Investigating the genetics and virulence of European foulbrood, a bacterial pathogen of honey bees

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Abstract

European foulbrood (EFB), a disease of honey bee larvae (*Apis mellifera*), is caused by *Melissococcus plutonius*. This bacterium has a worldwide distribution, found wherever honey bees are kept. EFB outbreaks vary in severity, with treatment options of shook swarm, oxytetracycline antibiotic application and colony destruction available to manage this disease. *M. plutonius* bacterial isolates have previously been differentiated using a multi-locus sequence typing scheme (MLST), with a focus on tracing the source of new EFB outbreaks. A putative toxin gene, melissotoxin A, has been shown to correlate with virulence in previous larval infection studies. Whole genome sequencing of approximately 50 *M. plutonius* isolates suggests that current strain typing (ST) methods are likely insufficient to separate the most common isolates in the UK, such as ST3. Statistical analysis of the relationship between isolate possession of a gene, melissotoxin A, and type of treatment used on the EFB outbreak, showed a negative relationship between the toxin presence and incidence of colony destruction. Using laboratory reared honey bee larvae, *M. plutonius* virulence was tested at the strain level. Within these experiments, it was found that isolates possessing the melissotoxin A gene did not cause a significantly higher larval mortality rate, and expected virulence phenotypes, based on clonal complex type, wasn't always observed for strains tested. Anecdotally, treatment success of EFB with OTC may vary dependent on strain type present in the infection. Isolates of *M. plutonius* were tested for OTC resistance *in vitro* by using minimum inhibitory concentration (MIC) assays and several antibiotic resistant strains were identified. A complete understanding of all aspects of EFB, and the causative bacteria *M. plutonius*, is essential to improve treatment strategy, and therefore honey bee health, in the future.

Acknowledgments

Many people have had an impact upon me during this challenging period of my life, and I will attempt to thank them properly now.

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Author's Declaration

I declare that this thesis, aside from the exceptions detailed below, is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.

Chapter 1:

- Images of honey bee diseases courtesy of The Animal and Plant Health Agency (APHA), Crown Copyright.
- Figure 1.4. Changes in treatment strategy of EFB in the UK over time created by Prof. Giles Budge.

Chapter 2:

- 16 *M. plutonius* isolates were originally cultured, extracted, sequenced, and assembled by Dr Edward Haynes, Fera Science Ltd.
- Sequencing services and automated bioinformatic pipelines (Trimmomatic , Samtool, Bedtools, Kraken, BWA-mem, SPAdes, QUAST) for the remaining 46 *M. plutonius* isolates was provided by MicrobesNG (http://www.microbesng.uk), which is supported by the BBSRC (grant number BB/L024209/1).
- Further bioinformatic analysis pipelines in section 2.4.10. (SPAdes, Snippy, Kraken, Nullarbor) exc by Sam McGreig, Fera Science Ltd.

Chapter 3:

- Protocol for grafting, feeding and checking larvae is adapted from OECD Test Guidance Document 239 (2016): Honey Bee Larval Toxicity Test following Repeated Exposure, courtesy of Dr Emma Wright and Selwyn Wilkins, Fera Science Ltd.
- Set up of larval rearing experiments was performed by the author with assistance from Sally Ponting and Kirsty Stainton.
- Confirmation of EFB infection in larvae during experiment using microscopy performed with assistance from Dr Kirsty Stainton, then Fera Science Ltd.
- Map of 2018 UK EFB outbreaks Figure 3.9. by Victoria Tomkies, Fera Science Ltd. with data from Prof. Giles Budge.

Chapter 4:

- MIC assay protocol design based on Clinical and Laboratory Standards Institute (CLSI) guide, 2017.

1. Introduction

This introduction provides an overview of the honey bee, *Apis mellifera*, its value as a pollinator, the different threats the species face from pathogens, an introduction to the bacterium *Melissococcus plutonius* which causes European foulbrood (EFB), diagnosis and treatment, disease pathogenesis, the genetics of *M. plutonius* and a summary of previous EFB research. Additionally, the aims of this thesis and the research undertaken are presented.

1.1. An introduction to honey bees (*Apis mellifera*)

1.1.1. Biology and geographic origin of honey bees

The European, or western honey bee, is a flying, eusocial insect belonging to the genus *Apis*, in the family Apidae. Colonies of honey bees produce a nest structure made of wax, which contain the queen, her brood and their food in hexagonal shaped cells (Seeley, 1989), all of which are housed in wooden structures maintained by beekeepers. Honey bee queens are responsible for producing both fertilised and unfertilised eggs that become workers and drones respectively, or future virgin queens, and populate the hive with genetically related cooperating individuals (Page & Robinson, 1991). The specifics of honey bee development are further explained in Chapter 3.

A. mellifera is one of eleven bee species belonging to this genus (Crane, 2009), including the Asian honey bee (*A. cerana*) and the giant honey bee (*A. dorsata*). The species *A. mellifera* is also split into various complex subspecies because of large amounts of both natural and human-induced hybridisation over time

(Meixner, *et al*., 2013). The majority of *Apis* species originate from the continent of Asia, however *A. mellifera* is native to Europe, Africa, and the Middle East (Han, *et al*., 2012). It was therefore assumed that *A. mellifera* also arose from Asia and expanded from there into other continents over millions of years. However, a recent study of the mitochondrial DNA of 18 *A. mellifera* subspecies supported an African or Middle Eastern origin instead, colonising Europe later through Asia Minor and the Strait of Gibraltar (Tihelka, *et al*., 2020). Overall, the fossil record of *A. mellifera* remains limited (Baker & Chmielewski, 2003) and only half of known *A. mellifera* subspecies have sequenced genomes (Tihelka, *et al*., 2020), so it therefore remains there are still questions regarding the origin of the honey bee in Europe.

1.1.2. Beekeeping: history and present

Honey bees have co-existed with humans for thousands of years (Crane, 1999), with early humans hunting for honey throughout both Africa and Europe. Beekeeping has been recorded as early as 3,000 years ago, with apiary-like structures discovered at an archaeological site in northern Israel (Bloch, *et al*., 2010). Additionally, more advanced beekeeping relics have been discovered in ancient Greek sites, with examples of smoking pots and honey extractors being recovered (Graham, 1975). As a result of this long historical co-existence with humans, nearly all western honey bee populations now need to be carefully managed to ensure survival. Colonies of *A. mellifera* subspecies exist in the wild, but these are rare in Europe and are often described as feral if found (Kohl & Rutschmann, 2018). The subspecies *A. mellifera scutellata*, the East African lowland honey bee, survive successfully in their native arid environment and specific traits such as having smaller average colony sizes (McNally &

Schneider, 1996) and behaviours such as swarming more often, appear to reduce mortality from pathogens (Locke, 2016), particularly *Varroa*. This is despite maintaining overall high colony densities in an area (Moritz, *et al*., 2007). Additionally, beekeeping of the Africanised honey bee, a cross breed of *A. mellifera scutellata* and other *A. mellifera* subspecies, is now popular in some countries in Central and South America (Winston, 1992), despite exhibiting less favourable traits such as absconding as a colony in response to stress and overall higher levels of aggression. This is due to hybrid subspecies colonies outperforming the Western counterpart, having increased levels of both disease resistance and honey production (Livanis & Moss, 2010).

In Europe between 1965 and 2005, there was an observed decline in the number of managed honey bees, specifically in central European countries (Potts, *et al*., 2010). However, in more recent years there has been a great uptake in interest in hobby beekeeping in the UK. In 2013, there were approximately 29,000 beekeepers registered with the National Bee Unit's (NBU) database Beebase managing around 126,000 hives, compared to 15,000 beekeepers managing 80,000 hives in 2008. In 2019, the latest NBU count, there were approximately 264,000 honey bee hives in the UK, which suggests an ever further rise in beekeepers in the UK population (statistics obtained from NBU). In addition to the NBU, there are many excellent organisations in the UK which aid individuals to learn the essentials to be successful in their beekeeping, such as the British Beekeepers Association, the Bee Improvement and Bee Breeders Association and the Bee Farmer's Association, overall strengthening the field with improved knowledge and practical skill.

1.1.3. The importance of the honey bee

In the present day, *A. mellifera* remain ecologically and economically important because of both their sizable contribution to worldwide crop pollination, importance to agricultural services, and because of their specific by-products such as honey and beeswax (Gallai, *et al.*, 2009). In California, in the United States, almond pollination is the biggest example of managed pollination in the world, with 70% of commercial hives in America used for this one type of crop (Lee, *et al*, 2019). Pollinators such as bees are critical to other types of food production, with estimations of a value exceeding £200 million per year in the UK alone (Carreck & Williams, 1998; Mwebaze, *et al*., 2010). Apples, oilseed rape and greenhouse-pollinated tomatoes are particularly valuable in this calculation based on historic estimated values of crops to the economy (Carreck & Williams). Additionally, there is now a strong cultural movement associated with “saving” the honey bee, and this, coupled with increasing environmental awareness and responsibility, has resulted in a higher value placed on preserving our natural ecological systems (Aryal, *et al*., 2020).

As a result, the protection of bees is critical for food security, and attempting to unravel the relative importance and interaction of all threats to the honey bee must begin with a thorough understanding of each factor affecting their decline (Vanbergen, *et al*., 2013). In 2006, Colony Collapse Disorder (CCD) began to be observed as a phenomenon (Ellis, *et al*., 2010), mainly in the USA. CCD is characterised by an unexplained sudden loss of adult bees from hives without any obvious disease but rather vague symptoms. Populations are now also under threat from multiple major pathogens, which will now be discussed.

1.1.4. Threats to honey bees from pathogens

There has been an observable decline in bee numbers for several decades (Potts, *et al*., 2010), but the overall cause, if there is one, is unclear and complex. It has been hypothesised that general hive stress may be a contributory factor in some cases, exacerbated by the presence of extra risk factors, for example parasites (Evans & Cook, 2018), fungi (Higes *et al*., 2007), viruses (De Miranda & Genersch, 2010), bacteria (Forsgren, 2010; Genersch, 2010a), and pests (Genersch, 2010b; Zhu, *et al*., 2020). The National Bee Unit is a recognised centre of excellence for providing advice and scientific research in bee health based in England and Wales (https://nationalbeeunit.com/). This centre of excellence for bee research facilitates the management and control of bee diseases and pests, trains beekeepers, provides laboratory support and diagnostic service, and employs seasonal Bee Inspectors with the goal of reducing or eliminating disease in the field, such as the examples discussed below.

Figure 1.1. Female adult Varroa mite (left), honey bee displaying DWV symptoms (middle) and ropiness test for AFB on a larva using a matchstick (right). Images courtesy The Animal and Plant Health Agency (APHA), Crown Copyright.

1) Parasites

Honey bees have several types of pathogenic parasite, but the most important, and deadly, is *Varroa destructor*, an ectoparasitic mite, that infests both *A. mellifera* and *A. cerana* (Figure 1.1., Zhou, *et al*., 2004). *Varroa* mites are now reportable at the apiary level in the UK as of 2021 (Bee Diseases and Pests Control Order 2021 amendment (England), similar order in place in Scotland and Wales). This parasite feeds by attaching to the exterior of the bee, consuming the fat from the body (Ramsey, *et al*., 2019), and weakening the individual through loss of mass. The life cycle of the mite is intrinsically linked to existence within a hive, with all stages of the life cycle dependent on honey bees for survival (Evans & Cook, 2018). Additionally, *Varroa* mites may act as disease vectors for other pathogens, and contribute greatly to the stress of a colony if large loads are present (Wilfert, *et al*., 2016). As a result, *V. destructor* parasite presence is closely monitored by bee inspectors and may be treated with chemicals such as Varroacides, or by trapping mites in a comb structure (Rosenkranz, *et al.,* 2010). Another potential threat is the parasitic small hive beetle (Hood, 2004), *Aethina tumida*, which is currently not present in the UK, is still a notifiable disease and thus imports from affected countries are carefully monitored for the parasites presence.

2) Viruses

There are many viruses that impact the health of honey bees but there are a couple that are more commonly found in the UK. Deformed wing virus (DWV) causes deformities in the adult bee, specifically the wings indicated by the name, but also the abdomen and legs (Figure 1.1., De Miranda & Genersch, 2010). DWV is particularly associated with the presence of previously

mentioned *Varroa* mites (Wilfert, *et al*., 2016), with high levels of mortality likely in a colony where both pathogens are found to be present, and a reduced probability of survival over winter (Dainat, *et al*., 2012). Chronic bee paralysis virus (CBPV) affects the nervous system of the adult bee, causing motility problems which can result in ejection from the hive by other bees (Ribière, *et al*., 2010), and this sudden loss in numbers will lead directly to the collapse of the colony. Problematically, nearly all honey bee viruses have limited treatment options at present, although there are a multitude of research avenues currently being explored in this area.

3) Fungi

Two major fungi exist that infect and cause disease in honey bees. Nosema disease is caused by two species of fungus, *Nosema apis* and *Nosema ceranae*, also categorised as microsporidian parasites (Fries, *et al*., 2013). *N. apis* has been found in European honey bees for over one hundred years (Fries, 2010), but *N. ceranae* evolved to infect the Asian honey bee (*A. cerana*) instead, with infections in *A. mellifera* only relatively recently documented (Higes*, et al*., 2006). Nosema affects all castes of the adult bee, shortening their lifespan considerably which will likely result in a weaker colony (Fries, *et al*., 2013). Chalkbrood disease is caused by the fungus *Ascosphaera apis* and affects the larval stage of the bee (Aronstein & Murray, 2010), which ultimately causes starvation. Both *Nosema* fungi and *A. apis* produce spores that are easily spread amongst hives by adult honey bee behaviour such as defecation, drifting and honey robbing (Genersch, 2010b) which commonly transmit many of the honey bee diseases discussed here.

4) Pests

Pests of honey bees can range from life threatening to the bee, to a nuisance to the beekeeper. The yellow legged Asian hornet (*Vespa velutina nigrithorax*), a highly invasive species originating from Southeast Asia, is a newly emerging threat to honey bees in the UK (Jones, *et al.,* 2020). This hornet is extremely successful at colonising new areas and has become widespread in much of mainland Europe, predating honey bees. In 2016, the yellow legged hornet was discovered in the UK for the first time, and subsequently destroyed, with one other incidence in 2017, and as a result the situation is now being closely monitored (Jones, *et al.,* 2020). The wax moth larvae (*Galleria mellonella*), a minor pest in contrast, does not directly harm the honey bee but instead feeds on beekeepers stored wax and honeycomb and damages equipment and frames (Wojda, *et al*., 2020).

5) Bacteria

There are two important bacterial pathogens of *A. mellifera*, both of which affect the larval stage of the bee. American foulbrood, caused by *Paenibacillus larvae*, is an incurable and notifiable disease that causes high mortality in hives, and as a result is exclusively destroyed when discovered (Genersch, 2010a). AFB disease symptoms include a foul odour, brown larvae with “ropey” texture and uneven mottled brood pattern (Figure 1.1.). Additionally, *P. larvae* produces heat- and freeze-resistant spores that can survive in an environment for more than 50 years, only sporulating when conditions become favourable (Genersch, 2010a). The second bacterium *M. plutonius*, which causes European foulbrood in honey bees, is the focus of this thesis and is discussed later in this introduction.

1.1.5. The honey bee gut microbiome

The honey bee microbiome, the natural bacterial community residing in the gut, is intrinsically linked to individual health, as is also often the case for both plants and other animals including humans (Cho & Blaser, 2012; Mueller & Sachs, 2015; Valdes, *et al*., 2018). For honey bees, the microbiome is linked to several vital functions, such as modulating immunological processes, protection from disease, larval development and nutrient metabolism (Raymann & Moran, 2018). Interestingly, in comparison to other insect species, the honey bee microbiome is highly conserved, consisting of only a few core bacteria species (Moran, 2015). These include *Snodgrassella alvi*, *Gilliamella apicola*, *Bifidobacterium* and *Lactobacillus spp.* Other rarer species that make up the "non-core" may also be present at any one time, including pathogenic bacteria (Moran, 2015). Worker bees emerge with an absence of any gut bacteria and then acquire their bacteria communities by between four to six days of adulthood, with species composition largely like those of the nurse bees within the colony due to their close association with the larval stage (Powell, *et al*., 2014).

Probiotics administered prophylactically have been suggested as a potential solution to 'unhealthy' hives, such as a study of AFB aiming to counter *Paenibacillus larvae* infection using *Lactobacillus spp.* administered through a "BioPatty" in the food (Daisley, *et al*., 2020). A similar strategy has been trialled against *N. ceranae*, with some success (Borges, *et al*., 2021). As multiple honey bee pathogens are found in the gut environment as previously detailed, novel strategies such as administering bacteria with proven probiotic properly may be

ideal candidates to treat disease in the future, whilst also avoiding any potential for problems such as antibiotic resistance to arise.

1.2. An introduction to *M. plutonius*, the causative bacteria of EFB in honey bees

1.2.1. Classification and microbiology of *M. plutonius*

M. plutonius is a gram-positive facultative anarobic bacterium belonging to the family Enterococcaceae, discovered in 1912 as an unculturable bacteria that could only be observed initially under a microscope (White, 1912). However, around forty years later, advances in the field of microbiology allowed further study and characterisation of the microbe, which was consequently named *Streptococcus pluton* (Bailey, 1957). This taxonomic classification was revised in 1982 by Bailey to *Melissococcus pluton*, which remains the only species in the genus *Melissococcus*, due to discovering dissimilarities with existing *Streptococcus spp.* (Bailey & Collins, 1982). The final alteration to *Melissococcus plutonius* was in 1998 consistent with universal changes in phylogenetic nomenclature (Truper & de Clari, 1998). Morphologically, *M. plutonius* appear as white-ish round colonies when grown on M110 agar plates, and as cocci shaped under the microscope, forming pairs or chains (White, 1912; Forsgren *et al*., 2013), and are considered fastidious organisms to grow under laboratory conditions, with relatively slow growth. This has led to *M. plutonius* to be relatively understudied in comparison to other honey bee diseases (Forsgren *et al*., 2013).

1.2.2. Diagnosis and treatment of EFB in honey bee hives

Initially, if disease is suspected, an inspection of the hive will be performed. EFB is a notifiable disease, and suspected infection is required to be reported by law in England and Wales (The Bee Diseases and Pests Control (England) Order

2006; The Bee Diseases and Pests Control (Wales) Order 2006). Visually, EFB signs include irregular brood capping and unusually positioned or discoloured larvae (Figure 1.2., Forsgren, 2010).

