this suggests that there is crosstalk between regulators that control 6mA and histone methylation, and possible cooperation between the two in transmitting epigenetic information.[160,161,164]

1.4.2.3 6mA in eukaryotic mtDNA

Although convincing evidence of 6mA in mammals remains to be established, it has been reported that mitochondrial DNA (mtDNA) may be enriched for 6mA, which may further increase under stress conditions.[162] *In vitro*, Hao *et al* reported that the presence of 6mA reduced the transcription activity of the mitochondrial transcription complex.[162] Furthermore, METTL4 was shown to accumulate in the mitochondria, more specifically in the matrix, and can catalyse 6mA mtDNA methylation and reduce mtDNA transcription and thus mtDNA copy numbers.[162] The knockdown of METTL4 also decreased 6mA levels in mtDNA but was shown not have any significant effect on RNA m6A levels in mt-rRNAs, mRNAs, and small RNAs suggesting METTL4 acts on mtDNA and not RNA.[162] Overall, there is growing evidence of the function of METTL4 as a mammalian 6mA methyltransferase, however more research is still required to further validate 6mA in mammals as the small quantities of methylation found in genomes requires extreme precision in analysis with little room for inaccuracy.[165]

1.4.3 DNA methylation in eusocial bees

1.4.3.1 5mC methylation in eusocial bees

Insects, in particular Hymenoptera, have become increasingly used for epigenetic research due to the significant role methylation plays in their genome.[56,128,151,166] As described earlier, the morphological differences including size, longevity, reproductivity, physiology and behaviour between female queens and workers vary considerably despite having nearly identical genomes.[56,57] Caste determination and differentiation between workers and queens are determined by the quality and quantity of food given to the developing larvae.[56,57] This early-life environmental influence determines the developmental trajectory of females and displays phenotypic plasticity which makes eusocial bees ideal model species for studying epigenetics.[35,56,57]

DNA methylation in bees occurs almost exclusively at CpG sites and is sparse amongst genomes with less than 1% of CpG dinucleotides being methylated.[36,151,160,168] In contrast to vertebrates, DNA methylation patterns are primarily localised in intragenic regions and associated with gene activation.[36,128,151,166-168] Furthermore, in honeybees, the methylated exons are found in genomic regions with low CpG ratios.[36] These hypomethylated genes have been associated with caste-specific expression and associated with developmental processes.[34,53,70,168]

There is evidence that CpG clusters in methylated genes are associated with gene splicing in bees; methylated cytosines could provide the information to established and regulate splicing patterns during development and differentiation.[36,70] It may also be considered that gene body CpG methylation in honeybees is kept mostly invariable in the germlines to preserve function and methylation patterns over generations.[167]

DNA methylation has previously been linked to caste differentiation with the knockout of *DNMT3* in larvae that received nutrition that would normally initiate worker production was shown to lead to queen-like adults.[169-171] This established the importance of DNA methylation in caste differentiation in

honeybees.[34,169] However more recently, despite suggestions of the role of methylation in caste development, the study by Cardoso-Junior *et al* found that gene body methylation was colony-specific and not affected by social or environmental cues in honeybees, but rather by genotype.[166] Furthermore, the colony-specific methylation patterns were found to be inherited from drones to their worker daughters, and there is no evidence of global DNA methylation reprogramming during embryogenesis which coincides with other invertebrates.[166,172]

Methylation patterns were found to be similar between the brain and ovary of bees and differentiated in only a small number of regions.[166] This suggests that DNA methylation is not associated with differential gene expression between tissue types or behaviour-related morphology variation in bees.[166] These findings are consistent with that reported by Marshall *et al* who found no evidence of genome-wide methylation differences between sterile and reproductive workers, and an inconsistency in the relationship between methylated genes and expression.[34] This suggests that DNA methylation may not have a direct response on gene expression changes leading to the phenotypic variation between reproductive and sterile workers in *B. terrestris*.[34] Furthermore, no association was found between methylation, gene expression, or alternative splicing during honeybee development which further correlates with the findings that methylomes are maintained during development and suggests gene body methylation is homeostatic.[173] However, an exception has been shown in the brain of honeybees with some evidence of differential methylation linking to differential gene expression.[173] DNA methylation has also been associated with behavioural plasticity and the transition between honeybee nurses and foragers.[171] Together this demonstrates the importance of studying epigenetics in a tissue-specific manner as one tissue cannot be extrapolated to represent the species as a whole.

Despite little evidence for the role of gene body methylation in caste differentiation it has been shown that DNA methylation is vital for honeybee viability; the knockout of the *DNMT3* gene, which is key in honeybee

development, is associated with death during early development.[166] Overall, *DNMT3* gene expression has a link with honeybee development and potentially caste differentiation, however the lack of differences in the methylomes between castes suggests that demethyltransferase genes may have a greater role beyond that of DNA methylation solely.[34,166]

In bumblebees, methylation has been shown to be associated with the reproductivity of workers.[34,128] Bumblebees can alter their reproductive status depending on their environment; in the absence of a queen, workers are divided into reproductive and non-reproductive sub-castes.[128,167] The DNA methylation profiles in the head tissue have been shown to differ between reproductive and non-reproductive workers in queen-less colonies.[34,167] Reproductive workers have also been shown to go back to sterility if returned to the queen suggesting that bumblebee worker reproductively is a plastic epigenetic process.[167]

DNA methylation has also been found to be fundamental in memory formation and preservation and is likely a conserved mechanism in memory consolidation in bees.[174,175] DNMT inhibition has been shown to affect the rate of extinction learning.[174-176] Memory extinction is the process of gradually diminishing a conditioned response over time as the response uncouples from the stimulus.[177] This is particularly important in honeybee foragers as they need to recall the location of floral resources and then remove these memories once other food sources are available.[174]

Epigenetic modifications may also have a role in genomic dosage compensation of haplodiploid insects such as bees. In the red imported fire ant, *Solenopsis invicta*, DNA methylation profiles were highly diverged between diploid females and males compared to haploid males.[178] Furthermore, genes that were found to be associated with dosage compensation in *Drosophila* were differentially methylated and expressed between haploid and diploid ants.[178] These findings suggest that DNA methylation may have an important role in the regulation of genes in response to differences in genomic dosage and help to achieve ploidy compensation in haplodiploid hymenopterans.[178]

Overall, 5mC DNA methylation is a crucial epigenetic mechanism in eusocial bees and is involved in a wide variety of functions including development, reproduction, and cognitive function. With an abundance of research in 5mC methylation it further supports the question as to whether other types of methylation such as 6mA may play a part and the necessity for this research to be undertaken.

1.4.3.2 Methyltransferases and demethylases in bees

In mammals, the majority contain the complete set of DNMT enzymes (DNMT1, DNMT2, DNMT3A and DNMT3B), however in contrast to vertebrates, many insects have lost certain demethyltransferase genes including DNMT1 and DNMT3 which have been lost in Diptera (e.g. fruit flies, house flies), Ephemeroptera (mayflies), Odonata (e.g dragonflies, damselflies), Orthoptera (e.g. grasshoppers, locusts, crickets), Lepidoptera (e.g butterflies and moths), and more.[151] Currently, only three other insect orders besides Hymenoptera have been found to contain the complete set, Coleoptera (beetles), Hemiptera (tree bugs) and Blattodea (cockroaches and termites).[151,170,179] It appeared that the complete set of DNMT enzymes were maintained in insect species that show the greatest phenotypic plasticity and sociality.[151] The honeybee genome has been found to encode two DNMT1 (DNMT1a and DNMT1b) enzymes and one DNMT3 enzyme.[173,180,181] The contribution of both DNMT1a and DNMT1b to the maintenance of DNA methylation is still relatively unknown and more research needs to be done to address the role of these genes.[151,181]

DNMT3 is the least common methyltransferase enzyme found in insects and is not always found in conjunction with the presence of DNMT1.[181] The loss of the *de novo* methyltransferase, DNMT3, in many insect species and the substantial association between the maintenance methyltransferase, DNMT1, and the presence of DNA methylation and a lack of correlation between DNMT3 and DNA methylation suggests that DNMT3 may not be a necessary enzyme for DNA methylation or that DNMT1 is able to compensate for its

absence.[181] This has been shown *in vitro* in insects; DNMT1 may compensate for DMNT3 and acts as a *de novo* methyltransferase and DNMT1 positively correlates with DNA methylation.[151,181,182] Furthermore, in honeybees both DNMT1 and DNMT3 were shown to actively methylate DNA at CpG sites.[151,183] Biergans *et al* found that DNMT1b was upregulated after cognitive training suggesting the enzyme may have an important role in bees during memory formation, further supporting the possibility that DNMT1 and DNMT3 may have different roles across different species.[180]

The higher expression rate of *de novo* methyltransferase DNMT3 compared to the maintenance methyltransferase, DNMT1, seen in bees and the association of DNMT3 on genome-wide DNA methylation in honeybees further supports its importance in the phenotypic plasticity of bees.[170,179,181]

In contrast to methyltransferase enzymes, there is relatively little known about demethylase activity in bees and other invertebrates.[184] So far one member of the Tet enzyme family has been identified in bees and other insects.[185] In honeybees, Tet has the highest expression and is found to be as equally fundamental as DNMT enzymes for long-term memory formation.[171,180,184] *Tet* is alternatively spliced and expressed through honeybee development and during adult life.[184] Furthermore, it has been found to convert 5mC to 5hmC.[180,184,185]

In bumblebees, the *Tet* gene has been shown to have the highest expression in drone testes.[185] In mammals, Tet proteins are involved in spermatogenesis by regulating the maintenance of pluripotency and proliferation of stem cells in the male germline.[185] Hence, it could be considered that Tet has an important role in spermatogenesis in bumblebees by regulating demethylation in the testes.[185] However, it should be considered that Tet may not be the only enzyme associated with demethylation in insects as other potential enzymes may be able to act as demethylases.[180,186] Overall, it is clear more research is required to understand the demethylation enzymes and their role in the methylomes of bees.

1.4.4 DNA methylation and stress

1.4.4.1 Environmental stressors and methylation in bees

The association between environmental stressors and DNA methylation is a relatively poorly studied area of epigenetic research. However, multiple stressors including thermal, social, and chemical have been shown to affect DNA methylation and gene expression in a variety of organisms. For example, heat stress has been shown to alter DNA methylation variation in cockroaches suggesting DNA methylation may provide a dynamic response to changes in temperature.[187]

In eusocial bees, one form of stress which has been associated with changes in gene expression are insecticides. Neonicotinoids have been shown to alter gene expression in honeybee worker larvae, workers and queens.[70,124,188] In bumblebees, the brain of workers exposed acutely to 10ppb imidacloprid showed differential gene expression compared to the control, however there was no significant effects on CpG methylation levels.[70] However, it may be that chronic exposure is necessary to elicit changes in CpG methylation which would correspond with current findings in honeybees where chronic exposure of 5ppb and 200ppb imidacloprid induced increases in global DNA methylation.[189] In honeybees, it has also been shown that DNA methylation increases with age, and so it could be suggested that imidacloprid accelerates senescence on the epigenetic level.[189]

Stochastic DNA methylation induced by environmental stressors can occur only several hours after exposure to the stimuli.[187,190] An example of this was shown in honeybees where the exposure of intruders to the hive elicited defensive behaviours, such as aggression, and altered DNA methylation in the brain as soon as a couple of hours post exposure.[171]

In summary, the stability of stress-induced methylation variation can vary from hours to several generations.[190] Hence, it is important to establish whether insecticides and other stressors can alter DNA methylation in bees, as negative

heritable epigenetic changes may have subsequent long-term implications on bee populations.[70]

1.4.4.2 6mA methylation in DNA and stress

There have been several studies in recent years associating stress with changes in 6mA methylation. In human mitochondria, hypoxia-induced stress elevated METTL4, and subsequently increased 6mA methylation by three-fold. This increase suggests that 6mA may have a role as a regulatory mark in mammalian mtDNA.[162] The knockdown of METTL4 affected mitochondrial respiration by increasing the basal respiration in response to hypoxia.[162] Complex III showed high activity which is the main mitochondrial site for ROS production.[162] Interestingly, these effects were not seen in cells which lacked mtDNA hence it has been suggested that METTL4 can modulate mitochondrial respiration through its role on mtDNA.[162] Overall, this may suggest that METTL4, and hence 6mA methylation, is important in maintaining mitochondrial function in mammals.[162]

Other studies have also showed that 6mA may be elevated under stress in eukaryotes.[158,160,164] In *C. elegans*, the METTL4 ortholog, DAMT-1 was shown to respond to mitochondrial stress as *damt-1* null *C. elegans* failed to upregulate 6mA following stress compared to wild type.[160] The mitochondrial stress response significantly increased 6mA methylation and was found to be necessary for the transmission of mitochondrial stress adaptation in progeny.[160] 6mA methylation marked genes that were associated with mitochondrial stress response and promoted transcription thus alleviating stress in progeny.[158,160] Hence, it could be concluded that 6mA is a transgenerational epigenetic mark in *C. elegans*.

In mice, environmental stress significantly increased 6mA levels in the prefrontal cortex of the brain, and the genes associated with stress-induced 6mA changes (intragenic 6mA loss) overlapped with upregulated neuronal genes related to neuropsychiatric disorders in mice.[158,164] In contrast, however, the unicellular eukaryote *Tetrahymena thermophila* showed decreased levels of

6mA when exposed to stress via starvation, albeit asymmetric 6mA methylation sites with a high methylation frequency did increase despite the overall reduction in 6mA.[191] These contradicting methylation responses to stress could be associated with the differences in characteristics between unicellular and multicellular eukaryotes. Generally, 6mA in unicellular eukaryotes is thought to be preferentially located in ApT dinucleotides and is associated with actively transcribed genes, whereas this is the opposite for multicellular eukaryotes.[191]

Overall, there is evidence that 6mA methylation is a dynamic response to stressors in eukaryotes and is unique to each eukaryotic species. Further research is required to determine how stressors can impact methylation and the downstream effects.

1.5 Thesis aims

From the literature discussed, it is apparent that there is substantial evidence of the effects of insecticides on flight performance and cognitive functioning in bees. However, less is known about the effects on mitochondrial functioning, particular in non-*Apis* species. The vast morphological and phenotypical differences between bee species and castes makes it important to study other species so we can better understand the effect that insecticides, such as neonicotinoids, have on our pollinators. The first study of this thesis aims to address this by using the Oroboros Oxygraph-2K for high-resolution respirometry to analyse the effects of the neonicotinoid, imidacloprid, in the brain and thoracic muscle of *Bombus terrestris.*

The use of tethered flight mills enables the evaluation of flight activity in a laboratory-controlled environment. Therefore, by using this approach the relationship between flight performance and mitochondrial function in the same individual bee can be analysed, which has never before been accomplished. Additionally, employing this methodology allows for the utilisation of bees as a model in broader research concerning xenobiotics, potentially impacting exercise performance and mitochondrial function. This

thesis aims to showcase this within the second study by determining the effects of exercise (using flight as a proxy) and the antipsychotic drug, olanzapine, on mitochondrial function using *B. terrestris* as the model organism.

Finally, the presence of 6mA methylation in eukaryotic DNA remains disputable and more evidence is required to convince the scientific community in one way or another. Epigenetic modifications, particular 5mC DNA methylation, have been accurately studied in eusocial bees and it could be argued that they are prime model systems for elucidating as to whether 6mA methylation may be present. Therefore, the final study of this thesis aims to determine the possible presence and quantity of 6mA methylation in genomic DNA and mtDNA of eusocial bees. In addition, it also aims to elucidate a potential function of 6mA methylation in bees and review the likelihood of 6mA methylation in eukaryotic DNA in general.

Overall, the research presented within this thesis aims to provide a greater insight into the mitochondrial function and epigenetics of eusocial bees. Furthermore, it aims to elucidate the effects that exercise, and other stressors, may have with the use of novel methodologies.

1.6 Hypothesis

Based upon current literature and previous research which has guided the experiments in this thesis, I firstly hypothesise that mitochondrial function in *B. terrestris* is influenced by caste-type, flight activity, and the exposure of the neonicotinoid, imidacloprid. The research presented in chapters one and two aim to accept or reject this hypothesis. Secondly, based on the importance of environmental factors in the epigenetics of eusocial bees, I hypothesise that 6mA methylation, if present, may be associated with environmental influence or stressors in *A. mellifera* and *B. terrestris*. The third chapter in this thesis aims to address this hypothesis.

2. Methods

During the course of the research described in this thesis I used three primary methods to obtain the genomic and mitochondrial data: high-resolution respirometry, the immuno-southern (iSouthern) method, and third-generation sequencing. All methods used frozen or fresh tissue taken from either the thoracic muscle or head of the bee.

2.1 Ethical statement

The use of bumblebees and honeybees used in the research of this thesis are not covered by the Animal (Scientific Procedures) Act (ASPA). However, all studies within this thesis were conducted in an ethical manner. Bees were handled with the upmost care and with the most appropriate and ethical approach. Only the necessary number colonies were purchased in each study to help minimise insect use. When euthanasia was required, the method was either via freezing or amputation of the head.

2.2 High-resolution respirometry (HRR)

To determine the mitochondrial functioning in bees I used high-resolution respirometry (HRR). This allows the precise measurement of oxygen consumption in a closed chamber. As the sample consumes oxygen during mitochondrial respiration the oxygen concentration in the chamber decreases. This approach was first constructed in 1955 by Chance and Williams.[192] However, advancements in the field have led to the development of modern instruments such as the Oroboros Oxygraph (Oroboros® Instruments, Innsbruck, Austria). The Oxygraph-2k can detect oxygen consumption at a much higher accuracy by using highly sensitive electrodes; therefore, enabling the detection of even subtle changes that can suggest mitochondrial dysfunction.[193] Furthermore, the Oxygraph-2k uses substrate-uncoupler-inhibitor titration (SUIT) protocols to measure oxygen consumption in various respiratory states and analyse numerous mitochondrial pathways.[194] The ability to use a variety of mitochondrial preparations including isolated mitochondria,

tissue samples, and living cells further allows its application to be applied in an expanding number of studies, including those I present in this thesis.[194]

A standard SUIT protocol, such as that used in chapter 3 of this thesis, measures different respiratory states including, routine, LEAK, electron transport capacity (ET capacity) and residual oxygen consumption (ROX). During routine respiration, the tissue or mitochondrial sample is added to the chamber and left to stabilise before the oxygen consumption measurement is taken. To measure the LEAK state, oligomycin is added to the chamber to inhibit ATP synthase and thus respiration is associated with the proton leak through the inner mitochondrial membrane. The maximum electron transport capacity can be determined by titrating the uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP) into the chamber in a stepwise manner until the optimum concentration of uncoupler is added. To determine the ROX (or non-mitochondrial respiration), the complex III inhibitor, antimycin A, is added to inhibit the electron transport system. Hence, any oxygen consumption in this state can be determined as non-mitochondrial and respiration values can be corrected for ROX as the baseline state.

Substrates such as pyruvate, malate, proline and succinate can also be included in protocols to determine respiration levels with these various substrates and elucidate how different complexes and pathways are functioning. The complex I inhibitor, rotenone, and the complex II inhibitor, malonate, can also be added to determine the functioning of the ETS after inhibition of complex I and complex II.

To normalise the data, the same amount of sample was added to the chamber. 1 mg of muscle tissue or one whole brain was added to the chamber as preliminary studies deemed these quantities appropriate.

Using the oxygen flux values, additional information can be obtained such as the flux control ration (FCR). The FCR can be calculated using the DatLab software (v7.4.0.4, Oroboros) and is defined as the ratio of oxygen flux at the different respiratory states (e.g. routine and LEAK) that are normalised

between 0 and 1. The lower limit (0) is set by the ROX state (inhibition by Antimycin A) and the upper limit (1) is set by the maximum ET capacity (CCCP). In addition to the FCR, the spare respiratory capacity (SRC) can also be determined. The SRC is the capacity to reach additional ATP demands that are greater than that of routine respiration. It can be calculated into a quantitative figure using the formula, SRC = [(ET capacity specific flux)/(Routine specific flux × 100)].

2.3 iSouthern

The immuno-southern (iSouthern) is a technique that Assoc. Prof. Reinhard Stöger established and is based on the combination of southern blotting and specific antibody binding to identify an antigen of interest. In this method, DNA is first separated by fragment size using restriction enzymes followed by gel electrophoresis. During the study described in chapter five of this thesis the restriction enzymes, MboI, DpnI and Sau3AI (New England Biolabs®) were used, alongside a water control. These enzymes all have the recognition site, GATC, and are adenine methylation-sensitive. DpnI cleaves at the recognition site when the adenine is methylated, whilst MboI cleaves when adenine is not methylated. Sau3AI is not methylation-sensitive and will cleave irrespective of methylation making it a suitable control enzyme to demonstrate that the DNA can be fully cleaved by restriction endonucleases. The use of water to replace the enzyme is another method of control to ensure enzymatic activity and efficacy.

Once the DNA is digested, gel electrophoresis is used to determine whether the DNA has been fragmented by the enzymes or water control and suggests whether 6mA methylation may be present. The gel is then transferred to a nitrocellulose membrane via Southern blotting. The membrane is then washed in a primary antibody of interest followed by a secondary antibody that in conjugated with horseradish peroxidase (HRP). Using enhance chemiluminescence (ECL) the binding of the primary antibody to the DNA can then be visualised (**Figure 2.1**).

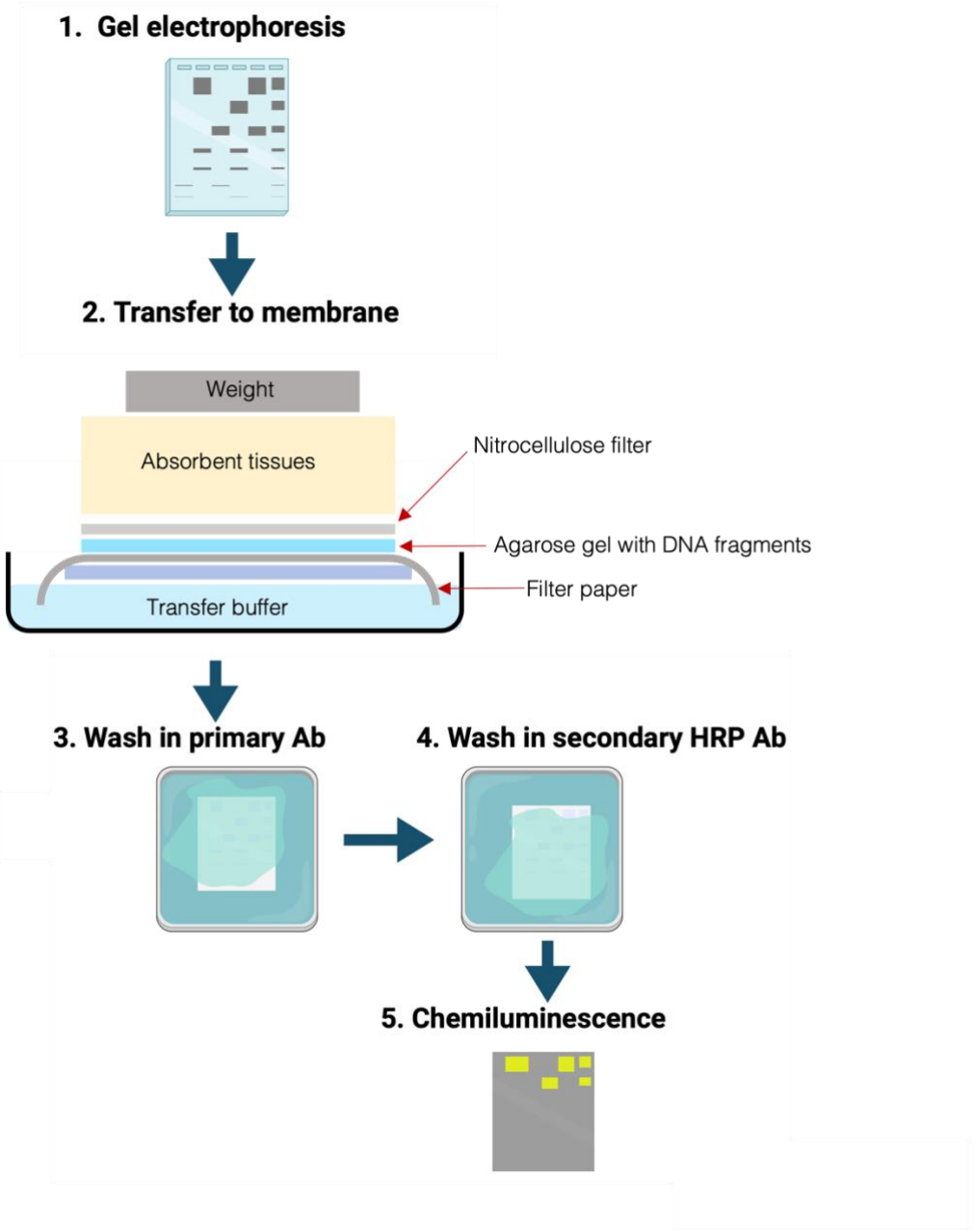

Figure 2.1: Diagram of the iSouthern method. Firstly, the DNA is separated by size via gel electrophoresis. A Southern blot is then performed to transfer the DNA fragments to a membrane. The membrane is then washed in a primary antibody followed by a secondary antibody labelled with HRP. The HRP causes the fragments to glow when exposed to chemiluminescent substrates and therefore indicates where the antibody is bound in the DNA.
Image created with BioRender.com

2.4 Third-generation sequencing

Over the past two decades next generation sequencing (NGS) has revolutionised genomic research and allowed scientist to sequence DNA and RNA at high accuracy.[195] However, NGS provides average read lengths of ≤ 300 bp which can be problematic when trying to assemble long repetitive regions of a genome with reads that are shorter in length than these regions.[195,196] To address this, a new era of sequencing termed third-generation sequencing (TGS) from companies including Oxford Nanopore Technologies and Pacific Biosciences emerged.[197] Unlike previous sequencers, TGS can produce ultra-long read sequencing (> 500 bp) in real-time at a single base accuracy.[195,196,198]

In 2014 Oxford Nanopore first released the MinION; a small portable sequencer capable of sequencing ultra-long reads in real-time.[198] The Oxford Nanopore sequencing technology involves measuring the changes in electrical current as the DNA passes through a protein nanopore. As each of the four different nucleic acids (adenine, thymine, cytosine, and guanine) pass through the pore they produce a unique disruption in the electric current. Using basecalling algorithms the raw signal is then translated to reveal the DNA or RNA sequence (**Figure 2.2**). Furthermore, epigenetic modifications can also be detected as methylated nucleotides elicit a different electrical signal than their corresponding canonical base.

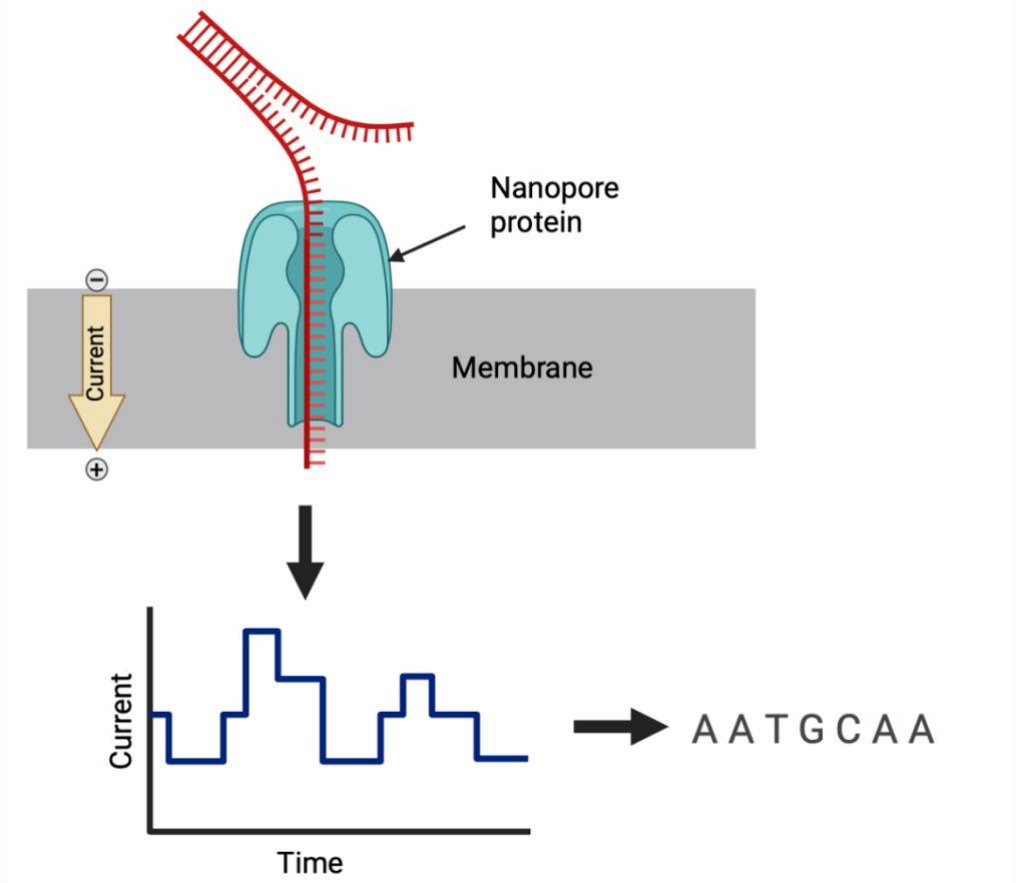

Figure 2.2: Diagram demonstrating nanopore sequencing. As the DNA flows through the protein nanopore each nucleotide base is identified by a characteristic disruption in the current. The raw electrical signal is then decoded into the DNA sequence.
Image created with BioRender.com

All DNA sequencing described in this thesis was performed using the Oxford Nanopore MinION (Oxford Nanopore Technologies) using the rapid sequencing kit.

DNA used for sequencing was first measured using the NanoDrop spectrophotometer to determine the quantity and quality. A minimum of 100 ng of DNA was required to run the sequencing for the R10.4.1 flow cells and corresponding kit and ~400 ng of DNA was required to run the sequencing with the R9.4.1 flow cells and kit.

Once sequenced, reads were analysed with basecalling softwares including Guppy (v 6.1.1)(Oxford Nanopore Technologies) or Dorado (v 0.5.1) (Nanoporetech). During basecalling, reads were filtered by phred q-score and those with a mean q-score < 7 (error probability of 0.1995) failed to meet the threshold and were removed. The Phred quality score (q-score) is used to calculate the accuracy of base identification during sequencing. Hence, for our analysis only reads with a basecall accuracy of $\geq 80.0\%$ (q-score ≥ 7) were used for analysis.

Genome coverage and mean sequencing depth were also determined. The mean sequencing depth refers to the number of times a base was covered during sequencing. A low sequencing depth means the sequencing is less reliable as the base was only sequenced a few times as opposed to a high depth which would mean greater sequencing accuracy.

3. Chapter 1: Acute imidacloprid exposure alters mitochondrial function in the bumblebee flight muscle and brain

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3.1 Abstract

Mitochondria are intracellular organelles responsible for cellular respiration with one of their major roles in the production of energy in the form of ATP. Activities with increased energetic demand are especially dependent on efficient ATP production, hence sufficient mitochondrial function is fundamental. In bees, flight muscle and the brain have particularly high densities of mitochondria to facilitate the substantial ATP production required for flight activity and neuronal signalling. Neonicotinoids are systemic synthetic insecticides that are widely utilised against crop herbivores but have been reported to cause, by unknown mechanisms, mitochondrial dysfunction, decreasing cognitive function and flight activity among pollinating bees. Here we explore, using high-resolution respirometry, how the neonicotinoid imidacloprid may affect oxidative phosphorylation in the brain and flight muscle of the buff-tailed bumblebee, *Bombus terrestris*. We find that acute exposure increases routine oxygen consumption in the flight muscle of worker bees. This provides a candidate explanation for prior reports of early declines in flight activity following acute exposure. We further find that imidacloprid increases the maximum electron transport capacity in the brain, with a trend towards increased overall oxygen consumption. However, intra-individual variability is high, limiting the extent to which apparent effects of imidacloprid on brain mitochondria are shown conclusively. Overall, our results highlight the necessity to examine tissue-specific effects of imidacloprid on respiration and energy production.

3.2 Introduction

Intensification of agriculture has driven a global increase in pesticide use and fragmentation of pollinator habitats, often leading to a sparsity of resources within the foraging range of bees.[199,200] This requires bees to fly greater distances, thus increasing the energy demand of foraging. At the cellular level, energy is produced in the form of adenosine triphosphate (ATP) *via* oxidative phosphorylation (OXPHOS) within mitochondria.[93] During OXPHOS, ATP is synthesised *via* the electron transport chain located in the inner mitochondrial membrane; electrons are transported *via* a series of carriers and protons are pumped into the intermembrane space producing a proton gradient which drives ATP synthesis *via* ATP synthase.[201] It has been reported that the rate of electron transfer between OXPHOS enzymes in bees are the highest measured in any animal.[113]

Carbohydrate, predominately in the form of trehalose, is the main source of energy in the nervous system and flight muscles of bees, however tissue-specific differences have been shown.[110,113] In the brain, evidence of β-oxidation to metabolise fatty acids and contribute to the high energetic demands has been reported, however there is little evidence of fatty acids fuelling flight in the thoracic muscle.[110] Furthermore, glycogen is stored in the flight muscles which may be utilised to extend flight duration.[113] These differences reinforce the importance of looking at multiple tissue types when analysing the mitochondrial function in bees.

In bees and other Hymenopterans, the brain and flight muscle have particularly high densities of mitochondria to facilitate the substantial energy demands of neuronal signalling and flight activity.[93,110,113] There is increasing evidence of an association between brain mitochondrial OXPHOS and behaviour in bees, suggesting that alterations in brain mitochondrial function, such as those caused by neonicotinoids, could induce changes in behaviours, such as aggression.[108] External factors that affect mitochondrial function may have the greatest impact within tissues with the highest energy demands.

Neonicotinoids are a widely used group of systemic insecticides which have been shown to affect pollinator learning, memory, homing, and flight capacity.[202,29] These compounds target nicotinic acetylcholine receptors (nAChRs); the most prevalent excitatory neurotransmitter in the central nervous system of insects.[11,203] Neonicotinoids impair mitochondrial function and structure in bumblebees and honeybees[9,23,204] and exposure to the neonicotinoid imidacloprid leads to premature flight exhaustion and altered cognitive performance.[16,205] This may be associated with a transient excitatory affect caused by the overstimulation of nAChRs by imidacloprid and potentially lead to a reduction in flight activity and foraging capacity.[16] The decline in flight activity associated with pesticide exposure may be due to insufficient ATP production in the flight muscle leading to premature exhaustion and flight inactivity. As mitochondria are responsible for the majority of ATP production during insect flight, an imidacloprid-induced impairment of mitochondrial function may impact on flight performance and neuronal signalling.[206]

Here we seek to determine if and how acute exposure to field-realistic doses of imidacloprid may affect mitochondrial function and OXPHOS in the bumblebee, *Bombus terrestris*. Using high-resolution respirometry we analyse mitochondrial respiration in the brain and flight muscle of female worker bees.[207] We analyse the oxygen consumption of three different respiration states: (i) Routine—the rate at which OXPHOS occurs in cells in the physiological coupling state, where ATP synthesis is coupled with the electron transport chain (ii) LEAK— a non-phosphorylating resting state of uncoupled respiration by the inhibition of ATP synthase; hence, oxygen consumption is associated with proton leak through the inner mitochondrial membrane and not *via* ATP synthesis-linked respiration; and (iii) maximum electron transport capacity (ET capacity)—the maximum rate of the electron transport pathway when not coupled to ATP synthase and at an optimum concentration of uncoupler.[207] By analysing these three states we aim to identify if and how imidacloprid may affect the OXPHOS system.

3.3 Materials and Methods

3.3.1 Bee husbandry

Commercial queen-right *B. terrestris audax* colony were obtained from Biobest®(Westerlo, Belgium) between November 2020 and June 2021, and maintained at 26˚C and 33% relative humidity. We fed the colony and age-matched laboratory reared bees on 2.0 M sucrose solution and pollen (purchased from Agralan, UK) provided *ad libitum*. To create cohorts of bees of a similar age, pupae were harvested and stored in a separate plastic container adjacent to the colony until eclosion. Once eclosed, bees were placed into cohorts of 1–3 bees and contained in plastic deli pots (115 × 75 mm) until used for high-resolution respirometry (HRR).

3.3.2 Imidacloprid treatment

Individual bees were taken from their cohort and placed in a queen marker tube where they were starved for 1 h to increase the likelihood of feeding on the diet subsequently provided. For the insecticide exposed bees, a 2.92 M sucrose solution containing 10 ppb imidacloprid was then used to feed the bees. This field- realistic concentration was selected as it has been previously reported in pollen and nectar, foraging bees, and inside colonies.[16] For the control bees, feeding was with 2.92 M sucrose solution only. Bees were allowed to feed for 10 mins and the quantity consumed was recorded. Any bees which did not feed during this period were excluded from the experiment. Thirty minutes after the end of feeding, bees were cold anesthetised (flight muscle $n = 4$, and brain $n = 7$; for both control and IMD groups).

3.3.3 Flight muscle and brain tissue dissection

Cold-anaesthetised bees were killed by decapitation. The thorax was photographed to later measure the inter-tegular distance (representing body size) for each bee using ImageJ software[208]. The brain or a small section of

flight muscle were removed, using a scalpel and forceps, and then individually weighed in 1.5 ml tubes. Individual bees representing biological replicates were used for each tissue type sample. Then 100 µl of MiR05 buffer (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM Lactobionic acid, 20 mM Taurine, 10 mM KH_2PO_4 , 20 mM HEPES, 110 mM D- Sucrose, 1g/L BSA, pH 7.1) per 1 mg of muscle, or one whole brain, was added and the tissue mechanically homogenised. Then 100 µl (1 mg) of muscle tissue homogenate and all the brain homogenate were transferred into a high-resolution respirometry chamber for analysis.

3.3.4 High-resolution respirometry

High-resolution respirometry (HRR) was carried out using an Oroboros Oxygraph-2k (Oroboros® Instruments, Innsbruck, Austria). The respirometer electrodes were calibrated daily to ensure oxygen concentration remained consistent for the duration of the experiment. All respiratory analyses were performed at 35°C as previous studies have reported this close to the average thoracic and head temperature of *B. terrestris* during flight.[102,209] Oxygen consumption of one brain and 1 mg flight muscle were analysed using the SUIT-003 protocol.[207,210] Routine respiration was measured followed by the addition of 1.0 µl of 5 µM oligomycin to determine LEAK state (inhibition of ATP synthase). Uncoupled maximal electron transport state was then measured by subsequent 10.0 µl titrations of 0.5 µM carbonyl cyanide m-chlorophenyl hydrazone (CCCP). Finally, 1 µl of 2.5 µM Antimycin A to determine the residual oxygen consumption (ROX) (**Figure 3.1**).

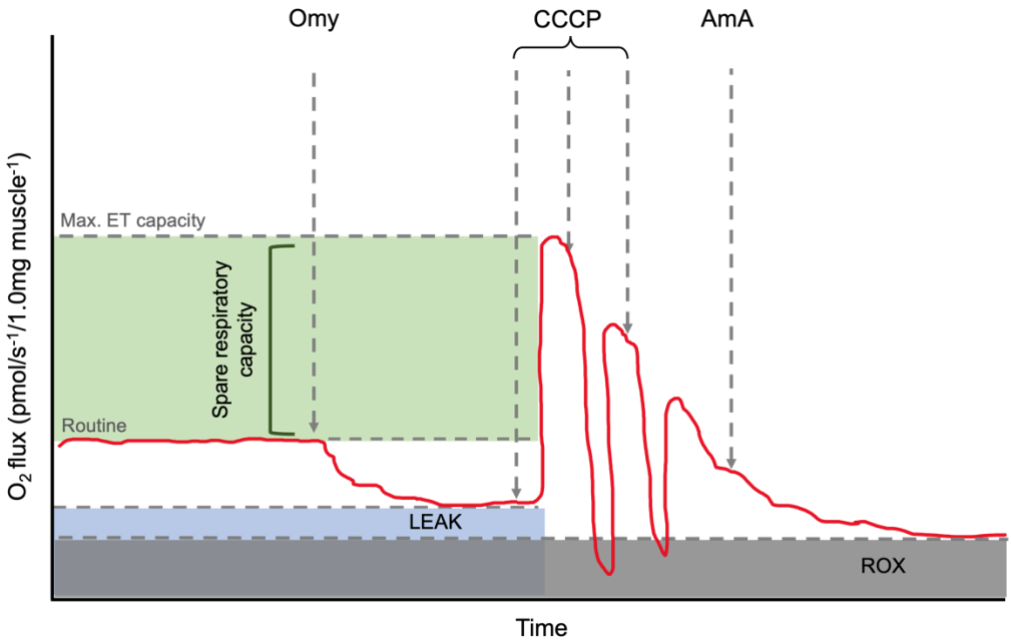

Figure 3.1: Annotated representation of the O2k oxygraph output. The red line is the oxygen flux. Routine is shown before the addition of Omy. LEAK state can be determined after the inhibition of ATP synthase by Omy. The maximum electron capacity is determined as the highest peak after CCCP titrations. The spare respiratory capacity and its relative value can be calculated *via* the calculation, [(ET capacity specific flux)/(Routine specific flux × 100)]. AmA, a complex III inhibitor, is added to determine the residual oxygen consumption (ROX) as the baseline state and allow for background correction. Abbreviations: Omy: oligomycin; CCCP: carbonyl cyanide m-chlorophenyl hydrazone; AmA: antimycin A.

3.3.5 Data analysis

Raw data outputs were acquired using the O2k-Software (DatLab v7.4.0.4, Oroboros), for data acquisition and analysis to determine instrumental background corrected oxygen flux values. Data were subsequently analysed using R Team.[211] Unpaired Student's *t*-tests were performed to determine whether there were differences between the mean oxygen flux at routine, LEAK and maximum ET capacity states, as well as the mean flux control ratios (FCRs) for routine and LEAK. The FCR can be defined as the ratio of oxygen flux in the different respiration states (routine and LEAK) that are

normalised to obtain lower [residual oxygen consumption (ROX)] and upper (maximum ET capacity) limits of 0–1.[212] Each of these tests used adopted a Type I error rate of $\alpha = 0.5$, with interpretations controlled for multiple comparisons as detailed in the supplementary materials. Oxygen consumption was also analysed in relation to the amount of sucrose consumed, bee age, and bee size (inter-tegular distance, ITD). There was no significant difference found in mean bee age or size between treatment groups in either tissue (**Supplementary Table S3.1**). However, as a range of ages were used in both groups, one-way ANOVAs were used to check for potential age-related effects on mitochondrial function: age did not significantly affect any of the respiration states in the flight muscle or brain. To determine whether the amount of diet (sucrose solution with or without imidacloprid) consumed correlated with oxygen flux, Pearson's correlation coefficients were calculated; there was no effect in either the flight muscle or brain (**Supplementary Table S3.3**).

3.4 Results

3.4.1 Flight muscle

Acute oral exposure of imidacloprid in worker bees significantly increased routine respiration in flight muscle (Oxygen flux: $t_{df=6} = -3.58$, $p = 0.012$; FCR: $t_{df=6} = -2.50$, $p = 0.047$, **Figure 3.2**; **Table 3.1**, note that the difference in FCR did not remain significant after correction for multiple comparisons, **Supplementary Table S3.1**). There were no significant differences found between the LEAK and maximum ET capacity states when comparing exposed and control bees (LEAK: oxygen flux, $t_{df=6} = -0.96$, $p = 0.376$; FCR, $t_{df=6} = -1.45$, $p = 0.196$. Maximum ET capacity: oxygen flux, $t_{df=6} = 1.72$, $p = 0.136$, **Figure 3.2**; **Table 3.1**).

The spare respiratory capacity (SRC) was also calculated for the flight muscle. The SRC describes the mitochondrial capacity to reach additional ATP demands that are greater than routine respiration levels and can be expressed as a quantitative value using the formula, SRC = [(ET capacity specific flux)/(Routine specific flux × 100)][213] (**Figure 3.1**). Hence, the mean SRCs were 0.069 and 0.035 for the control and imidacloprid groups respectively. This may be explained by the increase in routine respiration but not the maximum ET capacity by imidacloprid which would result in a lower SRC.

