Studies on the novel effects of feeding non-thermally treated honeybee gathered pollen on the colony stability and outputs of commercially-reared bumble bees (*Bombus terrestris*) for pollination services

by

John Eakins BSc. MSc.

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Abstract

Pollination is a vital resource, carried out mainly by animals, which without could threaten food security on a global scale. Of the agricultural crops that are produced globally, animal pollination is responsible for 40%.

Among animal pollinators, insects such as the honey and bumblebee are considered some of the most important for pollination of foods such as almonds, apples, strawberries, plums and blueberries. Commercial colonies of both are sold species are sold every year, which includes the global importation to over 50 countries of one million bumblebee colonies for the purpose of pollination. This practice of using commercially produced bee colonies for pollination is worth an estimated $14 billion to the US economy and $15 billion to the European economy per annum.

Despite the largescale use of commercial colonies, wild bees are still seen as major contributors to the pollination of crops and flowering plants. However, their survival is undertreat, due to a multitude of factors, such as climate change, pesticides, habitat lose and disease with the latter being considered as some of the major drivers. One of the major concerns with disease spread has been brought about by the introduction of commercial colonies. Commercial colonies are marketed as pathogen free, however many studies have found that this is not always the case. Disease carrying bees have been known to forage large distances and then disperse pathogens on flowers, which in turn act as reservoirs. Separate studies in both Canada and Ireland found that certain bee parasites were more prevalent in wild bees the closer they were to commercial greenhouses and that this lessened the further away the bees were captured. Wild bees who then forage on these flowers then pick up the pathogens and bring them back to their nests, contaminating the hives, a practice known as pathogen spillover. Furthermore evidence has also being found that suggests not only are commercial bees a source of contamination, but so is the pollen that they are fed on.

Commercial pollen is collected by honeybees and fed to both commercial honey and bumblebees colonies. Studies have found that it is often contaminated with parasites such as *Crithidia bombi* and *Nosema* species as well as some viruses. This suggests that commercial pollen could be a major driver in the spread of pathogens from commercial colonies to wild colonies and could be responsible.

This study looked at the possibility of using novel technologies for a purposes of reducing pathogen load within commercial pollen to help try prevent pathogen spillover to wild communities, and slow down the decline in species.

To do this, pulse-UV (PUV) technology was first examined and then Electron-beam (e-beam) irradiation at a dose of 100kGy and pollen filtration.

To examine how treated pollen affected bumblebee, 14 day old colonies were fed either pollen that had been irradiated with 100kGy dose of e-beam of pollen or pollen that had been filtered and compared against colonies fed on non-treated pollen for period of 30 days.

Bumblebee colonies fed on either e-beam treated or filtered pollen produced significantly less larvae and pupae, which also meant they produced less female bees and had a complete absence of males in their colonies.

On a molecular level they displayed an altered proteome and microbiome to those fed on non-treated pollen. Some of the key findings were a downregulated immune system, altered metabolism and the upregulation of toxicity associated proteins in bees fed treated pollen types compared to the control.

Altered abundances in the 3 " core" microbiota were observed in bees fed on either e-beam treated or filtered pollen, with treated colonies having a lower abundance of *Snodgrassella* and a greater abundance of *Gilliamella* and *Lactobacillus* compared to the control.

Altering pollen composition has a negative impact on bumblebee colonies, affecting reproduction, fatbody protein synthesis and the microbiota that inhabit bumblebee digestive tracts.

To conclude, this constitutes a potentially staggering new finding in the field of managed pollination and ecosystem services which suggests in part that super clean pollen may prevent occurrence of infection in commercial bumblebees; however, applying high level disinfection or mechanical disrupting (filtration) pollen can change the critical reproductive output of the commercial bumble bee colonies. It is not possible to fully state that all bee infectious agents were inactivated in pollen at 100 kGy due to limited infection model; but high level disinfection appeared applicable based on colony number observations. E-beam is gaining in popularity as a method of choice for the industrial sterilization industry due to desirable bulk

treatment properties; however, future studies are required to identify an appropriate dosage (kGy) that reduces bee pathogens yet also retains normal bumblebee colony reproductive outputs. Currently, ca. 150 tonnes of pollen are gathered across Europe of various floral types that concentrate parasitic and viral threats when fed to commercial bumble bees. This time EPA co-funded study aligns with key strategies for address food production and security including Food Vision 2030 (Ireland) and the UN Sustainable Development Goals.

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List of abbreviations

Abs	Absorbance abs
ATP	Adenosine triphosphate
AFB_1	Aflatoxins
AFB	American foulbrood
AMPs	Antimicrobial peptides
Apis mellifera	honeybee
AA	Arachidonic acid
ARGO2	Argonaute
B.cereus	*Bacillus cereus*
BGRP	Beta-glucan receptor proteins
BQCV	Black queen cell virus
Bombus terrestris	Bumblebee
Callow	newly emerged bee
CO_2	*Carbon dioxide*
CBPV	Chronic bee paralysis virus
COP II	Coat protein complex II
CoQ	coenzyme Q
CoA	coenzyme A
CCD	Colony collapse disorder
CFU/mL	Colony forming units per millilitre
C. bombi	*Crithidia bombi*
Cyp	Cytochrome
DPCoA	cytosolic dephosphoCoA
DAMPs	Damage Associated Molecular Patterns
Dredd	Death related ced- 3/Nedd2-like
Drone	Male
DWV	Deformed wing virus
DAFM	Department of Agriculture, Food and Medicine
DMAPP	dimethylallyl pyrophosphate

DTT	dithiothreitol
dsRNA	double stranded RNA
E-beam	electron beam
ER	endoplasmic reticulum
EDTA	Ethylenediaminetetraacetic acid
E.coli	*Escherichia coli*
FA	fatty acids
Female	bumblebee worker/nurse
FSC-A	Forward scatter area
GlcCer	glucosylceramide
GRAM positive	ve^{+}
Gram negative	ve-
Gyne	future queen
HFD	High fat diet
H_2O_2	Hydrogen peroxide
IMI	imidacloprid
Imd	Immunodeficiency
IGF	Insulin like growth factor
ITS	Internal Transcribed Spacer
IAA	Iodoacetamide
IPP	isopentenyl pyrophosphate
IAPV	Israeli acute paralysis virus
JNK	Jun-kinase
kGy	Kilogray
LTs	leukotrienes
LC-MS	liquid chromatography mass spec
LPS	lipopolysaccharide
MS	Mass spectrometry
MAMPs	Microbe Associated Molecular Patterns
miRNA	microRNA
NAD	nicotinamide adenine dinucleotide

NADPH	
NO	Nitrogen Oxide
N. apis	Nosema apis
N. bombi	*Nosema bombi*
N. ceranae	*N. ceranae*
OTUs	operational taxonomic units
OXPHOS	Oxidative phosphorylation
PAMPs	Pathogen-Associated Molecular Patterns
PRRs	Pathogen Recognition receptor
P. larvae	*Paenibacillus Larvae*
PO	phenoloxidase
PBS	Phosphate buffer saline
piRNA	piwi-interacting RNA
PEDV	porcine epidemic diarrhea virus
PI	Propidium iodide
PGs	prostaglandins
PUV	Pulse UV technology
ROS	reactive oxygen species
RFC	Relative fold change
RPM	Revolutions per minute
rRNA	Ribosomal RNA
RNAi	RNA interference
SBV	Sacbrood virus
SFD	Saturated fatty diet
siRNA	short- interfering RNA
SSC-A	Side scatter area
Sbm	Sobremes
SEM	Scanning electron microscopy
SSDA	statistically significant differentially abundant
dH_20	Sterile water
TCA cycle	The Tricarboxylic acid cycle

X g	Times gravity
tRNA	Transfer RNA
2DGE	2 dimensional gel electrophoresis
Vg	Vitellogenin
V. destructor	*Varroa destructor*
UGT	UDP-Glucuronosyltransferases
UV	Ultra violet light
W. anomalus	*Wickerhamomyces anomalus*

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Declaration

I hereby declare the work contained within this thesis, submitted to Technological University of the Shannon: Midlands Midwest for the degree of Doctorate of Philosophy, has not been accepted for the award of any other degree in any other higher education institute, and is entirely my own work and to the best of my knowledge, contains no work previously written or published by another party (except in the case of referenced material)

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John Eakins Date

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Chapter 1 Introduction

1.1 Pollination

Pollination is an ecosystem service and a vital resource provided by many insects, with 87.5 % of angiosperm species benefiting reproductively from these interactions (Theodorou *et al.*, 2020). Among the most important pollinators these are bees, particularly as the commercial honeybee (*Apis mellifera*) and various wild and commercial *Bombu*s species. Wild bumblebees are also recognised as efficient pollinators on a par with if not better then commercial honey bees (Kleijn *et al.*, 2015). A bumblebee queen will found her colony in spring and will carry out the foraging herself until the workers can take over in late spring and summer. She will then spend her time laying eggs to produce more workers until autumn, when the focus switches to producing new queens and males (Lozier, 2017). The bumblebee has also been adapted to commercial pollination especially for tomatoes with 95% of colonies been sold for that purpose in 2006 (Biodiversity and Series, 2009).

Animal pollination is required for up to 40% of the global crop production (Krams *et al.*, 2022). Pollination of crops in the United States alone is estimated to be worth in excess of $14 billion (Reeves *et al.*, 2018) which is provided by honeybees. Commercial honeybees are used to pollinate crops such as blueberries and apples (Al Naggar *et al.*, 2018). They are particularly important to the Californian almond industry that rely heavily on honeybee pollination to produce 80% of the almonds on the world market (Staveley *et al.*, 2014).

In Europe, 78% of all flowering plants are pollinated by animals, with 84% of crops such as strawberries, plums, cucumbers and rapeseed oil being carried out by insects, worth 15 billion euro per year. The honeybee is biggest contributor to pollination in Europe (Commission, 2018). This species is extremely important on a global scale for crop production, but evidence of regional declines in both Europe and the USA are mounting (Murphy and Stout, 2019). In Ireland pollination is required for rape seed oil, which is worth 3.9 million euro to the Irish economy (Commission, 2018). Ireland has 97 species of wild bees, 20 of them bumblebees and the rest solitary bees with only one species of managed honeybee. Wild bees in Ireland provide the bulk of pollination services to various crops and fruit (Biodiversity and Series, 2009), making them invaluable to the economy and for food variety.

1.2 The importance of bees for pollination

Pollinators play a major role not just in the pollination of crop species, but also in maintaining a functioning eco system where without animal plant pollination these plants that rely on pollinators would eventually disappear (Commission, 2018; Khalifa et al., 2021). While pollination is not exclusively insect based, commercial bee keeping including, honey, bumble and solitary bees are the most important pollinators of global crops. The western honeybee alone is estimated to be worth $15-$20 billion to the US economy, pollinating > 90% of the almond, blueberry and apples (Al Naggar *et al.*, 2018). In the EU there are 2000 species of wild bees that exist with conflicting reports as to whether managed honeybees supplement or substitute wild pollinators (Commission, 2018). The bumblebee *Bombus terrestris* is the second most important commercial pollinator after the honeybee, with 1 million colonies being exported to 57 countries globally per annum (Ings *et al.*, 2010).

In the UK insect pollination is valued at £ 92.1 million to the apple industry with wild pollinators calculated to being responsible for 75% of that value (Commission, 2018). Ireland relies heavily on bees to pollinate oilseed rape which is worth € 3.9 million per annum (Biodiversity and Series, 2009; Commission, 2018). Bumblebees are seen as more efficient pollinators for fruits such as tomatoes, blueberries and strawberries due to their ability to buzz pollinate (Biodiversity and Series, 2009) with half the market value of pollinated strawberries being valued at € 1 Billion in the EU (Commission, 2018).

1.3 The causes of bee decline

Recent losses in pollinator abundance and diversity is threatening food security and bees as with many pollinator insects are currently under treat across the world (Peso *et al.*, 2018). The decline in honeybees was first noticed in 2006 and this then became referred to as colony collapse disorder (CCD) (VanEngelsdorp *et al.*, 2017 ; Tong *et al.*, 2018). Since then losses have been observed in colonies in several countries. These losses are occurring across all species types with 37-65% of species been a conservation concern (Murray *et al.*, 2012). A multitude

of factors such as pesticides, parasites, viruses, climate change, habitat loss and a lack of quality nutrition have all been linked with poor colony survivorship (Runckel *et al.*, 2011; Tong *et al.*, 2018; Reeves *et al.*, 2018; Peso *et al.*, 2018). The speed of decline of bee pollinators is alarming that becomes an even bigger cause for concern when the benefits they provide to humans are weighed up, both from a nutritional and economical point of view.

One of the biggest factors in bee decline is their susceptibility to parasites and viruses, coupled with their limited immune system (Walderdorff *et al.*, 2018). Bumblebees tend to be dominated by the parasite *Crithidia bombi* while *Nosema ceranae* and *Varroa* mites tend to infect honeybees. There is evidence to suggest that these pathogens can cross taxa (Graystock, Goulson and Hughes, 2015). This has come about as a result of using non-native bee species for commercial pollination (Koch et al., 2017)

Parasites can be transmitted horizontally (faecal/oral route), with flowers acting as an intermediate transmission hub by foragers (Folly *et al.*, 2017; Gegear, Otterstatter and Thomson, 2006; Palmer-Young *et al.*, 2016; Graystock et al., 2015; Koch et al., 2017). These foragers then bring the parasite back to the colony, where the Larvae can act as a reservoir to infect naïve workers (Folly *et al.*, 2017). Honeybees (*Apis mellifera*) used as commercial pollinators can be infected by numerous pathogen such American Foulbrood (AFB) a highly infective Gram positive bacteria that spreads among the larvae and ultimately leads to death (Ribeiro *et al.*, 2019). This creates the possibility of infection being carried to wild bees from commercial bees and destroying wild communities if these parasites and viruses learn to jump species.

C. bombi is a trypanosome which shares its life cycle with one host. It is unable to survive for long periods outside the host so it needs to be able to infect quickly (Schmid-Hempel *et al.*, 2018). *C. bombi* lives in the gut of the bumblebee (Gegear, Otterstatter and Thomson, 2006); (Palmer-Young *et al.*, 2016) and is considered relatively benign. However it can affect the queens by castrating them, hampering their ability to form a new colony (Palmer-Young *et al.*, 2016; Schmid-Hempel *et al.*, 2018). It has also being shown to affect foraging ability (John Naughton *et al.*, 2017) with a study by *Gegear et al* (2006) showing that parasite bees were unable to learn colour of rewarding flowers leading them to conclude it affects the central

nervous system. It is linked to increased mortality but generally at times of stress, when food supplies are low (Gegear *et al*., 2006; John Naughton *et al.*, 2017).

Nosema ceranae is a fungal parasite that shortens the life span of the bee by causing energetic stress (Youngsteadt *et al.*, 2015).It has also supresses the immune system, affects foraging behaviour, impacts on the synthesis of vital molecules and cellular signalling. It is transmitted via the faecal/oral route or within a food source such as pollen, nectar or honey. Once inside, it germinates in the midgut lumen and injects sporoplasm into the cytoplasm of the host cell. Meronts mature into primary spores that can go on and infect adjacent cells. Mature spores are then released into the midgut lumen via cell lysis, they can then be expelled from the body where they are free to infect from the faeces (Burnham, 2019).

The midgut acts as defensive barrier, not only essential for detoxification and digestion but also for immune responses. *N. ceranae* also has the capability of interfering with cellular responses within the midgut, namely apoptosis. Infections have been known to inhibit apoptosis, which in turn aids *Nosema* prevalence within the host (Kurze *et al.*, 2018).

Nosema infections have been traditionally treated with antibiotics such as fumagillin, this has since been banned by the European Union due to fears over its toxicity, which leaves a gap in the market for technologies that can contain the spread of the parasite (Burnham, 2019). There are also studies that show exposure to imidacloprid increases the levels of *N. ceranae* within the colony (Mcmenamin *et al.*, 2016).

Honeybees are also susceptible to the mite *Varroa destructor,* which jumped from its native host *Apis cerana* to *Apis mellifera* (Youngsteadt *et al.*, 2015; Goulson and Hughes, 2015). It has the ability to reproduce in worker cells, making it extremely costly to those infected. It dampens the immune response and vectors viruses, playing a major role in colony collapse. Varroacides are chemicals used by beekeepers to treat colonies and prevent the spread of *Varroa* and *Varroa* associated viruses. Currently it is the best available treatment to reduce over wintering losses (Haber, Steinhauer and vanEngelsdorp, 2019).

Another major source of parasites and viruses that infect bees is the commercial pollen that is supplied to commercially available bee colonies for crop pollination services. These include

the above mentioned *N. ceranae*, and *Crithidia mellificae, Apicystis bombi* and viruses Deformed wing virus (DWV), Israeli acute paralysis virus (IAPV), Chronic bee paralysis virus and Sacbrood virus (SBV) (Pereira et al., 2019).Black queen cell virus (BQCV) can stop colonies from being formed by preventing a new queen from emerging from the hive (DeGrandi-Hoffman and Chen, 2015). DWV has a broad host range, capable of infecting both honey and bumblebees (Goulson and Hughes, 2015). It is a 30nm positive sense single strand RNA virus that can be transmitted both horizontally (the faecal/oral route) and vertically (parent to offspring) and the virus can be found in the midgut lumen, without causing damage to the midgut cells. When transmitted vertically it can be rather symptomless with no obvious negative impact, however when vectored (used as a transfer vessel) via *Varroa destructor* it can be fatal. This virus can cause death in the pupae and as the name suggests lead to deformed wings in adult bees that emerge (de Miranda and Genersch, 2010) .

Another virus that can replicate in *V. destructor* is IAPV, a member of the *Aparavirus* family and also a single stranded positive sense RNA virus (Levitt *et al.*, 2013). IAPV as the name suggests causes paralysis in bees (McMenamin *et al.*, 2018). Experiments using high titres of IPAV 1 X 10^8, saw an increase in *B. terrestris* mortality after 20 days, with bees appearing disorientated and falling over. The viral particles were detected in the head, gut and fat body (Piot *et al.*, 2015). IPAV also seems to be more prevalent in colonies where food supplies are low (Brutscher *et al*., 2015), which feeds into the theory that nutrition is key to mounting a good immunological response. Bees do however have a mechanism to try and cope with viral infections, known as RNAi. This works by neutralizing the viral strand as its replicating and marking it for degradation (Cappelle *et al.*, 2016).

Bacterial diseases are another cause for concern in *Apis mellifera*. *Paenibacillus Larvae* is a Gram positive bacteria that causes the highly contagious disease American foulbrood (AFD). It is spread horizontally to larvae, where they germinate in the midgut. Once mature they will infect the haemocoel causing death. Spores are formed and released across the hive once *P. larvae* has used up all the nutrients available. The fact that *P. larvae* produce spores makes it an extremely difficult organism to treat (Ribeiro *et al.*, 2019). Once a hive is diagnosed with *P. larvae*, they are marked for destruction to contain the spread (Pereira et al., 2019)

1.4 The insect immune system

Bees lack an adaptive immune system, so must rely on a humoral and innate cellular immune system (Erler, Popp and Lattorff, 2011) as well as behavioural strategies or social immunity to keep pathogen risk at a minimum (Evans and Spivak, 2010). However recent studies have shown that immune priming can act as a form of memory and even cross generations, better preparing them for future parasite attacks, with offspring constitutively expressing genes involved in antimicrobial peptides (AMPs) and beta-glucan receptor proteins (BGRP) (Barribeau, Schmid-Hempel and Sadd, 2016).

Behaviours such as grooming, undertaking and temperature control are strategies employed to reduce pathogen stress within the hive. Some bee species such as *Apis cerana* and *Apis mellifera* will groom each other for the removal of mites such as *Varroa,* which also requires damaging the mite via biting during removal. This strategy however is thought to favour some viruses such as *Chronic bee paralysis virus* as a mode of increased transmission (Evans and Spivak, 2010). Similar models to grooming include hygienic behaviour, a system that removes infected workers or pupae from the colony (DeGrandi-Hoffman and Chen, 2015). Honeybees will also remove their dead from the hive to reduce the risk of pathogen spread. There is even some evidence to suggest that honey bees will come together to raise the hive temperature if they suspect the larvae have been compromised with certain pathogen such as Chalkbrood. Raising the temperature 1^0C can create an environment unsuitable for Chalkbrood (Evans and Spivak, 2010).

The bee exoskeleton and digestive tract act as physical and chemical barriers to pathogens (DeGrandi-Hoffman *et al*., 2015) in much the same way as the skin and digestive tract does in humans. If pathogens evade either of these barriers, there are other lines of defence. The cellular immune system, which uses melanisation, phagocytosis and encapsulation. The humoral immune system which secretes antimicrobial peptides (AMPs) (Erler *et al*., 2011), reacts to macromolecules (Walderdorff *et al.*, 2018) and utilises various cellular pathways (Erler *et al*., 2011).

Pathogens have surface structures called Pathogen-Associated Molecular Patterns (PAMPs) that can be identified by Pattern Recognition Receptors (PRRs) of cells, which initiate a

signalling cascade and activate a cellular immune response, of phagocytosis, encapsulation. Initiation of phenoloxidase (PO) will regulate synthesis of melanisation (DeGrandi-Hoffman and Chen, 2015). Proteolytic cleavage of prophenoloxidase to its active form PO results in a melanin coat being deposited on the pathogen surface marking it for encapsulation (Roger *et al.*, 2017).

The main signalling pathways employed upon recognition of PAMPs, Damage Associated Molecular Patterns (DAMPs) or Microbe Associated Molecular Patterns (MAMPs) (Evans and Spivak, 2010) are the Toll, Jak/STAT and Immunodeficiency (Imd) (Roger *et al.*, 2017)and JNK (jun-kinase) pathways (Erler, Popp and Lattorff, 2011). The toll PRRs recognise and bind bacterial and fungal PAMPs. Upon activation a serine protease cascade results in the cleavage of pro-Spaetzle its active form Spaetzle which then binds the toll receptor. This leads to dimerization and recruitment of MyD88, Tube and Pelle. Pelle degrades the NF-kB inhibitors which allows the transcription factors Dorsal-1a and dorsal-1B to translocate to the nucleus to increase expression of AMPs (McMenamin *et al.*, 2018). The Imd pathway is activated by gram negative bacteria. Activation of adaptor protein Imd results in the *Relish* phosphorylating the IKK complex, *Relish* is cleaved by Death related ced- 3/Nedd2-like (Dredd) and transcriptionally regulates AMP expression (McMenamin *et al.*, 2018).

Several studies have been carried out to gain a better understanding of the immune genes involved upon infection. *Hemomucin* is upregulated in *B. terrestris* when challenged with *C.bombi. Hemomucin* is a surface receptor glycoprotein that is thought to act as a surface receptor molecule, inducing an immune response upon recognition (Schlüns *et al.*, 2010).

AMPs are mainly synthesised in the fat body (C. *et al.*, 2010), Abaecin, apidaecin and defensin are usually expressed upon activation of Toll or Imd, in response to transcription factors *Dif* or *dorsa*l and *relish,* with *relish* being the major transcription factor of the Imd pathway. In *B. terrestris* defensin 1 and hymenoptaecin are greatly expressed in response to lipopolysaccharide (LPS) or wounding with dorsal and prophenoloxidase not involved (Erler *et al.*, 2011). Phagocytosis is initiated through the Toll and also the JAK/STAT pathway (Walderdorff *et al.*, 2018).

In response to viral infection, there is evidence that bees use RNA interference (RNAi) as a part of their immune response (DeGrandi-Hoffman and Chen, 2015). There are 3 pathways

in RNAi, short- interfering RNA (siRNA), microRNA (miRNA) and piwi-interacting RNA (piRNA). Replicating viruses produce double stranded RNA (dsRNA), which is recognised by RNAse 111 and cleaved into short 21 – 22 bp strands. These short strands are bound by the endoribonuclease part of the protein, RNA- induced silencing complex (RISC) known as Argonaute (ARGO2). The passenger strand is then released and the other strand acts as a guide for RISC to target other complementary viral strands within the cytoplasm for cleavage (Brutscher *et al*., 2015). Studies have shown the effectiveness of RNAi in protecting pollinators from viruses. In honeybees orally administered IPAV specific and nonspecific dsRNA were effective at lowering the viral titre and prolonging the lifespan. Non-specific dsRNA was more effective in bumble bees, than the specific, however it could not prevent IAPV infection, but could lower viral titre and increase lifespan (Piot *et al.*, 2015).

Maintaining an immune response is costly in invertebrates (Barribeau *et al.*, 2016), and this can be intensified by poor diet. A study has shown that during times of nutritional stress, *B. terrestris* produces less prophenoloxidase (Roger *et al.*, 2017). This could result in a reduced immune response upon pathogen challenge as, due to lower levels of PO and less melanin.

Another stressor with the potential to impact immunity are pesticides. Neonicotinoids have been demonstrated to inhibit phagocytosis in both bumble and honeybees, with bumblebees being particularly susceptible. This could be due to half-life, as imidacloprid (IMI) has a longer half-life in bumblebees, 10 hours compared to 4 in honeybees. The addition of a pathogen increased the impact of IMI on hemocytes, further reducing phagocytosis in bumblebees. Bumblebees are impacted more by IMI and LPS combined, and this can cause a decrease in Nitrogen Oxide (NO) which acts as a signalling, and cytotoxic component in defence against bacteria and parasites. IMI had no effect on bumblebee ability to produce H_2O_2 (a reactive oxygen species) while it did impact on honeybees ability (Walderdorff *et al.*, 2018).

These studies give further insight into the multifactorial stressors that impact bees. In conditions of poor nutritional content, such as a poor-quality pollen that is contaminated with pesticides, then bees are more likely to succumb to parasites and viruses, due to an inability to mount an adequate immune response.

1.5 The bee microbiota

There is an increasing focus on the microbiome of the bee. Honeybees and bumblebees share closely related species. The hindgut of the honeybee is host to 95% of its microbiota, while the crop (foregut) and midgut contain few bacteria. The hindgut compromises of the ileum, which is home to species such as *Gilliamella apicola*, *Frishella perrara* and *Snodgrassell*a, while the rectum is dominated by *Lactobacillus-4, 5* and *Bifidobacterium* (Moran, 2015) .

The species comprising the microbiome remain constant under many stresses, however infection with *C. bombi* can alter its makeup. Bumblebees placed under nutritional stress and subjected to immune priming have been reported to show no change in the microbiome. However bumblebees challenged with *Nosema bombi* or *C. bombi* did exhibit a change in microbiota diversity (Koch, Cisarovsky and Schmid-Hempel, 2012). Studies have also indicated that the absence of total microbiota have led to increased levels of *C. bombi* infections, with Betaproteobacteria decreasing the likelihood of an infection (Koch and Schmid-Hempel, 2011). Further experiments using *Bombus impatiens* as a test model revealed that *Apibacter*, *Lactobacillus-5* and *Gilliamella* may be responsible for conferring resistance to *C. bombi*, the latter two comprising of the core group of gut microbiota in both honey and bumblebees. Other core members include *Bifidobacterium, Bombiscardivia, Lactobacillus-4* and *Snodgrassella,* with *Snodgrassella* being subject to conflicting reports as to its ability to offer resistance to *C. bombi*. *Apibacter* demonstrated the greatest ability to inhibit *C. bombi* infection (Mockler *et al.*, 2018).

Nosema ceranae, a honeybee parasite that resides in the midgut, has also demonstrated subtle effects on the gut microbiome with an increase of two *Gilliamella*. There are a number of different strains of *Gilliamella* that live in the honey bee, with different genetic makeup and possible functions and how exactly the slight upregulation of these two strains impacts on the honeybee is not quite known (Rubanov *et al.*, 2019).

The microbiome also plays a role in increasing the survival of bees exposed to metalloids. Metalloids are groups of elements that lie between metals and non-metals, exhibiting some properties of both (Dixon and Vasiliu, 2019). When they get into the environment they can contaminate soil and pose a risk to human health (Li *et al.*, 2017). The metalloid selenate can be found in contaminated soils and this exposes bees foraging on nectar and pollen.

Experiments exposing *Bombus impatiens* to selenium, found that bees without a microbiome had were more likely to succumb to selenate poisoning than those that had. The study also found it was the core gut bacteria that increased the survivorship, however to determine the exact mechanisms may require more study (Rothman *et al.*, 2019).

A lack of normal gut microbiota has also been linked with a decrease in the production AMPs, though this study showed variance in some of the trials in survivorship between those lacking a microbiota and those that had, upon *E.coli* challenge. This study also found evidence of the enzyme Cu-Zn superoxide dismutase in the bees that lacked the microbiota. This enzyme converts superoxide radicals to H_2O_2 helping to reduce toxicity (Kwong, Mancenido and Moran, 2017).

1.6 The commercial bee industry

The commercial bee industry is estimated to be worth around £55 million per year and was born out of the need for the pollination of commercial crops (Biodiversity and Series, 2009). Commercial pollinator bees were first introduced in the 1980s to aid the mass production of crops. These commercial hives are now imported in over 50 countries (Murray et al., 2013). In Ireland commercial bees are relied upon directly for the pollination of apples, rapeseed oil and strawberries and indirectly, with the bee pollinated foods that are imported (Murphy and Stout, 2019).

While the overall objective is to try prevent colony losses and bee decline in native species, there is a growing body of evidence that would suggest commercial bees are contributing to the problem. Commercial bees are marketed as pathogen free, however this is not always the case. Studies carried out by researchers in England found that both commercial bees and pollen were carrying pathogens (Graystock et al., 2013). Parasitic species such as *Apicystis bombi, Crithidia bombi, Nosema bombi, Nosema ceranae* and deformed wing virus were all found in commercially produced bumblebees, while *Nosema apis, Paenibacillus larvae* and *Ascosphaera* were three additional species detected in the commercial pollen (Graystock et al., 2013).

Flowers act as hotspots for the sharing of parasites. Parasites are passed on by commercial bees that act as reservoirs of pathogens dispersals flowers. These are then picked up by wild bees and brought back to the hive (Graystock, Goulson and Hughes, 2015).

Separate studies carried out in both Canada and Ireland found that *Crithidia bombi* and *Nosema bombi* were more prevalent in wild bees near commercial greenhouses and that this lessened the further away the bees were captured from the greenhouses (Murray et al., 2013; Colla et al., 2006). These findings highlight the issues that arise with the use of commercial bees, and the real need to tackle this problem to try limit pathogen spillover.

While government agencies regulate for the importation of foreign species of bee to prevent the introduction of disease such as the ban on importation of *Bombus terrestris* by the United States (Winter and Adams, 2006), it is clearly only part of the problem. Commercial bees have repeatedly been shown to be compromised with pathogens, as has commercial pollen (Graystock, Yates, Sophie E F Evison, *et al.*, 2013). Commercial pollen has been found to contain *N. ceranae*, *Crithidia mellificae, Apicystis bombi* and viruses Deformed wing virus (DWV), Israeli acute paralysis virus (IAPV), Chronic bee paralysis virus, Sacbrood virus (SBV) (Pereira et al., 2019) and Black queen cell virus (BQCV) (DeGrandi-Hoffman and Chen, 2015). What is currently unknown is whether the bee, or the pollen is the main source of the parasites.

Bombus terrestris has also established itself in at least 2 countries outside its native environment Japan and Chile. This is due to the importation of commercial *Bombus* colonies (Ings *et al.*, 2010). In Japan introduction of *B. terrestris* impacted negatively on native species by competing for food resources and nesting sites seeing a large reduction in *B. h. sapporoensis* queens. There was also concerns about the introduction of parasites such as *Nosema bombi*. A study that investigated hybrid production between invasive *B. terrestris* and the native *Bombus ignitus* resulted in the production of 566 eggs, but only 22 hatched (Tsuchida *et al.*, 2019a).

1.7 Commercially sold pollen

Commercial pollen is sourced from honeybees and this is then used to feed to bumblebees (Pereira, Meeus and Smagghe, 2019). It is possible that the commercial bees are initially pathogen free and that the pollen they are fed is the source of the contamination. Pollen has been recognised as a potential issue and attempts have been made to address this. Various studies have detected both infectious viruses and parasites in commercial pollen (Graystock et al., 2013; Pereira et al., 2019). Various studies have compared sterilisation techniques with gamma irradiation at present offering the best possible solution to inactivate and kill various parasites and viruses present (Simone-Finstrom et al., 2018) . However this latter approach is currently deemed far from a perfect practice (Graystock et al., 2016). It is important that other technologies for the sterilization of commercial pollen are explored. A technology that is cost effective, efficient and can significantly reduce the microbial burden of pollen is more likely to be adopted by the commercial pollen industry, thus reducing the potential for pathogen spillover into wild communities.

1.8 Bumblebees (*Bombus terrestris)*

Figure 2 ***Bombus terrestris***

Bombus terrestris belongs to the genus *Bombus* (Biodiversity and Series, 2009) numbering around 250 species (Watrous, Duennes and Woodard, 2019). They are efficient pollinators and have the ability to forage in low temperatures and adverse weather conditions. This makes them more advantageous compared to the honeybee, who can't forage in temperatures below 14^{O}C (Stern *et al.*, 2021). Other advantages *B.terrestris* has over the commercially available honey bee (*Apis mellifera*) is its ability to carry more pollen on its body (Knapp *et al.*, 2019) due to them having a body size twice that of the honey bee (Stern *et al.*, 2021), some also have longer tongues and frequent more flowers, being up to 2.5 times more efficient at pollinating some crops than honeybees (Biodiversity and Series, 2009). Bumblebees also have the ability to buzz pollinate. Roughly 6-8% of angiosperm plants are buzz pollinated (Ings *et al.*, 2010; Nevard *et al.*, 2021). Buzz pollination is thought to have evolved 45 times within bees, the action involves bees grabbing the anthers with its mandibles and using its flight muscles to produce thoracic vibrations which allows for allows for pollen release from the flower (Nevard *et al.*, 2021).

The life cycle of a bumblebee colony starts with a newly mated queen. A single monogynous queen will mate with a male (Di Pietro *et al.*, 2022) and found a colony of usually around 8 eggs which she will care for until adult worker emerge (Biodiversity and Series, 2009). Workers will then take over foraging duties with bumblebees foraging on a variety of plant species which helps create a diverse diet for developing larvae (Watrous, Duennes and Woodard, 2019). Not only do worker forage and look after the brood but they will also take up a role in nest defence (Di Pietro *et al.*, 2022). The queen and workers both take an active interest in looking after the colony until " the competition point" where workers will start laying eggs which is then followed by the queen laying haploid (male) eggs known as "the switch point" (Di Pietro *et al.*, 2022). At this point the queen will also lay eggs that develop into gynes (future queens) (Biodiversity and Series, 2009). Bumblebee colonies tend to have a male bias (Bourke, 1997) once they start to be produced in the late summer to early autumn, with the main role of males being to mate with newly emerging gynes (Watanabe and Sasaki, 2022a).

Surveys have been carried out across the island of Ireland by both Teagasc and the Department of Agriculture, Food and Medicine (DAFM) and found that in 2013, commercial bumblebees were being used to pollinate strawberries (60%), apples (25%) and tomatoes

(20%) (Biodiversity and Series, 2009). Despite the advantages of bumblebees for commercial pollination, there are also associated risks with the use of commercial pollinators. Commercial pollinators can escape and compete with native species for resources and along with pathogen spillover (Murray *et al.*, 2020) there's also the risk of hybridisation (Tsuchida et al., 2019).

1.9 Research Purpose

1.9.1 Research Aim

The overarching aim of this novel research is to reduce bee pathogen spillover from commercially reared bumblebee colonies to wild bee communities in order reduce or mitigate against complex diseases that will help pollination services.

1.9.2 Inter-related research objectives

1. To investigate the effectiveness of novel non-thermal technologies for the treatment of honey bee gathered pollen
2. To investigate the potential novel relationship between application of physical treatment(s) to pollen (including high level disinfection) on subsequent bumblebee colony reproductive outputs
3. To determine the impact of treated-pollen fed bumblebee colonies on stress and immunity responses from an important proteomics perspective
4. To investigate the impact of treated-pollen on the microbiome of consuming bumblebees

To achieve this the focus was on commercial pollen which is a source of contamination and pathogen infection for commercially reared bees. Pollen was sourced from a commercial supplier and was treated with PUV, this however was deemed to be inadequate for the purpose of the project, so the focus was then switched to using a singular accumulative dose of e-beam to sterilize the pollen. A second method of reducing pathogen loads was taking a more simplistic approach was filtering out the spores.

The different pollen types were then fed to bumblebee colonies over a period of a month. In total twelve colonies were divided into three groups and fed on either untreated pollen

(control group), e-beam treated pollen or filtered pollen. Over the course of the month the colonies were monitored for reproductive output as well as colony weight. Newly emerging bees (callows) were tagged and returned to the hive to access the proteome and microbiome.

Visual observations of the colonies provided insight into the reproductive output, colony development and bee size. Proteomic analysis of the fatbodies of tagged bees shed insight into bee health and stress in the treated pollen groups compared to the control groups. Significant differences in protein regulations were detected and highlighted between the different groups.

Finally microbiome studies on bees between the three groups would determine if there had been a significant difference between the abundance and presence of bacterial and fungal species present in the guts of bees.

Taken together the results of this project may determine if it was a beneficial to feed bees on treated pollen in the fight against bee decline. Many other studies have investigated treatments such as gamma irradiation at reducing pathogen presence within commercial pollen. However, these studies failed to assess any on bee health. It is envisaged that the outputs of this research will inform pollination and ecosystem service management for Europe with a global sustainability orientation.

Chapter 2 Sterilization of commercial pollen

2.1 Introduction

Bees are one of many pollinator insects under treat in the world today (Peso *et al.*, 2018). The decline in bees was first reported in 2006 in honeybees. Reports of losses in adult workers, excess brood and weaken or dead colonies were observed, which became known as colony collapse disorder (CCD) (vanEngelsdorp et al., 2009 ;VanEngelsdorp et al., 2017; Tong et al., 2018). Colony collapse disorder decimates hives by an average of 30% each year (Tong *et al.*, 2018), with causes most being attributed to a multitude of factors including pesticides, parasites, viruses, climate change, habitat loss and a lack of quality nutrition (Runckel et al., 2011; Reeves et al., 2018; Peso et al., 2018).

However the decline of pollinators is not limited to honeybees with 37-67% of bee species on the conservation concern list in Europe (Murray et al., 2013). This is an alarming statistic as the benefits to humans are well documented in terms of crop pollination. Pollination of crops in the United States alone is estimated to be worth in excess of $14 billion (Reeves *et al.*, 2018) which are provided by honeybees (*Apis mellifera*). They are particularly important to the Californian almond industry which rely heavily on honey bee pollination to produce 80% of the almonds sold on the world market (Staveley *et al.*, 2014). In Europe, 78% of flowering plants are pollinated by animals with 84% of crops such as strawberries, plums, cucumbers and rape being carried out by insects, worth €15 billion per year. The honeybee is biggest contributor to pollination in Europe (Commission, 2018). In Ireland pollination is required for rape, which is worth €3.9 million to the Irish economy (Commission, 2018). There are 97 species of wild bees in Ireland, with 20 species of bumblebees and the remaining being solitary bees. There is only 1 type of managed honeybee. Wild bees in Ireland provide the majority of pollination services to various crops and fruit (National Biodiversity Data Centre, 2015), making them invaluable to the economy and food variety

Decline in bumblebee species is in part attributed to habitat loss, due to intensive agriculture (Fitzpatrick et al., 2007; Theodorou et al., 2016), and also pathogen spillover from commercial bees to wild communities. Pathogen spillover occurs when infected bees escape confinement,

and interact with wild communities resulting in the spread of disease (Colla et al., 2006; Pereira et al., 2019). In 2013 it was noted that as much as 11% of species could be at risk of dying out and a study carried out in Ireland found that, pathogens in wild communities could be detected up to 2km away from greenhouses that harboured commercial bumblebees (Murray et al., 2013).

Commercial bees are fed on a diet of nectar and pollen, with pollen being the main source of protein, lipids, vitamins, phenolic compounds and flavonoids (Annoscia et al., 2017), however pollen fed to commercial bumblebees is sourced from honeybees (Meeus et al., 2014). One study found that commercial pollen can act as a source for disease, with seven parasites including *Apicystis bombi* and *Nosema ceranae* and four viruses being identified within the pollen itself (Pereira et al., 2019). Therefore, a potential solution to the prevention of pathogen spillover is the decontamination of commercial pollen.

Current methods of sterilization of commercial pollen include gamma irradiation, using a dose of 16.9KGy leads to a 100-1000x reduction of Israeli acute paralysis virus (IAPV). It was proposed that this dose would also be sufficient to kill *Nosema apis* (Meeus et al., 2014). Other studies found that 25KGy of gamma irradiation inactivated *Ascosphaera apis*, *Nosema ceranae* and Deformed wing virus (DWV), Black queen cell virus (BQCV) and Chronic bee paralysis virus (CBPV). Despite this success a limiting factor with this technology is the length of time it takes to sterilise the pollen. A standard dose of 25KGy takes 9h 45 min (Simone-Finstrom et al., 2018).

2.1.1 Pulsed UV light technology (PUV)

One potential non thermal treatment that could cut the length of time required for pollen sterilization is Pulse UV technology (PUV). UV light has the ability to inactivate viruses (Gómez-López *et al.*, 2021) and complex waterborne parasites, such as *Cryptospordium parvum* and *Giardia* species (Garvey et al., 2015). PUV was also reported previously to inactive the bumblebee parasite *Crithidia bombi* by members of a research team at Technological University of the Shannon (Naughton et al., 2017). However, the aforementioned study

focused on treatment of very small sample sizes in petri-dishes where no commensurate studies were conducted on colony stability or functionality post feeding.

Ultraviolet light is dividing into 4 bands in between a wave length of 100-400nm on the electromagnetic spectrum, UV (100-200nm), UV-C (200-280nm), UV-B (280-315nm), UV-A (315-400nm) (Gómez-López *et al.*, 2021). PUV delivers broad spectrum light over the wavelength range 200 to 1200 nm where the lethal action is attributed to irreversible effects on the treated pathogen including lipid peroxidation, membrane permeabilization, DNA damage, reactive oxygen species (ROS) generation, apoptosis and necrosis where these "multi-hits" increase with fluence (Farrell et al., 2011). PUV technology use xenon bulbs to generate the high intensity polychromatic flashes of light from stored electrical energy in capacitors. Measurement is determined by fluence (F, J/cm^2) defined as "the total radiant energy traversing a small transparent imaginary spherical target containing the point under consideration, divided by the cross section of this target" (Gómez-López *et al.*, 2021). The fluence rate is (E) constant over time (t) in seconds $F=Et$, then for specific PUV technology the equation is

$$F=F_p n$$

With F_p denoted as the fluence per pulse and n the number of pulses (Gómez-López *et al.*, 2021).

PUV has been the subject of intensive investigation for over a decade as a potential surface disinfection method for foods due to its ability to rapidly kill complex microbial pathogens and spoilage species including yeast and moulds (Hayes *et al.*, 2013). Commercially, the company Claranor has sold over 400PUV units for food packaging disinfection (Jureka, Williams and Basler, 2021). PUV has been reported to deactivate SARS-CoV-2 on plastic and glass with a 34.9mJ/cm^2 dose and on stainless steel with a 52.5mJ/cm2 dose (Jureka, Williams and Basler, 2021).

PUV has also been reported to detoxify complex chemical hazards at high fluence levels such as aflavotoxin B_1 (AFB_1) found in infected cereals, nuts and oilseeds (Kutasi *et al.*, 2021). Aflatoxins have been documented to increase the risk of diseases such as liver and lung cancer

(AFB_1) (Kutasi *et al.*, 2021), thus PUV could potentially be used to reduce microbial and parasite load found within commercial pollen, due to the similar size of pollen balls. However, previous researchers have reported that high voltages (such as use of 800 V or 900 V) representing large UV doses per pulse are required to effectively kill recalcitrant food and waterborne parasites (Rowan, 2019). The potential for PUV to be applied to the decontamination of commercial pollen presented an exciting prospect due to its' ability to be used in-situ as a portable bespoke system. Due to its' ability to store energy and release this in ultrashort bursts (sub-microsecond), PUV is capable of disinfecting surfaces in very short treatment times compared to similarly treated standard low-pressure UV technologies (Rowan, 2019).

2.1.2 Electron beam (E-beam)

Electron beam or e-beam is a nonthermal sterilization method that uses electrons and is commonly used for sterilizing medical devices and drugs (Silindir and Özer, 2009). Electron-beam (E-beam) uses high energy electrons emitted via an accelerator or ^{60}Co isotope as its radiation source (McEvoy and Rowan, 2019). It has advantages over gamma-treatments which include short exposure periods (usually minutes), fast cycle times, flexible batch size, good radiation dose distribution, simple validation process, no quarantine period and enables real-time monitoring. However, drawbacks to e-beam compared with using gamma-technology, include limited material penetration and degradation, along with the former using uniform packaging.

E-beam is cost-effective as processing speeds result in lower sterilization costs for low density materials along with greater flexible process as it can operate at different dose ranges and density of products with in a given process run. E-beam is not dependent on radioactive isotopes that decay over a long period of time. E-beam offers benefits in terms of environmental acceptance as it operates as an On/Off linear accelerator technology using standard electricity; thus, negating the need to relying upon radioactive material (McEvoy and Rowan, 2019). E-beam is able to operate as and when required, so as to optimize efficiency of treatments. This infers that the e-beam pollen penetration by accelerated electrons is less than gamma-radiation, which will mean a wider dose range than gamma. Thus, pollen packaged into discrete managing sizes to enable lower-density treatment. E-beam has been

used as a continuous process technology for the sterilization of medical devices, drugs (Silindir & Özer, 2009), and potentially for use on fresh foods (Marin-Huachaca *et al.*, 2002).

When compared with gamma irradiation for the treatment of sprouts and seeds, higher kGy doses of e-beam were needed to achieve the same levels of sterilization as gamma due to lack of penetration. E-beam treatments did achieve a 5-log reduction of four bacterial pathogens including the endospore *B. cereus* in fresh sprouts using 3.65kGY (broccoli) and 4.8 KGy (red radish sprouts) (Trudeau *et al.*, 2016). E-beam has also shown efficacy in reducing the load of porcine epidemic diarrhea virus (PEDV) found in contaminated feed (Trudeau *et al.*, 2016). These findings highlight the variability in susceptibility of different pathogens to E-beam, and other physical sterilization technologies (Franssena et al., 2019; Rowan, 2019), where there is a gap in our understanding of different sensitivities that includes parasitic stages (Franssena 2019; Gerard et al., 2019). E-beam has also shown to produce minimal changes to nutritional composition of treated food, such as powdered infant formula. It also showed efficacy at reducing *Bacillus* spores in the powdered infant milk formula (McFadden *et al.*, 2016). It is also used by NASA to irradiate foods being brought to space with the FDA requiring a minimum dose of 4KGy for frozen of packaged meats (Bhatia *et al.*, 2018).