Figure 1.2. Uneven brood pattern of EFB and shrunken larvae in cells. Courtesy The Animal and Plant Health Agency (APHA), Crown Copyright.

As brood disease symptoms may be present without EFB, additional diagnosis is routinely performed in the field using Lateral Flow Devices (LFDs) which incorporate a monoclonal antibody to indicate the presence of *M. plutonius* within a sample (Tomkies, *et al*., 2009), giving a positive or negative result. Further samples are usually sent for laboratory testing at facilities such as the NBU in the UK, using both microscopy and specific PCR methods (Forsgren, *et al*. 2013). Treatments of milder cases of EFB, or larger colonies, include shook swarm; the transfer of adult bees to a new hive, and an antibiotic, oxytetracycline (OTC), now less commonly used than in previous decades (Figure 1.3., Budge, *et al*., 2010). Using OTC as a treatment option is further discussed in Chapter 4. If a more virulent or persistent infection is noticeably present, or if the colony is very small, then livestock will sadly be destroyed.

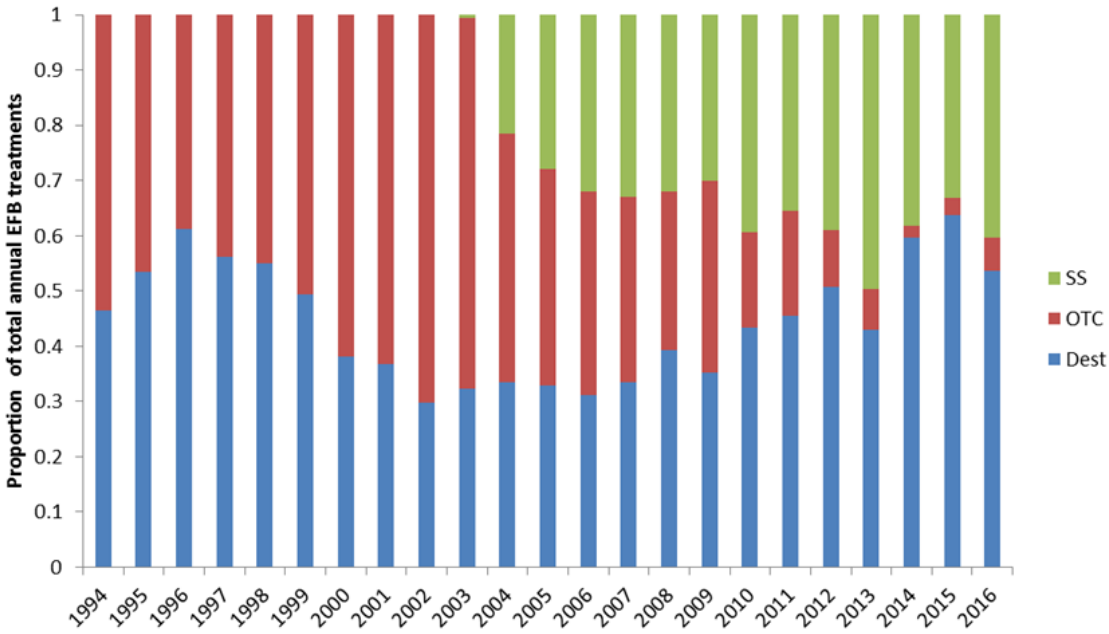

Figure 1.3. Changes in treatment strategy of EFB in the UK over time; Budge, (unpublished). Figure key: SS = shook swarm, OTC = Oxytetracycline treatment Dest = hive destroyed.

1.2.3. EFB pathogenesis

Proliferation of *M. plutonius* occurs in the gut of the honey bee larva, transferred within infected food by worker or nurse bees (Forsgren, 2010), with larvae dying when they are around three to five days old. Commonly, these EFB vector bees encounter *M. plutonius* through contact from their beekeeper, who may for example have shared contaminated equipment with others (Forsgren, 2010), but can also face infection thorough normal honey bee behaviour, such as foraging and honey robbing, and thus is difficult to eliminate entirely through improved husbandry practices. The exact mechanism in which EFB causes damage and mortality to honey bee larvae is unknown. It may be that the bacterium competes with its host for nutrients in the midgut (Bailey, 1981) or that the invasion and establishment of *M. plutonius* in the gut membranes may be harmful because of a reduction of the larvae's gut surface area and therefore

ability to absorb nutrients. Alternatively, it may be that there is a secretion of harmful compounds by the bacterium that directly impacts the host tissues and overall health. The types of genes that may be important in infection and mortality during an EFB outbreak are discussed further in Chapter 2: Sequencing and comparison of *M. plutonius* isolates from European foulbrood outbreaks in the United Kingdom.

1.2.4. Genetic differentiation of *M. plutonius* isolates

Multi Locus Sequence Typing (MLST) has been used to investigate evolutionary relationships among many bacterial strains. It relies on allelic profiling of specific house-keeping genes, with multiple conserved genes used to differentiate isolates (Maiden, *et al*., 1998). Relatively recently, a MLST scheme was developed for *M. plutonius* (Haynes, *et al*., 2013) identifying 35 sequence types (ST) (now 41) based on 4 housekeeping genes (Figure 1.8.). The use of sequence typing in a study of EFB outbreak (in a previously disease-free area) allowed the introduction to be traced back to the source of infection. The first whole genome sequence of *M. plutonius*, isolate ATCC 35311, was published in 2011 (Okumura, *et al*., 2011) and the first comparative genomics of *M. plutonius* was published in August 2018 (Djukic, *et al*., 2018), which focused on isolates originating from Norway and Switzerland. Whole genome sequencing will be discussed further in Chapter 2.

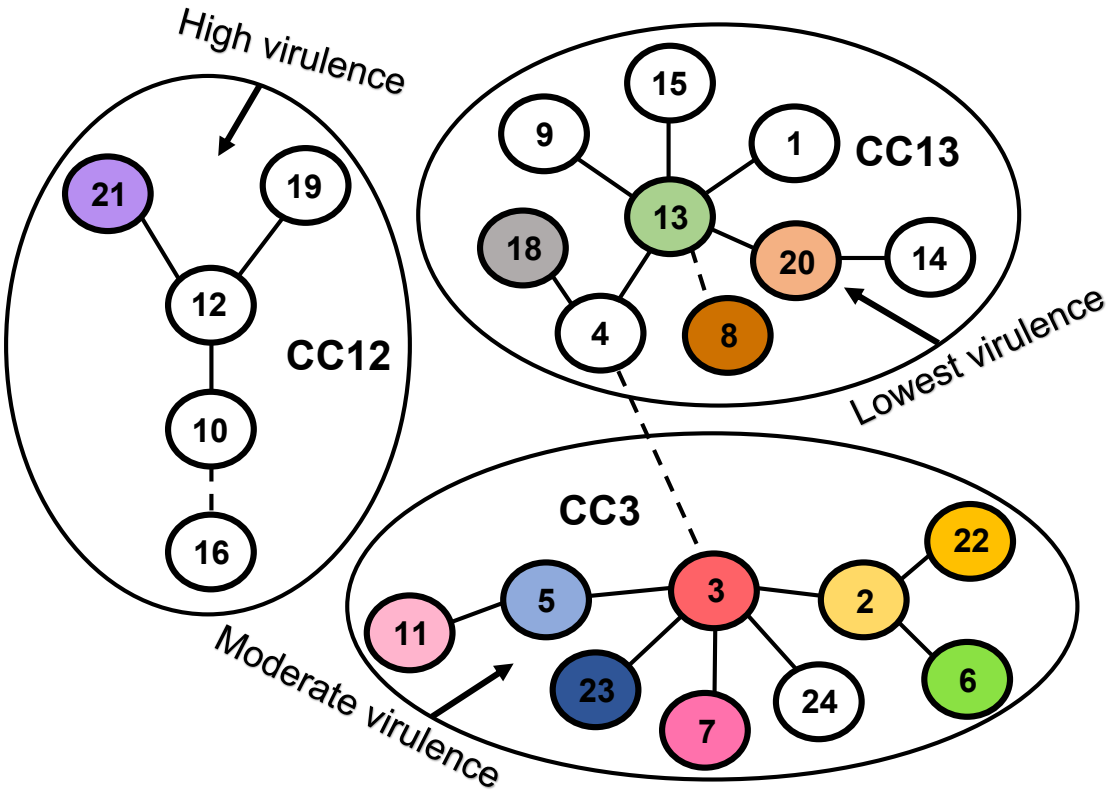

Figure 1.4. The genetic relationship of a selection of *M. plutonius* ST types, clustered into clonal complexes (CCs), a filled line represents allelic difference in one of four housekeeping genes, a dashed line represents two (Haynes, *et al*., 2013).. Coloured filled circles are strain types correspond to types used within this thesis.

1.3. The aims of this thesis

1.3.1. Improving the genetic understanding of *M. plutonius*

At the start of this PhD, there were no comparative genomics studies of *M. plutonius* published, and relatively few whole genome sequences available to analyse. As a result, there was little to no understanding of the bacterial genetics outside MLST typing, with a focus instead on disease outbreak tracing. This thesis aims to sequence additional whole genome sequence of *M. plutonius* of UK origin, identify the types of genes that may be playing a role in more virulent EFB infections and see if current strain typing methods are sufficient to differentiate bacterial isolates to an informative level.

1.3.2. Understanding the link between the genetics of *M. plutonius* and real EFB disease outbreaks and larval infections

There has been one published study that has included *M. plutonius* isolates of UK origin (Grossar, *et al*., 2020) in artificially reared honey bee larval infection experiments. Since this PhD project began, several studies have hypothesised that a type of toxin, “melissotoxin A” may be important in infection. This part of the thesis aims to validate if this is the case, and whether previously defined clonal complex always predicts isolate virulence as believed.

1.3.3. Testing the resistance of *M. plutonius* isolates to OTC

Although usage of antibiotics to treat EFB has dwindled in recent years, isolates used in this thesis originate from 2010-2012, and some of these were indeed subjected to an OTC treatment regime. Anecdotally, treatment failure is unusually high in ST5 isolates treated with OTC (Budge, unpublished). The last study of *M. plutonius* antibiotic resistance, around two decades ago (Waite, *et*

al., 2003b), found no isolates tested were resistant. Antibiotic resistance can evolve rapidly, and an updated knowledge of any emergence in a pathogen is essential when deciding on disease treatment regimens for in the future.

2. Sequencing and comparison of *M. plutonius* isolates from European foulbrood outbreaks in the United Kingdom

2.1. Abstract

Advances in next generation sequencing technology in the past few decades have made whole genome sequencing of bacteria both more easily accessible and cheaper. As a result, most bacterial species important in world epidemiology have now been fully sequenced, *M. plutonius* included. The genetics of *M. plutonius* have remained relatively understudied to date, but a recently developed MLST scheme that separates isolates into sequence types (STs) has been published (Haynes, *et al*., 2013). 46 UK *M. plutonius* isolates were selected for sequencing in this study, with varying ST type and county origin. These were supplemented by 16 isolates previously sequenced. Whole genome sequencing, followed by assembly, alignment and gene annotation revealed some differing gene content amongst these isolates and the presence of several types of bacteriophages. Numbers of core and accessory genes present in strains were identified in a sub-set of isolates, and relatedness to each other explored phylogenetically. Chi-squared analysis of the relationship between isolate possession of a toxin gene, melissotoxin A, and treatment regime used on the original EFB outbreak showed a negative relationship between having this toxin and colony destruction. Genetic studies undertaken at the whole genome level are required to fully understand bacterial relationships and disease dynamics.

2.2. Introduction

2.2.1. Next generation sequencing of bacterial isolates

Genome sequencing, determining the DNA sequence of an organism in it's entirety, is becoming easier, cheaper, and faster (Loman, *et al*., 2012). Sequencing has advanced extremely rapidly in the last few decades (Parkhill, 2013), providing new molecular methods to explore a range of important biological questions in fields ranging from ecology (Ekblom & Galindo, 2011) and plant biology (Egan *et al*., 2012) to biochemistry and functional genomics (Morozova & Marra, 2008). Advances in next generation 'sequencing by synthesis' technologies (Mardis, 2008), such as Illumina and Ion Torrent sequencing, have allowed the collection of previously inaccessible raw DNA data (Mardis, 2008), the whole genome sequence. There are advantages and disadvantages to the sequencing platforms available such as: read length, cost per base, and run time, all of which are important to take into consideration when choosing a technology (Loman, *et al.,* 2012).

2.2.2. Genomic analysis of bacteria: the core genome vs the accessory genome

Bacterial strains within a specific species have both a conserved set of "core" genes and a variable number of extra genes, termed "accessory' genes (Segerman, 2012), and the ability to categorise these as such has become increasingly easy with advances in whole genome sequencing technology and analysis pipelines. The comprehensive collection of genes present within a bacterial species has been termed the 'pan-genome' (Tettelin*, et al*., 2005), with core genes defined as such if they are ubiquitous in that species and found within all genomes studied, and additional genes categorised by their lower

relative commonality as accessory genes. Core genes are often essential to the bacterial function and survival (Tettelin*, et al*., 2005). Accessory genes, however, are only present in a sub-section of the strains of interest (Tettelin, et al., 2005), and may be involved in more niche functions such as virulence, antimicrobial resistance, or metabolism of novel substances (Croll & McDonald, 2012). An understanding of which genes are necessary for survival, and which are “bonus” genes offering beneficial functions, are useful when studying a bacterial species in depth, particularly in the context of pathogenesis (Tettelin*, et al*., 2005).

2.2.3. Genetic comparisons of *M. plutonius* isolates

Early studies revealed only small amounts of inter-isolate sequence variation in isolates of *M. plutonius* (Djordjevic, *et al*., 1999) due in part to the limited technological capabilities to differentiate bacteria on a deeper level, at the time. The first whole genome sequence of *M. plutonius*, isolate ATCC 35311, was published in 2011 and provided the first reference strain to build on for future studies (Okumura, *et al*., 2011). This reference genome consists of 1,891,014 bp (plus a plasmid of 177,718 bp), with a total of 1,773 protein-coding genes, and an additional 150 genes within the plasmid (total 1,923 genes). The reference also contains a single prophage-like sequence, and the closest bacterial species to *M. plutonius* defined using BLAST, is *Enterococcus faecalis* V583. A published reference genome is essential for any bacterial species, for both identifying important genes and other features, and for facilitating new comparative genomics studies amongst newly sequenced *M. plutonius* isolates, to act as a scaffold for the assembly of reads.

With advances in technology, more recent studies then discovered that isolates do vary more widely in both genotype and phenotype, with the additional identification of an ‘atypical’ strain of *M. plutonius*, DAT 561 (Arai, *et al*., 2012). This ‘atypical’ strain grows readily without the addition of potassium phosphate to growth media and maintains virulence when cultured in a laboratory setting, so has been used to carry out several experiments thus far. Strain typing of *M. plutonius* isolates became possible with the creation of an MLST scheme, as introduced in Chapter 1, and these strain types were later categorised into three clonal complex types that vary in their pathogenicity (Budge, *et al*., 2014).

The first comparative genomics of *M. plutonius* was published in 2018 by Djukic, *et al*., focusing on isolates originating from Norway and Switzerland. This study identified the following types of genes**:** bacteriocins, bacteria cell surface- and host cell adhesion-associated proteins, an enterococcal polysaccharide antigen, an epsilon toxin, proteolytic enzymes, and capsule-associated proteins, that they hypothesised to play a role in bacterial pathogenesis. They verified that several of these genes were expressed *in vivo*, including the epsilon toxin (‘melissotoxin A’), by sampling larvae exhibiting EFB symptoms and performing RT-PCR (Djukic, *et al*. 2018).

2.2.4. Adhesion and biofilm formation genes

Pathogenic organisms often target host tissue at mucosal barriers, such as the gastrointestinal tract, as is the case for *M. plutonius* (Forsgren, 2010) which lives and proliferates in the gut of the larvae. Bacterial adhesion is a species-host specific phenomenon. Micro-organisms are adapted to invade surface tissue, using adhesins to facilitate binding to host cell receptors and to avoid

elimination by the host organism (Chhatwal, 2002). It is therefore reasonable to assume that genes that promote the binding of bacterial cells to the larval gut epithelium may be involved in the progression of EFB disease.

Biofilms are populations of microorganisms that are encapsulated by a secreted extracellular slime. These polysaccharide matrices protect the bacteria from substances such as antimicrobial compounds and from attack by the host immune system (Dunne Jr., 2002). The formation of a *M. plutonius* biofilm within the gut walls of the larval host may restrict nutrient acquisition so therefore, differences in genes that regulate the formation of biofilms may be important in pathogenicity.

2.2.5. Mosquitocidal toxin – 'Melissotoxin A'

A toxin, "melissotoxin A', was identified in the comparative genomics study (Djukic, *et al*., 2018) as having sequence similarity to a different mosquitocidal toxin and found on the plasmid of *M. plutonius*. The mosquitocidal toxin that melissotoxin A shares a similarity to is a virulence factor in the bacteria *Lysinabacillus sphaericus* (Carpusca, *et al*., 2006). This bacterium was found to have insecticidal properties in the 1960s and has been used in previous studies as a biological control (Porter, *et al*., 1993). Interestingly, this bacterium and toxin targets the larval stage of the mosquito with high specificity (Wirth, *et al*., 2013), binding to the midgut cells and causing direct tissue damage. Another gram-positive soil dwelling bacterial species, *Bacillus thuringensis*, is used commercially as a biocontrol agent on pest insects (Jouzani, *et al*., 2017). This species invades the gut environment and releases toxins to cause rapid host mortality. Hypothetically, melissotoxin A may be playing a similar role in EFB

infections as these two examples. A recent study showed that the presence of this toxin does appear to cause increased virulence in larval infection assays (Grossar, *et al*., 2020). Three isolates possessing melissotoxin A genes caused increased brood mortality, relative to others tested within in these experiments.

2.2.6. Bacteriophages

Bacteriophages play a role in infection by being vectors for virulence factors such as toxins and have been found in most bacteria, including *Escherichia coli* (Oelschlaeger, *et al*., 2002) and *Salmonella enterica* (Kropinski, *et al*., 2007). No previous literature exists discussing the bacteriophage present in *M. plutonius* isolates. The bacteriophage species in *P. larvae* (the cause of American foulbrood in honey bees) have been described (Merrill, *et al.,* 2014), with 48 types of phage currently sequenced and annotated (Stamereilers, *et al*., 2018). Some of these have been trialled for use in phage therapy of honey bee hives (Brady*, et al*., 2017) and it is hoped these *P. larvae* bacteriophages may help combat AFB as a biocontrol agent.