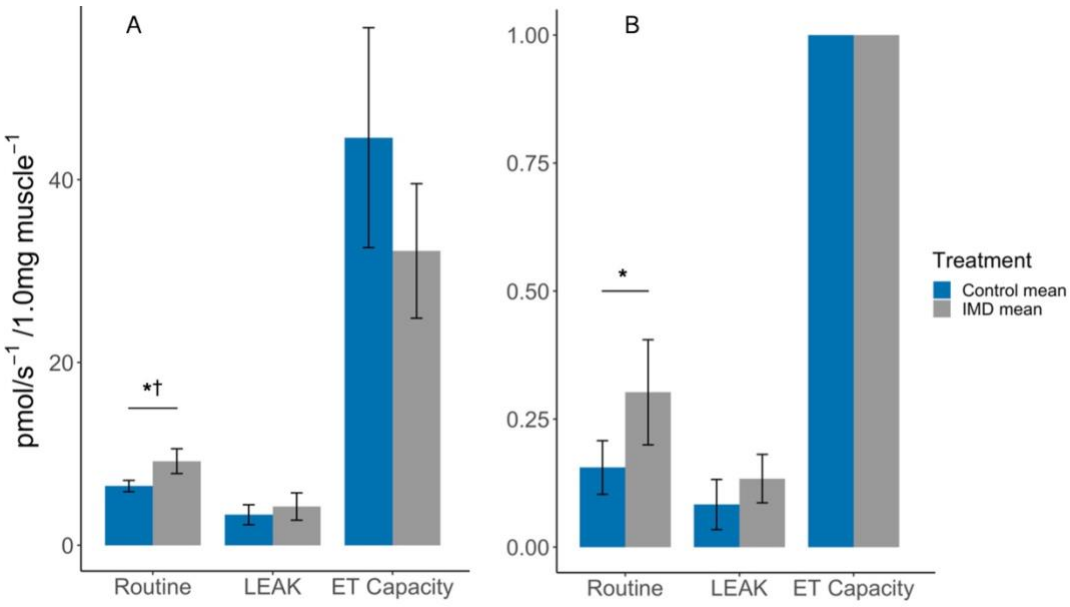

Figure 3.2: Effects of imidacloprid on flight muscle mitochondria at Routine, LEAK, and maximum ET capacity. States **(A)** Mean oxygen flux; and **(B)** Mean FCR, in flight muscle of imidacloprid and control worker bees. An unpaired Student's *t*-test was performed to determine significance between treatment groups. **p* < 0.05. *p*-values marked with † were also significant after controlling for Type I error rates. Error bars show 95% confidence interval. *n* = 4 for each of the control and imidacloprid groups. Routine: routine respiration rate; LEAK: electron/H^+ leak; ET capacity: maximum electron transport capacity.

Table 3.1: Mean oxygen flux and flux control ratio values in the brain and flight muscle of imidacloprid and non-imidacloprid fed worker bees.

	Flight Muscle			Brain		
Routine	Control n = 4	IMD n = 4	*p*	Control n = 7	IMD n = 7	*p*
Oxygen flux						
Mean (s.e.)	6.48 (0.32)	9.20 (0.69)	0.012*†	2.75 (0.60)	7.23 (2.07)	0.060
Variance	0.40	1.91		2.54	30.02	
FCR						
Mean (s.e.)	0.16(0.03)	0.30(0.05)	0.047*	1.18 (0.38)	0.99 (0.13)	0.639
LEAK						
Oxygen flux						
Mean (s.e.)	3.33 (0.56)	4.24 (0.76)	0.376	1.62 (0.37)	4.82 (1.74)	0.097
Variance	1.24	2.32		0.97	21.24	
FCR						
Mean (s.e.)	0.08(0.03)	0.13(0.02)	0.196	0.73 (0.26)	0.60 (0.15)	0.67
ET Capacity						
Oxygen flux						
Mean (s.e.)	44.60 (6.14)	32.21 (3.75)	0.136	3.11 (0.59)	6.59 (1.12)	0.017*†
Variance	150.83	56.38		2.42	8.76	

No flux control ratio (FCR) data are provided for the maximum electron transport capacity (ET capacity) as this is set to 1 to obtain the FCR for the routine and electron/H^+ leak (LEAK) states. *p*-values were obtained from unpaired Student's *t*-tests, with significance: **p* < 0.05. *p*-values marked with † were also significant after controlling for Type I error rates (**Supplementary Table S3.1**). IMD: imidacloprid; s.e.: ±1 standard error.

3.4.2 Brain

In brain tissue we found that the oxygen flux for imidacloprid fed bees had a significantly higher maximum ET capacity ($t_{df=12} = -2.76$, $p = 0.017$), but not in Routine and LEAK ($t_{df=12} = -2.08$, $p = 0.060$ and $t_{df=12} = -1.80$, $p = 0.097$, respectively) (**Figure 3.3**; **Table 3.1**). However, there were no significant differences between the treatment groups in terms of FCRs (Routine: $t_{df=12} = 0.48$, $p = 0.639$; LEAK: $t_{df=12} = 0.43$, $p = 0.674$). The SRC values were similar for both groups; 0.011 and 0.009 for control and imidacloprid treated bees, respectively.

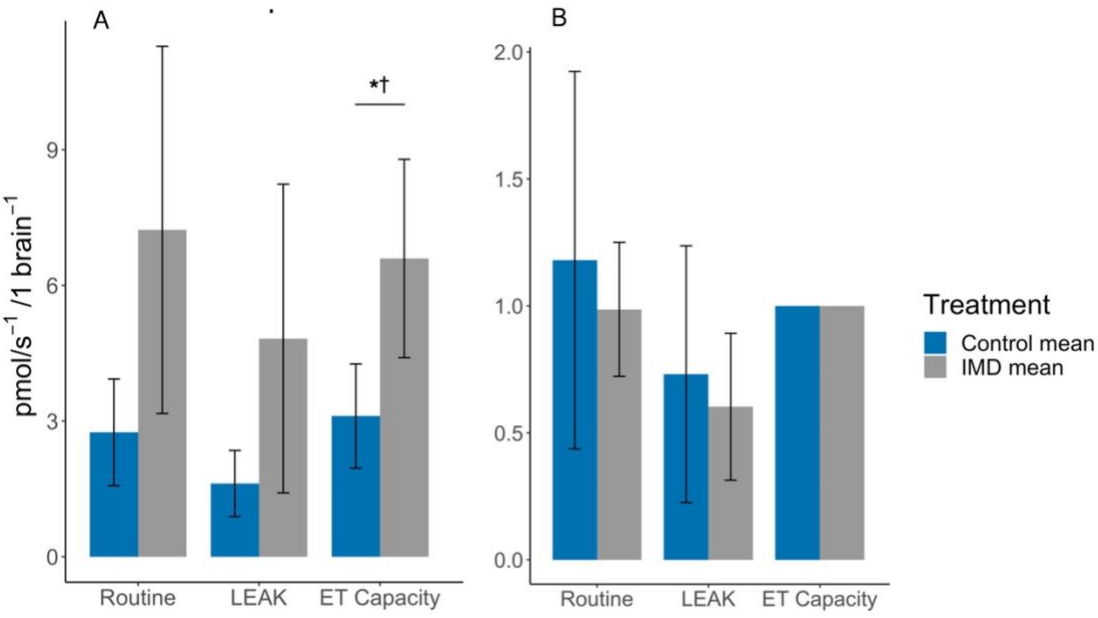

Figure 3.3: Effects of imidacloprid on brain mitochondria at Routine, LEAK, and maximum ET capacity. (A) Mean oxygen flux; and **(B)** Mean FCR, in the brains of imidacloprid and control worker bees. An unpaired students *t*-test was performed to determine significance between treatment groups *$p < 0.05$. *p*-values marked with † were also significant after controlling for Type I error rates. Error bars show 95% confidence interval. $n = 7$ for control and IMD groups. Routine: routine respiration rate; LEAK: electron/H^+ leak; ET capacity: maximum electron transport capacity.

3.5 Discussion

Acute imidacloprid exposure at the established field-realistic concentration of 10 ppb has been previously shown to affect flight activity in *B. terrestris* by increasing short-term velocity and decreasing the overall duration and distance flown.[16,214,215] This study has further shown that exposure to imidacloprid, at a concentration of 10 ppb, increases routine oxygen consumption in the flight muscles of workers. Imidacloprid targets acetylcholine receptors and can result in over-stimulation of the nervous system, initiating an excitatory response.[203] We suggest that an increase in oxygen consumption and respiration initiated by this response enables the short-term increase in flight velocity that has been previously reported.[16,72] The enhanced flight velocity in turn may lead to premature exhaustion resulting in a shorter flight duration.[16,216,217] Hyperactivity caused by neonicotinoids, such as imidacloprid, could also result in long-term muscle exhaustion and impaired energy metabolism as shown with thiamethoxam by Tosi *et al*.[72]

The higher routine oxygen flux and low spare respiratory capacity detected in the flight muscle of bees exposed to imidacloprid may be further factors contributing to the negative effects on flight activity. The SRC is defined as the mitochondrial capacity to reach additional ATP demands that are greater than routine respiration levels, and to thus prevent ATP crisis.[213] This is important in times of stress, exercise, and increased workload when the extra capacity is needed. Hence, the lower SRC in the imidacloprid-exposed bees, which was approximately half of the value for unexposed control bees, suggests a greater mitochondrial dysfunction during times of high ATP demand that is above the routine respiration rate, such as during flight. Imidacloprid increased routine respiration but did not affect the maximum ET capacity; the knock-on effect is likely a diminished SRC. We currently do not know for how long the effects of acute imidacloprid exposure on mitochondria functions last and if the resulting increases in routine respiration are transient. Therefore, the duration of lowered SRC will need to be determined in future follow-up studies.

In the brain, we detected no significant difference in routine and LEAK oxygen consumption between imidacloprid- exposed and control bees, however the maximum ET capacity in imidacloprid-exposed group showed a greater oxygen consumption. This indicates that acute imidacloprid-exposure results in elevated levels of oxygen consumption and thus, in higher maximum respiration rates. However, there was no detected difference in routine or LEAK FCRs, which are likely to be associated with the higher mean routine and LEAK oxygen consumption values. In addition, the FCR values suggest that the routine and LEAK values are in a similar ratio to the maximum ET capacity for both treatment groups. The similarity in the SRC values (0.011 for the control treatment and 0.009 for imidacloprid exposed bees) also illustrates this; these low values indicate that the routine respiration rate in the brain is at maximum ET capacity, as a SRC value of 0.01 represents that the oxygen consumption during routine and maximum ET capacity are equal. Therefore, it appears that imidacloprid increases overall oxygen consumption in the brain; however, due to high inter-individual variance, our results remain inconclusive. This high variability could be associated with the quantity of nAChRs in the brain, which is much greater than in the flight muscle and may therefore explain why there is lower variance among the flight muscles of imidacloprid-exposed bees and among control groups. Cognitive ability such as learning and memory varies among individual bees; this suggests that the brain is particularly susceptible to interspecific variation.[218,219] Hence, a possible variation in the number of neonicotinoid target sites among individual bees may lead to more variability in the response of imidacloprid on mitochondrial function.

Bees are exposed to imidacloprid both acutely and chronically, hence it is important to elucidate the mitochondrial function of both acute and chronic exposure. Chronic exposure of imidacloprid can lead to neuronal dysfunction.[9] Neurons require high levels of ATP, and a stable mitochondrial membrane potential is crucial to sustain ATP production at this level and thereby normal neuronal function.[9] Moffat *et al*.[9] also reported that acute exposure of 10nM (2.5 ppb) imidacloprid did not reach concentrations in the brain to induce membrane depolarisation. In contrast, 2-day chronic exposure of 1 nM led to

an increased sensitivity of bumblebee neurones to acetylcholine, and elicited mitochondrial depolarisation of neurons.[9] Our findings are in agreement with Nicodemo and colleagues[23], who reported tissue-specific sensitivity to acute imidacloprid exposure in the head and thorax of honeybees and negative effects on ATP synthesis. Our results will help resolve how acute imidacloprid exposure causes a different signature of mitochondrial activity compared to effects of chronic exposure. Moreover, it may now be possible to delineate where acute exposure ends and chronic effects begin.

An aspect of our study is that the worker bees were kept in an enclosed colony and were unable to fly. While this allowed a great deal of experimental control, it should be considered that exercise, such as foraging flights, can increase maximum ET capacity. Hence, mitochondrial function from laboratory-reared non-flying worker bees may vary from that of wild, free-flying and foraging worker bees. Another consideration is that social interactions may also impact brain energetics and mitochondrial function; all bees were kept in small cohorts within small containers from the time of adult eclosion, thus removing them from wider colony interactions and pheromones.[108] This may impact brain mitochondrial respiration and behaviour, which may in turn not provide a true representation of *B. terrestris* in a natural or agricultural environment. The effects of acute imidacloprid were only analysed at one time point, shortly after exposure; potential long-term implications of acute exposure on the mitochondrial function in bees remain to be determined. For example, whether mitochondria exposed to imidacloprid return to pre- exposure functioning and, if so, how long such recovery may take.

In this study we have further scrutinised how imidacloprid affects the different mitochondrial respiration states. We found that imidacloprid did not affect LEAK, indicating an increase in oxygen consumption is not associated with proton leak and thus imidacloprid does not seem to have an inhibitory effect on ATP synthase. The increase in routine respiration in the muscle suggests imidacloprid may affect the rate at which electrons flow through the electron transport chain, and thereby increases the rate closer to the maximum ET capacity *via* utilisation of the SRC. A similar mechanism of increased electron

transport rate could also explain how imidacloprid increased the maximum ET capacity in the brain. The SRC values were much lower in the brain compared to the flight muscle. This difference between the two tissues could explain how imidacloprid was able to increase the maximum ET capacity in the brain and not the flight muscle as only a slight increase in the rate would be capable of surpassing the maximum ET capacity. The exact mechanism by which imidacloprid affects the electron transport chain requires further investigation.

Overall, we found that acute exposure to imidacloprid, *via* feeding, increased the routine respiration rate in the flight muscle and showed a similar, marginally non-significant, trend towards higher respiration rates in the brain of workers. The higher routine respiration rates in the flight muscle may lead to short-term increases in oxygen consumption and potentially increase ATP production equating to a longer-term deprivation of energy. These results accord with those of previous studies which have shown flight duration and distance to be shorter in bumblebees exposed to imidacloprid. Negative impacts of flight activity will increase exhaustion and mortality of individual bees during flight and this reduced foraging capacity will lead to a reduction in pollination services within natural and agricultural landscapes. The broad range of responses in the brain by individual bees makes it difficult to draw clear conclusions from the measurements. Hence, more research is required to establish the impact of imidacloprid on the brain and elucidate possible explanations for the inconsistencies observed.

These results further contribute to our understanding of how sub-lethal levels of imidacloprid mediate tissue-specific effects on respiration and energy production and thereby impacts pollinator fitness. This study will provide a foundation for further research into the link between mitochondrial function and neonicotinoid exposure, as well as assist in the development and assessment of novel pesticides that minimise harmful effects on non-target species.

3.6 Author contributions

CS and RS designed the experiment with the additional support of LC and BE. Bee husbandry, laboratory work, and data collection were performed by CS. HRR was performed by CS with the support of LC and BE. Data analysis was performed by CS with supplementary analysis by CS and IH. Figures were prepared by CS. Paper was written by CS and RS with additional editorial assistance by LC, BE, IH and TD. All authors contributed to the article and approved the submitted version.

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3.9 Appendix A

3.9.1 Controlling for multiple comparisons

We tested for effects on brain and muscle tissue using 10 closely related statistical hypothesis tests on data from the same set of bees. Here we use the false discovery rate (FDR) procedure to control for Type I errors.[220,221] We treated results presented in Table 1 of the main paper as family of tests and set the family-wide α-value to a relatively stringent 0.10, which may in consequence generate Type II errors. The intially significant result for an effect of imidocloprid on flight muscle FCR was no longer significant but the other two signfiicant results from separate analyses retained their significance (**Supplementary Table S3.1**).

Supplementary Table S3.1: **Multiple comparisons testing of results reported in the main paper.** Results are reported in order of increasing *p*-value. FDR was set to 0.10.

Tissue and measure tested	*p*-values (from Table 2.1)	Benjamini-Hochberg significance
Flight muscle routine oxygen flux	0.012	Significant
Brain ET capacity oxygen flux	0.017	Significant
Flight muscle routine FCR	0.047	Not significant
Brain routine oxygen flux	0.06	Not significant
Brain LEAK oxygen flux	0.097	Not significant
Flight muscle ET capacity oxygen flux	0.136	Not significant
Flight muscle LEAK FCR	0.196	Not significant
Flight muscle LEAK oxygen flux	0.376	Not significant
Brain routine FCR	0.639	Not significant
Brain LEAK FCR	0.67	Not significant

3.9.2 Analysis of the morphology of bees and external factors on HRR results in each treatment group

Supplementary Table S3.2: Mean ages, sizes, and sucrose consumed before high-resolution respirometry (HRR) analysis in worker bumble bees. Intertegular distance (ITD) was used as a measure of body size. Unpaired Student's *t*-tests found no significant difference in mean age (flight muscle: $t_{df=6} = -0.42$, $p = 0.691$; brain: $t_{df=12} = -0.47$, $p = 0.644$), ITD (flight muscle: $t_{df=6} = 0.41$, $p = 0.696$; brain: $t_{df=12} = -0.21$, $p = 0.840$) or the amount of sucrose consumed (flight muscle: $t_{df=6} = 0.18$, $p = 0.866$; brain: $t_{df=12} = -1.1$, $p = 0.278$) between treatment groups for both tissues.

	Flight muscle		Brain	
	Control n = 4	IMD n = 4	Control n = 7	IMD n = 7
Age (days)				
Mean	16	17	11	11
Range	14-20	15-20	5-14	10-13
Mean ITD (mm)	5.87	5.76	5.50	5.56
Mean sucrose consumed (mg)	81.9	77.0	48.0	59.5

Supplementary Table S3.3: **Correlation between sucrose consumption and oxygen consumption at three respiration states.** Pearson's correlation coefficient was calculated to determine association between the amount of sucrose consumed and oxygen flux and different respiration states.

	Flight muscle			Brain		
	t-statistic	*p-value*	*cor*	t-statistic	*p-value*	*cor*
Routine	$t_{df=6} = -0.446$	0.671	-0.179	$t_{df=12} = -0.58$	0.572	-0.165
LEAK	$t_{df=6} = -0.135$	0.897	-0.055	$t_{df=12} = -0.48$	0.639	-0.138
ET capacity	$t_{df=6} = 2.18$	0.072	0.666	$t_{df=12} = -0.26$	0.800	-0.075

4. Chapter 2: Use of the buff-tailed bumblebee as a model for xenobiotic research and development: Effects of olanzapine on acute exercise and mitochondrial function

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4.1 Abstract

Olanzapine is a second-generation antipsychotic used to treat schizophrenia; however, it has been associated with an increased risk of the development of metabolic syndrome in patients. Traditionally, invertebrates such as *Drosophila* are used in research studies, however, here we demonstrate the use of the bumblebee, *Bombus terrestris*, as a model organism for the analysis of olanzapine on exercise performance and mitochondrial function. Using a combination of tethered flight mills and high-resolution respirometry, along with the O2k-Fluo Smart-Module to measure precise ATP concentrations, we analyse the effects of olanzapine on flight performance and mitochondrial respiration and ATP production in bumblebees. In addition to this, we compare the mitochondrial function between two female castes and analyse the effects of electron transport inhibitors on ATP production to gain a greater insight into the bioenergetics of bumblebees. We show that olanzapine is associated with declines in flight velocity and mitochondrial function, particularly during routine respiration in the brain of *B. terrestris*. We also highlight the significant levels of ATP synthesised via glycolytic pathways in the brain of *B. terrestris* workers. Finally, we show that female queens have a significantly higher rate of mitochondrial oxygen consumption compared to workers, yet they exhibit similar changes in ATP synthesis in response to substrates and inhibitors suggesting oxygen consumption does not always correlate with ATP production.

4.2 Introduction

4.2.1 Atypical antipsychotics and metabolic syndrome

Second-generation or atypical antipsychotics were first introduced in the 1990s and are commonly used to treat psychiatric disorders including schizophrenia and bipolar disorders.[222,223] Compared to first-generation antipsychotics, which primarily targeted the dopamine D2 receptor, atypical antipsychotics bind to a greater variety of receptors including serotonin, muscarinic and histamine which results in much fewer extrapyramidal symptoms, such as dyskinesias, akinesia and akathisia.[224,225] However, the improved efficacy and reduced extrapyramidal symptoms of atypical antipsychotics is accompanied with an increased risk in metabolic syndrome (MetS) which is characterised by weight gain, hyperglycaemia, hypertension, dyslipidaemia, and type II diabetes.[222,223,226-228]

MetS is three-fold higher in patients with schizophrenia that are undergoing drug treatment compared to the general population.[223,227] Therefore, understanding the relationship between these antipsychotics and the development of MetS is not only fundamental in the development of antipsychotic drug research but also enables doctors to prescribe the most suitable treatments for each patient and increase drug adherence.

4.2.2 Olanzapine

Olanzapine is considered to have a much higher efficacy compared to other antipsychotics on the market and is one of the most frequently prescribed antipsychotics, however it is also associated with the highest risk of developing MetS.[222,226,227,229,230] The greatest increases in weight gain are seen in patients receiving olanzapine and clozapine and these two drugs in particular are associated with greater risks in hyperglycaemia and insulin resistance.[225] Congruent with olanzapine, clozapine is highly effective at treating the symptoms of schizophrenia, however it is also found to cause drug-induced MetS in patients.[226] Therefore, it appears that the most

efficacious drugs at treating psychosis also tend to have the greatest metabolic adverse effects.[225,231]

Olanzapine predominately acts via antagonism of the dopamine D2 receptor and the serotonin receptors, $5HT_{2A}$ and $5HT_{2C}$.[230,232] However, other receptors including D1, D3 and D4 dopamine receptors, $5HT_3$ and $5HT_6$ serotonin receptors, $\alpha 1$ adrenergic, histamine H1 and muscarinic receptors may also be targeted.[230,232]

4.2.2.1 Dopamine receptors in bees

Dopamine receptors are G protein coupled receptors (GPCRs) that consist of two classes that are found in both vertebrates and invertebrates; D1-like - and D2-like receptors.[233-235] D1-like receptors increase intracellular cAMP levels whilst D2-like receptors decrease cAMP in the presence of dopamine but despite this there is evidence that these receptors act in cohesion.[233-236]

In honeybees there are at least three different receptors; *Am*DOP1 and *Am*DOP2 which are D1-like receptors, and *Am*DOP3 which is a D2-like receptor.[233,234,237] These three receptors have been found in the mushroom body of adult worker brains.[233,234]

Dopamine acts upon the CNS and has been shown to regulate motor activity and olfactory learning in various animals, including honeybees and solitary bees.[235,238-240] Flight activity has been associated with dopamine in the brain and haemolymph of drone, worker and queen honeybees.[238] Unmated queens have been shown to have higher dopamine levels in the brain when performing mating flights, but this level decreases once egg laying begins.[238] Dopamine levels in the brain of workers were also found to be significantly higher than that in young nurse bees suggesting foraging and flight activity may be associated with changes in dopamine.[237,238] Farkhary *et al* found that the antipsychotic and dopamine receptor antagonist, flupenthixol, delayed flight initiation and reduced flight distance, duration, and velocity.[238] Dopamine has been associated with locomotor activity, not only with flupenthixol but also

with the dopamine receptor agonist 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene and suggests that a decrease in flight performance is associated with a dopamine receptor blockade.[233,238] Similar findings have also been reported in the bumblebee, *B. terrestris*. The dopamine antagonist, fluphenazine, slowed foraging initiation time and increased the time to complete tasks.[241]

4.2.2.2 5-Hydroxytryptamine (serotonin) in bees

Serotonin and its function in bees has not been widely researched and to date there is little known. In mammals there are seven 5-HT receptor classes; 5-HT_1 to 5-HT_7.[242] All seven receptors (apart from 5-HT_3 which is a ligand gated ion channel) are GPCRs; 5-HT_1 and 5-HT_5 couple to $G_{i/o}$ proteins and inhibit the synthesis of cAMP, whilst 5-HT_2 couple to $G_{q/11}$ proteins which activate β - phospholipase C enzymes subsequently initiating the hydrolysis of inositol phosphates causing an increase in cytosolic Ca^{2+}.[242] 5-HT_4, 5-HT_6 and 5-HT_7 are coupled to G_s proteins which increase cAMP.[242]

There are four 5-HT receptors that are known in honeybees; Am5-HT_{1A}, Am5-$HT_{2\alpha}$, Am5-$HT_{2\beta}$ and Am5-HT_7.[235,242,243] Am5-HT_{1A} has been located in neuronal tissues whilst Am5-HT_2 and Am5-HT_7 are more widespread and been detected in neural and peripheral tissues.[242,243]

The second-generation antipsychotic, clozapine, is structurally and pharmacologically similar to olanzapine and has been shown to be an effective antagonist for both Am-5$HT_{2\alpha}$ and Am-5$HT_{2\beta}$ receptors.[242] Furthermore, the blockade by Am-5$HT_{2\alpha}$ and Am-5$HT_{2\beta}$ by 5-HT antagonists was associated with an increase in sting responsiveness to shock.[235,242,244] This suggests that serotonin may have a role as a down-regulator in sting responsiveness.[235]

In male carpenter bees serotonin was shown to be associated with the timing of territorial flight behaviour during mating.[245] Serotonin levels increased later in the day which was proposed to be associated with males performing their territorial flights when females are most likely to be out foraging.[245] In

honeybees, foragers have been shown to have much higher levels of serotonin (along with dopamine) compared to nurse bees.[246] This serotonin increase is most prominent in the antennal lobe of the brain, which is involved in olfactory learning which is vital in foraging.[246]

Overall, understanding the role of serotonin in eusocial bees is still very much in its infancy, and with very little to no research performed in bumblebees it is an area of interest that requires further research in a variety of species.

4.2.3 Mitochondrial dysfunction and atypical antipsychotics

The increase in MetS in patients taking second-generation antipsychotics, such as olanzapine and clozapine, has been associated with mitochondrial dysfunction.[223,226,227,247] However, these attributes of mitochondrial dysfunction are also heavily associated with the development of MetS separately to anti-psychotic treatment.[226] Impairments in energy production, mitochondrial morphology and number are often found in patients with MetS.[226,227]

The exact mechanism of how antipsychotic drugs affect mitochondria, and subsequently induce metabolic side effects is still to be elucidated. However, several hypotheses have been proposed including, disruption in glucose and fatty acid metabolism, increase in ROS production and alterations in the electron transport system (ETS) and oxidative phosphorylation (OXPHOS) pathway.[225,226,227,247] In the brains of mice and rats, clozapine was shown to alter mitochondrial function and expression of proteins of the ETS and OXPHOS.[226,247]

Clozapine and olanzapine have been associated with increases in ROS production.[226,227] Clozapine has been shown to cause mitochondrial depolarisation and alter mitochondria morphology by initiating swelling.[226] Damaged mitochondria produce increased levels of ROS which further damages healthy mitochondria.[226,247] Hence, this increase in swelling could be

a potential mechanism to prevent ROS damage subsequent to increases in ROS production by the ETS.

Disruptions in glucose and fatty acid metabolism have been linked to the use of atypical antipsychotics.[227] Glucose is the main source for ATP production in the brain and is metabolised during both glycolysis and oxidative phosphorylation.[229,248,249] In schizophrenia, glucose metabolism is thought to be altered by the impaired oxidation of glycolytic proteins and dysfunction of ATP production.[229] Other studies have found that impaired plasma glucose levels were associated with antipsychotic treatment as non-diabetic schizophrenia patients presented with glucose dysregulation after starting antipsychotic medication.[250] The association between second-generation antipsychotics, such as olanzapine, on glucose intolerance has been linked to the wider receptor binding, in particular the binding to serotonin receptors.[250] Antagonism of the serotonin receptor 5-HT_{1A} was shown to decrease pancreatic β-cell responsiveness which in turn decreases insulin secretion and initiates hyperglycaemia.[250,251]

In summary, atypical antipsychotics are associated with the development of MetS, of which mitochondrial dysfunction is often a hallmark. However, these relationships are complex and may often have interactive and amplification effects. Nevertheless, understanding the impact of antipsychotics on mitochondrial function and the subsequent effects of MetS can enable the development of novel drugs and approaches that minimise these negative effects.

4.2.4 Metabolic syndrome and management by aerobic exercise

In patients with MetS, exercise could be a key factor in the management of their symptoms. The WHO guidelines currently recommend at least 150-300 minutes of moderate or 75-150 minutes of vigorous, aerobic physical activity a week for adults to keep healthy.[252]

There is a strong association between MetS and physical inactivity and poor cardiovascular fitness.[253] Furthermore, olanzapine has been associated with negative effects on muscle strength and aerobic endurance.[254] Moderate to vigorous exercise reduces insulin sensitivity, dyslipidaemia, hypertension and the overall risk of developing MetS.[228] Exercise has been shown to improve mitochondrial respiration and increase respiratory capacity.[255,256] It is therefore important to elucidate whether the development of drug-induced MetS, and associated mitochondrial dysfunction, can be mitigated via aerobic exercise in patients being treated with atypical antipsychotics.

4.2.5 *B. terrestris* as models for mitochondrial research

Eusocial bees make excellent models for mitochondrial research as the high metabolic demands of flight require efficient mitochondrial and OXPHOS functioning.[93,101,256] Furthermore, the use of tethered flight mills allows for a controlled analysis of flight performance.[16,257] Utilising these factors and methods we have developed a comprehensive study that enables us to gain an understanding of how olanzapine affects mitochondrial function and aerobic exercise in one model species.

In this study, we used the buff-tailed bumblebee (*B. terrestris)*, as an insect model. We firstly determined whether olanzapine affects overall flight performance. Secondly, we measured the effects of olanzapine and acute exercise (flight) on mitochondrial respiration and ATP production in the brain. Thirdly, we analysed the effects of olanzapine on flight performance. Finally, we compared ATP production in the brain between female bumblebee castes, taking into account the effects of ETS inhibitors on ATP production, to gain a greater insight into the bioenergetics of bumblebees (**Figure 4.1**).

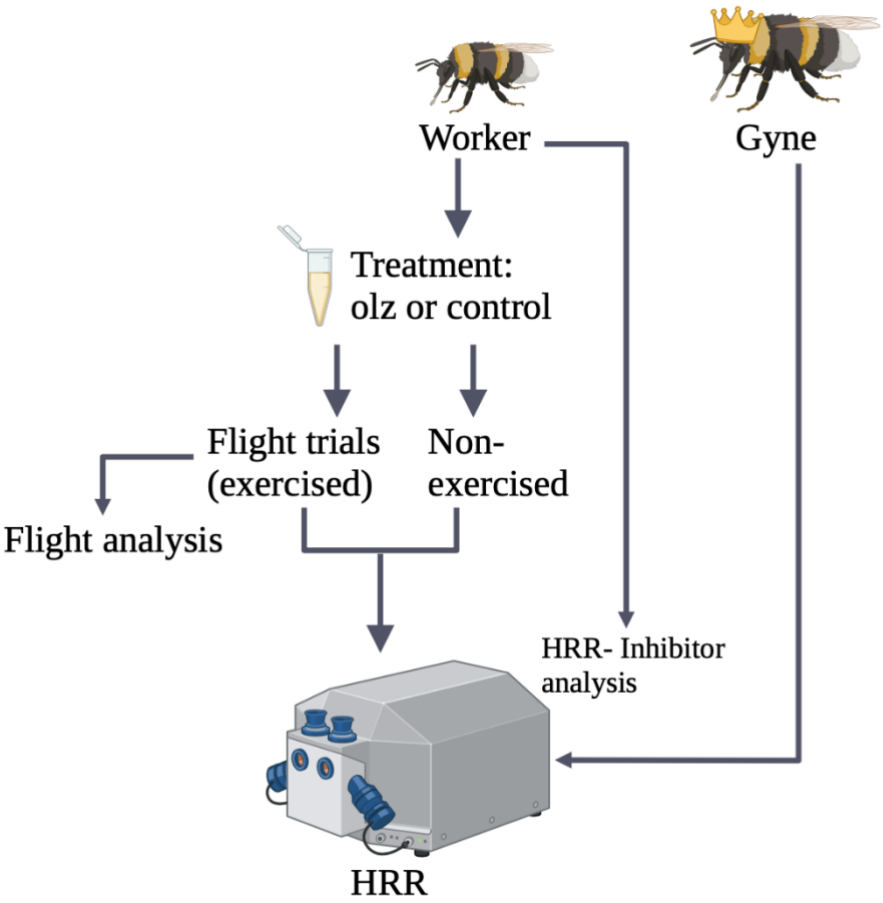


Figure 4.1: Diagram showing the experimental design. Workers were treated with olanzapine or the control before either undergoing a flight trial (exercised) or remaining enclosed in their pots (non-exercised). The brain tissue from exercised and non-exercised workers that received olanzapine, or the control were analysed by HRR. Flight data from workers which underwent flight trials was analysed to determine the effects of treatment on flight. The effect of ETS inhibitors on mitochondrial respiration and ATP production was analysed via HRR in the brain of non-exercised workers taken from the colony. Gynes were taken from the colony (non-exercised) and the brain was analysed via HRR to elucidate differences between *B. terrestris* castes.

Olz: olanzapine; HRR: High-resolution respirometry

Image created with BioRender.com

4.3 Methods

4.3.1 Bee husbandry

There were five queen-right *B. terrestris audax* colonies (A-E) used throughout the experiment. The first colony was purchased from Biobest® (Westerlo, Belgium) in March 2023 and the last in November 2023. Colonies were kept at 26ºC, 33% RH. Colonies were provided with sucrose supplied from Biobest®, and were given pollen (purchased from Agralan, UK) *ad libitum*. Generally, one colony was purchased at a time to limit the number of colonies required for the experiment and use the smallest number of bees as possible. The colony each bee derived from was noted to account for any colony variation.

4.3.2 Antipsychotic administration

Worker bees were removed from the colony and placed in plastic pots (115 x 75 mm) in cohorts of four. They were left without access to sucrose or pollen for a minimum of one hour. Once starved, the bees were placed into individual queen marker tubes and fed either 0.05 µg olanzapine dissolved in 2 µl DMSO or 2 µl of DMSO (control treatment). The 2 µl solution of 0.05 µg olanzapine + DMSO or DMSO were combined with 18 µl of 2.0 M sucrose solution, making the total volume 20 µl. The final olanzapine concentration in the sucrose solution was 8.0 µM. This concentration was deemed appropriate based on preliminary analyses and previous antipsychotic concentrations that have been used in *Drosophila*.[258] Only bees which consumed the 20 µl (complete dose of olanzapine or control) were used for flight trials and those that did not were excluded from the experiment. Bees were then left overnight in their cohorts with 2.0 M sucrose solution and pollen *ad libitum*.

4.3.3 Flight trials

The following day after treatment exposure the bees were immobilised by cooling and metal tags were superglued using a cyanoacrylate adhesive to the thorax between the wings (**Figure 4.2**). Bees were placed back in their respective pots for at least an hour before undergoing the flight trial.

The flight mills were developed by Lim *et al.*[259] The setup was similar to that described previously by Jyothi *et al* and consisted of four mills.[260] Each mill had a thin metal arm suspended between two magnets, which enabled low resistance movement in a circular trajectory with a circumference of 50 cm (**Figure 4.3**). The bee was attached on one end of the arm by the metal tag which slid tightly into a small plastic attachment on the mill. A small plastic disc with black and white stripes was attached to the centre axis which rotated with the arm. The light detector, which was connected to a computer, measured the number of turns of the disc and the computer calculated the distance flown and speed of flight for each mill. The distance flown (to the nearest 10 cm) was recorded and logged into a computer file and updated in five second intervals.

Attachment onto the mill took place under red light and was switched to white light once the trial begun. When not in flight, bees were rested on a plastic stand, which allowed them to remain attached to the mill but not suspended in the air.

Figure 4.2: Image of a bumblebee with the steel tag glued onto the thorax. Tags were made from steel wire with a small loop at the end which was superglued with a cyanoacrylate adhesive to the thorax of the bee.

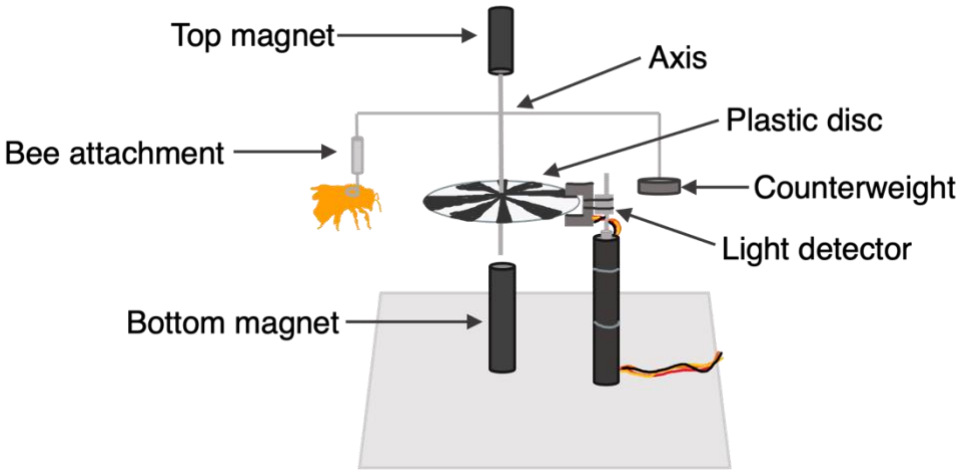


Figure 4.3: A schematic diagram of the tethered flight mill. The bee was attached to one arm of the mill via the metal tag and a counterweight was attached to the other arm. Magnets enable low friction suspension during flight with minimal resistance. The flight mill was connected to a computer which recorded in the speed and distance flown for each mill.

For the initial flight experiment, workers were allowed to fly for up to one hour. Bees were allowed a maximum of two stops during flight before the test was terminated on the third stop, or when an hour had elapsed. Bees were given three attempts to start flight and those that did not were marked as not initiating flight. For the second part of the experiment using high-resolution respirometry (HRR), only bees which flew for a minimum of five minutes (300 seconds) were included as 'exercised' bees.

Once the flight trial had ended, the bees which were not to be used for HRR were removed from the mills and were placed into a marked pot to be frozen. Bees undergoing HRR analysis were placed back into the plastic pot with sucrose and pollen *ad libitum* until analysis. HRR analysis took place within the same or the next working day but no longer than 24 hours after the termination of the flight trial. The thorax was photographed once the bees had been frozen or after they had been used for HRR and the inter-tegular distance (ITD) was measured using ImageJ software.[208]

4.3.4 High-resolution respirometry (HRR)

4.3.4.1 Bombus terrestris brain dissection

Bees were cold-anaesthetised before the brain was cut open and dissected using a scalpel and forceps. Individual brains were pooled; pool sizes ranged from 1-4. For each brain in a sample, 50 µl of magnesium-free MiR05 (60 mM lactobionic acid, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM D-Sucrose, 0.5 mM EGTA, and 1 g/L BSA (fatty acid free), pH 7.1) buffer was added and the tissue mechanically homogenised in a 1.5 ml microcentrifuge tube with a plastic pestle. Once homogenised, 50 µl (the equivalent of one brain) of the sample was added to the chamber for analysis.

4.3.4.2 Measurement of oxygen consumption

High-resolution respirometry (HRR) was performed using the Oroboros Oxygraph-2k (Oroboros® Instruments, Innsbruck, Austria). The electrodes were calibrated daily to ensure measurements remained consistent for the duration of the experiment. Respiratory analysis was performed at 35°C which has been reported to be close to the temperature of the head of *B. terrestris* during flight.[209]

For HRR analysis, the oxygen consumption at routine levels was first measured followed by the addition of the substrate's pyruvate and malate in the concentrations of 5 mM and 2 mM, respectively. Next 10 mM of proline was added, followed by titrations of the uncoupler, carbonyl cyanide m-chlorophenyl hydrazone (CCCP). Uncoupled maximal electron transport state was measured by titrating 5 mM CCCP, dissolved in ethanol, in an initial 5 µl titration (final concentration of 12.5 µM) followed by 2 µl titrations (final concentration of 5 µM) until the maximum oxygen concentration was reached. 1.5 µM rotenone was then added followed by 10 mM succinate. Finally, 7.5

µM of Antimycin A was added to determine the residual oxygen concentration (ROX)(**Supplementary Figure S4.1**).

4.3.4.3 Measurement of ATP

To measure the production of ATP, the Oroboros O2K-Fluo Smart-Module was used in conjunction with the measurement of oxygen consumption described in *4.3.4.2* (**Supplementary Figure S4.1**). The blue fluorescence-sensor with the MgG/CaG filter set was used to measure ATP production. When bound to free Mg^{2+}, which is released during ATP-ADP exchange across the inner mitochondrial membrane, Magnesium Green™ (MgG)(Thermo Fisher Scientific, M3733) fluoresces at a maxima of ~506/531 nm.[261] By determining the different dissociation constants for ADP-Mg^{2+} and ATP-Mg^{2+}, as well as factoring in the initial concentration of Mg^{2+}, the change in mitochondrial ATP concentration throughout the protocol can be calculated.

To determine the dissociation constants for ADP-Mg^{2+} and ATP-Mg^{2+}, the K_d of both ADP and ATP to Mg^{2+} using MgG was calculated in magnesium-free MiR05 buffer. This was done by first adding 1.1 µM MgG, followed by 5 nM oligomycin, 1 µM carboxyatractyloside (CAT), and 50 µM P1, P5-Di(adenosine-5')pentaphosphate (Ap5A). The fuel substrates (pyruvate, malate, proline, and succinate) and the mitochondrial sample (homogenised brain) were then added at the same concentrations as used in the respirometry protocol. A ten-step titration of 0.1 mM $MgCl_2$ was then performed which was followed by 19 stepwise titrations of ADP (0.25 mM per titration) and 11 stepwise titrations of ATP (0.2 mM per titration) in two separate assays. The K_d of ADP and ATP were then calculated using the Oroboros calibration spreadsheet which uses a least squares fitting method described in Chinopoulos *et al*.[262]

Shortly before the brain sample was added to the chamber for analysis, 1.1 µM MgG was added followed by 1 mM $MgCl_2$. The raw Mg^{2+} fluorescence values were recorded in two second intervals throughout the duration of the protocol.

4.3.4.4 Effect of ETC inhibitors on ATP production in B. terrestris

To determine the effect of the three electron transport chain inhibitors, rotenone, malonate and Antimycin A on ATP production an additional protocol was performed on *B. terrestris* workers. Bees were taken directly from the colony before dissection. All methods and concentrations of substrates and inhibitors remained as described in sections *4.3.4.1-4.3.4.3*. Once routine respiration was established, the substrates pyruvate and malate were added, and then proline. Rotenone was then added to determine the effects of complex I inhibition followed by the complex II substrate succinate. 5 mM of the complex II inhibitor malonate was then titrated which was followed lastly by the complex III inhibitor, Antimycin A (**Supplementary Figure S4.2**).

4.3.5 Data analysis

4.3.5.1 HRR and ATP data acquisition

HRR oxygen consumption data outputs were obtained using the templates provided with DatLab (v7.4.0.4, Oroboros) and used to calculate the background corrected oxygen flux values and flux control ratios (FCRs). The FCR is defined as the ratio of oxygen flux at the different respiratory states that are normalised between 0 and 1. The lower limit (0) is the oxygen consumption in the ROX state (inhibition by Antimycin A) and the upper limit (1) is the oxygen consumption at the maximum ET capacity (CCCP).[212]

K_d values and ATP production were determined using the templates provided by DatLab (v7.4.0.4, Oroboros) which followed the methods described previously.[262] To determine the changes in ATP production in comparison to routine levels, the K_d values for ATP and ADP were used to convert the fluorescent signal of free Mg^{2+} to ATP concentration.[262] The first Mg^{2+} value for routine ATP production was set to zero as the ADP levels were unknown. After the addition of each chemical a mean ATP value was calculated by

taking an average value at equilibrium, as shown in **Figure 4.4**. To increase accuracy, averages were only calculated using the Mg^{2+} values after 20 seconds had elapsed since the addition of another substrate or inhibitor. Furthermore, to account for variation, an average ATP concentration for routine was calculated; this was then subtracted from the mean ATP concentration calculated after the addition each substrate or inhibitor to determine the mean change in ATP concentration for each state.