2.1.3 Flow cytometery as a diagnostic tool

To test cell viabilty and vulnerabity to e-beam irraridation treatment, the use of diagnostic tools with this ability would be required. Flow cytometry can utlilize flourescent dyes to determine viabilty. Cells are incubated with DNA binding dyes such as SYBR safe which confirms their presence, excluding small debris, and Propidium iodide (PI), also a DNA binding dye to determine viabilty, as it only diffuses across memebranes of damaged cells. Cells incubated with dyes are passed in single file through a laser and visble light scatter is measured in two different directions, forward scatter (FSC) which detects cell size and side scatter (SSC) which detects the internal complexity of the cell (Büscher, 2019). By combining these dyes, it is possible to determine cells presence i.e SYBR+ = cells present, PI+ = dead/damaged cells and PI- = live cells.

Figure 2 Flow cytometer used for data analysis of cells and spores. Image referenced from miltenyibiotec. *https://www.miltenyibiotec.com/US-en/products/macsquant-analyzer-10.html#gref*

Aim for this chapter

The overarching aim of this chapter was to assess and potentially develop PUV and/or e-beam as appropriate modalities for sterilizing commercial pollen fed to bees with the intention of reducing commensal pathogens that would ultimately reach the colonies through consumption. Consequently, this would essentially reduce or eliminate unwanted transmission (or spillover) of complex bee pathogens to wild native bee communities. However, it is also important to commensurately consider and negate any potential negative toxicological effects, post treatment in commercial bumblebee that would influence decision to deploy these disinfection or sterilization modalities for this purpose, such as by commercial bee colony producers (Chapters 3-5).

The first test was to determine the capabilities and limitations of PUV and e-beam technologies in reducing biological load in commercial pollen. Use of bacterial endospores were used to assess lethality of PUV and E-beam as this approach has been accepted by the Sterilization Industry where bacterial spores (or bioindicators) are considered representative of more resistant organisms in the treated sample ((McEvoy and Rowan, 2019; Mcevoy, Maksimovic and Rowan, 2023). *Bacillus* species are also found ubiquitously including

contaminated food and soil; thus, there is a high likelihood that these aerobic spore-formers contaminate pollen.

Bacillus cereus at concentrations above $1x10^4$ cells or spores/mL can cause food poisoning, with symptoms such as diarrhoea and vomiting (Sarkinas *et al.*, 2018). Thus the objectives of this study were:

(a) to artificially- spike commercial pollen with *B. cereus* cells and determine the effectiveness of PUV at reducing loads of *B. cereus* CFU/mL on nutrient agar plates. To extract spores found within commercial pollen, and treat them with PUV based on findings from pre-screening bacterial inactivation studies.

(b) to access the effectiveness of that determined PUV dose using Flow cytometry a means of real time determining microbial death without reliance on conventional plate counts.

(c) to use an initial dose range for e-beam coupled with Flow cytometry. A predetermined range of 1.5, 5, 10 & 25KGy was based off the literature to determine the effectiveness of e-beam at treating spores found within commercial pollen.

(d) to access and determine the optimum dose of e-beam irradiation required to kill spores found in commercial pollen.

2.2 Materials and methods

2.2.1 Pollen

Pollen was sourced from Koppert (#10500 II) and came in grain form shipped on ice. Pollen was stored at -20^{o}C as per instruction.

2.2.2. Pre-screening studies on the effectiveness of PUV and E-beam using *Bacillus cereus* cells

Bacillus cereus cells were grown on nutrient agar plates by aerobic cultivation for 48 h at 37^{o}C with the view to generating a standard curve of cell concentration versus optical density. The standard curve of vegetative cells (figure 2) was generated by suspending *Bacillus cereus* (strain ATCC 11778) in PBS that would inform the working stock sample regime of pre-determined cell numbers for future PUV treatments. The concentration of the *B. cereus* stock was determined by measuring the absorbance @625nm and dividing by the length of the cuvette 0.4 cm. Each *B. cereus* stock varied in absorbance to create a wide range and was subjected to serial 1/10 dilutions, when then a standard pour plate method was used to enumerate, where colony counting was conducted to equate absorbance to cell number or CFU/mL. Plate counts were correlated to O.D, which allowed for the determination of concentrations of *B. cereus* cells to be used for PUV testing.

Optical Density = Absorbance @625nm / 0.4

Equation 2 the standard curve was obtained by measuring the absorbance of *B. cereus* in PBS, plating the microbes on nutrient agar and growing them at 36^{o}C for 18 h. Absorbance was correlated to plate counts, CFU/mL.

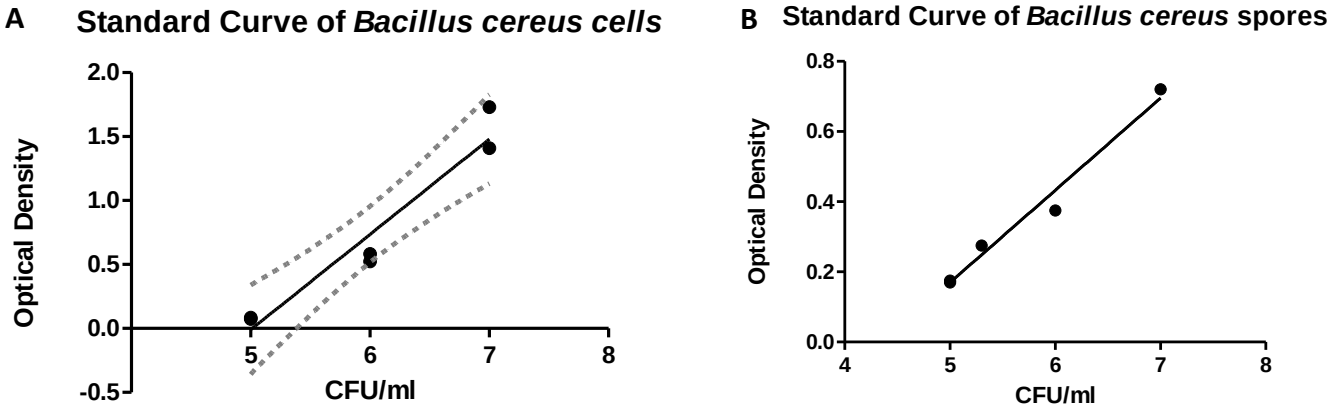


Figure 2.2.1: A standard curve of *Bacillus cereus* cells (A) and spores (B) suspended in PBS was created by using various concentrations of *B. cereus* ATCC 11778 cells. Values of $1X10^7$, $1X10^6$ & $1X10^5$ CFU/mL(X- axis) are plotted here against their Optical Density (OD) reading (Y-axis). This allowed unknown samples to be quantified by inputting their OD into the slope of the line before any subsequent experimentation was carried out. It meant there was no longer a need to carry out serial dilutions of each sample.

Generation of *Bacillus cereus* ATCC 11778 spores

To generate *Bacillus cereus* spores, an environment needed to be created which favoured spore formation over growth. The identify of *B. cereus* cells was confirmed by Gram reaction, growth on *B. cereus* selective agar (Oxford Media) and by confirming characteristic profile using API 50 CHB/20 E physicochemical gallery (API Biomerieux, France) as described by Rowan (2019). *B. cereus* ATCC 1178 was selected as it is a whole-genome sequenced strain that will also support and enable future comparative genomic and bee infection studies.

A sporulation media was prepared (Table 2) and 100 µL was added to nutrient agar plates and allowed to settle for 10 min. A single microporous bead of *B. cereus* cells, strain ATCC 11778 that had been cryogenically frozen and stored at -80^{o}C was streaked across each nutrient agar plate using a disposable inoculation needle and the plates were there after incubated aerobically for 72 h at 37^{o}C. Once the 72 h incubation period at 37^{o}C had elapsed the plates were wrapped in parafilm and allowed to sit on the bench for 5 d at room temperature. On the sixth day 2 plates were harvested for quantification purposes.

Table 2: **Sporulation media** was generated using the following formula. This was used to try reduce the length of time it took for sporulation to occur naturally**.**

Name	**Molecular Weight**	**Chemical composition**	**Molar Concentration**	**Quantity**
PBS				10 mL
Calcium Chloride	110.984g/mol	$CaCl_2$	.0005M	.0005 g
Potassium Chloride	74.5513g/mol	KCl	0.0268M	.02 g
Manganese Chloride	197.905g/mol	$MnCl_2.4H_2O$	0.015M	.03 g
Magnesium Sulphate	246.474g/mol	$MgSO_4.7H_20$	0.2M	.5 g

2.2.3 B.cereus spore harvesting

To harvest the *B.cereus* spores for quantification, 5 mL of sterile PBS was added to the plate to aid resuspension. The 5 mL suspension was then transferred to a 50 mL falcon tube and the process was repeated, generating a 10 mL spore suspension. The suspension was topped up to 20 mL with PBS and then incubated in a water bath at 70^{o}C for 20 min to try kill off any remaining vegetative cells.

The spore suspension was allowed to cool to room temperature and topped up to 40 mL with PBS. The cooled spore suspension was then transferred to redcap tubes 10 mL in each and centrifuged at 4000 x g for 15 minutes. The supernatant was removed and the pellet was suspended in 5mls of PBS and centrifuged again at 1780 x g for 15 min. The supernatant was then discarded and the pellet suspended in 5 mL of PBS.

To test to see if a higher yield of intact spores could be generated from the same sample solution, centrifugation steps that employed weaker G forces were used for the Flow cytometry protocol.

Samples were centrifuged at 2172 x g instead of the 6000 x g that was used previously and for vegetative cells. Samples were also incubated with SYBR safe for 15 min instead of 10.

A positive control of dead cells was also introduced to allow aid determination of alive versus dead ratio of spore suspensions using the fluorescent dye. This suspension of dead cells was achieved by taking a *Bacillus cereus* cell suspension of $1x10^6$ CFU/mL and heat killing it in a water bath at 70^{o}C for 20 min. After, the suspension was allowed to cool to room temperature, 1 mL of the suspension of dead cells was transferred to a sterile eppendorf tube and subjected to the same centrifugation and staining protocol as described in this chapter.

2.2.4 Initial streak plating and pulse UV adjustments

Based on the literature (Rowan, 2019), the voltage was set at 800v and the pulse time was set at 1 pulse per second (p/s), this meant every second the machine used 800v to generate a dose of UV. This could be measured using a Thermopile power detector (Model: XLP12-3S-H2-IN, Gentec-EO, Quebec, Canada) coupled with Integra software to convert it to a unit, 10.84mW/cm^2. Information obtained from previous work carried out by (Fitzhenry, 2019) informed the decision making on how best to calculate the dose measurement. However the length of time needed and the log reduction that could be obtained was yet to be determined.

Initial experimentation focused on optimizing the amount of pulses needed to kill *Bacillus cereus* vegetative cells. The time was set to either 30, 60, 90, 120 or 150 s. Plates were streaked with *Bacillus cereus* cells on nutrient agar. Plates were then pulsed at their allotted time and incubated at 37^{o}C for at least 18 hours and re-examined the next day for colony growth. Control samples of non-treated *Bacillus cereus* were incubated for at least 18 h.

This was later refined to aliquot 200 µL of a pre-determined concentration *B. cereus* cells into a 96 well plate. The plate was placed in the centre of the PUV machine and pulsed for the allotted time (30, 60, 90, 120, and 150). After the selected time, 100µL was taken from the well and allowed to recover on nutrient agar plates using a standard pour plate method at 37^{o}C for 18 h. Survivors were enumerated against control plates to determine level of microbial log reduction achieved.

2.2.5 Pulsing for 150 s and using a pour plate method

Pre-determined populations of *Bacillus cereus* cells of approximately $1x10^5$, $1x10^6$ and $1x10^7$ CFU/mL were prepared in 1 mL Eppendorf, 100 µL was poured onto a nutrient agar plate and incubated at 37°C for at least 18 h to act as control. The remaining volume from each Eppendorf was separated into 200 µL aliquots on a 96 well plate and pulsed at 800v for 150 s. This allowed for all samples to be performed in triplicate. Thereafter, 100 µL of each pulsed well was transferred and poured on a plate, which was then incubated overnight at 37°C. After this an aerobic incubation period, microbial colonies from untreated and pulsed samples were enumerated.

2.2.6 Initial PUV experimentation with pollen

To test the efficacy of pulse UV against contaminated pollen samples, 1 g of pollen was added to 9 mL of $1x10^6$ CFU/mL of *Bacillus cereus* ATCC 11778 cells suspended in PBS. The samples were homogenised through mechanical agitation and vortexing. Once a uniform solution was reached 100 µl aliquots were incubated at 37°C overnight on nutrient agar plated to act as controls, while pulse experiments were set up in triplicate using 200 µL of spiked sample in a 96 well plate. The samples were pulsed for 150 s before 100 µL from each well was poured onto a nutrient agar plate and allowed to incubate overnight at 37°C. Plates were counted and compared after this incubation period.

2.2.7 Treatment of pollen for experimentation with Pulse UV.

In order for PUV to be effective in practical terms it was deemed necessary that the integrity of the pollen was maintained during PUV treatment.

To spike the pollen, a $1x10^6$ CFU/mL concentration of *B. cereus* cells was prepared in PBS and placed into a plastic spray bottle. The bacterial PBS solution was then sprayed onto the dried pollen. It was vital not to spray too much as this would compromise the integrity of the pollen.

The samples were then separated out into weigh boats each containing 1 g of pollen. One boat would act as positive control while the other boat were treated with PUV for 150 s.

2.2.8 Flow cytometry (FCM) reagents and protocols

Table 2.1 SYBR safe Sigma SYBR Safe DNA gel stain (Sigma Aldrich) was used to generate various working stock solutions. SYBR safe was diluted in either dimethyl sulfoxide (DMSO) or PBS.

SYBR safe	1st dilution in DMSO	1st dilution in PBS	2nd dilution in PBS cell suspension	Final concentration
0.5µL	49.5 µL		5/495 µL	1000X
1µL	49 µL		5/495 µL	5000X
1.5µL	48.5 µL		5/495 µL	3300X
10µL		9990 µL	10/990 µL	10000X

2.2.9 Flow Cytometry and optimization of SYBR Safe

B. cereus cells were chosen at a concentration range of $1x10^5$ to $1x10^3$ CFU/mL and centrifuged at 6000 x g for 10 min then suspended in Flow buffer. SYBR safe was added at various quantities according to table 2.1 and incubated in the dark for 10 min. The cells were centrifuged again at 6000 x g, the supernatant was decanted, and the cells were suspended again in 500 µL of Flow buffer.

Samples were run on a MACSQuant Analyser 10 and analysis was carried out using Miltenyi Macs quantify.

2.2.10 E-beam treatment of suspected fungal spores and pollen associated microbial community

Commercial pollen obtained from Koppert contained a microbial community, predominatly made up of suspected fungal spores. These spores were extracted using a filtration method described in chapter 3. Fungal spore samples were set up in triplicate for sterilization. A total

of 24, 10 mL samples were prepared, 12 samples were the “stock” (undiluted microbial bioburden filtered from commercial pollen in sterile water) and 12 samples were diluted 1:10. The range used was 1.5 KGy, 5 KGy, 10 KGy and 25KGy. All samples were treated at Steris Tullamore on the 21st Janruary 2020. They were then prepared for Flow cytometry and checked for viability using SYBR safe and PI stains.

A second round of e-beam sterilization included higher doses of 50, 75 and 100KGy. These additional ranges were included based on the results obtained from the first round of testing using the initial dose ranges. The broader and higher dose range generated more visual results, based on Flow cytometry results using viability dye.

Due to COVID-19 pandemic circumstances operations were suspended after one round of the broader dose range. After consultation with Steris, experiments were refined to a single dose of e-beam. Based on the data gathered from the previous 2 experiments it was decided that anything under 25KGy would simply be ineffective and that perhaps 100KGy might be the most effective dose at damaging the populations present.

A total of 5 separate extractions were carried out and diluted in sterile water to a concentration of $1x10^6$ cells/mL and samples were divided up into triplicates of 10 mL aliquots in 15 mL redcap clear plastic tubes. Steris received the samples on the 30th March 2021 and had them processed and ready for analysis on the 1st of April 2021, with samples been kept refrigerated before and after treatment. A 100KGy accumulative dose of 33.5KGy x 3 was administered instead of a direct 100KGy dose. This was done at the discretion of Steris based on their knowledge and understanding of the technology.

The samples (a total of 32), were taken back to the lab to be analysed using flow cytometry techniques.

2.2.11 Preparation of cells for Flow cytometry

The suspected fungal spore samples returned from Steris and control samples of suspected fungal spores were prepped the following way. 1 mL samples were taken and centrifuged at 3000 x g for 5min to pellet. They were then resuspended in 1 mL PBS, with 50 µL SYBR safe and 50 µL PI. Samples were incubated in the dark at room temperature for 1 h. Samples were then centrifuged at 6000 x g for 5 min. The supernatant was decanted, and the pellet was resuspended in 1 mL of PBS. The samples were then centrifuged at 6000 x g for 5 min. This step was repeated. Finally, the pellet was resuspended in 500 µL of Flow buffer for analysis.

Flow cytometry was carried out on Macs quantify Flow cytometer. The following axis were selected on three dot plots:

- SSC-A (Side scatter area) vs FSC-A (Forward scatter area)
- SSC-A vs B1-A (SYBR safe)
- SSC-A vs B3-A (PI)

The Flow cytometer was set to take up 50 µL of each sample and wash the column in between each sample analysis.

2.2.12 Flow cytometry gating strategy

A gating strategy was developed using two dyes, SYBR safe, which was used to determine the presence of cells and PI which is used to determine viability. Control populations of cells are used to aid the gating strategy, these include positive and negative controls. A negative control is the same population of cells, minus any fluorescent dye, while a positive control is a population of the same cells that is incubated with the dye and will be positive register a positive signal. Using a negative control (figure 2.2.2) cells are passed in single file and detected by a laser. However, cells can sometimes adhere to each other, meaning a falsely enhanced signal, a doublet (2 cells stuck together) needs to be removed from analysis. For single cell fluorescence SSC-A is directly proportional to SSC-H, which appears as a diagonal on the dot plot, anything that deviates from this diagonal is excluded using a gate to create a population of single cells only. This population is now labelled as singlets. The selected

"singlet" population is now used to determine the gates for SYBR+ and PI+ cell populations that have been incubated with the fluorescent dyes.

The singlet population is set to SSC-A on the y-axis and B1-A (SYBR) on the x-axis. The area that appears with no cells present is gated and this gate is labelled SYBR+. When this gate is applied to cells that have been incubated with the dye, the SYBR+ cells will now appear within that gate. The same steps are then applied to determine PI+ cells, using the negative control singlet population, SSC-A is selected on the y-axis and B3-A (PI) is selected on the x-axis. The area clear of cells is then gated and labelled PI+. This gate is then applied to cells that have been incubated with PI and any cells that appear in that gate are noted as PI+.

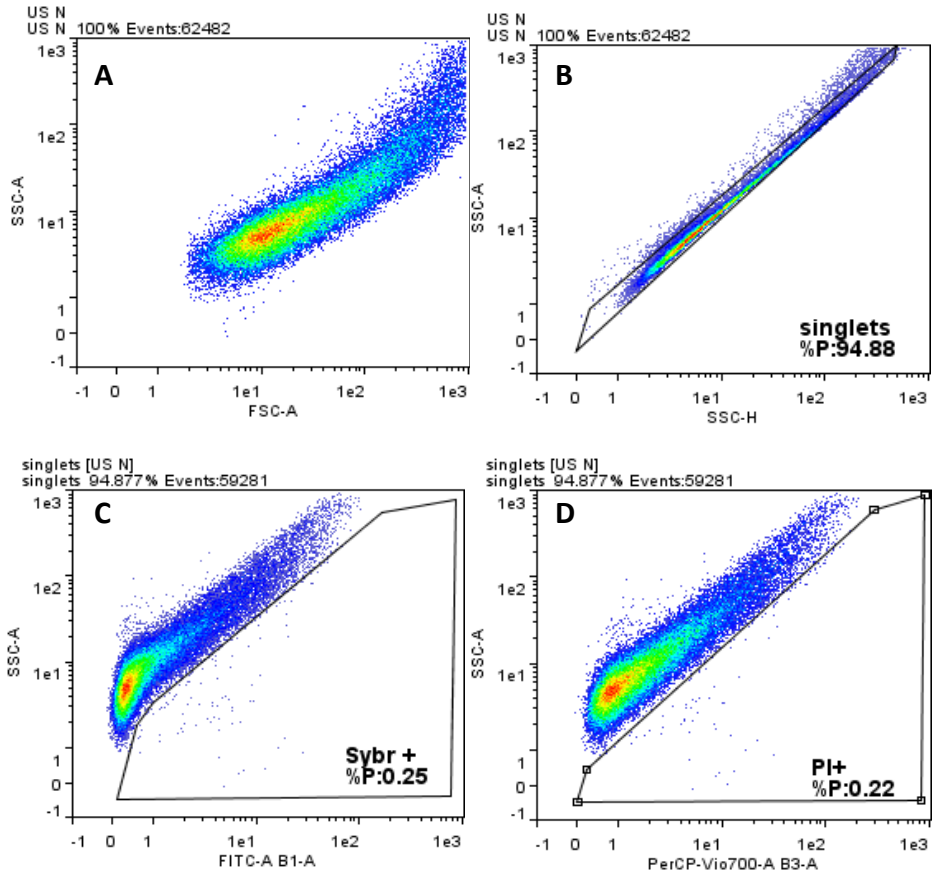


Figure 2.2.2 Using Flow cytometry to confirm *Bacillus cereus* spores and viability: Lays out the how a gate is determined for a Flow cytometry-based experiment. Unstained sample *B. cereus* spore populations (A) are used, and the parameters adjusted to side scatter area versus side scatter height, this allows us to gate for a singlet population (B) excluding any doublet cells. Next the gated singlet population is used and the SYBR safe channel is selected (C) and the PI channel (D). As both these populations contain no dye, this area is gated and labelled as SYBR+ (C) or PI+(D). This means anything that shows up in these gates for stained samples has taken up the dye.

2.2.13 Flow cytometry analysis

Data obtained was analysed using Flowlogic software v 8.5. Cell counts and cells with a positive fluorescent dye signal were measured using the tools built into the software. Gates were set to analyse SYBR+ and PI+ cells.

PRISM software was used to carry statistical analysis on the data. Statistical significance was determined using unpaired Mann-Whitney t-Test and the following label were attached * ($p \leq 0.005$), ** ($p \leq 0.01$), *** ($p \leq 0.001$).

2.3 Results

The generation of a standard curve allowed for the use of bacterial samples within a certain range to be used for experimentation with PUV treatment. Initially a concentration of $1x10^6$ CFU/mL was selected, the voltage was set at 800v, but the time period was adjusted. Replicates of the same *Bacillus cereus* cell sample were pulsed at one pulse per second (p/s) for time periods of 30, 60, 90, 120 & 150 s. Pulsed bacterial cell samples were then tested for growth using pour plate methodology. Following incubation, plates were counted, and the most effective time period was chosen for further study. Effectiveness was determined by the number of colonies that grew after PUV treatment. The time period of 150 s which saw a total reduction in *Bacillus cereus* was deemed to be the most effective and the most suitable starting point.

2.3.1 PUV treatment of *Bacillus cereus* cells

To test the sterilization capabilities of PUV technology vegetative suspensions of *Bacillus cereus* cells were prepared in aseptic conditions. The concentration was set between $1x10^6$ and $1x10^7$ CFU/mL. Samples were prepared in 200 µL aliquots in a 96 well plate, in triplicate. This allowed all samples to be treated simultaneously.

Samples were then treated with varying doses of PUV. The voltage was set to 800v @ 1pps. Samples were then treated at varying 30s time periods, from 30 s up to 150 s. When a

particular sample had reached its time quota, 100 mL was taken from the well and poured onto nutrient agar plates and incubated at 37°C for a minimum of 18 h. Plate counts were used to determine the efficacy of the PUV treatment, with all samples being carried out in triplicate. *Bacillus cereus* cells treated with PUV exhibited varied reductions in growth. A total reduction of $1x10^6$ CFU/mL was observed on agar plates when *B. cereus* cell aliquots were treated with 120 and 150 s of PUV. A $1x10^5$ CFU/mL reduction was observed when *B. cereus* cell aliquots were pulsed with 90 s of PUV (Figure 2.2).

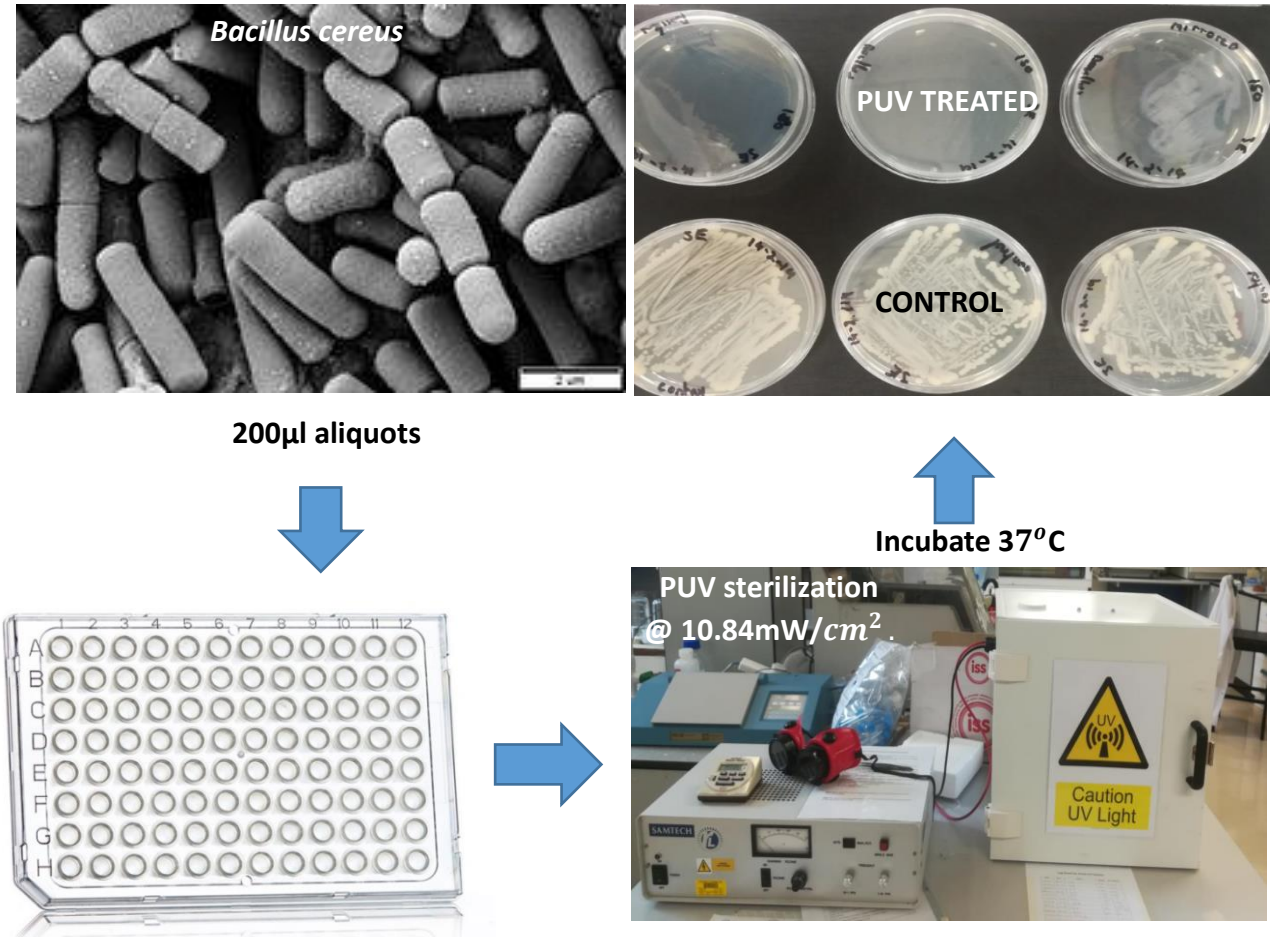


Figure 2.5 Schematic of PUV treatment process of *Bacillus cereus* cells: The above schematic depicts the process by which *Bacillus cereus* cells were subjected to PUV treatment. Cells are prepared at known concentrations ($1x10^7$-$1x10^5$ cells/mL) and aliquoted in 96 well plates and treated with PUV before being allowed to recover on nutrient agar plates for a minimum of 18 h at 37°C.

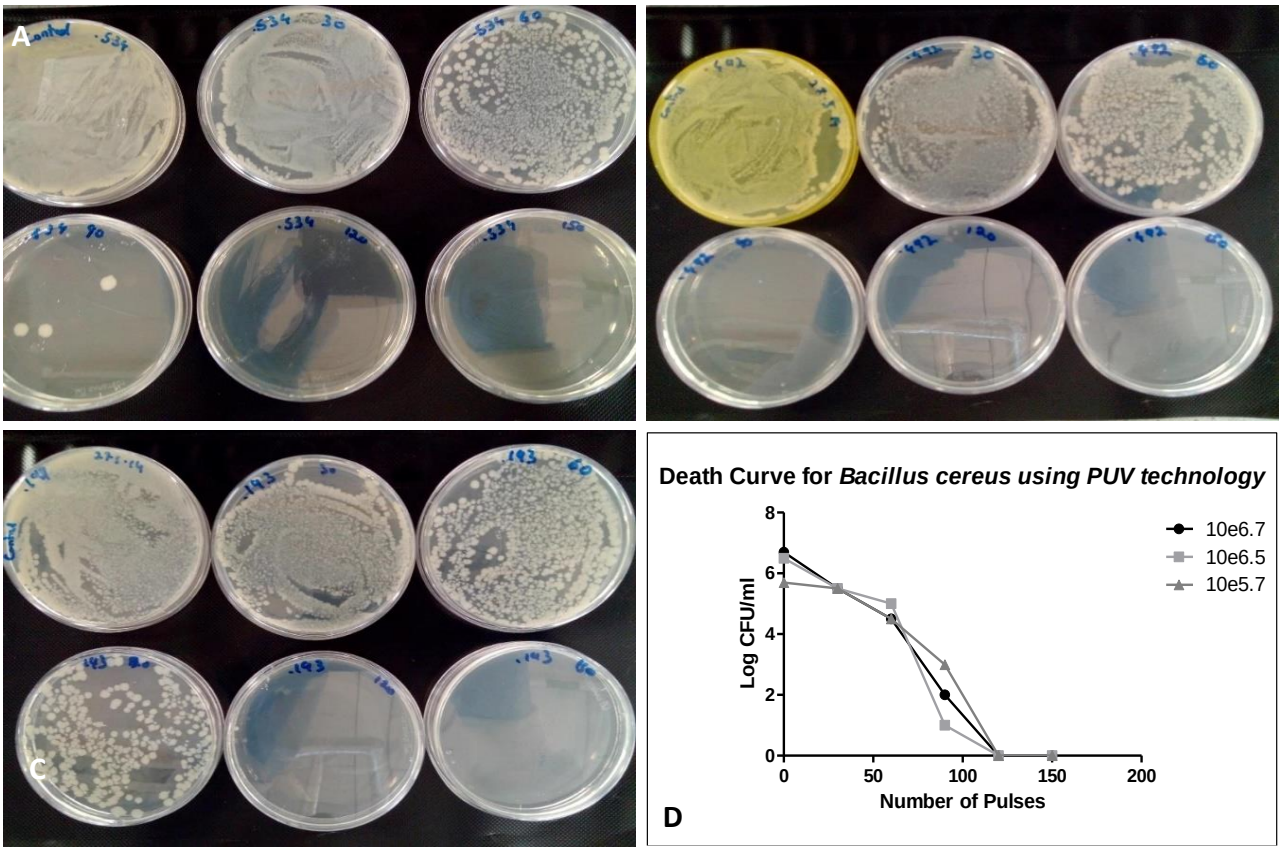


Figure 2.6 Generation of a death curve for *Bacillus cereus* cells using PUV technology: shows the effects of PUV technology on fixed concentrations of *Bacillus cereus* cells at different time periods. It is visually evident in A, B & C that as the time period of the PUV treatment increases so does the efficacy in reducing bacterial loads. Each sample shows 6 individual plates of a specific concentration and the number of pulses increasing left to right. In all 3 samples, time periods of 120 & 150 s (bottom middle and right of each pic A-C) were enough to completely eliminate *B. cereus* loads within the $1x10^5$-10^6 CFU/mL range. The death curve graph (D) shows the reduction for all 3 samples at each time period when treated with PUV. Each line is a representation of each individual sample of *Bacillus cereus* cells over the course of 150 pulses. As the number of pulses increases so does the reduction in the number of bacteria. At 120 and 150 pulses *Bacillus cereus* is at undetectable levels.

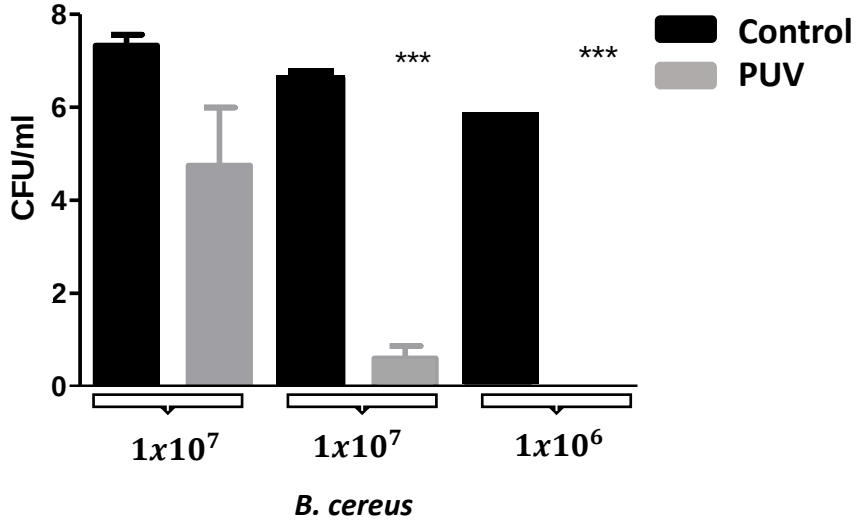


Figure 2.7 *Bacillus cereus* cells suspended in PBS and treated with PUV reduces bacterial loads with reduction influenced by cell concentration**.** *Bacillus cereus* cells suspended in PBS at concentrations of $1x10^7$ & $1x10^6$ CFU/mL, displayed reductions in CFU/mL depending on the starting concentration. *B. cereus* cell concentrations at 1 x 10^6 CFU/mL showed a complete reduction in numbers, while concentrations above $1x10^6$ CFU/mL showed partial reductions in cell numbers, when pulsed for 150 s. Reductions were based on colony counts after incubation on nutrient agar plates. All samples (n=5) are shown before (pre-pulse) and after PUV treatment (post-pulse) with significant log reductions observed. Analysis was carried out using a one-way ANOVA ($p \leq 0.001$).

PUV at a dose of 1p/s for 150s was successful at achieving a $1x10^6$ CFU/mL reduction in *Bacillus cereus* vegetative cells. Reductions were calculated based off enumeration of colonies post PUV treatment. Pulsed cells were allowed to recover at 37°C for 18h on nutrient agar using a standard pour plate method. Statistically significant reductions were observed between the control and PUV groups ($p \leq 0.001$) using a one way-ANOVA, CFU/mL reductions of *B. cereus* cells at $1x10^6$ and $1x10^5$ CFU/mL were observed (Figure 2.3).

Next pollen was spiked with *B. cereus* cells to see if similar results could be obtained. Pollen was mixed with concentrations of *B. cereus* cells ($1x10^7$ & $1x10^6$ CFU/mL) in water and homogenised. The liquid pollen solution was then taken up and 200 µL aliquots and pipetted into 96 well plates in triplicate and treated with PUV for 150 s. Once treated 100 µL of the solution was plated on agar plates and allowed to recover at 37°C for a minimum of 18 h.

The ability of $1x10^6$ *B. cereus* cells mixed with pollen and treated with 150 s of PUV to recover on nutrient agar was similar to what was observed in earlier experiments (Figure 2.3). A reduction in numbers from $1x10^6$ to $1x10^1$ CFU/mL was observed, while a reduction in CFUs for higher concentrations at $1x10^7$ to 10^4 CFU/mL was also achieved (Figure 2.4).

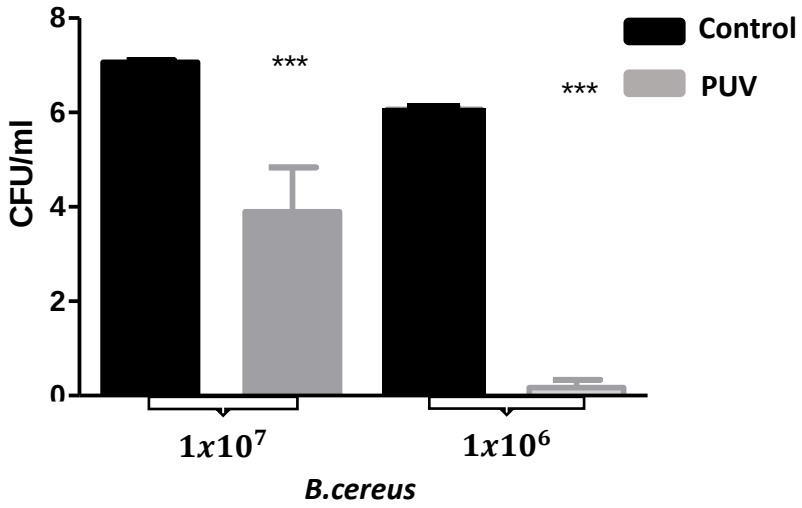


Figure 2.8 PUV treatment of pollen contaminated with *Bacillus cereus* shows pollen samples contaminated with *B. cereus* cells and homogenised pre and post PUV treatment. There is a significant reduction in *B. cereus* cells, with a greater reduction being observed in samples at a concentration of $1x10^6$ CFU/mL. A $1x10^3$ CFU/mL reduction is seen in samples that had a concentration of $1x10^7$ CFU/mL, with a 95% confidence level when using a one-way ANOVA. Samples at a concentration of $1x10^6$ CFU/mL also showed significant reductions using a one-ANOVA ($p \leq 0.001$).

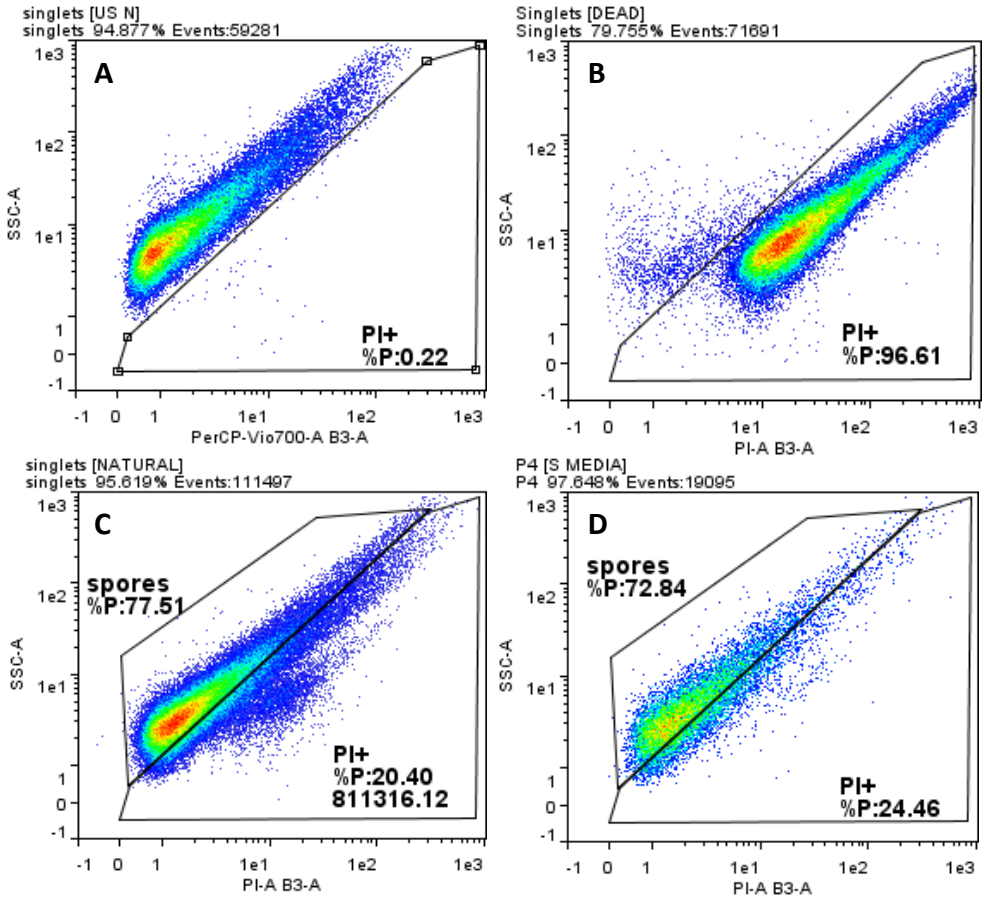


Figure 2.5 Determination of percentage *B. cereus* spores harvested using PI: Depicts 4 dot plots to try determine the percentage population of *B. cereus* spores from the harvest using viability dye PI as the determinant. First gates are set using an unstained control population (A), which are then transferred across to the other spore samples that had been incubated with PI. To determine the ability of the dye to adhere, a sample of spores were killed (B), and the dot plot shows that 96% of the *B. cereus* spore population are PI+ (B). The percentage difference in spore generation between natural sporulation (C) and chemical induced means (D) can be seen by adding in an extra gate which shows 77% and 72% PI- of the populations believed to be viable spores respectfully.

To determine the yield of *B. cereus* spores Flow cytometry and the use of fluorescent dyes that stain DNA was used. The gates were determined using unstained populations. Populations of *B. cereus* spores were prepared using the Flow protocol without the dye which allows for the determination of *B. cereus* spores that are present with the dye (Figure 2.5). The use of viability dye PI allows for the discrimination between spores and viable cells with intact spores being PI negative (PI-).

PUV analysis of microbial spores extracted from pollen.

Suspected fungal spores extracted from pollen were divided into aliquots of 200 µL at a concentration of $1x10^6$ cells/mL and treated with PUV for 150s at 1p/s. This was done in a 96 well plate and cells were then prepared for Flow cytometry analysis as described in the materials and methods section above. Viability was determined using PI where cells that take up the dye are determined to be damaged or dead. The average population of cells that were PI+ was 56.9675% (control) and 19.3475% (PUV) (Figure 2.6) and this was determined to be significant ($p \leq 0.05$) using a Mann –Whitney U unpaired t-test.

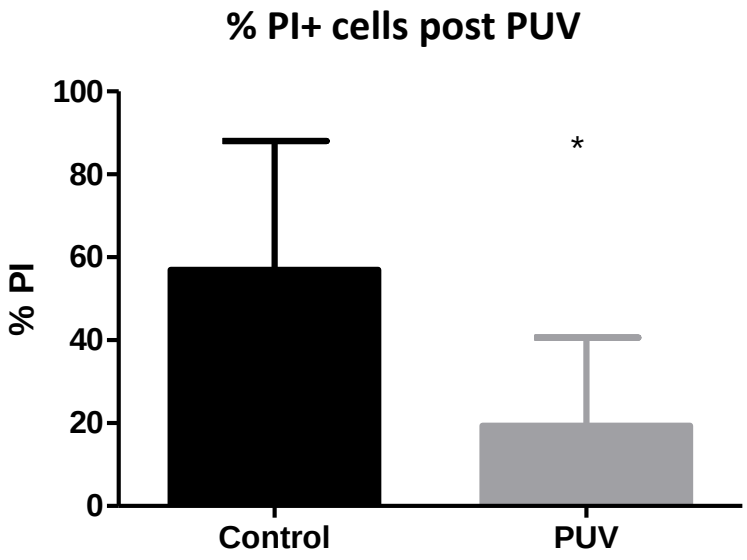


Figure 2.6 percentage of PI+ spores extracted from pollen and treated with PUV shows the percentage of PI+ pollen extracted spores that were treated with PUV. There was a significant difference recorded ($p \leq 0.05$) using a Mann-Whitney unpaired t-test with cells treated with PUV having taken up a lower percentage of the viability dye PI.

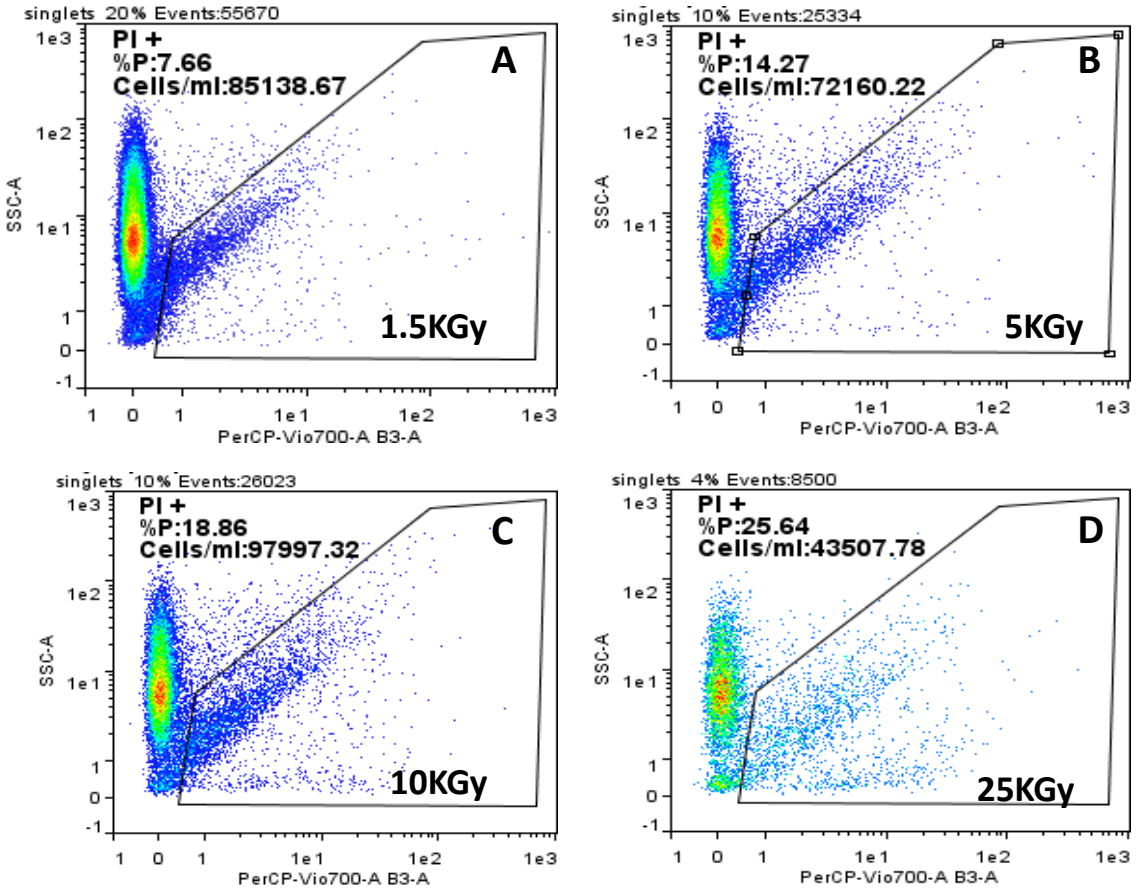


Figure 2.7 Dot plots of PI+ suspected fungal spores extracted from pollen and treated with varying doses of e-beam irradiation. The percentage of PI+ cells increased with the dose given. The lowest dose given was 1.5KGy (A) and the highest dose was 25KGy (D). To complete the range doses of 5KGy (B) and 10KGy (C) were also trialled.