2.3. Aim of study

This study sequences 46 new genomes of *M. plutonius* isolates, originally taken from historical EFB outbreaks in the UK. Once assembled and annotated, the types of genes present in *M. plutonius* isolate genomes that may be important in larval infection are identified and discussed. These isolates are then compared phylogenetically, with SNP differences and core and accessory genomes estimated, and an algorithm gives a measurement of genomic sequence similarity. The presence or absence of specific prophage regions is also explored. A toxin gene is investigated further as to potential relevance in infection by comparing presence and absence to the EFB treatment regime applied at time of infection. An improved overall knowledge of how isolates differ by strain type or genetic feature, would be advantageous to inform future management of EFB outbreaks and to provide a foundation to any wet-lab experimental works.

2.4. Materials and Methods.

2.4.1. Selecting *M. plutonius* isolates for sequencing

Isolates were taken from the established collection at Fera Science Ltd., which was created from EFB disease samples received to the NBU from outbreaks in the UK around a decade ago, between 2010-2012. Isolates can be identified only at the county level due to data protection, and the treatment type used on hive(s) once EFB presence was discovered is mostly known. Over the duration of this PhD, over 100 isolate DNA extractions were collected for further analysis, but many were not found to be suitable for sequencing due to issues such as contamination or lack of quality or quantity of DNA extracted. Repeated attempts were made to extract sufficient DNA or remove contamination from isolates, such as changes to lysis steps and extraction methods, longer growth periods and consultation with microbiology experts at Fera, but still many isolates within the collection could not be prepared for whole genome sequencing.

2.4.2. Culturing of *M. plutonius* isolates

46 isolates of varying ST, UK origin and outbreak treatment were sent for sequencing (Table 2.1.). The culturing method of *M. plutonius* is fully described in ‘Standard methods for European foulbrood research’ (Forsgren, *et al*., 2013). Isolates were grown on M110 agar anaerobically with added cysteine hydrochloride at 37°C for approximately 2 weeks.

Table 2.1. Metadata of *M. plutonius* isolates used in this study. Isolates within a study ID were cultured, extracted, and sequenced by Edward Haynes (unpublished). Treatment key: SS = shook swarm, OTC = oxytetracycline treatment, DEST = destruction of hive or if no treatment is listed it is unknown.

Study ID	Protect ID (P)	ST	CC	Origin	Year	Treatment
1	7707	3	3	Dyfed (Wales)	2011	DEST
3	7821	3	3	Surrey	2011	SS
5	8157	3	3	Greater London	2011	SS
10	8261	3	3	Mid Glamorgan	2012	SS
11	8279	3	3	Devon	2012	OTC
13	8284	3	3	Berkshire	2012	SS
15	8348	3	3	Devon	2011	OTC
17	7715	5	3	North Yorkshire	2011	SS
19	7917	5	3	Derbyshire	2011	-
20	7970	5	3	Suffolk	2011	SS
21	7810	5	3	Norfolk	2011	SS
22	8234	5	3	Cambridgeshire	2012	DEST
23	8322	5	3	Lincolnshire	2012	DEST
28	8325	5	3	Norfolk	2012	SS
29	8107	5	3	Suffolk	2011	SS
30	8257	5	3	Suffolk	2012	SS
36	8414	3	3	Kent	2012	DEST
38	8185	3	3	Manchester	2012	DEST
46	8289	2	3	Somerset	2012	DEST

47	8456	2	3	Greater London	2012	DEST
48	7516	3	3	West Sussex	2010	-
49	7523	3	3	Avon	2010	DEST
51	7915	3	3	Lincolnshire	2011	DEST
53	8527	3	3	Gwent (Wales)	2012	SS
55	7606	5	3	Essex	2010	DEST
58	7595	6	3	Greater London	2010	SS
60	7511	7	3	Surrey	2012	DEST
62	7531	7	3	Devon	2010	-
63	7928	7	3	Suffolk	2011	SS
64	8265	7	3	Oxfordshire	2012	SS
66	7534	8	13	North Yorkshire	2010	DEST
67	7604	8	13	North Yorkshire	2010	DEST
71	7641	13	13	Norfolk	2010	DEST
73	7611	11	3	Suffolk	2010	SS
75	7935	11	3	Suffolk	2011	OTC
81	8423	13	13	Suffolk	2012	SS
84	8251	22	3	Gloucestershire	2012	SS
85	8371	22	3	Oxfordshire	2012	SS
86	8115	23	3	Mid Glamorgan	2011	OTC
90	8518	23	3	South Yorkshire	2012	DEST
91	8081	7	3	Hampshire	2011	SS
106	7993	5	3	Norfolk	2011	DEST
113	7613	5	3	Suffolk	2010	OTC
116	8070	5	3	Suffolk	2011	OTC
118	8305	5	3	Suffolk	2012	DEST

119	7596	13	13	Oxfordshire	2010	DEST
n/a	8111	2	3	Dorset	2012	SS
n/a	8176	2	3	Dorset	2012	SS
n/a	8282	2	3	Cornwall	2012	SS
n/a	8450	2	3	Oxfordshire	2012	DEST
n/a	7911	6	3	Greater London	2011	SS
n/a	8078	7	3	Oxfordshire	2011	OTC
n/a	8101	7	3	Greater London	2011	SS
n/a	7955	13	13	Norfolk	2011	SS
n/a	8473	18	13	Scotland	2012	SS
n/a	8061	20	13	Dyfed (Wales)	2011	OTC
n/a	8448	20	13	Dyfed (Wales)	2012	SS
n/a	7746	21	12	Mid Glamorgan	2011	DEST
n/a	7892	21	12	Mid Glamorgan	2011	SS
n/a	7780	22	3	Norfolk	2011	OTC
n/a	7814	23	3	Kent	2011	DEST
n/a	8364	23	3	West Yorkshire	2012	DEST

2.4.3. Confirmation of bacterial identity

Bacterial DNA extractions were performed using the DNeasy Blood and Tissue kit (Qiagen) and standard gram-positive bacterial protocol, including the pre-lysis step. Universal standard 16S bacterial primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) were used for PCR amplification. Each reaction consisted of 2.5 µl of 1x Taq buffer (15 mM $MgCl_2$), 0.5 µl of 2mM dNTPs, 0.25 µl of GoTaq® Polymerase (5U/µl), 0.5 µl of 0.2mM primer 27F, 0.5 µl of 0.2mM primer 1492R and 0.5 µl of DNA template, made to a final volume of 25 µl with DNA-free water. PCR conditions were an initial denaturation at (95°C, 3 min) then 30 cycles of denaturation (95°C, 30 secs), primer annealing (55°C, 30 secs) and primer extension (72°C, 1 min 30 secs) followed by a final extension cycle (72°C, 5 min). Aliquots of PCR products (5 µl) were analysed by gel electrophoresis in a 1% (mg/ml) agarose gel and then purified using QIAquick purification kit standard protocol. Samples were sent for Sanger sequencing at Source BioScience (Sanger, *et al*., 1977). Sequences were initially viewed using Sequence ScannerTM version 1.0 to assess quality of samples and then confirmed as *M. plutonius* using BLAST (Altschul, *et al*.,1990).

2.4.4. Whole genome sequencing of *M. plutonius* isolates

DNA content was verified before being sent to MicrobesNG using a Qubit® dsDNA HS Assay kit and quantified on a Qubit® Fluorometer (ThermoFisher Scientific). Isolates that met the threshold of DNA (>1ng/µl) were sent for sequencing at MicrobesNG using Illumina MiSeq, with a target of >30X read coverage. Reads were trimmed using Trimmomatic, then the quality assessed

by in-house scripts (MicrobesNG, unpublished), Samtools (Li, *et al*., 2009) and Bedtools (Quinlan & Hall, 2010). Genomes were first assembled using Kraken (Wood & Salzberg, 2014) to identify the most closely related bacterial species, and then the raw reads were mapped to this reference using BWA-mem (Burrows-Wheeler alignment) (Li & Durbin, 2010). Reads were then also mapped by a de novo assembly using SPAdes (Bankevich, *et al*., 2012). Assembled contigs were annotated using RAST (Aziz, *et al*., 2008), using ATCC35311 as a reference genome (Okumura, *et al*., 2011). Assembly metrics (Table 2.2.) were calculated using QUAST (Gurevich, *et al*., 2013). Additionally, 16 isolates were provided by Edward Haynes (Fera Science Ltd., unpublished) sequenced and assembled, and these were also used for some aspects of this part of the study (Table 2.1.).

2.4.5. Identification of genes of interest

Putative regions of interest as described in the introduction were manually discovered using the programme Artemis (Carver, *et al*., 2012) by searching through the annotated sequences (Appendix 1) and the labels of the protein regions given by RAST reported. Additionally selected protein coding region functions were investigated using KEGG (Kanehisa & Goto, 2000).

2.4.6. Identification of OTC resistance genes

Genome annotations were viewed using Artemis (Carver, *et al*., 2012). Whole genome sequences were examined for known tetracycline resistance genes (Roberts, 2005), and presence or absence was noted. ResFinder, an online tool that identifies resistance genes from partial or total gene sequences, was also used to check for the presence of any resistance genes (Bortolaia *et al*., 2020).

Nullarbor, a tool described later, (Seemann, *et al*.) also gives information on genomic matches to known resistance genes. Antibiotic resistance genes are discussed more fully in Chapter 4.

2.4.7. Identification of bacteriophage

To identify prophage sequences in the genomes, a web-based tool called PHASTER was used (Arndt, *et al*., 2016). Detailed tables listing information such as region position, and closest matching prophage identity were available as an output (Table 2.5.).

2.4.8. Statistical analysis: Chi-squared test

This statistical analysis was used because of small sample sizes (<500 isolates). Chi-squared is intended to test for independence, using observed and expected probabilities to estimate if specific variables are linked. In this case, the test is if EFB outbreaks that were destroyed (and therefore presumed more severe) are more likely to possess the 'Melissotoxin A' gene, than those that were treated by some method (OTC or shook swarm combined) (Table 2.6.).

2.4.9. Average nucleotide identity of isolates

Average nucleotide identity (ANI) was calculated for a sub-set of *M. plutonius* isolates using the OrthoANIu algorithm, which uses USEARCH instead of BLAST (Yoon *et al.,* 2017), and a pairwise matrix was created to allow easy comparison (Figure 2.1.).

2.4.10. Further bioinformation analysis of *M. plutonius* isolates

A total of 46 paired-end reads which were sequenced by the author, were re-assembled with the SPAdes assembler (Prjibelski, *et al*. 2020), using the 'careful' parameter to minimise short indels and mismatches. Subsequently, the trimmed paired-end reads were analysed with the Nullarbor tool (Seemann, *et al*.), which identifies any sequence contamination, assembles the reads into genomes and generates core SNP phylogenies. Additionally, two isolates, P7917 and P8107 were filtered through Kraken manually (Wood & Salzberg, 2014), to exclude reads of non-*M*. *plutonius* origin before performing this part of the analysis, due to the genome contamination from other bacterial species found within the sequencing data (Table 2.2.). Nullarbor includes the Roary pipeline (Page, *et al*., 2015) to calculate the pan genome of bacterial isolates. The re-assembly of the 46 newly sequenced bacterial genomes was a pre-requisite for this further analysis as the Nullarbor tool does not work with pre-assembled genomes, only raw reads. A separate table of assembly metrics is provided for the re-assembled *M. plutonius* isolates (Appendix 2). Unfortunately, as mentioned, Nullarbor is only able to use raw fasta files and not assembled genomes, and therefore the genomes provided by Dr Edward Haynes at Fera have not been included in this analysis.

2.5. Results

2.5.1. Whole genome sequencing and assembly quality metrics

Overall, most of the bacterial isolates of *M. plutonius* that were successfully sequenced were done so to a good standard. Apart from three, all isolates were sequenced to a minimum coverage of 30X (Table 2.2.), with some isolates even exceeding 200 times coverage of the genome size. However, Kraken identified the presence of small amounts of sequence from other species, *Staphylococcus* and *Streptococcus* in three of the DNA extractions (Appendix 2). Subsequently, the assemblies of these three isolates (P7917 (ST5), P8107 (ST5) and P8185 (ST3)) were of lower quality than all other isolates. Apart from these specific assemblies, all other *M. plutonius* assemblies were assembled in the range of 7-53 contigs, with a size ranging from ~2.01Mbp to ~2.1Mbp and with high N50 scores (Table 2.2.). It is likely that the majority, if not all, genetic sequence present will be available for automatic annotation.

2.5.2. Identification of genes or proteins of interest

Using the annotations generated by RAST (Aziz, *et al*., 2008), putative genomic regions related to proteins involved in toxin production, bacteria-bacterial warfare, host resistance and biofilm formation were identified in the sequences of *M. plutonius* isolates. Some were ubiquitous in all isolates; some were variable as described below. Many of the putative gene regions or proteins previously described that could be important in EFB infection as were also found here (Djukic *et al*., 2018). A table list of protein clusters and other regions of interest is in Appendix 1.

Table 2.2. Metrics of genome sequencing and assembly from isolate DNA sent to MicrobesNG. †The length of the shortest contig in the set of largest contigs that together constitute at least half of the total assembly size. *Isolates have sequence similarity to another bacterium and therefore have DNA sequence contamination.

Study ID	Isolate ID	ST	Number of reads	Mean coverage	GC%	Assembled size (bp)	Number of contigs	N50†
1	P7707	3	796803	141.8	31.09	2064990	23	292401
3	P7821	3	331334	69	31.09	2065091	10	405861
5	P8157	3	408483	87.5	31	2062660	31	326481
10	P8261	3	489222	98.8	31.09	2065714	15	326481
11	P8279	3	340654	58.4	31.1	2062529	30	292515
13	P8284	3	340654	58.4	31.09	2065002	16	204302
15	P8348	3	427843	81.1	31.14	2107533	13	326481
17	P7715	5	252929	57.4	31.09	2048069	19	209016
19	P7917	5	435123	45.3	32.04	3903469	**1534***	**47575***
20	P7970	5	489836	97.4	31.09	2069976	12	326481
21	P7810	5	341581	70.9	31.09	2065523	13	405882
22	P8234	5	296954	59.2	31.1	2066288	26	326480
23	P8322	5	291573	60.6	31.09	2047586	10	326480
28	P8325	5	387559	80.5	31.1	2049117	15	292402
29	P8107	5	566125	55.3	31.67	4384644	**718***	**15274***
30	P8257	5	399683	79.1	31.09	2066777	11	405858
36	P8414	3	146990	31.5	31.12	2045534	30	200911
38	P8185	3	78290	**15**	31.38	2117401	**168***	154295
46	P8289	2	553648	103.3	31.1	2066521	10	326479
47	P8456	2	1421193	271.2	31.1	2066521	12	326480
48	P7516	3	1050963	209.6	31.1	2047248	7	405870
49	P7523	3	2150625	432.8	31.09	2047469	7	405811
51	P7915	3	240287	86.6	31.09	2062718	19	326482
53	P8527	3	727313	137.1	31.09	2064870	14	405882
55	P7606	5	1004297	203.7	31.09	2047829	9	405882
58	P7595	6	1092989	218.3	31.09	2047158	8	405881
60	P7511	7	1091125	213.7	31.09	2010007	12	326480
62	P7531	7	951021	200.2	31.09	2026681	14	405970
63	P7928	7	621050	119.6	31.04	2022121	22	326481
64	P8265	7	285099	60.5	31.04	2023860	11	326480

66	P7534	8	261780	55.2	31.09	2039709	10	326548
67	P7604	8	286430	64.5	31.13	2039035	11	330730
71	P7641	13	440537	99	31.14	2078836	11	326479
73	P7611	11	413172	91	31.11	2057061	20	326479
75	P7935	11	89098	**19.4**	31.11	2055892	34	326480
81	P8423	13	69614	**15**	31.17	2077011	53	95712
84	P8251	22	381215	83.8	31.14	2071591	19	326480
85	P8371	22	323871	65.7	31.1	2060666	20	204913
86	P8115	23	177761	38.7	31.11	2064436	28	326480
90	P8518	23	357549	71.8	31.13	2070606	19	326693
91	P8081	7	223774	50	31.06	2023763	12	405881
106	P7993	5	660314	134.1	31.09	2066622	12	326658
113	P7613	5	379698	36.6	31.09	2046301	12	326480
116	P8070	5	438085	89.2	31.09	2056458	15	292402
118	P8305	5	402583	77.2	31.09	2064210	13	326480
119	P7596	13	271254	50.2	31.15	2095171	23	292811

Table 2.3. Putative protein coding regions related to adhesion and attachment were identified by RAST annotation (Aziz, *et al*., 2008)

Annotation name by RAST	**Theoretical protein in the region**	**Citation**	**Locus tag in reference genome**	**GenBank protein accession ref**
Fibronectin/ fibrinogen-binding protein	FnBP	Hymes & Klaenhammer, 2016	MPTP_1180, MPTP_1181	BAK21631.1 BAK21632.1
Chitin binding protein	CBP	Frederiksen *et al*., 2013.	MPTP_0965	BAK21424.1
Cell wall surface anchor family protein	LPXTG_anchor	Siegel *et al*., 2017	MPTP_0195	BAK20694.1

All 62 isolates possessed these three genomic regions (Table 2.3) and the CDS region was annotated as the above in the reference ATCC 35311.

The region corresponding to chitin binding proteins was further investigated using KEGG genome browser (Kanehisa & Goto, 2000)**,** and showed this region encodes pathways related to sugar metabolism, with the enzyme alpha-glucosidase (EC:3.2.1.20).

Aggregation promoting factor, AGF, a protein domain related to biofilm production was found to be present in all isolates. Interestingly, the number of CDS annotated as above varied from two to six in some isolates. Investigation of the reference genome using KEGG genome browser suggested although the reference genome possesses two protein regions annotated as AGFs, one is a pseudogene (genome region 1774670..1776474), whilst the other protein region (NCBI protein ID: BAK22143) was assigned to the motif LysM domain, associated with breaking down peptidoglycan (Buist, *et al*., 2008).