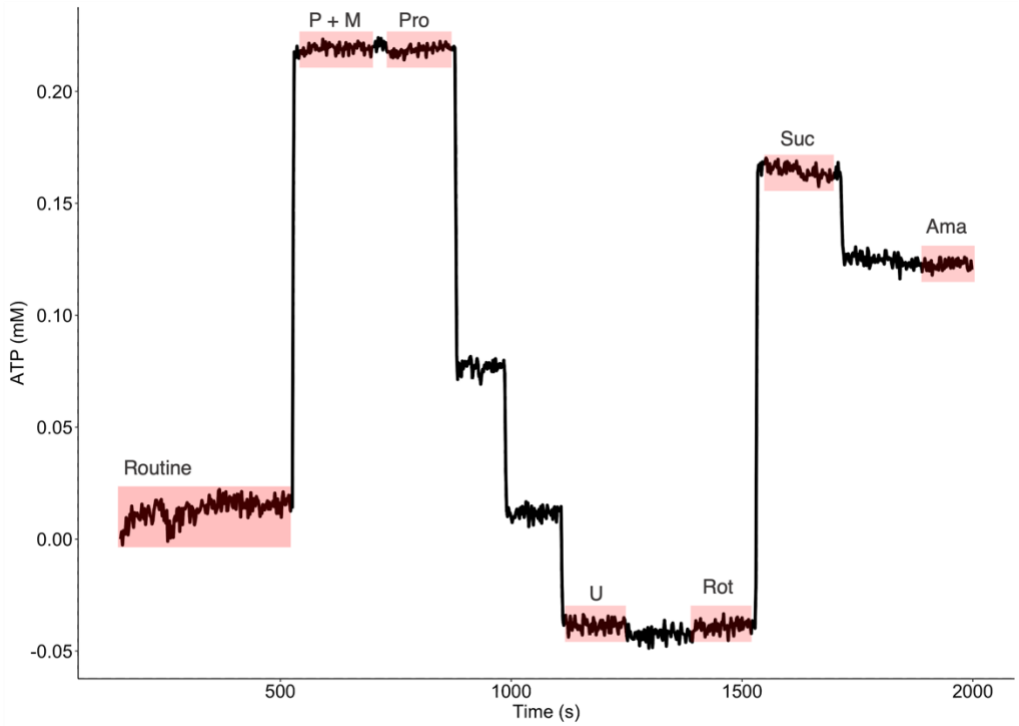


Figure 4.4: Annotated representation of the DatLab software output graph from the O2k fluo-module showing ATP concentration (mM) of *B. terrestris* worker brain. The red shading demonstrates where the mean ATP values were calculated. No averages were calculated using the values within 20 seconds before or after the addition of another substrate or inhibitor. For inhibitors Rot and Ama, the last 40 seconds (excluding the 20 seconds before the addition of Suc) were used to calculate the mean ATP concentration. Abbreviations - P+M: pyruvate + malate; Pro: proline; U: CCCP 'uncoupler'; Rot: rotenone; Suc: succinate; Ama: Antimycin A

4.3.5.2 Statistical analyses

All subsequent data and statistical analyses were performed using R v4.2.1.[211] For the analysis of flight data, statistical analyses including unpaired Student's *t*-test, one-way ANOVA and Kruskall-Wallis tests were done to determine whether colony or flight mill affected flight. Logistic regression was performed to determine the effect of olanzapine on flight initiation and linear regression was used to analyse flight performance. Two-way ANOVA were used to analyse the effects of olanzapine and exercise on oxygen consumption and ATP production in the presence of various substrates and inhibitors.

4.4 Results

4.4.1 Effects of olanzapine on *B. terrestris* flight

4.4.1.1 Olanzapine and flight performance

96 worker bees underwent a flight trial, of which 42 (43.8%) initiated flight and 12 (12.5%) flew for > 5 minutes. Of those 12 that flew, there was no significant difference between olanzapine and control on the distance flown (Wilcoxon rank sum test: Z = -0.243, W=16, p-value = 0.8763), flight duration (Z = -1.383, W = 9, p-value = 0.193), or mean velocity (unpaired Student's *t*-test: $t_{df=10}$ = 0.896, p-value = 0.391) (**Supplementary Figure S4.3**).

4.4.1.2 Effect of colony and flight mills on flight statistics

There was no effect of colony on flight duration or distance, however there was an effect on the mean velocity of flight. Bees from colony B flew significantly faster than those from colony A (unpaired Student's *t*-test: $t_{df=10}$ = -2.68, p-value = 0.023) (**Figure 4.5**).

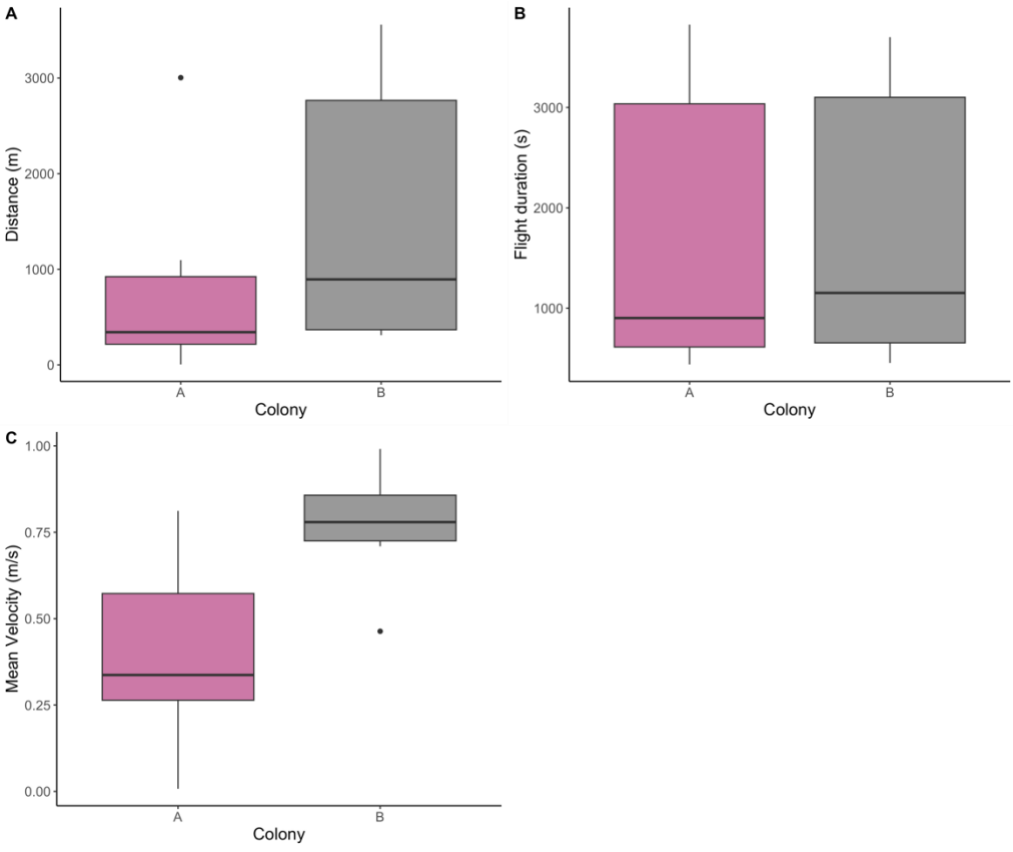


Figure 4.5: Boxplots of the flight statistics between colony A and colony B. The distance flown and flight duration between colony A (olanzapine: n = 3; control: n = 3) and colony B (olanzapine: n = 2; control: n = 4) did not vary, however, the mean velocity of flight in colony B was significantly faster ($t_{df=10}$ = -2.68, p-value = 0.023).

One-way ANOVA and Kruskall-Wallis showed there was no effect of the flight mills (1-3) on the distance flown, flight duration or mean velocity (**Supplementary Figure S.4.4**).

4.4.1.3 Olanzapine and flight initiation

More than half (56.3%) of workers did not initiate flight (flew < 5 minutes). To determine whether olanzapine had any effect of flight initiation a binomial logistic regression was performed to predict the probability of flight initiation by worker size (ITD) and treatment type. We found that neither size nor treatment affected the probability of flight initiation (**Supplementary Figure S4.5**)

4.4.1.4 Olanzapine and mean velocity of flight

Of the bees which flew > 5 minutes (n = 12), a linear model was performed to analyse the relationship between distance flown and mean velocity of flight between treatments (**Figure 4.6; Table 4.1**). We found that distance flown, treatment and colony had a significant effect on the mean velocity of flight. Assuming the distance flown remained constant, the coefficients suggested workers that received olanzapine flew 0.211 m/s slower than the controls, and bees in colony B flew on average 0.226 m/s faster than bees from colony A. The linear model also showed that for each metre flown, it was associated with an increase in velocity of 0.0001530 m/s.

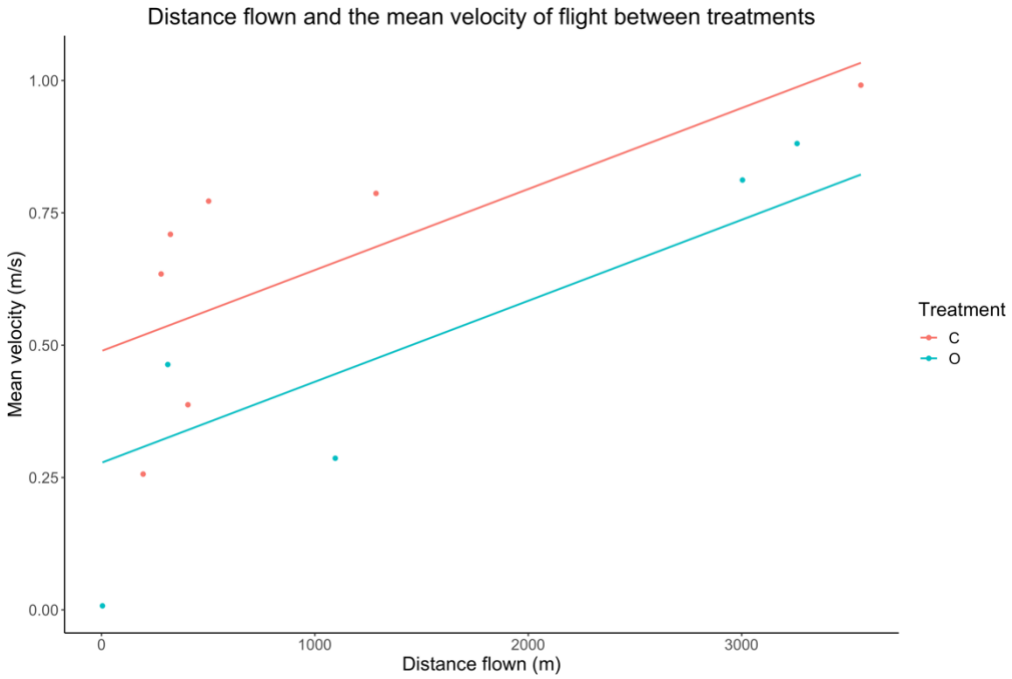


Figure 4.6: Linear model showing the relationship between distance flown and mean velocity of flight between olanzapine and control. Mean velocity (m/s) and distance flown (m) was calculated for each individual bee that flew > 5 minutes. The model 'Mean velocity ~ Distance + Treatment + Colony' was determined to be the best fit. Adjusted R-squared = 0.761. Olanzapine: n = 5; control: n = 7.

Table 4.1: Results from linear model analysing the effects of distance flown, olanzapine and colony on mean velocity of flight. Model call: Mean velocity ~ Distance + Treatment + Colony. *p <0.05, **p <0.01, ***p <0.001.

Explanatory variable	Coefficient estimate	Std. Error	95% CI	t-value	p-value
Distance flown (m)	0.000153	0.0000366	(0.0000813, 0.00022)	4.16	0.003 **
Treatment: Olz	-0.211	0.0909	(-0.389, -0.033)	-2.32	0.049 *
Colony: B	0.226	0.0908	(0.0482, 0.404)	2.49	0.038*

4.4.2 Effect of olanzapine on mitochondrial respiration in exercised and non-exercised workers

A two-way ANOVA was carried out to determine if there were any significant differences in the oxygen flux (background corrected) and FCR at each respiratory state between bees treated with olanzapine and control and exercised and non-exercised (**Supplementary Figure S4.6; Supplementary Figure S4.7**)

4.4.2.1 Bee size and oxygen consumption

The mean intertegular distance (ITD) of the bumblebee workers that were used for HRR was 5.31 mm (SD = 0.48). We used Pearson's correlation coefficient to assess whether the ITD (or the mean ITD calculated from pooled groups) had any effect on oxygen flux or FCR. We found no correlation between ITD and oxygen flux at any the respiratory states with the exception of CCCP which showed moderate positive correlation ($r(24) = 0.46$, $p = 0.019$)**(Supplementary Table S4.1)**.

For the FCR we found that there was a significant negative correlation between ITD and the substrates proline and succinate; $r(24) = -0.46$, $p = 0.017$ and $r(24) = -0.43$, $p = 0.027$, respectively **(Supplementary Table S4.1)**.

4.4.2.2 Effects of exercise and olanzapine on oxygen flux

We found that there was a significant interaction between exercise and treatment in routine respiration ($F(1, 26) = 6.49$, $p = 0.017$) (**Supplementary Figure S4.8**). Tukey's HSD post hoc test was then performed and showed that there was a significant difference in oxygen flux between; *i*) olanzapine and control treated non-exercised bees, *ii*) exercised and non-exercised bees treated with the control, *iii*) exercised bees treated with the control and non-exercised bees treated with olanzapine, and *iv*) workers that were treated with olanzapine and controls that exercised (**Table 4.2**)(**Figure 4.7**).

The mean oxygen flux in non-exercised bees that were treated with olanzapine was 13.09 pmol/s^{-1}/$brain^{-1}$ less than those treated with the control. In control treated bees the effect of exercise increased the mean oxygen flux of routine

respiration by 20.38 pmol/s^{-1}/$brain^{-1}$, and in exercised workers, olanzapine decreased routine respiration by an average of 31.44 pmol/s^{-1}/$brain^{-1}$ compared to the control (**Table 4.2**).

Table 4.2: Tukey's HSD results from a two-way ANOVA looking at the significant interaction between olanzapine and exercise on the oxygen flux of routine respiration in *B. terrestris*. *p <0.05, **p <0.01, ***p <0.001

Oxygen flux – routine respiration				
Group comparisons	Difference in means	Lower CI	Upper CI	p-value
Olz Non-exercised : Control Non-exercised	-13.09	-25.03	-1.15	2.78 x 10^{-2}*
Control Exercised : Control Non-exercised	20.38	5.88	34.87	3.57 x 10^{-3}**
Olz Exercised : Control Non-exercised	-11.06	-24.76	2.64	1.46 x 10^{-1}
Control Exercised : Olz Non-exercised	33.47	19.24	47.70	<0.001***
Olz Exercised : Olz Non-exercised	2.03	-11.39	15.45	9.75 x 10^{-1}
Olz Exercised : Control Exercised	-31.44	-47.17	-15.70	<0.001***

Exercise or treatment did not have any significant effect on oxygen flux after the addition of pyruvate and malate, proline, or at maximum ET capacity. However, exercise did significantly affect oxygen flux after the addition of the complex I inhibitor, rotenone ($F(1) = 6.97$, $p = 0.014$). Bees that exercised had an oxygen flux that was on average 7.715 pmol/s^{-1}/$brain^{-1}$ lower than those that did not fly (**Figure 4.7**) (**Supplementary Table S4.2**).

Neither exercise or treatment significantly affected the mean oxygen flux after the addition of succinate, however, the interaction between exercise and treatment was nearly significant ($F(1, 26) = 3.59$, $p = 0.069$).

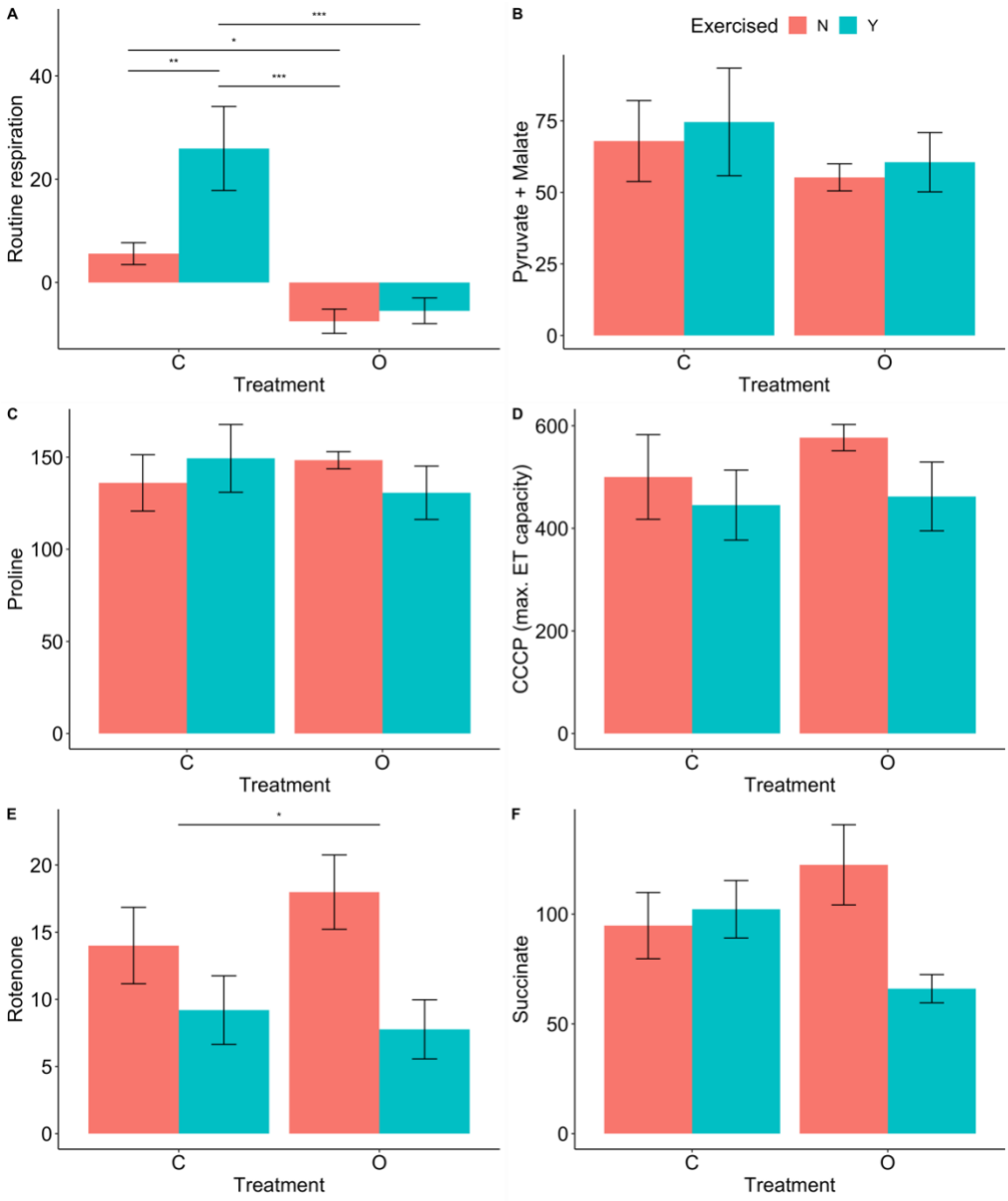


Figure 4.7: The effect of olanzapine treatment and exercise on the mean oxygen flux at each respiratory state in the brain of *B. terrestris* workers. Bees were fed 0.05 µg of olanzapine or a control followed by exposure to exercise (flight) or not. Brain tissue was analysed via high-resolution respirometry. Bar graphs showing the mean oxygen flux at each state: A) routine; B) pyruvate and malate; C) proline; D) CCCP (max. ET capacity); E) rotenone; and F) succinate. *P*-values indicated post-hoc analysis for differences where there was a significant two-way ANOVA effect. **p* <0.05, ***p* <0.01, ****p* <0.001. Error bars showing the mean ± standard error. y-axis = oxygen flux (pmol/s^{-1}/$brain^{-1}$). Olanzapine exercised: n = 6; control exercised: n = 5; olanzapine non-exercised: n = 10; control non-exercised: n = 9.

4.4.2.3 Effects of exercise and olanzapine on the FCR

The two-way ANOVA showed treatment and exercise had a significant effect on the FCR at routine respiration, and there was a significant interaction between the effects of treatment and exercise on routine ($F(1, 26) = 5.77$, $p = 0.024$) (**Supplementary Figure S4.9**). Tukey's HSD post-hoc showed that there was a significant difference in routine FCR between *i*) exercised and non-exercised controls, *ii*) control workers that exercised and olanzapine workers that did not exercise, and *iii*) between olanzapine and control workers that exercised (**Table 4.3**)(**Figure 4.8 A**).

The mean routine FCR was 0.04 higher in exercised controls compared to non-exercised controls, whilst for exercised workers treated with the control it was on average 0.07 higher than non-exercised bees that were treated with olanzapine. Between exercised workers, there was also a similar decrease in the bees that received olanzapine compared to controls (0.07) (**Table 4.3**).

Table 4.3: Tukey's HSD results from the two-way ANOVA looking at the significant interaction between olanzapine and exercise on the FCR of routine respiration in *B. terrestris*. **p* <0.05, ***p* <0.01, ****p* <0.001

Flux Control Ratio (FCR)				
Group comparisons	Difference in means	Lower CI	Upper CI	*p*-value
Olz Non-exercised : Control Non-exercised	-0.03	-0.05	0.00	7.1×10^{-2}
Control Exercised Control Non-exercised	0.04	0.01	0.08	9.1×10^{-3}**
Olz Exercised : Control Non-exercised	-0.023	-0.05	0.01	2.03×10^{-1}
Control Exercised : Olz Non-exercised	0.07	0.04	0.01	<0.001***
Olz Exercised : Olz Non-exercised	0.00	-0.03	0.03	9.96×10^{-1}
Olz Exercised : Control Exercised	-0.07	-0.10	-0.03	<0.001***

For the FCR of the substrates pyruvate and malate there was no significant effect of treatment or exercise, however exercise was close to significance

(F(1) = 2.99, p = 0.096). After the addition of proline the two-way ANOVA reported a significant effect of treatment on the FCR (F(1) = 4.32, p = 0.047), with the mean FCR for olanzapine treated bees being 0.04 less than controls (**Figure 4.8 C**). Exercise and the interaction between exercise and treatment were not significant. There was no significant effect of exercise or treatment on the FCR of rotenone, or after the addition of the complex II substrate, succinate.

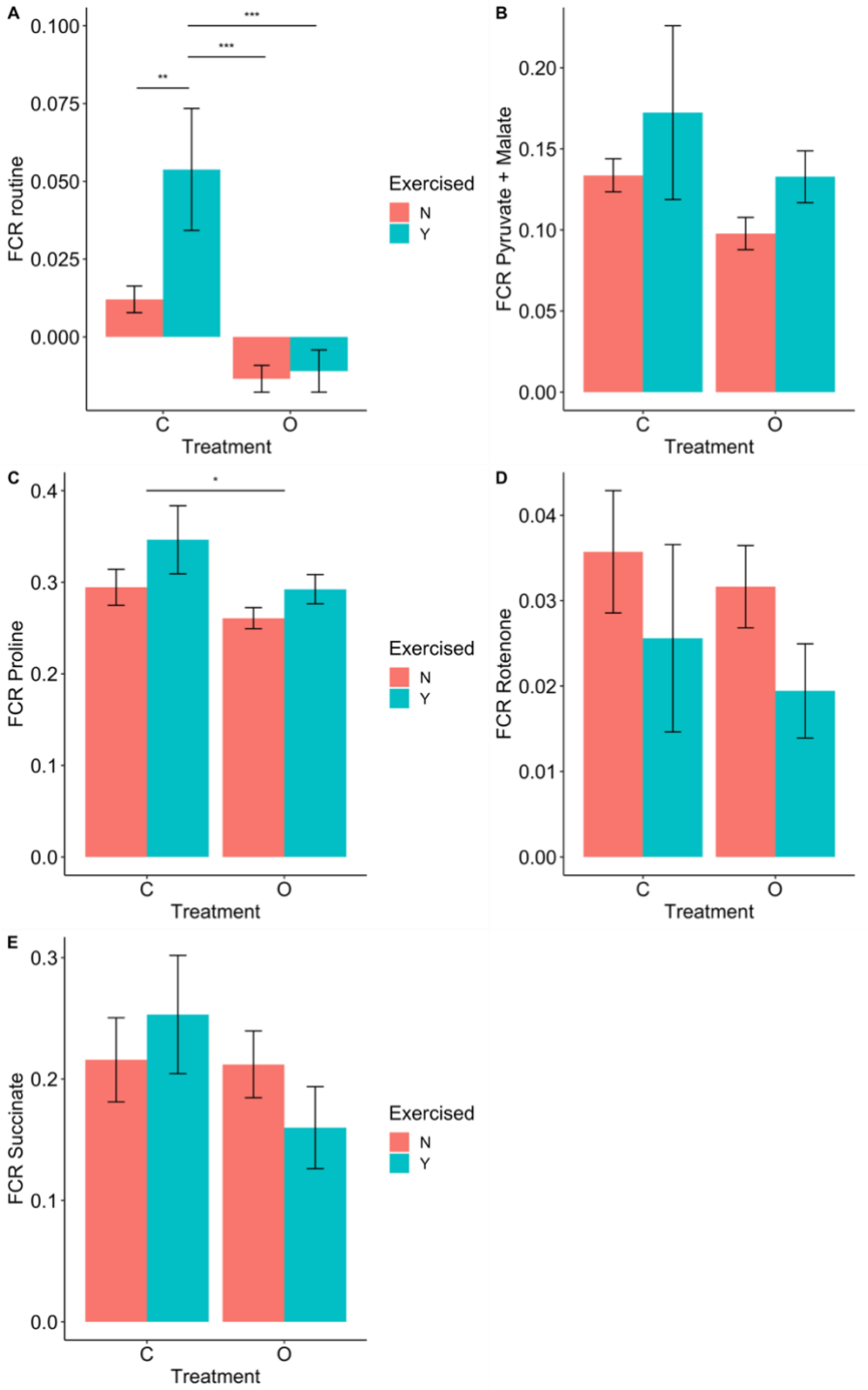


Figure 4.8: The effect of olanzapine treatment and exercise on the mean FCR at each respiratory state in the brain of *B. terrestris* workers. Bees were fed 0.05 µg of olanzapine or a control followed by exposure to exercise (flight) or not. Brain tissue was analysed via high-resolution respirometry. Bar graphs showing the mean FCR at each state. A) routine; B) pyruvate and malate; C) proline; D) rotenone; and E) succinate. *P*-values indicated post-hoc analysis for differences where there was a significant two-way ANOVA effect. **p* <0.05, ***p* <0.01, ****p* <0.001. Error bars showing the mean ± standard error. Olanzapine exercised: n = 6; control exercised: n = 5; olanzapine non-exercised: n = 10; control non-exercised: n = 9.

4.4.3 Effects of olanzapine and exercise on ATP production in *B. terrestris* workers

4.4.3.1 ATP production at different respiratory states

To look at the effects of olanzapine and exercise on ATP production at the different respiratory states two-way ANOVA were performed. Overall, our results show that in all groups pyruvate and malate increased mean ATP concentration to the highest concentrations from the routine production (0.250 – 0.302 mM). However, the addition of succinate also increased the ATP concentration a similar amount (0.258 - 0.311 mM) from the mean concentrations reported after the inhibition of complex I by rotenone (**Table 4.4**).

Despite increasing oxygen consumption, proline appeared to have little effect on ATP production. Proline increased ATP concentrations from pyruvate and malate by 0.004 mM for both non-exercised control bees and exercised olanzapine treated bees. However, for non-exercised olanzapine treated bees and exercised control bees' proline decreased the ATP concentration by 0.004 mM and 0.017 mM, respectively (**Table 4.4**).

After uncoupling, ATP production fell below that of routine respiration as expected. The inhibition of complex I by rotenone then caused a further decline in ATP production but only by 0.001 – 0.038 mM (**Table 3.4**).

Antimycin A decreased the ATP production, however, despite the inhibition of complex III there was still a relatively high concentration of ATP being produced that was above the initial routine levels (0.090 – 0.193 mM)(**Table 4.4**).

Table 4.4: Mean ATP concentration (mM) change from routine ATP concentration for each respiratory state in the brain of *B. terrestris*. Mean ATP concentrations values are calculated as a change (increase or decrease) from the ATP concentration at routine

	Non-exercised, Control (n = 7)		Non-exercised, Olz (n = 8)		Exercised, Control (n = 3)		Exercised, Olz (n = 4)	
Respiration state	Mean (s.e)	Variance	Mean (s.e)	Variance	Mean (s.e)	Variance	Mean (s.e)	Variance
Pyruvate + Malate	0.288 (0.046)	0.015	0.250 (0.039)	0.012	0.281 (0.023)	0.002	0.302 (0.055)	0.012
Proline	0.292 (0.045)	0.014	0.246 (0.038)	0.011	0.264 (0.028)	0.002	0.306 (0.057)	0.013
CCCP	-0.157 (0.035)	0.008	-0.099 (0.027)	0.006	-0.050 (0.045)	0.006	-0.139 (0.056)	0.013
Rotenone	-0.158 (0.039)	0.011	-0.108 (0.032)	0.008	-0.088 (0.017)	0.001	-0.169 (0.075)	0.022
Succinate	0.153 (0.037)	0.009	0.150 (0.019)	0.003	0.219 (0.016)	0.0007	0.142 (0.028)	0.003
AmA	0.097 (0.035)	0.009	0.108 (0.013)	0.001	0.193 (0.013)	0.0005	0.090 (0.027)	0.003

We found that exercise and olanzapine had no significant effects on ATP concentration at all respiratory states (**Figure 4.9; Table 4.4**). That being said, the interaction between treatment and exercise at the maximum ET capacity (after the addition of CCCP) showed a trend towards significance (described as a p-value > 0.05 and <0.1) ($F(1, 18) = 3.20$, $p = 0.090$). This was also found for the interaction between treatment and exercise after the addition of Antimycin A during the ROX state ($F(1, 18) = 3.80$, $p = 0.067$), as seen in bar graph F in Figure 4.9.

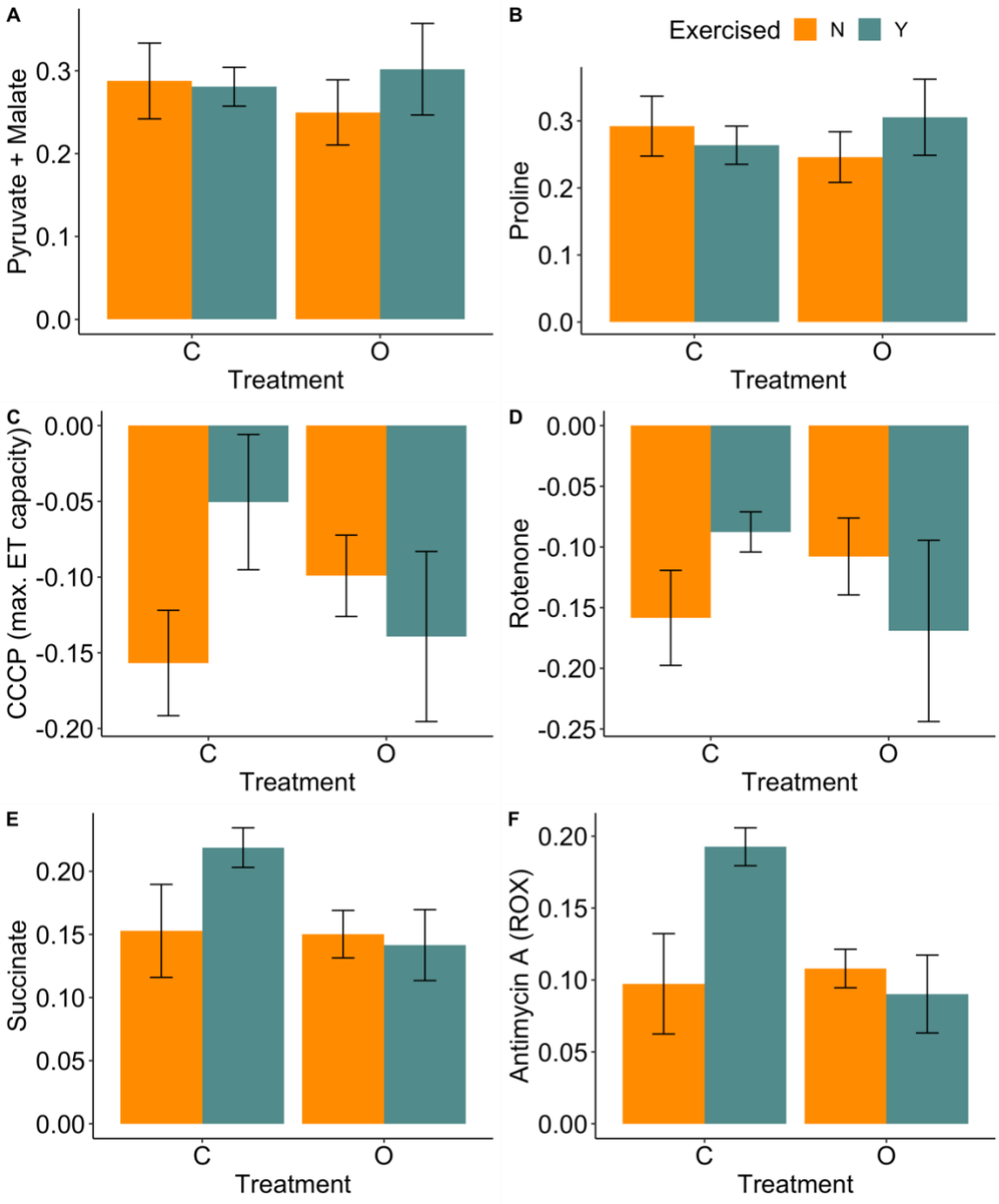


Figure 4.9: The effect of olanzapine and exercise on the ATP concentration (mM) change from routine ATP concentration at each respiratory state in the brain of *B. terrestris* workers. Bees were fed 0.05 µg of olanzapine or a control followed by exposure to exercise (flight) or not. Brain tissue was analysed via high-resolution respirometry using the O2K fluo-module and magnesium green. Bar graph of the ATP concentration (mM) change from routine ATP production at each state: A) pyruvate and malate; B) proline; C) at the maximum electron transport capacity, D) after inhibition of complex I by rotenone, E) addition of succinate, and F) inhibition of complex III by Antimycin A (residual oxygen consumption (ROX) state). Error bars showing the mean ± standard error. Non-exercised: olanzapine, n = 4; control, n = 3. Exercised: olanzapine, n = 8; control, n = 7

4.4.3.2 Flight performance and the effects on ATP production

The flight data was analysed from bees that were used for HRR (n = 7) to determine if there was any relationship between distance flown, flight duration, or mean velocity of flight on ATP concentration at the different respiratory states. Using linear regression, we found that the mean velocity of flight and olanzapine had a significant effect on the ATP concentration after the addition of succinate and in the ROX state (**Figure 4.10; Figure 4.11**).

The linear model suggests that bees that received olanzapine had an average reduction in ATP concentration of 0.069 mM, after the addition of succinate, compared to workers that received the control. The model also showed that for each metre per second increase in velocity this was associated with a decrease of 0.195 mM in ATP (**Table 4.5**).

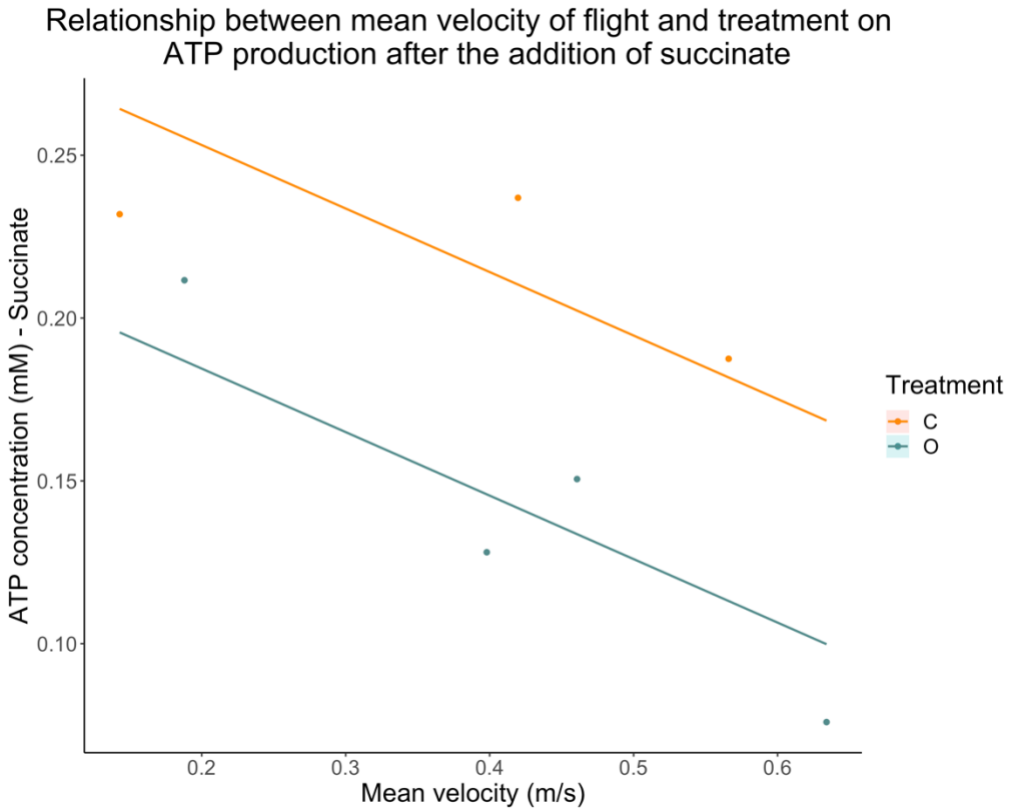


Figure 4.10: Linear regression showing the relationship between the mean velocity of flight and ATP concentration (mM) after the addition of succinate between olanzapine and control bees. The model call 'ATP(succinate) ~ Mean velocity + Treatment' was selected to be of best fit. Adjusted R-squared = 0.747. Olanzapine (green), n = 4; control (orange), n = 3

Table 4.5: Results from linear model analysing the relationship between the mean velocity of flight and ATP production after the addition of succinate between olanzapine and control bees. Model: ATP(succinate) ~ Mean velocity + Treatment.

*p <0.05

Explanatory variable	Coefficient estimate	Std. Error	95% CI	t-value	p-value
Mean velocity	-0.195	0.068	(-0.384, -0.006)	-2.872	0.045 *
Treatment: Olz	-0.069	0.023	(-0.133, -0.005)	-2.980	0.041 *

Assuming the mean velocity remained constant, the workers that received olanzapine had an average ATP concentration of 0.09 mM less at the ROX state, compared to workers that received the control. Furthermore, the linear model suggested that for each metre per second increase in velocity this was associated with a decrease of 0.184 mM (**Table 4.6**).

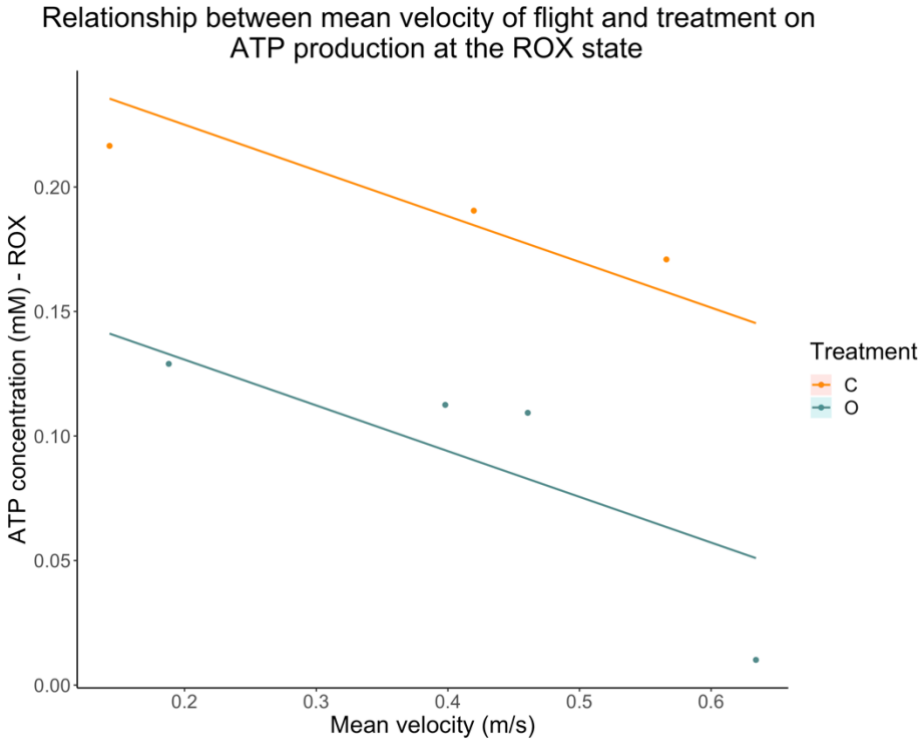


Figure 4.11: Linear regression showing the relationship between the mean velocity of flight and ATP concentration (mM) at the ROX state between olanzapine and control bees. The model 'ATP(Antimycin A) ~ Mean velocity + Treatment' was selected to be of best fit. Adjusted R-squared = 0.823. Olanzapine (green), n = 4; control (orange), n = 3

Table 4.6: Results from linear model analysing the relationship between the mean velocity and ATP production (mM) between olanzapine and control bees at the ROX state. Model call: ATP(Antimycin A) ~ Mean velocity + Treatment. *p <0.05

Explanatory variable	Coefficient estimate	Std. Error	95% CI	t-value	p-value
Mean velocity	-0.184	0.065	(-0.364, -0.003)	-2.822	0.048 *
Treatment: Olz	-0.094	0.022	(-0.156, -0.033)	-4.273	0.013 *

The flight duration and distance flown did not significantly impact ATP concentration changes in relation to routine production at any of the respiratory states.

4.4.4 Mitochondrial respiration and ATP production variation in different female castes

We compared the oxygen flux, FCR and ATP production in the brain of unmated *B. terrestris* queens (gynes). We also compared the oxygen flux and FCR with that of the control and unexercised worker bees. It should be noted that there were some differences between samples as the control workers were fed DMSO whereas the gynes were removed straight from the colony. However, we believed it was still of interest to analyse the differences.

4.4.4.1 Oxygen flux between gyne and worker B. terrestris

An unpaired Student's *t*-test was performed to determine any significant differences between gynes and workers at each respiratory state with the exception of routine respiration where an unpaired Wilcoxon rank sum test was done as the data did not meet the conditions for normality and required non-parametric analysis. There was a significant difference in oxygen flux at all states except after the addition of rotenone (**Supplementary Figure S4.10).**

4.4.4.2 FCR in non-exercised workers and gynes

The FCR is standardised against the maximum ET capacity and ROX, and therefore may be of more value when comparing castes as it could be expected that gynes have a greater level of oxygen consumption due to their phenotypical differences.

Unpaired Student's *t*-tests were performed to determine differences between castes for all respiratory states except for succinate, where the criteria were not satisfied, and an unpaired Wilcoxon rank sum test was used. We found that there was a significant difference between castes at routine respiration ($t_{df=13} = 4.07$, $p = 0.001$) and for the substrate's pyruvate and malate ($t_{df=13} = 3.00$, $p = 0.010$) (**Figure 4.12**). Interestingly this was not seen for the substrate proline.

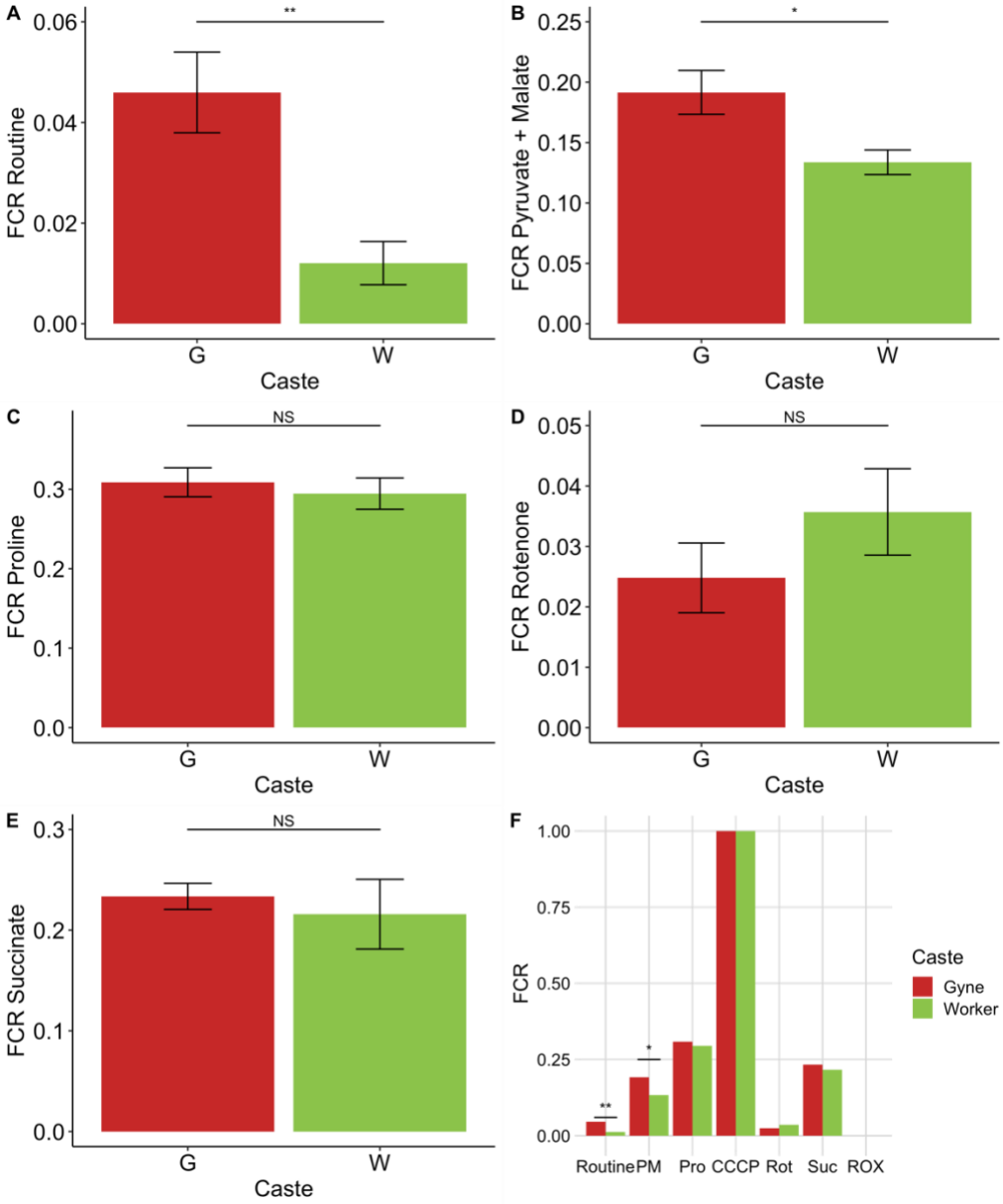


Figure 4.12: The effect of olanzapine and exercise on the FCR at each respiratory state in the brain of non-exercised *B. terrestris* workers and gynes. Brain tissue was analysed via high-resolution respirometry. Bar graphs of the FCR for gyne (red) and worker (green) *B. terrestris* at each respiratory state. A) Routine respiration; B) pyruvate + Malate; C) proline; D) rotenone; E) succinate; and F) combined plot. Error bars showing the mean ± standard error. To determine FCR the CCCP (maximum ET capacity) is set to 1 and the ROX is set to 0. *p <0.05, **p <0.01, ***p <0.001. Workers: n = 9; gynes: n = 6.