Initial experiments selected a low dose range of e-beam irradiation to focus on (figure 2.7). The increase in PI+ cells increased as the dose increased. At 25KGy PI+ cells had increased to 25.64% compared to the lowest dose 1.5KGy which had a cell population that was PI+ 7.66%.

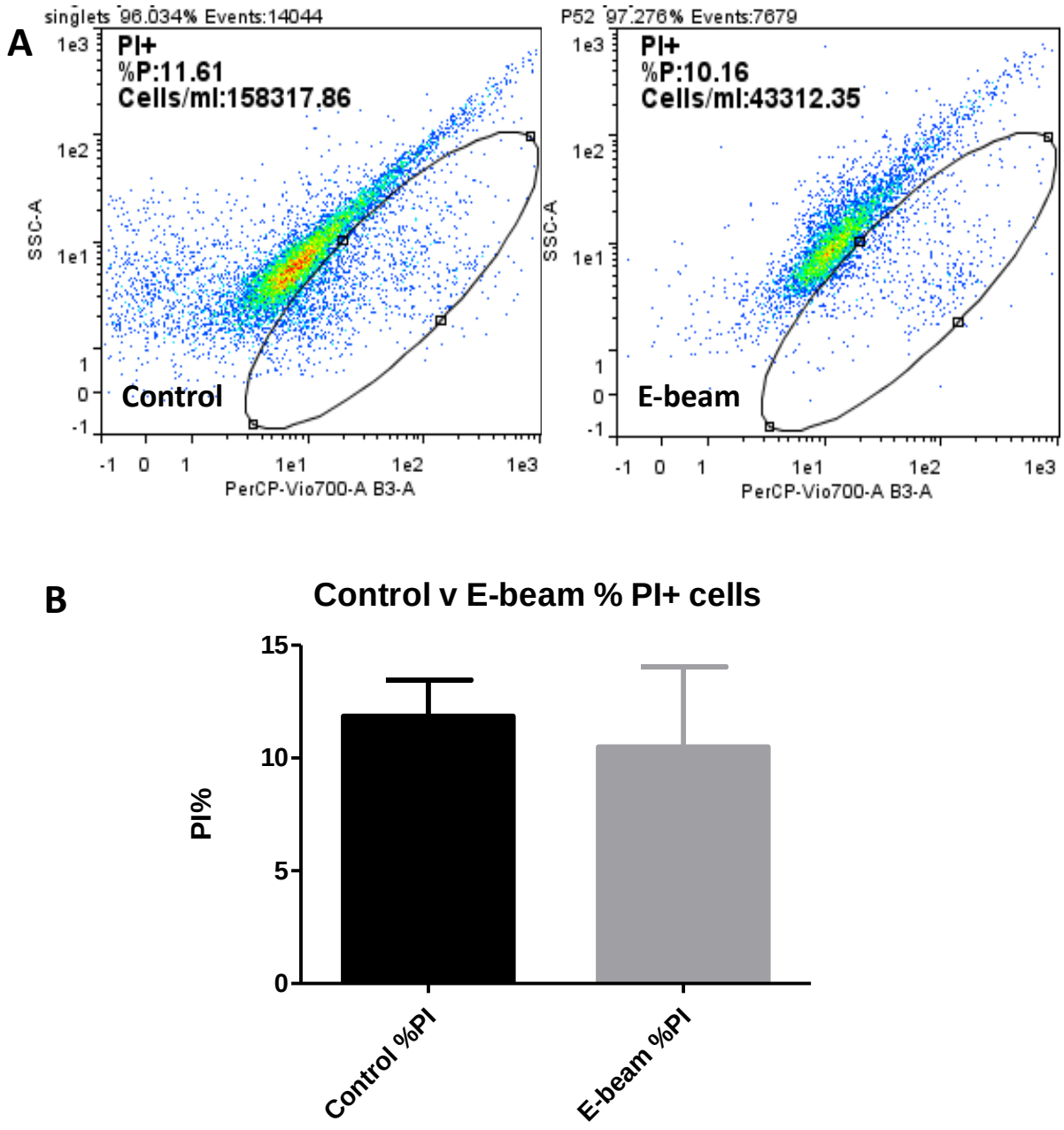


Figure 2.8 Dot plots and bar charts of PI-stained control and e-beam treated suspected fungal spore samples extracted from pollen. Dot plot images depict a higher percentage PI+ spores in control samples compared to e-beam treated samples (A). The bar chart (B) depicts the percentage of PI+ spores extracted from pollen between control and e-beam treated samples (n=5). Treated spore dominant samples were hit with a 100KGy accumulative e-beam dose. There was no significant difference recorded between the 2 samples sets based on data analysed using a t-test.

Suspected fungal spore samples extracted from pollen were incubated at room temperature with PI for 1 h in dark conditions and prepared for Flow cytometry analysis as described in the materials and methods section. A gating strategy was determined also as described in the materials and methods section. There was no statistical difference noted between control and e-beam treated samples (Figure 2.8) detected using a t-test. Overall, the control population had a slightly higher percentage of PI+ cells and significantly higher percentage of SYBR+ cells ($p \leq 0.001$) (Figure 2.9 & 2.10). However, e-beam treated pollen samples produced no fungal growth post treatment, compared to untreated control pollen which was completely covered in white fungal matter nine months post treatment (Figure 2.11).

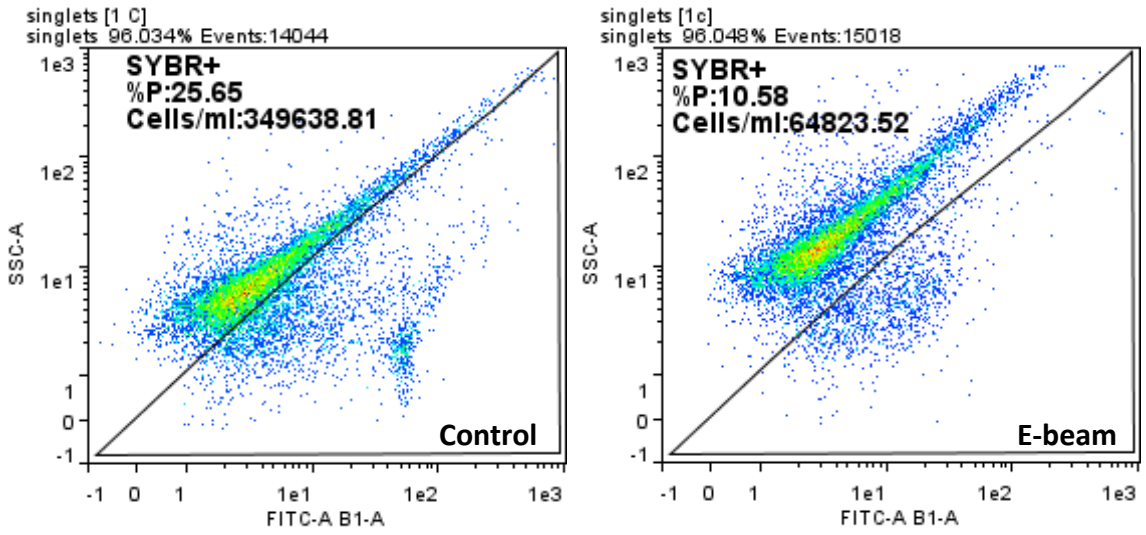


Figure 2.9 Dot plot images of SYBR stained control and e-beam treated suspected fungal spore samples: shows a higher percentage of SYBR+ cells present in the control compared to the e-beam treated sample.

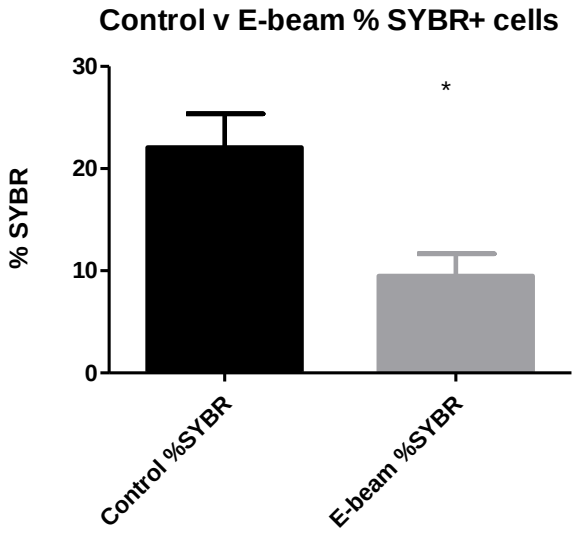


Figure 2.10 Percentage of Sybr+ pollen extracted spores of control and e-beam treated showed that there was a statistical significance ($p \leq 0.001$) between the percentage of SYBR+ cells in control and e-beam treated samples, analysed using a t-test. Control samples had a higher population of SYBR+ cells, than e-beam treated samples, which might be suggestive of a more vegetative presence pre-treatment.

Figure 2.11 image of control and e-beam treated pollen 9 months post experiment: depicts the control the pollen samples used for testing post colony experiment. Both samples were kept refrigerated for 9 months post colony experiment. The control (left) was covered with fungal growth, while e-beam treated pollen (right) had no growth and was visually the same as the day it was treated.

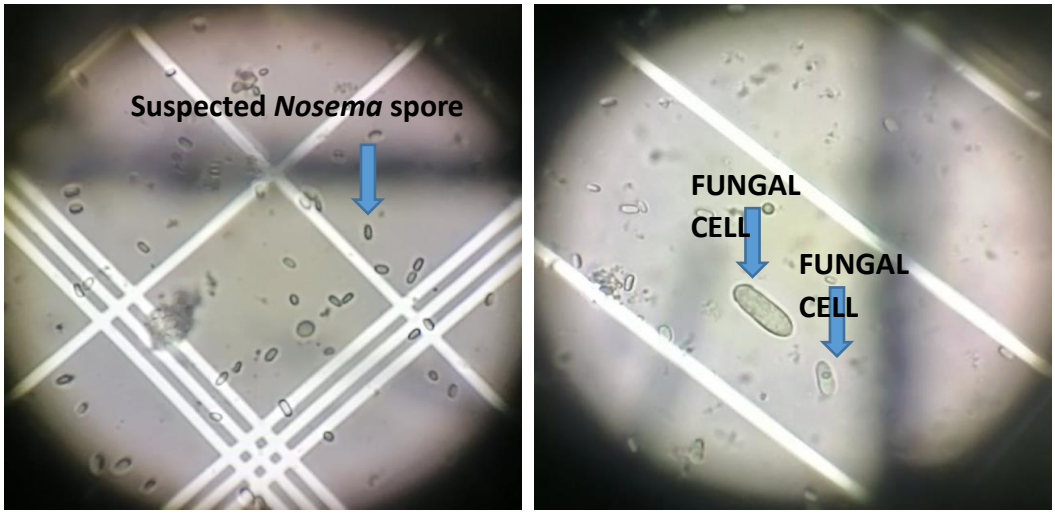


Figure 2.12 microscopic images of microbial bioburden extracted from pollen: microscopic images show suspected *Nosema* spores (left) as extracted from untreated commercial pollen. These spores were extracted in quantities of $1x10^6$ cells/mL using a developed filtration method. The image on the right depicts vegetative cells that are suspected to be fungal in origin based on proteomic ITS sequencing.

2.4 Discussion

Bee species are under threat globally with two species of bumblebee going extinct in the UK and eight having decreased in abundance since 1940 and another four species going extinct in Europe (Graystock et al., 2013). Along with habit loss (Fitzpatrick *et al.*, 2007) pathogen spillover from commercial colonies to wild communities remains one of the biggest challenges facing pollinators today (Colla et al., 2006; Graystock et al., 2013; Murray et al., 2013). Pollen sourced from commercial suppliers is known to be contaminated with pathogens (Graystock et al., 2013) and new approaches are required if pathogen spillover into wild communities is to be prevented (Colla *et al.*, 2006). Pathogens reduce the fitness of bees with pathogens such as *Apicystis bombi* reducing the fatbody of bumblebee workers and over-wintering queens, reducing their survival (Graystock, Goulson and Hughes, 2015). Current methods of pathogen reduction in commercial pollen make use of gamma irradiation (Simone-Finstrom et al., 2018), however this is time consuming so it's worth exploring more time efficient methods.

PUV had been previously demonstrated to reduce levels of *C. bombi* in the faeces of infected bees (Naughton et al., 2017) so it was tested to see if it could offer a more time efficient

alternative to gamma irradiation. Initial experiments to test its effectiveness showed promising signs of reducing the spore forming bacteria *Bacillus cereus*. Reductions that reduced the concentration of *B. cereus* vegetative cells by $1x10^6$ CFU/mL were achieved using a voltage of 800v at 1p/s for 150 s. This was determined by allowing cells treated with PUV to recover on nutrient agar plates over night at 36^{o}C. Mixing *B. cereus* cells with pollen and homogenising the solution before carrying out the same method of PUV treatment also showed signs of bacterial reduction using an agar based streak plate method of recovery.

Suspected *Nosema* spores were predominantly isolated from commercial pollen using a filtration method, described in ***chapter 3***. The use of viability and DNA dyes PI and SYBR presented the opportunity to distinguish between vegetative living/dead cells and spores post treatment using Flow cytometry. Flow cytometry settings and dye protocols were optimised using *Bacillus cereus* vegetative cells and spores.

Spores extracted from pollen were divided into 200 µL aliquots of $1x10^6$ cells/mL and treated with PUV for 150s at 1p/s the same as *B. cereus* cells. They were then analysed using Flow cytometry to determine the effectiveness of PUV. Results showed a slight but significant difference in PI+ cells, between the control and PUV treated spores extracted from pollen. However, it was the control population that had taken up significantly more of the viability dye. It was envisaged that any effects attributed to PUV would have shown the opposite, with a higher percentage of PI+ cells being observed in the PUV group. It is possible that the spores extracted from commercial pollen were more robust than *B. cereus* spores. These spores extracted from pollen were much larger in size than the bacterial spores and it is possible that a shielding effect is also helping to protect them. It doesn't help explain though why a higher percentage of PI+ cells was observed in the control group compared to the PUV treated group. The filtration allows for any organism smaller than 5µm to pass into the liquid medium. This could be suggestive of excessive DNA damage in the PUV population which is why a fluorescent signal is not being seen. Propidium iodide needs DNA to stain, so a signal can be detected, if there is no DNA present there is no DNA to stain and therefore no signal to be detected. While samples viewed immediately after extraction were predominantly spores, excess samples stored in the fridge displayed agglomerated growth 7-14 days post storage. These could then be viewed using light microscopy highlighting many different

morphologies, which meant there was a vegetative cell presence being washed out along with the spores.

E-beam is traditionally used for sterilizing medical devices (Silindir & Özer, 2009), it uses a beam of electrons to sterilize and has potential for use in the fresh food industry (Marin-Huachaca *et al.*, 2002). It was therefore a candidate technology to examine its potential as method of sterilizing fresh pollen. Once isolated spores are diluted to concentrations of $1x10^6$ cells/mL and placed in 15 mL plastic tubes. Treated samples were hit with a 100KGy dose of e-beam and then processed for Flow cytometry by incubating with viability dye PI and permeability dye SYBR safe.

Determination of effectiveness of e-beam was based on the theory that damaged or dead spores would take up the viability dye PI. The second assumption was that the dominant species present in the sample were spores, this was tested by using permeability dye SYBR safe. Control samples showed higher percentages of SYBR+ cells compared to e-beam treated spores. This indicated that there was some level of vegetative cells present in the sample, however the majority were spores. Microscopic analysis of the spores determined *Nosema* species as the most likely candidate, either *N. apis* or *N. ceranae* due to commercial pollen being collected by honeybees (Graystock et al., 2016). Differences in SYBR+ cells present could be down to e-beam treatment, with a destruction in part of vegetative cells present, leaving behind DNA too damaged for SYBR to adhere to. If so it would still be considered low for the high dose (100KGy) that was given. The mean overall percentage value for SYBR+ cells is similar to the mean of PI+ cells taken up by e-beam samples 10.49%, so it's very possible that the 2 populations are the same, vegetative and non-viable. This might also correlate to the control populations, but if it does that would mean that what is being seen is a population that was damaged or destroyed at some stage during the experimental analysis process. There was no statistical difference between either PI+ incubated cell populations, control or e-beam. This could be in part to a number of reasons, 1) a longer incubation time is required, 2) the treatment had no effect or 3) the dyes selected are not most suitable for determination of viability of these spores. It was possible to determine using PI the difference between *B. cereus* viable and non-viable spores, however the suspected fungal spores appear more robust than their microbial counterpart.

Effectiveness of the e-beam treatment is based on the amount of PI taken up by the spores, in this case the percentage of PI+ cells from the e-beam treated group was lower than the control, which indicates that whatever effectiveness e-beam might have had, it was not being detected through Flow cytometry. One difference to note was a higher cell count in the control compared to the e-beam treated PI percentages, but this was also not significant. However, in order to assess the effects of e-beam treated pollen on bee health, commercial pollen was treated with the same 100KGy dose of e-beam that the microbial samples were. The pollen had all been pooled and a 1 kg sample treated in Steris. Post exposure it was kept refrigerated in a separate box alongside untreated control samples. Control samples began to develop fungal growth a few months after refrigeration, which continued until its disposal 9 months post colony experimentation. Pollen that had been treated with e-beam did not, which does seem to suggest that whatever fungal organisms were present within the pollen were being inactivated by the e-beam dose. It is therefore possible that early perceived effectiveness of e-beam using viability dye PI was based off analysis of vegetative cells. There was a time delay between sample delivery and sample sterilization upon arrival at Steris and that time delay varied based on how busy the company was during that period. Later studies carried out by researchers with TUS discovered that PI does not penetrate the spore coat. This finding does add weight to the possibility that the PI+ population within the e-beam treated samples that formed the basis of the dose determination were in fact a mix of vegetative and spores.

This would seem to be backed up by visual evidence of the pollen itself post treatment. Future work could focus on optimising a Flow cytometry staining protocol for diagnostic purposes to determine the viability of spores. This would help inform future decision making better regarding the lowest possible dose of e-beam required for spore destruction.

2.5 Conclusion

PUV shows promise as a sterilization method for vegetative bacterial cells in liquid medium at low volumes, however it most likely lacks the penetrative power to inactivate more robust microorganisms such as fungal spores. The use of e-beam at a dose of 100KGy showed no significant ability to inactive fungal spores based on analysis using Flow cytometry, which is possibly limited at determining the effects of either PUV or e-beam due to the unsuitability of the DNA staining viability dye PI. This seems to be reflected by the lack of fungal growth on e-beam treated pollen 9 months post exposure compared to control pollen that exhibited high amounts of white fungal growth. The use of colony counts to determine viability for *B. cereus* cells after PUV treatment was an effective visual to determine effectiveness. Unfortunately, this was not an option to determine viability for suspected fungal spores extracted from pollen. This meant relying on Flow cytometry to determine effectiveness. Flow cytometry was inconclusive and perhaps a cell line that allowed for the growth of vegetative cells from suspected fungal spores might be a more reliable visual determinant. Overall, it would appear that e-beam was effective at inhibiting fungal species that reside inside commercial pollen at a dose of 100kGy, but more work needs to be carried on staining for flow cytometry optimization if it is to be used as a diagnostic tool.

Exploratory doses of e-beam were based on previous studies and decisions were made based off limited results using that dose range. E-beam irradiation of spores extracted from commercial pollen only showed signs of increased PI+ spore populations at a dose of 25kGy. Due to the onset of a global pandemic, access was restricted to Steris for both health and safety reasons and due to a backlog of orders from clients. Steris is the only e-beam treatment plant in Ireland, so adjustments had to be made in order for them to accommodate the project. For this reason, they granted the use of a singular dose only. The dose chosen was based on a very limited study and was the dose believed to generate the maximum effect in terms of reducing the viability of pollen borne spores. No upper limits have been determined for the use of e-beam with doses such as 44kGy used to irradiate food going into space to feed astronauts (Bhatia *et al.*, 2018). It was also decided prior to studies involving bumblebees that a singular dose would be chosen to reduce the number of colonies that would ultimately be sacrificed post experimentation. Had the pandemic not occurred a greater number of

experiments using a dose range from 1.5kGy-100kGy might have generated a greater insight into what lower doses might be viable for the treatment of commercial pollen. It does appear that a 100kGy dose of e-beam is successful at preventing microbial activity within pollen and the effects of this are explored in chapters 3-5.

Chapter 3 The effects of treated pollen on reproductive output in the bumblebee *Bombus terrestris*

3.1 Introduction

Bees require a diet of carbohydrates, lipids and proteins in order to carry out their basic everyday functions. Bumblebees need a wide variety of flowering plant species to meet their required nutritional intake (Becher *et al.*, 2018) while it has been shown that bumblebee species such as *Bombus impatiens* a protein to lipid ratio of 5:1 from the pollen they forage is required (Vaudo *et al.*, 2018). Nectar is a source of sugar and carbohydrates and is used to generate ATP for flight and other metabolic activities (Stabler *et al.*, 2015). The proteins and lipids come from the pollen (Annoscia et al., 2017a; Tarpy et al., 2018) which in the wild can vary in nutrient content (protein in pollen varies between 2.5 and 61%) (Alaux, *et al*., 2011) and also in availability. It also provides minerals, vitamins and flavonoids (Annoscia *et al.*, 2017a). *B. terrestris* colonies have been shown to grow quicker when there is a good diversity among floral species present (Vaudo *et al.*, 2018).

A nutritionally rich pollen diet is essential for the bees in order to mount a successful fight against, parasites, microsporidia, bacteria and viruses (Alaux *et al*., 2011; Huang, 2012). Pollen is digested by bees in the midgut. Protein in the pollen is broken down into amino acids which are needed for further protein synthesis in the bee. Vitellogenin (vg) is synthesized in the fat body and protects bees from oxidative stress. Unsaturated fatty acids found in pollen are thought to be essential to fighting infection and have been reported to have antimicrobial activity. Pollen rich in unsaturated fatty acids has been shown to mitigate the negative effects of the mite *Varroa destructor* and help reduce viral burden (Annoscia *et al.*, 2017).

Bumblebee species such as *B. terrestris* have demonstrated similar abilities to negate the effects of parasites when given a high protein diet in the form of pollen. Antimicrobial peptides (AMPs) were upregulated in *B. terrestris* fed on protein rich pollen, when challenged with the gut parasite *Crithidia bombi*. *C. bombi* is a parasite that lives in the gut of *B. terrestris* and is spread through the colony by oral uptake of infected faeces and can affect the queens ability to start a new colony after hibernation (Brunner, Schmid-Hempel and Barribeau, 2014).It also affects learning ability, foraging ability and increases mortality when colonies

come under stress. Sunflower pollen was also shown to have beneficial effects when it comes to reducing loads of *C. bombi* and *Nosema ceranae* infections in bees. *N. ceranae* is an intracellular pathogen that affects honeybees and has been linked to colony collapses. However honeybees fed on sunflower pollen had a 4 times greater mortality rate when compared to those fed on buck-wheat pollen, suggesting a trade-off between immunity and lifespan (Tarpy *et al.*, 2018).

In Ireland the majority of crop and wild flower pollination is carried out by wild bees, and these wild bees are facing issues such as food shortages and habitat loss (National Biodiversity Data Centre, 2009; National Biodiversity Data Centre, 2015). Intensive agriculture has had a negative impact on bee survival in many ways. Bumblebees have been shown to develop a preference for neonicotinoids(Arce *et al.*, 2018) and these pesticides have been detected in pollen and bee bread (Tong *et al.*, 2018). These pesticides then have an opportunity to synergize with pathogens which can lead to immune suppression and low sperm counts (Peso *et al.*, 2018). This, along with a decline in wild flowers due to habitat loss has resulted in reduced availability of pollen which is contributing to the decline of bee populations. The dominance of a cropland environment also reduces the amount and variability of floral pollen sources as well as nesting sources and also has been shown to impact on pollen delivery.

While the overall objective of this project is to prevent bee declines, there is a growing body of evidence that would suggest commercial bees are also negatively impacting on native bee populations. Commercial bees are marketed as pathogen free, however this is not always the case. Studies carried out by researchers in England found that both commercial bees and pollen were carrying pathogens (Graystock *et al.*, 2013). Parasitic species such as *Apicystis bombi, Crithidia bombi, Nosema bombi, Nosema ceranae* and deformed wing virus were all found in commercially produced bumblebees, while *Nosema apis, Paenibacillus larvae* and *Ascosphaera* were three additional species detected in the commercial pollen (Graystock, Yates, Sophie E F Evison, *et al.*, 2013).

In addition pathogen spillover can occur between commercially reared bees and their native counterparts. This generally occurs when commercial and native bees forage in the same

areas, with visited wildflowers acting as a parasite hotspot. Both these flowers and the commercially reared bees themselves act as reservoirs of pathogen dispersal flowers. These are then picked up by wild bees and brought back to their colony or hive (Graystock *et al.*, 2015).

Separate studies carried out in both Canada and Ireland found that *Crithidia bombi* and *Nosema bombi* were also more prevalent in wild bees near commercial greenhouses and that this lessened the further away the bees were captured from the greenhouses (Murray et al., 2013; Colla et al., 2006). These findings highlight the issues that arise with the use of commercial bees, and the real need to tackle this problem to try limit pathogen spillover.

While government agencies regulate for the importation of non-native species of bee to prevent the introduction of disease as seen with the ban on importation of *B. terrestris* by the United States (Winter and Adams, 2006), it is clearly only part of the problem. Commercial bees have repeatedly been shown to compromise pathogens and parasites, as has commercial pollen (Graystock et al., 2013) which can have an impact on native bees. What is currently unknown is whether the bee, or the pollen represents the main source of disease as it is possible that the commercial bees are initially pathogen free and that the pollen they are fed is the source of the contamination. Pollen is collected by honeybee foragers and gets caught in traps placed at the front or underneath the hive entrance. The traps are designed in such a way that large pollen is scraped from the foragers legs as it passes through the mesh design. Pollen gathered here can then be sold on to the commercial bee industry (Hoover and Ovinge, 2018). Pollen collected by honeybees is then used to feed commercially reared bumblebees and therefore increases the risk of pathogen transfer (Meeus et al., 2014**;** Graystock et al., 2016)**.**

The pathogen/parasite contamination of pollen used to feed commercial colonies has been recognised as a potential issue and attempts have been made to address this. Various studies have compared sterilisation techniques (Simone-Finstrom et al., 2018), with gamma irradiation at present offering the best possible solution. However this latter approach is currently deemed far from a perfect practice (Graystock et al., 2016). Gamma has been demonstrated to reduce the infect ability of RNA virus Israeli acute paralysis virus (IAPV) at a concentration of 16.9KGy which was enough to prevent oral infection, however how this would work in a real world situation was not possible as they did not know the concentration

of viral particles within Honeybee pollen (Meeus *et al.*, 2014). Gamma irradiation up to 10KGy has been demonstrated to change no physiochemical properties within the pollen, but did cause a minor colour change and doses of 7.5KGy reduced bacteria, molds and yeast (Yook, Lim and Byun, 1998) Gamma irradiation can inactivate *Nosema ceranae, Ascosphaera apis* and Deformed wing virus*,* which makes it a highly powerful tool to help prevent pathogen spill over from contaminated pollen. However it can only partially reduce infectivity of Black Queen cell paralysis virus. This still offers a promising partial solution to reducing some of the stresses bees' face (Simone-Finstrom et al., 2018).

In separate studies by (Graystock et al., 2016) it was found that a 16.KGy dose of gamma could remove Deformed wing virus, Israeli acute paralysis virus, Sacbrood virus and *N. ceranae* and rendered *C. bombi* and *Ascosphaera* non-infectious. It was unable to deactivate *A.bombi*. Gamma has probably to this point presented itself as the best option for pollen sterilization, however a few issues remain. It doesn't inactivate all the pathogens and viruses present, it can't be done *insitu* and the procedure is time consuming, for example 9 hours and 45 minutes exposure at 25kGy of gamma irradiation (Simone-Finstrom et al., 2018).

It is important that other technologies for the sterilization of commercial pollen are explored. Such technologies must be cost effective, efficient, scalable and significantly reduce the microbial burden of pollen and therefore would be more likely to be adopted by the commercial bee industry. This industrial level sterilization of pollen would ensure that commercial colonies are healthy, disease free and pose less of a threat of pathogen spillover into wild communities. One technology with significant potential is e-beam an approach commonly used for the sterilization of medical devices and drugs (Silindir and Özer, 2009). This involves using an accelerator to generatean electron-beam and signifocant reductions in microbial load with very little exposure time (Silindir and Özer, 2009). It has also shown efficacy at reducing *Bacillus* spores in powdered infant milk formula (McFadden *et al.*, 2016).

A specific dose of e-beam to treat commercial pollen was used, with the aim of reducing microbial loads within the pollen. The dose chosen was 100kGy and this was based on prelimiary results obtained using a range that went from 1.5kGy – 100kGy. Using Flow cytometry as an analytical tool it was determined that a dosage of 25kGy was only beginning to induce death of spores extracted from pollen (Chapter 2). A global pandemic prevented us from obtaining more results that might have helped define a smaller dose that could have

been effective. For doses of 1.5, 5, 15 and 25kGy it was possible to carry out repeat tests. However doses of 50, 75 and 100 were only varied out once. This limited our knowledge on what was the lowest dose that could be used to achieve the maximum benefit and a decision was made to go with what appeared to the most effective dose 100kGy.

Pollen was obtained from a commercial supplier and divided into 3 tubs. One was subdivided and sterilized in Steris and transported to NUIM. The other two would be used as the control and the other would be filtered. Bees were then fed on a diet of either untreated control pollen, e-beam treated pollen or filtered pollen for 30 days. This was suplemented with 30w/v sucrose solution.

The aim of this chapter was to determine the impact of feeding e-beam treated pollen on *B. terrestris* and the individual and colony level. Sterilised pollen was provided to colonies as the main pollen source and compared to colonies fed on untreated pollen. In addition it was also decided to assess the effects of washed pollen (filtered) on *B.terrestris* colonies, the development of which is described in the materials and methods section. The impact on colony reproduction and output was determined by i) measuring the colony weight; ii) counting the number of egg clumps, larvae and pupae produced; iii) counting males and females; iv) measuring the relative size of individual bees and assessing brood colouration. In addition the microbiome of representative bees from each colony was conducted using Amplicon sequencing for the Internal Transcribed Spacer (ITS) region and 16S which permitted the detremination of fungal and microbial profile respectively; (v) Mass spectrometry to determine any changes in th eprotein profile of the colonies.

3.2 Materials and Methods

3.2.1 E-beam pollen sterilization

Untreated pollen was sourced from a commercial pollen company in the Czech Republic and shipped to TUS labs on ice. Upon arrival it was stored at -20^{o}C until it was needed as per instructions laid out by the company. It was divided into 3 lots of 1 Kg per lot.

For treatment via e-beam the pollen was weighed out into equal measures and placed in clear see-through takeaway boxes 17 x 11.5 x 5.5 cm. 1 Kg on total of pollen was sterilized using an accumulative dose of e-beam totally 93.9KGy on site at Steris Tullamore. A total of 20 boxes containing 50 g of pollen each was sterilized. Each box had the lid sealed with parafilm prior to treatment and was transported to and from Steris on icepacks in a polystyrene box.

Treated samples were brought to NUIM, all boxes were opened and the pollen was all placed into a singular container and stored in the fridge at 4^{o}C.

3.2.2 Filtered (washed) pollen

Untreated pollen was washed by mixing fresh pollen with sterile water at a ratio of 1:10. The mix was homogenised through physical stirring and the filtered through a Buchner funnel using 5 µm Whatman filters. The pollen was then removed from the filter paper and blotted dry using standard Whatman filter paper until a large quantity of the excess water was removed. The filtered pollen was then transferred to a clean transparent box and stored at 4^{o}C in a clear see-through takeaway box. Filtered pollen was made up on average 2-3 times a week.

The filtered liquid was examined under light microscope conditions and were found to be predominantly spores. These spores were enumerated using haemocytometer counts and treated with PUV or e-beam as described in chapter 2.

3.3.3 Untreated (control) pollen

Untreated pollen sourced for Czech Republic was stored at 4^{o}C for the duration of the colony experiment.

3.3.4 Colony set up

To test the effects of treated pollen on commercial bees, e-beam technology was chosen. After treated pollen was brought back to the lab, the samples were pooled and stored in a singular box in the fridge for the duration of the experiment. Untreated pollen was homogenised with sterile water and filtered in order to move microbes in the region of $1x10^{6}$ cells/mL. Bees were fed on either control (untreated pollen), e-beam treated pollen or filtered (washed) pollen.

Bumblebee (*Bombus terrestris*) colonies were sourced from Agrilan UK and collected from Parcelforce depot in Belfast and transported to NUIM, Maynooth.

The first week the colonies were fed daily with 4 g of pollen and 8 g of pollen for each week after. Observations were made on each colony every second day, for a total of 30 days. The number of egg clumps, larvae and pupae were recorded during these observations and the weight recorded once every week.

Colonies 1-4 were fed untreated pollen and served as the control, colonies 5-8 were fed the same commercial pollen that had been treated with a 100KGy dose of e-beam, while colonies 9-12 were fed on washed pollen (filtered). All colonies were fed on pollen that had come from the same batch, and washed pollen was made up every three days, using sterile water and a 5µm filter paper.

After 18 days had elapsed, callows produced from the colonies were taken and tagged using a colour coded numerical system and returned. These bees were then extracted and snap frozen in liquid nitrogen 14 days after and stored at -80^{o}C.

Samples of snap frozen bees were later subjected to gut extractions and DNA extracted from the gut. These samples were then sent to Novogene for ITS sequencing and 16S sequencing to determine the microbial presence in the microbiome.

On day 30 the experiment was terminated. Colonies were placed into the -20^{o}C to induce sleep and eventual death to each colony.

Each colony was subsequently examined and typed for sex, intertegular distance measured and thorax width measured.

3.3.5 Thorax and intertegular distance measurement

Post 30 day experimentation colonies were euthanized and examined. Each individual colony was then accessed for sex, intertegular distance and thorax width.

Differences between male and females were observed on the basis of the presence or absence of a stinger. Female bees have a stinger, males do not. Queens or gynes were observed based on size, these bees are much larger than workers or nurse bees.

Intertegular distance was recorded using an electronic calliper by measuring the distance between where the wings attach to the thorax. The thorax was measured using an electronic calliper to record the width.

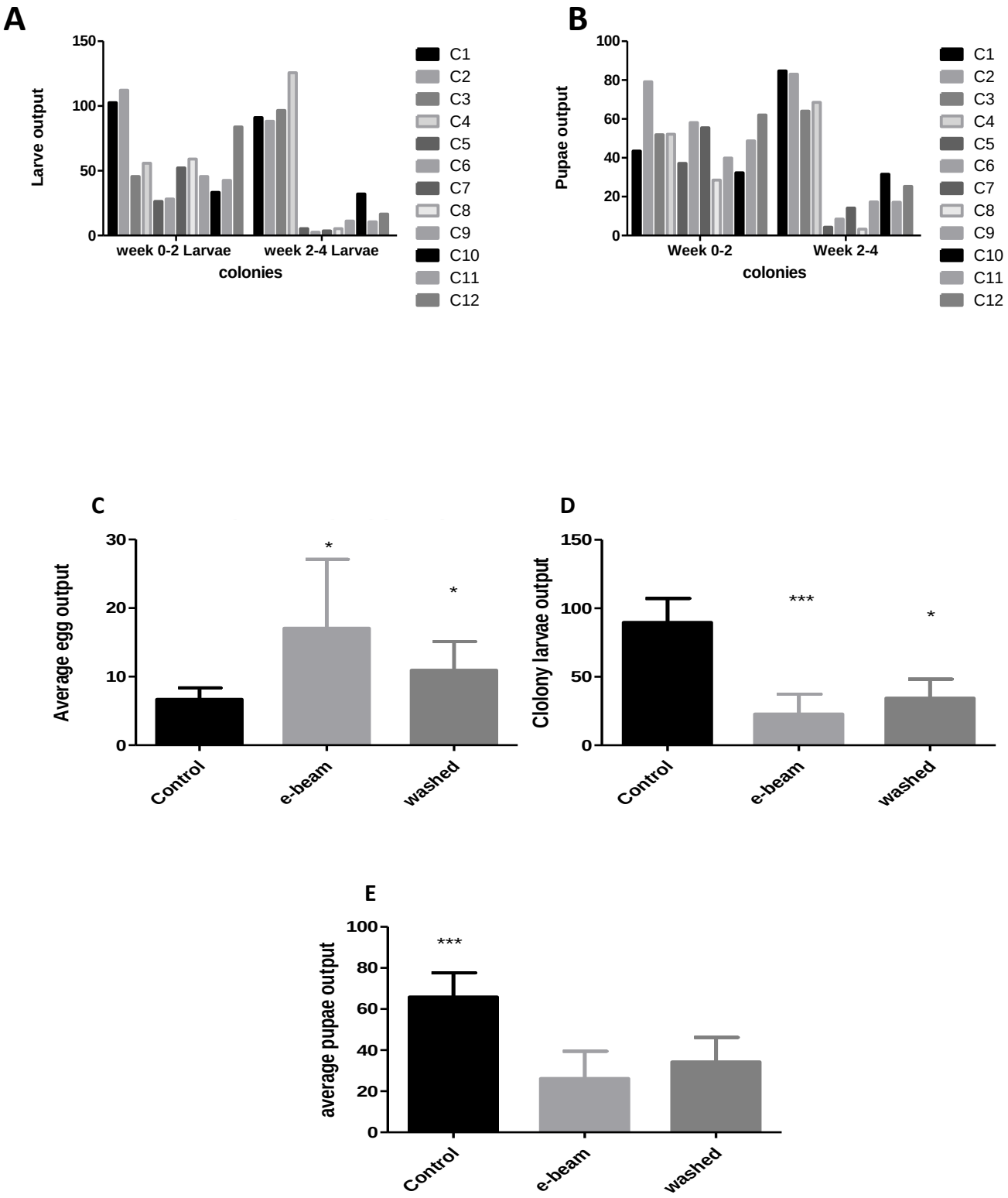
Bees scattered on the outskirts of the colony that were decomposing were denoted as dead and were not typed for sex or had any measurement carried out.

3.3 Results

Reproductive output

Bumblebee colonies were observed every second day for changes in brood development with the changes in eggs clumps, larvae and pupae numbers noted and recorded. This was done through visual observations and counting and carried out with limited lightening to avoid stressing the colonies. Colonies were kept in a dark room and counting was done in red light conditions.

Fluctuations in numbers were observed in all colonies, however a reduction in the number of larvae and pupae being produced in both treated colonies was noted after two weeks (figure 3A&B). This coincided with a change in colour in the eggs, larvae and pupae already present. These colonies failed to recover in terms of pupae output, with eggs still being produced, but failing to develop into larvae. Control colonies continued to produce eggs, larvae and pupae up to the experiment endpoint, with no note in colour change observed in these colonies.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colonies
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C4
C5
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C7
C8
C9
C10
C11
C12
B
Pupae output
100
80
60
40
20
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Week 0-2
Week 2-4
colonies
C1
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C4
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average pupae output
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Control
e-beam
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Figure 3 Colony reproductive output: A decline in the number of Larvae produced by both treated colonies compared to control was noted after week 2 (A), this was accompanied by a decline in the number of pupae being produced by both treated colony populations compared to control groups (B). Both e-beam and filtered groups produced significantly more eggs on average ($P \leq 0.05$) than control groups (C), however control groups produced significantly more Larvae when compared to e-beam ($P \leq 0.001$) and filtered ($p \leq 0.05$) (D) as well as significantly more pupae ($P \leq 0.001$) (E)compared to both treated groups. Colonies were analysed using a one-way ANOVA repeated measure test.

Intertegular distance and Thorax width

After colony experiment endpoint each colony was examined and each individual bee was extracted and was typed for sex, with intertegular distance and thorax width measured using a digital calliper. Bees that had died during the experiment and were already decomposing were not measured, but were included in the overall colony count and denoted as dead. The measure of intertegular distance is a good indicator of bee size (Cane, 1987). Body size can influence a range things such as geographic range, metabolic rates, growth rates and dispersal ability (Kendall *et al.*, 2019). A significant difference was noted in the intertegular distance and thorax width of both treated bee groups when compared to bees fed on non-treated pollen ($P \leq 0.001$). A significant difference was also noted between e-beam treated colonies and washed colonies in thorax width ($p \leq 0.05$), but not intertegular distance.

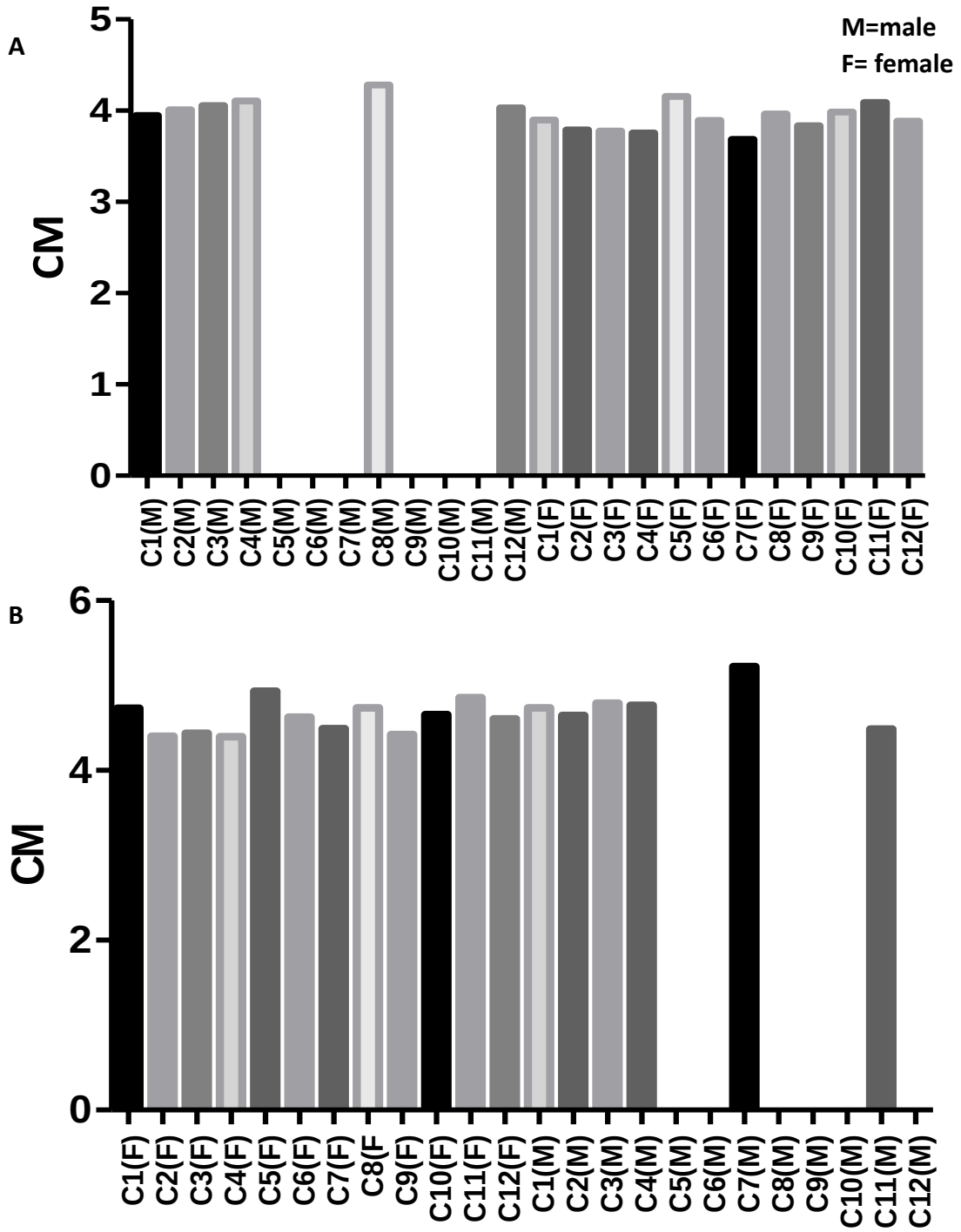


Figure 3.1 Intertegular distance and thorax width of bumblebees: (A) depicts the average intertegular distance of each individual colony (1-12) male and female (A) and average Thorax width (B) of each individual colony male and female. Only 2 colonies in the treated groups produced males colony 8 (11 males) in the e-beam group and colony 12 (2 males) in the filtered group. All four control groups produced males with colonies 1 and 2 producing more males than females (237:148 & 284:204).