Melissotoxin A

Isolates either possessed the melissotoxin A gene, annotated as “Mosquitocidal toxin” or it was absent from the genome. The absence was verified using BLAST (Altschul, *et al*.,1990) to check that the sequence was not present, instead of simply not annotated by the method use. The variation of presence of this gene amongst *M. plutonius* strains was also found in other genetic studies including Djuvic *et al*., 2018 (Table 2.4.).

Table 2.4. Number of isolates of each ST sequenced that possess the melissotoxin A gene and the total of isolates sequenced for that ST total.

Strain type	Isolates with toxin	Total number isolates sequenced
2	6	6
3	8	13
5	7	14
6	0	2
7	5	7
8	0	2
11	0	2
13	2	4
18	0	1
20	2	2
21	0	2
22	3	3
23	1	4
Total	**34**	**62**

Other genes of interest

Several other protein coding regions were identified that may be interesting to further study outside of this thesis. These include groups of genes that may encode siderophore production and colicin V production proteins.

Identification of bacteriophage

Three types of intact bacteriophage sequence were identified in these isolates. These were identified by PHASTER (Arndt, *et al*., 2016) as being most closely related to the following phage 'species': Lactob_Sha1, Lactob_phig1e and Lister_P35 (Table 2.5). Only ST7 isolates had no whole phage sequence present at all, and this was applicable to all seven strains sequenced (Table 2.5.).

There was also at least one partial phage sequence, Lactoc_Tuc2009 or Lactoc_TP901 present in all isolates except both ST21 strains and one ST23 strain P8364. Upon further inspection, both these partial phage sequences were identical although differently labelled by the PHASTER database algorithm (Arndt, *et al*., 2016). This was also the case for two of the partial phage sequences found in the two ST21 isolates Strept_315.6 and Lactob_jlb1 (Table 2.5.). This suggests that both matches have equal similarity to the sequence present, but none are identical due to the specific *M. plutonius* phage sequences not yet being present on the GenBank database.

Interestingly, some ST3 isolates possessed a partial sequence for another phage, Paenib_Vegas, which has been found to be able to infect *P. larvae*, the cause of AFB in honey bees. Three additional partial phage sequences found in isolate P8107 (ST5) were of *Staphylococcus* origin and likely due to contamination (Table 2.2, Appendix 2). Additionally, two isolates had two intact copies of Lactob_Sha1 phage species, P8176 (ST2) and P8348 (ST3).

Table 2.5. Closest matches from GenBank and properties of three types of complete bacteriophage (dark grey) and five partial bacteriophage sequences (light grey) present in *M. plutonius* genomes from PHASTER (Arndt, *et al*., 2016). Phage grouped together appear the same when examined structurally in PHASTER.

Phage present	**Host species**	**Region length (Kb)**	**GenBank ID**	**ST present**
Lactob_Sha1	*Lactobacillus sp.*	28.6 – 44.4	NC_019489	2, 3, 5, 6, 11, 13, 21, 22, 23
Lactob_phig1e	*Lactobacillus plantarium*	36.1 – 47.5	NC_004305	8, 13,18, 20
Lister_P35	*Listeria sp.*	38.8/37.2	NC_009814	13
Lactoc_Tuc2009/ Lactoc_TP901	*Lactococcus lactis*	10.8 – 11.6	NC_002703/ NC_002747	All except 21 and P8364 (23)
Paenib_Vegas	*P. larvae*	12.8	NC_028767	3
Strept_315.6/ Lactob_jlb1	*Streptococcus pyrogenes/ Lactobacillus sp.*	10.3	NC_004589/ NC_024206	21
Lactoc_bIL311	*Lactococcus lactis*	15.9	NC_002670	21
Staphy_SA780	*S. aureus*	11.2	NC_048711	21

2.5.3. Chi-squared test of independence on Melissotoxin A and hive treatment vs. destruction

There was a significant relationship between treatment type and possessing the melissotoxin gene (Table 2.6.) (X^2 (1, N = 62) = .011, p < .05), and we reject the null hypothesis the relative proportions of the isolates destroyed are the same regardless of the presence of melissotoxin A. Hives were more likely to be treated with shook swarm or antibiotics if the isolate present has the melissotoxin A gene. Three of the isolates could not be used as one was the strain type and the other's treatment was unknown (Table 2.1.).

Table 2.6. Proportions of *M. plutonius* isolates for treatment type and presence or absence of melissotoxin A. Isolates where treatment type was not known was not included in this analysis (three isolates).

	Present	**Absent**	**Total**
SS/OTC	26	11	37
Destruction	8	14	22
Total	34	25	59

2.5.4. Whole genome sequence similarity

Overall, all isolates tested were extremely genetically similar, with ANI values of greater than 99% for all isolates in this study (Figure 2.1.). The CC3 and CC13 isolates had ANI % values ranging between 99.75 – 99.86, except for the P7531 ST7 CC3 isolate that showed a greater similarity of 99.89 – 99.92 %. Additionally, the two isolates from ST13 shared less genetic similarity to the more common CC3 isolates tested (Figure 2.1.) than the others within CC13. The only two isolates evaluated that belong to CC21 (P7746 and P7892) were

the most different on average the other isolates (Figure 2.1.), although these two isolates showed an ANI of 100% to each other. This difference is also reflected in the number of core SNP differences observed amongst isolates (Figure 2.3.). Several isolates from differing ST groups showed an ANI value of 100% to each other when using this algorithm.

2.5.5. Phylogenetic tree and pairwise distance of core SNPs

Some of the STs grouped together as expected based on their core SNP phylogeny, including isolates ST5, ST7, ST8, ST11 and ST13. ST8 and ST13 grouped most closely with the reference isolate ATCC 35311, which is also a CC13 isolate (Figure 2.2., Figure 2.3). However, the ST3 isolates used in this study split into two separate distinct clades (Figure 2.2., Figure 2.3). Additionally, one ST2, one ST6 and the two ST22 strains grouped together, with one ST2 strain being most closely related to the ST6 strain, and the other than the other two ST22 strains present. A pairwise estimate quantifying the average number of core SNP differences was also generated (Fig 2.4.).

2.5.7. Core and accessory genome of *M. plutonius*

The number of combined core and accessory genes in present total ranged from 1825-1945 genes for most strains of *M. plutonius* (Figure 2.5.). Analysis using Roary suggests that 1709 of genes present within the *M. plutonius* genome are ‘core’ genes, present within 99%≤ of strains (Table 2.7.)

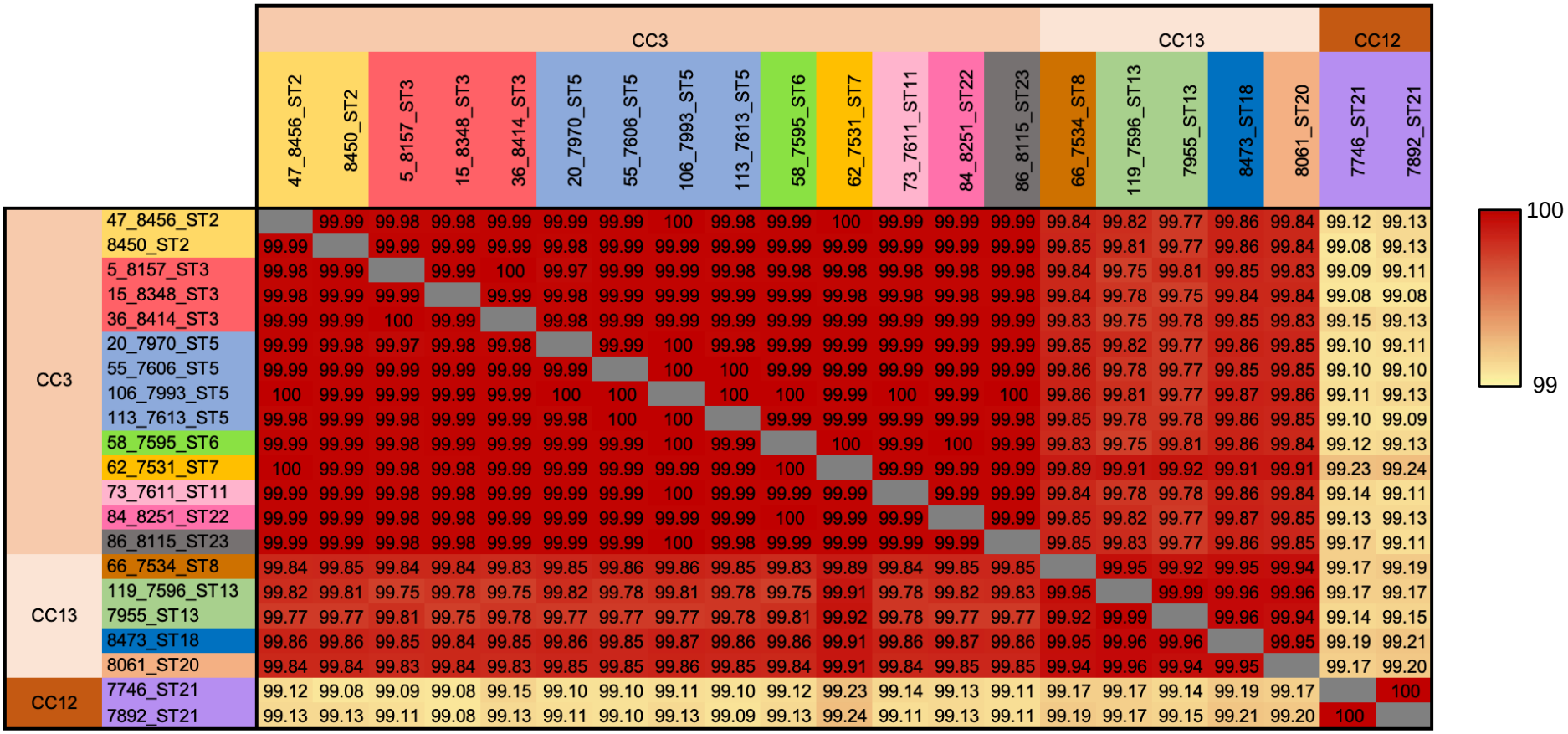

Figure 2.1. Average nucleotide identity matrix for selected *M. plutonius* isolates calculated using the OrthoANIu algorithm (Yoon, *et al*., 2017).

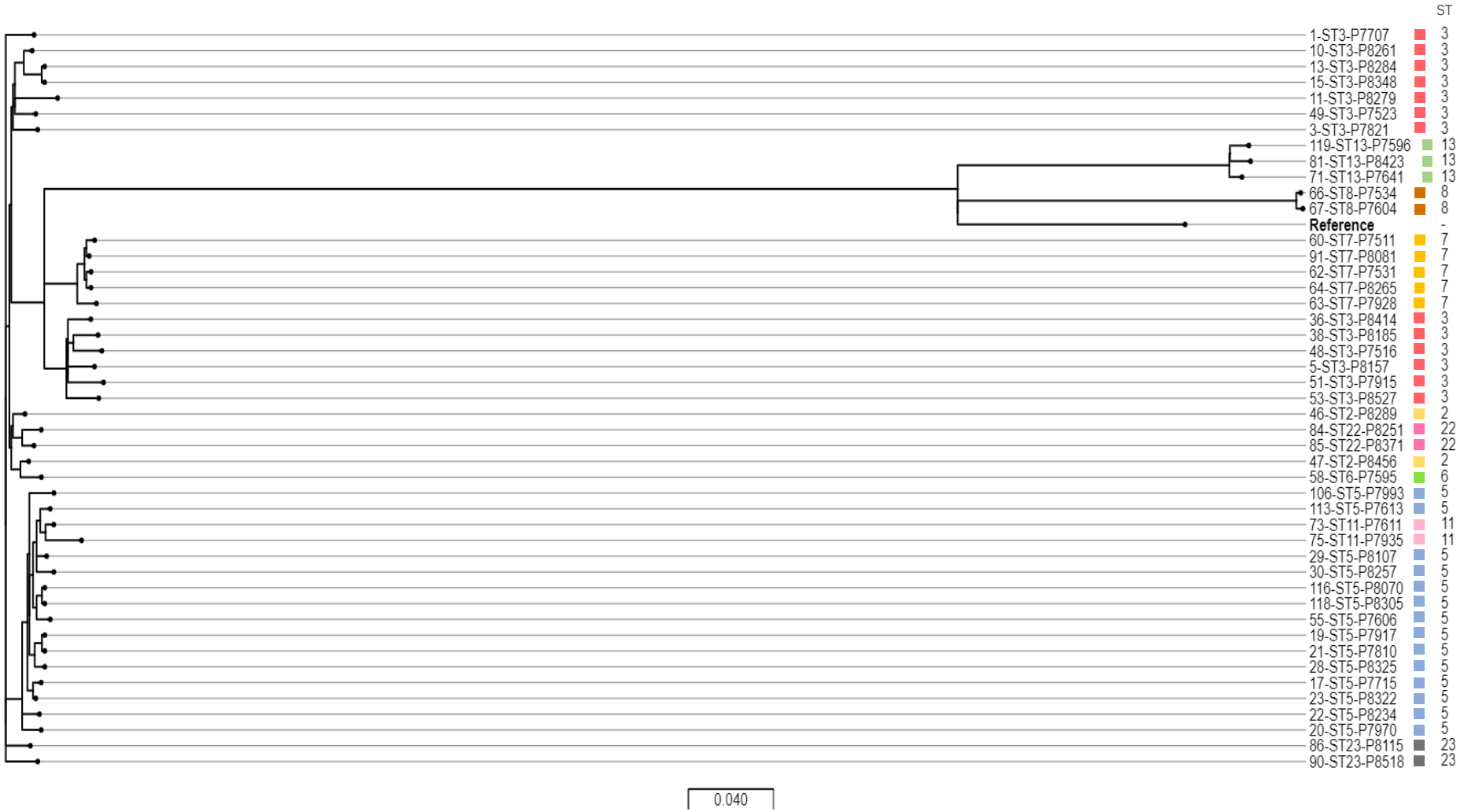

Figure 2.2. Phylogenetic tree of *M. plutonius* isolates based on generated core SNP matrix generated by Nullarbor, including strain type (ST) key. Scale 0.040 substitutions per base.

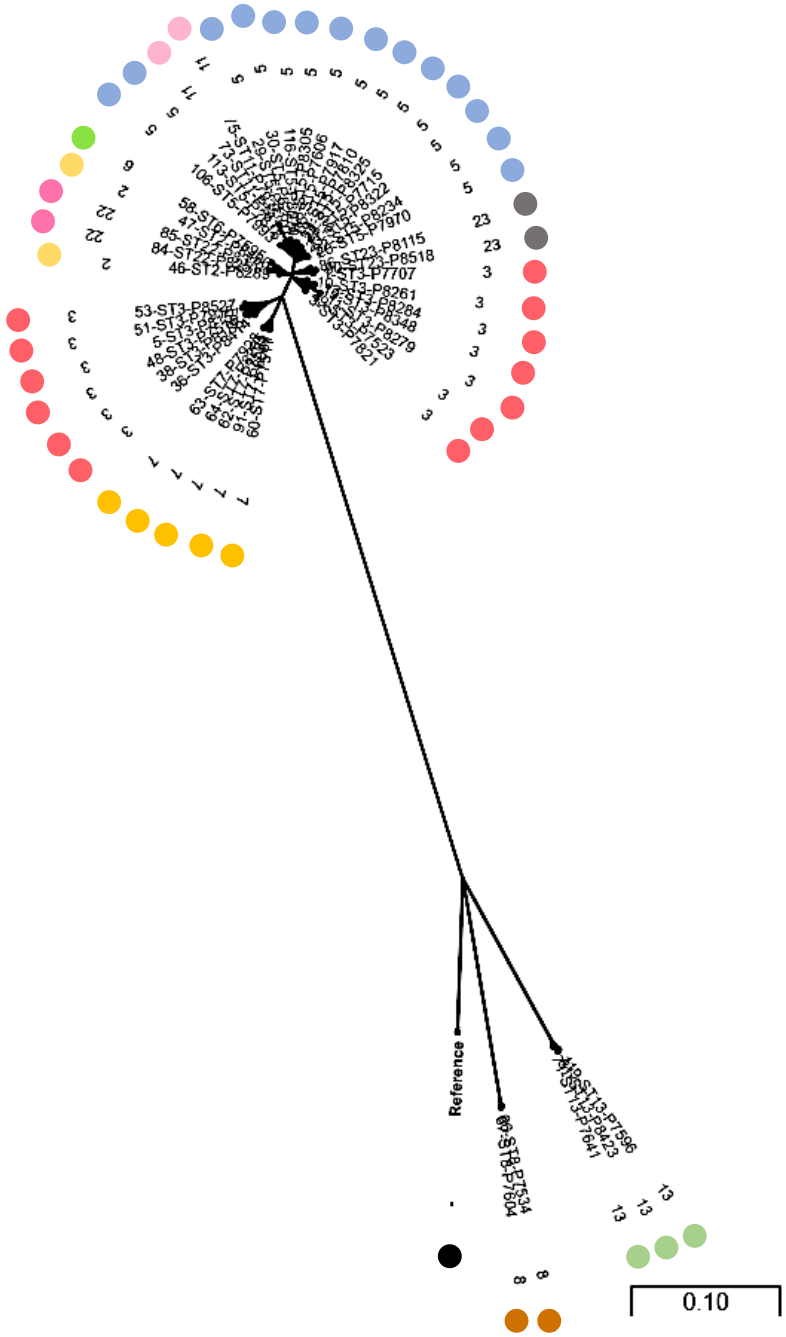

Figure 2.3. Unrooted phylogenetic tree generated using core SNP analysis created using Nullarbor including strain type classification. Scale 0.10 substitutions per base.