4.4.4.3 ATP production in B. terrestris gynes

Unpaired Student's *t*-tests were performed to determine differences in ATP production changes from routine levels between gynes and workers. Despite significant differences in oxygen flux and FCR, we found no significant difference in ATP concentration at any of the respiratory states between castes (**Figure 4.13**).

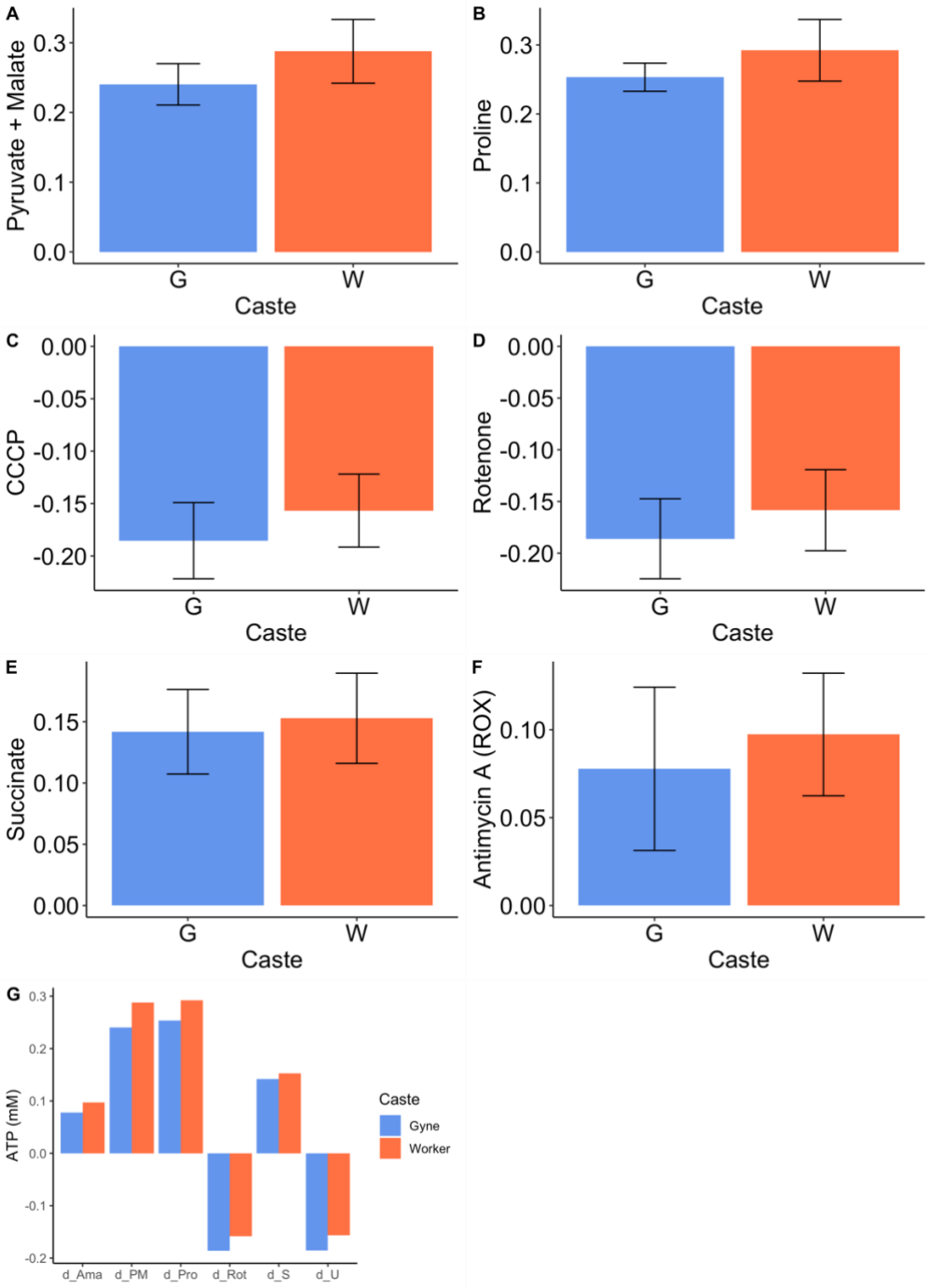


Figure 4.13: The effect of olanzapine and exercise on the ATP concentration (mM) change from routine ATP concentration at each respiratory state in the brain of non-exercised *B. terrestris* workers and gynes. Brain tissue was analysed via high-resolution respirometry using the O2K fluo-module and magnesium green. Bar graphs of ATP concentration (mM) change from routine ATP levels between *B. terrestris* gynes and workers at each respiratory state. A) pyruvate + malate; B) proline; C) CCCP (max. ET capacity); D) rotenone; E) succinate; F) Antimycin A (ROX); G) combined plot. Error bars showing the mean ± standard error. To determine FCR the CCCP (maximum ET capacity) is set to 1 and the ROX is set to 0. Workers: n = 7; Gynes: n = 3.
*p <0.05, **p <0.01, ***p <0.001

4.4.5 ETS inhibitors and ATP production in *B. terrestris* workers

To determine the effects of ETS inhibitors on ATP production in *B. terrestris* worker brain mitochondria, we looked at the mean ATP concentration (in relation to routine ATP levels) after the addition of each inhibitor. The complex I inhibitor, rotenone, only appeared to decrease mean ATP production slightly by 0.019 mM (5.9%). The complex II substrate succinate increased ATP production from routine the greatest, and subsequently malonate (the complex II inhibitor) then reduced this by 24.14%. The complex III inhibitor, Antimycin A then decreased ATP production further by 34.2% but still had a higher ATP concentration (0.217 mM) than routine respiration before the addition of the substrates (pyruvate and malate, and proline) (**Figure 4.14; Table 4.7**). ATP production from routine the greatest, and subsequently malonate (the complex II inhibitor) then reduced this by 24.14%. The complex III inhibitor, Antimycin A then decreased ATP production further by 34.2% but still had a higher ATP concentration (0.217 mM) than routine respiration before the addition of the substrates (pyruvate and malate, and proline) (**Figure 4.14; Table 4.7**).

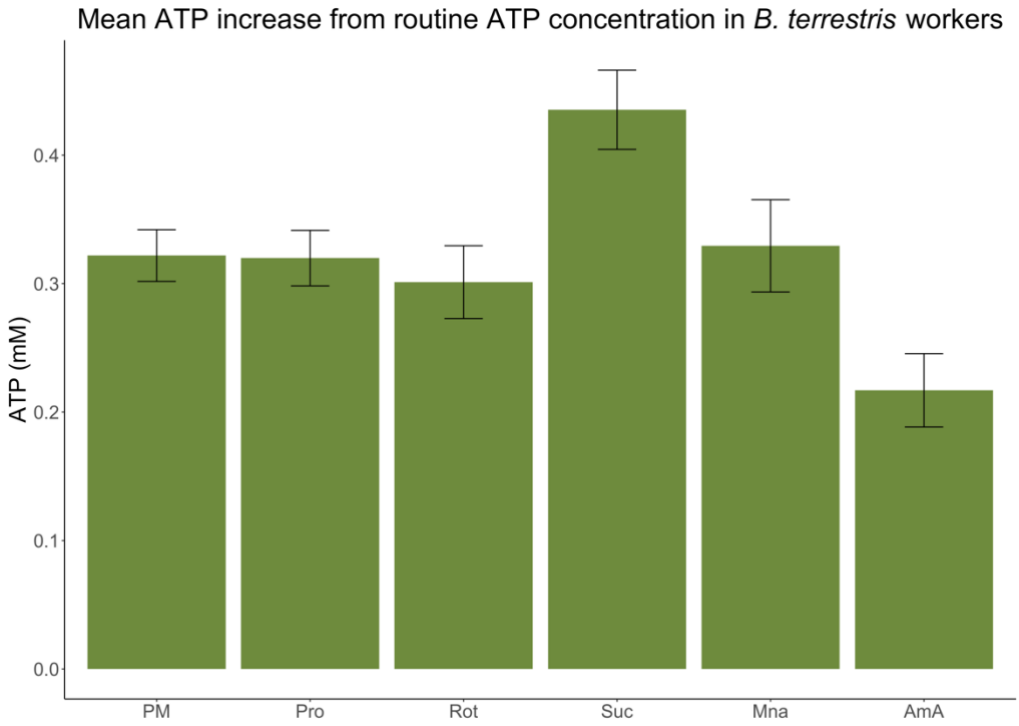


Figure 4.14: Effect of ETS inhibitors on ATP concentration (mM) increases from routine respiration concentration at different respiratory states in *B. terrestris* worker brain. Three inhibitors were added: Rotenone (the complex I inhibitor), malonate (the complex II inhibitor), and antimycin A (the complex III inhibitor). Bar graph showing ATP concentration increases from routine respiration production at different respiratory states in *B. terrestris* worker brain; n = 6. Error bars showing the mean ± standard error. Abbreviations: PM = pyruvate + malate; Pro = proline; Rot = rotenone; Suc = succinate; Mna = malonate; Ama = Antimycin A.

Table 4.7: Mean ATP concentration (mM) increase from routine at each respiratory state in *B. terrestris* worker brain mitochondria. n = 6

	Pyruvate + malate	Proline	Rotenone	Succinate	Malonate	Antimycin A
Mean ATP (mM)	0.322	0.320	0.301	0.435	0.330	0.217

4.5 Discussion

In this study, we have demonstrated the use of eusocial bees as a model to analyse the effects of xenobiotics. We used a combination of flight analysis and HRR, with the addition of MgG to measure ATP concentration, to gain insight into the effects of exercise and olanzapine on mitochondrial function in the brain of *B. terrestris*.

4.5.1 Effects of olanzapine on *B. terrestris* flight

Less than half of the bumblebees tested initiated flight (43.8%); and only around one in ten bees (n = 12, 12.5%) flew for more than five minutes. Neither worker size or treatment (olanzapine or control) affected the probability of initiating flight which suggests that this was due to an external factor, such as tagging, and that there is no bias between treatments.

This low sample size of flight data could reduce the reliability of the results; however, our findings suggest there is no difference in mean flight duration, distance flown or velocity between *B. terrestris* workers that received olanzapine or the control.

We found that there was no significant difference between colony A and colony B on the flight duration and distance flown. However, there was an effect on the mean velocity of flight with bees from colony B flying significantly faster than those from colony A. This could be associated with general colony health but as neither the distance flown, or duration of flight were significantly different it may suggest only a slight impairment.

Workers from colony B flew on average 0.226 m/s faster than bees from colony A, however there was an uneven distribution of olanzapine and control workers in colony B (n = 2 and n = 4, respectively), suggesting it could be associated with treatment type rather than colony. Nevertheless, differences in fitness between colonies are expected and replicate the natural variation found

in nature; therefore, by analysing bees from multiple colonies it may improve the reliability of our findings.

From the bees which flew for longer than five minutes (n = 12), olanzapine, total distance flown (m), and colony (A or B) had a significant effect on the mean velocity of flight. Bees treated with olanzapine flew 0.21 m/s slower than bees treated with the control. With a mean velocity of 0.65 m/s for the bees treated with the control this is a substantial decrease of a third (32.3%) in bees treated with olanzapine. These results suggest that olanzapine may be having a receptor blockade effect in the brain of *B. terrestris*. A subsequent decrease in dopamine levels by olanzapine may explain the decrease in flight velocity, which would be in agreement with the findings in the flight muscle of honeybees by the dopamine antagonist, flupentixol.[238] Our results, along with previous studies, provide evidence that maintaining sufficient dopamine is fundamental to facilitate optimum flight performance in bees.[238]

The distance flown was associated with mean velocity; the further the distance flown, the faster bees flew. It could be hypothesised that bees that fly for longer may fly at slower speeds than bees flying shorter distances. However, as these flight trials were time restricted this may elucidate why an increase in velocity would therefore result in a greater distance flown.

Overall, these results are important in providing a better understanding of the potential effects of olanzapine and other atypical antipsychotics on exercise performance in patients. Exercise releases dopamine and a ‘feel good’ response.[263,264] In patients taking these treatments it is possible that this response is being muted which may impact their ability to exercise at a high intensity as well as their overall motivation to partake in regular exercise which could subsequently increase their risk of developing MetS.

4.5.2 Effect of olanzapine on mitochondrial respiration and ATP production in exercised and non-exercised workers

Although oxygen consumption can be a valuable indicator of metabolic rates and ATP production there are various factors which can confound the assumption. Oxygen consumption is not a true index of ATP production and the amount of ATP per unit of oxygen consumed can vary among species and tissue-type.[265] Therefore, although oxygen consumption is a useful proxy for energy metabolism, using methods such as Magnesium Green™ to detect precise measures of ATP alongside, we can specifically understand the relationship between oxygen consumption and ATP production in different species and tissues.

There are limitations to this study however, as the initial ADP concentration is unknown the absolute ATP mM values cannot be determined. Therefore, the data can only be used to determine changes in ATP production in relation to routine (baseline) respiration ATP concentration.

4.5.2.1 Bee size and mitochondrial respiration

There was no correlation between ITD (or the mean ITD of bees used in pooled samples) and oxygen flux at any of the respiratory states, with the exception of maximum electron transport capacity which showed moderate positive correlation. For the FCR we found that there was a significant negative correlation between ITD and the substrates proline and succinate. These results could suggest that larger bees have a greater maximum ET capacity, albeit the oxygen flux after the addition of proline and succinate does not increase in relation to maximum ET capacity. This would subsequently reduce the flux control ratio for succinate and proline resulting in a negative correlation between the ITD and FCR. However, there was no correlation between size and mean oxygen flux after the addition of proline and succinate. These results suggest that size is most likely not a strong influence on oxygen consumption and may only have a minor effect on the mitochondrial bioenergetics in bees. Furthermore, as HRR was performed on tissue samples

that contained a pool of up to four brains, the possible effect of the ITD on mitochondrial respiration was likely to be mitigated by the use of pooled bees of varying of sizes.

4.5.2.2 Routine mitochondrial respiration

The oxygen flux of routine respiration was affected by the interaction between exercise and olanzapine. Our results suggest that under normal conditions (control), exercise increases routine oxygen flux five-fold by 20.38 pmol/s^{-1}/$brain^{-1}$. This also correlates with the FCR which was on average 0.04 greater in workers that flew compared to workers which did not fly. This increase in mitochondrial respiration after acute exercise correlates with previous studies in rats.[255] The high levels of oxygen consumption are likely to be associated with an increase in ATP production to meet the higher energetic demands that is prolonged, even after the termination of flight.

Bees treated with olanzapine did not show a post-exercise increase in oxygen consumption, which suggests olanzapine may hinder the effects of exercise on routine respiration. Furthermore, in exercised bees, olanzapine decreased the oxygen flux by 31.44 pmol/s^{-1}/$brain^{-1}$ compared to the controls. These findings provide evidence for olanzapine having an exacerbating effect on lowering routine mitochondrial respiration when in conjunction with not performing exercise in *B. terrestris* brain.

As we do not know the routine ATP concentration, we cannot specify whether exercise or olanzapine altered ATP concentration. However, as exercise and olanzapine significantly affected the oxygen consumption of routine respiration it could be hypothesised that the ATP production is significantly affected and therefore should be analysed in future studies.

4.5.2.3 Pyruvate and malate

The high ATP concentrations reported after the addition of pyruvate and malate along with the decrease after the inhibition of complex I by rotenone suggests that complex I has a substantial role in the synthesis of ATP in *B.*

terrestris brain which correlates with previous findings in bumblebee and honeybee flight muscle.[101,102,107]

Exercise or olanzapine did not have any significant effect on oxygen flux, FCR, or ATP concentration after the addition of pyruvate and malate, although the effect of exercise was close to significance for the FCR. It appears the addition of these substrates counteract any differences seen in routine respiration and increases respiration to a similar level for all groups. There is a slight indication that exercise may increase the utilisation of these substrates and thus respiration, however, these effects may require repeated exposure to aerobic exercise and should be something to consider in future research.

4.5.2.4 Proline - partial or complete oxidation and its use as a fuel in bees

The amino acid proline is found substantially in pollen and is highly soluble in both the muscle and haemolymph of bees making it a readily available fuel.[102,266] However, in honeybees it has been reported that as little as 0.1% of energy produced is via proline.[266] Other studies have also found proline to increase oxygen consumption in bumblebees[102,266], however, we are the first to report ATP concentration changes. Our results show a substantial increase in oxygen flux after the addition of proline, which corresponds with previous findings in bumblebees.[102] Interestingly, proline increased oxygen consumption but did not appear to change ATP concentration. Therefore, we find that *B. terrestris* have the ability to oxidise proline, however we found no evidence that proline is used as an important energy substrate in the brain.

The absence of an effect on ATP concentration could be associated with various reasons. One such explanation could be that the maximum ATP concentration was reached and so the concentration was unable to increase further. Furthermore, ATP synthase is a reversible enzyme and when ATP levels are high and the mitochondrial membrane potential is low, ATP hydrolysis is favoured over ATP synthesis.[267] Hence, this could explain why proline did not increase ATP concentration.

Another theory could be that proline is not primarily used as an energy substrate for OXPHOS in the brain and is oxidised for an alternative use. Proline is first oxidised by proline dehydrogenase (PDH) to form pyrroline-5-carboxylate (P5C) which is then converted to glutamate via pyrroline-5-carboxylate dehydrogenase (P5CDH).[102,266,268,269] The reversible transamination of glutamate and pyruvate forms alanine and α-ketoglutarate via alanine-aminotransferase (ALT).[266,268,270] Proline is therefore characterised as having an anaplerotic role as α-ketoglutarate can then integrate into the TCA cycle.[102,113]

The partial oxidation of proline to form alanine has been observed in insects.[266] In the flight muscle of bumblebees however, it has been shown that *B. impatiens* may oxidise proline completely as it did not lead to an accumulation of alanine.[266] Therefore, it has been suggested that in the flight muscle of bumblebees proline may be used during the initial states of flight to fuel OXPHOS.

Proline has been reported in high concentrations in the retina of honeybees and proline when in the presence of pyruvate has been shown to increase oxygen consumption.[268] Tsacopoulos *et al* also proposed that in honeybees alanine and proline fuelled the mitochondria in the photoreceptors.[268] There are few mitochondria in the glial cells of honeybees and hence glycolysis is the main producer of ATP.[268] Alanine is formed in these cells as a product of glycolysis and is then transported into the photoreceptors and transaminated back to pyruvate which can in turn enter the TCA cycle.[110,268,271] ALT has been located in the photoreceptors and an increase in alanine consumption after photostimulation provides evidence for the role of alanine in fuelling neurons in honeybees.[268]

Proline alone has been shown to have a much smaller effect on oxygen consumption, however when in combination of other substrates, in particular pyruvate, this enables the ETS to reach maximum capacity.[102] This suggests

that oxidation of proline to aid in the ETS may be dependent on pyruvate. As pyruvate is required to transaminate glutamate and pyruvate to alanine and α-ketoglutarate this may, in part, explain the increase in oxygen consumption of proline in the presence of pyruvate. The formation of alanine in the brain can then be used to aid mitochondrial respiration in photoreceptors by acting as a substrate to sustain energy in neurons, and α-ketoglutarate can enter the TCA cycle.[268] Alanine has also been proposed to be a neuronal signalling molecule in bees[248] and, with the addition of acetyl-CoA, resynthesise proline in the fat body of insects which would allow the storage of energy in lipid form.[266,272,273]

Glycolysis yields only two ATP molecules per glucose molecule which is much less than OXPHOS; therefore, the beneficial reasons for favouring glycolysis are limited. One such reason, however, is that glycolysis can produce ATP at a much faster rate. There are estimates of glycolysis being 100 times faster than OXPHOS.[274] This may be a crucial factor if alanine is fundamental in neuronal signalling in bees. Furthermore, if alanine is involved in neuronal signalling and produced via glycolysis, then we suggest that proline could act as an additional fuel for alanine production during times of high neuronal stimulation such as during flight.

High levels of proline have been shown to be detrimental to neuronal cells in the hippocampus of rats and facilitates cognitive impairment in humans and animals.[269,275] Decreased efficiency of PDH in *Drosophila* has also been linked to increased proline levels and sluggish behaviour.[269] A deficiency in PDH may also reduce glutamate levels and in turn neuronal signalling. Glutamate has been shown to act as a transmitter in *Drosophila* but also in the mushroom bodies of honeybees where it has been associated with memory formation.[269,276,277] Light stimulation was shown to increase the utilisation of glutamate made from proline and subsequently proline concentrations decreased in the stimulated retina.[268] The inner mitochondrial membrane in flight muscle has been shown to be permeable to proline but not glutamate.[268,278-280] This provides further evidence that proline may be associated with the transport of alanine and glutamate, as well as maintaining

levels of neuronal transmitters in the brain of bumblebees. Overall, high oxidation of proline would produce high quantities of glutamate, alanine and α-ketoglutarate and therefore potentially increase neuronal signalling and feed intermediates into the TCA cycle for OXPHOS. As we did not investigate the levels of alanine we cannot conclude as to whether alanine was accumulating the brain of *B. terrestris* but is something that should be considered for future analysis.

4.5.2.5 Maximum electron transport capacity

Neither exercise nor olanzapine affected the maximum ET capacity. One possible explanation for this could be that the acute treatment and singular flight is insufficient to alter the maximum ET capacity. In patients, the onset of antipsychotic drug-induced metabolic adverse effects takes weeks to months.[281,282] Hence, chronic exposure maybe required to elicit any change and is something to consider for future research. Furthermore, as the interaction between olanzapine and exercise was nearly significant and would be interesting to determine how much exposure to exercise and olanzapine would be required to see this interaction significantly affect the maximum ET capacity.

As MgG is measured using fluorescence it should also be considered that the yellow colour of the CCCP may affect the fluorescence signal and therefore accuracy of the ATP measurements. However, as all treatment groups received a similar amount of CCCP, an effect in the signal, if any, would likely affect all treatment groups and so we expect would have little consequence on the overall results.

4.5.2.6 Complex I inhibition

Rotenone significantly decreased the oxygen flux between exercised and non-exercised bees in both control and olanzapine treated workers. Bees that exercised had an oxygen flux that was on average 7.715 pmol/s^{-1}/$brain^{-1}$ lower than those that did not fly, suggesting inhibiting complex I was having a

greater effect in exercised bees. This could be associated with a utilisation of complex I substrates; pyruvate and malate stimulate a series of dehydrogenases resulting in the reduction of NAD^+ to NADH which feed electrons into complex I.

If exercise increases the utilisation of these substrates and complex I activity the addition of rotenone could result in a greater decline in oxygen flux. However, the FCR was not significantly different between groups, but this may be associated with the large variation in the data. Therefore, these results should be interpreted with caution, but we should still acknowledge the trends that exercise does appear to have an effect on mitochondrial respiration after the inhibition of complex I.

4.5.2.7 Succinate as an energy substrate in bumblebees

Succinate increased mitochondrial respiration the most for all groups which has also been reported previously in honeybees at temperatures between 36-45ºC.[115] It has been suggested that there is a metabolic switch in honeybees as mitochondrial respiration at different temperatures have been shown to favour different substrates and complexes. At flight temperature (~35ºC) honeybees were shown to favour the oxidation of succinate and G3P instead of complex I substrates.[93,115] However there have been other studies showing that succinate does not stimulate mitochondrial respiration in the flight muscle of honeybees at temperatures of 24ºC[101], or in *B. impatiens* at a temperature of 37ºC.[102] Our findings are congruent with that seen in honeybees at a similar temperature and show succinate oxidation is present in the brain of *B. terrestris* at 35ºC.[115]

Olanzapine and clozapine have been associated with the inhibition of succinate dehydrogenase in rat brain.[283] Although we found that neither exercise or olanzapine significantly affected the mean oxygen flux after the addition of succinate, the interaction was close to significance, and it should be considered there may be a potential effect of flight and olanzapine on complex II.

4.5.2.8 ATP production during the ROX state

After the inhibition of complex III by Antimycin A, ATP production was not significantly different between exercise and treatment. However, the interaction between treatment and exercise after the addition of Antimycin A was close to significance. Exercised bees that received the control appeared to have a much higher ATP concentration even after the addition of Anitmycin A. This trend could be associated with an increased rate of glycolysis in the brain that may occur with exercise.

Glial cells primarily produce energy via glycolysis; glucose is phosphorylated to glucose-6-phosphate and is subsequently incorporated into trehalose, glycogen and pyruvate.[249,268] Pyruvate is then transaminated into alanine via ALT, along with the oxidation of NADH to NAD^+, which enables glycolytic flux to be maintained.[268] Learning and memory are fundamental parts of foraging in bees and allow them to signal and return to areas with high floral resource.[284,285] Therefore, during flight it is likely that neuronal signalling is heightened which may increase glycolysis in the brain. This may explain the high levels of ATP production in exercised bees despite the inhibition of OXPHOS.

The higher levels of ATP were not seen in the bees which flew and that were treated with olanzapine. This could suggest that olanzapine may be having a dampening effect on the higher rates of glycolysis that are seen with exercise in the control bees.

In Arabidopsis, after the inhibition of complex I a change in alanine level was reported which is a characteristic of adaptive metabolic response when OXPHOS is impaired.[270] It has also been shown that proline oxidation may aid in production of ATP when complex I is inhibited in mice.[280] Hence, after complex I inhibition by rotenone this could have led to an increase in glycolysis in the brain.

Overall, after inhibition of the ETS, ATP concentrations do not fall back to the levels seen at routine respiration. This suggests that in the brain of *B. terrestris* glycolysis is a significant producer of ATP. Our results also suggest that

exercise may increase glycolytic rates. However, although the data suggested a possible trend towards significance, it was not statistically significant and so should be interpreted with caution and requires further research.

4.5.3 Flight performance and the effects on ATP production

Both olanzapine and the mean velocity of flight affected the ATP concentration after the addition of succinate and Antimycin A. ATP concentrations were significantly less at both respiratory states when bees received olanzapine. Olanzapine has been associated with alterations in the ETS and the inhibition of succinate dehydrogenase.[283] Therefore, if olanzapine has an inhibitory effect on succinate dehydrogenase in *B. terrestris* brain this may decrease the oxidation of succinate resulting in a decreased efficiency of OXPHOS and lower ATP concentrations compared to the controls.

A decrease in ATP concentration at the ROX state in bees that received olanzapine may be a consequence of dysfunction in the glycolytic pathway in the brain. Any ATP production after the inhibition of complex III cannot be produced via OXPHOS. Recent studies have shown that the glycolytic pathway is vulnerable to dopamine disruption.[286] In Parkinson's disease (PD), in which dopamine deficiency is a key aetiology, glycolytic pathways have been shown to be decreased, and subsequently by enhancing glycolysis it may be neuroprotective in PD.[286] Furthermore, dopamine has been shown to be able to bind to three glycolytic proteins in mice.[286] Olanzapine is a dopamine antagonist and any potential interaction between dopamine and glycolytic proteins in the brain of *B. terrestris* may lead to dysfunction of the glycolytic pathways which could be associated with the decrease in ATP concentrations that we have reported. More research is required to understand how dopamine antagonists such as olanzapine may interact with glycolysis, but it is clear that sufficient dopamine levels play a role in maintaining glycolysis in the brain.

For both succinate and Antimycin A the ATP concentration was associated with ~ 0.2 mM reduction for each metre per second increase in velocity during flight. However, flight duration and distance flown did not significantly

impact ATP concentration. This suggests that bees that flew faster produced less ATP in the brain after the addition of succinate and in the ROX state. As to why this may be, further research is required that is beyond the scope of this study. However, one hypothesis could be that bees which were able to fly at greater speeds have a more efficient OXPHOS pathway that favours the utilisation of complex I substrates. This could suggest that in the presence of succinate, a complex II substrate, lower ATP concentrations are seen. A more efficient ETS may also lead to a decreased requirement of ATP synthesis via glycolysis.

These results were gathered from a relatively small sample size (olanzapine, n = 8; control, n = 7). Flight data normally requires a high number of replicates due to high variation however obtaining this data is challenging to coincide with HRR. Therefore, these results provide evidence of a potential effect of olanzapine and exercise intensity on altering ATP production.

4.5.4 Mitochondrial respiration and ATP production variation in different female castes

Eusocial bees, and other social insects, show substantial phenotypic plasticity; gynes (unmated queens) and workers show significant variation in size, reproductivity and lifespan despite having almost identical genomes.[34] However, there is little research on whether these phenotypic differences correspond to differences in mitochondrial respiration. Our results suggest that the much higher levels of oxygen consumption seen in gynes is representative of the greater energetic demand. Gynes are much larger in size and so have more mitochondria and greater metabolic demands. We did not see any significant difference in the oxygen flux between castes after the addition of the rotenone (**Supplementary Figure S4.9**). However, this may be associated with high variance in the data as the mean oxygen flux in workers in much less than gynes which would be as expected.

The FCR which provides a greater insight into the effects of different substrates and inhibitors in relation to the maximum ET capacity and ROX

state, showed significant differences at routine respiration and after the addition of pyruvate and malate. Gynes had a much higher ratio than workers suggesting that at routine respiration and after the addition of pyruvate and malate they are using a higher percentage of their OXPHOS capacity than workers. Bumblebee workers are responsible for leaving the colony to forage for pollen and nectar and so must be capable of undertaking the energetically demanding activity. On the other hand, queens undertake flight at the beginning of their life cycle to mate and find a suitable nesting ground. Once they have established their colony they do not leave and focus on reproduction.[31,38] Therefore, there could be less genetic pressure on queens to have such a high mitochondrial capacity compared to foragers.

Interestingly, we saw no significant difference in FCR after the addition of proline or succinate or rotenone. If there is a higher demand for OXPHOS in workers compared to gynes then this provides further evidence that proline and succinate are not the primary substrates used for fuelling flight which corresponds with our previous results suggesting workers that flew faster have a greater utilisation of complex I substrates.

Although we do not know the routine concentration of ATP we hypothesise that it is higher in gynes compared to workers due to the significant differences in oxygen flux that we report. However, the ATP concentration at routine respiration is something that should be analysed in future studies to confirm this.

Independent of the ATP concentration at routine respiration, we found that there was no difference in the effect of the substrates and inhibitors between castes on ATP concentration. Neither gynes nor the non-exercised control workers were exposed to any kind of activity before analysis. However, it should be noted that the workers were fed a control sucrose solution containing DMSO whereas gynes consumed only sucrose. However, given our findings, we suspect that this had very little, if any, influence on the results.

In summary, we found significantly greater oxygen consumption in gynes which suggests they have a much greater energetic demand compared to workers. Gynes also used a higher percentage of their OXPHOS capacity for routine respiration and also in the presence of the substates pyruvate and malate, compared to workers. However, we report similar changes in ATP synthesis between workers and gynes in response to substrates and ETS inhibitors.

4.5.5 ETS inhibitors and ATP production in *B. terrestris* workers

We assessed ATP concentrations after the inhibition of all three ETS complex inhibitors, rotenone, malonate, and Antimycin A, on worker brain to determine the effects on ATP synthesis. All workers were taken straight from the colony and did not undergo any exercise. As we did not use CCCP all states were coupled.

Interestingly, rotenone appeared to have the least effect with the mean ATP concentration decreasing by ~6% from the mean concentration after the addition of pyruvate and malate and then proline. Succinate then increased ATP concentrations to the highest level, which malonate the complex II inhibitor, then reduced by a quarter. Together this suggests complex II and the succinate pathway is a significant contributor to ATP production. However as discussed, inhibition of complex I has also been suggested to increase glycolysis, and so rotenone could exacerbate the increase in ATP concentration seen after the addition of succinate. Nevertheless, the large decrease in ATP concentration after the addition of malonate demonstrates the contribution that complex II is having on ATP synthesis. Antimycin A decreased ATP concentration by an additional third, and consistent with our other results, ATP concentrations remained relatively high; 0.217 mM above concentration at routine level.

These results provide further confirmation that despite inhibition of ETS, ATP production is maintained via glycolysis in the brain of *B. terrestris*. Future

research which measures glycolytic markers, or the use of glucose isotope tracing may aid in further understanding the importance of glycolytic pathways in maintaining energetic homeostasis in the brain of bees.[287]

Despite complex I substrates reporting to be a predominant fuel for flight, inhibition of complex I in the brain had the smallest impact on ATP production. However, it should be considered that the bees had not partaken in any type of exercise or flight activity. Despite some controversy regarding the oxidation of succinate in some bee species, we show that in *B. terrestris* brain succinate is an important contributor to ATP production.

4.6 Conclusion

Overall, this study demonstrates the effect of olanzapine and acute exercise on mitochondrial function in *B. terrestris* brain. We show that flight activity is altered by olanzapine which corresponds with changes in OXPHOS, particularly during routine respiration. We also highlight the role of glycolysis as an important contributor to ATP synthesis in the brain of *B. terrestris*. We hope these findings will encourage future research so we can better understand neuronal signalling and bioenergetics in the brain of insects as well as improve our understanding of the relationship between MetS and atypical antipsychotics in patients.

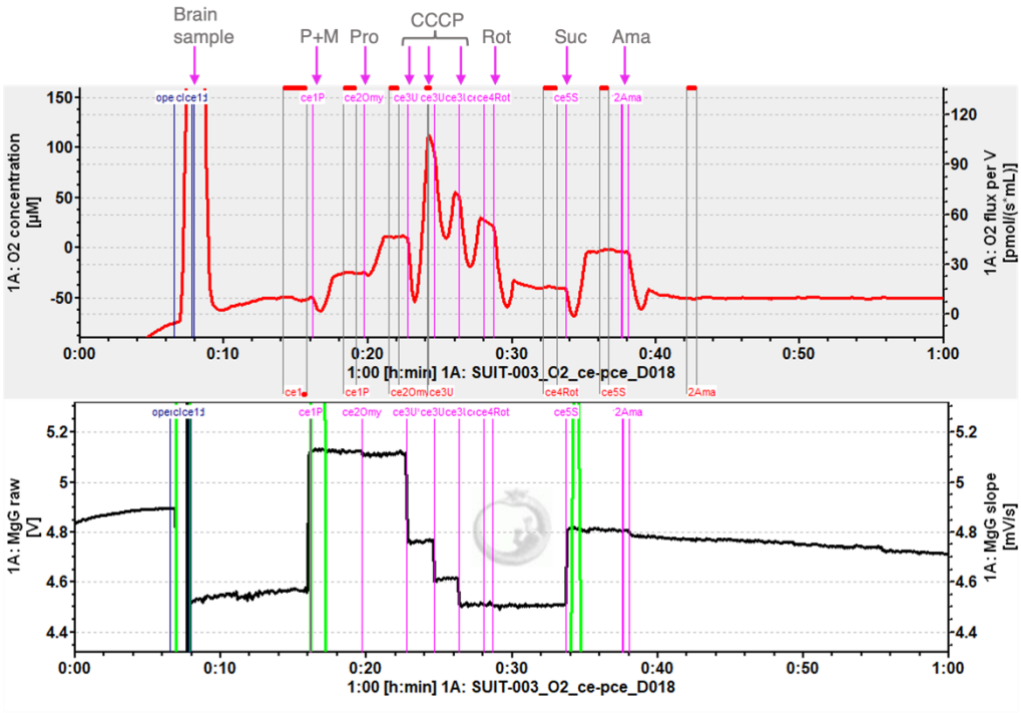
4.7 Author contributions

CS, LC and RS designed the experiment. CS performed all bee husbandry, laboratory work, data collection and analysis. The paper was written by CS with editorial assistance by LC and RS.

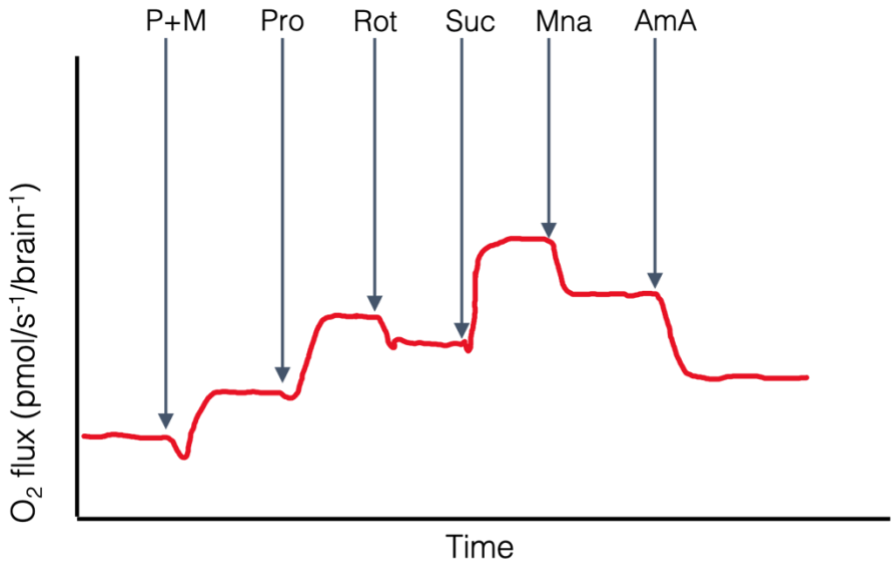
4.8 Funding

CS is a PhD candidate funded by the Future Food Beacon/Graduate Centre for International Agriculture, University of Nottingham, and Rothamsted Research

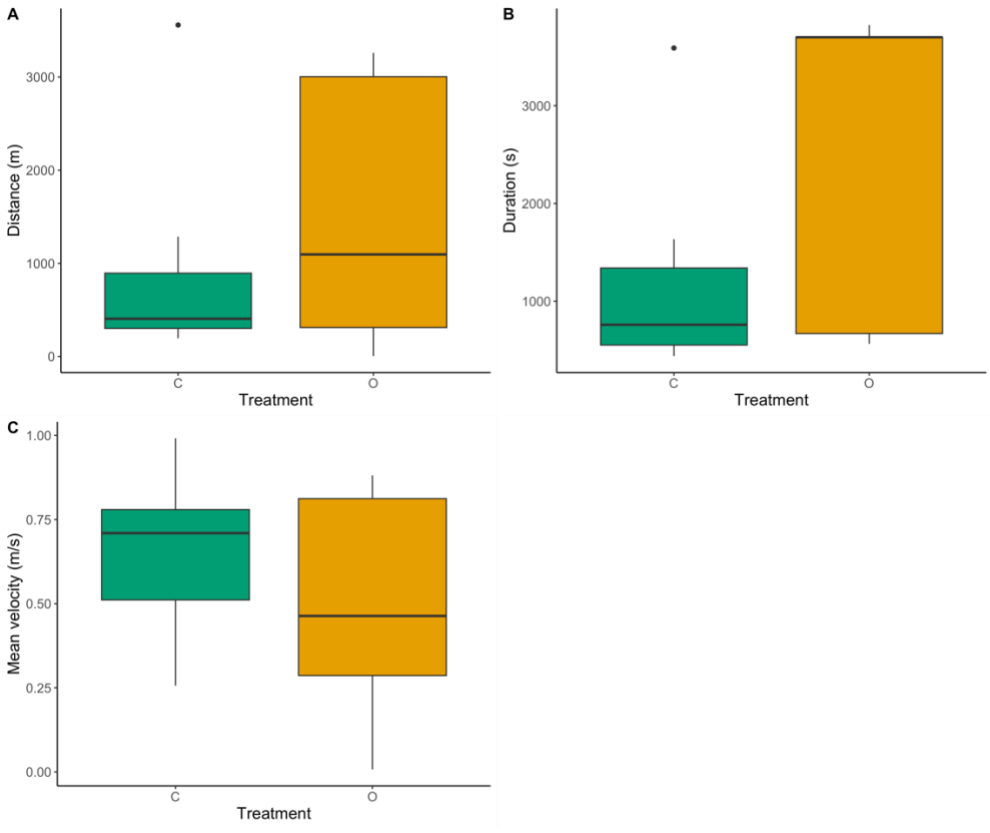
4.9 Appendix B



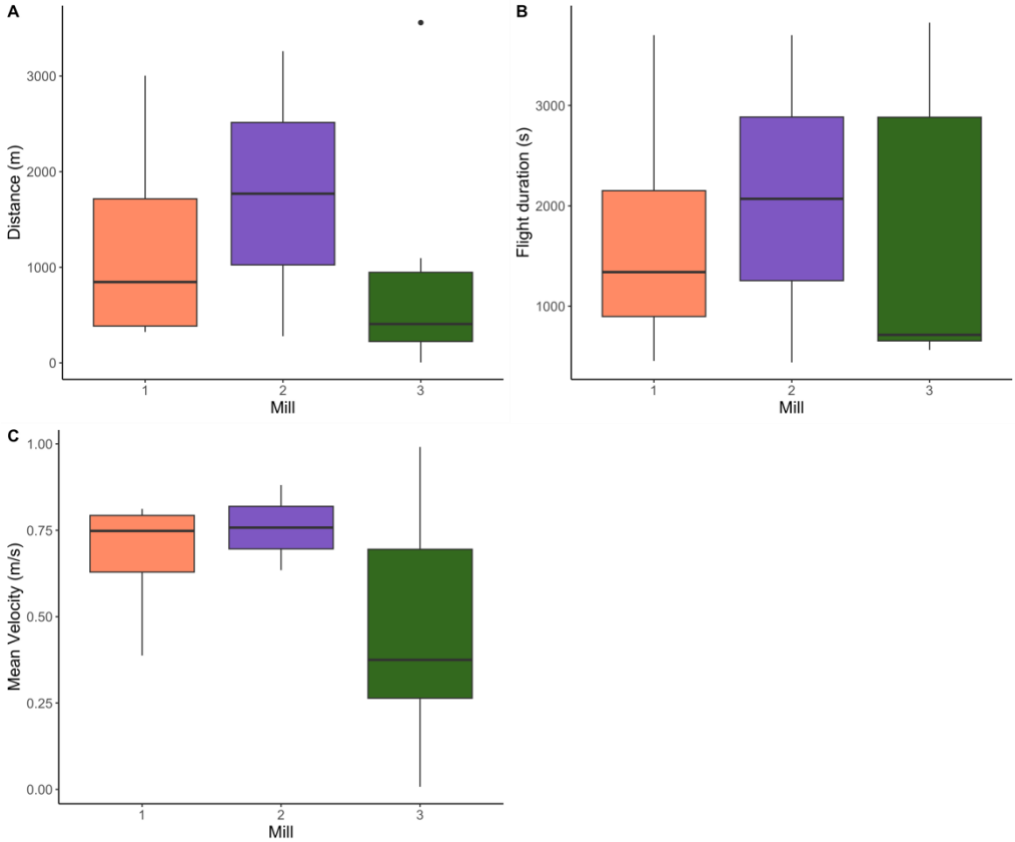
Supplementary Figure S4.1: Image of the O2k-oxygraph output measuring oxygen consumption along with MgG fluorescence. Top graph shows the O_2 flux (pmol/s^{-1}/$brain^{-1}$) as the red line. Bottom graph shows the MgG raw (V) as the black line. Pink arrows and lines indicate the addition of the substrate or inhibitor labelled in grey. X-axis for both graphs is time (minutes). Oxygen concentration (µM) and MgG slope (mV/s)(green line) are not visible in the figure.