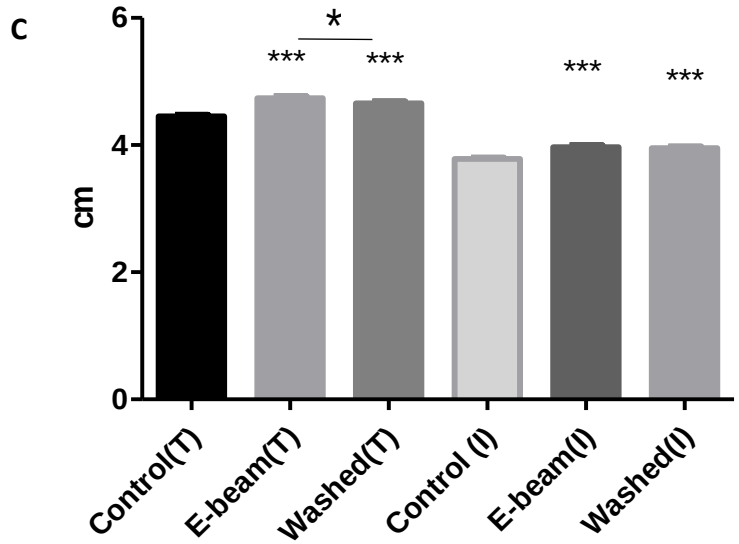


Figure 3.1 (C): shows the overall average measurement of intertegular distance and thorax width of female bees only (C) per treatment. There was a significant difference in both intertegular distance and thorax width between control and both treated populations in female bees ($p \leq 0.001$) and a difference between thorax widths E-beam treated and washed pollen treated colonies ($p \leq 0.05$). Analysis was carried out using a paired T-test and a one way ANOVA Tukeys test.

Colony numbers and sex

Colonies were examined post experiment and typed in terms of males and females with a few exceptions. Bumblebees that had died during the course of the experiment were included in the overall count, but were not typed in terms of sex due to the poor condition of their bodies. Female bees that were deemed to be larger than average were denoted as gyne/queen, these were seen mainly in the control colonies.

Male bumblebees also largely failed to emerge from the treated colonies with one or two exceptions in a few colonies producing extremely low numbers of males. Control groups produced males in all 4 colonies with ranges from 160 - 284 and females ranging from 102 - 231. When comparing control groups to e-beam treated groups, these colonies produced females ranging from 81- 110 and males ranging from 0-11, with only a single colony producing males. There was a similar pattern in the filtered group with only a single colony producing males (range 0 – 2) and females ranging from 127 – 212. Larger than average bees

were denoted as queens or gynes, each colony had 1 queen and any other bee of similar size within that colony was labelled as a gyne. In the control group three of the four colonies produced gynes with a range of 4 – 30. In the treated groups only filtered managed to produce a singular gyne, which was found in colony 11. Overall control colonies produced more gynes/queens, male and female bees when compared to treated groups. In total control groups produced 781 females, 856 males and 39 queens/gynes, this is compared to e-beam producing 379 females, 11 males and 4 queens/gynes and filtered producing 661 females, 2 males and 5 queens/gynes. There was a significant difference between the number of males produced in control groups compared to both treated groups ($P \leq 0.001$) and also a significant difference in the number of females produced between control groups and e-beam groups ($p \leq 0.01$) and filtered groups ($p \leq 0.05$).

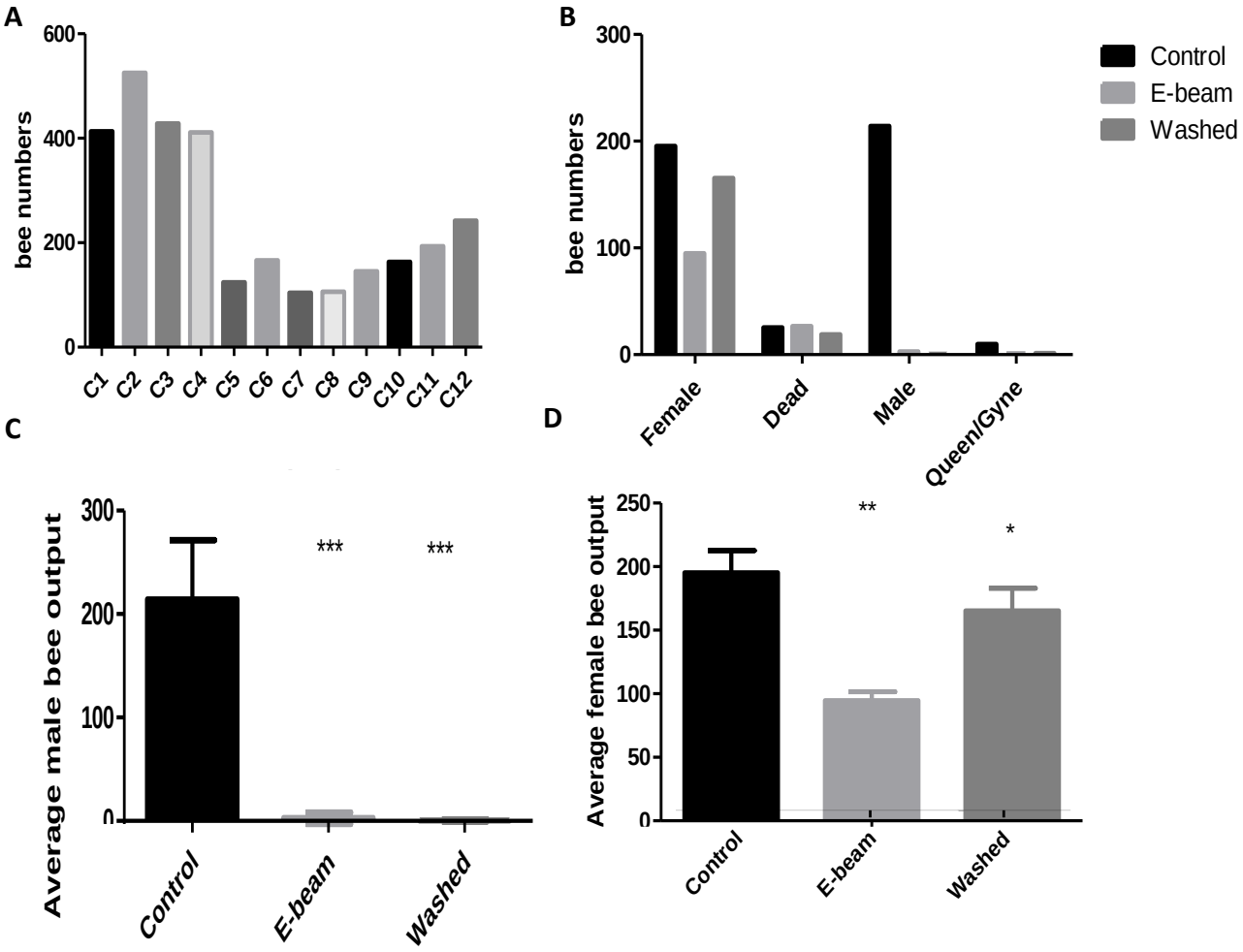


Figure 3.2 Differences in sex and colony numbers per treatment group: The differences observed in terms of individual colonies total bee counts (A) and the categories they were divided into via treatment (B). Treated colonies as a whole failed to produce male bees(C), whereas control colonies produced more males compared to females. A significant difference was observed for the number of males and females produced per treatment compared to the control. A difference was noted between the numbers of females produced by washed colonies compared to E-beam treated colonies, but no difference was noted for male populations, between the treatments. This was analysed using a one way ANOVA, Tukeys method.

Colony weights

Upon setup each individual colony was weighed to determine the baseline colony weight. Each colony was then weighed at specific time points over the 28 day period and the weights recorded. All colonies had a starting weight between 0.572 kg and 0.593 kg. Over the course of the 28 days all colonies gained weight, with some gaining more than others.

There was no significant difference between the weights gained by any of the treatments, however the control colonies as a whole weighed 50% more than the washed colonies and 25% more than the e-beam treated colonies. The e-beam colonies also weighed 35% more than the washed colonies, with washed colonies failing to gain the most weight as a whole. The control colonies gained the most weight almost doubling, with e-beam putting on 0.3 kg over the course.

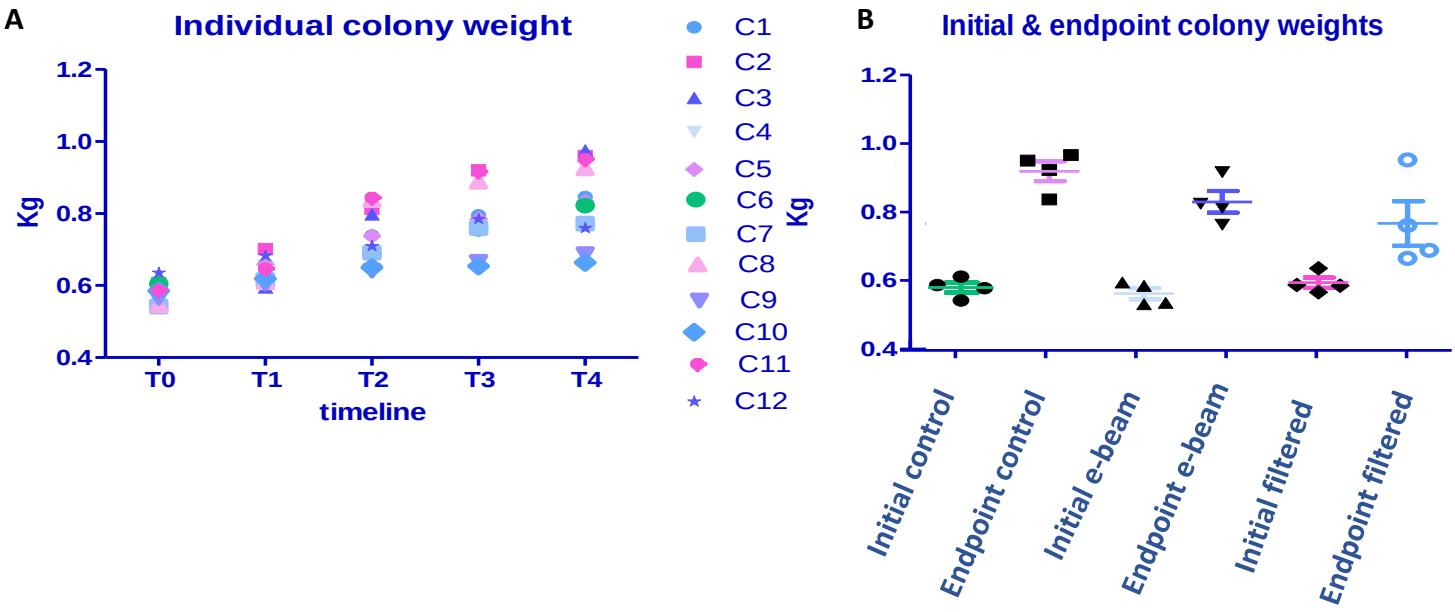


Figure 3.3 colony weights recorded over time, and treatment: The weights gained by individual colonies over the course of the time line (A), each colony is represented by its own individual key C1-C12. The initial baseline weight per treatment and endpoint weight are displayed in (B) for each individual colony per treatment. There was no significant difference recorded using Tukeys one way ANOVA.

3.4 Discussion

Pathogen spillover from commercial bee stocks into wild communities is a leading cause of concern, with both the bees themselves (Pereira et al., 2019) and the pollen they are fed on (Graystock, Yates, Sophie E F Evison, *et al.*, 2013). With bee communities in decline globally (Fitzpatrick *et al.*, 2007) with Egypt losing half its honeybee colonies between 1990 and 2018 (Al Naggar *et al.*, 2018). The decline in insect pollinators is a multi-faceted story with habitat loss (Fitzpatrick *et al.*, 2007) and pesticide use (Yang *et al.*, 2019) also implemented as causative agents in bee decline. In an effort to try reduce pathogen spillover, the focus of the study was on the sterilization of pollen using e-beam irradiation a technology traditionally used in medical appliance sterilization (Mine and A. Yekta, 2009).

Pollen sourced from a commercial supplier in the Czech Republic was treated with a dose of e-beam (100KGy) and then stored at $4^{O}C$ for the duration of the experiment. In addition to this two separate groups were set up, a control group that was fed on the same pollen only untreated and a third group that was fed untreated pollen that had been filtered through a 5µm filter to remove large quantities of spores present in the pollen.

Bumblebee colonies were fed over a 30 day period 8g of either untreated pollen, e-beam irradiated pollen or washed pollen and supplemented with a continuous supply of 30%w/v sucrose solution. A number of colony outputs were measured over the duration of the experiment. The measurements were colony weight and reproductive output. The number of eggs, larvae and pupae were recorded every second day while colony weight was recorded once a week. In total each group consisted of four individual colonies, with a total of twelve colonies being monitored.

Bees fed on treated pollen either e-beam treated or filtered displayed a poorer reproductive output compared to colonies fed on untreated controls, producing less larvae, pupae, both females and males along with fewer queens.

Observations at early time points indicated that there was no difference between treatments in terms of reproductive output. All twelve colonies produced similar levels of eggs, larvae and pupae. At 12 days however a change in the overall colouration of eggs, larvae and pupae

fed on treated pollen fed (e-beam & filtered) was observed within the colonies. After this point in these colonies a failure to produce new larvae and pupae was observed highlighting a major impact of the filtered and e-beam treated pollen on the colony. Although continued production of egg clumps was observed, their viability could not be ascertained and their failure to become larvae would suggest that they were not viable. Control colonies did not produce as many eggs as either e-beam or filtered groups, however this was most likely due to eggs that were produced in control colonies developing into larvae and eventually pupae. This would explain why more eggs were observed in both e-beam and filtered groups as eggs that failed to develop were still being counted along with any new eggs that were produced. Control colonies also produced significantly more larvae than bees fed on e-beam treated pollen ($p \leq 0.001$) and filtered pollen ($p \leq 0.05$), and significantly more pupae than bees fed on both e-beam treated and filtered pollen ($p \leq 0.001$). This is the first sign that both treated pollen types were negatively impacting on colony development.

Control colonies showed no signs of decline in terms of reproductive output right up to day 30, with callows still being observed in the colonies. Control colonies also produced more female and male bees compared to treated colonies, with both e-beam and washed (filtered) colonies failing to produce males with the exception of a few colonies. Overall treated colonies produced just 13 males (11 e-beam & 2 washed), compared to 856 males in the control group. There were also more males than females in the control group. The treated colonies also failed to see the emergence of gynes, with on average just one queen per treatment compared to ten gynes/queens on average per control colony. E-beam treated colonies fared the worst in terms of female output producing half as many females as the control and only two thirds the amount of females as those fed on filtered pollen.

This further backs up the suggestion that a reduction in development occurred in terms of colony output for bees that were fed on either e-beam irradiated pollen or washed pollen. Male bumblebees (drones) are produced from unfertilised haploid eggs. The length of time it takes for a drone to develop takes on average 24-28 days and this may vary due to temperature, number of workers attending to the eggs and quality and quantity of nutrients available (Belsky, Camp and Lehmann, 2020). The primary role of drones is to mate, however they also contribute to thermoregulation of the colony. Honeybee drones will reach sexual maturation after 1 week and leave the colony to mate with virgin queens and

will then return to the mother colony. Honeybees will mate with a single queen whereas bumblebee drones will mate with multiple gynes and will not return to the colony once they've left. The major role of the bumblebee drone is to mate (Watanabe and Sasaki, 2022).

By contributing to raising the temperature within the colony they play a role in pupal incubation (Cameron, 1985) raising the temperature 4-6^{O}C inside the nest (Dill, 1991) , *B. griseocollis* carries out similar tasks to workers by raising the temperature which is crucial to pupal warming (Cameron, 1985; Belsky, Camp and Lehmann, 2020).

The loss of bumblebee drones in both e-beam and filtered pollen groups could potentially be down to diet. In both groups this was the only variable and even though both methods employed very different techniques, both aimed at reducing microbial bioburden within the pollen, it seems the removal of these microbes has had a very similar effect. A possible explanation is that these microbes contribute to the nutritional status of the colony as a whole and that colonies in a nutritional deficit lack the ability to produce eggs that can mature. It is most likely that bees in these colonies were under stressed conditions and were solely focused on survival and unable to attend to the eggs in order for them to develop into larvae, pupae and eventually mature bees. Not only was there a failure to produce males but both treated groups also produced significantly fewer females. Female bees act as foragers and nurses looking after the brood (Belsky, Camp and Lehmann, 2020) and if these nurses are stressed then it's plausible that they are unable to care for the brood properly and could be why a decline in reproductive output was observed. The larvae and pupae became discoloured after week 2, which could have hinted at a decay within the enclosures. It would seem to suggest that the altered pollen was not sufficient to raise a brood on and this played a major role in the decline of the colony and ultimately the failure of both groups to produce males.

Colonies that lose their queen have been shown to veer towards a male bias (Bourke, 1997), however this is not what was observed. In the control colonies where each colony produced more than one gyne, a bias towards drones was observed overall. The groups fed on treated pollen either e-beam or filtered had on average only one queen per colony and failed to produce male bees.

All colonies were weighed on day 1 of the experiment and at regular specific time points up until day 30 of the experiment. All colonies weighed approximately the same, which was a good marker as a baseline. It allowed us to see the direct effects of the various pollen samples on the colonies. All colonies gained weight however the greatest weight gain was seen in the treated experimental colonies. Filtered colonies put on the least amount of weigh with control colonies gaining almost twice the amount of weight in the same time period. Visual observations over the course of the experiment suggested that the bees from all three groups were eating the pollen so the failure of 2 of the groups to make the same weight as the control group, could be down to possible nutrient loss in both treatments, with a difference in the type of nutrient loss, varying between each of the treated groups. This does contrast however with the larger Thorax width and intertegular distance observed in both e-beam treated pollen colonies and filtered pollen groups. The most likely cause for the loss in weight is probably down to the failure to reproduce. These treated groups had lower amounts of female bees as well as producing no males, this would go some way to accounting for the lower endpoint weights seen.

There was a slight difference noted in both thorax width and intertegular distance between treated and control colonies that was deemed significant. In *Drosophila* downregulation of amino acid transporter gene Sobremes (Sbm) during the larval stage leads to an increase in body size with smaller brain lobes (Manière *et al.*, 2020). This could be a possible explanation here, however as brain sizes were not measured for the bumblebees in this study it cannot be said for certain. Male bees were only produced in the control colonies so therefore no comparisons could be made against control colonies. On average female bees in the control group shared a smaller Thorax and intertegular distance compared to both the treated groups. There was no difference recorded between either of the treated groups with treated groups matching almost identical on average in terms of intertegular distance and thorax width recorded. This might suggest that bees in treated colonies are on average larger than those in the control colonies. Given that the sucrose available was consistent across all colonies, the change could be down to the differences in pollen, however how it actually impacts is yet to be determined. If it is assumed both treatments reduced the number and type of pathogens present, perhaps this is contributing to the gain in size, however further testing would be required to confirm or deny.

3.5 Conclusion

Overall, both treatments had an effect on the colony in terms of weight, negatively impacting reproductive output and a failure to produce drones which would impact the queen founding a new colony. What's interesting is that both treatments despite being very different exerted similar effects on the groups. In the case of filtered pollen, microbes are physically extracted, these microbes were predominantly spores and were suspected to *Nosema* based on size and morphology. Other microbes were also extracted using this method and these could be visualized via agglomerated growth in the tubes they were stored in. Each filtered extract was stored at 4^{o}C in sterile water, so the growth was attributed to microbes coming directly from the pollen and these could be visualised using a light microscope a week after storage. It also suggests that some nutrients were also being leeched into the water during the filtration process in order to sustain growth. These microbes were believed to be fungal and yeast in origin.

E-beam treated pollen, showed no fungal growth 9 months post treatment compared to control pollen and these colonies exhibited an almost identical reproductive profile to that of the filtered colonies, which could suggest that the fungal microbes and bacterial species that lie within commercial pollen actually contribute in a positive way in the initial establishment of a colony and removal impacts negatively.

A future study might feedback the extracted bioburden from colonies fed on filtered pollen and compare it to colonies that are fed solely on filtered pollen to see if the extracted microbes are the source of the negative reproductive impacts.

Chapter 4 A proteomic assessment of the effects of sterilised pollen on the *Bombus terrestris* fatbody.

4.1 Introduction

The pollination of certain crops has become a multibillion dollar both in the USA ($14 B) (Reeves *et al.*, 2018) and Europe (€15B) (Commission, 2018). Commercial crop producers rely on pollinators such as bees to pollinate a variety of crops including blueberries, apples (Al Naggar *et al.*, 2018), almonds (Staveley *et al.*, 2014), strawberries, cucumbers, rape and tomatoes (Commission, 2018; Murphy & Stout, 2019) with the latter being predominately pollinated by commercial bumblebees (Department of Agriculutre, 2018). Bumblebees are seen as the second most important commercial pollinator behind the managed honeybee with 1 million colonies being exported globally for pollination purposes annually. Out of the 57 countries that commercial bumblebees are sold in 16 are outside its native habitat (Ings *et al.*, 2010). They are seen as more efficient pollinators (Goulson and Hughes, 2015). Bumblebees are fed on commercial pollen which is sourced from honeybees (Pereira, Meeus and Smagghe, 2019). This commercial pollen however has been found to be contaminated with a variety of parasites and viruses which can impact negatively on bee health (Graystock, Yates, Sophie E.F. Evison, *et al.*, 2013). One study found evidence of the bumblebee parasite *Apicystis bombi* in commercial pollen (Pereira, Meeus and Smagghe, 2019), which is an area of concern as pathogen spillover from commercial colonies into wild communities is becoming a common issue (Murray *et al.*, 2013).

Commercial pollen is a source of pathogen contamination (Graystock, Yates, Sophie E F Evison, *et al.*, 2013) which can contribute to the spread of disease into wild communities, a process known as pathogen spillover (Murray *et al.*, 2013). Efforts have been made to reduce the pathogen load of the pollen and the potential for spillover, with the primary method used commercially being irradiation of pollen (Graystock et al., 2016) and also the reduction of some viruses (Meeus et al., 2014). Gamma irradiation has been shown to inactivate some fungal species such as *Nosema ceranae* and *Ascosphaera apis* as well as deformed wing virus (Simone-Finstrom *et al.*, 2018).

For this project alternatives to gamma irradiation, such as PUV technology, which can be housed *in-situ* and e-beam were investigated. Although PUV was quick and effective at inactivating vegetative *Bacillus* cells, it was unable to penetrate the coat of the fungal spores extracted from commercial pollen. E-beam treatment of spores showed more promise (Chapter 2) so commercial pollen obtained from a supplier was treated to an accumulative dose of 100KGy on site at Steris Tullamore. This was later fed to the bees over a period of 30 days to determine if there were any health benefits to be obtained including pathogen inactivation or load reduction.

Two separate treatments were examined against control colonies. Treatment one involved pollen subjected to an accumulated 100KGy e-beam dose, while treatment two extracted the microbial community including fungal spores from the pollen by way of *in-situ* filtration. This was carried out in the lab, by weighing out set amounts of pollen, mixing it with fixed amounts of sterile water and filtering the microbes out through 5µm filters. The pollen was then blotted dry and fed back to the bees. The control group was fed on commercial pollen that was not treated and all three pollen types were stored at $4^{O}C$ for the duration of the 30 day project.

Using visual observations over the course of the 30-day period it was possible to gather data relating to the reproductive output of each individual colony, the weight, sex and body size of each individual bee (see chapter 3). In both treated groups, those fed on e-beam treated pollen or filtered pollen reproductive output was down compared to the control group. These groups also failed to produce gynes and drones, while overall colony weight was also reduced. The evidence was suggestive of a negative impact on colonies fed on both types of treated pollen. In order to investigate whether the quality of pollen is affected by e-beam or filtration and causes any changes to the bumblebee at the molecular level, mass spectrometry (MS) based proteomics was conducted. In addition, the effects of pollen on the microbiome of bees fed treated and control pollen was assessed by DNA amplicon sequencing (chapter 5).

Snap frozen bees had their fat bodies extracted in order to determine if there had been any changes to the colonies between the treatments in terms of protein up regulation or down regulation. The molecular assessment of the treated pollen was conducted on the bumblebee fatbody, a tissue analogous in function to the mammalian liver and adipose tissue. The insect fatbody performs functions that are deemed similar to how the liver functions in humans

(Ament *et al.*, 2011). Its main roles in invertebrates are energy storage, utilization and detoxification (Chan *et al.*, 2011), hormone regulation and is an important mediator of the immune response (Ramsey *et al.*, 2019). It can vary in colour between insects, with white and tan in the moth *Plodia interpunctella* and blue and white in the worm *Helicoverpa zea* (Haunerland and Shirk, 1995). The subcuticular fatbody is made up of a multilayer of cells that surround each abdominal segment (Strachecka *et al.*, 2021). The morphology of the fatbody varies between insects but in general it is structured as thin lobes of tracheated tissue suspended in the haemolymph (Haunerland and Shirk, 1995). It is mainly made up of trophocytes and oenocytes, trophocytes are mesodermal in origin and oenocytes are ectodermal in origin (Inoue *et al.*, 2022). Oenocytes maintain homeostasis and detoxification while the trophocytes provide energy for synthesis of hemolymph proteins and metabolism (Inoue et al., 2022).

The fatbody also plays a key role in metabolism, of carbohydrates, lipids, amino acids, nitrogen metabolism and protein synthesis. Excess nutrients are stored here utilizing and synthesising fat and glycogen, as well as acting as a nutrient sensor. The level of nutrients present can impact growth and egg development (Arrese and Soulages, 2011).

As fatbodies are the primary site for protein synthesis (Inoue *et al*., 2022) fluctuations and alterations in the proteomic profile in response to variations in the diet should be detected here which makes it a good target for detecting potential effects caused by changes in the diet. Fat bodies are also the main production area of antimicrobial peptides, which are needed for fighting against pathogen infections (Alaux *et al.*, 2010). The immune response in bees is also dependent on nutrition, with bees deprived of pollen having reduced responses to pathogenic attack (Brunner, Schmid-Hempel and Barribeau, 2014). Pollen is the main source of protein and lipids in the bee diet (Annoscia et al., 2017) and in animals protein is required in order to obtain amino acids and essential amino acids. Amino acids are needed to generate ATP, cell signalling, protein production and new amino acid production (Stabler *et al.*, 2015). Poor quality pollen has also being demonstrated to affect brood rearing and worker mortality (Huang, 2012).

In honeybees, the biochemistry of the abdomen has been shown to be remodelled during the transition to foraging (Chan *et al.*, 2011). It also plays a role in hormone regulation, immune response, nutrient and energy storage and energy utilization as well as detoxification

(Inoue *et al*., 2022). It's role in detoxification (Inoue *et al*., 2022; Chan et al., 2011) and protection against oxidative stress (Strachecka *et al.*, 2021) make it a primary target for determining stress as some cytochrome P450 (CYP) monooxygenases are mainly expressed here (Guo *et al.*, 2012). CYP enzymes play an integral role in metabolism of drugs, pesticides and plant toxins (Guo et al., 2012; Rand et al., 2015) and are found in almost all organisms. Some are conserved and have housekeeping roles, involved in biosynthesis, activation and deactivation of 20-hydroxyecdysone (an essential growth hormone) and cuticle formation (Sztal *et al.*, 2012). P450 genes involved in development are more likely to be found in stable clades, while those that are involved in xenobiotic metabolism are more likely to be in an unstable form (Darragh, Nelson and Ramírez, 2021).

Dissection of fatbodies from bees fed on either non-treated, e-beam treated or filtered commercial pollen after 14 days would permit a molecular level insight into the effects of treated pollen on protein synthesis to be obtained. In addition if the microbial composition (both pathogenic and mutualistic) is altered by the treatment their differences may also be represented in the host proteome. For example the intracellular pathogen *Nosema bombi* has been associated with affected lifespan, colony growth and a reduction in reproduction (Cordes *et al.*, 2012). The spores identified in commercial pollen were suspected to be *Nosema* based on microscopic analysis, so it was theorised that a reduction in spores should correlate with a positive effect on bee health. This would then be reflected in the proteome of bees fed on treated pollen, compared to the spore heavy untreated commercial pollen. An increase in immune related antimicrobial peptides was expected in control groups while a reduction was expected in the treated groups. The quality of the pollen could also be assessed through the metabolic pathways as bees require pollen for protein and amino acid synthesis (Stabler *et al.*, 2015) and fluctuations in amino acid synthesis could be suggestive of a lack of nutrition quality. However poor quality nutrition can also affect production of antimicrobial peptides (Brunner *et al*., 2014) so this could present challenges in data analysis should the immune response be decreased in treated pollen groups. This would then present the question of whether the immune genes are downregulated because of poor quality pollen or because of a less parasited pollen product. Results here would then need to be taken in conjunction with other results already obtained. In chapter 3 it was discussed how the pollen fed to bees, were suggestive of altered reproductive output. Previous work has suggested

that poor quality pollen can have a negative effect on brood rearing (Huang, 2012), so perhaps this was already a sign of what to expect as bees fed on either e-beam treated pollen and filtered pollen produced fewer bees compared to bees in the control group.

Proteomics is the terms giving to the study of proteins that are found within a cell, tissue or organ. The term was first coined in 1995 and focuses on protein identification, function, modifications, localization and protein-protein interactions. By examining the proteome of a cell, a snapshot of the protein environment at that particular moment in time can be obtained (Graves and Haystead, 2002). In order to do this certain tools are required that will allow the identification of the proteins that are present. This was traditionally carried out by using techniques such as 2 dimensional gel electrophoresis (2DE) coupled with mass spectrometry (MS) (Bantscheff *et al.*, 2007). Mass spectrometry is used not only for proteomics, but also for metabolomics and microbiome analysis due to its ability to both identify and quantify (Alseekh et al., 2021; Bauermeister et al., 2022).

The examination of the proteome of a cell has its advantages over genes or mRNA as not all genes will be expressed and not all mRNA will be expressed as they are coded due to alternate splicing and proteins themselves are then subject to post translational modifications, with structure and folding being heavily tied to function (Graves and Haystead, 2002). The use of liquid chromatography mass spec (LC-MS) has been advancing the research in the biomedical field allowing for precision quantitative proteomic profiling (P. and Nita-Lazar, 2019). An LC-MS experiment can be broken down into various steps, including cell lysis using buffers to disrupt the cell membrane and protease inhibitors to prevent protein degradation. Proteins are digested into peptides by chemical or enzymatic means such as trypsin enzymes, purified and then introduced into the chromatography system to separate the peptides based on hydrophilicity/hydrophobicity binding capacities to a c18 column. The peptides are released by increasing gradient of acetonitrile into the mass spectrometer whether their masses of the fragmented ions are measured. The MS functions by 1) the ion source assigns a charge to each peptide 2) the mass analyser measures the mass to charge ratio (*m/z*) and 3) the detector captures the ions and measures their intensity. Data is acquired through a series of peaks filtering out background noise and then input into software for identification (Karpievitch et al., 2010).

Previous proteomic work carried out in this lab by (Cullen *et al.*, 2023) analysed using MS digestive tracts to determine the changes in the proteome of bumblebees exposed to glyphosate. The aim was to use a similar approach to observe any potential changes in the fatbody proteome of bees fed on various pollen types.

Chapter aims

The aim of this chapter was to determine if there was any changes in the protein profile in the fatbodies of bees fed on either e-beam treated pollen or filtered pollen compared to those fed on untreated pollen and if so, what could the potential changes mean in terms of bees health. Bumblebee colonies were fed on either control e-beam treated pollen of filtered pollen for a 30 day period. After 2 weeks newly emerged bees were removed from the colony, knocked out using CO_2, tagged with a coloured number that was glued between the wings on their thorax. They were then returned safely to their colony once they started to wake up. They were then extracted from the colonies after 2 weeks and snap frozen in liquid nitrogen. The fatbodies were dissected under a dissecting microscope. Cells were collected, lysed and total protein was quantified. The proteins were digested into and analysed using quantitative mass spectrometry-based proteomics to determine differences in protein abundances and the pathways and processes that affected by different treated pollen. It was anticipated that the results obtained here would help shed light on the findings obtained from the colony level experiment where a reduced reproductive output was observed in colonies fed on treated pollen.

4.2 Materials and Methods

Bumblebee (*Bombus terrestris*) colonies were sourced from Agralan in the (UK).

The first week the colonies were fed daily with 4 g of pollen and 8 g of pollen for each week after. Observations were made on each colony every second day, for a total of 30 days. The number of egg clumps, larvae and pupae were recorded during these observations and the weight recorded once every week. Bees had *ad libitum* access to feeding tubes filled with 40% (w/v) sucrose. All colonies were kept at 20 ± 2°C and 58 ± 5% relative humidity for the duration of the experiment and were continuously kept in the dark. Pollen (8g) was administered to each colony every 30 days.

Colonies 1-4 were fed untreated pollen and served as the control, colonies 5-8 were fed the same commercial pollen that had been treated with a 100KGy dose of e-beam, while colonies 9-12 were fed on washed pollen (filtered). All colonies were fed on pollen that had come from the same batch, and washed pollen was made up every three days, using sterile water and 5µm filter paper.

After 18 days had elapsed, callows produced from the colonies were taken and tagged using a colour coded numerical system and returned. These bees were then extracted and snap frozen in liquid nitrogen 14 days after and stored at -80^{o}C. Snap frozen bees were later used for fatbody and digestive tract dissections that were used for proteomics and microbiome sequencing, respectively.

On day 30 the experiment was terminated. Colonies were placed into the -20^{o}C to introduce sleep and eventual death to each colony.

4.2.1 Proteomic sample preparation

20 bees were removed from each colony and place in 1.5 mL tubes, snap frozen and stored at -80^{o}C until required. The fatbodies from thawed bees were extracted for proteomic analysis. Bees were first pinned to a wax bed and the connection between the thorax and the abdomen was severed. Abdomens were positioned slightly stretched and once the abdomen

was firmly pinned sterile scissors were used to cut around the edge of the cuticle to allow the ventral side of the abdomen to be peeled back to reveal the contents of the haemocoel. The wax bed was then placed under a dissection microscope for removal of the organs and collection of the fat cells.

Fat cells were collected from both dorsal and ventral sides of the abdomen for every dissection. Sterile tweezers were used to gently pry the fat cells from muscle and trachea. Once the fat cells were dissected they were removed to ice-cold lysis buffer comprising 6M urea, 2M thiourea and 1 tablet of Complete™, Mini Protease Inhibitor Cocktail (Roche Diagnostics), snap frozen in liquid nitrogen and kept at -20°C. Once the fatbodies were dissected from all bees, all samples were thawed on ice and homogenised for 30 seconds each using a motorised pestle. Samples were centrifuged at 10000rpm for five minutes to pellet any remaining cellular debris and the supernatants were aliquoted in to clean 1.5 mL tubes and stored at -80°C.

Protein quantification was conducted using Qubit® Quant-IT™ protein assay kit on a Qubit® fluorometer version 2.0 following manufacturer guidelines. 100 µg of protein was removed from each sample and processed with a 2-D clean up kit (GE HealthCare), following manufacturer guidelines, to remove biological impurities. The resulting pellet was resuspended in 50µl resuspension buffer (6M urea, 0.1M Tris HCl, pH 8.0), of which 20 µL was used for protein digestion. 115 µL of 50mM ammonium bicarbonate was added to each sample. Proteins were reduced and alkylated by adding 0.5M dithiothreitol (DTT) at 56°C for 20 min followed by 0.5M iodoacetamide (IAA) and incubating at room temperature for 15 min in the dark. 1 µl of 1% (w/v) Protease Max (Promega) and 1µl sequence grade trypsin (Promega) were added to each sample and incubated at 37°C for 16 hours. Subsequently, 1 µl of 100% trifluoroacetic acid (TFA) was added to each sample to terminate digestion and the samples were incubated at room temperature for five min and centrifuged at 10,800rpm for 10 min. The resulting supernatants were purified using Pierce C18 spin columns (Thermo Scientific) following manufacturer guidelines and the eluted purified peptides were dried down using a speedy vacuum concentrator (Thermo Scientific Savant DNA 120) and stored at 4˚C. Peptides were resuspended in a volume of loading buffer (2% (v/v) acetonitrile and 0.05% (v/v) TFA) to yield a concentration of 0.5 µg/µl, sonicated for

two minutes and centrifuged at 13,400 rpm for five minutes. The supernatant was used for LC MS/MS.

4.2.2 Mass spectrometry

1 µg of peptide mix for each sample was eluted onto the Q Exactive (Thermofisher Scientific, USA) high resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. An increasing acetonitrile gradient was used to separate peptides on a Biobasic C18 Picofrit™ column (200mm length, 75mm ID), using a 120-min reverse phase gradient at a flow rate of 250nL/min. All data were acquired with the mass spectrometer operating in automatic data dependent switching mode. A high-resolution MS scan (300-2000 Dalton) was carried out using the Orbitrap to select the 15 most intense ions prior to MS/MS. MaxQuant version 1.6.17.0 (www.maxquant.org) was used for protein identification and LFQ normalisation of all MS/MS data. The Andromeda search algorithm in MaxQuant was used to correlate all MS/MS data against protein reference sequences obtained from the National Centre for Biotechnology to correlate the data against the protein reference sequences derived from the *B. terrestris* genome (Sadd *et al.,* 2015) obtained from the National Centre for Biotechnology Information (NCBI) repository (17,508 entries, downloaded September 2021).

4.2.3 Proteomic Data Analysis

Perseus version 1.6.1.1 was used for data visualisation and statistical analysis. Normalized LFQ intensity values were used as a measure of protein abundance. The data was filtered for the removal of contaminants and peptides identified by site. LFQ intensity values were $\log_2$ transformed and samples were allocated to groups corresponding to treatment. Proteins absent from any samples in at least one group were not used for further analysis. A data imputation step was conducted to replace missing values with values that simulate signals of low abundant proteins chosen randomly from a distribution specified by a downshift of 2.1

times the mean standard deviation (SD) of all measured values and a width of 0.1 times this SD. Normalized intensity values were used for principal component analysis. A two-sample T-test was performed using a cut-off value of $p \leq 0.05$ to identify statistically significant differentially abundant (SSDA) proteins. Volcano plots were produced by plotting –Log p-values on the y-axis and Log_2 fold-change values on the x-axis to visualize differences in protein abundance between treatment groups. Hierarchical clustering of SSDA proteins was performed using z-score normalised intensity values to produce a heat map of protein abundance.

4.3.4 Functional Annotation

The Search Tool for the Retrieval of Interacting Genes/proteins (STRING) version 11 (Szklarczyk *et al.,* 2019) (www.string-db.org) was used to map protein-protein interaction networks. Protein sequences were inputted into the STRING database and protein-protein interactions were analyzed using the homologous *Apis mellifera* match for each identified *B. terrestris* protein. STRING clusters were inspected for the protein sets of higher or lower abundance in fatbodies from bees fed E-beam-treated or filtered pollen with respect to the control pollen. The gene ontology categories enriched with each set (Fisher's Exact test; $p<0.05$) were obtained for molecular function, biological process, cellular compartment, KEGG term and others where appropriate to determine the pathways and processes affected by the different pollen treatments

4.3 Results

In total 2,833 proteins were identified from the *B. terrestris* fatbodies, with 1,575 proteins remaining after filtering for contaminant and reverse hit peptides and proteins not found in all 5 replicates of at least one group (Appendix File 1). PCA on all proteins determined variance of 45.9% in component one and 16.7% in component two (Figure 4 PCA). All three groups resolved as distinct clusters highlighting that the e-beam treated and filtered pollen caused global changes to the fatbody proteome in comparison to the untreated control pollen.

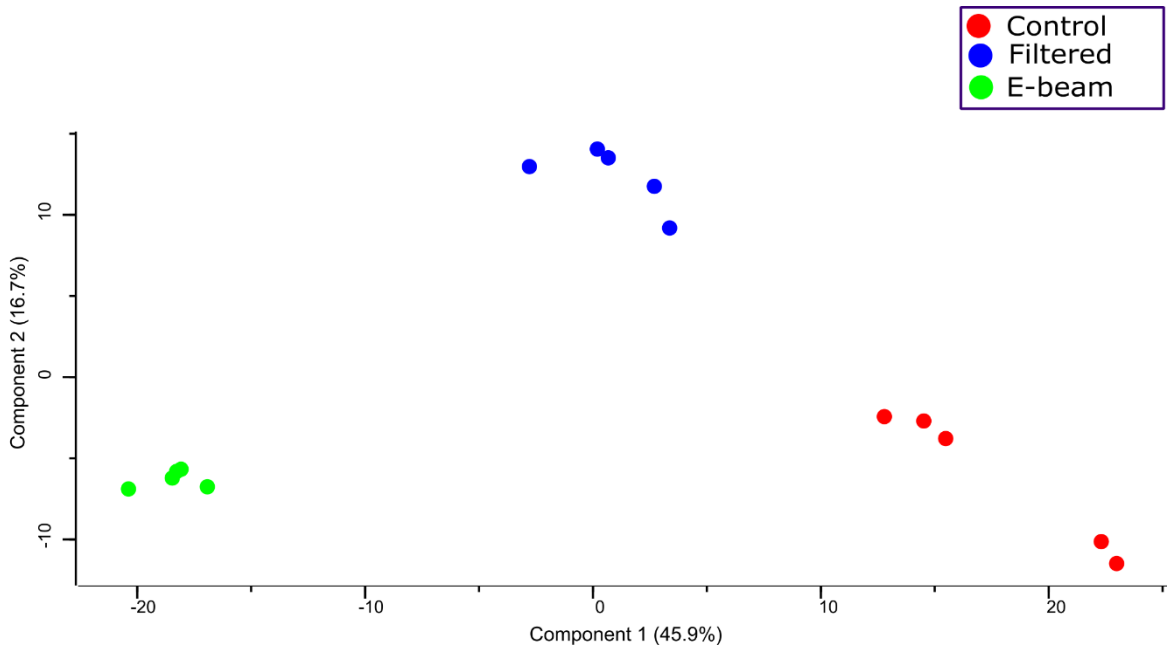


Figure 4 Principal component analysis of the 15 samples from the three groups. The three groups (fatbodies from control-, e-beam treated or filtered pollen fed bees) are distinctly resolved indicating a clear and distinct effect of each treatment on the fatbody proteome.

4.1 Two sample T-tests

Two sample t-tests were performed amongst treatment groups to determine statistically significant differentially abundant (SSDA) proteins ($p \leq 0.05$, S0 = 0.1) and their relative fold differences. STRING analysis was conducted on SSDA lists to identify pathways, processes, GO, KEGG terms and protein networks that were enriched in one protein set over another.

4.2 E-beam Treated Pollen vs Control

A total of 177 SSDA proteins (Appendix 2; Figure 4.2 Volcano 1) were identified between fatbodies from e-beam treated pollen fed and control bees (RFC range: + 21.7 to – 169.2). There were 103 proteins with increased abundance and 74 with decreased. The top 15 proteins that were upregulated (Table 4.2.1) were associated with toxicity, immunity, translation and sex hormone production. The top 15 proteins that were downregulated in colonies fed on e-beam treated pollen were associated with bee venom, chitin degradation, exoskeleton, peptidoglycan recognition, cellular respiration and heat shock.

In the proteins that were in higher abundance in the e-beam compared to the control were Cytochrome b5 (RFC 6.9) which is part of the cytochrome p450 enzymes. Vitellogenin (RFC 6.4) and Uncharacterized protein (RFC 21.1) and also Cytochrome p450 (RFC 4.3). Cytochrome enzymes are generally associated with detoxification.

Proteins that were in lower abundance in the e-beam treated pollen fed group included melittin (RFC-168.9), Chitinase 2 (RFC-10.5) and proteins involved in defence and immunity such as the serine proteinase Chymotrypsin (RFC-4.3) and Peptidoglycan recognition protein (RFC-4.6). It is suggestive of an upregulation of detoxification proteins within the e-beam treated pollen fed group coupled with a downregulation of proteins involved in immunity. This could be mean a possible trade-off between toxicity and immunity due to the dose of e-beam given to the pollen.

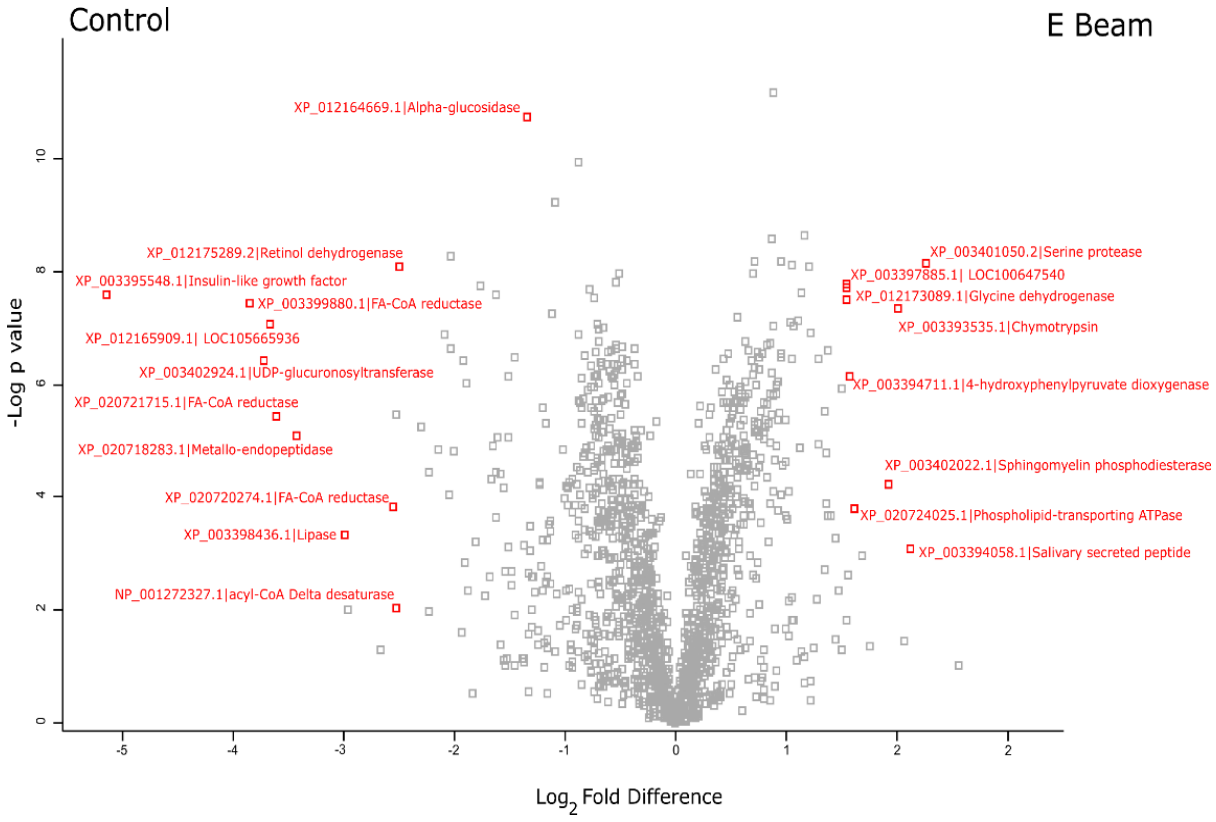


Figure 4.1 Volcano plot of e-beam v control pollen fed bees. Volcano plot analysis of fatbodies from bees in the control group and e-beam group. Both treatments were compared based on –log p value and $\log_2$ fold difference. The proteins with the greatest fold change are annotated.