	1	10	106	11	113	116	118	119	13	15	17	19	20	21	22	23	28	29	3	30	36	38	46	47	48	49	5	51	53	55	58	60	62	63	64	66	67	71	73	75	81	84	85	86	90	91	REF
1-ST3-P7707	0	27	39	40	37	34	34	535	33	33	32	34	32	34	31	29	34	35	30	39	59	63	23	25	65	29	61	66	63	37	32	61	59	62	59	577	578	530	39	54	536	32	28	26	30	58	521
10-ST3-P8261	27	0	38	29	36	33	33	528	12	12	31	33	31	33	30	28	33	34	21	38	52	56	18	20	58	18	54	59	56	36	27	54	52	55	52	570	571	523	38	53	529	27	23	25	29	51	514
106-ST5-P7993	39	38	0	51	22	19	19	546	44	44	19	19	25	19	24	16	19	20	41	24	70	74	34	36	76	40	72	77	74	22	43	72	70	73	70	588	589	541	24	39	547	43	39	37	41	69	532
11-ST3-P8279	40	29	51	0	49	46	46	541	35	35	44	46	44	46	43	41	46	47	34	51	65	69	31	33	71	31	67	72	69	49	40	67	65	68	65	583	584	536	51	66	542	40	36	38	42	64	527
113-ST5-P7613	37	36	22	49	0	13	13	542	42	42	17	17	23	17	22	14	17	10	39	14	68	72	32	34	74	38	70	75	72	16	41	70	68	71	68	584	585	537	10	25	543	41	37	35	39	67	528
116-ST5-P8070	34	33	19	46	13	0	0	541	39	39	14	14	20	14	19	11	14	11	36	15	65	69	29	31	71	35	67	72	69	9	38	67	65	68	65	583	584	536	15	30	542	38	34	32	36	64	527
118-ST5-P8305	34	33	19	46	13	0	0	541	39	39	14	14	20	14	19	11	14	11	36	15	65	69	29	31	71	35	67	72	69	9	38	67	65	68	65	583	584	536	15	30	542	38	34	32	36	64	527
119-ST13-P7596	535	528	546	541	542	541	541	0	534	534	539	541	539	541	538	536	541	542	531	546	521	522	526	528	528	530	524	529	526	544	535	526	524	527	524	312	313	15	546	561	19	533	529	533	537	523	256
13-ST3-P8284	33	12	44	35	42	39	39	534	0	0	37	39	37	39	36	34	39	40	27	44	58	62	24	26	64	24	60	65	62	42	33	60	58	61	58	576	577	529	44	59	535	33	29	31	35	57	520
15-ST3-P8348	33	12	44	35	42	39	39	534	0	0	37	39	37	39	36	34	39	40	27	44	58	62	24	26	64	24	60	65	62	42	33	60	58	61	58	576	577	529	44	59	535	33	29	31	35	57	520
17-ST5-P7715	32	31	19	44	17	14	14	539	37	37	0	14	18	14	17	3	14	15	34	19	63	67	27	29	69	33	65	70	67	17	36	65	63	66	63	581	582	534	19	34	540	36	32	30	34	62	525
19-ST5-P7917	34	33	19	46	17	14	14	541	39	39	14	0	20	0	19	11	8	15	36	19	65	69	29	31	71	35	67	72	69	17	38	67	65	68	65	583	584	536	19	34	542	38	34	32	36	64	527
20-ST5-P7970	32	31	25	44	23	20	20	539	37	37	18	20	0	20	17	15	20	21	34	25	63	67	27	29	69	33	65	70	67	23	36	65	63	66	63	581	582	534	25	40	540	36	32	30	34	62	525
21-ST5-P7810	34	33	19	46	17	14	14	541	39	39	14	0	20	0	19	11	8	15	36	19	65	69	29	31	71	35	67	72	69	17	38	67	65	68	65	583	584	536	19	34	542	38	34	32	36	64	527
22-ST5-P8234	31	30	24	43	22	19	19	538	36	36	17	19	17	19	0	14	19	20	33	24	62	66	26	28	68	32	64	69	66	22	35	64	62	65	62	580	581	533	24	39	539	35	31	29	33	61	524
23-ST5-P8322	29	28	16	41	14	11	11	536	34	34	3	11	15	11	14	0	11	12	31	16	60	64	24	26	66	30	62	67	64	14	33	62	60	63	60	578	579	531	16	31	537	33	29	27	31	59	522
28-ST5-P8325	34	33	19	46	17	14	14	541	39	39	14	8	20	8	19	11	0	15	36	19	65	69	29	31	71	35	67	72	69	17	38	67	65	68	65	583	584	536	19	34	542	38	34	32	36	64	527
29-ST5-P8107	35	34	20	47	10	11	11	542	40	40	15	15	21	15	20	12	15	0	37	12	66	70	30	32	72	36	68	73	70	14	39	68	66	69	66	584	585	537	12	27	543	39	35	33	37	65	528
3-ST3-P7821	30	21	41	34	39	36	36	531	27	27	34	36	34	36	33	31	36	37	0	41	55	59	21	23	61	23	57	62	59	39	30	57	55	58	55	573	574	526	41	56	532	30	26	28	32	54	517
30-ST5-P8257	39	38	24	51	14	15	15	546	44	44	19	19	25	19	24	16	19	12	41	0	70	74	34	36	76	40	72	77	74	18	43	72	70	73	70	588	589	541	16	31	547	43	39	37	41	69	532
36-ST3-P8414	59	52	70	65	68	65	65	521	58	58	63	65	63	65	62	60	65	66	55	70	0	26	50	52	28	54	24	29	28	68	59	50	48	51	48	566	567	516	70	85	522	59	55	57	61	47	510
38-ST3-P8185	63	56	74	69	72	69	69	522	62	62	67	69	67	69	66	64	69	70	59	74	26	0	54	56	26	58	28	33	32	72	63	54	52	55	52	566	567	517	74	89	523	63	59	61	65	51	510
46-ST2-P8289	23	18	34	31	32	29	29	526	24	24	27	29	27	29	26	24	29	30	21	34	50	54	0	14	56	20	52	57	54	32	21	52	50	53	50	568	569	521	34	49	527	19	15	21	25	49	512
47-ST2-P8456	25	20	36	33	34	31	31	528	26	26	29	31	29	31	28	26	31	32	23	36	52	56	14	0	58	22	54	59	56	34	13	54	52	55	52	570	571	523	36	51	529	23	19	23	27	51	514
48-ST3-P7516	65	58	76	71	74	71	71	528	64	64	69	71	69	71	68	66	71	72	61	76	28	26	56	58	0	60	30	35	34	74	65	56	54	57	54	572	573	523	76	91	529	65	61	63	67	53	516
49-ST3-P7523	29	18	40	31	38	35	35	530	24	24	33	35	33	35	32	30	35	36	23	40	54	58	20	22	60	0	56	61	58	38	29	56	54	57	54	572	573	525	40	55	531	29	25	27	31	53	516
5-ST3-P8157	61	54	72	67	70	67	67	524	60	60	65	67	65	67	64	62	67	68	57	72	24	28	52	54	30	56	0	31	30	70	61	52	50	53	50	568	569	519	72	87	525	61	57	59	63	49	512
51-ST3-P7915	66	59	77	72	75	72	72	529	65	65	70	72	70	72	69	67	72	73	62	77	29	33	57	59	35	61	31	0	35	75	66	57	55	58	55	572	573	524	77	92	530	66	62	64	68	54	517
53-ST3-P8527	63	56	74	69	72	69	69	526	62	62	67	69	67	69	66	64	69	70	59	74	28	32	54	56	34	58	30	35	0	72	63	54	52	55	52	570	571	521	74	89	527	63	59	61	65	51	514
55-ST5-P7606	37	36	22	49	16	9	9	544	42	42	17	17	23	17	22	14	17	14	39	18	68	72	32	34	74	38	70	75	72	0	41	70	68	71	68	586	587	539	18	33	545	41	37	35	39	67	530
58-ST6-P7595	32	27	43	40	41	38	38	535	33	33	36	38	36	38	35	33	38	39	30	43	59	63	21	13	65	29	61	66	63	41	0	61	59	62	59	577	578	530	43	58	536	30	26	30	34	58	521
60-ST7-P7511	61	54	72	67	70	67	67	526	60	60	65	67	65	67	64	62	67	68	57	72	50	54	52	54	56	56	52	57	54	70	61	0	6	17	6	568	569	521	72	87	527	61	57	59	63	3	512
62-ST7-P7531	59	52	70	65	68	65	65	524	58	58	63	65	63	65	62	60	65	66	55	70	48	52	50	52	54	54	50	55	52	68	59	6	0	15	2	566	567	519	70	85	525	59	55	57	61	3	510
63-ST7-P7928	62	55	73	68	71	68	68	527	61	61	66	68	66	68	65	63	68	69	58	73	51	55	53	55	57	57	53	58	55	71	62	17	15	0	15	569	570	522	73	88	528	62	58	60	64	14	513
64-ST7-P8265	59	52	70	65	68	65	65	524	58	58	63	65	63	65	62	60	65	66	55	70	48	52	50	52	54	54	50	55	52	68	59	6	2	15	0	566	567	519	70	85	525	59	55	57	61	3	510
66-ST8-P7534	577	570	588	583	584	583	583	312	576	576	581	583	581	583	580	578	583	584	573	588	566	566	568	570	572	572	568	572	570	586	577	568	566	569	566	0	3	307	588	603	313	575	571	575	579	565	272
67-ST8-P7604	578	571	589	584	585	584	584	313	577	577	582	584	582	584	581	579	584	585	574	589	567	567	569	571	573	573	569	573	571	587	578	569	567	570	567	3	0	308	589	604	314	576	572	576	580	566	273
71-ST13-P7641	530	523	541	536	537	536	536	15	529	529	534	536	534	536	533	531	536	537	526	541	516	517	521	523	523	525	519	524	521	539	530	521	519	522	519	307	308	0	541	556	16	528	524	528	532	518	251
73-ST11-P7611	39	38	24	51	10	15	15	546	44	44	19	19	25	19	24	16	19	12	41	16	70	74	34	36	76	40	72	77	74	18	43	72	70	73	70	588	589	541	0	21	547	43	39	37	41	69	532
75-ST11-P7935	54	53	39	66	25	30	30	561	59	59	34	34	40	34	39	31	34	27	56	31	85	89	49	51	91	55	87	92	89	33	58	87	85	88	85	603	604	556	21	0	562	58	54	52	56	84	517
81-ST13-P8423	536	529	547	542	543	542	542	19	535	535	540	542	540	542	539	537	542	543	532	547	522	523	527	529	529	531	525	530	527	545	536	527	525	528	525	313	314	16	547	562	0	534	530	534	538	524	251
84-ST22-P8251	32	27	43	40	41	38	38	533	33	33	36	38	36	38	35	33	38	39	30	43	59	63	19	23	65	29	61	66	63	41	30	61	59	62	59	575	576	528	43	58	534	0	14	30	34	58	519
85-ST22-P8371	28	23	39	36	37	34	34	529	29	29	32	34	32	34	31	29	34	35	26	39	55	59	15	19	61	25	57	62	59	37	26	57	55	58	55	571	572	524	39	54	530	14	0	26	30	54	515
86-ST23-P8115	26	25	37	38	35	32	32	533	31	31	30	32	30	32	29	27	32	33	28	37	57	61	21	23	63	27	59	64	61	35	30	59	57	60	57	575	576	528	37	52	534	30	26	0	28	56	519
90-ST23-P8518	30	29	41	42	39	36	36	537	35	35	34	36	34	36	33	31	36	37	32	41	61	65	25	27	67	31	63	68	65	39	34	63	61	64	61	579	580	532	41	56	538	34	30	28	0	60	523
91-ST7-P8081	58	51	69	64	67	64	64	523	57	57	62	64	62	64	61	59	64	65	54	69	47	51	49	51	53	53	49	54	51	67	58	3	3	14	3	565	566	518	69	84	524	58	54	56	60	0	509
Reference	521	514	532	527	528	527	527	256	520	520	525	527	525	527	524	522	527	528	517	532	510	510	512	514	516	516	512	517	514	530	521	512	510	513	510	272	273	251	532	517	251	519	515	519	523	509	0

Figure 2.4. Pairwise core SNP distance estimates amongst isolates analysed in Nullarbor, with the number of average differences displayed.

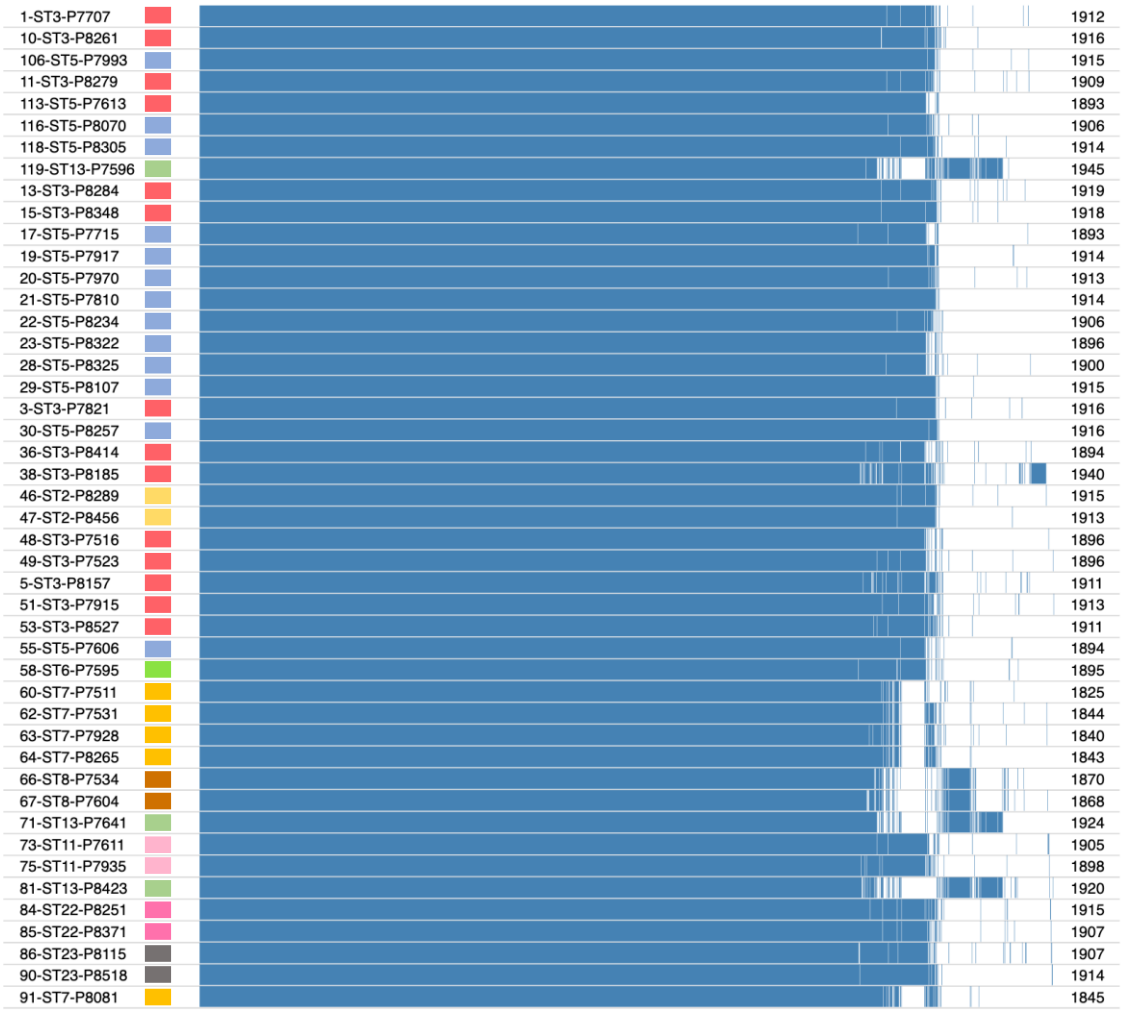

Figure 2.5. Genome alignment of *M. plutonius* isolates, with the total number on the right showing the estimated genes present in each isolate in total for both core and accessory genes (total also includes total of genes present on plasmid).

Table 2.7 Numbers of core and accessory genes estimated by Roary pipeline (Page, *et al*., 2015) for the 46 isolates tested within Nullarbor (Seemann, *et al*.)

Ortholog class	Definition	Count
Core genes	99% ≤ strains ≤ 100%	1709
Soft core genes	95% ≤ strains < 99%	49
Shell genes	15% ≤ strains < 95%	172
Cloud genes	0% ≤ strains < 15%	289
Total	**0% ≤ strains ≤100%**	**2219**

2.6. Discussion

2.6.1. Context of this study

EFB is a destructive and widespread honey bee disease, and a full understanding of the genetic differences of *M. plutonius* isolates is essential to the effective treatment of already infected hives. The publication of a comparative genomics study of *M. plutonius* last year (Djukic, *et al*., 2018) is the first step to understanding the genomic content of this bacterium. However, as this paper concludes, **"**The following discussion is based on the pure presence of the identified genes in the *M. plutonius* genomes and experimental evidence is needed to develop a fully functional infection model". Linking the genetic information to real infection within the affected organism is essential to fully understand disease progression.

Firstly, this study has attempted to identify genetic features amongst the genomes of 62 isolates originating in the UK, that may play a role in *M. plutonius* pathogenesis. This includes gene regions that encode for attachment and biofilm formation proteins, and the presence of bacteriophage sequence. Additionally, the similarities and relationships amongst strains was investigated, both phylogenetically and by including analysis of core and accessory gene numbers. Lastly, this chapter explored the relevance of the presence of a toxin gene, 'melissotoxin A', that is expressed in honey bee larval tissue (Djukic, *et al*., 2018), and causes increased *M. plutonius* virulence in larval infection experiments (Grossar, *et al*., 2020) to how field EFB infections were treated at the time it was originally found (Table 2.1.). How the infection was contained is assumed to correlate to EFB severity, with shook swarm or antibiotic OTC as

less severe and destruction by burning more severe. Overall, the quality of the 46 genomes newly sequenced met the standards required for this kind of study, apart from the three previously described (Table 2.2.) and these can be useful for further research of *M. plutonius* in the future beyond this thesis.

2.6.2. Comparative genomics – experimental improvements

There are several key improvements that could be made to this thesis chapter in terms of the analysis methods undertaken. A more comparative approach across all the 62 genomes, focusing on elucidating the key gene differences between isolates of strain types or clonal complexes expected to have low virulence versus those that have high virulence in previous studies, would have likely allowed identification of new candidate virulence genes of interest in an unbiased way. This study has only identified the level of similarities, but not which specific gene types instead differ. An improved approach would also have allowed the discovery and exploration of genes and genomic regions that were not annotated by RAST, that would subsequently be missed by examining the putative genetic content manually as was carried out in this chapter. Any genes that seemed to correlate with phenotypically variation could then have had been further explored in this chapter in a more functional way, for example with KEGG **(**Kanehisa & Goto, 2000), a tool useful in predicting true gene function. Use of the Gene Ontology (GO) Enrichment Analysis (Mi, *et al*., 2019) would also have given further information about evolution of protein coding families within *M. plutonius*, which is a further missed opportunity for expansion of the genetic component of this thesis. There are a number of command-line based programmes that can be used for whole genome sequencing and comparison, either on a gene by gene basis or across the whole genome, for

example ISMapper (Hawkey, *et al*., 2015) which identifies insertions transposon sites in short read data, and Gubbins (Croucher, *et al*., 2015) that looks for recombination in nucleotide sequences, but due to lack of programming experience and the inability to learn these skills within the timescale of this PhD the possibilities of some of these useful tools were unfulfilled.