Supplementary Figure S4.2: Annotated representative O2k graph output from high-resolution respirometry using the inhibitor titration protocol. Red line represents oxygen flux (pmol/s^{-1}/$brain^{-1}$). Arrows demonstrating when the substrate or inhibitor was added in the O2k chamber. Abbreviations: P+M: pyruvate + malate; Pro: proline; Rot: rotenone; Mna: malonate; and AmA: Antimycin A

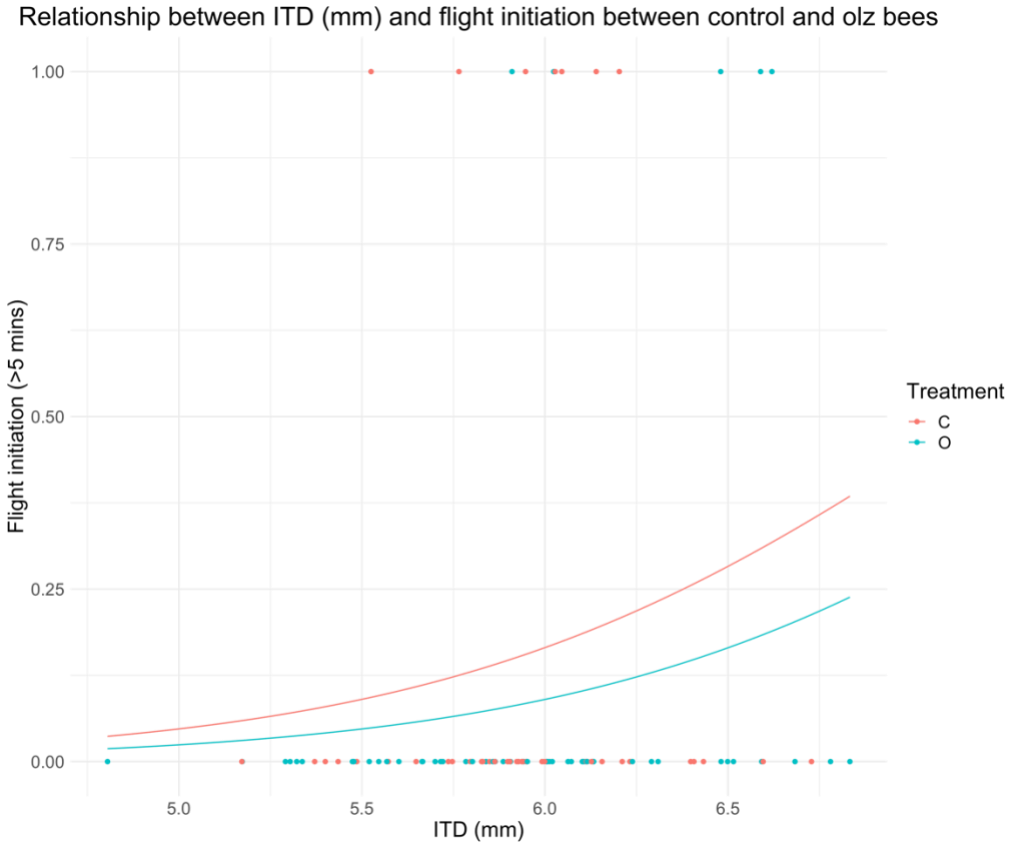


Supplementary Figure S4.3: Effect of olanzapine on flight performance in *B. terrestris* works that flew > 5 minutes. Flight distance, duration, and mean velocity were measured using a tethered flight mill attached to a computer which calculated the distance flown to the nearest 10cm and speed of flight**.** Bees were fed 0.05 µg of olanzapine or a control. Boxplots of flight statistics between olanzapine and control *B. terrestris* worker bees that flew > 5 minutes. A) Total distance flown (m); B) Flight duration (s); C) Mean velocity (m/s). No significant difference was found between workers exposed to olanzapine (O) or control (C). Olanzapine: n = 5; Control: n = 7.

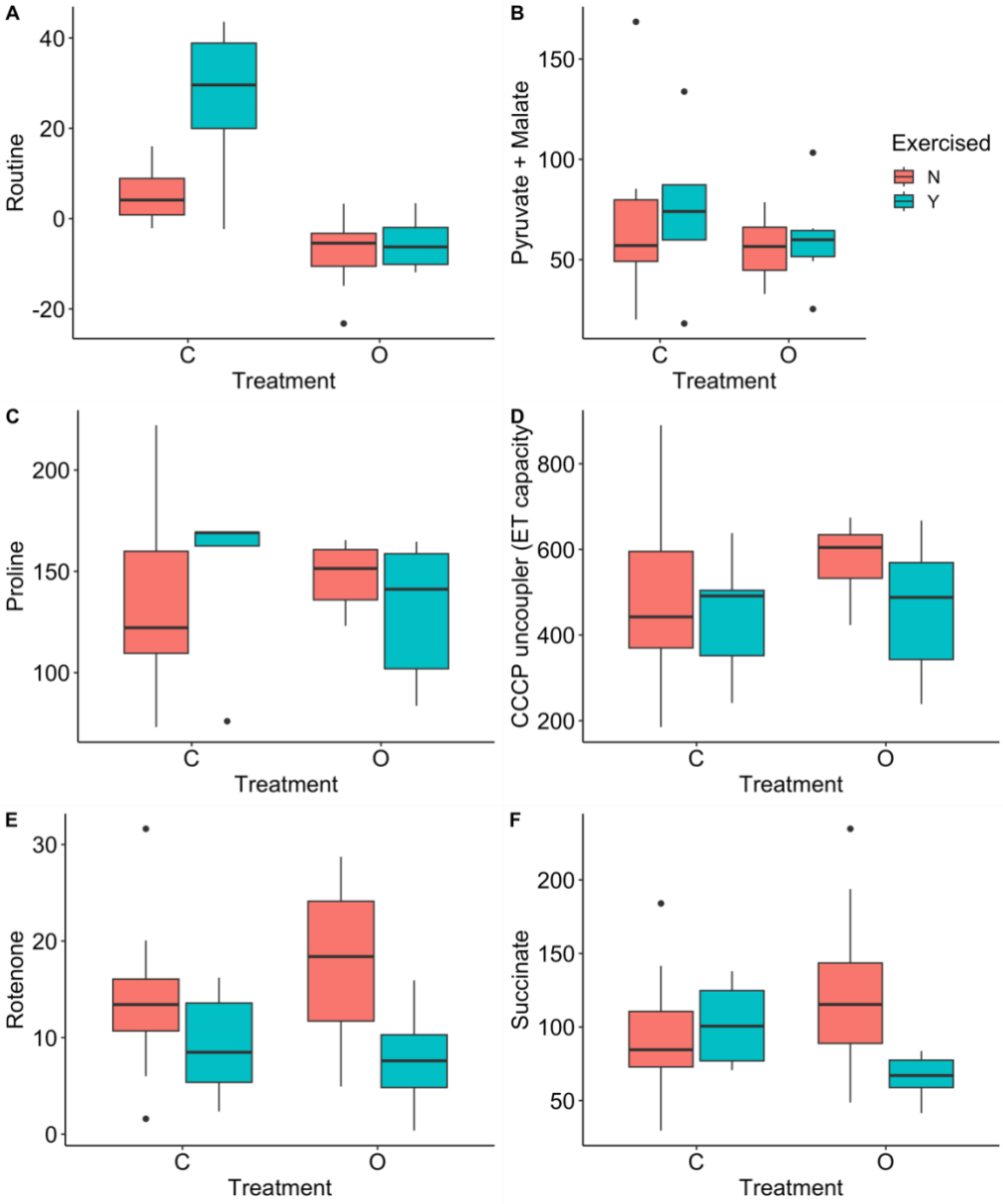


Supplementary Figure S4.4: Effect of mill number on flight performance in *B. terrestris* works that flew > 5 minutes. Flight distance, duration, and mean velocity were measured using a tethered flight mill attached to a computer which calculated the distance flown to the nearest 10cm and speed of flight**.** Boxplots showing the flight statistics between flight mills. There was no different in distance flown,

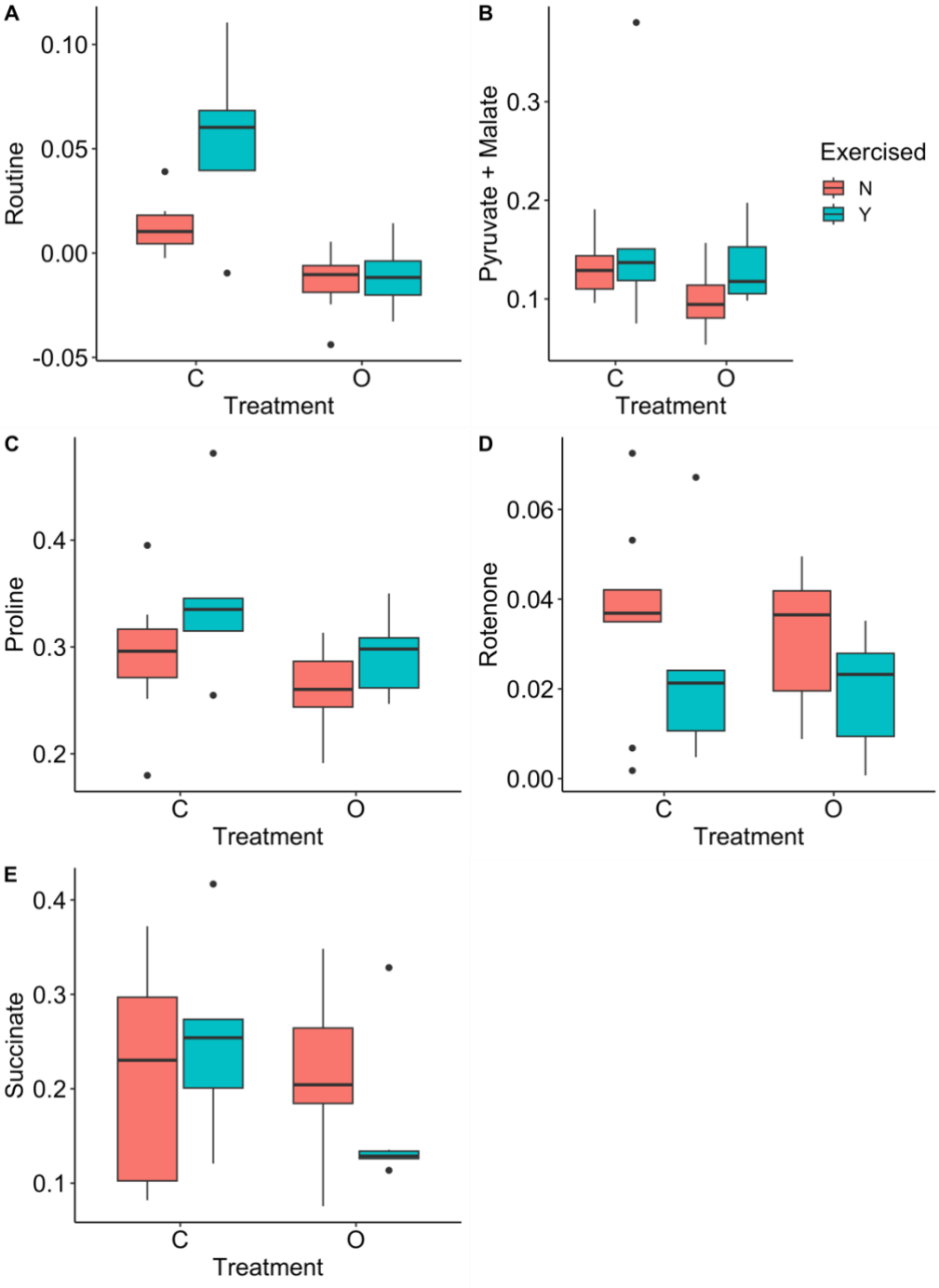
flight duration or mean velocity between mills 1-3. Distance flown: Kruskal Wallis: chi-squared = 0.667, df = 2, *p*-value = 0.717. Flight duration: Kruskal Wallis: chi-squared = 0.130, df = 2, *p*-value = 0.937. Mean velocity: One-way ANOVA: F-value = 1.102, *p*-value = 0.400. Mill 1 (pink): n = 4; mill 2 (purple): n = 2; mill 3 (green): n = 6



Supplementary Figure S4.5: Logistic regression between intertegular distance (ITD) and flight initiation between workers exposed to olanzapine and control. ITD (mm) (used a proxy for bee size) and olanzapine did not significantly affect the probability of flight initiation (*p*-value of coefficients: ITD = 0.114; Treatment (olanzapine) = 0.282). Model call selected as best fitting: Flight initiation ~ ITD + Treatment. Hosmer-Lemeshow goodness of fit: Statistic = 9.407, df = 8, *p*-value: 0.30915. y-axis: 0 = uninitiated flight, 1 = initiated flight. Olanzapine: n = 53; control n = 43



Supplementary Figure S4.6: Effect of exercise and olanzapine on the oxygen flux in *B. terrestris* worker brain. Bees were fed 0.05 µg of olanzapine or a control and exposed to exercise via the flight mill or not. Boxplots showing the oxygen consumption (background corrected) in worker bumblebees between treatment type (olanzapine and control) and exercise. A) Routine; B) Pyruvate + Malate; C) Proline; D) Uncoupler (ET capacity); E) Rotenone; and F) Succinate. Abbreviations: C = control, O = Olanzapine, Y = Exercised, N = Not exercised. Olanzapine exercised: n = 6; control exercised: n = 5; olanzapine non-exercised: n = 10; control non-exercised: n = 9.

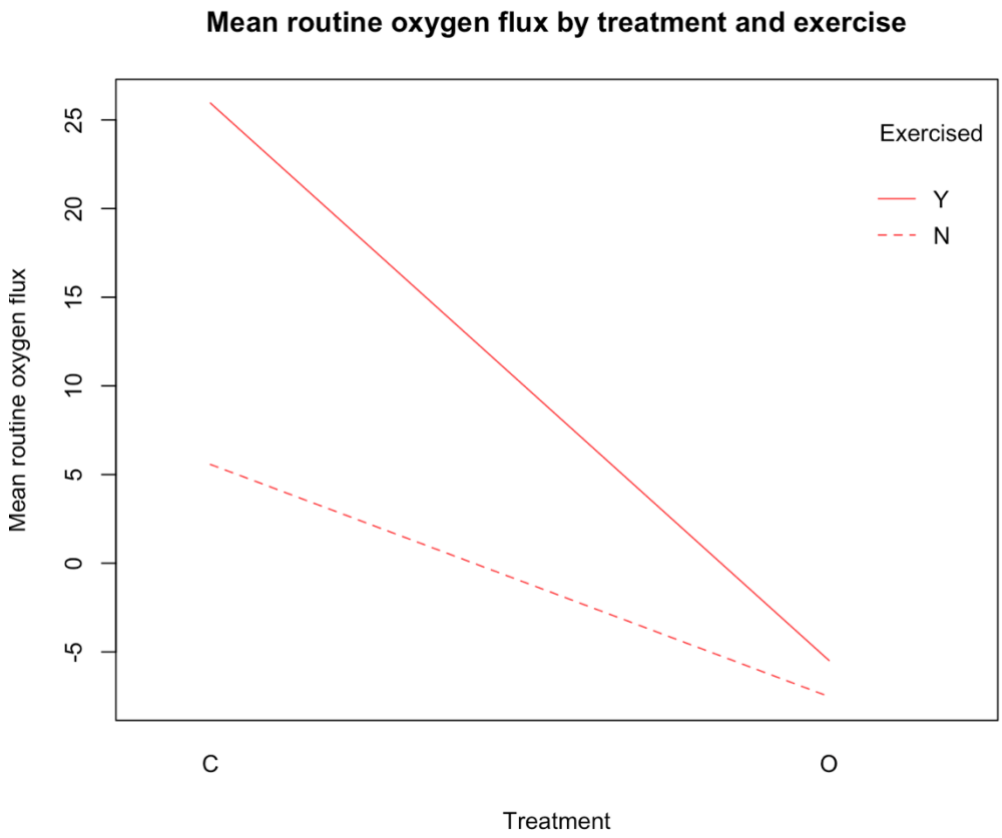


Supplementary Figure S4.7: Effect of exercise and olanzapine on the FCR in *B. terrestris* worker brain. Bees were fed 0.05 µg of olanzapine or a control and exposed to exercise via the flight mill or not.

Boxplots showing the flux control ratios (FCR) in worker bumblebees between treatment type (olanzapine or control) and exercise. A) Routine; B) Pyruvate + Malate; C) Proline; D) Rotenone; and E) Succinate. To calculate the FCR the ET capacity (uncoupler) is set to 1 and the ROX (AmA) is set to 0. Abbreviations: C = control, O = Olanzapine, Y = Exercised, N = Not exercised. Olanzapine exercised: n = 6; control exercised: n = 5; olanzapine non-exercised: n = 10; control non-exercised: n = 9.

Supplementary Table S4.1: Pearson's result showing the correlation between intertegular distance (ITD) and oxygen flux and FCR at the different respirations states. df = 24 for all tests. *p <0.05

Respiration state	Pearson	
	r	*p*-value
Oxygen flux		
Routine	-0.01	0.951
Pyruvate + Malate	0.17	0.403
Proline	0.20	0.330
CCCP	0.46	0.019*
Rotenone	-0.004	0.984
Succinate	-0.09	0.648
FCR		
Routine	-0.11	0.6027
Pyruvate + Malate	-0.13	0.5148
Proline	-0.46	0.017*
Rotenone	-0.25	0.2265
Succinate	-0.43	0.027*

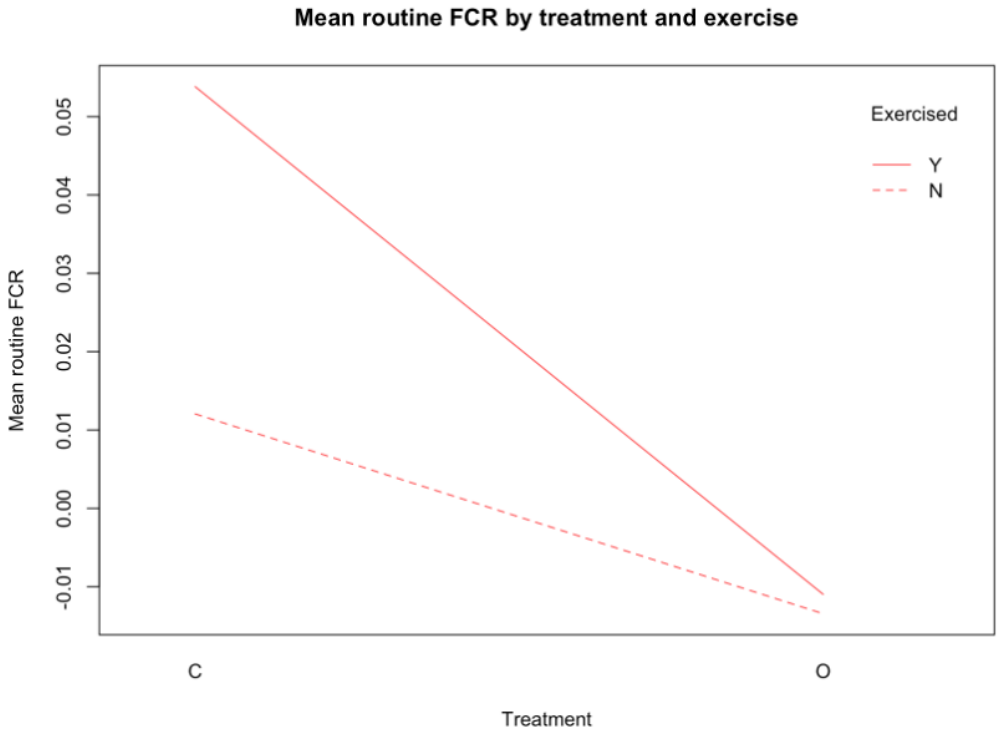


Supplementary Figure S4.8: Interaction between exercise and treatment at routine respiration oxygen consumption.

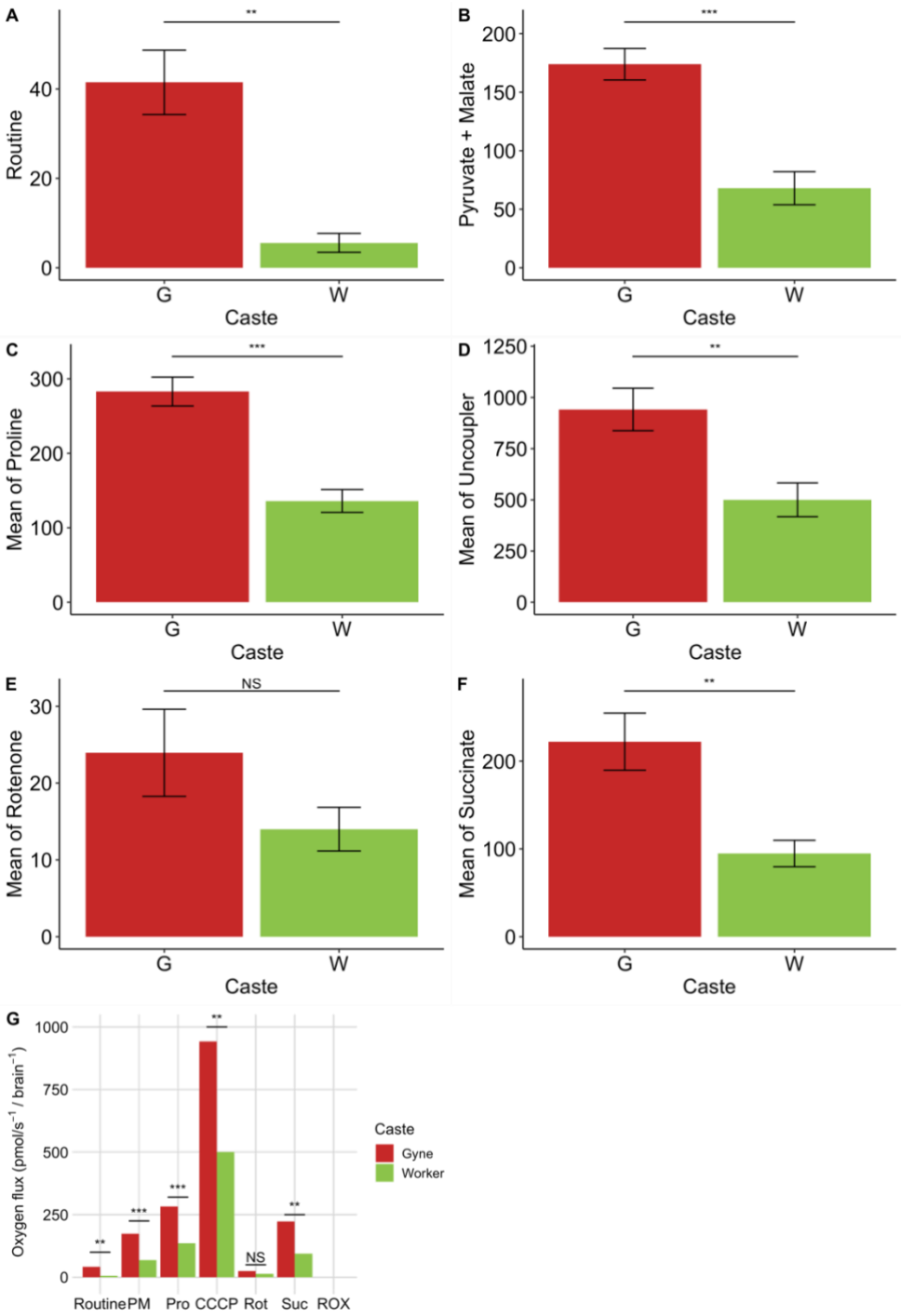
Abbreviations: C = control, O = Olanzapine.

Supplementary Table S4.2: Mean oxygen flux and flux control ratio (FCR) values in *B. terrestris* worker brain of exercised and non-exercised bees treated with olanzapine and control. No FCR data is provided for the maximum ET capacity (CCCP) as this value is set to 1 to obtain the ratios for the other respiratory states listed.

Respiration state	Non-exercised, Control (n = 9)		Non-exercised, Olz (n = 10)		Exercised, Control (n = 5)		Exercised, Olz (n = 6)	
	Mean (s.e)	Variance	Mean (s.e)	Variance	Mean (s.e)	Variance	Mean (s.e)	Variance
Oxygen flux								
Routine	5.57 (2.12)	40.60	-7.52 (2.35)	55.24	25.95 (8.14)	331.46	-5.49 (2.49)	37.08
Pyruvate + Malate	67.95 (14.14)	1800.12	55.25 (4.74)	224.56	74.62 (18.80)	1767.81	60.55 (10.37)	645.25
Proline	136.00 (15.28)	2101.12	148.31 (4.67)	218.09	149.32 (18.37)	1688.02	130.66 (14.47)	1256.66
CCCP	500.13 (82.43)	61149.29	576.85 (25.57)	6535.77	445.35 (68.22)	23267.70	462.13 (67.08)	26999.21
Rotenone	14.00 (2.84)	72.77	17.98 (2.77)	76.60	9.20 (2.55)	32.60	7.77 (2.20)	29.09
Succinate	94.75 (15.08)	2045.49	122.46 (18.27)	3336.19	102.19 (13.08)	855.67	66.04 (6.43)	248.36
FCR								
Routine	0.01 (0.004)	0.0002	-0.01 (0.004)	0.0002	0.05 (0.020)	0.002	-0.01 (0.007)	0.0003
Pyruvate + Malate	0.13 (0.010)	0.0002	0.10 (0.010)	0.001	0.17 (0.054)	0.014	0.13 (0.016)	0.002
Proline	0.29 (0.020)	0.003	0.26 (0.012)	0.001	0.35 (0.037)	0.007	0.29 (0.016)	0.002
Rotenone	0.04 (0.007)	0.0005	0.04 (0.005)	0.0002	0.03 (0.011)	0.0006	0.02 (0.006)	0.0002
Succinate	0.22 (0.035)	0.011	0.22 (0.028)	0.008	0.25 (0.049)	0.012	0.16 (0.034)	0.007



Supplementary Figure S4.9: Interaction between exercise and olanzapine at routine respiration flux control ratio (FCR).
Abbreviations: C = control, O = Olanzapine.



Supplementary Figure S4.10: The effect of olanzapine and exercise on the mean oxygen flux at each respiratory state in the brain of non-exercised *B. terrestris* workers and gynes. Brain tissue was analysed via high-resolution respirometry. Bar graphs of the mean oxygen flux between gyne and worker bumblebees at the different respiratory states. A) Routine respiration; B) pyruvate + malate; C) proline; D) rotenone; E) succinate; F) combined plot

Unpaired Students *t*-test were performed between castes at each state, with the exception of routine where an unpaired Wilcoxon rank sum test was performed as the data was not normally distributed. Error bars showing the mean ± standard error. Workers: n = 9; gynes: n = 6. *p <0.05, **p <0.01, ***p <0.001

5. Chapter 3: Current approaches for the prediction of DNA N^6-methyladenine methylation in eusocial bees and the influence of endosymbiotic bacteria

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5.1 Abstract

In recent years there has been a growing number of studies reporting 6mA methylation in eukaryotic DNA. However, the levels of methylation are often very low which creates challenges in accurately detecting the prevalence of 6mA in eukaryotes. Eusocial bees have been the focus of epigenetic research due to their phenotypic plasticity and the strong environmental influence on caste determination. Therefore, in this study we use the bumblebee, *Bombus terrestris* and European honeybee, *Apis mellifera* as model organisms for elucidating the potential presence and roles of 6mA methylation in the genomic (nuclear) and mitochondrial DNA. We demonstrate the use of multiple techniques to detect and quantify 6mA methylation in bees including Oxford Nanopore sequencing, and a new approach we term the immuno-southern (iSouthern) which we establish as a reliable method for the detection of 6mA methylation in DNA. Using these different methods, we find evidence that 6mA DNA methylation can be present in eusocial bees and find it to be associated with infection of the bacterial endosymbiont, *Arsenophonus apicola*. We further report that *A. apicola* infection in bumblebees increases the number of highly methylated 6mA sites in the genes that are associated with cellular differentiation. Overall, we demonstrate the importance of identifying endosymbionts when performing DNA sequencing, particularly when detecting 6mA methylation in eukaryotic DNA samples.

5.2 Introduction

5.2.1 6mA in eukaryotes

6mA methylation is the most prevalent DNA modification in bacteria and is involved in mismatch repair, DNA replication and transcription.[141-143,159] However, in recent years there has been a great interest in a potential role of 6mA methylation in eukaryotic DNA and growing evidence of its presence.[138,141,158,160] In *C. elegans* and *Drosophila* levels of 6mA have been reported to range between 0.01-0.4% and 0.001-0.07%, respectively.[138,143,161] In *Drosophila*, 6mA was present in the gene bodies of transposons and found to be fundamental in transposon activation.[158,163] Active transcription has also been shown to be maintained by the demethylation of intragenic 6mA in genes associated with neuronal function in *Drosophila*.[158] Current evidence suggests that the presence of 6mA DNA can lead to different transcriptional outcomes depending on its position within a specific genomic region.

During development 6mA may also play a transient role; in the early stages of *Drosophila* development 6mA levels were found to be higher than in the latter stages.[143] This was also seen in pigs and zebrafish which showed 6mA levels reach a density of up to ~0.2 % during early embryogenesis.[157]

Studies have also found mitochondrial DNA (mtDNA) in mammals to be enriched for 6mA. Hao *et al* reported that 6mA in human mtDNA may be responsible for supressing mitochondrial gene expression.[162] Furthermore, stress (in the form of hypoxia) was also shown to elevate 6mA levels in the mtDNA by three-fold.[162]

5.2.2 DNA methylation in eusocial bees

Eusocial bees have been of particular interest in epigenetic research due to their phenotypic plasticity.[34] Considerable morphological and phenotypical differences including size, lifespan, behaviour and reproductivity are seen between female castes despite having near identical genomes.[34,56,57] Caste differentiation is predominately determined by the quality and quantity of food

received during the larval stage.[56,57,59] Hence, the influence of environmental factors has sparked much interest in the role of epigenetic factors in caste determination.

5mC methylation has been the focus of most epigenetic research in bees, with a particular bias towards the honeybee, *Apis mellifera*. 5mC is found to occur almost entirely at CpG sites with a prevalence of ~1% of dinucleotides being methylated in honeybees.[36,151,166,167] DNA methylation, if primarily found in genes, is often association with gene activation.[36,128,151,166-168] CpG clusters in methylated genes are also thought to be associated with gene splicing; it is considered that 5mC may regulate splicing patterns during caste differentiation.[36,70] However, the role of DNA methylation in caste differentiation is still under scrutiny. Cardoso-Junior *and* colleagues showed that gene body methylation in honeybees was colony-specific and affected by genotype rather than any social or environmental cues.[166] DNA methylation has also been associated with the initiation of reproductivity in workers when there is an absence of a queen, as well as being crucial in memory formation and preservation.[34,128,174,175]

Stress can also elicit changes in DNA methylation. For instance, in honeybees the exposure of intruders to the hive initiated aggressive behaviour which altered DNA methylation in the brain after only a couple of hours.[171] It is important to establish how environmental stressors such as threats, insecticides, or disease can alter DNA methylation in bees as a heritable epigenetic change can have long-term effects amongst bee populations.[70]

Based on our current understanding of DNA methylation in eusocial bees, we propose the use of honeybees and bumblebees as a suitable model organism for elucidating potential roles of 6mA DNA methylation, if present.

5.2.3 Current methods for detecting low level methylation

In eukaryotes, although 6mA methylation has been reported in DNA, levels are generally extremely low.[143,158] Recent studies have highlighted the need for

caution when detecting 6mA in eukaryotes as some techniques can overestimate methylation levels and be unreliable.[141] Therefore, in order to reliably identify 6mA methylation it is important to use methods that can detect low level methylation accurately. Currently, a variety of methods are used to detect 6mA methylation in DNA. One such method is the use of anti-6mA antibodies which bind to 6mA sites in RNA and DNA.[138] 6mA antibodies can be used in dot blotting or to immunoprecipitate methylated DNA for subsequent sequencing.[138] Detection with the use of antibodies, however, is not quantifiable or specific to 6mA as it will bind to other adenine base modifications such as 1mA or m6A in RNA.[138] Furthermore, any contamination in the sample will also be detected, which needs to be considered.[288]

The use of methylation-sensitive restriction enzymes is another method which has long been used to distinguish between methylated and unmethylated bases within or close to the DNA recognition sequence of particular restriction endonucleases.[138] Enzymes which are sensitive to methylated adenine are therefore able to accurately determine 6mA methylation at a single base resolution, with the caveat that only adenines in the recognition site of the enzyme will be identified.[138,288]

Liquid chromatography coupled with mass spectrometry (LC-MS/MS) is another approach, which provides the most sensitive detection of 6mA methylation and unlike the previously mentioned methods allows quantification of modified bases.[138,289] 6mA detection at very low levels by LC-MS/MS comes with the caveat that this modification is often detected as a result of contaminating bacterial DNA. Any detection should therefore be interpreted cautiously so not to misinterpret background levels likely to be derived from bacterial DNA contaminants.[138,141,288] For example, enzymes which digest the DNA into nucleosides prior to LC-MS/MS analysis have been found to contain 6mA methylated, bacterial DNA and may therefore distort results.[141]

A relatively new method which allows the quantification and detection of 6mA is third-generation sequencing, such as single molecule real time sequencing (SMRTseq) and nanopore sequencing. These long-read sequencing technologies can identify sequence reads and modified bases within sequence reads with reasonable accuracy. In the case of nanopore sequencing, individual bases are detected via an electric current signal as each nucleotide produces a unique characteristic disruption pattern to the current as it passes through the protein pore embedded in a electro-resistant membrane.[290,291] A methylated nucleotide will produce a different signal compared to its non-methylated counterpart which enables the identification and quantification of methylation.[197,290] The raw electric current signal can then be translated during basecalling into the respective DNA sequence.[291]

Sequencing can provide ultra-long reads and base-specific methylation detection at a genome-wide level, however, the algorithms and models used for basecalling use neural networks and machine learning models which are trained on real data.[197,291] Hence, basecallers are often influenced by the data they were trained on; the performance of this type of basecalling depends on whether the modifications and sequence motifs were represented in the training data.[288,289,291] This is particularly important in 6mA methylation as it is much less established, in comparison to 5mC methylation, particularly in eukaryotic genomes. Sequencing methods have also been shown to give more accurate results in organisms with a higher abundance of 6mA and decline in organisms with lower levels of methylation.[141]

The different methods and approaches of 6mA detection, including those described above, have been shown to yield varied levels of methylation measurements in eukaryotic genomes ranging from ~0.0006-0.6% of 6mA, which has led to much criticism of the techniques.[141,158,161,288] One concern is not taking into consideration the potential for bacterial contamination in samples which may lead to artefacts.[138,158,288] Another is the uncertainty regarding the possibility of the presence of 6mA derived from m6A in RNA.[158] Studies using LC-MS/MS have demonstrated that 6mA in mammalian DNA is found at such low levels it could be explained by the mis-incorporation of

recycled m6A from RNA via the nucleotide-salvage pathway.[158] Therefore, it is important to take into consideration the limitations of each method and have the right balance of sensitivity and specificity to prevent false positive results but still detect weak signals.[138]

In this study, we use a combination of techniques to cross-validate the results from different detection approaches and achieve both high sensitivity and specificity in the detection of 6mA methylation in DNA. The first approach is the use of methylation-sensitive restriction endonucleases. The DNA is first cut with one of three restriction enzymes, DpnI, MboI and Sau3AI (New England Biolabs®), which all have the recognition site, GATC. DpnI and Mbol are methylation-sensitive; DpnI cleaves at the recognition site when the adenine is methylated, whilst MboI cleaves when the adenine is unmethylated. Sau3AI is not methylation-sensitive and will cleave irrespective of methylation making it a suitable control enzyme to demonstrate that the DNA sample to be analysed can be completely cleaved by restriction endonucleases. This approach has also been described by Joshi *et al.*[154] Once the DNA is digested, gel electrophoresis then determines whether the DNA has been cut and fragmented and thus indicates the presence or absence of 6mA methylation. The second step is the method that Reinhard Stöger first termed the immuno-southern (iSouthern). The iSouthern combines the techniques of Southern blotting and specific antibody binding to determine the presence of an antigen of interest within DNA in this case, the N^6-methyladenine base. Hence, by transferring the DNA to a membrane via Southern blotting and then binding a primary 6mA antibody followed by a secondary antibody that is conjugated with horseradish peroxidase (HRP), it is possible to further identify 6mA methylation presence in the DNA. Although these two methods can give a strong indication of 6mA methylation presence, the third approach of DNA sequencing is able to provide a quantifiable result and affirm the presence of 6mA. With the use of Oxford Nanopore sequencing, a better understanding of the potential role of methylation in the bumblebee and honeybee genomes can be achieved by mapping the precise location of methylation sites. Overall, by combining these three approaches, *i*) methylation-sensitive restriction digest, *ii*) iSouthern, and *iii*) nanopore sequencing, a more reliable detection and

quantification of low-level 6mA methylation can be achieved allowing greater confidence in the findings of this particular DNA modification and its impact for future investigations.

5.2.4 Experimental aims

The presence of 6mA in eukaryotic DNA is still uncertain and its potential biological function remains to be elucidated.[292] We know 5mC methylation has an important role in eusocial bees, and there is growing evidence that supports the occurrence of 6mA methylation in higher eukaryotes. Therefore, it is pertinent to determine whether 6mA methylation is present in the genomic DNA (gDNA) or mitochondrial enriched (mtDNA) of honeybees and bumblebees and further elucidate its potential function in eukaryotes.

We aim to further contribute to our current understanding of 6mA methylation in eukaryotic DNA, and more specifically in eusocial bees. Firstly, using a combination of techniques, including iSouthern and Oxford Nanopore sequencing, we will determine whether 6mA is present in gDNA and mtDNA, in the eusocial bees, *Apis mellifera* and *Bombus terrestris*. Secondly, we will aim to explore previously unaccounted factors, such as bacterial endosymbionts, that may influence adenine modification in eukaryotic DNA. Finally, we will review current opinions regarding the presence of 6mA in eukaryotes.

5.3 Methods

5.3.1 DNA isolation

5.3.1.1 Bee collection and husbandry

Honeybees were collected throughout spring to late summer in the years 2020-2022 from two hives located at the University of Nottingham, Sutton Bonington Campus and stored in -80ºC. Live bumblebees were purchased from Biobest® (Westerlo, Belgium) and stored at 26°C and 33% relative humidity and were fed 2.0 M sucrose solution and pollen (purchased from Agralan, UK) *ad libitum.*

5.3.1.2 Arsenophonus apicola culture

To determine the role of endosymbiotic bacteria on the 6mA levels in bees we performed *Arsenophonus apicola* infection analyses. *A. apicola* infected and uninfected bumblebees, along with *A. apicola* plates, were provided by Professor Greg Hurst at the University of Liverpool. Bumblebees were dissected one week after *A. apicola* infection. *A. apicola* contained the Pom1-GFP plasmid and the presence or absence of infection in bumblebees was confirmed by dissecting their abdomen and analysing under a fluorescent microscope (**Supplementary Figure S5.1**).

5.3.1.3 Tissue dissection and DNA extraction

To extract nuclear and mitochondrial-enriched DNA fractions from the brain and flight muscle the method was similar to that described in Katyal *et al.*[293] The head and thoracic tissue from ~10-20 bees were dissected and pooled into separate tissue-types before being homogenised in mitochondrial extraction buffer (50 mM Tris-HCl (pH 7.4-7.6), 100 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 50 mM HEPES, 100 mM sucrose) using the gentleMACS Dissociator (Miltenyi Biotec). The homogenate was then briefly centrifuged to remove any debris before being centrifuged again at 800 g for ten minutes to remove any further debris. The supernatant was then removed and centrifuged for ten minutes at 1000 g to collect the nuclear DNA. The supernatant was separated

from the nuclear DNA pellet and centrifuged for 30 minutes at 13200 g to collect the mitochondrial-enriched DNA. The nuclear and mitochondrial enriched DNA fractions were stored at -80ºC until DNA isolation.

5.3.1.4 Genomic DNA isolation

To isolate genomic DNA from the nuclear fraction, it was first lysed at 56ºC overnight in a solution of 1x TEN9, 1% SDS, proteinase K (20 mg/ml) and RNase A (4 mg/ml)(Promega). After lysis, protein precipitation solution (Promega Wizard®) was added, and the solution was cooled on ice for ten minutes before being centrifuged for five minutes. All centrifugation was performed at 16000 g for both genomic and mitochondrial-enriched DNA isolation, unless otherwise stated. The supernatant was removed and added to 0.9 ml of isopropanol and 1.5 µl of GlycoBlue™ (Invitrogen™) and left at -20ºC for a minimum of 60 minutes. The solution was then centrifuged for seven minutes, and the supernatant was discarded. The DNA pellet was washed in 500 µl of 70% ethanol and centrifuged for a further two minutes The ethanol was discarded, and the DNA was briefly air dried before adding 40 µl of elution buffer (10 mM Tris-Cl, pH 8.5)(Qiagen).

5.3.1.5 Mitochondrial-enriched DNA isolation

To isolate the mitochondrial-enriched DNA, the protocol by Isokallio and Stewart[294] was partly followed (DNA extraction section, step 20-38).[294,295] In brief, the mitochondrial-enriched DNA fraction was lysed overnight at 56ºC in 400 µl buffer (20 mM Tris, 150 mM NaCl, 20 mM EDTA, 1% SDS (pH 8.75)), 5µl proteinase K (20 mg/ml) and 5 µl RNase A (4 mg/ml)(Promega). 100 µl of protein precipitation solution (Promega Wizard®) was then added followed by 500 µl choloform:isoamylalcohol (24:1). Briefly mixed by shaking, the solution was then centrifuged for ten minutes before the upper aqueous phase was transferred and added to 1 ml of ice cold 100% ethanol and 1.5 µl of GlycoBlue™ (Invitrogen™) and inverted five times to mix. The solution was then stored at -80ºC for 1-2 hours. After freezing, the DNA was

pelleted via centrifugation for 15 minutes. The supernatant was discarded, and the pellet was washed with 500 µl of 70% ethanol before being centrifuged again for a further five minutes. The ethanol was removed, and the pellet was briefly air dried before adding 25 µl of elution buffer (10 mM Tris-Cl, pH 8.5)(Qiagen©). The DNA was quantified using a NanoDrop spectrophotometer. Both the genomic and mitochondrial-enriched DNA were stored at 4ºC, or alternatively -20ºC for later analysis. *A. apicola* chromosome and plasmid isolation

5.3.1.6 A. apicola chromosome and plasmid isolation

A. apicola colonies were collected from the agar plates, provided by the University of Liverpool, and the DNA was isolated. To isolate the chromosomal (genomic) DNA the Qiagen DNeasy PowerSoil Pro kit was used (ID: 47014/Qiagen©). For plasmid isolation, the QIAprep Spin Miniprep Kit (50) (ID: 27104) was used.

5.3.2 Immuno-southern (iSouthern)

5.3.2.1 Enzyme restriction digest and gel electrophoresis

For each DNA sample, a master mix was generated, containing ~4 µl of rCutSmart® buffer (New England Biolabs®) and ~ 2 µg DNA; this was mixed before adding 9 µl each into four tubes; 1 µl of either DpnI, MboI, Sau3AI (New England Biolabs®) or water (control) was then added to the DNA and incubated at 37ºC for up to 12 hours. The digested DNA, along with ~30 ng of lambda dam+ DNA (λ+) (New England Biolabs, catalogue: N3011) and ~30 ng of lambda dam- DNA (λ-) (New England Biolabs: N3013), was then run on a 1% agarose gel containing 0.5 µg/ml ethidium bromide and then imaged.

5.3.2.2 Southern blot

After imaging, the gel was treated to UV radiation for two minutes before being washed in denaturing buffer (0.5 M NaOH, 1.5 M NaCl) for one hour at room temperature. The gel was then transferred to a nitrocellulose membrane at room temperature overnight. Once transferred, the membrane was rinsed in

40 mM NaPi for 1-2 minutes before being cross-linked via UV light exposure for two minutes.

5.3.2.3 6mA antibody binding

The membrane was blocked in a 5% non-fat milk 1xTBST (5% non-fat milk, 20 mM Tris, 150 mM NaCl, 0.1% Tween®20) solution for one hour at room temperature. It was then washed in 1xTBST for five minutes and repeated twice more before being incubated overnight at 4°C in the primary rabbit polyclonal 6mA antibody (1 mg/ml) (Synaptic Systems, 202 003). The membrane was then washed three times as before in 1xTBST before incubating in the secondary goat anti-rabbit IgG HRP antibody (1 mg/ml)(Invitrogen™, 31466) at room temperature for one hour. Finally, the membrane was briefly washed in 1xTBST three times and then imaged with chemiluminescence using the Pierce™ ECL western blotting substrate (Thermo Scientific™).

5.3.3 Oxford Nanopore sequencing

5.3.3.1 R.9.4.1 flow cell protocol

Sequencing was carried out using the MinION Mk1C from Oxford Nanopore with the SQK-RAD004 rapid sequencing kit. The R9.4.1 flow cell version was used which provides a raw read accuracy of up to 95%. ~400 ng of DNA was loaded into the flow cell and left to run for a minimum of 48 hours. A minimum sequence length of 200 bp was set.