Table 4.2.1 The top 15 SSDA proteins in bees from e-beam treated and control pollen fed colonies. Positive relative fold change (RFC) values indicate proteins with higher abundances in fatbodies from bees fed with the e-beam treated pollen whereas negative RFCs indicate proteins with reduced abundances in bees fed with the e-beam treated pollen in comparison to those fed the non-treated control pollen.

Accession number	Protein name	Relative fold change
XP_003397300.2	Uncharacterized protein	21.1
XP_012176245.1	Cytochrome b5	6.9
XP_012163499.1	Vitellogenin	6.4
XP_020721756.1	Uncharacterized protein	6.1
XP_003396646.1	Uncharacterized protein	5.0
XP_003393261.1	Mitochondrial coenzyme A transporter SLC25A42	4.3
XP_003395337.1	1-acyl-sn-glycerol-3-phosphate acyltransferase gamma	4.3
XP_003393699.1	Cytochrome P450 9e2	4.3
XP_003399652.1	PRA1 family protein 3	3.7
XP_003398970.1	long chain fatty acid transport protein 1	3.7
XP_012164498.1	ADP-ribosylation factor 2	3.5
XP_012164498.1	Chaoptin	3.2
XP_020723232.1	60S ribosomal protein L39	3.2
XP_003402576.2	Serine protease inhibitor 3/4 partial	3.2
XP_003401025.1	Ornithine amninotransferase	3.0
XP_020718386.1	Melittin	-168.9
XP_003395447.1	Epididymal sercretory protein E1	-17.1
XP_003400637.1	Probable chitinase 2	-10.5
XP_003395094.1	Uncharacterized protein	-9.8
XP_012165909.1	Uncharacterized protein	-9.2
XP_020718419.1	Glycine rich cell wall structural protein 1	-9.2
XP_003397852.1	Nicotinamidase	-9.2
XP_020718442.1	Transmembrane protease serine 9	-8.6
XP_012169980.1	Protein lethal(2) essential for life	-7.0
XP_12172148.1	Uncharacterized protein	-5.3
XP_003400160.1	Peptidoglycan recognition protein	-4.6
XP_003394953.1	Cuticle protein 16.5	-4.6
XP_012169306.1	Uncharacterized protein	-4.6
XP_020718419.1	Glycine rich cell wall structural protein 1	-4.6
XP_003402742.1	Chymotrypsin	-4.3

STRING analysis was conducted on all up and down regulated sets to identify pathways and processes that were generally affected by each treatment (Appendix file 3). The 103 proteins that were of higher abundance in e-beam treated pollen fed bees were associated with toxicity, cellular metabolic processes, transport, muscle development and peptidoglycan binding with four separate protein clusters were identified with this group (Figure 4.2). One cluster of proteins were associated with the molecular functions of detoxification (19/574) and oxidoreductase activity (19/574) and KEGG pathways involved in oxidative phosphorylation (6/86) and proton transport. The three other clusters were identified in Golgi vesicle transport (6/125) (4/37), tRNA aminoacylation (4/46) (5/61) and fatty acid degradation and scp-2 sterol transfer family (4/36).

The 74 proteins with decrease abundance in e-beam treated bees, could be resolved into clusters associated with muscle development, starch and sucrose metabolism, peptidoglycan binding and serine proteases and immunity (Figure 4.2.2B). Specifically, these included proteins associated with the molecular function of peptidase activity (10/334) and trypsin protein domains (7/63). There were three other separate clusters identified, one with both amylase, catalytic domain and amino acid permease (4/32) and glucan biosynthetic process and alpha amylase domain (3/12). One that had both actin cytoskeleton and striated muscle cell differentiation (5/124) and myofilament and tropomyosin (3/22). A final cluster with trypsin-like serine protease and immunity (9/117) and animal peptidoglycan recognition proteins homologous to bacteriophage T3 lysozyme and glycosyl hydrolases family 16 (2/5).

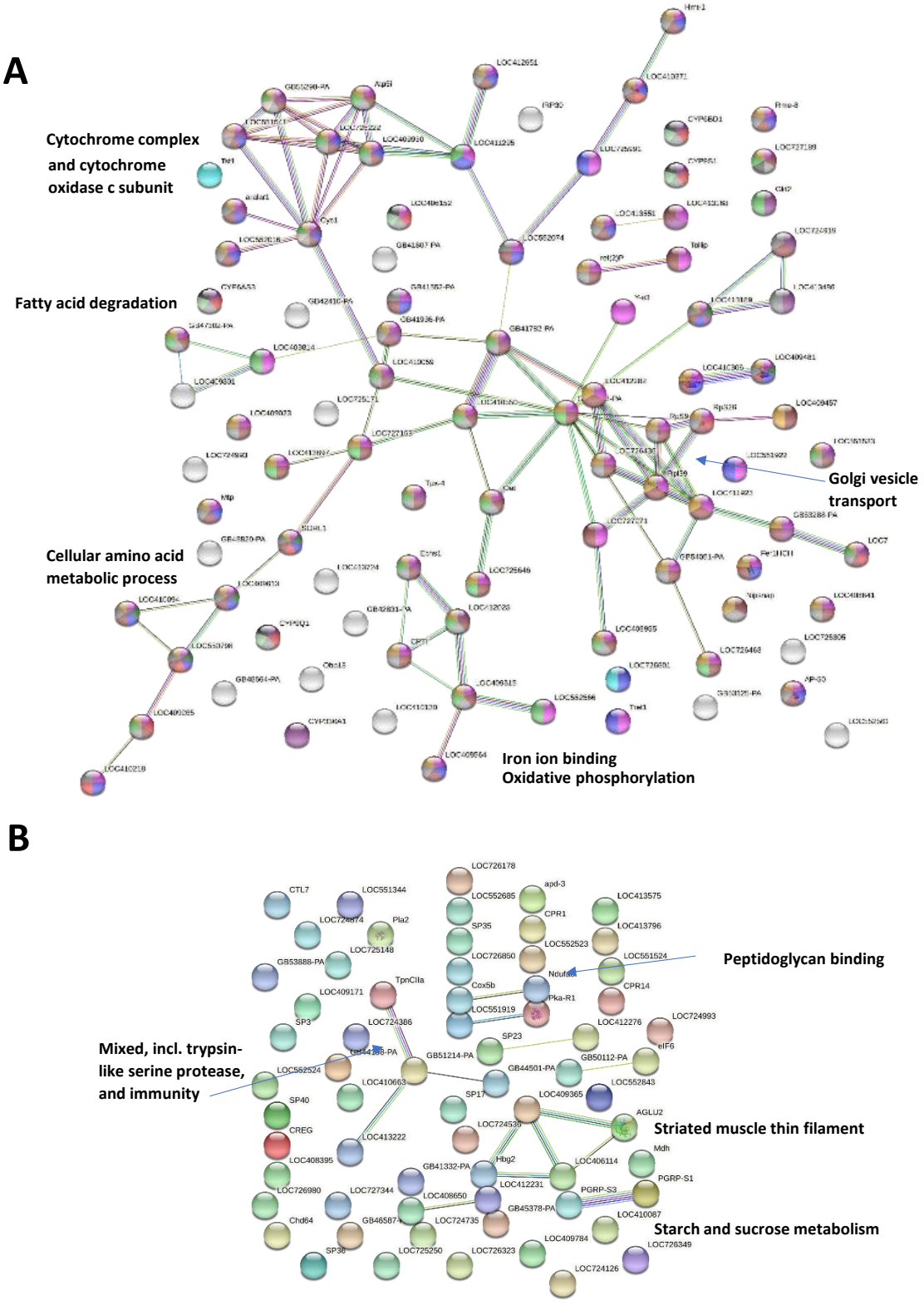


Figure 4.2 STRING images for the proteins that were of higher (A) or lower (B) abundances in fatbodies from bees fed upon e-beam treated or control pollen. These included proteins and pathways involved in oxidative phosphorylation and fatty acid degradation being upregulated (A), while proteins involved in immunity such as serine proteases and peptidoglycan binding being downregulated (B).

4.3 Filtered Pollen vs Controls

Quantitative mass spectrometry and statistical analysis identified 523 proteins that were SSDA (Appendix 4; Figure 4.3 Volcano 2) between fatbodies from filtered and control pollen fed bees (RFC range: + 47.5 to – 394.3). There were 337 proteins with increased abundance and 185 with decreased. The top 15 proteins that were upregulated (Table 4.2) were associated with toxicity, immunity, translation and sex hormone production. The top 15 proteins that were downregulated in colonies fed on filtered pollen were associated with bee venom, chitin degradation, exoskeleton, peptidoglycan recognition, cellular respiration and heat shock.

Figure 4.3 Volcano plot of filtered v control pollen fed bees. Volcano plot analysis of fatbodies from bees in the control and filtered groups. Both treatments were compared based on –log p value and log2 fold difference. The proteins with the greatest fold change are annotated

Table 4.2.2 The top 15 SSDA proteins in bees from filtered treated and control pollen fed colonies. Positive relative fold change (RFC) values indicate proteins with higher abundances in fatbodies from bees fed with the filtered pollen whereas negative RFCs indicate proteins with reduced abundances in bees fed with the filtered pollen in comparison to those fed the non-treated control pollen.

Accession number	Protein name	Relative fold change
XP_003395548.1	Insulin gf binding protein acid labile subunit	48.5
XP_020721715.1	Putative fatty acyl CoA reductase CG8306	17.1
XP_012164498.1	Chaoptin	16
XP_012163499.1	Vitellogenin	13.9
XP_020718283.1	Membrane metallo-endopeptidase 1	12.1
XP_003402924.1	UDP=Gluctonosyltransferase 2B15	11.3
XP_003399880.1	Putative fatty acyl CoA reductase	11.3
XP_012175289.2	Retinol dehydrogenase 14	9.8
XP_003396646.1	Uncharacterized protein	9.1
XP_003402892.1	Glucosylceramidase	8.5
XP_003395752.1	Ribosomal protein S29	8
XP_003397300.2	Uncharacterized protein	7.4
XP_020723232.1	Ribosomal protein	7.4
XP_003398436.1	Pancreatic lipase related protein 4	6.9
XP_003395762.1	Omega conotoxin protein 3	6.9
XP_020718386.1	Melittin	-388.0
XP_020718442.1	Transmembrane protease serine 9	-27.8
XP_003400637.1	Probable chitinase 2	-27.8
xp_012170989.1	Phospholipase A2	-18.3
XP_003402742.1	Chymotrypsin 1	-14.9
XP_003400160.1	Peptidoglycan recognition protein	-13.9
XP_003393535.1	Chymotrypsin 1	-12.9
XP_003398056.1	DNA-directed RNA polymerase II subunit RPB1	-10.5
XP_003394953.1	Cuticle protein 16.5	-9.8
XP_003394058.1	Probable salivary secreted peptide	-9.8
XP_003401050.2	Serine protease 53	-9.8
XP_003395094.1	Uncharacterized protein	-8
XP_003402225.2	Maltase A1	-8
XP_003399666.1	Collagen alpha (IV) chain	-7.4
XP_012165896.1	Uncharacterized protein	-7.4

String analysis on the proteins of higher and lower abundance in the fatbodies from bees fed on filtered and control pollen resolved a number of key pathways and process that were clearly affected. The 338 proteins that were upregulated compared to control colonies were associated with male sterility, biosynthesis of fatty acids, translation, processing and localisation to the endoplasmic reticulum and endoplasmic Golgi vesicle (Figure STRING 4.3.3A). One cluster of proteins was involved in COPI- coated vesicle budding (4/12) and oligosaccharyl transferase activity (4/6) with protein functions that included oxidoreductase activity (41/574). Another cluster identified had proteins involved in male sterility (5/11), (6/17) as well as KEGG pathways involved in fatty acid biosynthesis (3/10), fatty acid elongation (4/23) and N-Glycan biosynthesis (7/33). A third cluster identified had proteins that were involved in ribosome biogenesis (15/219), while a forth identified 5 local network clusters involved in aminoacyl-tRNA synthetase (5/10), ligase activity (9/32), (7/25) and tRNA aminoacylation (10/46) and eukaryotic translation initiation factor 2B complex (11/61).

The 138 proteins that were downregulated in filtered pollen fed bees compared to control colonies were involved in the cytoskeleton, oxidative phosphorylation, extracellular matrix and glycosphingolipid biosynthesis. Clusters involved in the extracellular matrix and cytoskeleton were down regulated as were clusters involved in cellular respiration and glycosphingolipid biosynthesis. In one cluster collagen containing extracellular matrix and thrombospondin type 1 domain was found (6/91) as well as a cluster of extracellular matrix and integrin complex (7/111), functions included serine-type peptidase (8/103) and endopeptidase activity (7/87).

A second cluster identified Glycosyl hydrolase family 18 (6/16) plus lysosome and transferase activity (8/112) with functions including carbohydrate binding (6/64) and hydrolase activity (13/65). A third cluster contained many local network clusters involved in actin cytoskeleton and striated muscle cell differentiation (17/124) (18/131) (15/102), actin binding (20/172), myofilament and myosin ii complex (8/15) myosin complex (4/5), muscle myosin complex (7/10) (tropomyosin (12/22) (14/27) with functions including actin binding (13/168), calcium binding (13/219) cytoskeletal protein binding (16/362) , striated muscle thin filament (4/6) and structural molecule activity (13/355). A fourth cluster had several local network clusters involved in NADH dehydrogenase activity (8/23), (3/11) and a NADH dehydrogenase complex (9/32). Other local network cluster were Respirasome (12/57), oxidative phosphorylation and

vacuolar proton-transporting V-type ATPase complex (14/101) and inner mitochondrial membrane protein complex (15/179). Functions of this cluster included oxidoreductase activity (28/574) and NADH dehydrogenase activity (9/28) and ubiquinone activity (4/15).

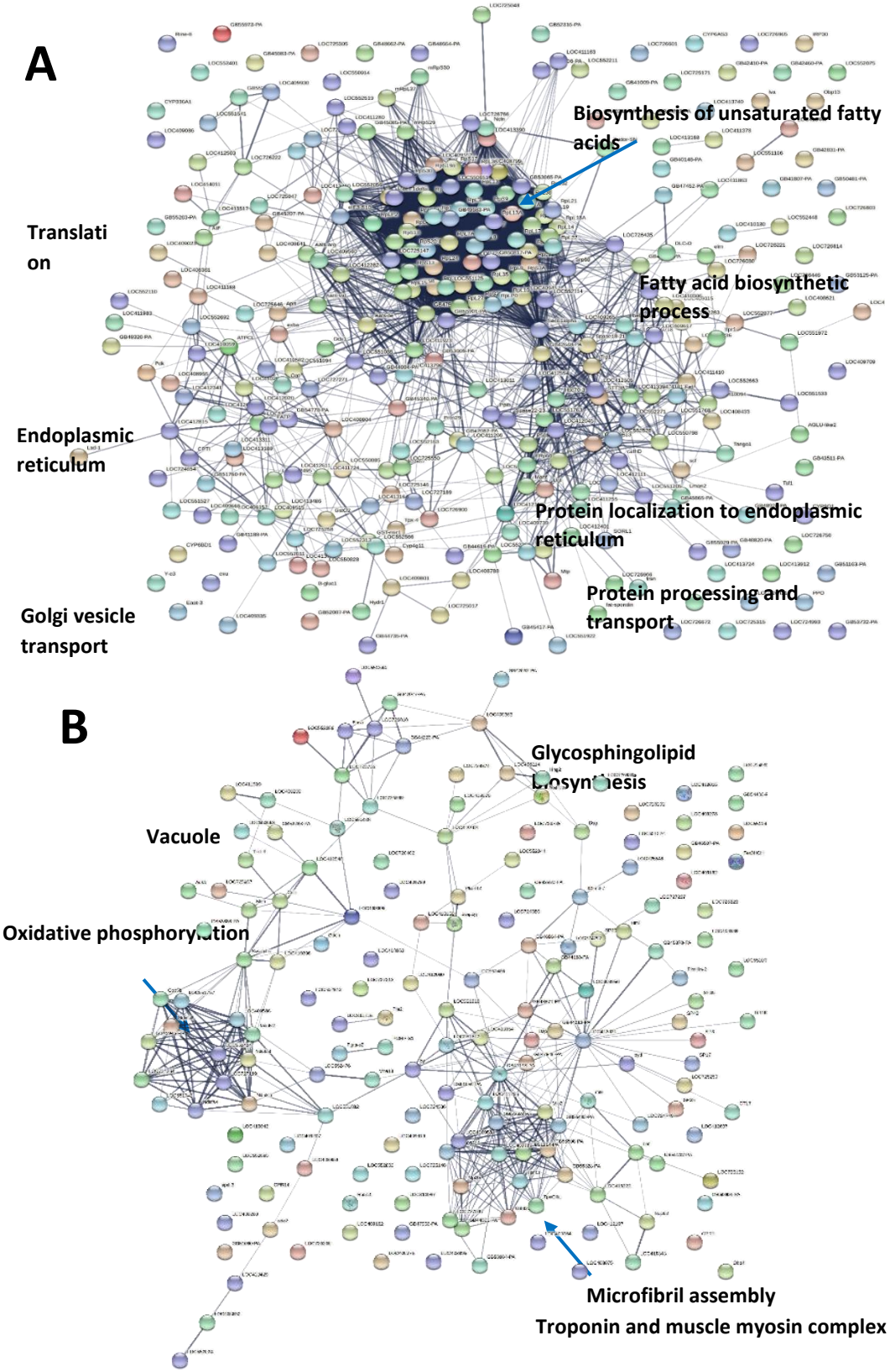


Figure 4.4 STRING images for the proteins that were of higher (A) or lower (B) abundances in fatbodies from bees fed upon filtered or control pollen. These included proteins and pathways involved in fatty acid and unsaturated fatty acid biosynthesis being upregulated (A), while proteins involved in oxidative phosphorylation and transport being downregulated (B).

4.4 Hierarchical clustering

In addition to the pairwise comparisons described above hierarchical clustering on the SSDA proteins was conducted to identify proteins and protein groups with similar expression profiles across all three groups. In total 7 clusters (A to G) were resolved (Figure 4.5 Heatmap), representing similarities and variations among the treatments. Where possible terms associated with KEGG pathways were chosen to represent the main pathways of a cluster (TABLE 4.2.2). In cluster A the Proteasome (18/37), peroxisome (4/59), TCA cycle (3/32) and amino acid biosynthesis (4/41) were some of the main pathways affected. In both treatments e-beam and filtered these pathways were upregulated while being down regulated in the control. In cluster B local network cluster identified COP II-coated vesicle cargo loading (3/5) as being upregulated in filtered colonies, while being downregulated in both the control and e-beam treated pollen colonies. KEGG pathways effected were also downregulated in both the control and e-beam treated pollen colonies, while been up in filtered pollen colonies included the Proteasome (6/37), Aminoacyl t-RNA biosynthesis (4/35) and the Ribosome (42/117).

Cluster C had an upregulation of local network cluster Cytochrome p450 and isoprenoid biosynthesis (6/106) in both e-beam and filtered colonies, while being downregulated in control colonies. KEGG pathways affected that were also upregulated in both e-beam and filtered colonies while being downregulated in control colonies were Aminoacyl-tRNA biosynthesis (6/35), N-Glycan biosynthesis (3/33) and fatty acid biosynthesis (2/10). In cluster D KEGG pathway Glycosphingolipid biosynthesis was somewhat normalised in e-beam treated colonies while being upregulated in filtered colonies and downregulated in control colonies (2/4). Co category biological process was again somewhat normalised in the e-beam treated pollen colonies while being downregulated in control colonies and upregulated in filtered pollen colonies, these included Tissue development (24/850), Ubiquinol-cytochrome-c reductase activity (3/5) and Aerobic respiration (8/51). In cluster E the Kegg pathways downregulated in e-beam treated and filtered colonies while being up in control colonies were Selenocompound metabolism (2/8) and Oxidative phosphorylation (4/86).

In cluster F some of the Kegg pathways affected were the insect hormone biosynthesis (2/15), Phagosome (3/58), Fructose and mannose metabolism (2/17) and lysosome (7/75). These pathways were somewhat normalised in the control colonies, while being upregulated in the

e-beam treated pollen colonies and downregulated in the filtered pollen colonies. In cluster G, Kegg pathways in both the control and filtered treated colonies, were somewhat normalised or downregulated, while e-beam pollen treated colonies had Kegg pathways upregulated. These included Oxidative phosphorylation (8/86), Endocytosis (5/102), Fatty acid degradation (3/23) and the Pentose phosphate pathway (3/19).

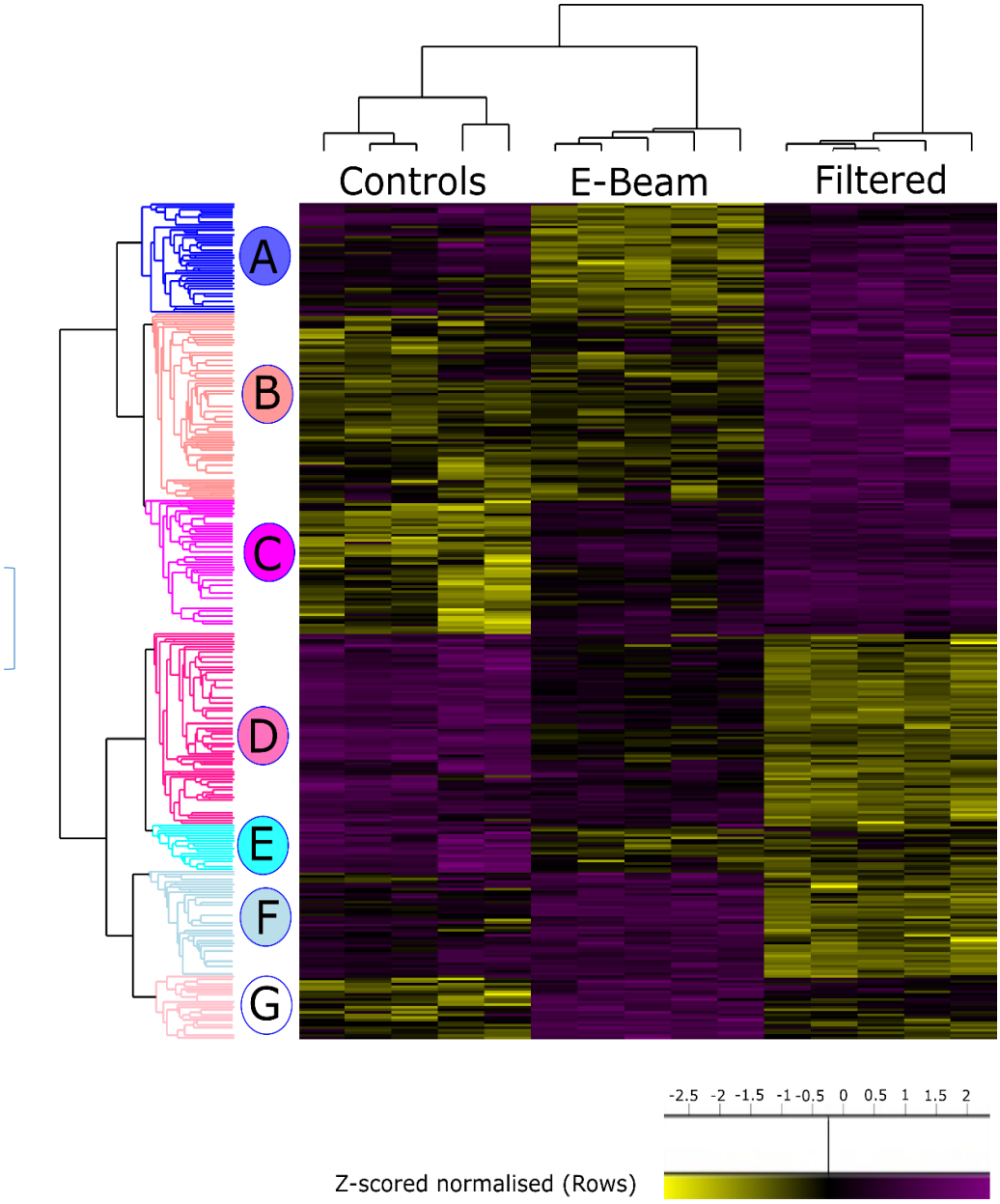


Figure 4.5 Heat map of treated clusters from fat body analysis highlighting the protein groups within the treated colonies. Mass spectrometry identified 7 clusters with the treatments with various proteins and pathways being either upregulated or downregulated. Differences are noted between the treatments in each cluster with yellow denoting downregulation and purple denoting up regulation. KEGG pathways affected are noted beside each cluster.

Table 4.2.3 Gene ontology categories for the major protein groups of each cluster identified by hierarchical clustering. Seven clusters were identified including A which were up in controls and filtered and down in e-beam, B which was down in control and e-beam, but up in filtered, C which was down in control, normal in e-beam and up in filtered, D which was up in control, normal in e-beam and down in filtered, E which was up in control and down in both e-beam and filtered, F which was normal in the control up in e-beam, and down in filtered and G which was down in the control, up in e-beam and down in filtered**.**

Cluster	GO Category	GO Term	Description	Count in Network	Strength	FDR
A	KEGG	ame03050	Proteasome	18 of 37	1.82	1.17E-23
	KEGG	ame04146	Peroxisome	4 of 59	0.97	0.0252
	Biological process	GO:0006090	Pyruvate metabolic process	4 of 38	1.16	0.0295
	KEGG	ame00020	Citrate cycle (TCA cycle)	3 of 32	1.11	0.0345
	KEGG	ame01230	Biosynthesis of amino acids	4 of 41	1.13	0.0084
B	Biological process	GO:0090110	COPII-coated vesicle cargo loading	3 of 4	1.8	0.006
	KEGG	ame03050	Proteasome	6 of 37	1.14	0.00027
	KEGG	ame00970	Aminoacyl-tRNA biosynthesis	4 of 35	0.99	0.0101
	KEGG	ame03010	Ribosome	42 of 117	1.48	8.01E-44
C	KEGG	ame00970	Aminoacyl-tRNA biosynthesis	6 of 35	1.3	4.28E-05
	KEGG	ame00510	N-Glycan biosynthesis	3 of 33	1.02	0.0404
	Biological Process	GO:0009062	Fatty acid catabolic process	4 of 32	1.16	0.0266
	Local network cluster	CL:13440	Cytochrome P450, isoprenoid biosynthetic process	6 of 106	0.81	0.0212
D	local network cluster	CL:16153	Glycosyl hydrolases family 18,	5 of 16	1.42	0.00025
	KEGG	ame00604	Glycosphingolipid biosynthesis - ganglio series	2 of 4	1.62	0.0206
	Biological process	GO:0003012	Muscle system process	6 of 33	1.18	0.0011
E	KEGG	ame00190	Oxidative phosphorylation	4 of 86	1.17	0.0118
	local network cluster	CL:7210	Troponin and muscle myosin complex	4 of 5	1.83	0.00016
	local network cluster	CL:12535	Cytochrome complex	4 of 15	1.35	0.0033
	Biological process	GO:0046034	ATP metabolic process	20 of 100	1.23	5.60E-14

	KEGG	ame00620	Pyruvate metabolism	4 of 30	1.05	0.0079
F	KEGG	ame00981	Insect hormone biosynthesis	2 of 15	1.28	0.0301
	Biological process	GO:0006909	Phagocytosis	5 of 42	1.23	0.0017
	KEGG	ame00051	Fructose and mannose metabolism	2 of 17	1.22	0.0355
	KEGG	ame04142	Lysosome	7 of 75	1.12	2.81E-05
G	biological process	GO:0006119	Oxidative phosphorylation	4 of 59	1.23	0.0112
	KEGG	ame04144	Endocytosis	5 of 102	1.09	0.0017
	biological process	GO:0006631	Fatty acid metabolic process	6 of 108	1.14	0.001
	KEGG	ame00030	Pentose phosphate pathway	2 of 19	1.42	0.0296

4.4 Discussion

Bees fed on either e-beam treated pollen or filtered pollen had exhibited changes in size and reproductive output when compared to bees fed on the same untreated pollen. Bees in both these groups failed to produce males, gynes and had fewer females present compared to control groups (Chapter 3). To determine if these effects were down to differences in the pollen that the colonies were fed on, a molecular approach was taken dissecting the fatbodies of 14 day old *B. terrestris* to see what differences if any had occurred on a molecular level. The fatbody in invertebrates functions in energy storage, utilization and detoxification (Chan *et al.*, 2011), hormone regulation and immune response (Ramsey *et al.*, 2019). By looking at what proteins had been upregulated and downregulated a greater understanding could be formed as to the possible effects feeding treated pollen to bees could potentially have.

It was discovered that, bees fed on both e-beam treated pollen and filtered pollen had more proteins associated with immunity downregulated such as peptidoglycan recognition protein and chymotrypsin compared to the control. Proteins associated with detoxification and stress were upregulated in e-beam treated pollen fed bees, cytochrome p450 and vitellogenin while proteins associated with growth and development such as insulin growth factor binding protein, vitellogenin and glucosylceramidase were upregulated in filtered fed colonies compared to the control colonies.

Overall we found that when bees were fed on either e-beam treated pollen or filtered pollen there was a difference in the fatbody proteomic profile compared to bees that were fed on untreated control pollen. These fluctuations in protein levels could help explain why a difference in both reproductive output and size in colonies, fed on either e-beam treated or filtered pollen was observed. Determining what those differences could mean not just only from a reproductive output point of view but from a potential health effects would require a deeper dive into the proteins and pathways affected. The literature was searched in order to determine the implications of these observations.

The effects of e-beam treated pollen on the fatbody proteome of *Bombus terrestris*

Proteins with increased abundance in bees fed e-beam treated pollen

Stress related protein upregulation

A number of proteins associated with stress were upregulated in comparison to the control in e-beam treated pollen fed colonies. A total of 104 proteins were upregulated in these colonies when compared to the control. Proteins such as vitellogenin (Vg) which are vital for egg maturation and embryonic development (Wu *et al.*, 2021). Vitellogenin is synthesised in the fatbody of insects and transported to oocytes via endocytosis (Mitchell, Sonenshine and Pérez De León, 2019; Wu *et al.*, 2021). It not only plays a role in egg maturation, but in *Apis mellifera* it also plays a roles in protecting against oxidative stress and extending lifespan, sensing fatbody sugars and can act as a pathogen recognition receptor (PRR) and transporter of pathogen derived molecules to offspring to confer immunity (Wu *et al.*, 2021).

Reproductive output of both larvae and pupae was down in e-beam treated colonies compared to control colonies. Bumblebees fed on e-beam treated pollen failed to produce males and produced less females compared to control groups. It is the plausible that vg was upregulated in response the stunted reproductive output within these colonies. E-beam treated pollen fed colonies did produce overall, more eggs than the control groups and this could be why an upregulation of vg was observed. Vitellogenin is critical to embryonic development and if more eggs are present, a possible explanation could be down to more vg being produced. However these eggs failed to mature into larvae, so it is likely that the higher numbers was down to the recounting of eggs that never developed. A more likely explanation is that vg upregulation was in response to stress conditions, as an upregulation in phagosome, lysosome and oxidative phosphorylation was observed in the heat maps too. This could be a result of poor nutrition and vg could be acting as sensor for sugar. Fatty acid degradation was upregulated in these colonies, so perhaps vg was being used as a sensor for the detection and recycling of lipid molecules. While it can't be said for certain, it is probably likely that it is not playing a role in pathogen recognition here, as a 100KGy dose of e-beam should be sufficient enough to kill whatever microbial life resided within the pollen. This is backed up by the lack of fungal growth on the e-beam treated pollen 5 months post experimentation.

Other proteins upregulated included members of the cytochrome p450 family cytochrome p4509e2. Cytochrome P450 enzymes metabolise toxins and promote tolerance. In *Apis mellifera* these enzymes play a role in detoxification of pesticides (Mao, Schuler and Berenbaum, 2011). It is possible that there is a toxic effect being observed from the sterilized pollen. This could be due to a change in molecular makeup of the pollen itself. E-beam treated pollen, visually differed from non-treated commercial pollen, it had a waxy glow, resembling plasticine and took on a darker colour. This could mean that it is somehow chemically different to non-treated pollen and this change is causing an upregulation in detoxification genes.

Further investigations would be required to know the exact mechanisms at play, but it does seem plausible that e-beam treated pollen is having an effect on bee health. This is most likely through mechanisms similar to starvation, and toxicity which would suggest that it is impacting negatively.

Metabolism

Colonies fed on e-beam treated pollen also had an upregulation in oxidative phosphorylation, endocytosis, fatty acid degradation and the pentose phosphate pathway (cluster G).

The pentose phosphate pathway is a key pathway in the formation of molecules for nucleic acid and amino acid biosynthesis while the non-oxidative pathway can supply glycolysis with the necessary metabolites required for normal function. It also converts glucose 6 phosphate an intermediate of the TCA cycle into NADPH which plays a role in antioxidant responses to stress (Cheung, Olin-sandoval and Grüning, 2015). NADPH is required for the scavenging of reactive oxygen species (ROS) which is critical to cell survival under stress conditions and also for fatty acid synthesis (Coates, Chin and Chung, 2011).

Here there also seems to a possible link between pathways, with the pentose phosphate pathway possibly generating fatty acids and then fatty acid degradation. The catabolism and biosynthesis of fatty acids would require the generation of ATP to maintain both pathways and this could be why there is also an upregulation of oxidative phosphorylation (Garrido-Pérez *et al.*, 2020). Endocytosis is used by cells to internalize nutrients molecules for recycling or degradation. It is also a mechanism cells use to communicate with their environment (Sigismund *et al.*, 2021).

All these pathways were downregulated in both the control and filtered group compared to the upregulated profile of bees fed on e-beam treated pollen, which suggests that e-beam is having an effect on the pollen that leads to a profile exclusive to those colonies and it could be down to a lack of vital microbes within the pollen or a change in the makeup of the pollen itself. It could be that NADPH is being used to combat toxicological effects of e-beam treated pollen and the cell is working overtime to breakdown various metabolites in order to recycle them into the TCA cycle to generate energy. Although the reason cannot be known for sure, it was observed that a dose of 100kGy dosage of e-beam, was having an effect on bees that was not witnessed in control or filtered fed groups.

Fructose and mannose metabolism was also upregulated. Both mannose and fructose are hexose sugars the same as glucose and can also be utilized for energy production within the cell. Fructose is metabolised through a process known as fructolysis with its metabolites entering glycolysis. Mannose metabolism is essential for post translational modifications of proteins, N-glycosylation. Glycoproteins play an import role in cells with some involved in iron transfer in the blood, neural development and communication. Mannose has also been shown in mice to have a positive effect on the microbiome leading to a lean phenotype (Lieu *et al.*, 2021). An upregulation in the colonies fed on e-beam treated pollen, could hint at either an excess of both sugars in the diet or a dependency on both sugars in the diet in order to maintain energy levels for normal function. Mannose and fructose metabolism was downregulated in the filtered group and normalised in the control, which suggests that e-beam treated pollen is altered in some way and this alteration is affecting the metabolism of bumblebees causing the upregulation of certain metabolic pathways in order to maintain homeostasis.

Another protein that was upregulated in e-beam treated pollen fed colonies was cytochrome b5. Cytochrome b5 are a family of reductase flavoproteins that regulate reduction oxidation (redox) reactions within a cell (J. Kim et al., 2016; Hall et al., 2022). This family exists as a single enzyme in Drosophila melanogaster CYB5R3 and acts as an electron donor by way of NADH oxidation. It plays a role in fatty acid elongation and cholesterol biosynthesis (Kim *et al.*, 2016) as well as being able to reduce heme and coenzyme Q (CoQ) (Hall *et al.*, 2022). These proteins can be found located on the endoplasmic reticulum (ER) (Borgese *et al.*, 1993), mitochondrial outer membrane or the plasma membrane (Kim *et al.*, 2016).

It is possible that it is playing a role in lipid homeostasis as fatty acid degradation was observed to be upregulated in two clusters when compared to the control in the heat maps. While in cluster C it was shown as normalised, this was still up compared to the control. In both clusters C and G fatty acid degradation was down in the control, which could be because the bees in control colonies were getting enough nutrients and therefore didn't need to resort to the breakdown of fat in order to maintain normal metabolic function. An upregulation of cytochrome b5 in e-beam treated pollen fed colonies could be a direct result of fatty acid degradation within these colonies and the need for fatty acid elongation and/or cholesterol biosynthesis. Cholesterol is essential to cell fluidity and integrity (Kim *et al.*, 2016). If bees in these colonies were facing some sort of a nutrient deficit, then it is possible that fatty acids are being broken down to generate ATP and a remodelling of degraded fatty acids is occurring to maintain lipid metabolism. Mitochondrial coenzyme A transporter SLC25A42 was also upregulated. This mitochondrial inner membrane protein transports both cytosolic dephosphoCoA (dPCoA) and coenzyme A (CoA), with CoA playing a role in fatty acid and cholesterol biosynthesis, fatty acid oxidation and amino acid metabolism (Philip L *et al.*, 2012). This suggests that there is a breakdown and restructuring of various fatty acids in order to maintain homeostasis.

Lysosomes can form pairings with phagosomes to remove damaged mitochondria, a process known as mitophagy. Within it there is macroautophagy and microautophagy, which are not limited to mitochondrial degradation, but also protein degradation and even endoplasmic reticulum (ER) removal (Yoo and Jung, 2018). Interestingly enough, disruption of mitochondrial respiration has been shown to increase lysosome biogenesis (Wong *et al.*, 2019). Digested lysosome materials are reused as building blocks in order to maintain cell homeostasis (Yang and Wang, 2021), so it's entirely possible that a feedback loop through degradation due to lack of nutrients is being created here in order to maintain survival.

All these proteins are downregulated in filtered pollen fed colonies, this could be due to a difference in the pollen make up due to the various treatment types. While essentially it's the same pollen, the microbial profile is most likely different. Spore forming microbes to the concentration of $1x10^6$ cells/mL were washed out, with most likely any smaller bacterial species that may be present. While e-beam pollen is technically microbe free, that doesn't mean that inactivated versions of the microbes are not present in the pollen. It is possible

some of these bacteria play a role in the recycling of metabolites generated through lysosomal activity. All three colony types had access to the same quantity and quality of sucrose solution, so this would suggest the differences in the TCA cycle upregulation is been driven by the pollen quality available or perhaps an over reliance on sucrose in order to maintain normal metabolic function.

Compared to the control and filtered colonies different protein profiles were observed, with heat maps only displaying similar profiles between e-beam and the control groups in cluster B and between e-beam and filtered groups in cluster E. In cluster E oxidative phosphorylation and selenocompound metabolism were both downregulated. The differences in profiles is suggestive of altered pollen composition and or nutritional value in both treated groups and that each treated group impacts the fatbody bee proteome based on how its treated.

Protein with decreased abundance in bees fed e-beam treated pollen

Melittin

Some of the proteins downregulated in e-beam treated pollen colonies were melittin. Melittin is a component of bee venom in honeybees with the related peptide in *Bombus* species known as bombolittins. It is a 26 amino acid residue that binds to cell membranes disrupting permeability (Guha *et al.*, 2021). It was downregulated in both e-beam treated and filtered pollen colonies, which would suggest that changes to the makeup of the pollen are the drivers behind its down regulation. It is possible that bees in these colonies are suffering some sort of nutritional deficit and have switched to survival mode only maintaining proteins essential for metabolism.

Immunity

Proteins that could be associated with inflammatory responses and immunity were also down regulated in e-beam treated pollen fed colonies. This could be in response to lack of pathogen presence. Proteins also associated with response to plant defences were also downregulated, which hint at changes to the pollen as well. Chymotrypsin's are a family of proteins that have developed in response to defence mechanisms produced by plants against insects that fed on them (Kim *et al.*, 2022). Serine proteases also fall under this umbrella (Dunse *et al.*, 2010). The protein fragments generated by these enzymes are then further digested by amino or carboxyl peptidases to generate amino acids for growth. It is then possible that mechanisms produced by pollen such as proteinase inhibitors and defensive enzymes (Kim *et al.*, 2022) have been deactivated by e-beam sterilization and this inactivation has led to a downregulation of proteins required to counteract it.

There is also a downregulation of peptidoglycan recognition proteins, which also could suggest a cleaner more pathogen free pollen. It is plausible that a lack of bacteria present in the pollen would no longer require the upregulation of pathogen recognition receptors (PRR's) to the same level as control colonies fed on non-treated pollen. Innate immunity utilises PRR's to activate immune pathways to generate peptides for the clearance of pathogens. These peptides such as defensins, attacins and cecropins are synthesised in the fatbody and secreted in the haemolymph. Upon invasion by bacteria either the *Toll* pathways (response to ve^{+} or fungi) or *Imd* pathways (response to ve-) are activated. Activation of Peptidoglycan receptors ultimately leads to the production of melanin (Kordaczuk, Sułek and Wojda, 2020). Melanin is generated through the enzyme phenoloxidase (PO) and leads to melanin being deposited around the pathogen, leading to its encapsulation and death via starvation (González-Santoyo and Córdoba-Aguilar, 2012).

The fact that these proteins are downregulated in e-beam treated pollen and not in the control hints that perhaps there is no need for them as the pollen is cleaner and in order to conserve energy, these pathways have been reduced. Nicotinamidase an enzyme that de-animates nicotinamide adenine dinucleotide (NAD) an electron donor central to glycolysis (Hunt, Lerner and Ziegler, 2004) was also downregulated. This could be because there is a lack of NAD available or because the nutrient quality is so poor that the necessary precursors for maintaining normal homeostasis are not available. In this case then perhaps the immune

channels are being downregulated in response to maintaining only what is essential for life. Fatty acid degradation was upregulated in cluster G so this might feed into this theory that starvation is playing a role in protein regulation and perhaps the immune pathways are being downregulated in favour of maintaining pathways for fatty acid degradation for ATP generation.

Cuticle formation

In insects cuticle layers make up the insect exoskeleton, which is shed during growth and development. This process is known as molting and the three layers that make up the exoskeleton are known as the outermost epicuticle, exocuticle and innermost endocuticle (Md Saheb Ali et al., 2020). The cuticle acts as a protective barrier as well as maintaining body morphology and integrity and is synthesised during the embryonic stage and then again at each larval change (Page and Johnstone, 2007). Chitin has also been demonstrated in human and some animal models to induce innate immune responses via macrophage activation and cytokine production (Lee, 2009). Chitinases facilitate the breakdown of chitin which is also a component of fungal cell walls (Oyeleye and Normi, 2018; Leoni *et al.*, 2019).

Both cuticle proteins and chitinases were downregulated in e-beam treated pollen fed colonies. Whether this is in relation to reproduction within the colonies or as a response to a lack of fungal pathogens present is unclear. This could signify a developmental retardation within the colonies, however both e-beam and filtered colonies produced larger bees compared to the control, so it is possible the downregulation of chitinase is more in response to a lack of fungus within the pollen. E-beam treated pollen exhibited zero fungal growth post experiment, compared to both control pollen and filtered pollen that exhibited extensive colonization by unknown fungal species.

Homeostasis and metabolism

Molecules essential for cell maintained and protein production were down regulated in bees fed on pollen treated with a 100kGy dose of e-beam. The TCA cycle and biosynthesis of amino acids was downregulated, as was Aminoacyl – tRNA biosynthesis, the proteasome and the ribosome. Amino acids are essential for growth and development during the larval to pupation stage (Manière *et al.*, 2020). Pollen has been shown to affect the levels of amino acids with non-essential amino acid levels being increased in pollen fed honey bees (Gage *et al.*, 2020). This could indicate that pollen composition has been altered in both e-beam treated and filtered samples or perhaps the nutritional content of the e-beam treated pollen was poor leading to a downregulation of the pathways involved. If pollen determines the level of non-essential amino acids then it would seem plausible that poor quality pollen or pollen significantly altered would not produce the same level of amino acids as good quality pollen. Studies have shown altered amino acid profiles in the brains of bees deprived of pollen (Gage *et al.*, 2020), however these were not quantified and the amino acid profile was not determined, so it was not possible to say if these profiles were altered between control and treated (e-beam & filtered) colonies. However, it is plausible due to the down regulation of amino acid biosynthesis in e-beam treated pollen colonies and upregulation of amino acid biosynthesis in filtered fed colonies. Further studies could determine if there was a change in amino acid profile and give a greater insight as to how it affects biosynthesis. The TCA also helps generate metabolites for the biosynthesis of proteins (Martínez-Reyes and Chandel, 2020) and this has been demonstrated to be affected by *Nosema Ceranae* infection independent of pollen deprivation. In pollen deprived bee higher levels of amino acids were observed in bees also infected by *N. ceranae* (Gage *et al.*, 2020). While there was no evidence of *N. ceranae* infection in bees, light microscope observations suggested the presence of *N. ceranae* spores within the pollen, which also could account for the baseline levels and elevated levels in the control and filtered colonies respectively. It could also explain why TCA cycle and amino acid biosynthesis was down regulated in e-beam colonies as a 100KGy dose of e-beam was expected to kill or inactivate all microbial life within the pollen. However it is also worth pointing out that the TCA cycle was also found to be upregulated in the heat map clusters which conflicts with it been downregulated on cluster A. This is also observed in the

filtered group with both being in opposite to each other in both groups which is perhaps most likely down to grouping, as these groups were more consistent in the control group in both clusters.

Aminoacyl tRNAs are responsible for adding the correct amino acid to its anticodon (Ibba and Soll, 2000), while ribosomes also play a role in protein synthesis with tRNA binding and translocation(Wilson and Cate, 2012). COP II helps facilitate protein transport from the endoplasmic reticulum (ER) through the generation of transport vesicles, and the physical deformation of the ER membrane (Sato and Nakano, 2007). The proteasome is a protein complex that degrades intracellular proteins (Tanaka, 2009).