It would also have been useful to perform a further analysis of other nucleotide variation besides core SNP differences such as insertion–deletion mutations (indels) or to have a deeper understanding of the evolution of genes and genetic features of *M. plutonius*. Indels are the most common type of Mobile Genetic Elements (MGEs) in bacteria (Siguier, *et al*., 2014), with the loss or gain of nucleotides often leading to the creation of non-functional pseudogenes (Danneels, *et al.,* 2018.). It is sometimes difficult to detect indels from short read whole genome sequence data (Shigemizu, *et al.*, 2013), without confirming true deletions with additional measures such as sanger sequencing, performing deeper sequencing with a higher genome coverage (Neuman, *et al*., 2013), using long read methods (Ahsan, *et al*., 2021). This information was not generated in the analysis with Nullarbor and it was not possible to perform a more complex bioinformatic analysis outside the capabilities of this thesis, later discussed in the General Discussion chapter. However, a loss of genes important to virulence in CC13 isolates could be a key in explaining the variation in disease symptom severity and mortality observed in EFB infections.

Additional insights may have also been gleaned if there had been more *M. plutonius* isolates available for analysis generally. Any preassembled isolates, for example the two belonging to ST21 (Figure 2.1.) and several used in larval

infection experiments later in Chapter 3 were not able to be analysed through Nullarbor pipeline (Seemann, *et al*.) in this chapter due to not having access to raw sequencing reads. A lot of *M. plutonius* isolates in the strain collection at Fera Science Ltd. were also unable to either be grown, were contaminated, or be extracted at sufficient DNA quantity or quality to be sequenced. In hindsight, extraction of new samples sent to the NBU in the first two years of the project (2016-2018) could have been prepared to add to the collection and used to supplement this research. As a lot of the analysis pipelines in this Chapter 2 were performed relatively late in the project, it has meant that opportunities to build on findings regarding *M. plutonius* key isolate relationships were not possible, and this also a fundamental weakness of this chapter.

2.6.3. Biofilm formation and adhesion proteins

Several gene regions mentioned in the comparative genomics study (Djukic, *et al*., 2018) have also been identified amongst the 62 UK isolates. These include genomic regions associated with fibronectin/fibrinogen binding proteins and chitin and cell wall binding proteins. As *M. plutonius* infection occurs in the gut tract of the bee larvae, it is possible that possessing copies of genes encoding these types of proteins will facilitate enhanced bacterial infection. However, a recent study on peritrophic matrix (PM) degrading proteins in the atypical *M. plutonius* strain DAT561 (Nakamura *et al*., 2021), concluded that for at least three of these protein encoding genes, they were in fact not essential to high virulence in larval infection studies. Knockout bacterial strains were created for genes encoding enhancin, chitin-binding domain-containing protein and endo-α-*N*-acetylgalactosaminidase and then tested *in vivo*. The mutant strains of *M. plutonius* DAT561, lacking the above functional proteins, could still kill honey

bee larvae in laboratory infection models and were therefore concluded to be dispensable in terms of virulence. It may be that this highly virulent CC12 strain has other modes of infection, and it highlights the need to focus on differing strain types within *M. plutonius* studies, particularly as the 'atypical' strain used here is not seen in field infections of EFB. It is not possible to complete studies such as the above, manipulating the genes of a bacterium, without first using whole genome sequencing and related methods to find the genes to test *in vivo* and validation of genetic features in this way is essential to 'proving' their relevance to pathogenesis.

Within the isolates studied, the number of copies of a biofilm formation protein region, annotated as a putative aggregation-promotion factors, varied from two to six annotated CDS regions. Originally described in *Lactobacillus plantarum strain 4B2*, aggregation-promotion factor genes increased the frequency of conjugation and aggregation of bacteria (Reniero, *et al*., 1992). *Lactobacillus acidophilus*, a bacterium found in the vaginal bacterial ecosystem, often protects the host from undesirable microbial colonisation by aggregating to form a barrier (Cribby*, et al*., 2008). However, the co-aggregation of this bacteria alongside other pathogenic *Lactobacillus* and *Lactococcus* strains, also increases the conjugation frequency of these co-infecting species and as a result can often worsen vaginal infections (Reniero, *et al*., 1992, Boris, *et al*., 1997). Co-infections of *Lactobacillus reuteri* and *E. coli* have also been observed in pig intestines, with the aggregation of multiple bacterial species contributing to disease virulence (Kmet, *et al*., 1995). However, when studying the two CDS regions annotated in the reference on KEGG, it appeared one of these regions was a pseudogene, perhaps caused by insertions or deletions as

mentioned above. *M. plutonius* has often been observed in the larval gut alongside other secondary invader bacteria, such as *Enterococcus faecalis* and *Paenibacillus alvei* (Forfisgren, 2010), and the ability of *M. plutonius* to co-aggregate with natural host bacteria may impact the progression of infection within the honey bee host.

2.6.4. The link between melissotoxin A and treatment severity

A gene, melissotoxin A, was identified previously as having 33% identity to a mosquitocidal toxin, found in *L. sphaericus*, and that has insecticidal properties (Porter, *et al.,* 1993). This toxin was also confirmed to be expressed *in vivo* within the honey bee tissue during larval infection (Djukic, *et al*., 2018), and caused the increased virulence levels of *M. plutonius* that utilised it (Grossar, *et al*., 2020). Nonetheless, within this study, using chi-squared statistical testing, there was no increased likelihood of destruction of a hive if the isolate causing the EFB outbreak possessed the melissotoxin A gene. Instead, the opposite was true, and hives with this toxin gene were more likely to be treated.

There may be several reasons for this. Firstly, the study is relatively small, with only 59 isolates used in this specific part of the study. An increase in the number of isolates studied may find that there is an increased chance of destruction if a toxin is present. Secondly, the choice of treatment regime is selected by a bee inspector based on how the hive looks in the field. This could be subjective to the inspector – either what they class as a severe infection or based on an inspector's previous experiences of EFB. Additionally, UK bee inspectors will routinely destroy very small (five frames or less) colonies with

EFB due to the chance of survival being very minimal regardless of treatment, and this data on colony size is not available to supplement the analysis.

It could also be theorised that having the toxin does cause the bacteria to kill more larvae in an infection, and that these diseased larvae die quickly and are removed from the hive by the worker bees before the inspector can observe them. Increased early mortality may also result in slower disease spread throughout the hive due to rapid removal of the dead, and therefore hives do possess over all less EFB disease as a result. There could be a trade-off between infection severity and infectivity, those that kill larvae more quickly would therefore be treated rather than destroyed for this reason due to less evidence of EFB being present upon pure observation. The high incidence of horizontal transmission of most honey bee diseases has been hypothesised to favour the evolution of lower virulence levels of pathogens (Fries & Camazine, 2001), even in the case of highly virulent AFB, to maximise the spread to new honey bee colonies.

Lastly to consider, it is of course possible, and probable, that in some isolates, this toxin gene may be non-functional or differentially expressed depending on environmental factors. Toxins are costly to produce (Horak & Tamman, 2017), and it may be that under unfavourable conditions, *M. plutonius* isolates repress the toxin secretion, and therefore mortality is unrelated to the presence or absence of this gene in the bacterial genome. With the absence of transcriptomic studies for the isolates tested in this way, it is impossible to conclude if the presence of this gene alone leads to higher larval mortality, as it may be that the *M. plutonius* simply did not express the toxin when the outbreak

was first established. Ideally, the expression of melissotoxin A could be verified in field infections, however as the isolates tested in this thesis are from historical EFB outbreaks, it would only be now possible to test the isolates retroactively in artificial experiments, further discussed in Chapter 3. Additionally, even if the toxin was shown to be expressed in laboratory larval infections, it still does not mean it was being utilised during the original disease event. Overall, we cannot make strong conclusions regarding the role of melissotoxin A in UK EFB outbreaks originating approximately a decade ago.

2.6.5. Bacteriophage

Three differing whole sequences of bacteriophage have been identified as present in *M. plutonius* for the first time, and these showed most similarity to Lactob_Sha1, Lacto_ph1ge and Lister_P35 (Ardnt, *et al*., 2016). Lactob_Sha1 and Lacto_ph1ge were both first isolated in *Lactobacillus plantarum*, which is a bacterium widely utilised in fermentation processes and the manufacture of dairy products (Liu, *et al*., 2015). *Lactobacillus* species are found very commonly within the gut microbiome of the honey bee and its larvae (Moran, 2015) so there may be some gene transfer occurring from the natural host microflora to *M. plutonius.*

The presence of a partial phage sequence (Table 2.4.), found in the honey bee bacterial pathogen *P. larvae*, also supports the idea that gene transfer may occur in the opposite direction, from host pathogen to host microbiome. It is most interesting that most of the isolates only contain one type of complete bacteriophage, and that almost all isolates of the same ST and therefore CC type have identical phage types to each other. CC3 and CC12 isolates, the two

most virulent clonal complex types, all have Lactob_Sha1 phage, except ST7. All CC13 isolates appear to have a complete Lacto_ph1ge, with two ST13 isolates also having Lister_35, and the other two Lactob_Sha1.

2.6.6. Comparing the overall sequence similarities of *M. plutonius* isolates

Average nucleotide identity analysis can be used as a tool to directly compare the relatedness of bacterial whole genome sequences, with similarity values of 95% generally used to assign species to isolates (Arahal, 2014). Analysis of ANI using the OrthoANIu algorithm (Yoon *et al.,* 2017), quantified how similar isolates belonging to CC3 were to each other, with values > 99.98 generated by the analysis tool. The CC3 isolates also showed less, although still extremely high, similarity to isolates from CC13. In contract the two ST21 isolates were the most significantly different from the other isolates, (whilst still above 99% ANI) and this may have been expected, as strain type DAT561 that also belongs to the same clonal complex 12 (Arai *et al*., 2012) is considered an “atypical” *M. plutonius* strain. Using isolates that are representative of as much diversity in *M. plutonius* as possible in studies of EFB is important therefore, as even within a highly similar bacterium there are genetic differences to consider.

Based on core SNP phylogeny, some of the isolates grouped together as expected within their strain type, and some did not. ST3, the most common in the UK, had two sub-groups on differing clades (Figure 2.2, Figure 2.3). This suggests that MLST typing under the current scheme does not capture all the variation within the genome sufficiently, and that ST3 may benefit from being split into more than one category group. This is particularly important as it is the dominant and most common strain present in the UK. A core genome MLST

(cg-MLST), which uses SNPs in genes that are present for all isolates of a species, would further improve the existing typing system (Maiden, *et al*, 2013), not only for tracing disease outbreaks, but for further exploring the genetic differences of *M. plutonius* isolates. If a more comparative and bioinformatic approach had been taken in this chapter as mentioned previously in this discussion, identification of candidate loci for a cg-MLST may have been discovered, as although there were core genes identified that were shared amongst isolates, which sets of genes are shared and which genes differ unfortunately is still unknown.

The reference genome ATCC 35311 CC13, groups phylogenetically with ST13 isolates here. However, these isolates, except for the ST21 CC12 isolates, are the most different from the rest of the isolates used in this study. This includes phylogenetically (Figure 2.2), within the ANI matrix (Figure 2.1.), and when considering SNPs (Figure 2.4.). References should generally be representative of most isolates within the species, as they are the scaffold used to build genomes from raw sequences, and if ATCC 35311 is less similar to isolates grouped within the more commonly found (in the UK) CC3 then it might be appropriate to introduce a new reference genome for this clonal complex group, as there is for CC12, DAT 561 (Arai, *et al*., 2012), especially as it represents the most commonly found strain types within the UK.

The dissection of bacterial pan-genomes, separating out genes essential for survival from those that are dispensable, is helpful when considering disease and virulence. Often the accessory genome is referred to as the “adaptive genome” and here there may be novel genes, acquired from the other bacterial

species present in the environment (Mira, *et al.,* 2010). As EFB disease progression happens within the gut environment, there is ample opportunity for horizontal transmission between honey bee microbiome strains to occur if advantageous to the pathogen. Overall, the estimated core genome of *M. plutonius* is highly conserved, however there are still clear differences to be explored genetically at the clonal complex level, which may aid further investigation into the virulence differences both in laboratory studies (Nakumura, *et al*., 2016; Lewkowski and Erler, 2019) and in field outbreaks (Budge, *et al*., 2014). It would be potentially more helpful to also further explore the 289 genes that are classified here as 'cloud' genes, and only present within between 0-15% of strains, may correlate to novel genes suitable for further studies of virulence of EFB. When studying whole genomes, it is important to classify both the core and accessory genome in analysis, to ensure true measures of within strain diversity is considered.

2.6.7. Conclusions

Isolates of *M. plutonius* are extremely genetically similar, with >99% average nucleotide identity, however there are clear differences in the ability of strains to induce death in EFB infection. There are variable genes identified amongst strains, for example the presence or absence of a toxin gene, 'melissotoxin A', and types of bacteriophage sequence present in the genome. Using treatment type as a proxy for disease severity showed there may be a negative relationship between 'melissotoxin A' and the appearance of EFB symptom severity, but with the absence of gene expression experiments, it is impossible to draw strong conclusions. Core SNP phylogenetic analysis suggests that current strain typing methods may not be sufficient to differentiate all *M.*

plutonius isolates into appropriate groups, necessitating more whole genome sequencing of bacterial isolates from EFB outbreaks in the future.

3. Testing the virulence of *Melissococcus plutonius* (European foulbrood) isolates using laboratory reared honey bee (*Apis mellifera*) larvae.

3.1. Abstract

European foulbrood disease outbreaks vary in severity and persistence, which in turn informs the choice of treatment. Knowledge of the genetic differences amongst *M. plutonius* strains are limited, with strain type (ST) and clonal complex (CC3, CC13 or CC12) currently in use to differentiate outbreak isolates. Although whole genome sequencing and comparison has been utilised in studies of *M. plutonius*, how this links to bacterial virulence in real infections is still unknown. Larval infection studies can be used to examine how *M. plutonius* isolates vary in their potential infection severity, and previous studies have shown those that are CC3 (the most common in the UK) to be intermediately virulent, with CC13 being the least virulent and CC12 as the deadliest clonal complex type. Three experiments using varying strains of *M. plutonius* did not reveal if the presence or absence of a specific putative toxin gene is important in infection. However, two of the experiments suggest that strain type, and therefore clonal complex, may not always reliably describe how virulent a strain is during *in vitro* infection. The ability to predict how severe an EFB infection will be from ST or CC is likely to be beneficial to treatment success in the future.

3.2. Introduction

3.2.1. An overview of honey bee development in the hive

A single honey bee colony comprises of a single mated queen, many thousands of unmated female worker bees, and in summer, several hundred male drone bees. All have four life cycle phases (egg, larva, pupae, and adult) which vary in their duration from egg laying to emergence as an adult, depending on the caste of bee. Queens emerge from pupation typically after 15-16 days, worker bees after around 21 days and drones after approximately 24 days (Crane, 2009). Queens are responsible for populating the hive with unfertilised eggs that become drones and fertilised eggs that become either worker bees, or when fed nutritious royal jelly, future virgin queens (Rembold, *et al*., 1974). Worker bees express age polyethism, meaning they transition between tasks as they age (Calderone & Page, 1988). Important early tasks include nursing the brood and cleaning out diseased or dead larvae. Whilst EFB affects the larval stage of the honey bee, worker bees are a vector of transmission, passing the causative bacteria directly to the larvae via their food, either from the environment, contaminated honey or from cleaning out diseased larvae (Forsgren, 2010).

3.2.2. Larval infection studies of *M. plutonius* to understand virulence

Previous studies have attempted to unravel how differing *M. plutonius* isolates may alter EFB disease progression in the honey bee. Mckee *et al*. (2012) established that disease severity was influenced by bacterial density of *M. plutonius* administered. Further investigations showed that strains of *M. plutonius* described as atypical are less fastidious in the lab, and when grown *in*

vitro, cause severe EFB symptoms in larvae (Arai *et al*., 2012). These 'atypical' strains were later characterised as CC12, as mentioned above (Budge, *et al*., 2014). Under manipulated experimental infection condition using a potassium rich diet, Nakamura, *et al.* (2016) confirmed field observations (Budge, *et al*., 2014) that CC13 was the least virulent clonal complex, followed by CC3 and finally CC12 the most virulent. A recent larval study of *M. plutonius* investigated CC3 and CC13 only, but also looked at the impact of host genotype (Lewkowski and Erler, 2019). Their data also followed that CC3 was the most virulent, with larval survival after infection with CC13 isolates not statistically different from the control. The most recent larval infection study suggested that host background contributes to the progression of disease, but that the most important factor in disease virulence was strain genotype, specifically a toxin gene (Grossar *et al*., 2020). Differences in growth dynamics were also observed but these did not explain virulence differences in the *M. plutonius* strains used in this case. The mechanism of infection and potential *M. plutonius* genes of importance are discussed in Chapter 2.

To explore if strains that contain the melissotoxin A gene are lethal, larval infection assays will be utilised. This type of experiment involves rearing <1-day old larvae in 48 well plates and infecting with *M. plutonius* via a dose within their food. The larvae will then theoretically either die of infection or develop into pupae and emerge as adult bees, with the inclusion of an uninfected control group to measure for natural mortality (Figure 3.1). Directly comparing how long differing strains take to kill could give further insight into the relative virulence of UK *M. plutonius* isolates and whether strain typing alone is an appropriate way to characterise the virulence of individual strains, given that there appears to be

some further grouping within the same strain type (Figure 2.2, Figure 2.3.). These types of artificial infection studies have already been used to test virulence of *M. plutonius* strains in laboratory reared larvae (Nakamura, *et al*., 2016). Observation at the genome level does not necessarily predict how the bacteria may behave during pathogenesis, so using a practical model of infection, albeit in the laboratory, is an appropriate next step.