Basecalling and alignment were performed using Guppy v 6.1.1 (Oxford Nanopore Technologies) with the Rerio basecall model 'res_dna_r941_min_modbases-all-context_v001.cfg'[296], to identify and tag methylated adenines. During basecalling, reads were filtered by phred q-score and those with a mean q-score < 7 (error probability of 0.1995) were deemed to have failed. Oxford nanopore uses the Phred quality score (q-score) to calculate the accuracy of base identification during sequencing.[297,298] Phred q-scores are determined using the formula $Q = -10 \log_{10} P$, and so q-scores are

logarithmically correlated to the error probability (*P*).[297,298] Hence, for our analysis only reads with a basecall accuracy of ≥ 80.0% (q-score ≥ 7) passed and were subsequently used for downstream analysis.

Once aligned to the reference genome, the reads which passed quality control were sorted and only the primary mapped reads were selected and indexed using Samtools[299]. The mapped and indexed reads were then analysed using the EPI2ME Labs programme 'Modbam2bed'[300]. This creates a bedMethyl file containing methylation frequency data and read coverage for the base of interest (6mA). The data contained in the bedMethyl files were then analysed using R (version 4.2.1).[211]

5.3.3.2 R.10.4.1 flow cell and kit 14 chemistry protocol

In the latter stages of the study the flow cells were upgraded to the R.10.4.1 version with the corresponding kit 14 chemistry. The rapid sequencing kit (SQK-RAD114) was used for all sequencing protocols. The R.10 series of nanopores contains a greater raw read accuracy of > 99%.

~100-150 ng of DNA was used to load into the flow cell and a minimum sequence length of 200 bp was set. Sequencing was left to run for a minimum of 48 hours and only stopped when the number of cumulative reads had levelled off.

Modified basecalling and aligning were performed using Dorado (v0.5.1)[301] with the model 'dna_r10.4.1_e8.2_400bps_sup@v4.3.0' to identify and tag methylated adenines. Unlike Guppy, reads are not filtered out based on q-scores and can be filtered in downstream analyses.

As described with the R.9.4.1 protocol, the primary aligned reads were then sorted and indexed with Samtools[299]. The reads were then converted to a bedMethyl file using Modkit[302]. The adenines were filtered and bases with low confidence, based on the predicted modification probabilities, and falling below the threshold of 0.8 and 0.75 for modified and canonical adenines,

respectively, were removed. The bedMethyl files were analysed using R (version 4.2.1).[211]

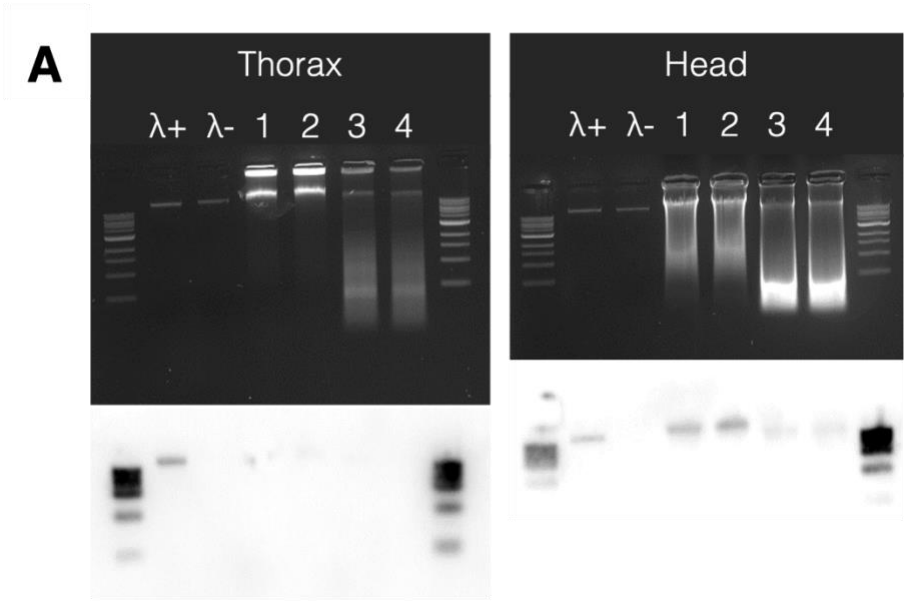
5.4 Results

5.4.1 iSouthern

5.4.1.1 Bumblebee iSouthern results

We found no indication of 6mA based on the methylation-sensitive restriction digests and subsequent iSoutherns in any of the *B. terrestris* (bumblebee) gDNA (nuclear enriched fraction) or mtDNA (mitochondrial enriched fraction) samples that were analysed. Both the undigested control DNA (DNA sample was treated with water, rather than a restriction enzyme) and DpnI enzyme showed a high molecular band whilst the MboI and Sau3AI enzyme had fragmented the DNA (**Figure 5.1; Figure 5.2; Figure 5.3**).

Although the bumblebee worker gDNA isolated from the head gave a signal from the antibody, this was seen in all four of the digests and was therefore considered to be background signal (**Figure 5.1**). Therefore, our analysis using the iSouthern approach suggests that 6mA is not present in *B. terrestris*.



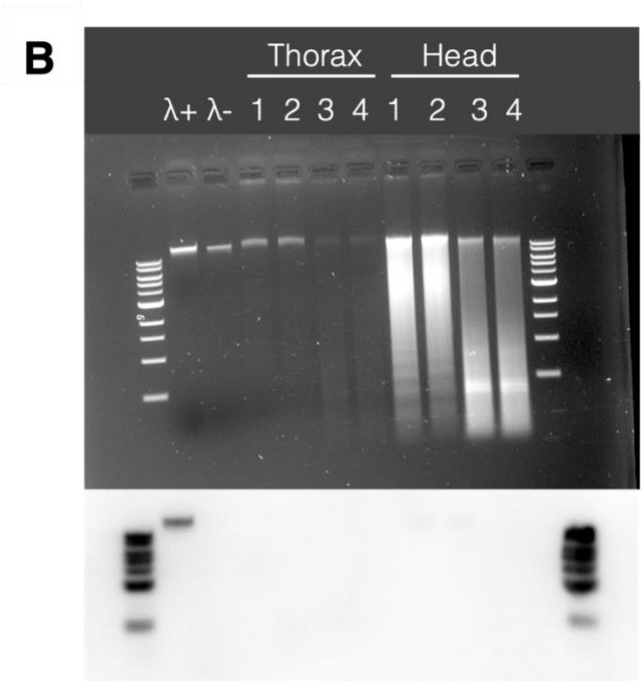


Figure 5.1: Image of gel (top) and iSouthern blot (bottom) suggesting 6mA is not present in the genomic DNA or mitochondrial-enriched DNA of *B. terrestris* worker head or thorax. A) genomic DNA from the nuclear fraction of *B. terrestris* workers; B) mitochondrial-enriched DNA from the mitochondrial fraction of *B. terrestris* workers. Ladders are 1kb. DNA was digested with either a water control or restriction enzyme.1: H_2O control; 2: DpnI; 3: MboI; 4: Sau3AI. λ-: 6mA unmethylated lambda DNA. λ+: 6mA methylated lambda DNA. Chemiluminescence was visualised with the use of the Pierce™ ECL western blotting substrate.

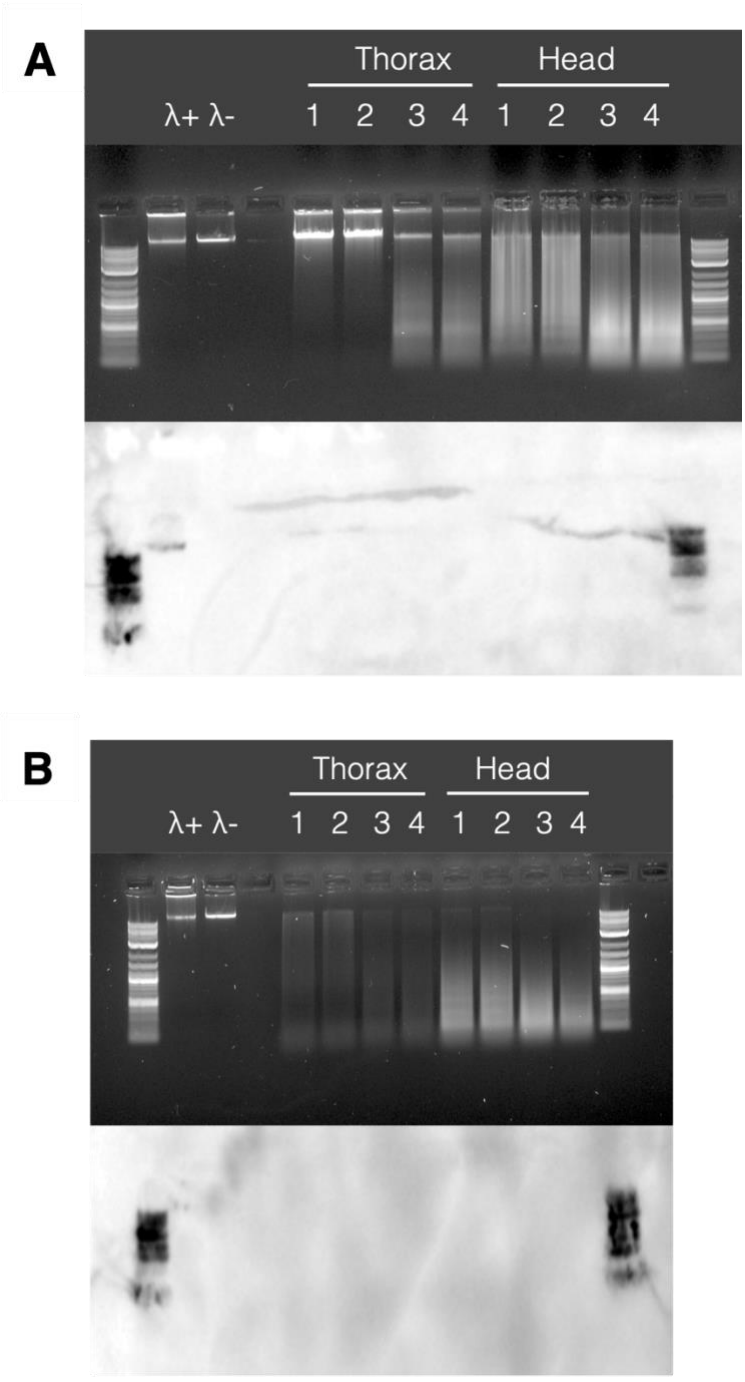


Figure 5.2: Image of gel (top) and iSouthern blot (bottom) suggesting 6mA is not present in the genomic DNA or mitochondrial-enriched DNA of *B. terrestris* drone head or thorax. A) genomic DNA from the nuclear fraction of *B. terrestris* drones; B) mitochondrial-enriched DNA from the mitochondrial fraction of *B. terrestris* drones. Ladders are 1kb. DNA was digested with either a water control or restriction enzyme.1: H_2O (control); 2: DpnI; 3: MboI;

4: Sau3AI. λ-: 6mA unmethylated lambda DNA. λ+: 6mA methylated lambda DNA. Chemiluminescence was visualised with the use of the Pierce™ ECL western blotting substrate.

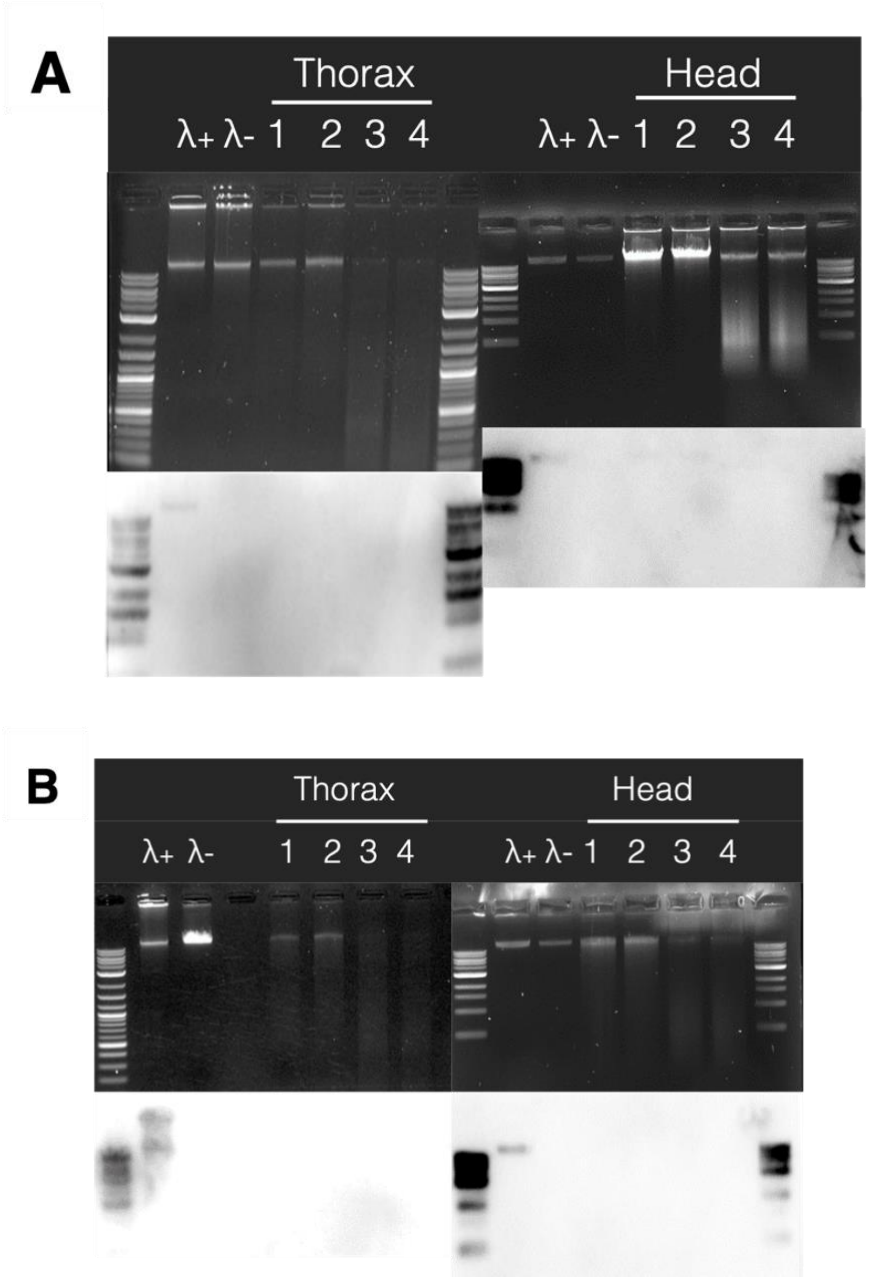


Figure 5.3: Image of gel (top) and iSouthern blot (bottom) suggesting 6mA is not present in the genomic DNA or mitochondrial-enriched DNA of *B. terrestris* queen head or thorax. A) genomic DNA from the nuclear fraction of *B. terrestris* queens. B) mitochondrial-enriched DNA from the mitochondrial fraction of *B. terrestris* queens.

DNA was digested with either a water control or restriction enzyme.1: H_2O (control); 2: DpnI; 3: MboI; 4: Sau3AI. λ-: 6mA unmethylated lambda DNA. λ+: 6mA methylated lambda DNA. Chemiluminescence was visualised with the use of the Pierce™ ECL western blotting substrate. Ladders are 1kb.

5.4.1.2 Honeybee iSouthern results

As seen in the bumblebees, the iSouthern results suggested there was no 6mA in honeybee forager or nurse gDNA or mtDNA (**Figure 5.4; Figure 5.5**). The gDNA taken from the head of nurses did give a faint signal from the antibody, however this is most likely due background signal as the control and DpnI DNA bands are so highly concentrated. This is further supported by the λ+ DNA providing a stronger antibody signal despite the much lower DNA concentrations than the gDNA bands indicated by the gel.

In contrast to the female castes, the honeybee drone gDNA and mtDNA iSouthern suggested the presence of adenine methylation in both tissues. The MboI treated DNA still showed a high molecular weight, undigested band whilst the Sau3AI digested DNA had been almost completely fragmented. The DpnI band for the mtDNA also appeared fainter than the control and MboI, suggesting the DNA had been cleaved at the adenine sites that were methylated. Furthermore, the antibody gave a strong signal for the MboI digested DNA as well as the control (**Figure 5.6**). To confirm, the iSouthern was repeated and the same result was obtained (figure not shown).

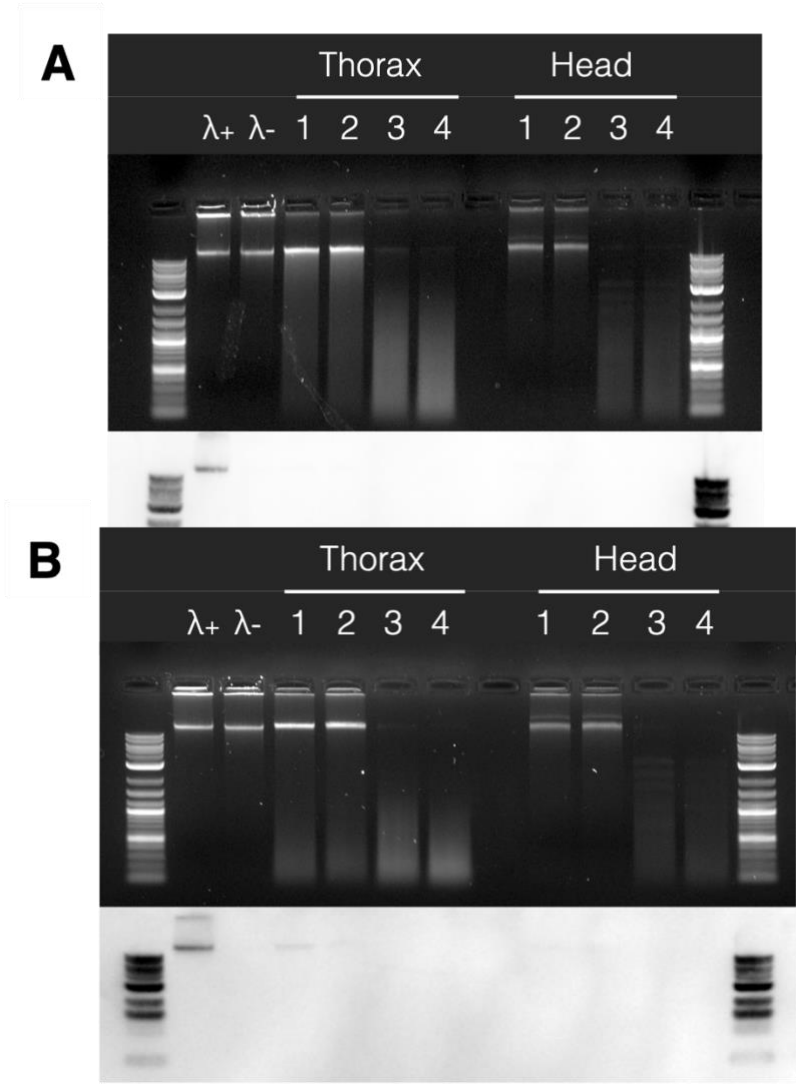


Figure 5.4: Image of gel (top) and iSouthern blot (bottom) suggesting 6mA is not present in the genomic DNA or mitochondrial-enriched DNA of *A. mellifera* forager head or thorax. A) genomic DNA from the nuclear fraction of *A. mellifera* foragers. B) mitochondrial-enriched DNA from the mitochondrial fraction of *A. mellifera* foragers. DNA was digested with either a water control or restriction enzyme.1: H_2O (control); 2: DpnI; 3: MboI; 4: Sau3AI. λ-: 6mA unmethylated lambda DNA. λ+: 6mA methylated lambda DNA. Chemiluminescence was visualised with the use of the Pierce™ ECL western blotting substrate. Ladders are 1kb.

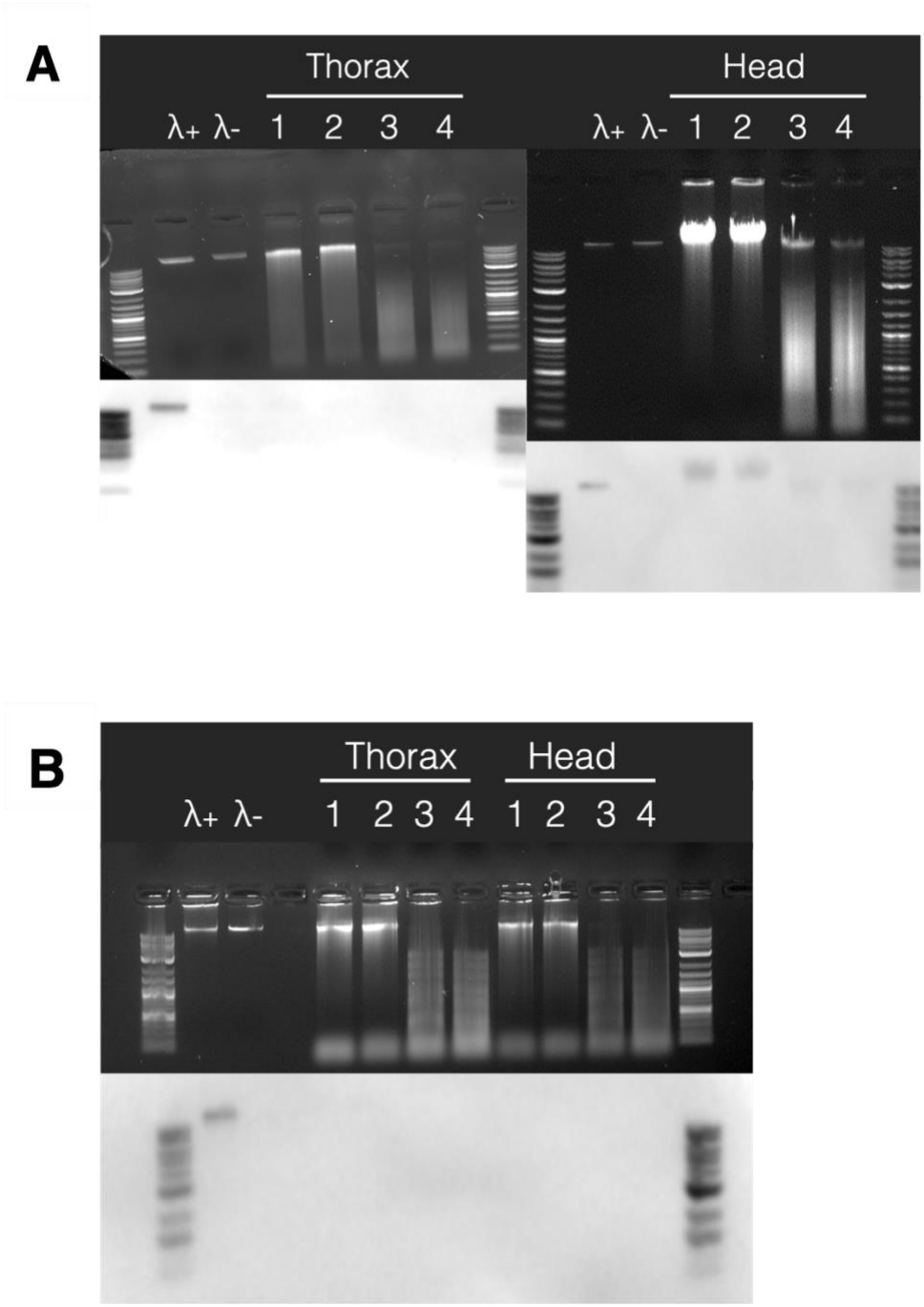


Figure 5.5: Image of gel (top) and iSouthern blot (bottom) suggesting 6mA is not present in the genomic DNA or mitochondrial-enriched DNA of *A. mellifera* nurse head or thorax. A) genomic DNA from the nuclear fraction of *A. mellifera* nurses. B) mitochondrial-enriched DNA from the mitochondrial fraction of *A. mellifera* nurses. DNA was digested with either a water control or

restriction enzyme.1: H_2O (control); 2: DpnI; 3: MboI; 4: Sau3AI. λ-: 6mA unmethylated lambda DNA. λ+: 6mA methylated lambda DNA. Chemiluminescence was visualised with the use of the Pierce™ ECL western blotting substrate. Ladders are 1kb.

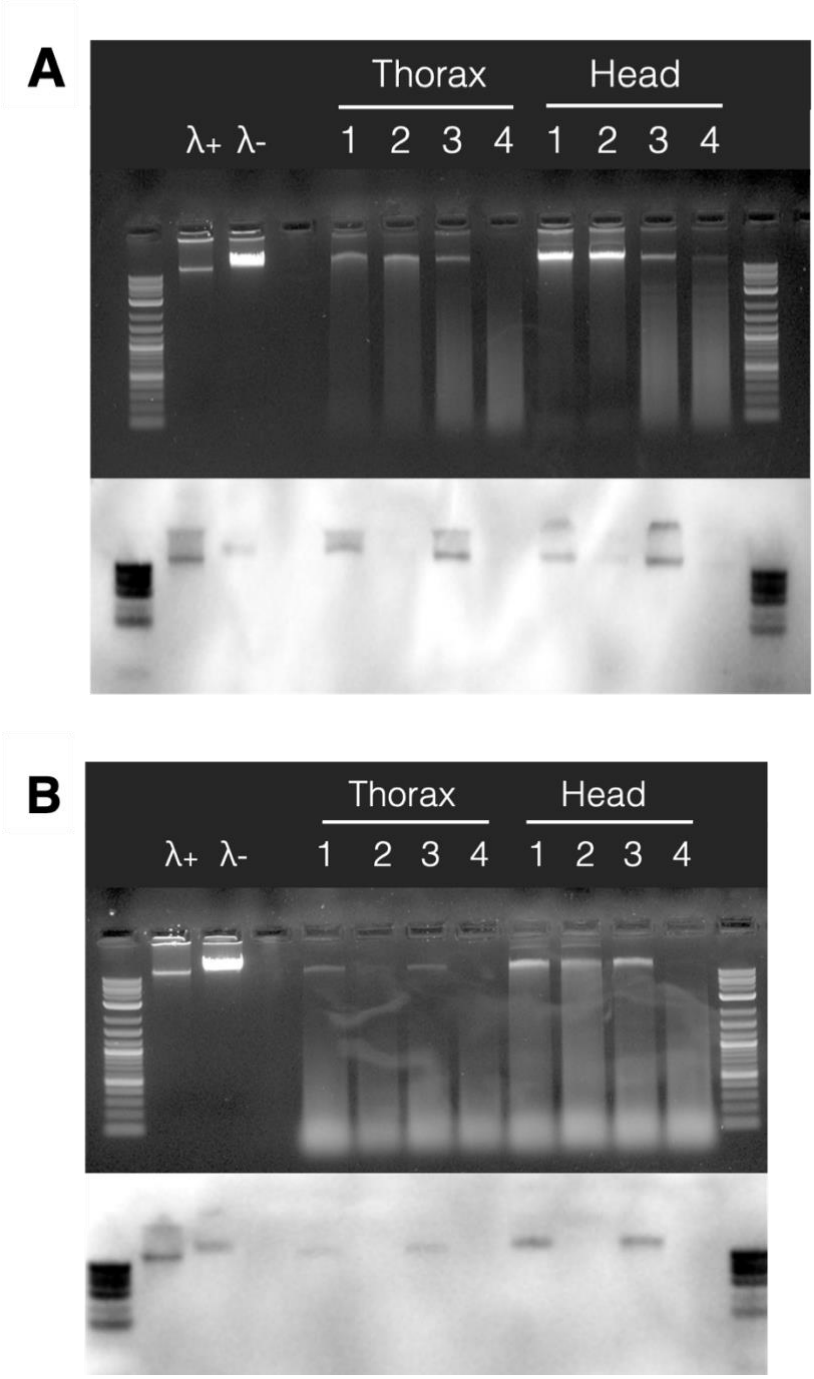


Figure 5.6: Image of gel (top) and iSouthern blot (bottom) suggesting 6mA may be present in the genomic DNA or mitochondrial-enriched DNA of *A. mellifera* drone head and thorax. A clear band can be seen after digestion with MboI (lane 3)

and a weaker band can be seen after the digestion of DpnI (lane 2) which suggests 6mA may be present. A) genomic DNA from the nuclear fraction of *A. mellifera* drones. B) mitochondrial-enriched DNA from the mitochondrial fraction of *A. mellifera* drones. DNA was digested with either a water control or restriction enzyme.1: H_2O (control); 2: DpnI; 3: MboI; 4: Sau3AI. λ-: 6mA unmethylated lambda DNA. λ+: 6mA methylated lambda DNA. Chemiluminescence was visualised with the use of the Pierce™ ECL western blotting substrate. Ladders are 1kb.

5.4.2 Nanopore Sequencing

To supplement the results from the methylation-sensitive restriction digests/iSouthern method and to enable the quantification of 6mA methylation levels in *B. terrestris* and *A. mellifera* we performed third-generation, Nanopore sequencing. This would in principle allow the identification of 6mA methylation at the precision of each singular base in DNA.

5.4.2.1 B. terrestris gyne mitochondrial-enriched DNA (thoracic muscle)

456,000 reads were processed during basecalling. Of those, 4739 (1.04%) reads failed quality checks (q-score < 7) and 451,261 (98.96%) reads passed. The average read q-score was 9.84.

There were 32,514 reads (50% of the total reads) primary mapped to the mitochondrial reference genome (NCBI GenBank: MK570129.1) and the mean mapping quality (MAPQ) was 57.1. The complete genome (17,232 bp) was covered and the 6mA density was 0.09% (**Table 5.1**).

5.4.2.2 B. terrestris gyne gDNA (thoracic muscle)

Less than a quarter of the total reads primary aligned to the *Bombus terrestris* reference genome (NCBI RefSeq: GCF_910591885.1). The mean sequencing depth of the adenine sites was 3.13 x and the 6mA density for the total adenines sequenced was 0.082% (**Table 5.1**).

5.4.2.3 A. mellifera drone mitochondrial-enriched DNA from the head and thoracic muscle

Given the previous research associating 6mA methylation in the mtDNA of eukaryotes[162] we analysed the mitochondrial-enriched DNA to determine if 6mA methylation was present.

Nearly three quarters of reads sequenced from mitochondrial-enriched DNA isolated from the head passed basecalling with a mean q-score of 9.54. For the thoracic muscle the sequencing data from the drone thorax genomic DNA was used and aligned to the mtDNA reference genome (NCBI GenBank: L06178.1). The mtDNA reference genome of *A. mellifera* is 16,343 bp in length with 7061 adenine bases. For both tissue types, ~9% of the total reads primary aligned to the reference genome and the complete genome was covered. Sequencing depth was high with adenines being covered ~240 x and ~170 x, for the thoracic muscle and head, respectively. 6mA density was similar between tissues at 0.15% in the thoracic muscle and 0.12% in the head (**Table 5.1**).

5.4.2.4 A. mellifera drone gDNA from the thoracic muscle and head

The *A. mellifera* drone thoracic muscle showed evidence of 6mA; the DNA was run on a gel and displayed a band after digestion with MboI suggesting 6mA presence (**Supplementary Figure S5.2**). To corroborate this finding an aliquot was taken from the same sample of gDNA and was then sequenced on the Nanopore platform.

Approximately 30% of the total reads primary aligned to the *A. mellifera* reference genome (NCBI RefSeq: GCF_003254395.2) for both the thoracic muscle and head gDNA. The thoracic muscle gDNA sequencing covered about a quarter of the genome whereas the head gDNA covered 15%. However, the mean sequencing depth of the adenine sites was only 1.12 x and 1.05 x times.

(**Table 5.1)**. The 6mA density was 0.22% and 0.14% for the thoracic muscle and head, respectively, with little variation between individual chromosomes (**Supplementary Figure S5.3**).

Table 5.1: 6mA methylation overview of nanopore sequencing runs.

All nanopore sequencing was performed with R.9.4.1 flow cells and the rapid sequencing kit SQK-RAD004. All primary mapped reads were filtered by MAPQ and those with <20 were removed*

*excluding *A. mellifera* drone MboI digested A

Species	Caste	DNA Sample/tissue	Ref genome	Primary mapped reads	% of reads mapped to ref	Mean MAPQ	Genome coverage (%)	A sites covered in ref (%)	Mean depth of A sites	Total canonical A's	Total modified A's	6mA density
B.terrestris	Gyne	Thoracic muscle	mtDNA	32514	49.57%	58.9	100%	100%	353.55 x	4865221	4402	0.090%
B.terrestris	Gyne	Thoracic muscle	gDNA	50533	23.27%	56.8	3.73%	1.88%	3.13 x	10177122	8330	0.082%
A. mellifera	Drone	Thoracic muscle	mtDNA	24059	8.81%	45.4	100%	100%	240.46 x	3100674	4499	0.145%
A. mellifera	Drone	Thoracic muscle	gDNA	92914	32.02%	57.9	26.70%	14.72%	1.12 x	21841031	47189	0.216%
A. mellifera	Drone	Head	mtDNA	10023	8.72%	59.0	100%	100%	170.03 x	2193570	2688	0.123%
A. mellifera	Drone	Head	gDNA	57039	29.35%	58.88	15.28%	8.23%	1.05 x	11738345	16304	0.139%
A. mellifera	Drone	MboI enzyme digested Head + Thoracic muscle	gDNA	182827	12.66%	9.02	0.61%	0.46%	23.29 x	13543132	24225	0.179%

5.4.3 MboI digested gDNA isolation: iSouthern and sequencing

There was insufficient genome coverage and very low sequencing depth, when sequencing the genomic DNA with our described Nanopore approach. Therefore, we decided to enrich for 6mA-containing DNA, by first digesting the *A. mellifera* drone DNA (from both the thoracic muscle and head) with the MboI enzyme. This enzyme cleaves the DNA when the adenine in the recognition site (GATC) is unmethylated and thus leaves a high molecular weight band containing 6mA sites. We then isolated this high molecular weight band from the gel for subsequent Nanopore sequencing (**Figure 5.7**). However, it should be considered that there will be 6mA sites located on low molecular weight bands between the MboI cleavage sites that would not be isolated and therefore lost during this enrichment approach.

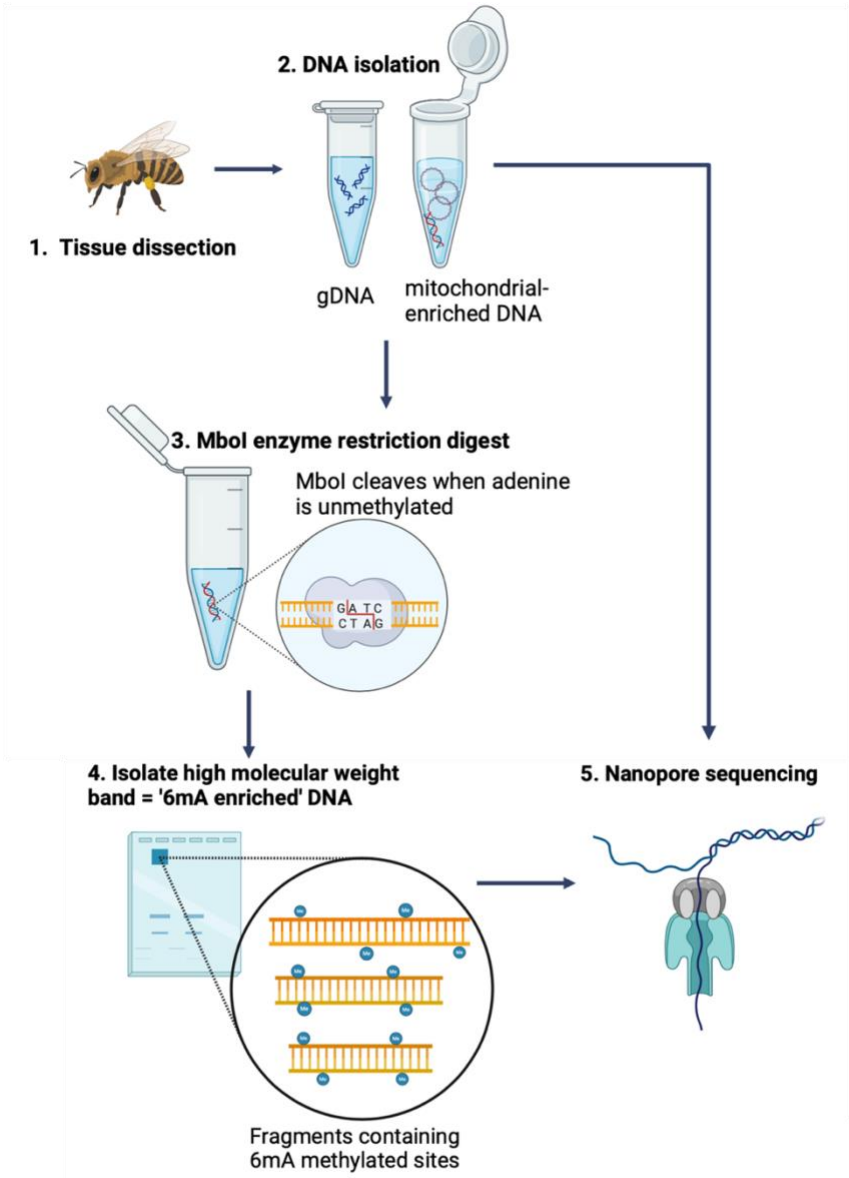


Figure 5.7: Diagram showing the isolation of 6mA-enriched DNA from the digestion with the MboI restriction enzyme. Head (brain) and thoracic muscle were dissected from the bee and the gDNA and mitochondrial-enriched DNA for both tissue types (described in section *5.3.1.3*) were obtained. Sequencing without enriching for 6mA could then be directly performed. To enrich the gDNA for 6mA, the gDNA was digested with MboI which cleaves the DNA at the target site 'GATC' when the adenine is unmethylated. Gel electrophoresis then separates the fragments, leaving a high molecular weight band that is enriched for 6mA in the GATC site. The band is then isolated and sequenced.

5.4.3.1 Nanopore sequencing

The average q-score of passed reads was 9.04, and of those 182,827 reads primary mapped to the *A. mellifera* reference genome. Only 0.61% of the total genome was covered, which was spread throughout each chromosome (1-16), with coverage ranging from ~0.3-1% (**Figure 5.8**). The mean depth across all sites were considerably higher (23.3 x) of this 6mA-enriched genomic DNA than the undigested genomic DNA (**Table 5.1**). The total 6mA density was 0.179%, which varied slightly between chromosomes (**Figure 5.8**; **Figure 5.9**).

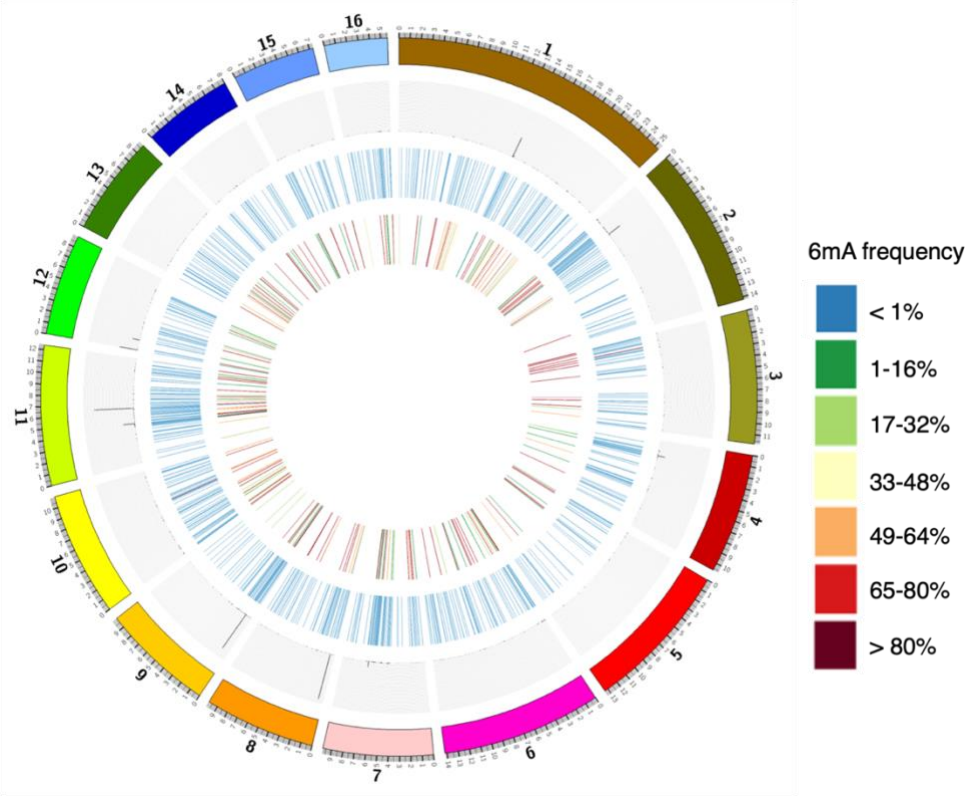


Figure 5.8: Circos plot showing the sequencing depth and 6mA frequency from *A. mellifera* drone 6mA-enriched gDNA for each chromosome. The gDNA was first digested with the MboI enzyme and the high molecular weight DNA was isolated to enrich for 6mA prior to sequencing. DNA was sequenced using the rapid sequencing kit (SQK-RAD114). Outer ring: line plot showing the sequencing depth; axis ranging from 0-7000. Middle ring: heatmap showing all adenine sites

sequenced and the methylation frequency (n = 675,792). Inner ring: Adenine sites with >0% methylation frequency only (n = 12,852).

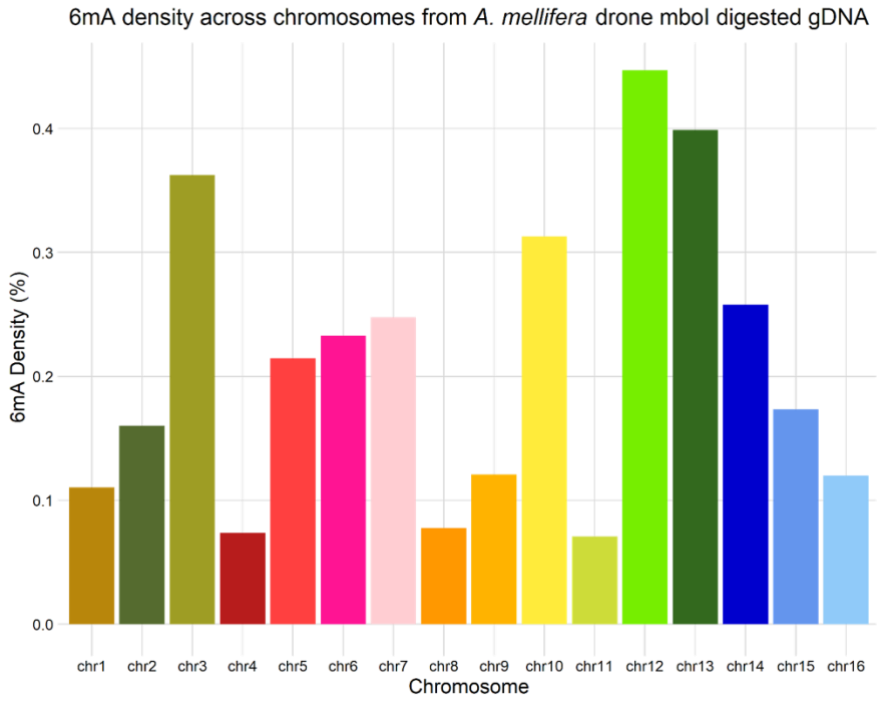


Figure 5.9: 6mA density across the genome of *A. mellifera* 6mA-enriched genomic DNA. The gDNA was first digested with the MboI enzyme and the high molecular weight DNA was isolated for sequencing. DNA was sequenced using the rapid sequencing kit (SQK-RAD114). Bar graph showing the 6mA density across each chromosome of the *A. mellifera* MboI digested gDNA. 6mA density (%) is defined as: $\left(\frac{Total\ modified\ A's}{Total\ canonical\ A's}\right)\ x\ 100$

5.4.3.2 Presence of DNA from bacterial endosymbionts

In our analysis only approximately 12% of the reads primarily mapped to the *A. mellifera* reference genome (**Table 5.1**). Therefore, to determine if and what other organisms the reads aligned to, we performed a *What's In My Pot* (WIMP) workflow analysis with the Epi2me platform (Oxford Nanopore

Technologies). The WIMP analysis enables species identification and quantification for the sample.

We found that nearly half the reads aligned to the gammaproteobacteria, *Arsenophonus nasoniae* (**Table 5.2**). In comparison, this was followed second by *A. mellifera* with just 16% of the reads from WIMP analysis aligning.

Table 5.2: Epi2me WIMP output from *A. mellifera* 6mA-enriched gDNA sequencing showing the top five classifications and the percentage of reads aligning to the species.