With no discernible differences noted between the control and the e-beam treated pollen fed colonies, it is possible that the bees in these colonies naturally down regulate these pathways. It could be assumed that there is no external factors such as pathogens driving the down regulation as the e-beam pollen is deemed to be pathogen free, while the control colonies were fed with commercial pollen, which based on haemocytometer counts had suspected parasitic spores present at least in the region of $1x10^6$ cells/mL. Therefore, if it was pathogen driven the downregulation would be expected in the control but not in the e-beam treated colonies.

The two major pathways that were downregulated in e-beam treated pollen colonies in cluster E were selenocompound metabolism and oxidative phosphorylation. Selenium is a trace element that is utilized by animals and humans to carry out biological functions in the form of selenoproteins (Lazard *et al.*, 2017)**.** Twenty two eukaryotic selenoproteins are known and at least half of these are implicated as having antioxidant functions (Shantanam & Mueller, 2018)**.** Mitochondria have the ability to generate reactive oxygen species (ROS)as a by-product of aerobic respiration (Nolfi-Donegan, Braganza and Shiva, 2020). The OXPHOS system allows for the transportation of electrons to oxygen to generate a transmembrane gradient to allow for the passing of molecules through. Deficiencies in this pathway have been found to modify protein secretions from the cell, reduction in mRNA and leptin (food intake and energy expenditure hormone) as well as pathological production of ROS (Garrido-Pérez *et al.*, 2020). ROS over production can be damaging to lipid membranes proteins and

nucleotides (Garrido-Pérez *et al.*, 2020)**,** while low levels are used for cell signalling (Nolfi-Donegan, Braganza and Shiva, 2020)**.** These two pathways could be playing into each other here, however in what way exactly can only be hypothesised. Both are down in treated groups (e-beam and filtered) compared to the control. It might be better understood if selenocompound metabolism was up in comparison to oxidative phosphorylation and then it could be theorized that an overproduction of ROS is driving selenocompound metabolism, but it appears both are down. These pathways are also up in the control, so it is possible that differences in pollen are what's driving the differences in the pathways. It is quite possible that the differences between the colonies is down to nutritional value of the pollen. It could also be down to mitochondrial copy number within the cells themselves with lower numbers in the cells of the bees that inhabit e-beam treated pollen fed colonies. Mitochondrial mass increases during respiration and that protein systems essential for this have their activity increased (Pfanner, Warscheid and Wiedemann, 2019). A more active colony might require more energy for everyday tasks such as feeding and attending to the brood, so it might be expected that these colonies would require more energy. A colony that was starved or less active could be trying to conserve energy of using other pathways such as the β-oxidative pathway to generate ATP, which is why a downregulation of oxidative phosphorylation in both e-beam treated pollen fed colonies was observed.

The effects of filtered pollen on the fatbody proteome of Bombus terrestris

Proteins with increased abundance in bees fed on filtered pollen

Bees in the filtered group were fed daily on commercial pollen that had been put through a filtration process to remove suspected parasitic spores. These colonies experienced reduced numbers in terms of less workers, drones and gynes compared to control groups. Investigations into how it affected these bees on a molecular level were conducted by carrying out proteomic analysis of dissected fatbodies of 14 days old bees. The fatbody proteomic profile between *Bombus terrestris* fed on filtered pollen differed to those fed on control pollen. Numerous proteins and pathways were upregulated including proteins involved in growth and development, Insulin growth factor binding protein (RFC 48.5), metabolism, such as amino acid biosynthesis, Aminoacyl-tRNA biosynthesis, biosynthesis of unsaturated fats (Kegg 7/27) and fatty acids (Kegg 3/10), fatty acid degradation and Putative fatty acyl CoA reductase CG8306 (RFC 17.1), (Kegg 5/12), Chaoptin (RFC 16), and toxicity UDP-Gluctonosyltransferase 2B15 (RFC 11.3) and Cytochrome p450.

Unsaturated fats and Fatty acids biosynthesis

Biosynthesis of fatty acids and unsaturated fats (Kegg 7/27) were upregulated in bees fed on filtered pollen. Bees fed on filtered pollen also had less diversity in their gut microbiome and a greater abundance of *Firmicutes* and *Bacteroidota* compared to control colonies, but less *Firmicutes* present compared to e-beam treated colonies (chapter 5). High *Firmicutes* and *Bacteroidota* levels are an indicator of a high fat diet (HFD) while intake of unsaturated fatty acids (UFA) leads to an increase in diversity of gut microbiota with a decrease in *Firmicut*es and *Bacteroidota*. An increase in saturated fatty acids (SFA) in the diet decreases microbiota diversity (Wang *et al.*, 2021).

Taking into account the lack of diversity of the microbiota present, the higher number of *Firmicutes* present and the upregulation of fatty acid and unsaturated fat biosynthesis, it suggests that bees fed on filtered pollen are living on a HFD/SFA diet. The upregulation in the biosynthesis of unsaturated fats is probably a direct result of nutrition defects within the

pollen. Similar microbiome profiles were observed in e-beam treated pollen fed bees, yet the two methods of reducing the bioburden present are very different. If e-beam treated pollen is taken as absolute zero in terms of microbes present post-treatment then the differences in microbiota numbers present could be down to the method. Which could mean that the microbes present within the pollen play a role in the bees diet and their absence is feeding into bees in both colony treatments experiencing HFD/SFA diets with the difference in *Firmicutes* present being down to the differences in bacteria present within the pollen itself. Upregulation of fatty acid biosynthesis along with fatty acid degradation and fatty acid acyl CoA reductases which reduce fatty acids to fatty aldehydes or alcohols (Lehtinen *et al.*, 2018) might suggest that the pollen diet is leading to a change in metabolism and a reliance on fat stores in order to maintain normal homeostasis.

Metabolism

The main pathways upregulated were the TCA cycle, amino acid biosynthesis, the peroxisome and proteasome. These pathways were up in filtered fed pollen colonies. Amino acids are essential for growth and development during the larval to pupation stage (Manière *et al.*, 2020). Pollen has been shown to affect the levels of amino acids with non-essential amino acid levels being increased in pollen fed honey bees (Gage *et al.*, 2020). This could indicate that pollen composition has been altered in filtered samples. An increase in amino acid biosynthesis in filtered colonies, coupled with an increase in proteasomal and TCA cycle activity might suggest that the bees' metabolism required more energy in order to break down the filtered version of the pollen. If pollen determines the level of non-essential amino acids then it would seem plausible that poor quality pollen or pollen significantly altered would not produce the same level of amino acids as good quality pollen. Studies have shown altered amino acid profiles in the brains of bees deprived of pollen (Gage *et al.*, 2020), however, these were not quantified or the amino acid profile determined, so it was not possible to say if these profiles were altered between control and filtered colonies. The TCA also helps generate metabolites for the biosynthesis of proteins (Martínez-Reyes and Chandel, 2020) and this has been demonstrated to be affected by *Nosema ceranae* infection independent of pollen deprivation. In pollen deprived bees higher levels of amino acids were observed in bees also infected by *N. ceranae* (Gage *et al.*, 2020). While there was no evidence

of *N. ceranae* infection in bees, light microscope observations suggested the presence of *N. ceranae* spores within the pollen, which also could account for the baseline levels and elevated levels in the control and filtered colonies respectively.

An upregulation of aminoacyl tRNA biosynthesis coupled with Cop II vesicle transport and a high ribosome presence could indicate an increase in protein production. Aminoacyl tRNAs are responsible for adding the correct amino acid to its anticodon (Ibba and Soll, 2000), while ribosomes also play a role in protein synthesis with tRNA binding and translocation(Wilson and Cate, 2012). COP II helps facilitate protein transport from the endoplasmic reticulum (ER) through the generation of transport vesicles, and acts in the physical deformation of the ER membrane (Sato and Nakano, 2007). The proteasome is a protein complex that degrades intracellular proteins (Tanaka, 2009).

Both amino acid biosynthesis and COP II vesicle transport was upregulated in cluster A and B compared to control samples, so it makes sense that translation and protein localisation and processing would also be upregulated along with vesicle transport. The Golgi apparatus acts as the main transport pathway for the secretion of proteins between the endoplasmic reticulum (ER) and the cell surface (Prydz, Dick and Tveit, 2008).

The upregulation in amino acid synthesis and in turn protein synthesis and trafficking, could be due to a need for maintenance of normal cell signalling. This could be disrupted due to a HFD/SFA diet and why there is an upregulation also in fatty acid degradation.

Whether the proteasome is upregulated in response to over production of proteins or is facilitating production of essential proteins is unclear, however it does seem plausible that these pathways are feeding into each other and driving protein production and degradation. It is not clear what is driving the response, because this was not observed in either the control or e-beam treated colonies. It would be expected that control is the standard that response is measured from, so the fact that a similar profile was observed in the e-beam would suggest that something else is going on with filtered pollen. It could be nutrition defect or perhaps the emergence of a microorganism now becoming dominant in the presence of a reduced spore count and the bees metabolism adjusting to that accordingly. Fungal growth was observed on both control pollen and filtered pollen, however the time period was months on the control compared to days to a week on filtered.

Some of the main pathways and functions that were affected in Cluster C were Aminoacyl tRNA and N-glycan biosynthesis, fatty acid degradation and cytochrome P450 and isoprenoid biosynthetic process. These were upregulated in filtered fed colonies and down regulated in the control colonies. Isoprenoids are a diverse group of molecules made from isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) produced from a pathways that uses acetyl-coenzyme A (CoA) (Guggisberg, Amthor and Odom, 2014). They are essential molecules of both primary and secondary metabolism with vitamins and steroids being but two (Rudolf *et al.*, 2021) as well as having a role in cell growth regulation and energy production (Guggisberg, Amthor and Odom, 2014). If this is taken in conjunction with fatty acid degradation, it mean that bees fed on filtered pollen are lacking in nutrients and that the bees metabolisms in these colonies are working harder in order to generate the energy necessary to carry out daily functions

Isoprenoid biosynthetic process was also upregulated. These consist of over 80,000 known compounds that are essential constituents of primary and secondary metabolism made up of steroids and vitamins (Rudolf *et al.*, 2021) as well regulation of cell growth, intracellular signalling and energy production (Guggisberg, Amthor and Odom, 2014)**.** It is possible that they are playing a role in energy production and cell signalling in conjunction with other pathways and proteins as part of the cycle of protein degradation and recycling.

Toxicity

Other proteins upregulated included members of the cytochrome p450 family cytochrome p4509e2. Cytochrome P450 enzymes metabolise toxins and promote tolerance. In *Apis mellifera* these enzymes play a role in detoxification of pesticides (Mao, Schuler and Berenbaum, 2011). It is possible that there is a toxic effect being observed from the filtered pollen. This could be due to a change in molecular makeup of the pollen itself.

UDP-Glucuronosyltransferases (UGT) help mediate the excretion of xenobiotics and endobiotics from the body via conjugation with glucuronic acid. These UGTs are part of a super family of enzymes that change depending on disease state, diet and age (Knights, Rowland and Miners, 2013). They are located in the membrane if the endoplasmic reticulum (ER)

removing lipophilic molecules, that originate internally or externally (Manevski *et al.*, 2013). Upregulation could hint at a toxicity element brought about by excess lipids.

Growth and development

Insulin like growth factor (IGF) plays a role in growth development, reproduction, stress resistance and lifespan, which are normally encoded by single genes in invertebrates (Reza, Ugorski and Suchański, 2021), with *Drosophila melanogaster* having insulin like peptide genes (Reza, Ugorski and Suchański, 2021). Insulin like peptides, such as IGF mediate metabolic homeostasis in response to nutritional resources available (Naoki Okamoto, 2018). In each of the clusters A-G, the proteomic profile among each treatment was different with proteins up regulated or downregulated according to pollen type being fed. Downregulation can affect energy homeostasis. Here IGF was upregulated compared to control populations which could be why larger bees were observed in filtered colonies compared to control colonies. Insulin acts to regulate carbohydrate and lipid metabolism, whereas IGF work to promote body and tissue growth during development, in response to the nutritional status (Naoki Okamoto, 2018). Amino acid biosynthesis is upregulated in filtered populations compared to both control (normal) and e-beam (down). Amino acid availability is key for larval growth and development in *Drosophila* (Naoki Okamoto, 2018) and this could also be the case here. However, both filtered and e-beam colonies produced larger bees compared to the control and amino acid biosynthesis was downregulated in e-beam pollen fed colonies so there could be other factors driving this.

Glucosylceramidase breaks down glucosylceramide (GlcCer) to glucose and ceramide. The main site for degradation is in lysozymes. GlcCer is a founding molecule in glycosphingolipid synthesis. These function in cell adhesion, recognition, growth, development and inflammation (Reza, Ugorski and Suchański, 2021). Deficiencies in glucosylceramidase can impair memory, and movement via accumulation of glucosylceramide (Reza, Ugorski and Suchański, 2021). Interestingly enough glycosphingolipid biosynthesis was downregulated in cluster D and was upregulated in control populations. This could suggest that glucosylceramidase was not directly involved in glycosphingolipid synthesis but rather this

was shift in metabolism to try generate as much glucose as possible for body maintenance and ATP generation.

Fatty acid degradation

Biosynthetic fatty acid process and biosynthesis of unsaturated fatty acids upregulated was detected in string while upregulated of fatty acid degradation was also observed. It could mean that bees fed on filtered pollen are lacking in nutrients and that the bees metabolisms in these colonies are working harder in order to generate the energy necessary to carry out daily functions. Acetyl CoA again plays a role here in the synthesis of fatty acids (FA) with carboxylation of the molecule to form malonyl CoA which is further condensed with Acetyl CoA (De Carvalho and Caramujo, 2018). This process can then be reversed to generate Acetyl CoA that can enter the citric acid cycle (TCA) (De Carvalho and Caramujo, 2018). In a state of starvation fatty acids are broken back down into acetyl CoA through a process known as β-oxidation in order to provide fuel for extrahepatic tissues (Rui, 2014), one 16 carbon molecule of FA can yield 129 ATPs, which is 3 times greater than the yield generated from a single glucose molecule. However it consumes more oxygen, generates (ROS) and is much slower (Poitelon, Kopec and Belin, 2020). Oxidative phosphorylation was however, downregulated in filtered colonies as was tissue development, so it is possible β-oxidative pathways were used. The upregulation of these 2 pathways in filtered pollen fed colonies, could suggest that what is being witnessed is due to nutrient deficiency and would align with what was observed in cluster B with an upregulation in possible protein synthesis. It might also hint that the nutritional content of e-beam treated pollen is better, taking that control colonies have all these pathways down regulated.

Pancreatic lipase was also upregulated in bees fed on filtered pollen compared bees fed on untreated commercial pollen. In mammals these lipases are made up of 7 subfamilies, playing a role in the digestion of dietary fats, converting triglyceride to diglyceride and eventually glycerol (Lim et al., 2022.).

Another protein upregulated that might suggest an excess of lipids is fatty acid acyl CoA reductase. Fatty acid reductases belong to a family of oxidoreductases and use either NADPH or NAD to reduce fatty acids to fatty alcohols. These are also localised in the membrane of the

ER. These fatty alcohols can protect against water loss in insects and are the main pheromone components in bees (Tupec *et al.*, 2017).

The breaking down of lipids for both synthesis of fatty alcohols or removal through excretion and its upregulation compared to the control suggest that something is driving this change in metabolism. While filtered pollen is not irradiated it is possible that its washing is somehow contributing to this upregulation of molecules responsible for lipid breakdown. It could be that certain microbes that would be present and utilised for macromolecules are no longer in abundance and so the shift has been to breakdown whatever is available and in this case lipids.

Photoreceptor cells

Two proteins were also identified that act on photoreceptor cells, chaoptin (RFC 16) a cell adhesion molecule expressed in photoreceptor cells in adult eyes (Krantz and Zipursky, 1990) and retinol dehydrogenase (RFC 17.1) a member of the short chain dehydrogenase/reductase family (SDR) which catalyses retinol through a NAD(P) dependent redox reaction in vitamin A metabolism (Hofmann, et al., 2018) .

Lack of vitamin A, can lead to impaired photoreceptor function and in turn night blindness. Vitamin A is sourced through plants as animals cannot synthesis it *de novo* (Deepshe, et al., 2021) .

Upregulation of enzymes that act upon photoreceptor cells could be down to an increase in the availability of vitamin A within the diet. However since the metabolism of vitamin A is essential for vision it could be that what is being observed is an increase due to a growth burst within the colonies. It is interesting that these proteins are upregulated in filtered colonies and not in control, but it is not possible to guess as to the possible implications. It could be that bees in filtered fed pollen colonies are developing at different rates and that's why an upregulation here is being observed here. It could that these bees are developing late and so these proteins are then upregulated where as in the control it's a more normalised pattern.

Proteins with a decreased abundance in bees fed on filtered pollen

String analysis of fatbodies from bees fed on filtered pollen and compared to controls highlighted pathways and proteins involved that were downregulated compared to control colonies. These included proteins and pathways involved in development, metabolism and immunity. Some of the main proteins and pathways that were downregulated included glycosphingolipid biosynthesis, fructose and mannose metabolism, muscle cell development, peptidoglycan recognition protein and chymotrypsin and melittin which was the most downregulated protein in colonies fed on filtered pollen. It was also the most downregulated protein in colonies fed on e-beam treated pollen too. Melittin is a component of bee venom known as bombolittins in *Bombus* species. It binds to cell membranes disrupting permeability (Guha *et al.*, 2021). It is possible it is not seen as a vital protein so in times of stress is not upregulated, or perhaps it has a role to play in immunity and it is why it is downregulated here, as other proteins associated with immunity were also downregulated.

Growth and Development

Proteins downregulated here were troponin, which forms a complex with tropomyosin. Thin filaments in skeletal muscle are activated and regulated by Ca2+ (Qiu *et al.*, 2003). A rise in cytosolic Ca2+ initiates contraction (Cao, Thongam and Jin, 2019). When Ca2+ binds tropomyosin-troponin complex the shift exposes the myosin binding site of actin (Qiu *et al.*, 2003), binding of myosin heads to actin thin filaments form cross bridges which activate myosin ATPase and allow the movement along the thin filament (Cao, Thongam and Jin, 2019).

Troponin T 10A isoform is a Ca2+ binding regulatory protein which is upregulated in mature foragers of *Apis mellifera* and is thought to affect flight performance (Schippers *et al.*, 2006). The extract isoform of troponin is not known however it forms a part of the supramolecular complex that plays a role in indirect flight (Qiu et al., 2003; Schippers et al., 2006; Cao et al., 2019) and a switch to a reliance on carbohydrates to power flight (Schippers *et al.*, 2006).

If an increase in troponin is a signal for the transition to foraging, then downregulation could hint at a delay in that transition. Fewer females in colonies fed on filtered pollen were observed, so this be another aspect tying in with a lower reproductive output and a delay in transition.

Motor proteins also play a role in vesicle transport such as endocytosis (Calábria *et al.*, 2011). Endocytosis was also downregulated in bees fed on filtered pollen so it is possible downregulation of these proteins is also affecting vesicle transport.

Many proteins, pathways and biological functions relating to muscle development, cardiac muscle development, muscle cell development and actin and cytoskeletal organisation were also detected. The down regulation of cytoskeletal structure and organisation could play a role in reduced mitochondrial reproduction. Actin facilitate the delivery of mitochondria into the bud which eventually divides into a daughter cell. The cytoskeleton also plays a role in mitochondrial distribution within a cell (Rizki and Boyer, 2015). This could play a role here leading to a reduction of mitochondria which in turn is leading to a reduction of aerobic respiration and overall reduction in cell signalling and tissue development. Mitochondria morphology and number play a role in energy production with elongated and fused mitochondria displaying a higher mitochondrial respiration (De Goede *et al.*, 2018). It is also possible that whatever is in effect here is also having an effect on the CNS, as glycosphingolipid biosynthesis was also downregulated compared to the control population of bees.

Glycosphingolipids play a role in maintaining normal functioning the central nervous system (Schengrund, 2020). They are made up of a carbohydrate linked to a hydrophobic aglycon and are minor components cellular membranes (Sandhoff and Kolter, 2003). The glycosphingolipids identified were of the ganglio series where they are highly concentrated in the CNS (Schwarzmann, 2018). If the downregulation of glycosphingolipids is taken in conjunction with the downregulation of motor protein function, then perhaps it's possible that bees fed on filtered pollen are experiencing developmental delays in terms of cognitive ability too. Glycosphingolipid biosynthesis was down in filtered pollen colonies. The ganglio series of this glycolipids can be found on the cell surface of eukaryotic cells and are abundant in neuronal cells, helping with normal function of the central nervous system (CNS) (Sandhoff and Kolter, 2003). They can also play a role in cell to cell signalling, and regulate response of signal proteins such as insulin receptors (Schwarzmann, 2018). Insulin signalling has a role to

play in regulating growth, reproduction, metabolism and stress response and a reduction in insulin signalling can be found during periods of starvation (Heier and Kühnlein, 2018). A reduction in reproductive output of colonies fed on filtered and e-beam treated pollen, was observed, which could suggest a disruption in insulin signalling, however downregulation of insulin molecules was not directly observed. An upregulation of insulin binding growth factor (RFC 48.5) was however, so perhaps insulin signalling is not having a major impact on growth here, however it is interesting that insulin binding growth factor is upregulated here and not in e-beam as bees in both these groups exhibited a slight but significant size difference compared to control groups (chapter 3). It would suggest that the size difference is not being attributed insulin binding growth factor, but some other protein(s) and pathways. Bees fed on filtered pollen also had a upregulation of vitellogenin (RFC 13.9) a protein associated with longevity (Alaux *et al.*, 2011). Colonies fed on filtered pollen, did not experience retarded growth or increased mortality but rather a decrease in numbers and sex and the pathways responsible for this would require further more refined and explorative measures to try and determine in the future.

Immunity

Several proteins that are associated with innate immunity were down regulated in bees fed on filtered pollen. These included serine proteases, chymotrypsin, peptidoglycan recognition, phospholipase and chitinase. Chitinases will breakdown chitin a component of the fungal cell wall (Oyeleye & Normi, 2018 ;Leoni et al., 2019), also activation of *Toll* will lead to a the transcription of drosomycin in the nucleus and other antimicrobial peptides that will act upon fungi and gram positive bacteria (Dziarski and Gupta, 2006). Insects respond to bacterial pathogens through the activation of either the *Imd* pathway for Gram negative bacteria or *Toll* pathway against gram positive bacteria (Dziarski and Gupta, 2006).

Phospholipase liberates arachidonic acid from glycerophospholipids. Arachidonic acid (AA) is a precursor molecule from leukotrienes (LTs) and prostaglandins (PGs) (Murakami and Kudo, 2002). Prostaglandins are known to play a key role in the mediating inflammatory responses by recruiting immune cells (Ricciotti et al., 2011).

Glycosyl hydrolases are a family of chitinases (Lee *et al.*, 2013). Chitin is a component of the fungal cell wall and its breakdown is carried out by chitinases (Oyeleye and Normi, 2018). Its downregulation could mean bee fed on filtered pollen are more susceptible to fungal species. Filtering pollen led to a more rapid fungal growth on the pollen surface compared to control samples. It could be possible that fungal species in filtered pollen are playing a more dominant role due to the filtration of other microbial species and are somehow inhibiting chitinases to facilitate their growth *in vivo*. However the exact mechanism here is unknown and would require further exploration to try validate this theory.

The fatbody proteomic profile is also similar to what was observed in the bees fed on e-beam treated pollen, with a downregulation of proteins involved in or that play a role in innate immunity. This could suggest that both treatments are effective in reducing bacterial, fungal and possibly parasite numbers. Phagosome and lysosome activity was also downregulated in filtered fed pollen colonies compared to both control and e-beam treated colonies which could further add weight to the theory that bees fed on filtered pollen are exposed to fewer microbes. It could be argued that filtered pollen is having a positive effect on the bee health in these colonies in terms of being beneficial towards immunity, however there seems to be upregulation of a lot of proteins involved with ATP generation and lipid degradation to suggest that these benefits are cancelled out by the bees need to generate energy through alternative pathways in order to survive. Bees in these colonies also for the majority of colonies failed to produce males or gynes, suggesting that the pollen is having an effect not only at a molecular level, but it is also translating into reproductive output and phenotype.

An alternative possibility is that immune genes are downregulated due to a lack of nutrients, as maintaining an immune response is costly in invertebrates (Barribeau et al., 2016). Therefore it is not impossible to think that bumblebees in treated colonies have downregulated their immune genes in response to favouring metabolism in order to survive. Further investigations would be necessary in order to determine if this is the case or not.

Stress and Metabolism

Selenocompound metabolism and oxidative phosphorylation were downregulated in filtered pollen colonies when compared to the control. Selenium is a trace element that is utilized by animals and humans to carry out biological functions in the form of selenoproteins (Lazard *et al.*, 2017)**.** Twenty two eukaryotic selenoproteins are known and at least half of these are implicated as having antioxidant functions (Shantanam & Muller, 2018)**.** Mitochondria have the ability to generate reactive oxygen species (ROS), as a by-product of aerobic respiration (Nolfi-Donegan, Braganza and Shiva, 2020). Oxidative phosphorylation allows for the transportation of electrons to oxygen to generate a transmembrane gradient to allow for the passing of molecules through. Deficiencies in this pathway have been found to modify protein secretions form the cell, reduction in mRNA and leptin (food intake and energy expenditure hormone) as well as pathological production of ROS (Garrido-Pérez *et al.*, 2020). ROS over production can be damaging to lipid membranes proteins and nucleotides (Garrido-Pérez *et al.*, 2020)**,** while low levels are used for cell signalling (Nolfi-Donegan, Braganza and Shiva, 2020)**.** These two pathways could be playing into each other here, however in what way exactly can only be hypothesised. Both are down in treated groups (e-beam and filtered) compared to the control. These pathways are also up in the control, so it is possible that differences in pollen are what's driving the differences in the pathways which could be down to nutritional value in the pollen.

A colony that was starved or less active could be trying to conserve energy by using other pathways such as the β-oxidative pathway to generate ATP, which is why a downregulation of oxidative phosphorylation in filtered pollen samples was being observed.

Energy metabolism in *B. terrestris* fed on filtered treated pollen was downregulated compared to that of bees fed on control pollen. The TCA cycle, the phagosome, lysosome and fructose and mannose metabolism, aerobic respiration and Ubiquinol-cytochrome-c reductase activity, and oxidative phosphorylation and pentose phosphate pathway.

Both mannose and fructose are hexose sugars the same as glucose and can also be utilized for energy production within the cell. Fructose is metabolised through a process known as fructolysis with its metabolites entering glycolysis. Mannose metabolism is essential for post translational modifications of proteins, N-glycosylation. Glycoproteins play an import role in

cells with some involved in iron transfer in the blood, neural development and communication. Mannose has also been shown in mice to have a positive effect on the microbiome leading to a lean phenotype (Lieu *et al.*, 2021).

Lysosomes can form pairings with phagosomes to remove damaged mitochondria, a process known as mitophagy. Within it there is macroautophagy and microautophagy, which are not limited to mitochondrial degradation, but also protein degradation and even endoplasmic reticulum (ER) removal (Yoo and Jung, 2018). Interestingly enough, disruption of mitochondrial respiration has been shown to increase lysosome biogenesis (Wong *et al.*, 2019), however a downregulation in lysosome activity was observed. It is possible that these are independent and that selective degradation of mitochondrial proteins is occurring via lysosome degradation activity (Wong *et al.*, 2019) and is perhaps somehow feeding into the TCA cycle. Digested lysosome materials are reused as building blocks in order to maintain cell homeostasis (Yang & Wang, 2021), so it is entirely possible that a feedback loop through degradation due to lack of nutrients is being created here in order to maintain survival.

Endocytosis, fatty acid metabolic process and the pentose phosphate pathway were all downregulated in bees fed on filtered pollen (cluster G).

The pentose phosphate pathway is a key pathway in the formation of molecules for nucleic acid and amino acid biosynthesis while the non-oxidative pathway can supply glycolysis with the necessary metabolites required for normal function. It also converts glucose 6 phosphate an intermediate of the TCA cycle into NADPH which plays a role in antioxidant responses to stress (Cheung, Olin-sandoval and Grüning, 2015). NADPH is required for the scavenging of reactive oxygen species (ROS) which is critical to cell survival under stress conditions and also for fatty acid synthesis (Coates, Chin and Chung, 2011).

The catabolism and biosynthesis of fatty acids would require the generation of ATP to maintain both pathways (Garrido-Pérez *et al.*, 2020). Endocytosis is used by cells to internalize nutrients molecules for recycling or degradation. It is also a mechanism cells use to communicate with their environment (Sigismund *et al.*, 2021). These are downregulated in filtered pollen fed colonies, and while it is essentially the same pollen, the microbial profile is most likely different. Spore forming microbes to the concentration of $1x10^6$ cells/mL were washed out, with most likely any smaller bacterial species that may be present. While e-beam

pollen is technically microbe free, that doesn't mean that inactivated versions of the microbes aren't present in the pollen. It is possible some of these bacteria play a role in the recycling of metabolites generated through lysosomal activity and their absence might explain the downregulation of the lysosome. It is also possible that filtered fed colonies are not under the same level of stress as e-beam fed pollen or control colonies and hence why this machinery is down regulated. All three colony types had access to the same quantity and quality of sucrose solution, so this would suggest the differences in metabolic processes is been driven by the pollen quality available.

Heat map cluster similarities

Similarities were observed between clusters (Figure 4.3.3) in the control group and e-beam group (cluster B), e-beam and filtered group (cluster E) and the control group and filtered group (cluster G). In all cases the proteins and pathways were downregulated with the oxidative phosphorylation (OXPHOS) pathway down regulated in both cluster E and F and downregulated in filtered in both those clusters, suggesting that filtered pollen could be in some way responsible for the downregulation of the OXPHOS pathway, even though it was observed in both the control and e-beam groups.

In cluster B some of the main pathways and molecules affected were Cop II vesicle transport, Aminoacyl t-RNA biosynthesis, the ribosome and the proteasome. These were downregulated in both control and e-beam treated pollen fed colonies while being upregulated in filtered fed pollen colonies. With no discernible differences noted between the control and the e-beam treated pollen fed colonies, it is possible that the bees in these colonies naturally down regulate these pathways. It could be assumed that there is no external factors such as pathogens driving the down regulation as the e-beam pollen is deemed to be pathogen free, while the control colonies were fed with commercial pollen, which based on haemocytometer counts had spores present at least in the region of $1X10^6$ cells/mL. Therefore if it was pathogen driven the downregulation would be expected in the control but not in the e-beam

treated colonies. Filtered pollen removes spores and possibly other bacteria present through a simply filtration step, it does not guarantee that the pollen is microbe free, whereas a 100kGy dose of e-beam is considered to have inactivated all microbial life present. It is possible that by reducing spore and microbial levels, it somehow allows for the upregulation these pathways. That a balance between, microbe free pollen and microbe enriched pollen is having an effect on the bees metabolism. It is also entirely plausible that this is completely independent of microbial content and perhaps nutrient driven, with filtered pollen suffering a nutrient lose during the process. An upregulation of Aminoacyl tRNA biosynthesis coupled with Cop II vesicle transport and a high ribosome presence could indicate an increase in protein production. Aminoacyl tRNAs are responsible for adding the correct amino acid to its anticodon (Ibba and Soll, 2000), while ribosomes also play a role in protein synthesis with tRNA binding and translocation(Wilson and Cate, 2012). COP II helps facilitate protein transport from the endoplasmic reticulum (ER) through the generation of transport vesicles, it also affects the physical deformation of the ER membrane (Sato and Nakano, 2007). The proteasome is a protein complex that degrades intracellular proteins (Tanaka, 2009).

Whether the proteasome is upregulated in response to over production of proteins or is facilitating production of essential proteins is unclear, however it does seem plausible that these pathways are feeding into each other and driving protein production and degradation. It is not clear what is driving the response, because this was not observed in either the control or e-beam treated colonies. It would be expected that control is the standard that response is measured from, so the fact that a similar profile was observed in the e-beam would suggest that something else is going on with filtered pollen. It could be nutrition defect or perhaps the emergence of a microorganism now becoming dominant in the presence of a reduced spore count and the bees metabolism adjusting to that accordingly. A fungal growth on both control pollen and filtered pollen was observed, however the time period was months on the control compared to days to a week on filtered.

The two major pathways affected in cluster E was selenocompound metabolism and oxidative phosphorylation both of which were downregulated in e-beam treated pollen and filtered pollen colonies when compared to the control. Selenium is a trace element that is utilized by animals and humans to carry out biological functions in the form of selenoproteins (Lazard *et al.*, 2017)**.** In eukaryotes some selenoproteins are known to have antioxidant functions

(Shantanam and Muller, 2018). The OXPHOS system allows for the transportation of electrons to oxygen to generate a transmembrane gradient to allow for the passing of molecules through. Deficiencies in this pathway have been found to modify protein secretions from the cell, reduction in mRNA and leptin (food intake and energy expenditure hormone) as well as pathological production of ROS (Garrido-Pérez *et al.*, 2020). ROS over production can be damaging to lipid membranes proteins and nucleotides (Garrido-Pérez *et al.*, 2020)**,** while low levels are used for cell signalling (Nolfi-Donegan, Braganza and Shiva, 2020)**.**

Downregulation of both here could affect cell signalling and transport of molecules and ultimately affect energy production. The TCA cycle was also downregulated in the e-beam group (cluster A) while fructose and mannose metabolism along with fatty acid degradation were up and normalised respectfully which could suggest that e-beam treated pollen is more nutrient deprived than filtered pollen. It does seem very possible that both pathways being downregulated here are as a direct result of the pollen being treated. Another possible explanation for how these groups (e-beam & filtered) are being affected in terms of energy and metabolism is down to the lack of microbes present in the pollen. Both treatments reduce microbial content in different ways and this could explain why similarities are seen in some pathways and not others. It is possible that microbes present in the pollen add to the nutritional value and are recycled for protein and energy metabolism. A difference in microbes present could cause a difference in what pathways are upregulated and downregulated as a consequence of what microbes are present.

In cluster G the control and filtered fed pollen colonies both had their fatbody protein profile down regulated while the colonies fed on e-beam treated pollen saw an upregulation of their fatbody protein profile. Some of the main processes and pathways involved were oxidative phosphorylation, endocytosis, fatty acid degradation and the pentose phosphate pathway.

The pentose phosphate pathway is a key pathway in the formation of molecules for nucleic acid and amino acid biosynthesis while the non-oxidative pathway can supply glycolysis with the necessary metabolites required for normal function. It also converts glucose 6 phosphate an intermediate of the TCA cycle into NADPH which plays a role in antioxidant responses to stress (Cheung, Olin-sandoval and Grüning, 2015). NADPH is required for the scavenging of

reactive oxygen species (ROS) which is critical to cell survival under stress conditions and also for fatty acid synthesis (Coates, Chin and Chung, 2011).

Here there seems to a possible link between pathways, with the pentose phosphate pathway possibly generating fatty acids and then fatty acid degradation. The catabolism and biosynthesis of fatty acids would require the generation of ATP to maintain both pathways and this could be why there is suggestion of an upregulation of β-oxidation (Garrido-Pérez *et al.*, 2020). Endocytosis is used by cells to internalize nutrients molecules for recycling or degradation. It is also a mechanism cells use to communicate with their environment (Sigismund *et al.*, 2021).

The profiles of control and filtered pollen fed colonies is similar, with both downregulated compared to the upregulated profile of the e-beam treated pollen colonies, suggesting that e-beam is having an effect on the pollen exclusive to those colonies and it could be down to a lack of vital microbes within the pollen or a change in the makeup of the pollen itself. It could be that NADPH is being used to combat toxicological effects of e-beam treated pollen and the cell is working overtime to breakdown various metabolites in order to recycle them into the TCA cycle to generate energy. Although the reason cannot be known for sure, it can be said that using e-beam at a dose of 100kGy is having an effect on bumblebee colonies that is not witnessed in control or filtered fed groups.

4.5 Conclusion

Bumblebees fed on either pollen treated with a 100KGy dose of e-beam or filtered exhibited a remarkably different proteomic profile from those fed on untreated commercial pollen. Some of the side effects witnessed were a switch to β-oxidation as a means to generate ATP as well as a downregulation of genes associated with immunity. It is plausible that in both pollen types (e-beam & filtered) the composition has been altered significantly enough to cause the proteomic shifts observed. Whether that is down to removal of key bacteria that might be recycled for key nutrients or because of physical alteration to the pollen itself will remain to be determined by future studies. However, it is likely in the case of e-beam treated pollen that both these factors are combining in synergy, where with filtered pollen it seems likely that key bacteria, fungi and possibly some nutrients are being washed from the pollen itself.

It is possible that by treating the pollen, key microbial species that help shape the gut microbiome and fatbody proteomic profile are being removed or killed which in turn is having an effect on reproductive output, metabolism, stress and immunity. The other explanation is that key nutrients are being washed out (filtered) or denatured (e-beam). Either way by attempting to remove potential harmful pathogens from the pollen, not only was the colony reproductive output affected, but also the fatbody proteomic profile, which appears to have had a negative effect on energy production and metabolism.

Chapter 5 Assessing the impact of sterilised pollen on the *Bombus terrestris* digestive tract microbiome.

5.1 The bacterial microbiome of bees

The insect gut is generally made up of 3 different regions, the foregut, lined with chitin made exoskeleton, often containing a separate crop for temporary food storage. The midgut which contains no exoskeletal lining an is the main area of digestion and absorption, and the hindgut which is also lined with an exoskeletal layer, that houses many chambers, including ones for fermentation and one for storage of faeces (Engel and Moran, 2013). The hindgut of the bee is known to host up to 95% of the microbiota, while the foregut and midgut contains few bacteria and fungi. The hindgut comprises the ileum, which is home to species such as *Gilliamella apicola*, *Frishella perrara* (in honeybees) and *Snodgrassell*a, while the rectum is dominated by *Lactobacillus-4, 5* and *Bifidobacterium.* The foregut tends to be dominated by *Lactobacillus* and *Acetobacteraceae*. A mature bee worker can be colonized by as many as 1 billion microbial cells (Moran, 2015), which are thought to play a role in macromolecule digestion, nutrient provisioning, protection against toxins that are acquired through diet and offer defence against parasites (Engel *et al.*, 2016).

Gilliamella apicola is a Gram negative, predominant fermentative bacteria with a complete glycolysis pathway and phosphotransferase systems allowing them to digest a carbohydrate rich diet as well as pectin encoding genes that could aid digestion of pollen polysaccharide walls to monosaccharides. *G. apicola* can metabolize mannose, xylose and arabinose (Zheng *et al.*, 2016). Studies carried out by (Zhang Guo, et al., 2022) identified 27 *Gilliamella* species and 7 species of *Snodgrassella*.

Snodgrassella is a *Betaproteobacteria* and is also one of the core bacteria that inhabit guts of honey bees and bumblebees (Cornet *et al.*, 2022) and forms a biofilm in the gut with *Gilliamella* growing on top and can trigger a host-beneficial immune response (Zhang Guo, et al., 2022).

Lactobacilli are Gram positive cocci or coccobacilli lactic acid bacteria that produce lactic acid. They produce lactic acid via carbohydrate metabolism and can be either homofermentative (85% of fermentation product is lactic acid) or heterofermentative (lactic acid, CO_2, ethanol

or acetic acid are produced in equimolar amounts) (Tannock, 2004). Studies involving *Lactobacillus* have found that its monocolonization of the honey bee gut can improve learning and memory with similar results using oral supplementation of *Lactobacillus* in bumblebees also seeing improved memory and increased levels of glycerophospholipids in the hemolymph (Zhang Mu, et al., 2022).

The species comprising the bee microbiome remain constant under many stresses, however infection with *C. bombi* can alter its makeup. Bumblebees placed under nutritional stress and subjected to immune priming have been reported to show no change in the microbiome. However bumblebees challenged with *Nosema bombi* or *C. bombi* did exhibit a change in microbiota diversity (Koch, Cisarovsky and Schmid-Hempel, 2012). Studies have also indicated that the absence of total microbiota have led to increased levels of *C. bombi* infections, with *Betaproteobacteria* decreasing the likelihood of an infection (Koch and Schmid-Hempel, 2011). Further experiments using *Bombus impatiens* as a test model revealed that *Apibacter*, *Lactobacillus-5* and *Gilliamella* may be responsible for conferring resistance to *C. bombi*, the latter two comprising of the core group of gut microbiota in both honey and bumblebees. Other core members include *Bifidobacterium, Bombiscardivia, Lactobacillus-4* and *Snodgrassella,* with *Snodgrassella* being subject to conflicting reports as to its ability to offer resistance to *C. bombi*. *Apibacter* demonstrated the greatest ability to inhibit *C. bombi* infection (Mockler *et al.*, 2018).

Nosema ceranae, a honeybee parasite that resides in the midgut, has also demonstrated subtle effects on the gut microbiome with an increase of two *Gilliamella* strains. There are a number of different strains of *Gilliamella* that live in the honeybee, with different genetic makeup and possible functions and how exactly the slight upregulation of these two strains impacts on the honeybee is not yet known (Rubanov *et al.*, 2019).

The microbiome also plays a role in increasing the survival of bees exposed to metalloids. Metalloids are groups of elements that lie between metals and non-metals, exhibiting some properties of both (Dixon and Vasiliu, 2019). When they get into the environment they can contaminate soil and pose a risk to human health (Li *et al.*, 2017). The metalloid selenate can be found in contaminated soils and this exposes bees foraging on nectar and pollen. Experiments exposing *Bombus impatiens* to selenium, found that bees without a microbiome were more likely to succumb to selenate poisoning than those that had. The study also found

it was the core gut bacteria that increased the survivorship, however to determine the exact mechanisms may require more study(Rothman *et al.*, 2019).

A lack of normal gut microbiota has also been linked with a decrease in the production AMPs, though this study showed variance in some of the trials in survivorship between those lacking a microbiota and those that had, upon *E.coli* challenge. This study also found evidence of the enzyme Cu-Zn superoxide dismutase in the bees that lacked the microbiota. This enzyme converts superoxide radicals to H_2O_2 helping to reduce toxicity (Kwong, Mancenido and Moran, 2017).

The fungal microbiome of bees

While there is a significant understanding of the bacterial microbiome in the bee gut and how it impacts health and nutrition, less is known about the fungal microbiome or "mycobiome" (Tauber *et al.*, 2019). Some fungal genera have been reported in pollen and bee bread suggesting that they may have some beneficial role in bee health and nutrition. It has also been suggested that the hive stored bee bread environment might actually favour fungal growth over bacterial growth (Disayathanoowat *et al.*, 2020). *Zygosaccharomyce*s has been identified in pollen and aged bee bread (Detry *et al.*, 2020), *Rhizopus* was also identified in pollen of *Trigona collina* colonies (T. Eltz, Brühl and Görke, 2002)**,** while *Wickerhamomyces* has been found to have acquired naturally in a study carried out on newly emerged bees (Tauber *et al.*, 2019). Yeast also influence larval development and provide organic nitrogen, vitamins and lipids to adult insects (Cappelli, Favia and Ricci, 2021).

Zygosaccharomyces consists of 6 different species, *Z. bailii, Z. bisporus, Z. kombuchaensis, Z. lentus, Z. mellis and Z. rouxi* (Dai *et al.*, 2020).It is a yeast often associated with food spoilage and has been identified and the different species have different tolerance levels with *Z. rouxi* capable of growing at low water activity and *Z. machadoi* thought to perhaps cause spoilage in the nests of stingless bees and *Z. siamensis* been found to be metabolically active in honey made by the European honey bee (Péter, 2022)**.**

Wickerhamomyces is found mainly in soil, fruit juices and plants with a high tolerance for the antifungal drug fluconazole (Zhang et al., 2021). It is a budding yeast in the class of

saccharomyces and has been identified in the gut of many insects including honeybees (Cappelli, Favia and Ricci, 2021). It has also been isolated from the midgut of honeybees (Tauber *et al.*, 2019) and has been demonstrated to cause bacterial lysis (Muccilli and Restuccia, 2015). *Wickerhamomyces anomalus* can induce this through the secretion proteins or glycoproteins known as killer toxins (KTs) (Giovati *et al.*, 2021). Studies carried out with newly emerged bee that naturally acquired *W. anomalus* found that upon challenge with *N. ceranae*, *W. anomalus* titres dropped and bees fed with *W. anomalus* had lower gene expression of immunity related transcripts and changed the relationships of gut microbiota. It was proposed that the presence of the yeast changed the environment to a more acidic one favouring *Lactobacilli* species (Tauber *et al.*, 2019).

Kazachstania belong to the family *Saccharomyctaceae* and have recently been classed as an invasive fungal infection (IFI) with 13 cases of fungal disease caused by the organism identified in humans (Kaeuffer *et al.*, 2022). There has been over 32 different species identified and species have been isolated from fruit, leaves soil, animals and even wastewater with some being heterothallic and some homothallic (James *et al.*, 2015). It has also been suggested that it plays a role in wine fermentation and can affect the *Saccharomyces cerevisiae* growth and metabolism by competed for nutrients (Santiago-Urbina, Arias-García and Ruiz-Terán, 2015).

Rhizopus belongs to the *Mucorales* that primarily infects through the air and can grow at temperatures of 37^0C due to them being thermotolerant (Nicolás *et al.*, 2020). *Rhizopus* was identified on corbicular loads of pollen gathered by foragers of the stingless bee *Trigona collina*. The fungi is known to spoil food and has been suggested to cause mortality among brood of nesting bees, though it is rare in honeybees (Eltz et al., 2002). It has also been demonstrated to have the potential to inhibit chalkbrood growth while *Penicillium* can also inhibit bacterial growth (Disayathanoowat *et al.*, 2020).

Genetic-based identification of microbial species

Internal transcribed spacer (ITS) has become more common in the past 15- 20 years for the identification of fungal species (Bellemain et al., 2010). The ITS region offers high species resolution for a broad range of fungi (Banos *et al.*, 2018). The ITS included 2 regions ITS 1 & 2 separated by a 5.8S gene with up to 250 ITS copies per cell making it an ideal target even when DNA quantities are low (Bellemain et al., 2010).There are an estimated 3.8 million

fungal species with roughly only 120,000 described (Banos *et al.*, 2018). The use of high throughput sequencing over the past few decades has reduced the cost (Nakatsu, Byappanahalli and Nevers, 2019) and effort compared to traditional sanger sequencing of the 16S region which required cloning, generating and assembling cloned genes, with two to three reads per clone. The 16S rRNA gene is highly conserved with 9 variable regions including the V4 region which was targeted in this study for species identification (Johnson *et al.*, 2019).