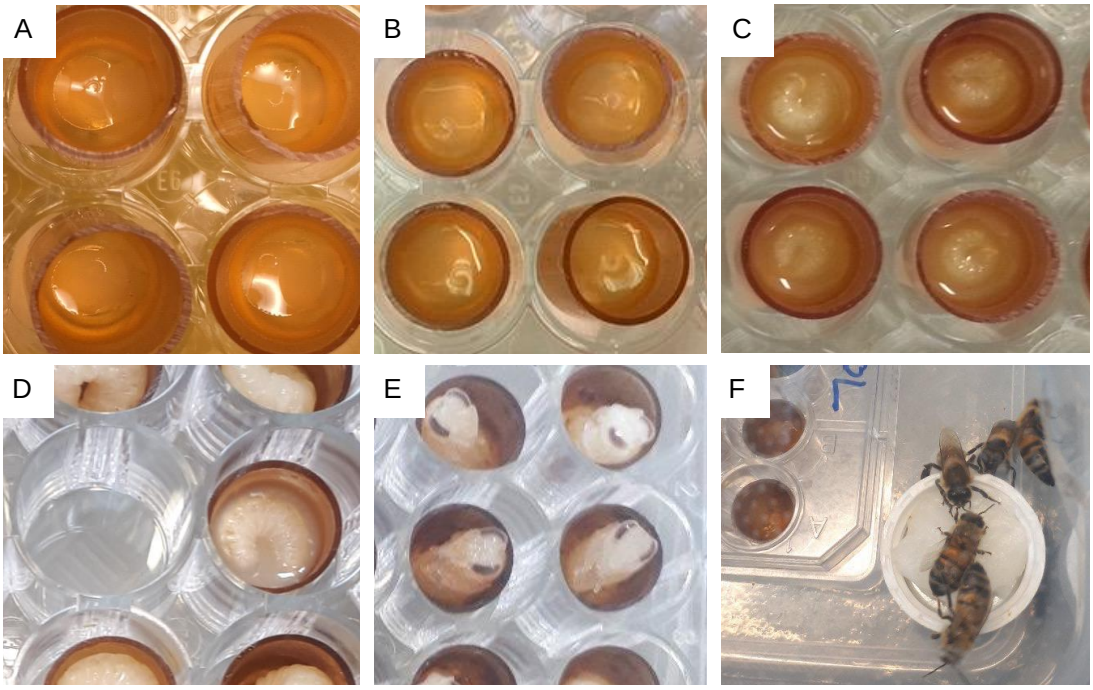

Figure 3.1. Honey bee workers developing in the lab: A) on day of grafting, B) two days post grafting, C) 4 days post grafting, D) 7 days post grafting E) day 14 post-grafting pupae and F) emerged adult honey bees.

3.2.3. Experiment 1: Pilot study to test infection model

Previous studies have observed that some strains are attenuated during artificial infection experiments, meaning that virulence is lost (Nakamura *et al*., 2016). Additionally, there had been no larval infection studies using *M. plutonius*, a generally fastidious bacteria, attempted at the National Bee Unit for

at least 5 years, and the larval grafting success of beginners is generally poor, creating challenges to successfully establishing this assay. To therefore both validate an infection protocol on a smaller scale and to test whether EFB could be induced artificially in the two typical clonal complex types, three strains were selected for the pilot experiment (Table 3.1.). As the gene content of UK *M. plutonius* isolates are very similar (see Chapter 2), focus was upon the putative melissotoxin A gene which can be present or absent within the same strain type. Isolates selected were all from the same origin and either CC3 (intermediately virulent) or CC13 (least virulent). One of the CC3 isolates had the toxin, as did the single CC13 isolate (Table 3.1.). If possessing this gene is important in EFB infection, we could expect that clonal complex predictions may not be as previously found, for example, showing a reduction in the virulence of CC3 P8325 and an increase in the virulence of CC13 P7955, due to an absence or presence of the MTX gene respectively.

Table 3.1. Metadata of *M. plutonius* isolates used in pilot experiment 1.

Isolate	**ST**	**CC/ Virulence^**	**Origin**	**Toxin?**
P7993	5	3 / MID	Norfolk	Yes
P8325	5	3 / MID	Norfolk	No
P7955	13	13 / LOW	Norfolk	Yes

^Virulence level determined by strain type/clonal complex based on previous studies (Budge, *et al*., 2014, Nakamura *et al*., 2016, Lewkowski and Erler, 2019)

3.2.4. Experiment 2: Does the toxin gene always matter, and does virulence vary within clonal complex?

A second larval experiment was conducted to further explore any differences in toxin presence and absence as the pilot data suggested it may be important in early infection progression (Table 3.2.). Two isolates belonging to two of the same clonal complexes were chosen again (CC3 and CC13), with both less virulent CC13 strains having a toxin gene present (Table 3.2.). A single CC12 isolate, P7746, was also introduced to ensure representation from all three clonal complexes and to test if the increased virulence prediction of this clonal complex previously observed was also followed.

Table 3.2. Metadata of *M. plutonius* isolates used in experiment 2.

Isolate	ST	CC / Virulence^	Origin	Toxin?
P8450	2	3 / MID	Oxfordshire	Yes
P7613	5	3 / MID	Suffolk	No
P7596	13	13 / LOW	Oxfordshire	Yes
P8061	20	13 / LOW	Dyfed	Yes
P7746	21	12 / HIGH	Mid Glamorgan	No

^Virulence level determined by strain type/clonal complex based on previous studies (Budge, *et al*., 2014, Nakamura *et al*., 2016, Lewkowski and Erler, 2019).

3.2.5. Experiment 3: Further exploration of clonal complex, ST, and virulence

The third experiment was intended to further explore if isolate clonal complex classification is an accurate predictor of virulence and severity of infection. Isolates included three from CC3, the most common clonal complex in the UK (Table 3.3.). ST3 (CC3) is also the most common strain type, spreading throughout most of the areas where EFB outbreaks have occurred in England and Wales. Often pathogenic bacteria can have a trade-off between infectivity (and therefore transmission) and virulence, and this may also be the case for *M. plutonius*. Additionally, one UK ST3 strain tested in the latest larval infection study was deemed 'avirulent' (Grossar, *et al*., 2020).

Table 3.3. Metadata of *M. plutonius* isolates used in experiment 3.

Isolate	ST	CC / Virulence^	Origin	Toxin?
P8157	3	3 / MID	Greater London	Yes
P8414	3	3 / MID	Kent	No
P7993	5	3 / MID	Norfolk	Yes
P7955	13	13 / LOW	Norfolk	Yes
P7746	21	12 / HIGH	Mid Glamorgan	No

^Virulence level determined by strain type/clonal complex based on previous studies (Budge, *et al*., 2014, Nakamura *et al*., 2016, Lewkowski and Erler, 2019)

3.3. Aim of study

Larval infection experiments are a useful way of experimentally testing the virulence levels of individual *M. plutonius* strains. At the time of these experiments, no other larval infection studies of isolates of UK EFB outbreak origin had been published, except for the inclusion of two isolates in Grossar, *et al*., 2020. Clonal complex type, based on strain type, has been used previously as a crude indicator of virulence level. Melissotoxin A has been identified as a candidate gene of interest (Grossar, *et al*., 2020), and the effect of the presence or absence of this gene will be tested in laboratory reared honey bee larvae. Additional gene expression studies will be needed in future projects to validate that genes of interest are used by the bacterium if they possess it in their genome. If strain virulence can be reliably determined in EFB infections, key differences in the genes of isolates causing higher mortality may be identified in future.

3.4. Methods

3.4.1. Collection of frames for larval grafting

Healthy and untreated hives without prior antibiotic treatment and with no recent Varroacide application were selected for each experiment. The queen was confined in each colony to a single frame, enclosed in a queen frame cage and left for four to five days to lay fresh eggs. Frames were inspected on the morning of grafting to ensure appropriate age larvae were present (~1 day old).

3.4.2. Preparation of larval food

Larval food was prepared based on the method in the OECD Test Guideline: 237 (2016): Honey Bee Larval Toxicity Test following Single Exposure and OECD Test Guidance Document 239 (2016): Honey Bee Larval Toxicity Test following Repeated Exposure. Basic diets comprised of glucose, fructose, and yeast with the addition of royal jelly (obtained from The Raw Honey Shop: www.therawhoneyshop.com).

Three types of diet solution were prepared A, B and C (Table 3.4.) and 0.22µm filter sterilised into 20ml scintillation vials. Diet solutions were stored at 0-10°C for up to one month.

Larval diet consisted of 50% of diet solution (A, B or C) and 50% royal jelly (w/w) prepared less than one week before use and was warmed in the incubator (34.5°C) at least 30 minutes prior to use

Table 3.4. Total % weight/weight of different components of larval diet solutions.

	Total of final diet solution (% w/w)			
Diet solution	**Glucose**	**Fructose**	**Yeast extract**	**Deionised water**
A	**12**	**12**	**2**	**74**
B	**15**	**15**	**3**	**67**
C	**18**	**18**	**4**	**60**

3.4.3. Preparation of artificial queen cells

Larvae were grafted and reared using crystal polystyrene grafting cells (artificial queen cells) placed in 48 well culture plates. Cells were raised in height to allow ease of grafting using dental roll soaked in Milton's sterilising solution (12ml Milton's/155g glycerol made up to 1L with demineralised water). Then assembled well plates containing cups were sterilised using UV light for a minimum of 20 minutes. Plates were warmed in the incubator prior to grafting (34.5°C).

3.4.4. Grafting of larvae

Larvae were assigned to treatment (bacterial isolate) post grafting. A positive displacement pipette was used to fill cups with 5µl of warmed larval diet. Young larvae (>1 day old) were removed from combs using grafting tools (Figure 3.2.) and placed onto larval diet, ensuring access to food but as not to submerge, as risk of drowning is high (Figure 3.2.). Larvae were kept in a hermetically sealed chamber within an incubator, set at 34.5°C and 65±5 % humidity, with a saturated solution of K_2SO_4 added to the bottom of the chamber. On day 8, the

solution was replaced with a saturated solution of sodium chloride (NaCl), intended to increase the relative humidity in the chamber, to provide an optimal environment for larval pupation.

Figure 3.2. Techniques to rear honey bees in the lab: collecting frames from hives (left), grafting set up with frame, lamp, grafting tool, 48 well plate and artificial queen cells (middle) and honey bee larvae two days post grafting resting on food (right).

Table 3.5. Number of larvae grated for each experiment

Experiment	Control	Treatment groups
1	50	80
2	80	80
3	80	80

3.4.5. Infection of larvae with *M. plutonius*

Previously adjusted cultures of comparable OD (600nm) value were added to individual larval diet A aliquots (19:1 food to inoculum ratio), with media used in place of bacterial for control groups. Plates of grafted larvae were randomly split into rows and assigned a treatment to remove any plate effect or any

differences in mortality caused by donor colony differences. An additional 5µl of prepared infected food or control was fed to groups of larvae to induce EFB infection. To estimate the dose of *M. plutonius* fed to larvae, food was diluted 1:10 and 1:100 and spread onto agar plates to estimate colony forming units (CFUs), which were counted after approximately 10 days (Table 3.6.).

3.4.6. Verification of infection of larvae with *M. plutonius*

Larvae were randomly selected on day 7 of each experiment to check for the presence of *M. plutonius*. Guts were dissected from larvae and samples stained using nigrosine as per the usual EFB diagnostic protocol at the NBU (OIE Terrestrial Manual, 2018). In all three experiments *M. plutonius* was observed under the microscope whilst the control groups were consistently free from infection.

3.4.7. Feeding, weight and mortality checks

Mortality observations were taken daily at approximately the same time, with the exception of day 1 post infection where larvae were checked twice to exclude those that were dead as result of grafting. Dead larvae were removed to prevent fungal growth. Larvae were also fed until day 6 on diet A, B or C with differing quantities (Table 3.7.). Larvae were followed until pupation in the pilot study, and until adult bee emergence in experiment 2 and 3. Once day 14 pupae observations were completed, each plate was placed into a plastic Tupperware (no lid), and then sealed inside a perforated bag to allow the emergence of adults to occur in a confined enclosure. Food was provided in the form of 50% W/V aqueous sucrose solution in an Eppendorf and

Ambrosia Bee Fondant for emerging individuals. Additionally, on day 7 of experiment 3, seven larvae were randomly selected for weight measurements from each treatment and the control group and then discarded from the experiment.

3.4.8. Survival curves and statistical analysis

All statistical analyses were performed using GraphPad Prism (version 8 or 9). Larval survival was evaluated by means of log-rank (Mantel-Cox) pairwise tests for treatment effect (strain). Additionally, a Kruskal-Wallis test with a post hoc Bonferri correction (Kruskal & Wallis, 1952) was used to compare the median survival in days of larvae firstly in each treatment group per experiment (individuals that were alive at the end point were given the maximum survival i.e. 13 or 15 days). Subsequently if significant, data was combined for isolates belonging to the same clonal complex group to test CC virulence levels, with a follow up Mann–Whitney *U* (two-tailed) test to test for pairwise significance of various treatments.

Table 3.6. Bacterial dose (CFU/ml) information for all strains of *M. plutonius* used in each experiment.

Experiment	Isolate	ST	CC	Toxin gene	CFU/ml in food‡
1	P7993*	5	3	Yes	2.35×10^4
	P8235	5	3	No	3.89×10^4
	P7995*	13	3	Yes	9.2×10^3
2	P8450	2	3	Yes	1.55×10^4
	P7613	5	3	No	1.9×10^4
	P7596	13	13	Yes	2×10^3
	P8061	20	13	Yes	2.13×10^4
	P7746*	21	12	No	8.4×10^3
3	P8157	3	3	Yes	2.5×10^3
	P8414	3	3	No	5×10^3
	P7993*	5	3	Yes	1.65×10^4
	P7955*	13	13	Yes	6.6×10^3
	P7746*	21	12	No	1.41×10^4

*Bacterial isolate was used in multiple experiments

‡Estimated from bacterial CFUs

Table 3.7. Diet type and volume fed to bees at differing ages during all experiments, days post infection (P.I.).

Age of bee (days)	Days (P.I.)	Diet type	Volume (µl)
4	1	A	5 (+5 bacteria or control)
5	2	A	10
6	3	B	20
7	4	C	30
8	5	C	40
9	6	C	50
10	7	N/A	None
11	8	N/A	None
12 (pupation)	9	N/A	None

3.4.9. Regression analysis of bacterial dose and mortality:

Simple linear regression analysis using GraphPad Prism (version 9) was performed to test if bacterial dose administered on day 0 predicted the final mortality rate of larval treatment groups. The null hypothesis is that the bacterial dosage variable has no correlation with the dependent variable mortality at the larval stage end point. To adjust for larval mortality not attributed specifically to *M. plutonius* infection, end point death percentages for each isolate were adjusted by subtracting the experiments control group mortality percentage on either day 13 post infection (Experiment 1) or day 15 post infection (Experiment 2 and 3), to ensure only the effect of the bacterial dose strength was analysed.

3.5. Results

3.5.1. Experiment 1: Pilot experiment to validate infection model

Doses of *M. plutonius* given to larval groups (CFU/ml of food) did not significantly predict end point larval mortality rate at day 13 post-infection, R^2 = .68, $F(1,1) = 2.13$, $p = .38$, although there was a positive regression coefficient value (Figure 3.4.). On the first day of the experiment, strains P7993 (ST5, CC3, toxin) and P7955 (ST13, CC13, toxin) had 13.89% and 15% mortality respectively. In contrast, strain P8325 ST5 (without toxin) had ~2.5% mortality, and the control group ~6.5% (Figure 3.3.). However, by the end of the experiment, both ST5 isolates had significantly different survival curves to the control (log-rank tests: P7993, P=.0237; P8325, P=.0286). None of the three survival curves of the *M. plutonius* infected larvae populations significantly differed from each other (Figure 3.3.). The ST13 survival curve also did not significantly differ from the control group (Figure 3.3.). Overall, the number of days of survival within the experiment did not significantly differ between all isolate and control treatments (Kruskal–Wallis ANOVA: $H_{ISOLATE}(3) = 4.32$, p = .23, N = 278).

By day 13 of the experiment (post infection), pupation should be occurring in larvae. Of the larvae that survived to this day, ~92% of control larvae were pupae (Table 3.8.). In contrast, ST13 surviving larvae were half as likely to be pupae, with the two ST5 infected larvae it was on average only ~10% (Table 3.8.). Individuals in the pilot study were culled before emergence, and therefore this specific data is absence for this initial experiment.

Table 3.8. Mortality and pupation data for pilot experiment. Pupation % measured as percentage of alive individuals that have pupated vs. still larvae on day 14, death % describes mortality percentage of individuals in that group on day 14 in the whole experiment.

Isolate	Total individuals start[§]	Larval number survival day 14	Death % of total individuals	Pupae at day 14
P7993 ST5 CC3 toxin	72	23	68	2
P8325 ST5 CC3	80	26	67.5	3
P7955 ST13 CC13 toxin	80	32	60	15
Control	46	26	43.5	24

[§]Individuals removed from experiment day 1 check if deceased at first check point

Table 3.9. Metadata for three isolates used in Experiment 1.