Total reads analysed	Total reads aligned to root (*A. melifera*)	Classified reads	Top five classifications (species)	Number of reads (% of total classified reads)
779,947	96,354 (16.01%)	601,823	*Arsenophonus nasoniae*	283,215(47.06%)
			Homo sapiens	91,737(15.24%)
			Escherichia coli	38,958(6.47%)
			Arsenophonus endosymbiont of *aphis craccivora*	32,159(5.34%)
			Enterobacterales	10,035(1.67%)

5.4.4 6mA methylation levels and the endosymbiont, *Arsenophonus apicola*, in bumblebees

B. terrestris workers were infected via abdominal injection with the endosymbiont, *A. apicola,* to determine whether bacterial presence affected 6mA methylation level. Infection was confirmed by the analysis of their abdomen under fluorescence to identify the presence of the green fluorescence protein (GFP) (**Supplementary Figure S5.1**). The head and thoracic muscle of workers with a confirmed *A. apicola* presence were dissected and pooled. Uninfected *B. terrestris* workers were taken directly out of the colony. All *B. terrestris* DNA was digested with the MboI enzyme and all sequencing was performed with the MinION using R.10.4.1 flow cells and the rapid sequencing kit (SQK-RAD114).

5.4.4.1 6mA methylation in A. apicola infected and uninfected B. terrestris

The WIMP analysis showed a strong presence of *A. nasoniae* in the infected bumblebee gDNA (42.02% of total reads analysed) and was not reported in the top five species for the uninfected bumblebee gDNA, indicating very low levels, if any (**Table 5.3**). This is also confirmed by the number of reads which mapped to the *A. apicola* reference genome from both the infected and uninfected gDNA. We found that 22.3% of the primary reads from the sequencing of the infected sample aligned to *Arsenophonus* whereas only 0.4% of the primary reads from the uninfected sample aligned (**Table 5.3**).

An iSouthern was also done using infected and uninfected bumblebee MboI digested (6mA-enriched) gDNA to determine if there was a presence of 6mA. There was a stronger signal in the thoracic muscle compared to the head and as expected the strongest response from the antibody binding was in the MboI digested DNA. There was also a signal from the control lanes in both tissues, however as this DNA had already been digested with MboI this could explain the response (**Figure 5.10**).

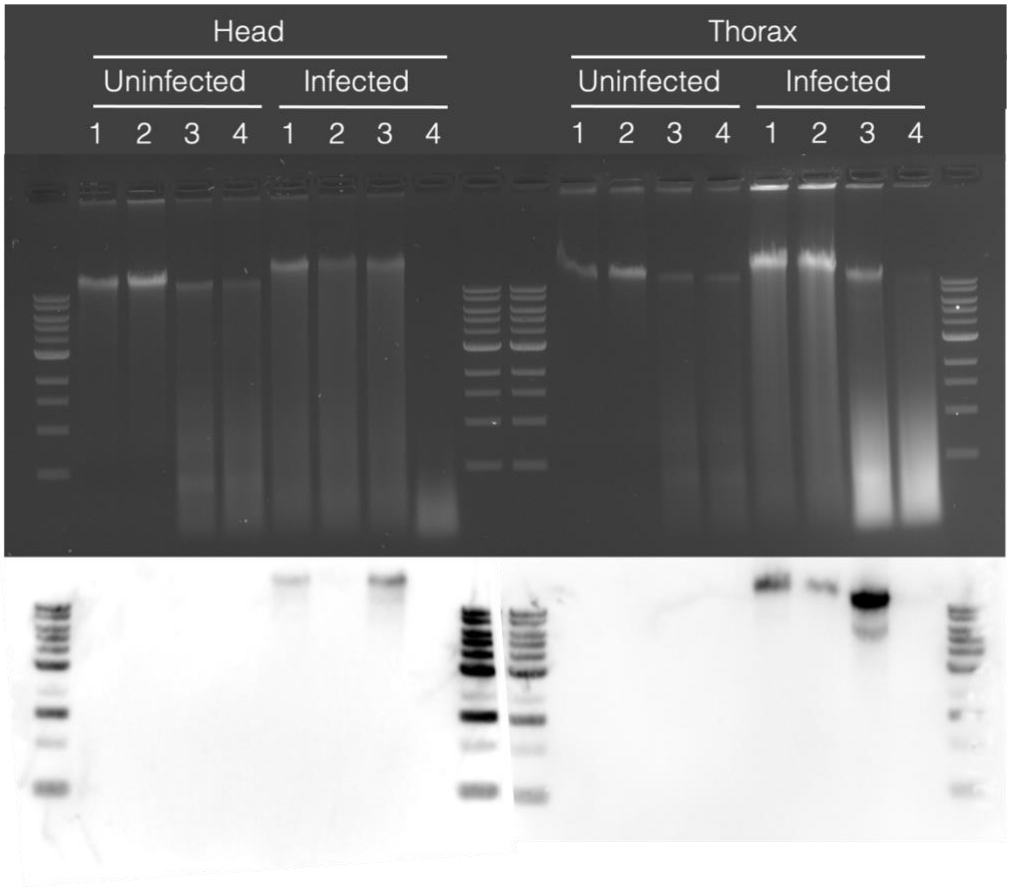


Figure 5.10: Image of gel (top) and iSouthern blot (bottom) from *A. apicola* infected *B. terrestris* worker genomic DNA and uninfected worker genomic DNA taken from the head and thorax. A stronger signal is seen in the thoracic muscle compared to the head with an indication of 6mA. DNA was digested with either a water control or restriction enzyme.1: H_2O (control); 2: DpnI; 3: MboI; 4: Sau3AI. Chemiluminescence was visualised with the use of the Pierce™ ECL western blotting substrate. Ladders are 1 kb.

Less than 10% of the total reads from the infected and uninfected bumblebee gDNA from the thoracic muscle aligned to the *B. terrestris* genome (**Table 5.4**). The total genome coverage was 2.2% and 1.2% for the infected and uninfected DNA, respectively. Overall depth across all sequenced adenine sites was low (2.21 x and 3.45 x), however similar regions had high sequencing depth between both samples (**Figure 5.11**).

Table 5.3: Epi2me WIMP output from the *A. apicola* infected *B. terrestris* worker gDNA and uninfected *B. terrestris* worker gDNA sequencing showing the top five classifications and the percentage of reads aligning. gDNA was isolated from the thoracic muscle.

A. apicola infected	Total reads analysed	Classified reads	Total reads aligned to root (*B. terrestris*)	Top five species (excluding root)	Number of reads (% of total classified reads)
Uninfected	425	342(80.47%)	114(33.33%)	*Stenotrophomonas maltophilia*	38(11.11%)
				Escherichia coli	38(11.11%)
				Escherichia coli KO11FL	29(8.48%)
				Enterobacterales	23(6.73%)
				Achromobacter xylosoxidans	13(3.80%)
Infected	742	583(78.57%)	154(26.42%)	*Arsenophonus nasoniae*	245(42.02%)
				Escherichia coli KO11FL	33(5.66%)
				Enterobacterales	31(5.32%)
				Escherichia coli	28(4.80%)
				Arsenophonus endosymbiont of Aphis craccivora	17(2.92%)

Table 5.4: Nanopore sequencing overview for *A. apicola* infected and uninfected *B. terrestris* worker MboI enzyme digested gDNA from the thoracic muscle. Reads were aligned to multiple reference genomes including the *B. terrestris* mitochondrial genome and *A. apicola* chromosome.

Species	Caste	*A. Apicola* infected	DNA Sample/tissue	Ref genome	Primary mapped reads	% of reads mapped to ref	Mean MAPQ	Genome Coverage (%)	A sites covered in ref (%)	Mean depth of A sites	Total canonical A's	Total modified A's	6mA density
B.terrestris	Worker	Infected	MboI enzyme digested thoracic muscle	gDNA	34619	5.93%	6.34	2.175%	1.39%	2.21 x	5545082	171070	3.09%
B.terrestris	Worker	Uninfected	MboI enzyme digested thoracic muscle	gDNA	22936	8.46%	11.92	1.17%	0.67%	3.45 x	4176895	115827	2.77%
B.terrestris	Worker	Infected	MboI enzyme digested thoracic muscle	mtDNA	1559	0.31%	8.76	59.25%	49.69%	7.37 x	51663	1949	3.77%
B.terrestris	Worker	Uninfected	MboI enzyme digested thoracic muscle	mtDNA	745	0.33%	15.4	56.81%	47.03%	4.93 x	32890	1043	3.17%
B.terrestris	Worker	Infected	MboI enzyme digested thoracic muscle	*A.apicola* chr	122846	22.26%	54.9	99.99%	159.95%	20.24 x	64629557	2057279	3.18%
B.terrestris	Worker	Uninfected	MboI enzyme digested thoracic muscle	*A.apicola* chr	840	0.38%	53.7	21.0523%	6.10%	1.08 x	222890	7037	3.16%
A. apicola	N/A	N/A	N/A	Chr	491369*	73.71%	59.4	100%	197.45%	94.77 x	375114527	10246071	2.73%

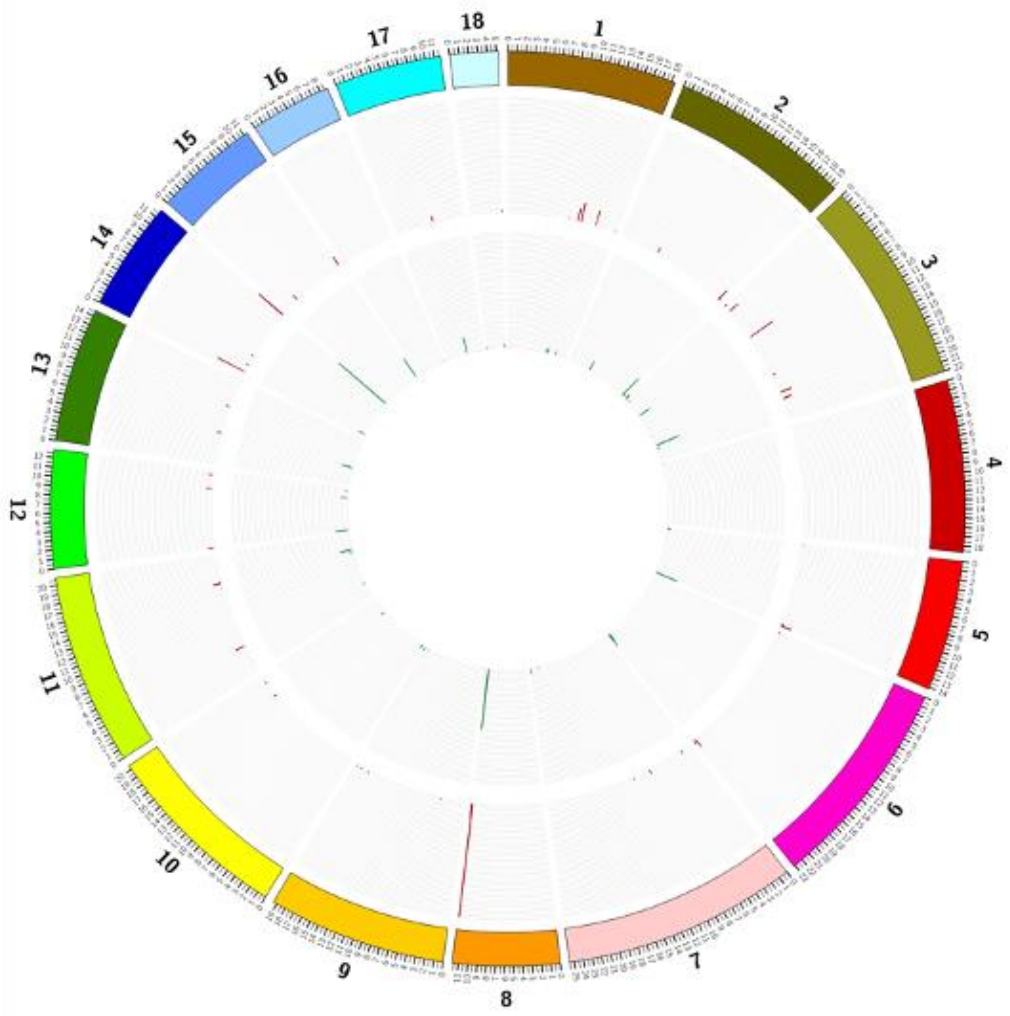


Figure 5.11: The sequencing depth from oxford nanopore sequencing across the genome from *A. apicola* infected and uninfected *B. terrestris* 6mA-enriched gDNA. DNA was digested with the MboI enzyme to enriched for 6mA prior to sequencing with the rapid sequencing kit (SQK-RAD114). Circos plot showing sequencing depth from infected and uninfected *B. terrestris* gDNA. Outer line plot (red) from bumblebee infected thoracic muscle gDNA. Inner line plot (green) from bumblebee uninfected thoracic muscle gDNA. All adenine sites were filtered and those with a depth of ≥10 x were removed from the data. Axis scale from 1-1000.

The 6mA density was 3.09% and 2.77%, for infected and uninfected, respectively. However, there were greater differences in 6mA density between infected and uninfected gDNA at the individual chromosomes, particularly on chr3 and chr15 (**Figure 5.12**).

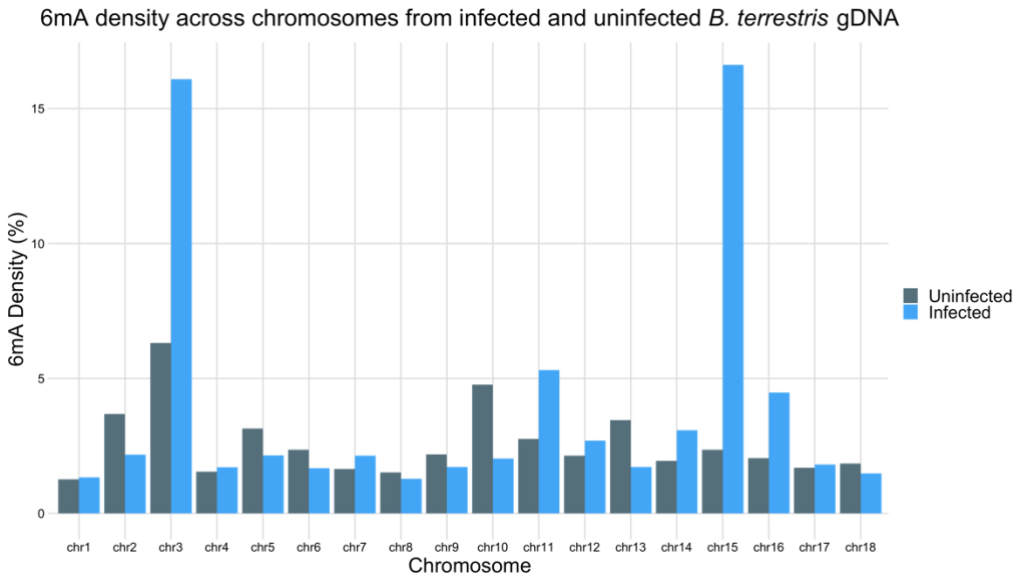


Figure 5.12: 6mA density across the genome of *A. apicola* infected and uninfected *B. terrestris* worker 6mA-enriched gDNA. gDNA was isolated from the thoracic muscle and first digested with the MboI enzyme to obtain the high molecular weight 6mA-enriched DNA prior to sequencing. DNA was sequenced using the rapid sequencing kit (SQK-RAD114). Bar graph showing the 6mA density across each chromosome of *A. apicola* infected and uninfected bumblebee workers. 6mA density (%) is defined as: $\left(\frac{Total\ modified\ A's}{Total\ canonical\ A's}\right)\ x\ 100$

Adenine sites with a sequencing depth with less than ten were then removed from the data (**Supplementary Figure S5.4**) Data was then also filtered to remove sites with 0%, < 25%, < 50% and < 75% 6mA frequency. Interestingly, the infected bumblebee gDNA had double the number of modified adenines in relation to the total number of sequenced sites, with a frequency ≥ 50% in samples of infected and uninfected bumblebees (0.093% and 0.046%, respectively) (**Supplementary Figure S5.7**). This was also the

case for adenines with a 6mA frequency ≥ 75%; n = 1813 (0.070%) and n = 486 (0.039%), for infected and uninfected *B. terrestris*, respectively.

The adenine sites with ≥ 75% of methylation was split unevenly between chromosomes with chr3 and chr15 having the greatest number of sites from the infected gDNA (**Figure 5.13**).

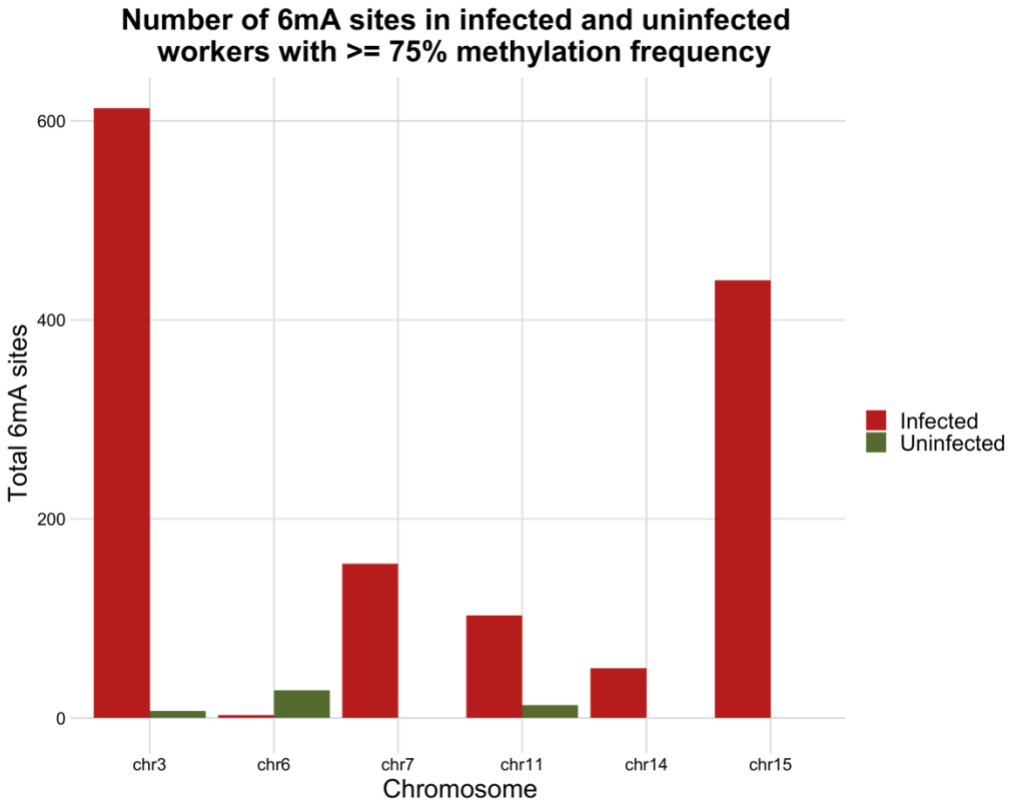


Figure 5.13: The number 6mA sites with a methylation frequency ≥ 75% in each chromosome from *A. apicola* infected and uninfected *B. terrestris* worker 6mA-enriched gDNA. gDNA was isolated from the thoracic muscle and first digested with the MboI enzyme to obtain the high molecular weight 6mA-enriched DNA prior to sequencing. DNA was sequenced using the rapid sequencing kit (SQK-RAD114). Bar graph showing the number of modified adenines with a frequency ≥ 75% for each chromosome. All adenines had a sequencing depth ≥ 10. Chromosomes without any sites in the infected or uninfected gDNA were removed.

The adenines with high methylation frequency (≥ 75%) were then analysed further and the 6mA sites found in both in the infected and uninfected *B. terrestris* gDNA were removed. 1425 modified adenines remained that were present in either the infected or uninfected gDNA; 1376 (96.56%) of these adenines were found in the infected gDNA (**Figure 5.13**). The modified adenines were separated into chromosomes and found to be clustered in gene regions (**Table 5.5**; **Figure 5.14**).

Table 5.5: Number of 6mA sites with ≥ 75% methylation located in either the *A. apicola* infected or uninfected *B. terrestris* 6mA-enriched gDNA. All adenines had a sequencing depth ≥ 10 x. Adenines on chr6 were not analysed for location as there were < 50 sites.

Chr	Infected modified A's	Uninfected modified A's	Gene Location	Gene ID	Ensembl description
3	613	7	18302728-18304752	ENSBTSG00005021760	Vomeronasal 1 receptor 13 (Vmn1r13)
			1293488-1295748	ENSBTSG00005035295	si:dkey-259j3.5
6	3	28	N/A	N/A	N/A
7	155	0	1669299-1671522	ENSBTSG00005020886	N/A
11	103	13	4690050-4692221	ENSBTSG00005028918	LncRNA
14	50	0	1856748-1857838	ENSBTSG00005022966	LncRNA
15	440	0	2426890 -2468445	LOC100644345	Semaphorin-5A

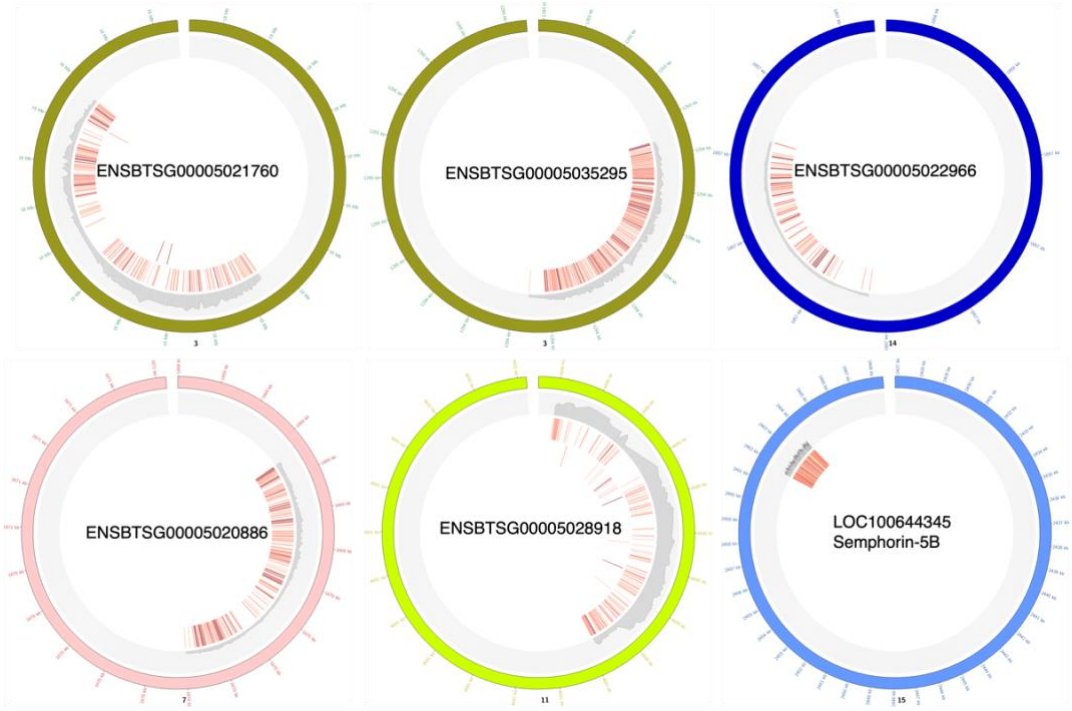


Figure 5.14: Circos plot of each gene containing a high number of 6mA sites with a frequency ≥ 75% from the sequencing of *A. apicola* infected and uninfected *B. terrestris* worker 6mA-enriched gDNA. gDNA was isolated from the thoracic muscle and first digested with the MboI enzyme to obtain the high molecular weight 6mA-enriched DNA prior to sequencing. DNA was sequenced using the rapid sequencing kit (SQK-RAD114). All adenines had a sequencing depth ≥ 10. Outer graph: line plot showing the sequencing depth; axis range from 0-100. Middle: heatmap showing the 6mA sites from infected *B. terrestris* gDNA. Inner: heatmap showing the 6mA sites from uninfected *B. terrestris* gDNA.
Heatmap colour increases with 6mA frequency; light red (~76-84%), red (85-93%), and dark red (93-100%).

5.4.4.2 Infected and uninfected B. terrestris reads aligned to mtDNA

The reads from the infected and uninfected gDNA were also aligned to the *B. terrestris* mitochondrial genome (**Table 5.4**). The mtDNA coverage for both the infected and uninfected samples was 59.3% and 56.8%, respectively. The

mean depth for infected and uninfected DNA of all the sequenced adenine sites was 7.37 x and 4.93 x and the 6mA density was 3.77% and 3.17%.

5.4.4.3 Infected and uninfected B. terrestris reads aligned to A. apicola chromosome

The infected and uninfected bumblebee gDNA reads were aligned against the *A. apicola* reference chromosome (NCBI reference sequence: NZ_CP084222.1). Nearly a quarter of reads (22.3%) from the infected *B. terrestris* mapped to the *A. apicola* genome, compared to just 0.4% of the reads from the uninfected gDNA sample (**Table 5.4**).

The 6mA density was similar between the infected and uninfected gDNA aligned to *A. apicola* chromosome; 3.18% and 3.16%, respectively. Only 21% of the chromosome was covered by the uninfected gDNA compared to almost complete coverage from the infected gDNA (**Figure 5.15**).

5.4.4.4 Arsenophonus apicola sequencing

Nearly three quarters of the reads from the *A. apicola* chromosome DNA sequencing aligned to the reference genome. The whole chromosome was covered, and the mean depth of adenine sites was 94.77 x. The 6mA density was slightly lower at 2.73% than the *B. terrestris* gDNA reads aligned to the *A. apicola*, which were 3.18% and 3.16% for infected and uninfected (**Table 5.4**; **Figure 5.15**).

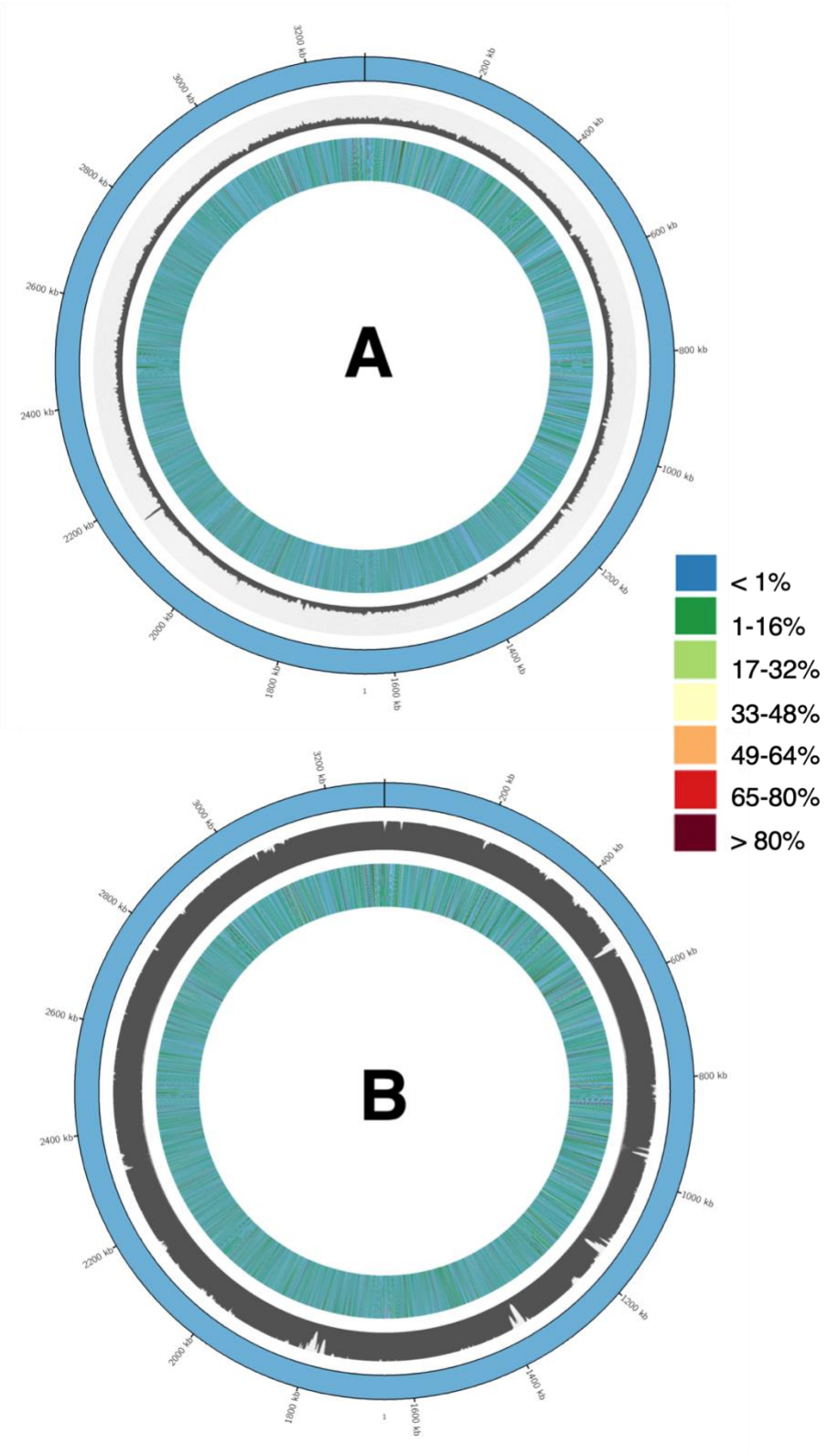


Figure 5.15: Circos plots of the *A. apicola* chromosome showing the sequencing depth and 6mA frequency of adenines. Chromosomal DNA was isolated from the bacteria using the Qiagen DNeasy PowerSoil Pro kit (ID: 47014). Circos plot A) Reads from the *A.*

apicola infected *B. terrestris* 6mA-enriched gDNA. B) Reads sequenced from *A. apicola* chromosomal DNA. DNA was sequenced using the rapid sequencing kit (SQK-RAD114). Both sequencing reads were aligned to the *A. apicola* reference chromosome NZ_CP084222.1. Outer line graph showing the coverage depth of adenines; axis ranging from 1-200. Inner heatmap showing the 6mA frequency of adenines.

The *A. apicola* chromosomal DNA was also digested with the restriction enzymes and analysed via gel electrophoresis (**Figure 5.16**). There was a high molecular band from the MboI digested DNA which was similar to the control, whilst the DpnI and Sau3AI DNA had been fragmented suggesting there was 6mA methylation present.

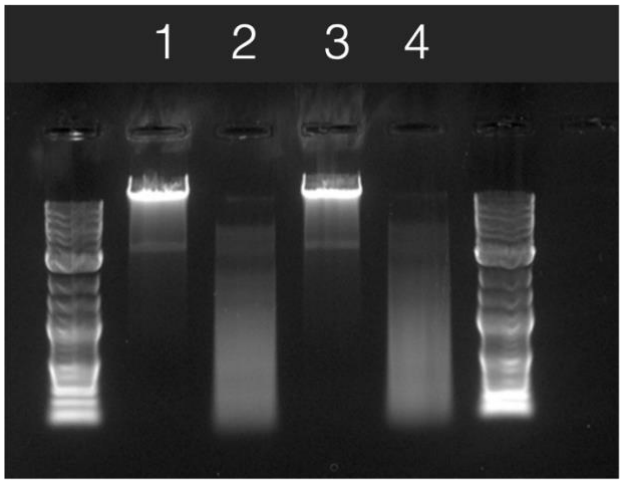


Figure 5.16: Image of the gel from the *A. apicola* chromosomal DNA that was used to infect *B. terrestris* workers showing presence of 6mA methylation. The strong signal from MboI (lane 3) digested DNA and very weak signal from the DNA digested with the DpnI (lane 2) enzyme suggests 6mA is present in *A. apicola* chromosomal DNA. DNA was digested with either a water control or restriction enzyme.1: H_2O (control); 2: DpnI; 3: MboI; 4: Sau3AI. Ladders are 1kb.

There are six *A. apicola* plasmids in the reference genome (NCBI reference sequences: NZ_CP084223.1; NZ_CP084224.1; NZ_CP084225.1; NZ_CP084226.1; NZ_CP084227.1; NZ_CP084228.1). All but one plasmid

(p3) was fully covered during sequencing and the 6mA density did not differ more than 1% between plasmids (**Figure 5.17**; **Supplementary Table S5.1**).

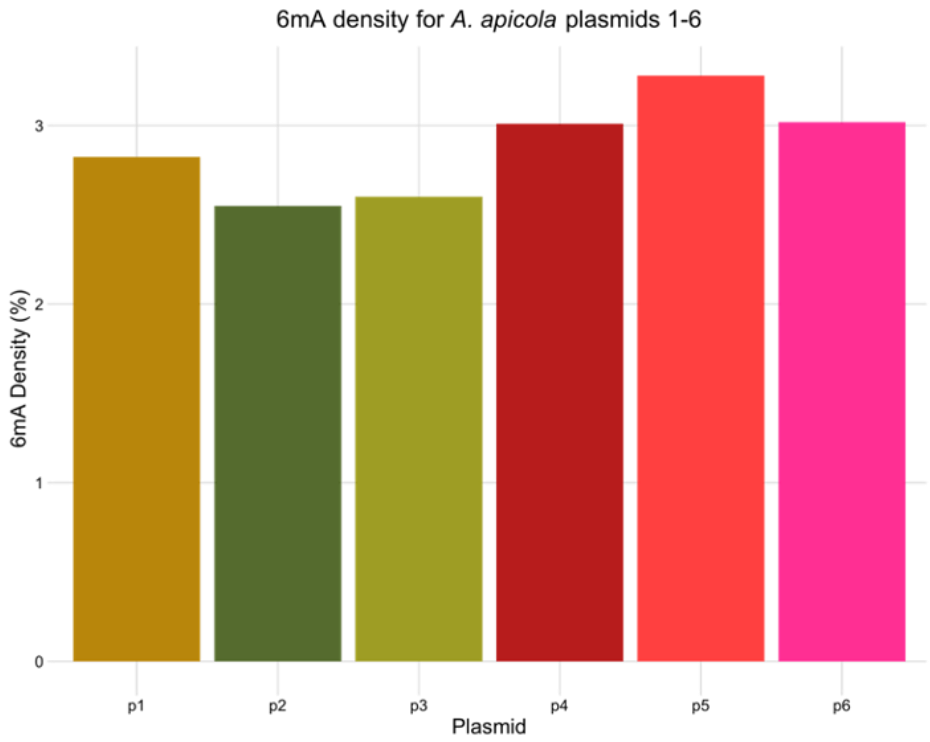


Figure 5.17: 6mA density across the six *A. apicola* plasmids. Plasmid DNA was isolated from bacteria using the QIAprep Spin Miniprep Kit (ID:27104). DNA was sequenced using the rapid sequencing kit (SQK-RAD114). Bar graph showing the 6mA density across each plasmid. 6mA density (%) is defined as: $\left(\frac{Total\ modified\ A's}{Total\ canonical\ A's}\right)\ x\ 100$

5.4.5 Determining relative 6mA levels in Oxford Nanopore sequencing

The Lambda dam+ DNA is considered to be ~50% methylated at the dam sites (GATC).[303] The Lambda+ DNA (NEB, Catalogue#: N3011) is extracted from a dam+ *E .coli* strain (C190).[303] The Dam methylase transfers a methyl group to the adenine in the sequence GATC and the EcoKI methylase modifies adenine in the sequences, AAC(N6)GTGC and GCAC(N6)GTT.

Lambda++ DNA was Lambda+ DNA (N3011) which had initially been digested with HindIII to yield eight fragments that could be used as molecular weight standards for agarose gel electrophoresis and a positive control for iSouthern experiments. To increase the 6mA methylation levels in this Lambda DNA sample, the EcoGII methyltransferase enzyme was added (NEB, catalogue#: M0603S). This is a non-specific methyltransferase that modifies adenines in any DNA sequence context. The amount of 6mA in the Lambda++ was not quantified but can be considered much higher than the Lambda+ DNA.

By nanopore sequencing, we found the 6mA density in the Lambda++ DNA was nearly 9x higher than the Lambda+ DNA, 1.41% and 0.16%, respectively. Both sequencing was done using the MinION R.9.4.1 flow cell and the Guppy modified basecalling described previously (**Supplementary Table S5.2**; **Supplementary Figure S5.8**).

5.5 Discussion

5.5.1 6mA methylation in *B. terrestris*

The iSouthern results indicate that 6mA methylation in the gDNA or mtDNA was absent in any of the DNA samples initially isolated from *B. terrestris* castes. Despite a similar partial band from both MboI and Sau3AI this is likely due to an incomplete digestion, as Sau3AI should cut the DNA regardless of the methylation status within the GATC recognition sequence. Further evidence that 6mA methylation is not present came from the iSouthern results. The 6mA antibody did not give a signal for any of the DNA bands, except for the lambda dam+ DNA (6mA positive) as expected.

These findings correlate with the results obtained by nanopore sequencing of both the mtDNA and gDNA taken from the thoracic muscle tissue of *B. terrestris* gynes (unmated queens). The gDNA sequencing did not provide enough depth or coverage however based on the methylation profile of the reads that were sequenced we reported a low level of 6mA. A similar 6mA density was seen in the mtDNA and the gDNA (0.08-0.09%), however, as only half of the reads aligned to the mtDNA and a quarter to the gDNA this suggests there was considerable contamination in the sample which may contribute to the levels of 6mA we found.

5.5.2 6mA methylation in *A. mellifera*

The iSoutherns gave a clear indication of 6mA presence in the gDNA and mtDNA of *A. mellifera* drones but not in any of the female castes we analysed. Our results showed a distinct band for the MboI digested DNA, where only high molecular weight DNA that contains 6mA in the GATC recognition remains undigested; this high molecular weight band corresponded with a strong anti-6mA antibody signal.

Given the indication of 6mA methylation in the drones of *A. mellifera* we then proceeded to sequence the gDNA and mtDNA from the thoracic muscle and head. The 6mA density in the mtDNA between the tissues was similar at

0.15% and 0.12%, for the thoracic muscle and head, respectively. This shows an 50% increase in the 6mA density from that reported in the *B. terrestris* mtDNA. The mean sequencing depth of the gDNA was 1 x; this reduces the reliability of the 6mA calls as the 6mA frequency (proportion of canonical and modified as a percentage) of an adenine could only have been 100% or be canonical.

Given the insufficient coverage and depth of the gDNA sequencing we proceeded to first digest the gDNA with the MboI enzyme to obtain high molecular weight DNA that was most likely to contain 6mA methylation (**Figure 4.7**). This also increased the chance of obtaining higher sequencing depth for the nanopore reads, which indeed is what we found.

The sequencing of MboI digested gDNA increased the mean depth to ~23 x. We covered 0.61% of the total *A. mellifera* genome, however as we digested the DNA prior this was not unexpected. The 6mA density was 0.18% which was similar to that found in the mtDNA. The sites with high sequencing depth were sporadic across the genome with the highest peaks in chr8, chr6 and chr11. These genomic locations (chr8, chr6 and chr11) were not the chromosomes where we had found the highest 6mA densities (chr 3, chr12, chr13), suggesting higher sequencing depths did not necessarily correlate with high levels of 6mA methylation (**Figure 5.8; Figure 5.9**). This is of particular interest as the DNA had been digested with MboI to enrich for 6mA-containing DNA; that is, the reads with the highest depths were expected to contain the greatest levels of 6mA DNA methylation. However, we did not observe this.

Only around 10% of the total sequencing reads of the 6mA-enirched DNA primarily mapped to the *A. mellifera* genome which suggests a large amount of contamination of DNA from other organisms. To determine what was in the composition of the sequenced DNA sample a WIMP analysis was performed. We found that the majority of reads matched to *Arsenophonus nasoniae* DNA. *Arsenophonus nasoniae* is a bacterium, that can cause a reduction of the male progeny in infected *Nasonia vitripennis* wasps. A closely related bacterial

species, *Aresenophonus apicola* has been shown to be a bacterial endosymbiont found in honeybees.[304,305]

A. apicola appears to be present in the gut and haemolymph of honeybees.[304] *A. apicola* infection in honeybees has been associated with poor health and colony collapse disorder (CCD), although more research is required to confirm this.[304,306,307] Research suggests that the prevalence of *A. apicola* infection in honeybees ranges from around a quarter to half of bees that are tested.[306,307] Infection is transmitted horizontally and is considered to be acquired via nectar and pollen when foraging and infection is spread via the oral-faecal route in the hive.[304-306] *Varroa destructor* as also been associated as a potential transmission vector as *A. apicola* has been found in the mite which feeds on the honeybee haemolymph.[306]

Our results show that nearly half of the reads in the honeybee gDNA sample aligned to *A. apicola* which correlates with previous studies that suggest when *A. apicola* is present in honeybees it can out-compete other bacteria.[307]

5.5.3 *Arsenophonus* infection and 6mA methylation in *B. terrestris*

To determine whether the presence of *A. apicola* infection altered 6mA methylation in bees we decided to infect *B. terrestris* workers with *A. apicola* that contained a GFP-expressing plasmid. The use of commercially purchased *B. terrestris* colonies, which seem to be uninfected, allowed for the controlled analysis, following *Arsenophonus* infection. Workers which had a confirmed presence of *A. apicola*, via fluorescence imaging, showed a positive indication of 6mA methylation from the iSouthern which was not seen in the uninfected workers. This confirms that *Arsenophonus* infection in bees provides a positive 6mA methylation result using the iSouthern approach. As to whether this is due to the bacterial DNA itself, or the effect of *Arsenophonus* on adenine methylation of the host nuclear DNA remains to be determined.

Nanopore sequencing between infected and uninfected *B. terrestris* worker gDNA that was digested with MboI (**Figure 5.7**) revealed that only a small proportion of the sequenced reads aligned to the *B. terrestris* reference

genome. However, the WIMP analysis clearly showed that in the infected workers ~40% of the reads aligned to the *Arsenophonus* genome, which was similar to our results derived from the honeybee drone samples. In the uninfected bumblebee workers, *Arsenophonus* was not reported in the top five species that the reads aligned to, providing further confirmation that the infection was not present. This was also confirmed by the very low number of reads which mapped to the *A. apicola* reference genome from the uninfected gDNA.

5.5.3.1 6mA methylation in gDNA of infected and uninfected B. terrestris

The 6mA density was slightly higher in the infected worker gDNA compared to the uninfected, 3.09% and 2.77%, respectively. However, this was an ~16-fold increase in 6mA density from that we reported in the honeybee drone. However, these differences are most likely due to variation between the flow cells and basecalling software protocols described in sections *5.3.3.1* and *5.3.3.2*; the sequencing protocol was updated to the newer R.10.4.1 flow cells for the analysis of *A. apicola* in *B. terrestris*. From these results it suggests that the R.10.4.1 flow cells with the dorado software and 6mA basecalling model is much less conservative than the R.9.4.1 flow cell with guppy basecalling. Therefore, we cannot make any comparisons between methylation levels between the results obtained from different sequencing protocols.

5.5.3.2 6mA methylation in mtDNA of infected and uninfected B. terrestris

We aligned the infected and uninfected gDNA nanopore sequencing reads to the mitochondrial genome to determine whether 6mA was present. We reported similar coverage for both at ~50% with a mean depth around 5-7 x. As the gDNA had been digested with MboI this may explain why a similar coverage was seen; the MboI enzyme would have cut the gDNA at the same sites which would result in the fragments aligning to the same regions of the mtDNA. As reported in the gDNA, the 6mA density was slightly higher in the infected worker mtDNA compared to uninfected; 3.8% and 3.2%, respectively, suggesting that infection may increase 6mA methylation.

5.5.3.3 6mA methylation levels in A. apicola

The *B. terrestris* gDNA from infected and uninfected bees gave a similar 6mA density of ~3.2% when aligned to the *A. apicola* reference genome, whereas the sequencing of the *A. apicola* chromosome gave a slightly lower density of 2.7%. We found the total number of adenine bases in the *A. apicola* chromosomal and plasmid DNA to be much higher than that recorded in the reference sequences. Mismatches during alignment, other bacterial contaminants, or variation from the reference genome may provide a potential explanation for the increase in adenine bases. The identification and research of *A. apicola* has been a relatively recent discovery and so the reference genome which was sequenced in 2021 may vary from the strain that we used to infect *B. terrestris*. Nevertheless, the sequencing analysis suggest that the 6mA methylation in *A. apicola* is at a similar prevalence to what we report in the infected and uninfected bumblebees.

5.5.3.4 Highly methylated adenines and A. apicola infection

We found a significant bias in the 6mA density between *B. terrestris* chromosomes. 6mA density increased to > 15% in the infected workers in chr3 and chr15 which was not found in the gDNA of uninfected specimen. As discussed, we found only a slight increase in the total 6mA density in the infected bees compared to uninfected; yet when filtering for sites that showed ≥ 75% 6mA frequency we found that the infected DNA had double the number of 6mA sites (in relation to the total number sequenced) compared to the uninfected DNA. This may suggest that the adenines with a high 6mA frequency can be considered to be truly methylated as they appear to be influencing the 6mA-positive iSouthern results in the infected gDNA. Therefore, by filtering out the sites with ≤ 75% 6mA frequency we were able to analyse these highly methylated sites of interest. We found 1425 modified adenines that were unique to either the infected or uninfected gDNA. Of these, all but 49 sites were unique to the infected workers only. Again, we found that chr3 and chr15 had highest number of 6mA sites.