ITS and 16s analysis of bumblebee gut DNA extracts returned a variety of bacterial and fungal genus in different abundances depending on the pollen type they were fed. This leads us to propose that altering the pollen composition either through irradiation or through filtration has an effect on the microbiome of the colony through dysbiosis. The gut microbiome and mycobiome appear to be quite susceptible to a number of factors such as pH, nutrition availability and the presence of other microbes. Factors that favour one will most likely allow it to grow at the expense of others. How this impacts the health and nutritional status of the host remains to be determined, but it is generally accepted that dysbiosis impacts negatively on health, immunity and behaviour (Disayathanoowat *et al.*, 2020).

Chapter aims

Lab reared bumblebees were fed on either untreated commercial pollen, commercial pollen treated with a 100KGy dose of e-beam or filtered pollen, pollen that was washed with sterile water and filtered through a 5µm filter to determine if there was a noticeable change in the gut microbiota community. It was hoped to determine if there was any potential health benefits for bees fed on either e-beam treated pollen or filtered pollen, by way of pathogen defence by measuring the abundance and type of microbiota present. Studies have shown that microbiota can have a positive effect on bee health and even protect against some pathogen infections (Koch and Schmid-Hempel, 2011; Mockler, 2018) and prevent against toxicity (Rothman *et al.*, 2019). This part of the project investigated if there was a shift in the core bacteria *Gilliamella*, *Snodgrassella* and *Lactobacillus*. It would also look at the lesser understood mycobiome and try further our understanding of the possible roles it may play in bee health.

5.2 Material and methods

5.2.1 Tagging callows

Newly emerged callows were tagged by removing them from the colony under red light conditions. The callows were anesthetised using CO_2 and had a colour coded numbered sticker attached to their back using nontoxic glue. Once the callows awoke they were then reintroduced into the colony.

Tagged callows were removed from the colony after 2 weeks. They were then snap frozen in liquid nitrogen in 1.5 mL Eppendorf tubes and stored at -80^{o}C until required.

5.2.2 Callow dissection for microbiome analysis

To dissect the digestive tract for microbiome analysis, bees were first pinned to a wax tray. Bees were dissected with the bee pinned to a wax tray. The bee was positioned with their head forward and a single pin was placed in the thorax. The bee was stretched slightly and another pin was placed through the stinger of the bee. The tray was placed under a dissecting microscope and a cut was made using a microscissors in the right side of the bee at the abdomen area. The exoskeleton was then peeled to one side and held back by pinning it to the wax tray. The gut was removed using a sterile (washed with 70% ethanol) tweezers to a single sterile eppendorf.

5.2.3 Gut preparation for DNA extraction

Bumblebee digestive tracts were homogenised in a sterile eppendorf in 200 µL of buffer ATL from the Qiagen DNeasy DNA extraction kit, using a motorised pestle.100 µL of lysozyme (10 mg/mL) was added and the solution was incubated at 36^{o}C for 40 min. Once the incubation was completed, 200µL of buffer AL and 20 µL of Proteinase K was added to each. Samples were inserted into a preheated incubator at 56^{o}C for 1 H. Once completed, 200µl of chilled RNA pure ethanol was added to each sample and vortexed. The solution was placed into a DNeasy spin column sitting and centrifuged at 8000rpm for 1 min. The supernatant and collection tube were discarded from each column and the column was placed into a new tube. 500 µL of Qiagen Buffer AW1 was added to each column and they were centrifuged again at 8000rpm for 1 min. 500 µL of Qiagen Buffer AW2 was added and all columns were centrifuged

for 3min at 14000rpm. Onto each column, 150 µL of Qiagen buffer AE was added and columns were then allowed to incubate at room temperature for 1 min and centrifuged at 8000rpm for 1 min to elute the DNA.

5.2.4 Library preparation, sequencing and data analysis

Fifteen samples (five samples per treatment group) of DNA extracted from digestive tracts of individual workers were sent to Novogene U.K. for 16S (v4) and ITS (ITS1-5F) amplicon-based metagenomic sequencing and data analysis. Novogene performed PCR amplification and generated libraries for each sample and the following section describes these steps.

Samples were sequenced on a NovaSeq 6000 using a pair-ended approach (250bp). Each sample met the requirement of having over 75% of reads with a Q score of at least 30 and generating 30,000 tags of raw data. Filtering was performed using Qiime (v1.7.0) on raw reads and chimera sequences were identified and removed using the SLVA138 database (http://www.arb-silva.de/) with the UCHIME algorithm. On remaining data, sequences with 97% similarity were clustered and organised into operational taxonomic units (OTUs) by Uparse (v7.0.1090) Representative sequences for each OTU were screened by QIIME using Mothur method against the SSUrRNA database from SILVA138. Phylogenetic relationships were determined between OTU groups using MUSCLE (v3.8.31). Standard sequence numbers were used to normalise OTU abundance information for each sample. All the following analyses were carried out on this normalised data.

QIIME was used to calculate several indices of alpha diversity (including observed-species and Shannon indices) and beta diversity (weighted and unweighted Unifrac). A Principal Component Analysis (PCA) and Principal Coordinate Analysis (PCoA) were performed and visualised on R (v2.15.3) using FactomineR and ggplot2 packages. Wilcoxon two rank sum tests were performed to test for significance between diversity estimates between wild and commercial bumblebee gut microbiomes in both bacterial and fungal communities. Anosim, MRPP and t-test analyses were also performed on R. All results were returned in a report.

5.3 Results

Bacterial diversity in the bumblebee digestive tracts

16S rRNA V4 region Novogene sequencing was used to generate data from 15 bee digestive tract samples (5 from each treatment group). Individual colony OTUs ranged from 1795-2111 (control), 747-1293 (e-beam) and 535-1544 (filtered). From this data it was possible to identify the presence of 'core' microbiota *Gilliamella*, *Snodgrassella* and *Lactobacillus* in all three treatment groups. These core bacteria varied in abundance depending on the type of pollen that the bees were fed. *Gilliamella* (0.3992) and *Lactobacillus* (0.2313) was most abundant in bees fed on e-beam treated pollen while *Snodgrassella* (0.3505) was most in bees fed on control pollen. Other bacteria identified within the treatment groups in varying abundance, included *Murbaculaceae*, *Bacteroide*, *Rubrobacter*, *Pseudomonas* and *Escherichia-Shigella*. These species were in much lower abundance being in bees fed on either e-beam treated pollen or filtered pollen compared to bees fed on control pollen (Appendix file 5)

Figure 5: Table of the top 5 bacterial species identifed per colony included the three 'core' bacteria *Gilliamella*, *Snodgrassella* and *Lactobacillus*. Abundance of thses bacteria varied from colony to colony, with bees fed on e-beam treated pollen having a greater abundnace of both *Gilliamella* and *Lactobacillus* compared to bees fed on either control pollen or filtered pollen. Bees fed on control pollen had a greater abundance of *Snodgrassella* compared to bees fed on e-beam and filtered pollen.

Taxonomy	*Gilliamella*	*Snodgrassella*	*Lactobacillus*	*Chloroplast*	*Mitochondria*
C1	0.334316	0.304899	0.105729	0.003004	0.00142
C2	0.229844	0.162689	0.175851	0.00284	0.000364
C3	0.16744	0.476216	0.123041	0.001493	0.000218
C4	0.310524	0.33306	0.178854	0.003204	0.00051
C5	0.242368	0.475707	0.039284	0.003477	0.000965
E1	0.378552	0.344565	0.16704	0.028526	0.011596
E2	0.43848	0.147907	0.162325	0.07105	0.034315
E3	0.428413	0.050298	0.367247	0.024248	0.009921
E4	0.341871	0.217155	0.311015	0.036626	0.016475
E5	0.408953	0.097155	0.327727	0.047276	0.01997
F1	0.324504	0.459469	0.127792	0.023374	0.009903
F2	0.33874	0.239801	0.318315	0.024248	0.011596
F3	0.494621	0.19664	0.224055	0.015965	0.007081
F4	0.395682	0.19613	0.305627	0.022628	0.011796
F5	0.279231	0.263157	0.181039	0.061857	0.029709

Bacterial diversity in the the bumblebee digestive tract

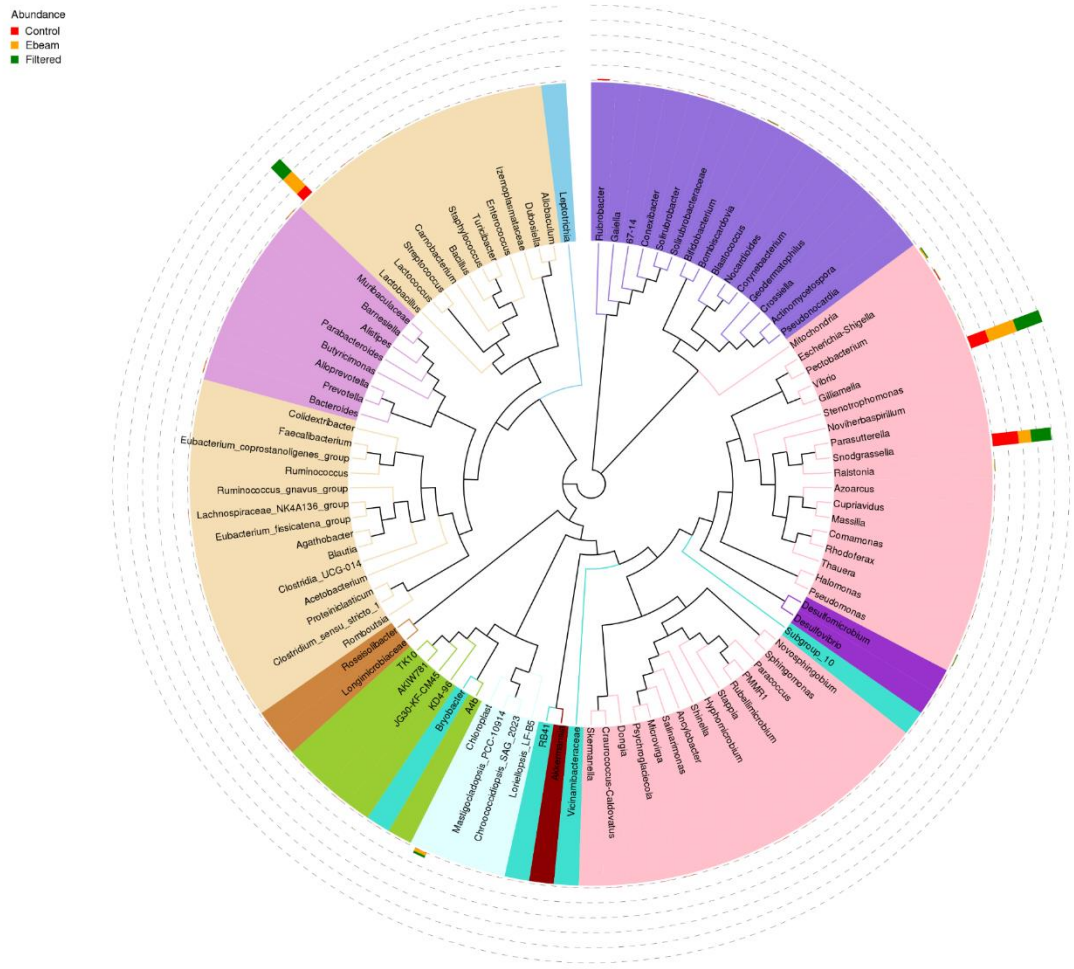


Figure 5.1: Cladogram of distributed microbiota per treatment: The cladogram in figure 5 the adundance of species present per treatment, control, e-beam and filtered.Three of the core bacteria Gillamella, Snodgrassella and lactobacillus are represented across all treatments. Chloroplast and mitochondria are in higher abundance in both e-beam treated pollen fed bees and filtered pollen fed bees.

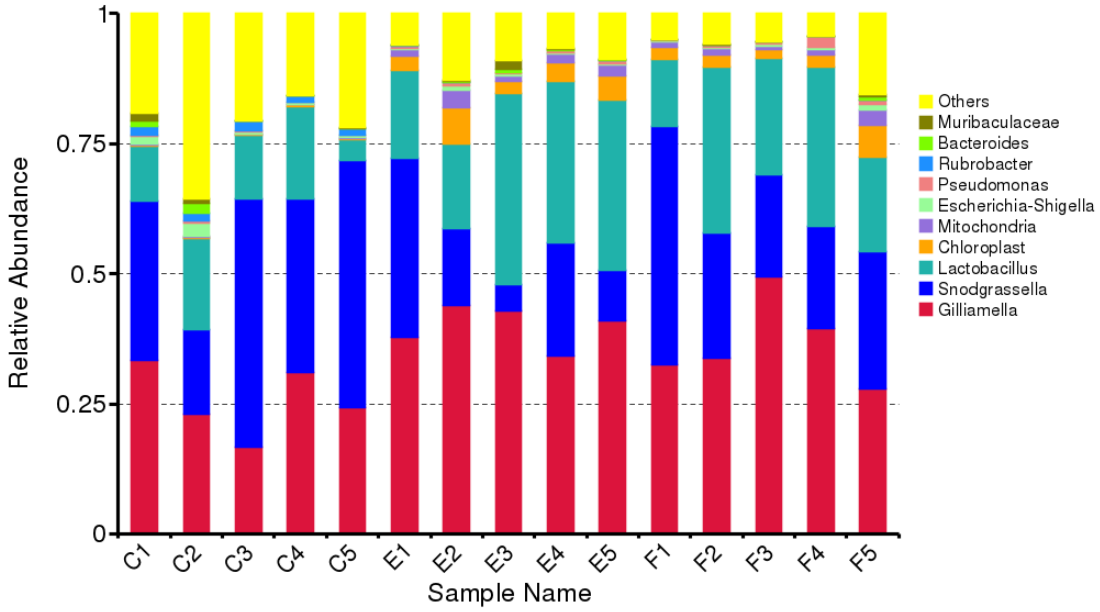


Figure 5.2: bacteria species identifed per colony :shows the diversity of microbiota present on an individual colony level. The abundance varied from colony to colony with a greater number of mitochondria present in both e-beam and filtered pollen fed colonies. These colonies also contained a higher number of chloroplasts compared to the control colonies. *Snodgrassella* is more prevenlant in the guts of bees fed on control pollen, while *Gilliamella* and *Lactobacillus* are more prevelant in the guts of bees fed on e-beam treated and filtered pollen.

Variance was noted in bacterial numbers and dominace across all three pollen types. The top 5 microbiota identifed in individual colonies were *gilliamella, snodgrassella, lactobacillus*, chloroplast and mitochondria. Overall *Gilliamella* had a higher range in filtered (0.2792-0.4946) compared to e-beam (0.3418-.4384) and control (0.1674-0.3343) . *Snograssella* had a higher range in control (0.1626-0.4757) compared to filtered colonies (0.1961-0.4594) and e-beam (0.0502-0.3445). *Lactobacillus* had a range of (0.3928-0.1788) in control colonies, (0.1670-0.3672) in e-beam treated pollen colonies and (0.1277-0.3183) in filtered fed pollen colonies. The range at which chloroplasts were detected at an individual colony level was control (0.0014-0.0034), e-beam(0.0285-0.0710) and filtered (0.0159-0.618). Mitochondria were detected at a range of (0.0005-0.0014) control, e-beam (0.0992-0.0343) and filtered (0.0070-0.02970).

Another bacteria that appeared to be more abundant in the control compared to the treated colonies was *Rubrobacter* control (0.0112-0.1936), e-beam (0-$7.28x10^{-5}$) and filtered (0.0001-$7.28x10^{-5}$).

Figure 5.3: Top 5 bacterial species at treatment level were the 'core' bacteria of *Gilliamella*, *Snodgrassella* and *Lactobacillus*. The relative abundance of these bacteria varied depending on the pollen type that the bees were fed.

Taxonomy	*Gilliamella*	*Snodgrassella*	*Lactobacillus*	*Chloroplast*	*Mitochondria*
Control	0.256898	0.350514	0.124552	0.002803	0.000695
E-beam	0.399254	0.171416	0.267071	0.041545	0.018455
Filtered	0.366556	0.271039	0.231365	0.029614	0.014017

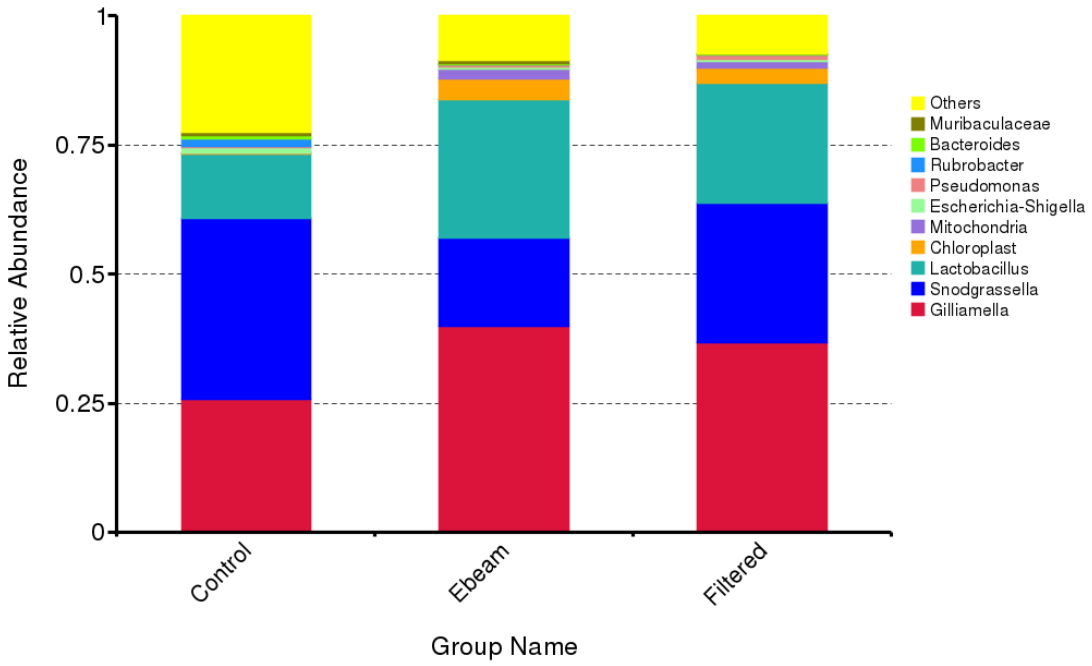
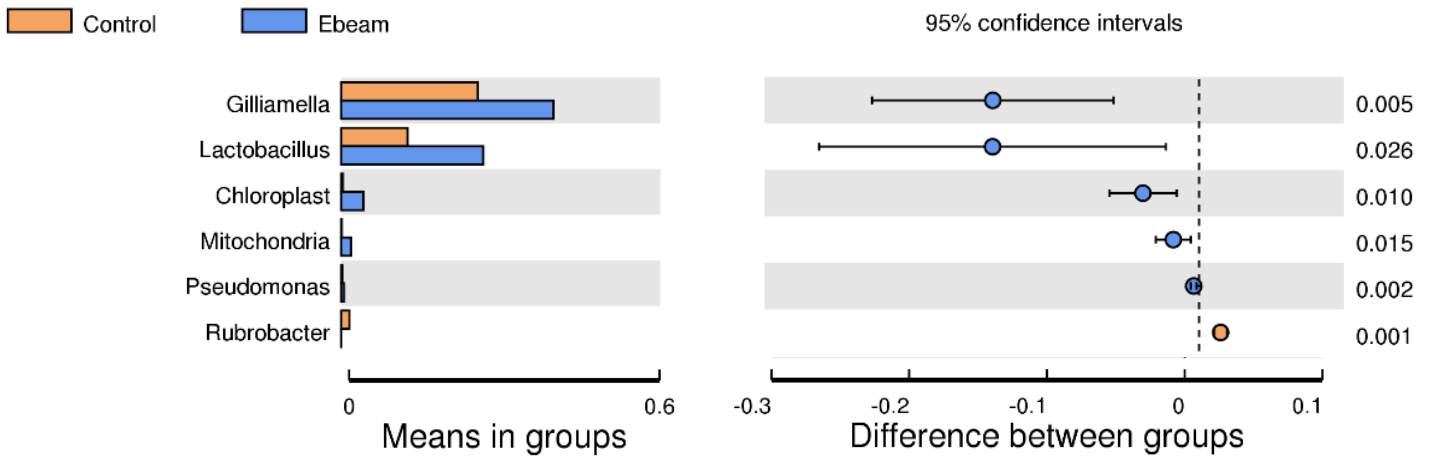


Figure 5.4: bacterial abundance and species per treatment: The relative abundance of the the microbiome varied depending on the type of pollen the colonies were fed. The three core bacteria *Gilliamella, Snodgrassella* and *Lactobacillus* made up the majority of the micriobiota present in the digestive tracts, with differences being observed in the numbers present depending on the type of pollen the bees were fed. Greater numbers of *Gilliamella* and *Lactobacillus* were observed in both e-beam treated pollen fed & filtered fed colonies compared to the control. Overall bees fed on control pollen had a greater number of *Snodgrassella* present in their gut compared to both filtered fed & those fed on e-beam treated pollen who had the lowest cell count.

A



B

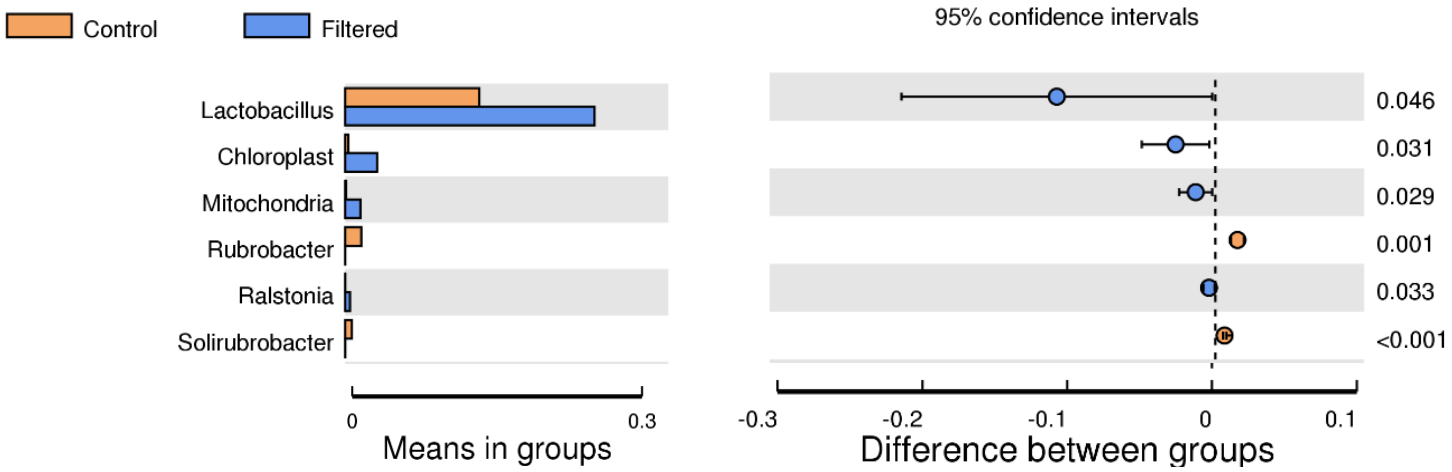


Figure 5.5: mean abundance of bacterial species per treatment: The mean abundance of microbiota species from the digestive tracts of bees fed on e-beam treated pollen compared to control pollen (A) and fed on filtered pollne (B). The confidenece interval of between group variation demonstartes the lower and upper confidence limits of the 95% confidence interval, with the centre representing the difference of the mean value.

The top 5 microbiota identifed in individual colonies were *gilliamella, snodgrassella, lactobacillus*, chloroplast and mitochondria. Grouped overall *Gilliamella* was more abundant in e-beam treated pollen fed colonies (0.3992) compared to filtered pollen fed colonies (0.3665) and control colonies (0.2568). *Snodgrassella* was more abundant in control colonies (0.3505) compred to e-beam (0.1714) and filtered (0.2710). *Lactobacillus* was more abundant in e-beam treated colonies (0.2670) compared to filtered (0.2313) and control colonies (0.1245). Chloroplasts were more abundant in e-beam treated pollen fed colonies (0.0415) compared to filtered (0.0296) and control colonies (0.0028). Mitochondria were also more abundant in e-beam colonies (0.0184) compared to filtered (0.0140) and control colonies (0.0006). Rubrobacter was more abundant in control colonies (0.0149) compared to filtered (0.0001) and e-beam (0.0000).

The mean abundant differences between species from various genera of microbiota found in the digestive tracts of bees fed on either untreated commercial pollen, e-beam treated commercial pollen or filtered commercial pollen were recorded with confidence intervals between the groups of a upper limit of 95%.

Fungi

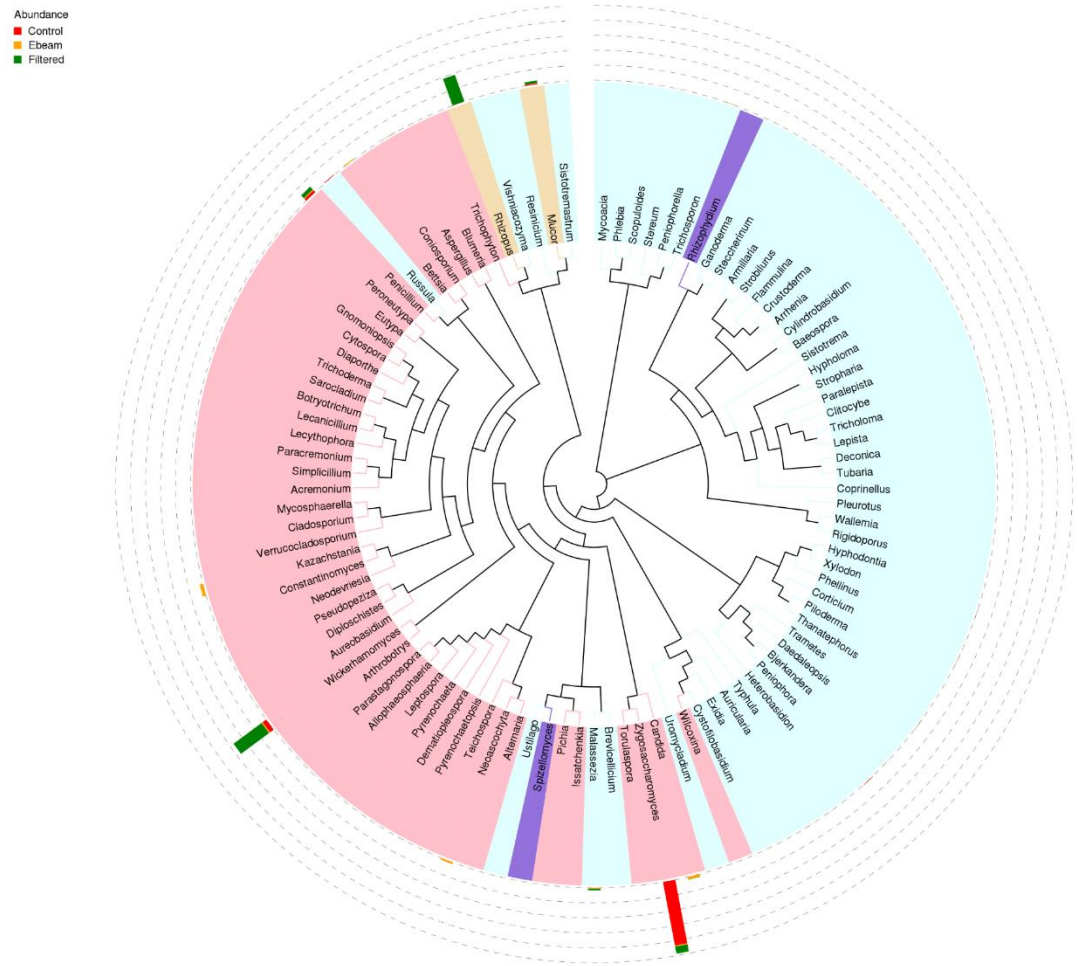


Figure 5.2.1 cladogram of fungi present per treatment: The cladogram in figure 5.2.1 shows the adundance of species present per treatment group. *Zygosaccharomyces* is the dominant fungus in colonies fed on control non-treated commercial pollen. *Rhizopus* and *Wickerhamomyces* are more prevalent in colonies fed on filtered pollen while e-beam treated pollen fed colonies tend to have no one species that is dominant. Results for bees fed on e-beam treated pollen showed that of the fungi detected not one species tended to dominate over the other, but rather unidentified species labelled as other made up the majority of what was present. The top 10 fungi present were made up of the above mentioned three along with *Kazachstania, Candida, Penicillium, Neoascochyta, Mucor, Malassezia* and *Bettsia* with everything else labelled as other.

Figure 5.2.2 table of fungi present in individual colonies depicts the top fungi found in each colony and the abundance at which they were found. Zygosaccharomyces, Rhizopus, Wickeramomyces, Kazachstania and Candida were all idenetified in varied abundance in the bumblebee gut.

Taxonomy	*Zygosaccharomyces*	*Rhizopus*	*Wickerhamomyces*	*Kazachstania*	*Candida*
C1	0.940056	0.001786	0.002891	0	0
C2	0.018196	0	0.048125	0	4.25E-05
C3	0.011734	0	0.017813	0	0.000978
C4	0.02317	0	0	0	0
E1	0.010586	0	0.010288	0	0.038475
E2	0.004464	0	0.00659	0.044512	0
E3	0.001063	0	0.003103	0.00744	0.00017
E4	0.000595	0	0	0	0
F1	0.002041	0.00068	0.010756	0	0.003614
F2	0.051994	0.000723	0.419012	0	8.50E-05
F3	0.054502	0.432446	0.08728	0	0.000425
F4	0.001828	0.000213	0.018578	0	0

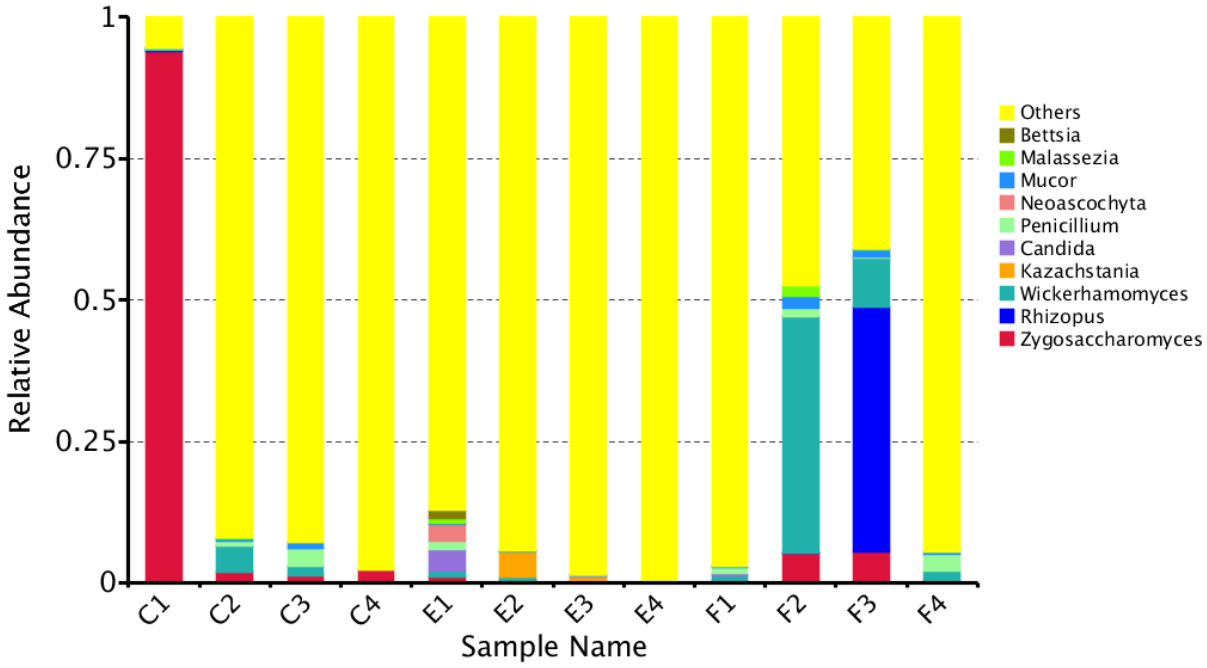


Figure 5.2.3 graph of fungi present in individual colonies : highlights the variance in the mycobiome on an individual colony level. Not only is the variance per pollen type fed, but it also differs from colony to colony within each pollen type fed. *Zygosaccharomyces* is dominant in colony 1 of the control yet much levels are observed the rest of the control colonies with only trace amounts found in the guts of bees fed on e-beam treated pollen and in 2 of the filtered fed colonies. Other yeast such as *Kazachstania* were only detected in 2 of the e-beam pollen fed colonies with *Rhizopus* only been found in filtered fed bee guts and one colony of the control.

The abundance and diversity of fungi present varied per colony and per pollen source. *Zygosaccharomyces* ranges in control colonies (0.0117-0.9400), e-beam (0.005-0.0105) and filtered (0.0018-0.545), *Rhizopus* ranged in the control (0-0.0017), e-beam (0) and filtered (0.0002-0.4324), *Wickerhamomyces* ranged in the control (0-0.0481), e-beam (0-0.0102) and filtered (0.0107-0.4190), *Kazachstania* ranged in the control (0), e-beam (0-0.0445) and filtered (0), *Candida* ranged in the control (0-4.25×10^{-5}), e-beam (0-0.3847) and filtered (0-8.5×10^{-5}). Other fungi present were *Penicillin* which ranged in the control (0-0.0306), e-beam (0-0.1471) and filtered (0.0024-0.0310), *Neoascochyta* which ranged in the control (0-0.0001), e-beam (0-0.2967) and filtered (0), *Mucor,* control (0-0.0106) e-beam (0-0.0023) and filtered (0.0007-0.0195), *Malassezia* control (0.0002-0.0008), e-beam (0.0001-0.0009) and filtered

(0.0013-0.0189) and *Bettsia* which ranged in the control (0), e-beam (0-0.0148) and filtered (0).

Figure 5.2.4 Table of Fungi present per treatment group with the varied abunsdance of fungi present. Bumblebee colonies fed on e-beam treated pollen had a more diverse mycobiome with no single fungal species dominating. *Zygosaccharomyces* was the most abundant in bees fed on control pollen. *Wickerhamomyces* was more abundant in bees fed on filtered pollen.

Taxonomy	*Zygosaccharomyces*	*Rhizopus*	*Wickerhamomyces*	*Kazachstania*	*Candida*
Control	0.248289	0.000446	0.017207	0	0.000255
E-beam	0.004177	0	0.004995	0.012988	0.009661
Filtered	0.027591	0.108515	0.133907	0	0.001031

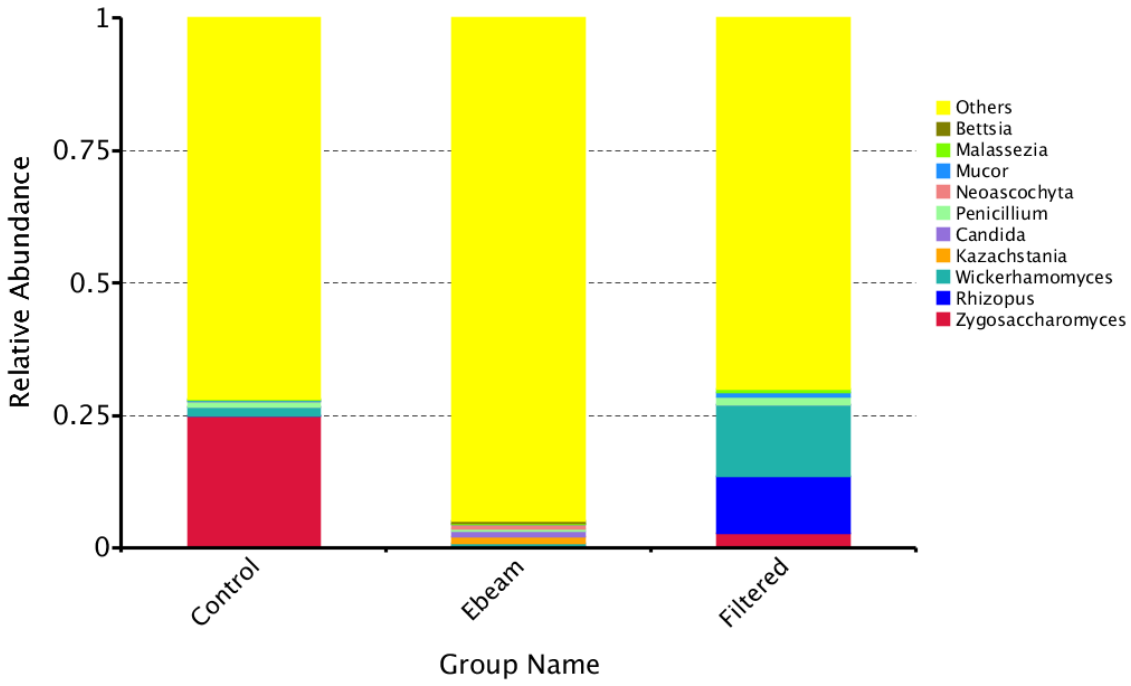


Figure 5.2.5 graph of Fungi present per treatment group: shows the grouped microbiota found in the gutrs of bees fed on either control, e-beam treated or filtered pollen. Bees fed on e-beam treated pollen had the greatest diversity yeasts and fungi present in the their mycobiome with only *Rhizopus* not present. Both control and filtred pollen fed bees displayed a similar profile with both *Kazachstania* and *Bettsia* not being detected, also filtered pollen fed bees had no *Neoascochyta* present with only trace amounts found in control colonies. These were all detected in bees fed on e-beam treated pollen, although not in every colony.

The abundance and diversity of fungi present varied per colony and per pollen source. *Zygosaccharomyces* averaged in control colonies (0.2482), e-beam (0.0041) and filtered (0.2759), *Rhizopus* ranged in the control (0.0004), e-beam (0) and filtered (0.1085), *Wickerhamomyces* ranged in the control (0.0172), e-beam (0.0049) and filtered (0.1339), *Kazachstania* ranged in the control (0), e-beam (0.0129) and filtered (0), *Candida* ranged in the control (0.0002), e-beam (0.0096) and filtered (0.0010). Other fungi present like Bettsia were not present in either the control (0) or filtered (0) while small amounts were detected in e-beam (0.0037). *Neoascochyta* was also found in e-beam (0.0074) with a much smaller amount found in the control (0.00003) and none detected in filtered (0). *Penicillium, Mucor* and *Malassezia* were detected in bees across all three pollen types. The quantities varied per pollen type the bees fed on*, Penicillium* in the control group (0.0094), e-beam (0.0042) and filtered (0.0147), *Mucor* in control (0.0003), e-beam (0.0011) and filtered (0.0087) and *Malassezia* (0.005) control, e-beam (0.0011) and filtered (0.00871). A total of 10 genus were identified with the rest denoted as other which made up the bulk of what was present in the bee gut mycobiome. In bees fed on control pollen 8 of the 10 were present, 9 of the 10 in bees fed on e-beam treated pollen and 7 of the 10 in the bees fed on filtered pollen. Bees fed on e-beam treated pollen also had the greatest number of unidentified fungi, while *Zygosaccharomyces* was the dominant yeast in control while bees fed on filtered pollen were colonized by *Wickerhamomyces* and to a lesser extent *Rhizopus*.

5.4 Discussion

Spillover of pathogens from commercially reared bees to wild communities is a thought to play a contributing role in the loss of colonies (Murray *et al.*, 2013; Graystock *et al.*, 2013). The pollen received from commercial suppliers contained spores most likely *Nosema* and other fungal and yeast species that grew in both low temperatures (4^{o}C) and at room temperature. By treating the pollen with 100KGy of e-beam it was hypothesized that the microbiological community within the pollen would be inactivated or killed and that this could pass on health benefits to the bees that feed on it. Likewise it was also thought that by filtering out large quantities of these spores that a less parasited bee gut would be observed and that this would result in health benefits for the bee.

Here newly emerging bees from each group were tagged and returned to the colony. These were then collected after 2 weeks and snap frozen and dissected at a later date. Newly emerged bees had fed solely on either untreated commercial pollen, 100kGy e-beam treated pollen or filtered pollen supplemented with 30%w/v sucrose solution. These bees would have microbiomes distinct to that group and any shifts in microbiome species of abundance should be evident.

The microbiome of bee is made up of core bacteria and can be host specific acquired through social transmission (Engel *et al.*, 2016). In mosquitos during larval development the gut bacteria is eliminated upon reaching the adult stage, however bees are thought to use oral trophallaxis or the oral faecal route in the direct or indirect transmission of microbes and even sometimes inoculated eggs with symbiotic microbes (Engel and Moran, 2013).

Our results identified various bacterial and fungal species in the guts of bees fed on commercial pollen, either untreated, treated with 100KGy of e-beam or filtered with sterile water. The microbiomes and mycobiomes of all three differed mostly in terms of abundance per treatment but also in terms of species present. Ten species of bacteria that were present in all treatment groups were identified in varying abundance and another 10 fungi that were present in some groups and not others also with varying abundance.

Bees fed on e-beam treated pollen had the greatest diversity of fungal species present with a total of 9, while filtered had the least amount present with 7 and control sat in the middle

with 8 detected species. The food spoiling yeast *Zygosaccharomyces* was the dominant strain present in bees fed on control pollen, however this was mostly due to high numbers in a single colony rather than the overall group, bumblebees fed on filtered pollen also displayed high numbers in 2 of the 4 colonies, but overall were largely colonized by *Wickerhamomyces*. *Zygosaccharomyces* has been detected in bee bread and offers a possible transmission route to newly emerging bees. It is a fermenting organism and has the ability to grow in pollen due to high osmotolerance (Detry *et al.*, 2020). Why it was less prevalent in e-beam treated pollen fed bees is not known, but it could be down to the lack of present due to how the pollen was treated. Having various yeast and bacteria colonize the gut in a symbiotic way can be beneficial to both, as the microbiomes get a stable environment and the bee gets aid of digestion with the use carbohydrate fermenters to break down complex sugars. However when an environment favours one over the other dysbiosis occurs (Tauber *et al.*, 2019).

It is plausible to believe that dysbiosis has occurred here, and the varying treatments carried out on the pollen is the reason for it. All bees were kept in the same conditions in the lab with a continuous supply of sucrose with the only variable being the type of pollen they were fed. *Lactobacilli* favour a more acidic environment and bees fed on e-beam treated and filtered pollen had higher quantities of *lactobacillus* in their guts compared to bees fed on control pollen, which could suggest that both processes could affect the communities in a similar way. *Lactobacilli* are one of the main organisms involved in converting bee pollen into bee bread through lactic acid fermentation (Barta *et al.*, 2022). Whether the pollen in both treatments enriched for *Lactobacilli* due to nutrient content, or increased number required for fermentation due to augmented pollen grain is not known. It could be that the presence of fungi helped create a more acid environment. However there is no clear dominant fungal species present in the e-beam group across all species, where as *Rhizopus* and *Wickerhamomyces* were both at elevated levels compared to both the e-beam and control group. This suggests that there is no clear evidence to point to the mycobiome environment influencing the microbiome environment. The only similarity between the e-beam group and filtered group was that both *lactobacillus* and *Gilliamella* were present at higher levels than in the control group. This is surprising as *Gilliamella* is known to have a symbiotic relationship with *Snodgrassella* with the two forming a biofilm with *Gilliamella* growing on top of *Snodgrassella*. Both are recognised as the dominant genus in bee guts. *Gilliamell*a can

degrade polysaccharides so acts as a nutrient network while the biofilm can trigger host beneficial immune responses (Zhengyi Zhang *et al.*, 2022). It is possible that in both guts the environment has changed to favour *lactobacillus* over *Snodgrassella* and a new relationship has formed between *Gilliamella* and *Lactobacillus*. In the control group *Snodgrassella* is the dominant genus with *Gilliamella* which could suggest that the appropriate biofilm has formed, whereas *Gilliamella* is present in far greater numbers than *Snodgrassella* in both e-beam and filtered groups. This could suggest that there is an excess of *Gilliamella* not partaking in biofilm construction in both groups and are either free moving or perhaps contributing somehow to the gut environment in a way that favours *Lactobacillus* growth.

One of the more notable outcomes was the relative increase in the presence of mitochondria in bees fed on treated pollen compared to those fed on control pollen. The primary function of mitochondria is the generation of ATP, but also plays a key role in apoptosis (Boengler *et al.*, 2017). Proteomic analysis of fatbodies of tagged callows (Chapter 4) showed a reduction in aerobic respiration and cytochrome-c reductase activity hinted that it could be down to a possible reduction in mitochondria number with bees fed on e-beam treated and filtered pollen, however microbiome analysis shows that mitochondrial numbers were higher in both e-beam treated pollen fed bees and filtered pollen fed bees compared to the control, so an alternative explanation could be that the morphology of the mitochondria present is different leading to lower levels of aerobic respiration (De Goede *et al.*, 2018). Mitochondria have evolved over millions of years, adapting to the role of being one of the ultimate symbionts for almost every species on the planet bar one *Monocercomonoides* (McCutcheon, Boyd and Dale, 2019). Symbionts offer a mutualistic exchange with their host offering nutrient breakdown and or defence against pathogens in exchange for a stable environment (Engel *et al.*, 2016), so the presence of chloroplasts in elevated numbers in both e-beam and filtered groups is interesting. Chloroplasts can produce Reactive oxygen species (ROS) which generally play a role in plant defence against bacterial plant pathogens (Lu and Yao, 2018), so their presence is somewhat conflicting. If they are acting in a symbiotic capacity then their genetic make-up is quite possibly altered to allow proliferation (McCutcheon, Boyd and Dale, 2019). It is also simply just a case that altered pollen structure has allowed for a build-up of chloroplasts within the gut and that the altered microbial community simply lacks the ability to break them down for food.

5.5 Conclusion

Feeding bees on either e-beam treated pollen at a dose of 100KGy or filtered pollen changes the relative abundance of *Gilliamella*, *Snodgrassella* and *Lactobacillu*s compared to the control. In both treatment groups bees fed on these pollen types favoured colonization of digestive tracts for *Gilliamella* and *Lactobacillus*, whereas the control group was favoured by *Snodgrassella* and *Gilliamella* respectively. All three core bacteria were present within the three groups in varying abundances. The mycobiome was less distinct with *Rhizopus* being the most abundant species in the filtered group and Zygosaccharomyces being the most abundant in the control group. Bees in the e-beam treated group were the most species enriched with no one species dominating over the other.

It is not known exactly how this impacted on bee health overall, however it is possible the shift in the microbiome played some role in colonies of the treated groups as they had a lower reproductive output, failed to produce males and new queens. Further investigations would be required to determine if the shift in microbiota abundance and diversity was the sole driver or played any role in the low reproductive output of treated colonies.

Chapter 6 Overview of the effects of e-beam treated and filtered pollen on *Bombus terrestris*

6.1 Introduction

Crop pollination has is the basis of multibillion dollar and euro industries in the USA ($14 B) (Reeves *et al.*, 2018) and Europe (€15B) (Commission, 2018) respectively. The production of a wide array of crops rely on pollinators such as bees including blueberries, apples (Al Naggar *et al.*, 2018), almonds (Staveley *et al.*, 2014), strawberries, cucumbers, rape and tomatoes (Commission, 2018; Murphy & Stout, 2019). Although the vast majority of crop pollination is conducted by wild and native bee species, an ever-growing proportion requires commercially bred and distributed bees, particularly honeybees and bumblebees. Commercial bumblebees are currently exported to 57 countries for pollination of crops with an estimated 1 million colonies sold per year (Ings *et al.*, 2010). Bumblebees are seen as more efficient pollinators than honeybees (Goulson and Hughes, 2015) and are fed on commercial pollen which is sourced from honeybees (Pereira et al., 2019). However commercial pollen has been found to be contaminated with a variety of parasites and viruses which can negatively impact bee health (Graystock et al., 2013). One study found evidence of the bumblebee parasite *Apicystis bombi* in commercial pollen (Pereira, Meeus and Smagghe, 2019b), which is highly concerning as pathogen spillover from commercial colonies into wild communities is becoming a common issue (Murray et al., 2013).

This project aimed to build upon previous studies that attempted to assess the potential of alternative technologies to sterilize commercial pollen to help try reduce the possibility of pathogen spillover from commercially sourced honeybees and bumblebees to wild communities. The initial candidate technology chosen was Pulse UV technology (PUV). UV light has the ability to inactivate viruses (Gómez-López *et al.*, 2021) PUV had demonstrated the ability to inactive *Crithidia bombi* (Naughton et al., 2017). However early experiments demonstrated a limitation of this technology to inactivate the fungal spore community that inhabited the commercial obtained from suppliers.

After this discovery the focus switched to the use of another non-thermal technology known as e-beam irradiation. Although early testing was inconclusive it was still chosen as the

candidate technology at which to move forward with. Bumblebees were fed on e-beam treated pollen and assessed against bees fed on untreated commercial pollen. A third "cleaning" treatment was also examined and that involved feeding bees with pollen that had been washed/filtered. This a simple technique that used the filtration of fungal spores based on size, capturing and retaining pollen grains while eluting spores into a conical flask suspended in sterile water.

Colonies of *B. terrestris* were sourced from Agralan UK and fed on commercial pollen that was either untreated, e-beam treated or filtered over a 30 day period. Colonies were monitored for reproductive output during this period and other growth and reproductive characters such as size and sex after the experiment had ended. Newly emerged bees were tagged after 14 days and returned to the colony for a further 10 days. These tagged bees were then recaptured and snap frozen and used to assess the impact of pollen treatment on the fatbody proteome and digestive tract microbiome in order to obtain molecular level insights.

6.2 Discussion

In chapter 2 the potential of novel candidate technologies PUV and then e-beam irradiation was investigated. Initial experiments used a surrogate bacterial species *Bacillus cereus* to test the potential of PUV as a candidate technology for sterilization of commercial pollen. Effectiveness was determined through streak plate analysis and the ability of *B.cereus* to recover post PUV treatment. Similar testing was carried out with spores of *B.cereus* and flow cytometry was used as a diagnostic aid with the use of fluorescent dyes to determine viability. Once a level of understanding of how flow cytometry might be used as a means of determining viability, spores were extracted from commercial pollen and treated with the same dose of PUV. However no observed differences were noted between spores treated with PUV and spores that were left untreated. This led to the conclusion that PUV lacked the penetrative power required to damage spores that were obtained from commercial pollen.

Once this was decided, a different technology was chosen and that was e-beam. A dose range of 1.5 – 25KGy was chosen based on the literature (Mcfadden *et al.*, 2016) and liquid spore solutions were prepared and then treated on site at an e-beam facility in the midlands of Ireland. Flow cytometry was again used to determine viability. Initial testing using this dose range indicated that perhaps the lower doses were ineffective at treating what were

suspected fungal spores, so a higher dose range of 50, 75 & 100KGy were chosen to run in conjunction with further testing of the lower dose ranges. In total 2 tests using a dose range of 1.5-25KGy were carried out and a singular experiment at a dose range of 50-100KGy was carried out. This was due to the COVID-19 pandemic that significantly limited access to host laboratories and to the sterilization plant. Based on flow cytometry analysis of our limited results a call was made to go with the upper most limit for the sterilization of future spores. Determination would be decided by flow cytometry analysis as a plate method recovery based analysis was not possible due to the suspected fungal spores also being suspected parasites, therefore they would need a cell line within which to grow.

The Flow cytometry analysis of suspected fungal parasite spores treated with 100KGy e-beam dose was inconclusive. There was little difference in terms of PI+ cells in control population and e-beam treated populations. When taken into account that pollen treated with a 100KGy dose had no fungal growth on it nine months post treatment, there is no reason to believe that the dose of e-beam given was not effective at killing fungal pathogens and if not the spores at least every other microbe that inhabited the pollen. Future work could be done to try determine the most effective fluorescent dyes that could be used to ascertain spore damage and death using Flow cytometry. It might be also worth exploring the use of scanning electron microscopy (SEM) to assess the damage done to the spore coat with different kGy doses of e-beam.

Despite the less than convincing evidence of e-beam efficiency at producing cell death to the spores, it was still decided to treat the commercial pollen with 100KGy of e-beam irradiation. In total 1kg of pollen was treated on site at the midlands facility then transported and stored in the lab at $4^{O}C$.

Another treatment was also chosen that might achieve the desired effect of removing spores from commercial pollen and this was filtration. This technique had been optimised over the course of the project in order to purify spore loads extracted from pollen, leaving behind potential contaminants such as pollen grains that might interfere with the flow cytometry machine. It was decided that along with setting up a bumblebee group that would be fed on e-beam treated pollen, a second group would be set up that would feed bumblebee colonies on pollen that had been washed i.e. with the high concentration of fungal spores filtered out, dried and then fed back to the bees, with lower fungal spore load.

A total of twelve bumblebee colonies were sourced from Agralan UK. These were kept in dark conditions with a constant supply of sucrose and fed on either e-beam treated or filtered pollen. The colonies were monitored every second day and fed every day on pollen. The aim was to observe if any colony/organismal level differences were exhibited in terms of reproductive output, colony size and colony weight. This was the first step in determining whether any possible beneficial or negative side effects were evident after feeding bees on a treated version of pollen (e-beam or filtered) when compared to untreated commercial pollen (control).

The reproductive output of bumblebees fed on either e-beam treated pollen or filtered pollen were similar, however they differed significantly from colonies fed on untreated commercial pollen. Bumblebees fed on either e-beam treated or filtered pollen, produced bees with a slight yet significant difference in thorax size, with these bees being slightly bigger. However bees in these colonies failed to produce larvae, pupae and ultimately adult bees to the same level as colonies fed on untreated pollen. This difference in reproductive output was noted after 14 days of feeding bumblebee colonies on either e-beam treated or filtered pollen. Bees in these colonies did not show any signs of being less active, however after a two week period these colonies did not look as abundant in terms of adult bees as control group colonies. Bumblebee colonies fed on treated pollen (e-beam or washed) produced less female bees, gynes and failed to produce males. These recordings were taken after a 30day period had elapsed and the colonies had been euthanized. Each bee was examined individually and sex and size was recorded. The similarity between both bees fed on e-beam treated pollen and filtered pollen was noted. In both these groups virtually no males were produced. Males play a role in incubation of the colony as well as mating (Watanabe and Sasaki, 2022b). A failure to produce males is most possibly evidence of stunt in colony stage development. Fewer females were also recorded within the treated colonies (e-beam and filtered) and this failure to produce offspring would most likely affect colony productivity with less workers to attend the brood and then less foragers to gather food from the developing brood. Also the failure to produce gynes (virgin queens) would most likely mean that new colonies would not be established. While this might not necessarily be a negative factor for commercial colony establishment or production, as they are only required for a single growing season, it does pose the question that if these effects are being observed at the colony and individual

organism level, then what is happening on a molecular level. What was also interesting were the similarities between the two treated groups, despite very different methods of removing or reducing potential pathogen loads. It poses the question as to whether or not a change in the chemical or nutritional makeup of the pollen was the driver, or perhaps the removal of other microorganisms that could be beneficial to bee nutrition.

To further assess the relationship between colony level observations and the pollen type they were fed, newly emerged bees (callows) were captured and tagged 14 days into the experiment, then allowed to return to the colony for a further 10 days. These tagged callows were then recaptured and snap frozen until they were needed. These Bumble bees would be representative of the individual colonies and groups and would form the basis of both our proteomic and microbiome analyses. Examination of the bee fatbody proteome resulted in differences being observed in pathways involved in stress, metabolism and immunity between Bumble bees fed on either untreated, e-beam treated or filtered commercial pollen. Again there was some similarities observed between bees fed on e-beam treated pollen and filtered pollen most notably in the downregulation of immune associated pathways and proteins. There was an observed switch to β-oxidation as a means to generate ATP in groups fed on e-beam treated and filtered pollen. This could be due to a lack of essential nutrients from both pollen types, a change in the chemical make-up of the pollen or alteration in the microbial community some of which could be offering nutritional benefits to the bees. To determine whether the observed effects were attributable to the loss of key bacteria that might be recycled for nutrients or due to the physical alteration to the pollen itself requires future studies.

Protein pathways associated with immunity were also downregulated in colonies fed on either e-beam treated or filtered pollen compared to control. This could be due to the fact that maintaining an immune response is very costly during times of nutritional stress (Barribeau *et al.*, 2015) or because both groups are subjected to reduced pathogenic microbe loads compared to bumblebees fed on untreated pollen. The observation of an increase in proteins associated with stress seems to support the former.

Finally the microbiome was examined, by extracting DNA from the digestive tracts of snap frozen 10 day old callows fed on either control, e-beam treated or commercial pollen. It was clear that even at a microbial level feeding bees on either e-beam treated pollen or filtered

pollen also affected the bumblebee microbiome. Changes in the relative abundance of *Gilliamella*, *Snodgrassella* and *Lactobacillu*s in the bee digestive tract compared to the control were observed in both treatment groups, bees fed on these pollen types favoured colonization of digestive tracts for *Gilliamella* and *Lactobacillus*, whereas the control group was favoured by *Snodgrassella* and *Gilliamella* respectively. All three core bacteria were present within the three groups in varying abundances. Changes were also observed in the mycobiome of the bee digestive tract with *Rhizopus* being the most abundant species in the filtered group and Zygosaccharomyces being the most abundant in the control group. Bees fed on e-beam treated pollen had the greatest diversity of species with no one species dominating over the other. How these changes in the microbiome and mycobiome impact bee health is not fully understood and would require further investigation to determine the overall effects.

6.3 Conclusion

There is evidence to suggest that by treating commercial pollen by either irradiating it or filtering it is having a negative impact not only on the reproductive output within the colony, but it is also affecting the bees on a molecular level. Along with differences in colony reproductive out, differences in the fatbody proteomic profile and microbiome profile were observed in bees fed on e-beam treated and filtered pollen when compared to those fed on control pollen. Based on the colony reproductive output and fatbody proteomic profile these effects could be taken as negative, decreasing the chances of survival due to low numbers and founding of future colonies.

While it could be argued that the dose of e-beam was excessive (100KGy) it is worth noting that similar results were observed in colonies fed on pollen that was treated in a very different way. While e-beam treated pollen observed no surface growth nine months post treatment, control pollen and filtered pollen did indicating that there may have been some level of fungus within both pollen types when they were being fed to the bees. This could mean that it is not reduction in fungal species within the pollen that was causing perceived negative effects but rather either the removal of key bacteria or a reduction in nutrient content, due to a change in chemical makeup.

Therefore it is concluded that feeding bees on pollen altered in anyway is not advisable and lacks evidence to support a positive benefit to bumblebee health and reproduction.

6.4 Implications of Findings and Future Research

- Electron beam treatment effectively sterilized honey pollen at 100 kGy, but it is important to conduct additional research to investigate the impact of e-beam treatment on pollen structure including potential changes to macro and micronutrients
- Treatment of pollen to be used for commercial bumblebees is very complex as it appears that physical non-thermal (e-beam) processes affects the pollen that influences colony reproductive outputs. Future research is required to determine the optimal impact of non-thermal treatment processes so as to mitigate against important colony reproductive changes in commercial bumblebees
- There is a possibly that super-clean or sterilized pollen is not appropriate for feeding commercial bumblebees – this needs further studies so as to investigate the implications of changes on bee stress and immune responses, and microbiome changes post treatments
- There is a potential to explore other non-thermal technologies such as vaporised hydrogen peroxide as used in the adjacent sterilization industry; however, this an oxidative gaseous process that may also affect pollen structure and its subsequent appropriate use for bee consumption
- There is a need to consider digital tools to interpret and analyse complex data sets generated in these pollination studies, such as the future use of machine learning.
- There is a need to inform regulation of honeybee harvesting from a future regulatory perspective.
- There is future potential for generating a beneficial bioactive/microbiome (probiotic) for feeding bumblebees where studies reveal desirable immunological and stress responses.
- There is need to combine technology-based projects with more ecological/ecosystem studies for effective, robust, concrete, plausible solutions
- There is a need to understand the relationship (interface) between top down strategic policies (such as Biodiversity Action Plan 2030; Food Vision 2030) and end-user needs or requirements – such as effective workable solutions for bee diseases informing pollination.

Dissemination and publications

Published

Goblirsch, M., Eakins, M., Rowan, N. (2021). Disease-mitigating innovations for the pollination service industry: Challenges and opportunities. Current Opinion in Environmental Science and health, Vol 22 https://doi.org/10.1016/j.coesh.2021.100265

Submitted (under review) -

Eakins, J., Lynch, M., Carolan, J., Rowan, N. (2023). Studies on the effect of nonthermally-treated pollen on colony reproductive output in commercial bumblebees (*Bombus terrestris*). Science of the Total Environment.

Future planned publications

Eakins, J., Lynch., M., Rowan, N.J. Carolan, J. (2023). The benefits of developing and applying an appropriate treatment technology for colony reproductive and functionality outputs in commercial bumble bees [Discussion paper]. Science of the Total Environment.

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Appendices

Appendix 1 representation of data protein change in relation to control group

Up in Ebeam	Down in Ebeam	Up in Fliltered	Down in Filtered
A0A088AQ81	A0A087ZZY4	A0A088AB74	A0A088A3U8
A0A088A5G3	A0A088AJM6	A0A088AD05	A0A088A5I7
A0A088ADL8	A0A088A008	A0A088AB76	A0A087ZTI8
A0A088ASV9	A0A088ABY8	A0A088ADL8	A0A088A299
A0A087ZU97	A0A088AF68	A0A087ZY35	A0A088AFL8
A0A088AQM9	A0A087ZQU2	A0A088AS11	A0A088AVC8
A0A088ALS3		A0A088AD06	A0A088A939
A0A088AVB4	A0A088ARM7	A0A088AQ58	A0A088A1B8
A0A088AUX7	A0A088AKM4	A0A087ZU97	A0A088A9C2
A0A088A3U0	A0A088AI70	A0A088APM5	A0A087ZUH7
A0A088A7D1	A0A088A2P5	A0A087ZTD7	A0A088A367
A0A088AB76	A0A088A042	A0A088AQ81	A0A088A768
A0A088A8G1	A0A088A8C7	A0A088A8G1	A0A088AAC0
A0A088ABM1	A0A088AES7		A0A088A0K0
A0A088AFG9	A0A088ATG4		A0A088A398
A0A088AVB4	A0A088A7H8	A0A088AVC4	A0A088AA70
A0A087ZWD2	A0A087ZTN5	A0A088A5G3	A0A088ATP0
A0A088AVA3		A0A088AS40	A0A087ZS68
A0A088ABM1	A0A088AD32	A0A087ZSU4	A0A088AUT2
	A0A088AQK1	A0A088AG75	A0A087ZV69
A0A088A5A6	A0A088ABN7	A0A087ZXU5	A0A088AKM9
A0A088AJD7	A0A087ZUY6	G9F9Z6	A0A088ABM1
A0A088ATH0	A0A088AUW4	A0A088ABM1	A0A088ASZ6
A0A088AFH7	H9K9P8	A0A088A4L9	A0A087ZUB8
A0A088ACJ9	A0A088A1R8	A0A087ZUE7	A0A088A902
A0A088ACK9		A0A088A8Y7	A0A087ZU85
A0A087ZVK5	A0A088AP40	A0A087ZR38	A0A088ACZ2
A0A088AL57	A0A088AKG2	A0A088A5E4	A0A088AKC5
A0A087ZXU5	A0A087ZZ55	A0A088AVB4	A0A088A2Z3
A0A087ZQW0	A0A088A0I6	A0A088A5K3	A0A088AUI9
A0A088A8Y7	A0A088AJ47	A0A088AS52	A0A088ARJ5
A0A088A8I9		A0A087ZTV4	A0A088A2R3
A0A088A2G0	A0A088AG76	A0A088A003	A0A088ARC7
	A0A088AEY3	A0A088AI42	A0A088A8B0
A0A088A4B1	A0A088A3B9	A0A088A108	A0A088AHD0
A0A088AV16	A0A088ABN3	A0A088ALS3	A0A088AKG2
A0A088A839	A0A087ZRC1	A0A088AIA2	A0A087ZXL3
A0A087ZSJ6			A0A087ZP36

Appendix 2 representation of data e-beam versus control group

Relative fold change	Apis UniProt number	Protein name					Difference
21.1121	A0A088AQ81	XP_003397300.2|uncharacterized protein LOC100644683					4.4
6.9644	A0A088A5G3	XP_012176245.1|cytochrome b5					2.8
6.498	A0A088ADL8	XP_012163499.1|LOW QUALITY PROTEIN: vitellogenin					2.7
6.0628	A0A088ASV9	XP_020721756.1|uncharacterized protein LOC100643625					2.6
4.9245	A0A087ZU97	XP_003396646.1|uncharacterized protein LOC100644966					2.3
4.287	A0A088AQM9	XP_003393261.1|mitochondrial coenzyme A transporter SLC25A42					2.1
4.287	A0A088ALS3	XP_003395337.1|1-acyl-sn-glycerol-3-phosphate acyltransferase gamma					2.1
4.287	A0A088AVB4	XP_003393866.1|cytochrome P450 9e2					2.1
3.7321	A0A088AUX7	XP_003393699.1|PRA1 family protein 3					2.9
3.7321	A0A088A3U0	XP_003399652.1|long-chain fatty acid transport protein 1					1.9
3.4822	A0A088A7D1	XP_003398970.1|ADP-ribosylation factor 2					1.9
3.249	A0A088AB76	XP_012164498.1|chaoptin					1.8
3.249	A0A088A8G1	XP_020723232.1|60S ribosomal protein L39					1.7
3.249	A0A088ABM1	XP_003402576.2|serine protease inhibitor 3/4, partial					1.7
3.031	A0A088AFG9	XP_003401025.1|ornithine aminotransferase					1.6

Appendix 3 representation of data protein abundance and reductions in e-beam group

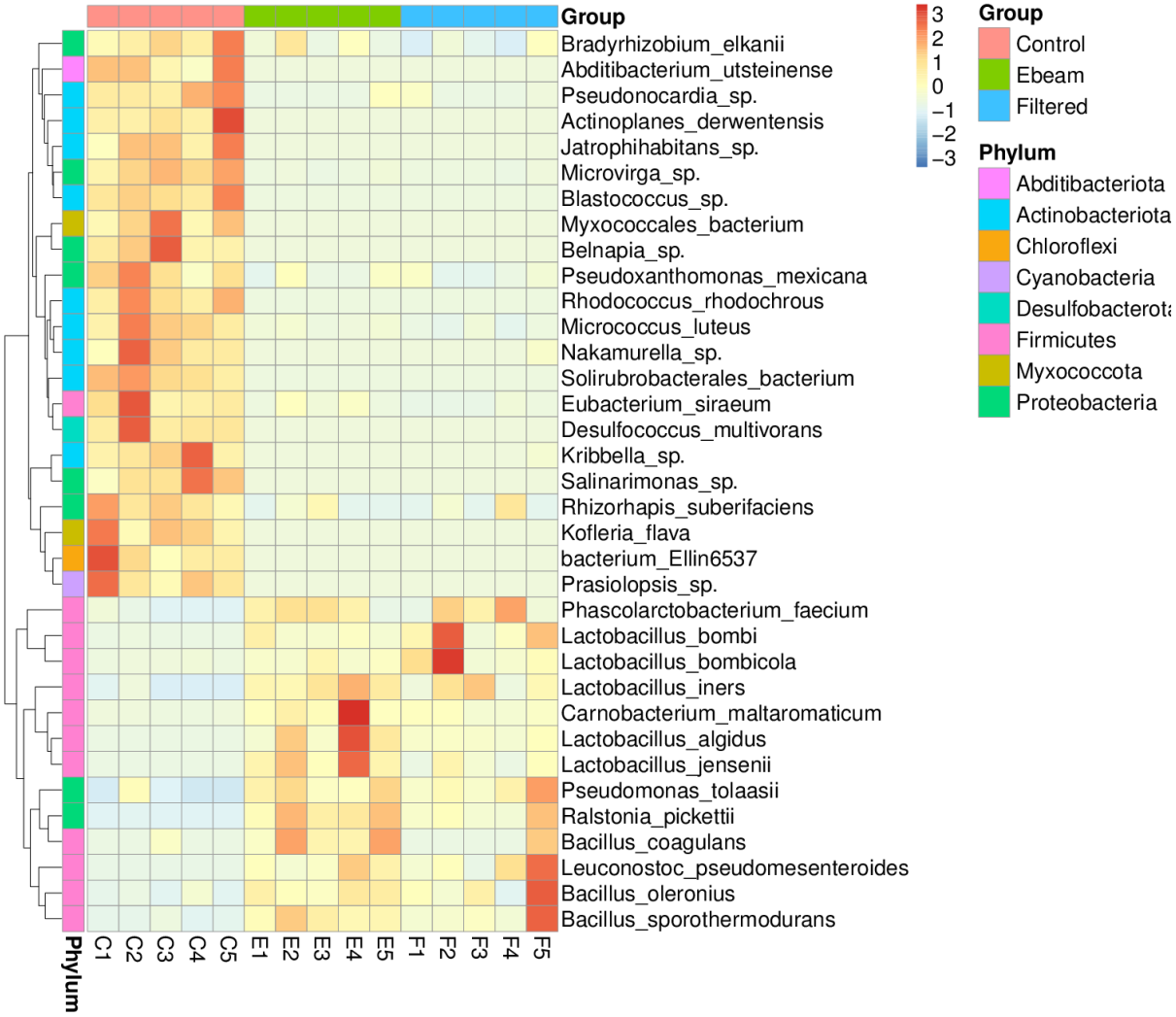
Down in Ebeam					GO Category	GO Term	Descrition	Count in Network	Strength	False Discovery Rate
A0A087ZZY4				Down in E-beam	Molecular Function	GO:0004553	Hydrolase activity, hydro	5 of 65	1.26	0.0026
A0A088AJM6					Molecular Function	GO:0016787	Hydrolase activity	20 of 1419	0.52	0.00051
A0A088A008					Molecular Function	GO:0003824	Catalytic activity	31 of 3453	0.33	0.0021
A0A088ABY8					Cellular component	GO:0005576	Extracellular region	24 of 659	0.93	1.82E-13
A0A088AF68					Cellular component	GO:0110165	Cellular anatomical entit	55 of 8868	0.16	0.0014
A0A087ZQU2					Local network cluster	CL:13148	Mixed, incl. gpi anchor r	6 of 145	0.99	0.0075
					Local network cluster	CL:13353	Mixed, incl. alpha amyla	4 of 32	1.47	0.0037
A0A088ARM7					Local network cluster	CL:13356	Glucan biosynthetic proc	3 of 12	1.77	0.0062
A0A088AKM4					Kegg pathways	ame01100	Metabolic pathways	12 of 820	0.54	0.014
A0A088AI70					Kegg pathways	ame00500	Starch and sucrose met	3 of 19	1.57	0.014
A0A088A2P5					Protein domain(smart)	SM00642	Alpha-amylase domain	3 of 5	2.15	0.0011
A0A088A042					Molecular Function	GO:0005509	Calcium ion binding	6 of 219	0.81	0.0437
A0A088A8C7					Cellular component	GO:0110165	Cellular anatomical entit	55 of 8868	0.16	0.0014
A0A088AES7					Cellular component	GO:0005865	Striated muscle thin fila	3 of 6	2.07	0.0016
A0A088ATG4					Local network cluster	CL:7190	Mixed, incl. actin cytosk	5 of 124	0.98	0.0286
A0A088A7H8					Local network cluster	CL:7201	Mixed, incl. myofilament	3 of 22	1.51	0.0223
A0A087ZTN5					Molecular Function	GO:0016787	Hydrolase activity	20 of 1419	0.52	0.00051

Up in Ebeam			GO Category	GO Term	Descrition	Count in Network	Strength	False Discovery Rate
A0A088AQ81		UP IN Ebe	Biological process	GO:0048193	Golgi vesicle transport	6 of 125	0.88	0.0397
A0A088A5G3			Molecular function	GO:0036094	Small molecule binding	22 of 1215	0.46	0.0021
A0A088ADL8			Molecular function	GO:0043167	Ion binding	31 of 2384	0.31	0.0111
A0A088ASV9			Cellular component	GO:0005789	Endoplasmic reticulum n	12 of 290	0.82	5.71E-05
A0A087ZU97			Cellular component	GO:0031090	Organelle membrane	29 of 893	0.71	2.12E-10
A0A088AQM9			Cellular component	GO:0098588	Bounding membrane of c	13 of 418	0.69	0.00027
A0A088ALS3			Cellular component	GO:0012505	Endomembrane system	26 of 1218	0.53	5.60E-06
A0A088AVB4			Local network cluster	CL:8587	Mixed, incl. golgi-associa	4 of 37	1.23	0.0179
A0A088AUX7			Subcellular locatization	GOCC:0016020	Membrane	27 of 2230	0.28	0.0497
A0A088A3U0			Subcellular locatization	GOCC:0110165	Cellular anatomical entity	75 of 8023	0.17	0.00016
A0A088A7D1			Subcellular locatization	GOCC:0005622	Intracellular	58 of 6518	0.15	0.0496
A0A088AB76			Biological process	GO:1902600	Proton transmembrane tr	6 of 75	1.1	0.004
A0A088A8G1			Biological process	GO:0044281	Small molecule metaboli	29 of 769	0.77	2.89E-11
A0A088ABM1			Biological process	GO:0098660	Inorganic ion transmemb	11 of 323	0.73	0.003
A0A088AFG9			Biological process	GO:0006812	Cation transport	11 of 380	0.66	0.0104
A0A088AVB4			Biological process	GO:0006811	Ion transport	16 of 587	0.63	0.00048
A0A087ZWD2			Biological process	GO:0044249	Cellular biosynthetic proc	20 of 1223	0.41	0.0206
A0A088AVA3			Biological process	GO:1901576	Organic substance biosy	20 of 1255	0.4	0.0269
A0A088ABM1			Molecular function	GO:0015078	Proton transmembrane tr	5 of 63	1.1	0.0143

Appendix 4 representation of data, protein detection in filtered group

Locus	Apis UniProt	Immun	Gene	Category	Peptid	Mol. weight [kDa	Score	Intensit	MS/MS coun	LFQ intensity Filt1	LFQ intensity Filt2	LFQ intensity Filt3	LFQ intensity Filt4	LFQ intensity Filt5
LOC100649621	A0A088AB74	immune	SLIT	immune_responsive	17	52.26	279.14	4.643E+09	73.00	27.83418	27.80686	28.12583	27.94843	28.48689
LOC100645534	A0A088AD05				17	57.30	199.27	2.446E+09	106.00	26.27265	26.2176	26.05238	26.61639	26.17729
LOC100650035	A0A088AB76	immune	SLIT	immune_responsive	13	100.72	323.31	7.58E+09	134.00	28.60728	28.15562	28.60102	28.57087	29.25746
LOC100650436	A0A088ADL8				117	202.48	323.31	4.18E+11	1819.00	30.48174	30.44352	30.64039	30.49333	31.01332
LOC100645190	A0A087ZY35	immune			24	85.69	163.16	3.816E+09	118.00	26.10627	25.98103	26.21601	26.12733	26.72468
LOC100647883	A0A088AS11				16	60.63	109.80	2.619E+09	62.00	26.31647	26.39341	26.39885	26.37466	26.61302
LOC100645650	A0A088AD06				14	57.92	206.11	2.719E+09	79.00	26.46986	26.50782	26.56455	27.00282	26.60781
LOC100644668	A0A088AQ58				12	40.34	138.90	1.94E+09	60.00	26.43188	26.48876	26.52362	26.60428	26.56989
LOC100644966	A0A087ZU97				3	11.08	27.67	2.801E+09	34.00	27.55003	26.89631	27.30513	27.22131	27.65895
LOC100643871	A0A088APM5				7	58.09	53.97	724140000	29.00	25.52151	25.43103	25.50803	25.80477	25.84261
LOC100643570	A0A087ZTD7				4	6.59	26.54	1.836E+09	42.00	26.33405	26.49062	26.37527	26.56945	26.73156
LOC100644683	A0A088AQ81				21	128.21	323.31	1.59E+10	161.00	27.04677	27.11555	27.22453	27.22453	27.36578
LOC110120071	A0A088A8G1				2	6.36	20.18	732480000	31.00	25.74037	25.55104	25.56877	25.65436	25.81029

Appendix 5 representation of data from 16s microbiome sequencing



Appendix 6 Published paper

Disease-mitigating innovations for the pollination service industry: Challenges and opportunities

Mike Goblirsch[1], John Eakins[2] and Neil J. Rowan[2,a]

Abstract

Commercially reared bumblebees are often deployed for fruit, vegetable, and seed crop pollination. Commercial bumblebee pollination contributes significantly to economic and nutritional security; thus, maintaining healthy stocks should be a priority for bumblebee producers. Honey bee–collected pollen is used as a nutritional source for bumblebee rearing, but potential contaminationofpollenwithpathogensrequiresmitigationtolimitspreadof infectious diseases. Gamma irradiation is the primary means of sterilizing pollen, but limitations, including off-site access to cobalt60, warrant exploration into alternatives. Sterilization technologies used inthe food safetyand medical device sectors, such as pulsed UV and electron beam, offer options with the potential to deliver safe, effective, and less restrictive mitigation. Adopting these alternatives could ultimately support healthy bumblebee stocks and reduce pathogen transmission to other bees.

Addresses

[1] USDA-ARS Thad Cochran Southern Horticulture Laboratory, 810 Hwy 26 W, Poplarville, MS, 39470, USA

[2] Bioscience Research Institute, Athlone Institute of Technology, Dublin Road, Athlone, Co. Westmeath, Ireland

Corresponding author: Goblirsch, Mike (michael.goblirsch@usda.gov) [a]

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Keywords

Decontamination, Sterilization, Emerging infectious diseases, Insect viruses, Pathogenic microbes.

Introduction

Contamination of pollen with pathogens: a source of opportunity

Bumblebees reared commercially, mainly Bombus impatiens and Bombus terrestris, are essential contributors to global food production. Visitation of greenhouse, high tunnel, and field crops such as tomatoes, peppers, cucurbits, and soft fruits by bumblebees results in highly efficient pollination. This pollination efficiency is partly explained by the ability of bumblebees to buzz pollinate or produce thoracic vibrations that trigger the release of pollen held tightly within the anthers of these flowering plants [1]. Moreover, bumblebee colonies can be produced year-round in commercial facilities, and containment of individual colonies in small, transportable units simplifies deployment to meet growers' demand. There are more than one million bumblebee colonies reared globally every year, and pollination by commercially produced bumblebees increases crop yield and quality, promoting economic and nutritional security [2e4].

Initiating bumblebee colonies artificially requires that queens be confined to small nesting boxes provisioned with food (Figure 1). Diet quality and quantity are essential for queen nesting success and subsequent colony growth [5e8]. Unlike managed honey bees
(e.g. Apis mellifera), artificial diets are not available to successfully rear bumblebee colonies [3]. Queens cannot forage freely during rearing confinement; therefore, their diet is provided to them and consists of sugar solution, which serves as a source of carbohydrates, and pollen harvested from honey bee colonies, which provides proteins, lipids, and micronutrients (Figure 2).

One concern of feeding commercially reared bumblebees honey beeecollected pollen is pathogens in pollen. Honey beeecollected pollen can be contaminated with viruses (e.g. black queen cell virus and deformed wing virus), bacteria (e.g. Paenibacillus larvae, the causative agent of American foulbrood), fungi (e.g. Ascosphaera apis, the causative agent of chalkbrood disease), Microsporidia (e.g. Nosema spp.), and protozoa (e.g. Crithidia spp.) [9e12]. Pathogens found in honey beeecollected pollen can infect bumblebees, which may pose a risk of transmission among managed and wild bee populations [9,10,13e18]. Although our understanding of the impact of pathogens on bee health is best characterized in managed bees [19,20], much remains unknown about their effects on several thousand species of wild bees [13,21e24]. As pathogens are a leading contributor to declining populations of both managed and wild bees [25e27], there is a precedent for mitigating infection

Figure 1

An early stage in the development of a bumblebee colony reared artificially. A queen incubates the brood raised atop a mass of honey bee–collected pollen. Two of the first workers have emerged to the adult stage and will assist the queen in caring for the brood. Photo Credit: Elaine Evans.

Figure 2

Collected pollen dislodged from the corbicula of honey bee foragers that have returned to their colony. A pollen-trapping device placed on the colony restricts the passage of returning pollen foragers into their nest, causing the pollen to become dislodged from their corbiculae. Significant quantities of pollen 'pellets' are harvested using this mechanism. Trapped pollen is the primary source of nutrition for rearing bumblebees. Photo Credit: University of Minnesota Bee Laboratory.

and transmission in honey beeecollected pollen provisioned to commercial bumblebee colonies.

Challenges and potentially disruptive pollen sterilization technologies

Reducing the incidence and spread of pathogens among bumblebee colonies reared commercially is a priority for producers. Goulson and Hughes [3] illustrate critical control points in the flow of pathogens among managed bees where abatement is possible and that could reduce transmission to other bees. Honey beeecollected pollen is a point for control in this scheme [3]. The most common approach to sterilizing pollen is exposure to gamma irradiation [28]. Although effective, there are drawbacks to this technology (see the following section). Limitations of gamma treatment prompt exploration of alternative technologies, especially those used in the medical device and food production sectors (Table 1), for their efficacy in inactivating bee pathogens. Before technologies are adopted to treat honey beeecollected pollen, studies should establish effective doses and determine whether there are adverse effects on nutritional quality and associated dietary microbiota [29e32].

Biological surrogates and complementary techniques to optimize sterilization processes for honey bee–collected pollen

Researchers have historically approached sterilization efficacy through biological surrogates, such as Bacillus spp. endospores [33e35] or oocysts of waterborne protozoa [36,37]. Biological surrogates are innocuous microbes exhibiting greater resistance to applied inactivation stresses and provide a safe substitute over intended target pathogens for validating sterilization processes [38]. For example, a biological surrogate is exposed to conditions of a sterilization process, and the inability of the surrogate to grow in culture after treatment confirms the process is effective. Biological surrogates used in the food safety and healthcare sectors could serve as calibrators for adapting sterilization processes against complex pathogens that affect bees [39,40]. Biological surrogates would help resolve factors mediating inactivation of target pathogens, such as highly infectious P. larvae spores. These factors are multifaceted and include operational (e.g. applied dosage, system configuration, nonthermal modality), environmental (e.g. temperature, pH, water activity), and biological considerations (e.g. amount of organic matter, diversity and abundance of parasites present, inclusion of recalcitrant life stages) [41,42]. The addition of highly sensitive and specific molecular techniques, such as quantitative polymerase chain reaction (qPCR), and cell culture could complement the use of surrogates and permit reliable post-treatment quantification of the pathogen load and reduction in viability and infectivity [38,41]. The appropriateness of complementary in vitro systems will depend on the cell line selected. In the case of bees, demonstrating inhibition of infectivity and growth of treated pathogens using cell lines established from bee tissues could be highly useful [43]. Moreover, modeling inactivation kinetics of treated-bee pathogens by flow cytometry would help evaluate sterilization modalities as it will provide real-

Table 1

Properties of different decontamination approaches considered for treating honey bee–collected pollen.[a]

Process considerations	Hydrogen peroxide vapor (VH_2O_2)	Ethylene oxide (EO)	Pulsed UV light	Moist heat	Electron beam	Gamma irradiation
Methodology	Penetration of sterilant gas	Penetration of sterilant gas	Surface irradiation	Penetration by uniform heating	Ionizing energy from electron beam	Irradiation using photons from radioisotopes
Efficacy of process	Efficacy confirmed by biological indicators and/or process monitoring	Efficacy confirmed by biological indicators and/or process monitoring	Variable, but efficacy confirmed by biological indicators or dosimetry	Efficacy confirmed by biological indicators and/or process monitoring	Efficacy confirmed by biological indicators	Process parameters confirmed using dosimetry
Penetration	Limited penetration; gaspermeable packaging/ product design required	Gas-permeable packaging and product design required	Limited penetration	Suitable for treatment of packaged products but depends on material sensitivity	Efficient penetration at bulk densities between 0.05 and 0.03 g/cc	Penetration at high densities (>0.4 g/cc)
Material compatibility	Good material compatibility except cellulose-based materials	Broad material compatibility	Broad material compatibility	Broad material compatibility, but heat can affect nutrients in pollen	Negative effects are less pronounced or eliminated based on packaging.	Broad material compatibility except plastics such as acetals, PTFE, polypropylene
Turnaround time	One-day processing	Conventional treatment requires 9–10 days.	Relatively short, typically 1 h depending on the dose	Relatively short, typically 1 h	Very short, several minutes depending on the dose	Relatively short, several hours depending on the dose
Process	Complex process that introduces VH_2O_2 under vacuum or aerosol	Complex process; variables include time, temperature, humidity, and [EO].	Simple, rapid process; delivery of UV (J/cm^2) in an enclosed chamber	Simple, rapid process; duration depends on time, temperature, and RH.	Complex process; variables include scan height, processing speed, number of passes, beamproduct alignment	Simple process; variables include time and isotope load.
Putative mechanisms of pathogen inactivation	Potent oxidizer of proteins, but mechanism is not fully understood.	Proteins, enzymes, and nucleic acid alkylation (targets sulfhydryl groups)	Irreversible damage to RNA affecting replication and infection	Thermal aggregation of viral nucleocapsid and membrane proteins	Extensive degradation of RNA and DNA — but yet to elucidate mechanisms properly	Extensive degradation of RNA and DNA molecules
Limitation	Complex process requiring monitoring and control, not for in situ application	Toxic residuals (carcinogenic and teratogenic), not recommended for in situ	Operator safety due to UV exposure, shading issues, can be used in situ	Limited by thermal sensitivity of materials (e.g. pollen)	Not often used in situ but more as an external contract service	Adversely affects material, not recommended for in situ

PTFE, polytetrafluoroethylene; RH, Relative humidity. [a]

Modified from the study by McEvoy and Rowan [31*].

time cellular and molecular mechanistic information underpinning the killing process [35,44].

Gamma irradiation

Although various sterilization technologies are applied toward mitigating pathogens found in honey beee collected pollen and equipment, gamma irradiation using cobalt-60 is the current standard [28]. Gamma irradiation causes irreparable breaks in nucleic acids and has been reported to inactivate several bee pathogens, including some but not all bee viruses [45e48]. Gamma treatment has been evaluated as safe for food production for more than 30 years (US Food and Drug Administration; URL: https://fda.gov/food/buy-store-serve-safefood/food-irradiation-what-you-need-know), and direct exposure of bees or nest materials does not affect bee survivorship [28,49]. Gamma treatment improves food safety and extends the shelf life by reducing or eliminating microorganisms. Furthermore, treatment does not make foods radioactive, compromise nutritional quality, or noticeably change taste, texture, or appearance (US Food and Drug Administration; URL: https:// fda.gov/food/buy-store-serve-safe-food/food-irradiationwhat-you-need-know). Gamma irradiation facilities can accommodate large batch sizes, and treatment is compatible with high-density materials, with excellent penetration into nonuniform packaging [33]. However, treatment must be conducted at regulated facilities, requires relatively long processing periods (hours), and potentially degrades products through the release of heat. Owing to the shortage of cobalt-60 supply, medical devices are given priority for gamma treatment, making it prudent to investigate alternative approaches for pollen sterilization.

Hydrogen peroxide in vapor form

Vaporized hydrogen peroxide (VHP) is an environmentally gaseous process used for sanitation of hospitals and health-care facilities [51,49]. The mode of action stems from the generation of free hydroxyl radicals that cause oxidation of DNA, proteins, and lipids [52]. It is effective against adenovirus and avian flu virus [53] and sporicidal when distributed evenly into areas where manual cleaning is impractical [54]. There are two types of VHP sterilization: exposure to 30e35% vapor produced by heating hydrogen peroxide (H_2O_2) or evaporation of H_2O_2 droplets from a 5e7% aerosol. These treatments have long been explored for use in factories for packaging and machinery sterilization [55] and decontamination of meat processing facilities, with varying, but potential, efficacy, against Listeria monocytogenes [51]. VHP is most efficacious on inanimate objects but would likely be unsuitable for pollen treatment as exposure to condensate or heat (55e60 C) would cause structural damage to pollen [55] and nutrient degradation (Eakins and Rowan, personal communication, December 9, 2020).

Moist heat

Moist heat uses either plant, process, or pure steam [56] and is used in the pharmaceutical industry for vaccine and medical device sterilization and in the food industry for pasteurization. Most vegetative microorganisms are inactivated between 55 and 65 C using moist heat, with more resistant microbes and spores requiring temperatures 70 C and 100 C, respectively, to achieve inactivation [56]. Owing to pollen's organic nature and denaturation of matrix proteins at >60 C [57], moist heat could be an obstacle, but further investigation is warranted. As mentioned previously, pollen will form a dough-like mass after exposure to condensate, which may provide opportunistic microbes a substrate for growth that leads to nutrient degradation and spoilage. Studies should determine if bees are attracted to pollen treated with moist heat.

Ethylene oxide gas

Ethylene oxide (EO) is a gaseous process traditionally used for sterilization of spices and now predominately for medical devices [58,59]. EO effectively diffuses through solid matter without causing damage to heat- or moisture-sensitive materials [58,60]. EO is an explosive, highly flammable gas and is highly toxic, carcinogenic, and mutagenic. It is an alkylating agent that interacts with biomolecules, such as nucleic acids and proteins. The addition of alkyl groups to these structures prevents regular cellular activity and inhibits microbial reproduction [61]. The compatibility of EO with moisture-sensitive products is of potential interest for pollen treatment. However, the generation of toxic byproducts, such as ethylene glycol, when EO interacts with water, would require further safety considerations [58,61]. Other potential drawbacks of EO include cost and cycle length [58]. Despite the compatibility with a broad range of materials, this modality will likely be reduced or replaced because of ongoing environmental and sustainability considerations.

Pulsed UV light

Pulsed UV (PUV) technology dissipates stored energy in ultrashort bursts of broad-spectrum light. Currently, PUV is used for high-throughput sterilization of packaging for the food industry [38]. PUV inactivates various complex pathogens [39,40], including those associated with bees [62].BriefPUVexposurereducestheviabilityofsurrogate oocysts of the trypanosome Cryptosporidium parvum

[37] and the trypanosome Crithidia bombi, a common bumblebee parasite [62]. PUVis considered nontoxic and environmentally friendly based on an increased understanding of the relationship between the UV dose and inactivation of cellular mechanisms [38,41,63]. PUV can be delivered from a fixed source in situ or in an adjustable configuration via a handheld device to achieve maximum exposure; however, penetration depth is limited by nontarget materials obstructing the flow of UV radiation [38]. These drawbacks could restrict usage to surface disinfection, but PUV has several advantages compared with gamma irradiation, including in situ application and relatively short processing time. Further studies are required to determine the potential of PUV for pollen sterilization.

Electron beam

High-energy electrons emitted from an accelerator (E-beam) are an alternative to gamma irradiation [33]. E-beam operates through standard electricity, negating the need for radioactive isotopes [33], and is a continuous process technology for sterilizing medical devices and pharmaceuticals [64]. E-beam reduces bacterial pathogens on fresh foods, including Bacillus cereus endospores using doses of 3.65 kGy (broccoli) and 4.8 kGy (red radish) [65]. It also reduces porcine epidemic diarrhea virus in contaminated feed [66] and causes minimal changes to powdered infant formula [67]. E-beam lacks the penetrative power of gamma sterilization, and as an in situ process, there is potential for recontamination of treated products during redistribution [65]. Despite these drawbacks, E-beam has several advantages compared with gamma irradiation and includes short exposure periods (minutes), fast cycle time, flexible batch size, even distribution of dose, simple validation, no quarantine, and real-time monitoring. Rapid processing of lowdensity materials and greater operational flexibility can make E-beam a cost-effective approach for pollen treatment.

Conclusions

Development and application of effective, nonthermal sterilization of contaminated pollen would be a potentially powerful tool to help sustain the health of commercial bumblebee stocks and reduce pathogen transmission to other managed and wild bees. Currently, there is a lack of efficacy data for emerging sterilization technologies, and research that addresses the complex morphology and culture requirements of bee pathogens is needed. This review highlights the potential benefits of alternatives to gamma irradiation for pollen treatment, but additional studies should address appropriate dosage, treatment configurations, and mechanistic information underpinning cellular and molecular damage to pathogenic microorganisms and viruses. There remains a reliance on using live bees to confirm treatment effect; however, advances in in vitro diagnostics may enable surrogate approaches as a screening tool. Novel processes will be informed by applying technology, policy, and society readiness level framework that considers the intended environment and sustainability of innovation. Ultimately, the deployment of sustainable decontamination technologies to treat honey beee collected pollen used to rear bumblebees would

contribute a vital countermeasure to reduce pollinator decline.

Author contributions

MG, JE, and NJR conceived and proposed the topics of the manuscript. MG, JE, and NJR wrote the article. MG and NJR provided funding and resources.

Equal employment opportunity (eeo)/nondiscrimination statement

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix 7