The modified adenines in each chromosome appeared to be in clusters and some were located in genomic regions that contain genes. Of the six genes we identified, two of them were not classified. Another two genes appeared to be long non-coding RNAs (lncRNA). LncRNA genes are described as > 200 nt and lack coding for the translation of proteins, however they have been found to evolve more rapidly than protein coding genes.[308-310,]The mechanistic information for the majority of lncRNAs is currently not known and the lack of annotation makes it difficult to elucidate the function of lncRNAs, however they have been commonly associated with the regulation of gene expression via chromatin scaffolding, cell differentiation, and tumorigenesis.[308,309] LncRNAs have also be implicated in the regulation of DNA methylation in cancer via multiple mechanisms, including, the recruitment of DNA methyltransferases (DNMT) and regulation of DNMT and Tet activity.[310] LncRNA expression in plants has also been shown to be influenced by environmental factors such as stress.[311] As to whether stress via the presence of endosymbionts such as *Arsenophonus* may affect lncRNAs may be something to consider in future research.[311]

On chr15 the *Semaphorin-5A* gene was shown to contain a smaller cluster of 6mA sites. Semaphorins are extracellular signalling proteins that are found in most tissues; they are involved in the regulation of cell shape and motility.[312] One of the genes on chr3 that contains high 6mA frequency sites appears to be an ortholog of the vomeronasal 1 receptor 13 (*Vmn1r13*) gene in mice. Gene ontology analysis showed that *Vmn1r13* belongs to the large family of G protein-coupled receptors (GPCRs) and in mouse may be involved in receptor signalling and hematopoietic progenitor cell differentiation. The other gene on chr3 with high 6mA frequency sites is an ortholog of the zebrafish *si:dkey-259j3.5*, also known as adhesion G protein-coupled receptor G2b (*adgrg2b*).[313,314] Mutations of a human ortholog, Adhesion G Protein-coupled Receptor G2 gene (ADGRG2) have been associated with congenital absence of vas deferens (CAVD), a major cause of obstructive azoospermia.[315]

5.5.3.5 Biological effects of bacterial-influenced 6mA modification in bee hosts

The three genes to which we found some description and the two lncRNA genes shared a common association with receptor binding and cell differentiation. Changes in cell differentiation have been shown to be influenced by the presence of endosymbionts.[316,317] Bacterial influence on host cell differentiation may cause cells to become less differentiated to facilitate bacterial transmission or become more differentiated to enable mutualistic interactions.[316] Alternatively pathogenic symbionts may increase host cell differentiation to initiate a metabolic state which is more beneficial for bacterial replication.[316] Furthermore, epigenetic modifications are a known mechanism that symbionts use to alter host cellular differentiation.[316] Bacteria are able to influence DNA methylation and histone post-translational modifications to alter accessibility to chromatin for transcription.[316] Hence, if lncRNAs are often associated with the control of gene expression through chromatin then this may explain why we found high levels of 6mA methylation in lncRNA genes. As to whether *A. apicola* can alter cell differentiation and metabolic states in bees requires further investigation and the short exposure of the infection (one week) in workers was likely to be insufficient to analyse these effects in the current study. Hence, it should be considered that a longer exposure time with *Arsenophonus* may be required to induce further modifications in DNA methylation and elucidate the biological consequences of infection.

To determine if the genomic regions with high 6mA levels in the infected bumblebees could have been also mapped to the *A. apicola* chromosome we searched by BLAST each of the six genes with high 6mA methylation against the *A. apicola* chromosome. There were no significant similarities suggesting that high-6mA level sequencing reads are indeed derived from the host genome and not from the *A. apicola* chromosome. We also found that the MboI enzyme cut the gDNA at similar GATC sites in the infected and uninfected workers which may suggest that the genome of *B. terrestris* contains conserved regions of 6mA methylation.

Overall, these results suggests that the presence of endosymbionts may alter 6mA methylation in host cells with a particular focus on genes which influence host cell differentiation. As to the mechanisms in which endosymbionts could initiate the methylation of adenines in the host is beyond the scope of this study and requires further research. However, as 6mA is the primary form of methylation in prokaryotes it seems plausible that they may target adenine over other base modifications.

5.5.4 6mA methylation in bees: should we really bee-lieve it?

5.5.4.1 iSouthern results and Arsenophonus presence

Arsenophonus infection is often acquired via horizontal transmission in the summer and autumn and peaks in prevalence around the months of August and September.[304] Infection is then subsequently lost throughout overwintering.[304] As we predict, if the presence of *A. apicola* in honeybees is associated with a positive 6mA methylation result in the iSoutherns then the seasonal shift of infection may explain why we did not see 6mA methylation in female castes. This was likely due to our collection times. As we were not previously aware of *A. apicola*, we were not considering the months in which we collected the various castes. Therefore, it was likely that we collected female nurses and foragers in early spring and drones towards mid-summer to the end. Furthermore, as we were collecting bees from 2020-2022, it is likely infection presence varied throughout the years. Furthermore, the lack of infection prevalence in *B. terrestris* may also be explained by the laboratory environment. Bumblebee colonies were obtained commercially from Biobest® and so were unlikely to be exposed to any environments in which they could have become infected with *Arsenophonus*.

Overall, we have shown a strong association between *Arsenophonus* infection and a positive 6mA methylation iSouthern result. In the absence of the endosymbiont, it appears eusocial bees do not show 6mA methylation via the iSouthern method.

5.5.4.2 High variation in 6mA levels between sequencing protocols

Our initial sequencing found that 6mA levels to be similar to that reported in other invertebrates. However, when switched to the R.10.4.1 flow cells and basecalling protocol we found it to be significantly higher, even in the absence of *Arsenophonus*. This suggests there is substantial variation between basecalling programmes and models. Furthermore, we found the 6mA density in the Lambda + DNA to be 0.16% which appears to be considerably lower than the ~50% methylation levels reported. However, the 6mA level was high enough to provide a positive iSouthern response and correlates with the 6mA density and iSouthern results for the honeybee drone MboI digested gDNA which gave a similar 6mA density (0.18%). Therefore, according to these results, the 6mA levels were higher in the honeybee gDNA than that in the Lambda dam+ DNA which theoretically suggests > 50% of the adenine sites are methylated in the MboI digested gDNA from the honeybee drone. Given these findings, we advise caution when interpreting 6mA results from third-generation sequencing and further advocate the use of multiple approaches when trying to quantify 6mA methylation.

The digestion of the gDNA with the MboI enzyme prior to sequencing may have also affected the reliability of our results. The MAPQ scores were much lower in the enzyme digested DNA. The shorter fragments produced by enzyme digestion may increase the chance of incorrect alignment or reads from bacterial contaminants aligning to the reference genome.

5.5.4.3 Methylation frequency of adenines and effects on 6mA levels

We have demonstrated there was clear association with the presence of endosymbiotic bacteria and the level of 6mA methylation for sites with high 6mA frequency (≥ 50% and ≥ 75%). This suggests that when analysing DNA methylation via these sequencing methods it is important to consider the 6mA frequency of sites and make appropriate assessments. Sites with low 6mA methylation frequency should be heavily scrutinised and are potentially more likely to be model errors and less reliable.

In summary, we find evidence that 6mA methylation may be present in the genome of eusocial bees, particularly when infected with *Arsenophonus*. As to

whether infection by other symbionts effects 6mA methylation requires further research. However, discovering that endosymbionts are a contributing factor, it should also be considered that sample contamination is a significant concern in analysis. Hence, we suggest further analyses via alternative methods, such as LC-MS/MS, should be performed to gather additional evidence to support our results that 6mA is present in eusocial bees.

5.5.5 Conclusions

This study is the first to analyse 6mA methylation in eusocial bees. Overall, we found infection with the endosymbiont, *A. apicola,* to be associated with 6mA methylation presence when using the iSouthern approach. Furthermore, the use of nanopore sequencing demonstrated that there was an increase in the number of highly methylated sites in *A. apicola* infected bees in genes that were associated with cellular differentiation. We propose the need for further research to determine whether the relationship between bacterial symbiosis and 6mA methylation may expand to other eukaryotic species and to elucidate the mechanisms in which this may occur.

5.6 Author contributions

CS and RS designed the experiment. Bee husbandry was performed by CS. GH and TS provided assistance with the *A. apicola* laboratory work. Data collection and laboratory work for the iSouthern analysis was performed by CS and RS. The methodology of the iSouthern was established by RS and QZ. CS performed Oxford Nanopore sequencing and bioinformatic analysis. CS performed all data analyses and prepared all figures. The manuscript was written by CS with the assistance of RS.

5.7 Funding

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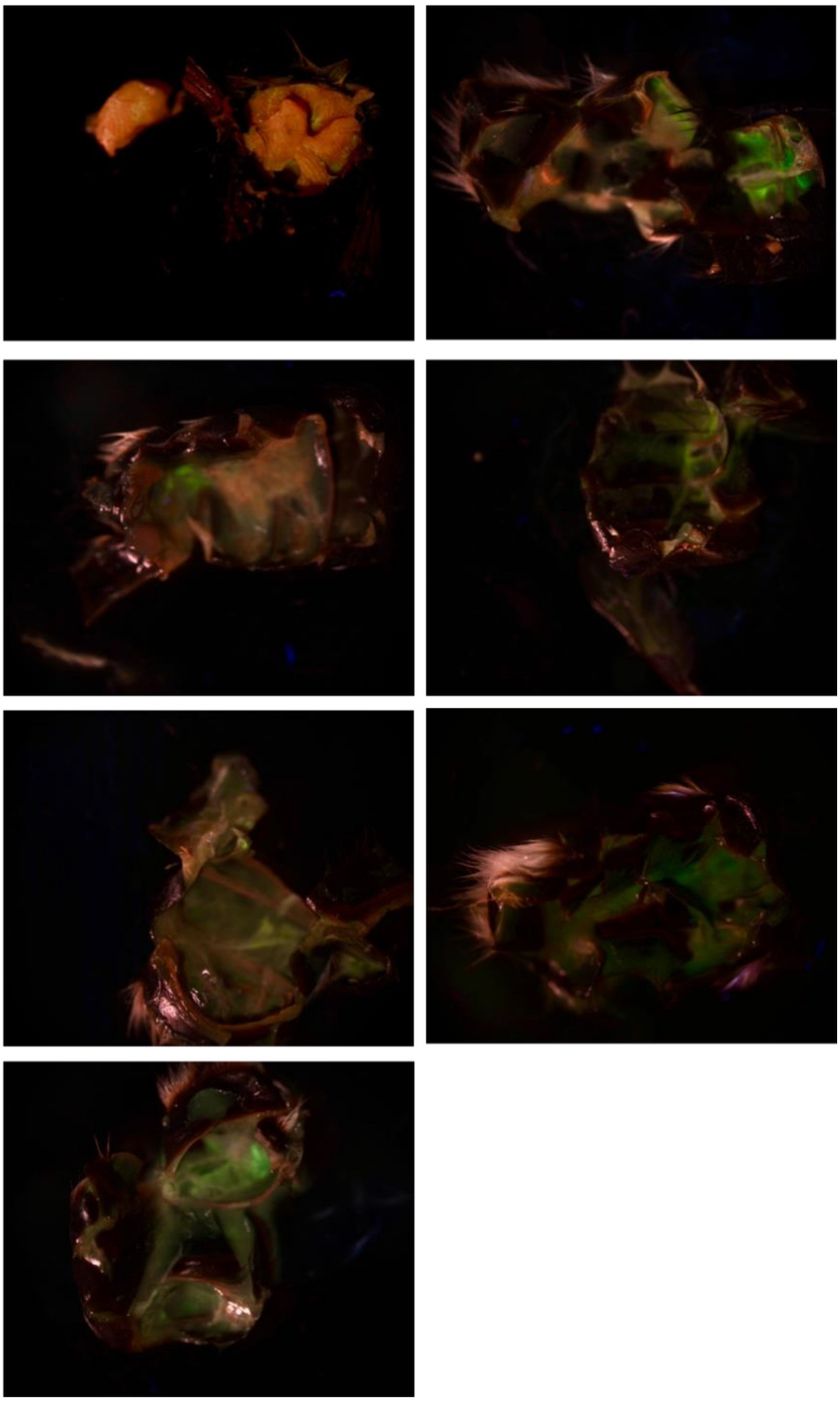
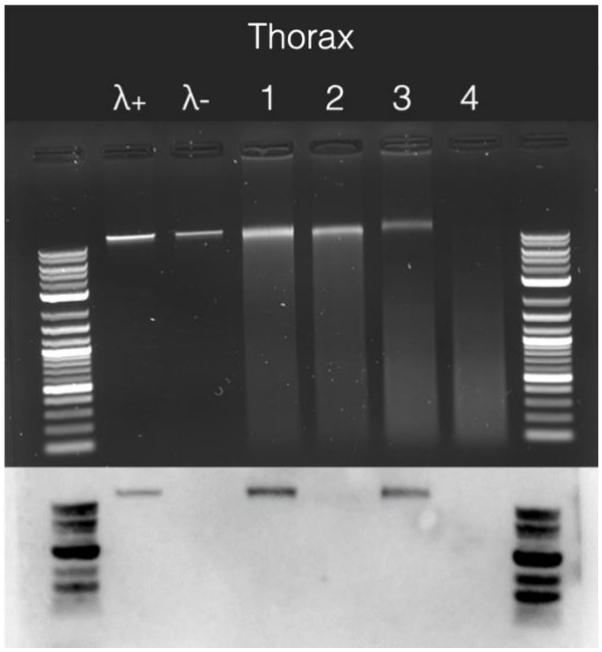
5.8 Acknowledgements

The authors would like to thank Adam Blanchard and Nathan Archer from the University of Nottingham for their support to the first author during the bioinformatic analysis.

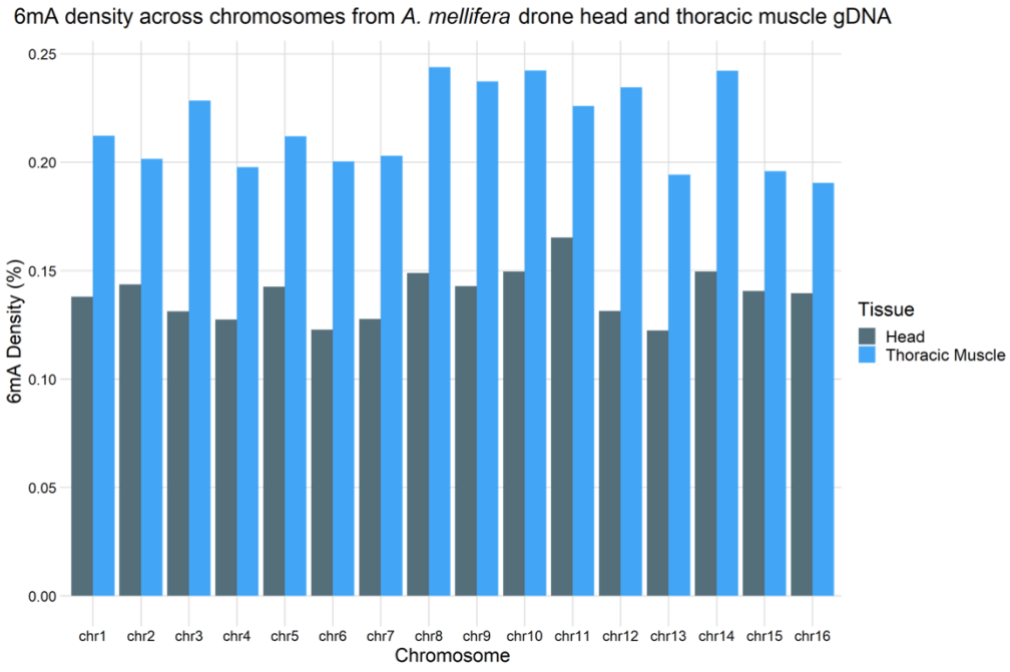
5.9 Appendix C



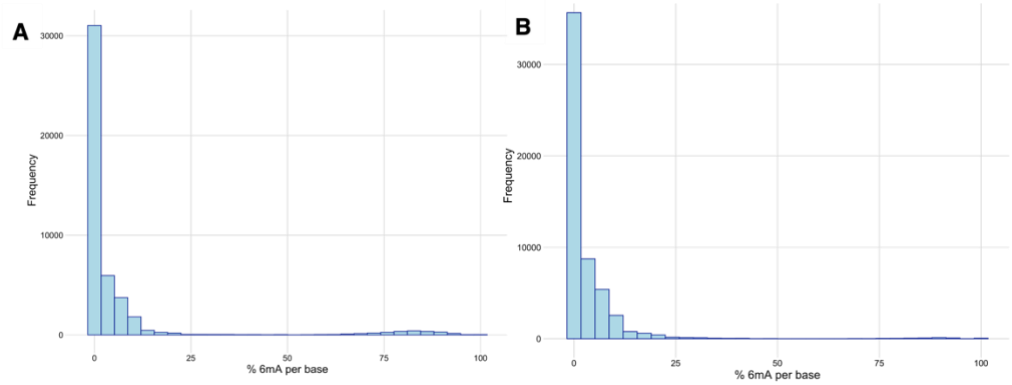
Supplementary Figure S5.1: Images of the infected *B. terrestris* showing the presence of *A. apicola* with GFP. Top left image showing the thoracic muscle, all other images showing the abdomen. Green light indicates *A. apicola* presence.



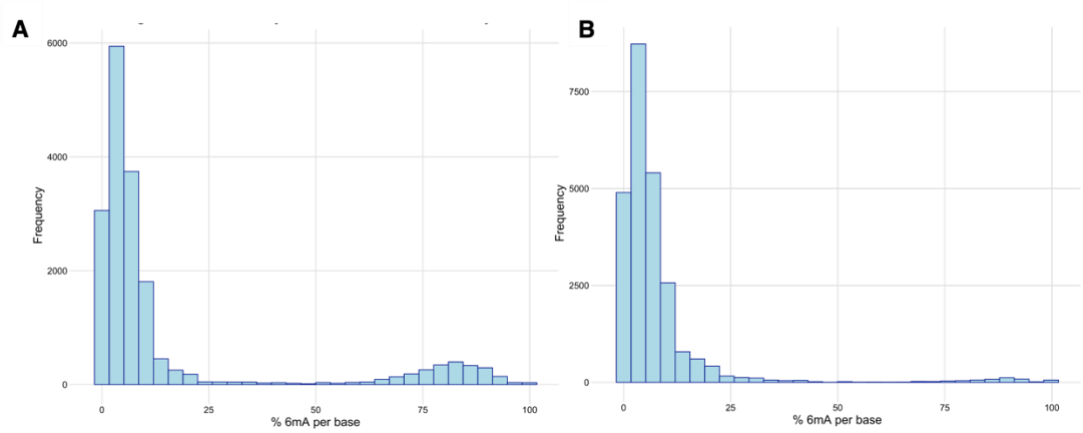
Supplementary Figure S5.2: Image of the gel from *A. mellifera* drone genomic DNA from the thorax that was then used for sequencing in section *5.4.2.4*. DNA was digested with either a water control or restriction enzyme.1: H_2O (control); 2: DpnI; 3: MboI; 4: Sau3AI. λ-: 6mA unmethylated lambda DNA. λ+: 6mA methylated lambda DNA. Ladders are 1kb.



Supplementary Figure S5.3: 6mA density for each chromosome in the *B. terrestris* drone gDNA isolated from the thoracic muscle and head. DNA sequencing was done using the rapid sequencing kit (SQK-RAD004)**.** Bar graph showing the 6mA density for each chromosome in the drone thoracic muscle (blue) and head (grey) gDNA. 6mA density (%) is defined as: $\left(\frac{Total\ modified\ A's}{Total\ canonical\ A's}\right)\ x\ 100$

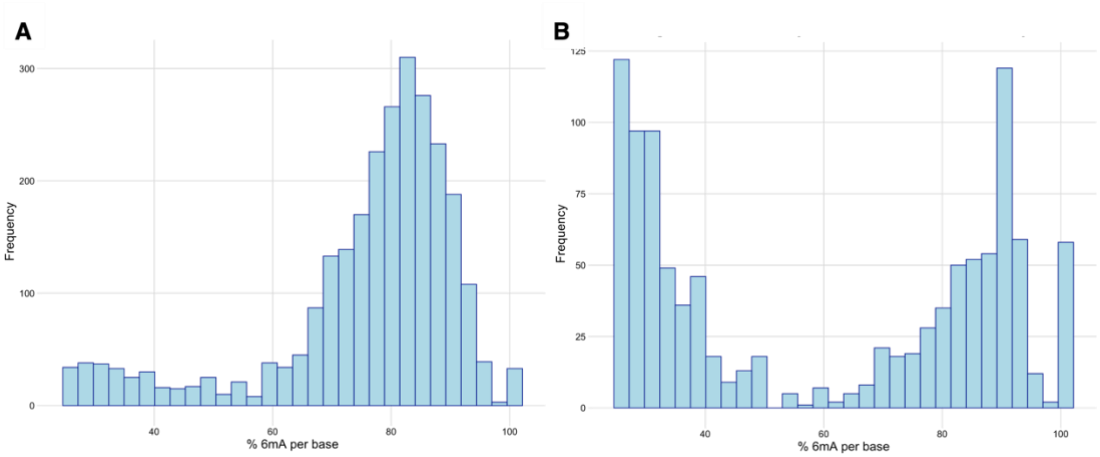


Supplementary Figure S5.4: Histogram showing the 6mA frequency in *A. apicola* infected and uninfected *B. terrestris* worker 6mA-enriched gDNA. 6mA frequency of adenine sites with ≥10 sequencing depth only. A) *A. apicola* infected *B. terrestris*, n = 46,068 (1.78%) and, B) *A. apicola* uninfected *B. terrestris*, n = 55,308 (4.45%). n (%) = number of adenines sites and the percentage of the total adenines sequenced

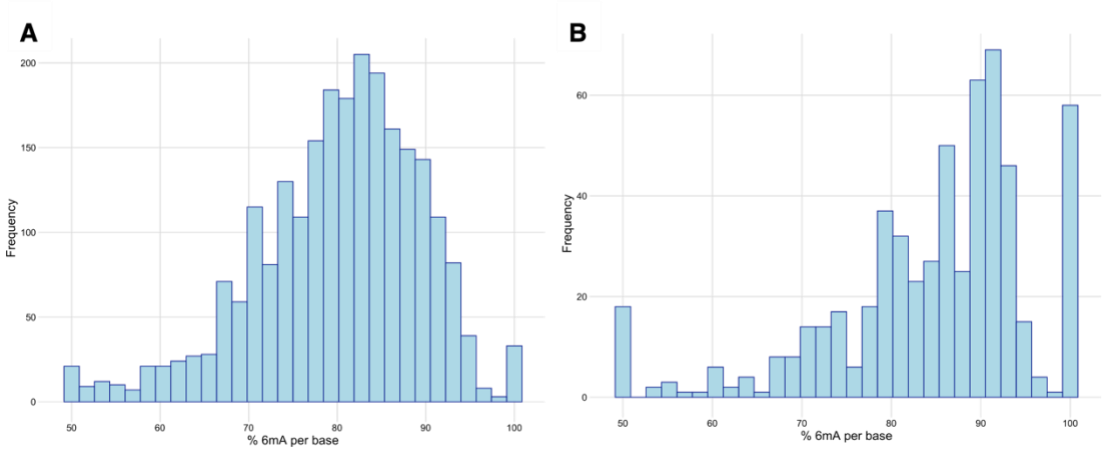


Supplementary Figure S5.5: Histogram showing the 6mA frequency >0% in *A. apicola* infected and uninfected *B. terrestris*

worker 6mA-enriched gDNA. 6mA frequency of adenine sites with ≥10 sequencing depth only. A) *A. apicola* infected *B. terrestris*, n = 18,096 (0.70%) and B) *A. apicola* uninfected *B. terrestris*, n = 24,538 (1.97%). n (%) = number of adenine sites and the percentage of the total adenines sequenced.



Supplementary Figure S5.6: Histogram showing the 6mA frequency ≥25% in *A. apicola* infected and uninfected *B. terrestris* worker 6mA-enriched gDNA. 6mA frequency of adenine sites with ≥10 sequencing depth only. A) *A. apicola* infected *B. terrestris*, n = 2637(0.102%) and B) *A. apicola* uninfected *B. terrestris*, n = 1060 (0.085%). n (%) = number of adenines sites and the percentage of the total adenines sequenced



Supplementary Figure S5.7: **Histogram showing the 6mA frequency ≥50% in *A. apicola* infected and uninfected *B. terrestris* worker 6mA-enriched gDNA.** 6mA frequency of adenine sites with

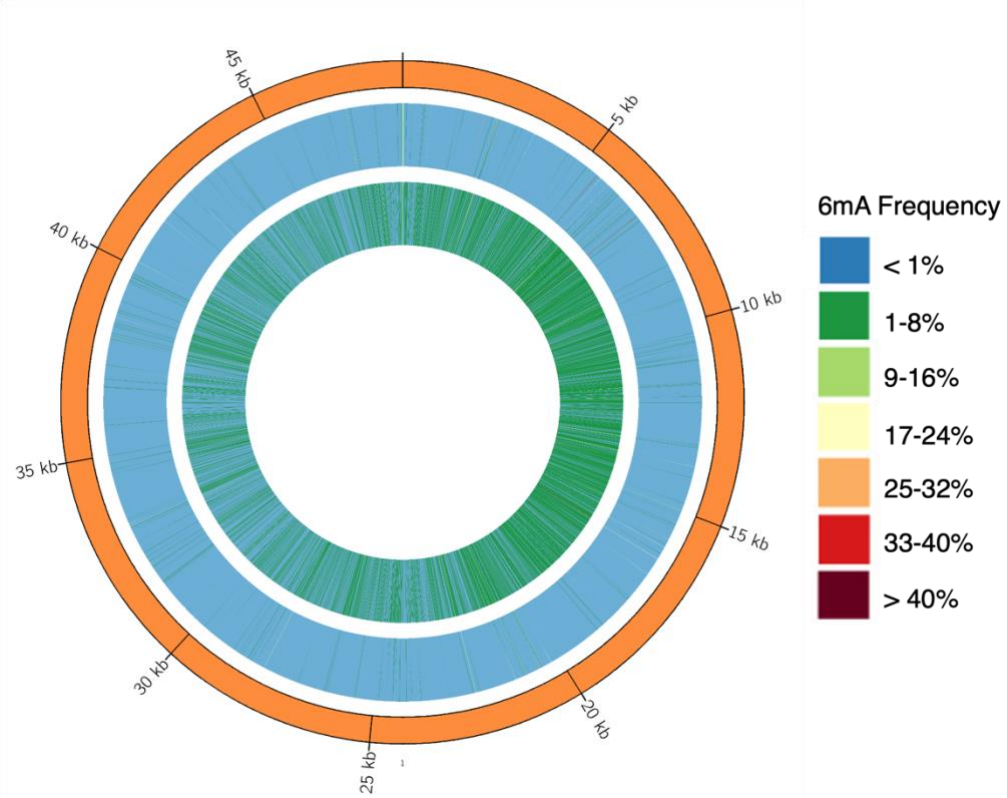
≥10 sequencing depth only. A) *A. apicola* infected *B. terrestris*, n = 2388 (0.093%) and B) *A. apicola* uninfected *B. terrestris*, n = 573. (0.046%) n (%) = number of adenines sites and the percentage of the total adenines sequenced.

Supplementary Table S5.1: Sequencing overview of 6mA methylation in *A. apicola* plasmids 1-6. Primary mapped reads were filtered by MAPQ score and those <20 were removed.

Plasmid	Primary mapped reads	% of reads mapped to ref	Mean MAPQ	Plasmid Coverage (%)	A sites covered in ref (%)	Mean depth of A sites	Total canonical A's	Total modified A's	6mA density (%)
p1	10367	1.34%	58.5	100%	192.59%	30.94 x	3259917	92058	2.82%
p2	5741	0.74%	58.1	100%	207.72%	39.67 x	2946901	75135	2.55%
p3	102	0.01%	40.3	46.78%	38.59%	3.10 x	37113	965	2.60%
p4	5118	0.66%	57.5	100%	215.40%	47.62 x	3034284	91310	3.01%
p5	5555	0.72%	57.2	100%	236.52%	49.85 x	3286061	107713	3.28%
p6	10915	1.41%	59.3	100%	296.66%	215.13 x	11636192	351261	3.02%

Supplementary Table S4.2: Nanopore sequencing overview of 6mA methylation between Lambda+ and Lambda++* DNA. *Lambda + was digested with HindIII and EcoGII

Sample	Ref genome	Primary mapped reads	% of reads mapped to ref	Mean MAPQ	Genome coverage (%)	A sites covered in ref (%)	Mean depth of A sites	Total canonical A's	Total modified A's	6mA density
Lambda	Lambda	53768	99.079%	59.8	99.99%	99.99%	2044.965	46379800	72076	0.16%
Lambda ++*	Lambda	165483	92.87%	58.5	100%	99.97%	1126.171	23430783	331261	1.41%



Supplementary Figure S5.8: Circos plot of the Lambda dam+ and Lambda ++* chromosomal DNA showing the sequencing depth and 6mA frequency of adenines. DNA was sequenced using the rapid sequencing kit (SQK-RAD004). Outer heatmap showing the 6mA frequency of adenines from Lambda + DNA (NEB #N3011). Inner heatmap showing the 6mA frequency of adenine from Lambda ++* DNA.

*Lambda++ DNA: Lambda+ DNA digested with HindIII and EcoGII

6. General Discussion

6.1 Effects of acute neonicotinoid exposure on mitochondrial function in bees

Several studies have demonstrated the adverse effects of neonicotinoids on locomotor activity in bumblebees.[318,319] Declines in flight duration and distance, along with short-term increases in flight velocity after acute exposure to imidacloprid have also been demonstrated with the use of flight mills.[16] However, understanding the mechanism in which neonicotinoids alter flight activity has yet to be determined. The first study in this thesis aimed to address this by elucidating the effects of acute imidacloprid exposure on mitochondrial function in the brain and thoracic muscle of bumblebees, and subsequently provide a potential explanation for the impairments in flight activity that have been reported.

The research presented showed imidacloprid increased routine respiration and lowered the spare respiratory capacity (SRC) in the flight muscle of workers, which was not evident in the brain, but rather the maximum ET capacity was increased. Previously there has been little research in the mitochondrial function of bees using HRR and in the studies which have been performed they have primarily focused on one tissue.[93,101] This study found the effects of imidacloprid on mitochondrial function were not ubiquitous throughout all tissues and demonstrated the importance in analysing mitochondrial function in a tissue-specific context.

The short-term increases in flight velocity associated with neonicotinoid exposure[16] may be explained by the findings we reported as the increase in routine respiration in the flight muscle and the marginally non-significant trend of higher respiration in the brain may facilitate this response. Furthermore, increases in flight velocity may subsequently lead to premature exhaustion and energy depletion which could explain decreases in flight distance and duration associated with neonicotinoid exposure.[16,216]

There is scope to improve upon this study and the methods we described. The mitochondrial respiration of the brain and thoracic muscle was analysed at a singular time point after acute exposure to imidacloprid. Although bees are acutely exposed to insecticides when foraging, it is likely that chronic exposure is most prevalent in agricultural landscapes, and so it is important to extend upon this research to determine the long-term effects of sub-lethal concentrations on mitochondria. Furthermore, we did not determine whether the impairments on mitochondrial function we reported were transient and if mitochondria were able to return to pre-exposure functioning. This is important to elucidate in the future, as it will aid in our understanding of the impact that neonicotinoids have on bees, even after their use has been discontinued.

6.2 Demonstrating the use of bees as models for xenobiotic research

Model organisms such as the fruit fly *Drosophila* and vertebrates such as rodents are commonly used for initial drug research and development studies. Bees are often overlooked as insect models for research beyond that of conservation studies, possibility due to public perception and their status as critical pollinators. However, with growing increases in the importation of commercial bumblebees for agricultural services[24] it seems reasonable that their use in scientific research beyond that of conservation purposes may also be utilised.

In the second chapter of this thesis, we have demonstrated how bumblebees are prime models for researching the effects of xenobiotics such as atypical antipsychotics on exercise and mitochondrial function. Unlike previous research, this study was the first to combine the use of tethered flight mills and HRR to directly analyse flight performance alongside mitochondrial function in the same individual bee. Furthermore, the use of the O2k-Fluo Smart-Module (Oroboros® Instruments, Innsbruck, Austria) and Magnesium Green (MgG) to determine ATP concentrations in bees has not been reported until now.

In the first chapter of this thesis, we found that acute exposure of imidacloprid, which induces neuronal excitation,[11] increased routine respiration in the flight muscle with a similar, yet non-significant trend in the brain, and has been previously shown to increase short-term flight velocity.[16] In this study we found that the olanzapine, a dopamine and serotonin antagonist, decreased routine respiration in the brain and reduced mean velocity of flight. Together, these two studies have showed that acute exposure of xenobiotics which alter neurotransmission have a direct effect on mitochondrial routine respiration and the velocity at which bumblebees fly.

The beneficial effects of exercise on mitochondrial function, including increased OXPHOS capacity and mitochondrial biogenesis, has been demonstrated in both vertebrates and invertebrates.[320-323] In bees, mitochondrial function has been shown to alter depending on foraging activity; OXPHOS capacity increases when nurse's transition to foragers to meet the high ATP demands required for flight.[97] Olanzapine appeared to inhibit changes in mitochondrial function associated with acute flight activity in the brain of bumblebees which may suggest that long-term treatment with this drug may have adverse effects on exercise performance. This is important to understand as the increased risk of developing MetS in patients receiving olanzapine and similar antipsychotics may be mitigated by exercise. If olanzapine prevents a beneficial increase in mitochondrial function and OXPHOS capacity, this may subsequently reduce exercise performance and potentially reduce the capacity for exercise to mitigate MetS.

This study has provided new insights into the effects of olanzapine on exercise and mitochondrial function which will aid in further research and help mitigate the adverse effect of these drugs in patients. Furthermore, we have showcased how bees can be valuable models for initial research studies which can help guide the development of larger studies in vertebrates.

This study could have been improved by increasing the sample size of bees which flew. Studies analysing flight data often require a high number of

replicates as variation in flight performance is often high.[16] However, this is often challenging, particularly in our study as bees were kept enclosed in small cohorts overnight before they participated in flight trials. This time away from the colony may have increased social isolation and stress levels which may have been associated with the high percentage of bees which did not initiate flight.

We focused on acute treatment and exercise, and so were unable to elucidate the long-term effects of olanzapine on exercise and mitochondrial function. Symptoms of MetS often take weeks to months before they become apparent in patients,[281,282] and therefore it is important to analyse the chronic exposure of olanzapine on exercise performance to enhance our understanding.

6.3 Measurement of ATP concentrations in *B. terrestris* and caste-specific differences

Using HRR alongside the MgG protocol we were able to analyse the effects of three ETS inhibitors, rotenone (complex I inhibitor), malonate (complex II inhibitor) and Antimycin A (complex III inhibitor) on ATP concentrations. Despite research suggesting in bees there is a bias towards substrates favouring complex I,[93] we found that rotenone had the smallest effect of ATP production. Therefore, it appears in bumblebees, that after the inhibition of complex I the ETS is able to maintain high functioning.

We showed that after inhibition of all three complexes of the ETS, ATP concentrations did not fall back to routine levels before the addition of substrates providing evidence of glycolysis being a significant contributor to ATP production in the brain of bumblebees.

The importance of analysing mitochondrial function between castes has been previously discussed. In the second chapter we aimed to develop our understanding of the mitochondrial function in the brain of two female bumblebee castes, workers and virgin queens (gynes). We found that despite gynes having significantly higher rates of oxygen consumption after the

addition of substrates, likely as a result of their increased size and subsequent higher energetic demand, the changes in ATP concentration from routine levels in response to substrates and ETS inhibitors were similar between castes. This demonstrated the disparity between oxygen consumption and ATP concentration and the importance of taking this into consideration in HRR studies.

The measurement of oxygen consumption to predict mitochondrial respiration rates and ATP production is a common approach, however our results suggest this should be interpreted with caution as it is not a true index of ATP production, as we demonstrated by the addition of the substrate proline which increased oxygen consumption but not ATP concentrations.

A key limitation to this study was that the initial ATP concentration at routine respiration was unknown and so the absolute ATP mM values could not be determined. Therefore, the data can only be used to calculate the changes (increase or decrease) in ATP concentration in relation to ATP concentration at routine.

6.4 Epigenetics in bees to identify and develop novel biomarkers

Epigenetic modifications are associated with environmental and behavioural influences.[324] Therefore, it is important to understand how behaviours linked to caste-type, or stressors such as disease and insecticides, can alter epigenetic marks and phenotypes in bees. Furthermore, this can lead to the development of DNA methylation as potential biomarkers for detecting diseases and other stressors.

The controversy around the presence of 6mA methylation in eukaryotes has been of particular interest to epigeneticists over the years. The third chapter presented in this thesis aimed to contribute to this research and, to our knowledge, is the first reported study to analyse the presence of 6mA methylation in eusocial bees. The chapter aimed to not only provide further

evidence to the debate regarding 6mA methylation in higher eukaryotes but also elucidate as to whether 6mA methylation could be associated with environmental influence or caste-specific behaviours in eusocial bees.

Within the methodology of the study, we established the immuno-southern (iSouthern) method to enable the tailored, relatively cost-effective analysis approach for the identification of 6mA methylated sites with the combined use of methylation-specific restriction enzymes and antibodies. Following on from this study, we hope the method will be used in a wider context to aid epigenetic research of other base modifications. In combination with the iSouthern we demonstrated the use of nanopore sequencing and bioinformatic software to detect 6mA modifications in mitochondrial-enriched DNA and genomic DNA. We used two different Oxford Nanopore flow cells and protocols during our nanopore sequencing; the R.9.4.1 and the newer R.10.4.1 flow cells. The basecalling software and model for 6mA methylating calling also varied. We found that these two protocols significantly altered the level of 6mA methylation reported. This demonstrates the impact that different bioinformatic analyses and pipelines can have on results, particularly when analysing methylation at such small quantities as found in eukaryotic DNA.

6.5 6mA methylation and the relationship between host and bacterial symbionts

Our understanding of *Arsenophonus* and its prevalence in bees is in its infancy. In recent years it has been suggested that *Arsenophonus* may be associated with colony collapse disorder in honeybees and an overall decline in bee health, however this requires further research.[304,306,307]

During our initial DNA sequencing of honeybees, we found evidence for the presence of 6mA methylation in eusocial bees, with more substantial levels in bees which were infected with the bacterial endosymbiont, *Arsenophonus apicola*. We concluded that the iSouthern approach gave a positive result for 6mA methylation when *A. apicola* infection was present in the bee. These findings may suggest that *A. apicola* levels were high enough to provide

significant contamination so that the bacterial DNA was identified, or that the presence of the endosymbiont was associated with an increase in 6mA methylation in bees.

Nanopore sequencing revealed 6mA methylation in both *B. terrestris* and *A. mellifera*, however as mentioned, the levels reported were highly dependent on the sequencing protocol used. As discussed, the iSouthern method was influenced by the presence of *Arsenophonus*; the nanopore sequencing showed higher levels of 6mA methylation in infected bumblebees compared to uninfected bees but only in 6mA sites which had high methylation frequency. This suggests that 6mA calls may not be reliable at sites with low frequencies and are more likely to be associated with a model error.

In *A. apicola* infected bees, genes that were associated with receptor signalling and cell differentiation contained clusters of 6mA methylation. The significance of methylating these genes may be linked to the influence of *A. apicola* on its host and the potential epigenetic mechanism in which it might alter molecular pathways to favour its survival. Therefore, developing our understanding of the symbiotic relationship between *Arsenophonus* and bees will be integral to answering these questions.

Third-generation sequencing protocols provide fast and accurate methods however they are still prone to errors and results can often be misinterpreted. Researchers need to be aware of the shortfalls to make appropriate interpretations of the data and to mitigate the chances of error. Multiple techniques should be used to determine the presence of 6mA methylation particularly when in such small quantities as reported in eukaryotes.

With the combined use of the iSouthern and nanopore sequencing we aimed to provide a more accurate and reliable approach for the detection and quantification of even small quantities of 6mA methylation in bees. However, these methods were not without their limitations. The iSouthern is unable to quantify the level of 6mA methylation and is particularly prone to contamination.

The methylation calling of Oxford Nanopore sequencing reads relies on machine learning models which are often trained on real data;[291,297] hence, methylation models may be influenced by data they are trained with and increase the chance of incorrect methylation calling in organisms that differ significantly. Furthermore, 6mA methylation is currently not as well studied in eukaryotes in comparison to other modifications, such 5mC methylation, and so these protocols are less established and potentially more prone to error.

We were unable to sequence the complete genome of *A. mellifera* or *B. terrestris* and at a high sequencing depth. Therefore, we decided to enrich the DNA for 6mA methylation by digesting it with the MboI enzyme, however, this caused the DNA to be cleaved into shorter fragments. One of the advantages of third-generation sequencing in the ability to sequence long reads, however we were unable to utilise this in our approach and is something that could be improved upon in future studies. In the 6mA-enriched DNA we also found the mapping quality to be much lower and therefore was more likely to have greater misalignments when mapping to the referencing genome. Considering the limitations of this study, to further validate our findings of 6mA methylation in eusocial bees the use of alternative methods such as liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) should be performed.

Overall, this study has brought to attention the presence of endosymbionts as potential and substantial contaminants of DNA samples during down-stream analyses, including sequencing and detection of DNA modifications. From our results we showed that not only does contamination from external sources need to be considered, but also the presence of intracellular symbionts within the organism. This is of particular importance when trying to identify low level methylation such as 6mA methylation in eukaryotes.

6.6 Thesis conclusions

The research presented in this thesis has demonstrated that mitochondrial function is altered by neonicotinoids and this effect is tissue-specific. These results may provide a possible explanation for the changes in flight activity that have been reported in previous studies. Furthermore, this thesis has shown that acute exposure to xenobiotics that affect neurotransmission in bumblebees subsequently affects mitochondrial respiration, particularly at routine states, which influences the velocity at which they fly. When using HRR it is important to recognise that oxygen consumption alone is not an accurate measurement of ATP production and techniques such as the MgG protocol described in this thesis should be used to determine more accurate results. This research has also shown that bumblebees make sufficient model organisms for research studies and can be used as an alternative to the more commonly used invertebrate species when appropriate.

Finally, this thesis has found that 6mA methylation may be present in *B. terrestris* and *A. mellifera* and has the potential to be utilised as a biomarker for the presence of the endosymbiont *A. apicola*. This research has also demonstrated the importance of identifying the presence of endosymbionts when performing DNA sequencing of the host species, particularly when analysing 6mA methylation in eukaryotes.

6.7 Future Directions and Perspectives

Following the research described within this thesis, there is a significant opportunity to utilise the methods and explore the findings in much greater depth. Firstly, there is a need to analyse the effects of novel insecticides on pollinators. Pesticide use is not likely to decline; with estimates of the global population reaching nearly 10 billion by 2050,[325] there is more pressure than ever to increase agricultural food production. However, this is generally in conflict with ensuring the protection of our environment and biodiversity. Often the effect of insecticides on pollinators and other species are analysed retrospectively but there is a desire to move away from retrospective studies. Alternatively, research, such as that described in this thesis, can be performed before an insecticide is authorised for commercial use to minimise the potential threats to pollinators. Hence, by using the methodology described in this thesis it is possible to understand how acute and chronic effects of novel insecticides can alter mitochondrial function in eusocial and solitary bee species.

In regard to olanzapine, there is a clear association between impaired exercise performance and mitochondrial function in the brain of bees. However, flight is also heavily dependent on efficient mitochondrial function in the flight muscle. Therefore, by researching the effects of olanzapine on other tissue-types, such as the flight muscle, this will improve our understanding of how atypical antipsychotics may impair exercise performance in patients. Furthermore, by elucidating the mechanisms in which neuronal function can alter mitochondria, and vice versa, this will help to elucidate how psychopharmacological drugs affect mitochondrial bioenergetics.

The use of bees as models to understand the relationship between exercise performance and mitochondrial function can also be utilised in future studies. In particular, the research of drugs which enhance or impair metabolic function or exercise can be analysed using the methodology described in this thesis.

Furthermore, this method is cost-effective and efficient whilst also limiting the use of vertebrates, which one can argue provides a more ethical approach.

With respect to ATP production and substrate utilisation in eusocial bees, there are still many unanswered questions. HRR alongside the measurement of ATP concentration has allowed us to gain new insight into the function of different substrates, however the role of proline in bumblebees remains to be determined. Developing our understanding of not only metabolic pathways, but also neuronal function in bees, may help to elucidate these questions.

The work described in the third chapter provides a foundation for further studies to explore the potential effects of endosymbionts on 6mA methylation and DNA sequencing practices. Given the greater depth of understanding of 5mC methylation in eukaryotes, it may be wise to determine if endosymbionts could also alter alternative DNA methylation modifications such as 5mC in host genomes. Furthermore, by performing studies that incorporate a more prolonged exposure to endosymbionts, this may bring a greater insight into the potential epigenetic and biological effects within the host.

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