Isolate	ST	CC / Virulence	Toxin gene?	Treatment in field
1) P7993	5	3 / Mid	Yes	Destruction
2) P8325	5	3 / Mid	No	Shook swarm
3) P7955	13	13 / Low	Yes	Shook swarm

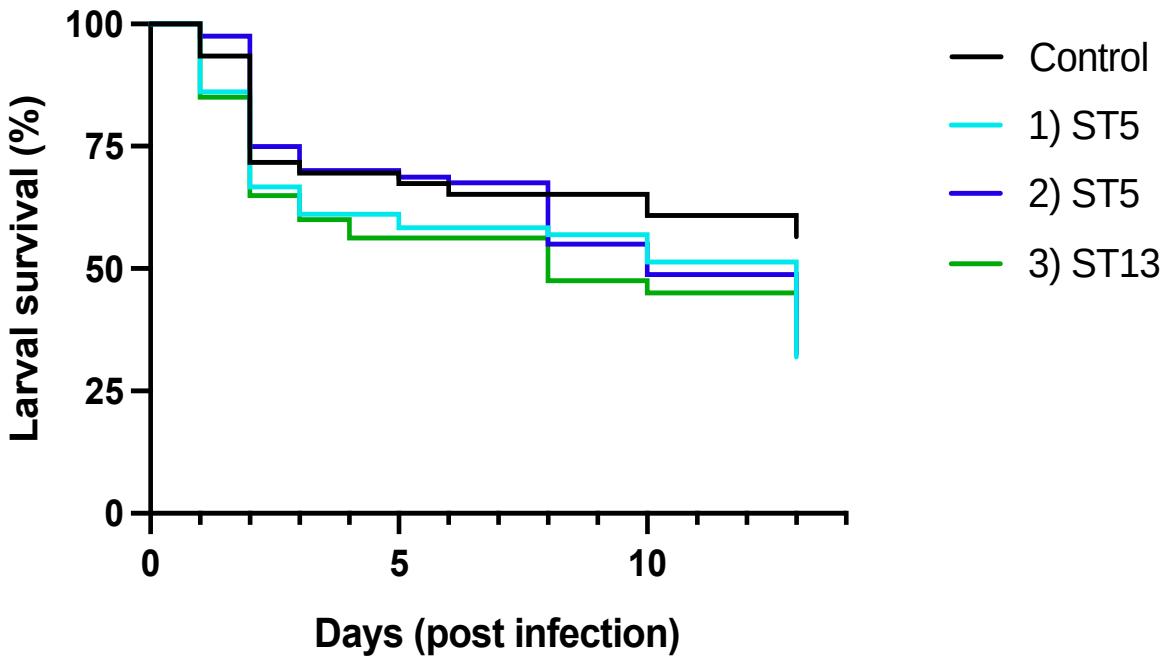

Figure 3.3. Honey bee larval survival rates over 13 days (14 days post infection) following infection with different strains of *M. plutonius*.

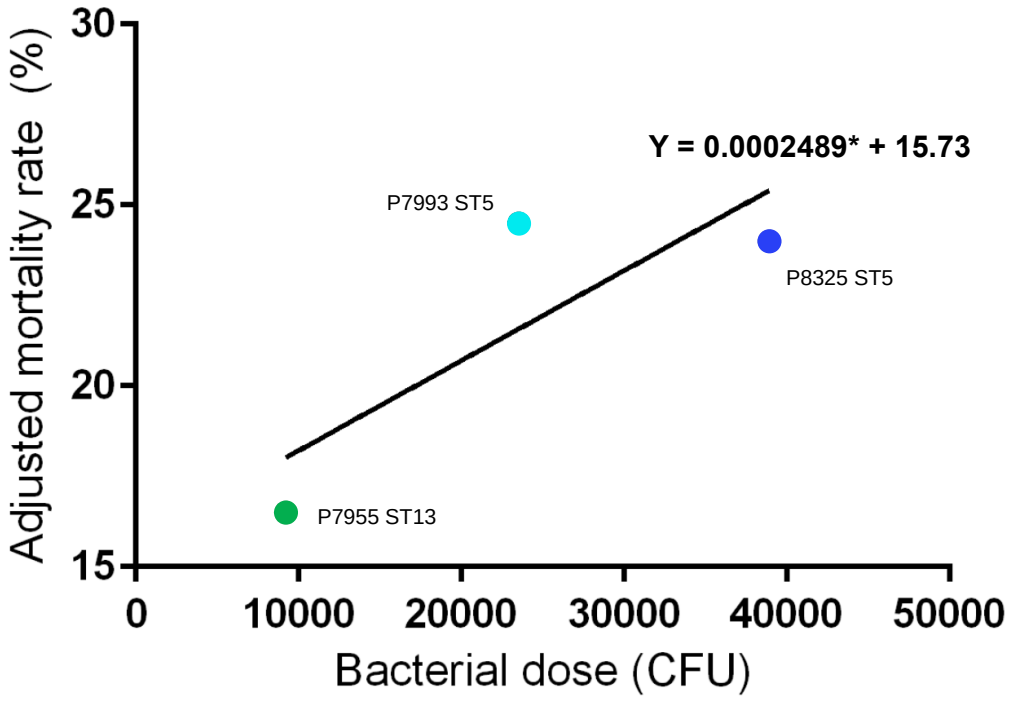

Figure 3.4. Linear regression of differing *M. plutonius* bacterial isolate dose administered on day 0 of experiment 3 compared against control adjusted experiment larval mortality percentage on day 15 post-infection.

3.5.2. Experiment 2: Is possessing a toxin gene important, and does virulence vary within a clonal complex?

In Experiment 2, again doses of *M. plutonius* given to larval groups did not significantly predict end point larval mortality rate, R^2 = .015, $F(1,3)$ = .047, p = .84 (Figure 3.6.) on day 15 post-infection. When considering the entire death curve, mortality rates of all larvae were initially slower than in Experiment 1 (Figure 3.3.; Figure 3.5.), perhaps because of less injury caused during manual grafting on day 1 of the assay. However, by day 7 post infection, P7746 (ST21, CC12, no toxin) had induced ~62% death (Figure 3.5.). In contrast, P8061 (ST20, CC13, toxin), P7956 (ST13, CC13, toxin) and the control had ~26%, 35% and ~30% mortality respectively. At day 15 post infection, only P8061 (ST20, CC13 toxin) had a survival rate that was not significantly different to the control over the whole survival curve (Figure 3.5.) unlike the second CC13 isolate P7956 (ST13, toxin) (log-rank: P=.0233).

The ST2 survival curve was not significantly different from any of the other infected groups except ST20 (log-rank: P= .0001) and ST5 was significantly different to both ST13 and ST20 (P=.0072; P=<.0001). The survival curves of ST2 (toxin), ST5 (no toxin) (both CC3) and ST21 (no toxin, CC12) did not significantly differ from each other (Figure 3.5.). The highest mortality of larvae on day 15 were those infected with ST5 (~89%), followed by ST21 (~86%). The mortality of the control group was ~49% (Figure 3.5.).

The Kruskal-Wallis test of variance indicated that there is a significant difference in the number of days of survival between the different isolate treatment groups (Kruskal–Wallis ANOVA: $H_{ISOLATE}(5) = 41.88$, , $p < .001$).

Isolates grouped into their clonal complexes and then reanalysed, also showed significant difference in survival between clonal complex groups (Kruskal–Wallis ANOVA: $H_{CC}(3) = 35$, $p < .001$).

Testing with Mann Whitney *U* analysis indicated that the mean ranks of the following clonal complex groups or control pairs are significantly different:

- Control and CC3 (Mann–Whitney $U = 4278$, $n_1 = 80$ $n_2 = 156$, $P < .001$ two-tailed)
- Control and CC12 (Mann–Whitney $U = 1825.5$, $n_1 = 80$ $n_2 = 78$, $P < .00001$ two-tailed)
- CC3 and CC13 (Mann–Whitney $U = 9104.5$, $n_1 = n_2 = 156$, $P < .001$ two-tailed)
- CC13 and CC12 (Mann–Whitney $U = 3717$, $n_1 = 156$ $n_2 = 78$, $P < .00001$ two-tailed)

CC3 & CC12 and Control & CC13 number of days survival were not significantly different to each other.

Adult bee emergence varied greatly between infected groups. Only ~7% of larvae infected with ST2, and 12.5% of larvae infected with ST5 (CC3 isolates), that survived to day 16 post infection then emerged as adults (Table 3.10.). In contrast, ~58% of ST13 (CC13) and ST20 (CC13) infected larvae emerged, which was a similar result to the control group (~55%). Although very few numbers of ST21 (CC12) survived to pupation and just over a third of emerged as adults (Table 3.10.).

Table 3.10. Number of larvae individuals at the start of experiment 2, number of larvae surviving to day 15 post infection, mortality % of individuals on day 15 P. I., post-pupation emergence numbers and % successful emergence of adult bees of those survived to pupation stage (survival until day 16).

Isolate	Total individuals start[§]	Survival until day 15 (pupae)	Death % of total by day 15	Individuals emerging pupation
P8450 ST2 CC3 toxin	80	14	82.5	1
P7613 ST5 CC3	77	8	89.6	1
P7956 ST13 CC13 toxin	79	24	69.6	14
P8061 ST20 CC13 toxin	79	36	54.4	21
P7746 ST21 CC12	78	11	85.9	4
Control	79	40	49.4	22

[§]Individuals removed from experiment day 1 check if deceased at first check point

Table 3.11. Metadata for five isolates used in Experiment 2.

	ST	CC / Virulence	Toxin gene?	Treatment in field
1) P8450	2	3 / Mid	Yes	Destruction
2) P7613	5	3 / Mid	No	OTC
3) P7596	13	13 / Low	Yes	Destruction
4) P8061	20	13 / Low	Yes	OTC
5) P7746	21	12 / High	No	Destruction

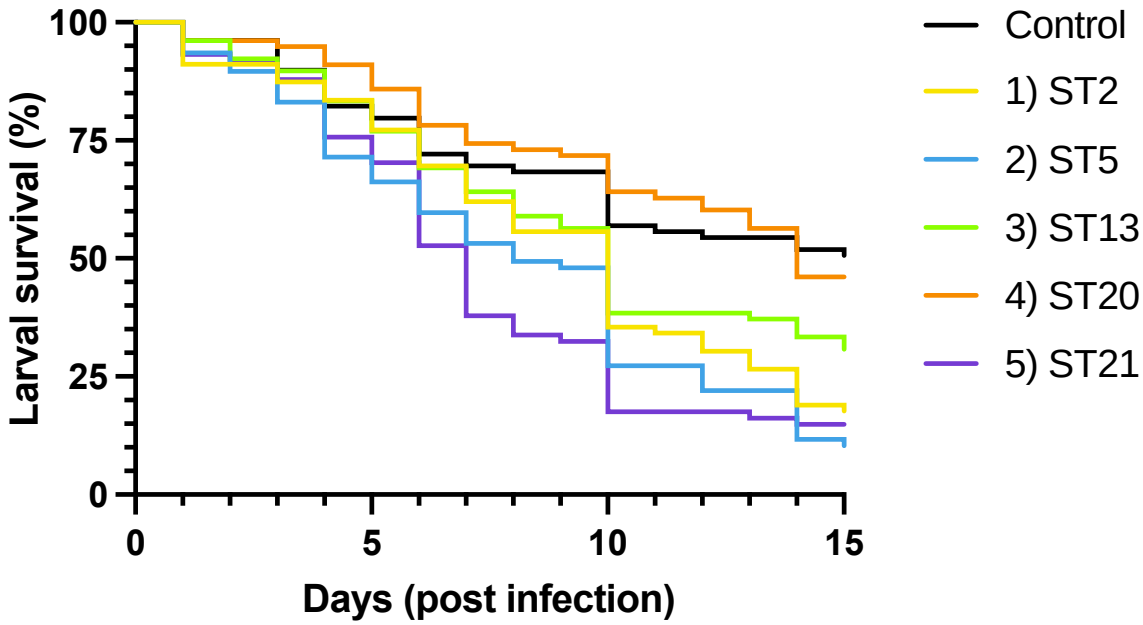

Figure 3.5. Honey be larvae survival over 15 days post infection with different isolate strains of *M. plutonius*.

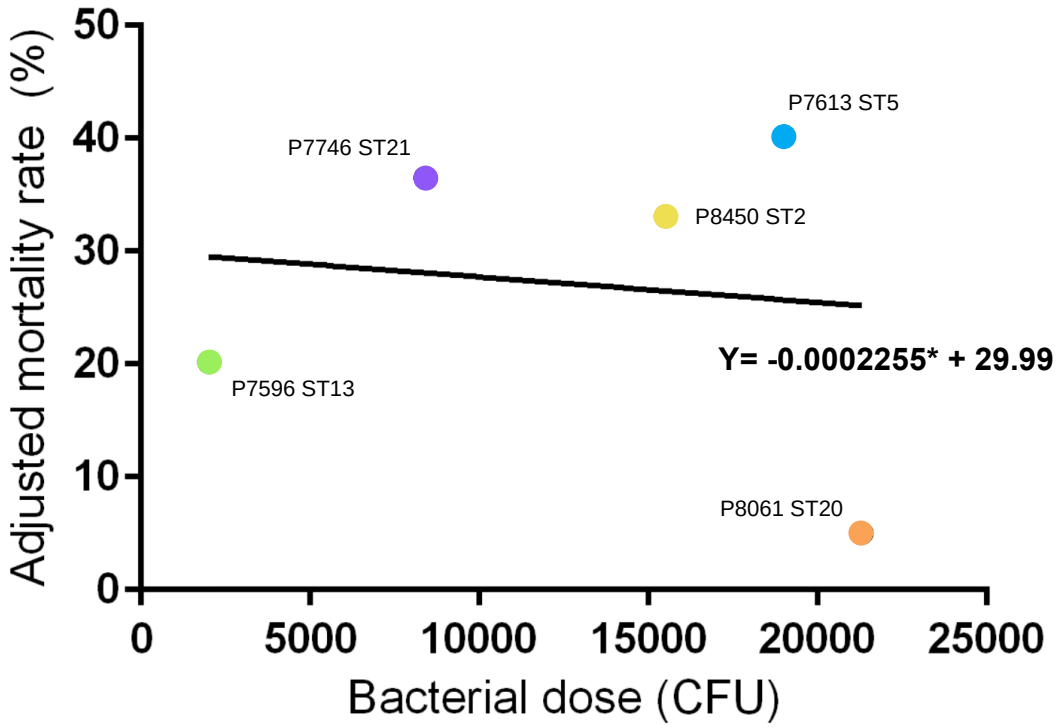

Figure 3.6. Linear regression of differing *M. plutonius* bacterial isolate dose administered on day 0 of experiment 2 compared against control adjusted experiment larval mortality percentage on day 15 post-infection.

3.5.3. Experiment 3: Further exploration of clonal complex, ST, and virulence

Regression analysis performed suggests that original starting doses of *M. plutonius* given to larval groups does not significantly predict larval mortality rate on day 15 post-infection, R^2 = .56, $F(1,3)$ = 3.85, p = .14 (Figure 3.8.). In experiment 3, disease again progressed slowly for all groups until between day 5 and day 6 when a ~34% increase in mortality in larvae infected with strain P7746 (ST21, CC13, no toxin) was observed (Figure 3.7.). Both ST3 CC3 survival curves were not significantly different from the control group or the ST13 CC13 strain. The ST13 CC13 strain was also different from the control group in this experiment as in the previous experiment 2 (log-rank, P=.0304). All survival curves were significantly different from ST21 (log-rank, P=<.0001). The ST5 CC3 survival curve was not significantly different from ST13 but was from the other two CC3 ST3 isolates (log-rank: P8157, P=<.0120; P8414, P=.0056) and from the control (log-rank: P=<.0001).

The highest mortality on day 16 of the assay was in the ST21 infected larval group (~95%) followed by ST5 (~58%) (Figure 3.7.). In contrast only ~30% of those in the control group were dead, followed by the two groups of ST3 infected larvae having mortality rates of ~41%. Again, in this experiment presence of melissotoxin A did not appear to correlate with larval survival rate.

The Kruskal-Wallis test indicated there is a significant difference in the number of days of survival between the different isolate treatment groups (Kruskal–Wallis ANOVA: $H_{ISOLATE}$ (5) = 90.25, p < .001) and clonal complex groups (Kruskal–Wallis ANOVA: H_{CC}(3) = 81.67, p < .001)

Performing pairwise Mann Whitney *U* tests indicated that the mean ranks of the following pairwise comparisons are significantly differed:

- Control and CC3 (Mann–Whitney $U = 4278$, $n_1 = 79$ $n_2 = 236$, $P < .05$ two-tailed)
- Control and CC12 (Mann–Whitney $U = 1303.5$, $n_1 = 79$ $n_2 = 80$, $P < .00001$ two-tailed)
- CC3 and CC12 (Mann–Whitney $U = 3935.5$, $n_1 = 236$ $n_2 = 80$, $P < .00001$ two-tailed)
- CC13 and CC12 (Mann–Whitney $U = 1197.5$, $n_1 = 79$ $n_2 = 80$, $P < .00001$ two-tailed)

As only one isolate for CC12 and CC13 was tested in the experiment, the CC3 group of ST3s (two isolates grouped) and ST5 single isolate were split and tested for significant differences:

- ST3 and ST5 (Mann–Whitney $U = 4644.5$, $n_1 = 157$, $n_2 = 79$, $P < .01$ two-tailed)
- ST3 and ST21 (Mann–Whitney $U = 2590.5$, $n_1 = 157$ $n_2 = 80$, $P < .00001$ two-tailed)
- ST5 and ST13 (Mann–Whitney $U = 2351.5$, $n_1 = 79$ $n_2 = 79$, $P < .01$ two-tailed)
- ST5 and ST21 (Mann–Whitney $U = 1345$, $n_1 = 79$ $n_2 = 80$, $P < .00001$ two-tailed)

ST3 isolates and the ST13 isolate showed no significant variance in the days survival compared to the Control group and each other.

Adult bee emergence also varied between infected groups in Experiment 3. Only 5% of larvae infected with P7746 (ST21, CC12, no toxin) emerged from the experiment as adult bees. In contrast, ~47% of both ST3 CC3 infected larvae groups emerged on day 22 (Table 3.12.). In contract, the other CC3 isolate, ST5 only had an approximately 27% emergence rate.

Table 3.12. Number of larvae at the start of experiment 3, number of larvae surviving to day 16 post infection, mortality % of individuals on day 16, post-pupation emergence numbers and % successful emergence of adult bees of those survived to pupation stage (survival until day 16).

Isolate	Total individuals start[§]	Survival until day 15 (pupae)	Death % of total by day 15	Individuals emerging pupation
P8157 ST3 CC3 toxin	78	44	43.6	34
P8414 ST3 CC3	79	46	41.8	21
P7993 ST5 CC3 toxin	79	30	62	17
P7955 ST13 CC13 toxin	80	41	48.8	20
P7746 ST21 CC12	80	4	95	0
Control	79	56	29.1	36

[§]Individuals removed from experiment day 1 post infection check if deceased at first checkpoint

Table 3.13. Metadata for five isolates used in Experiment 3.

Isolate	ST	CC / Virulence	Toxin gene?	Treatment in field
1) P8157	3	3 / Mid	Yes	Shook swarm
2) P8414	3	3 / Mid	No	Destruction
3) P7993	5	3 / Mid	Yes	Destruction
3) P7955	13	13 / Low	Yes	Shook swarm
5) P7746	21	12 / High	No	Destruction

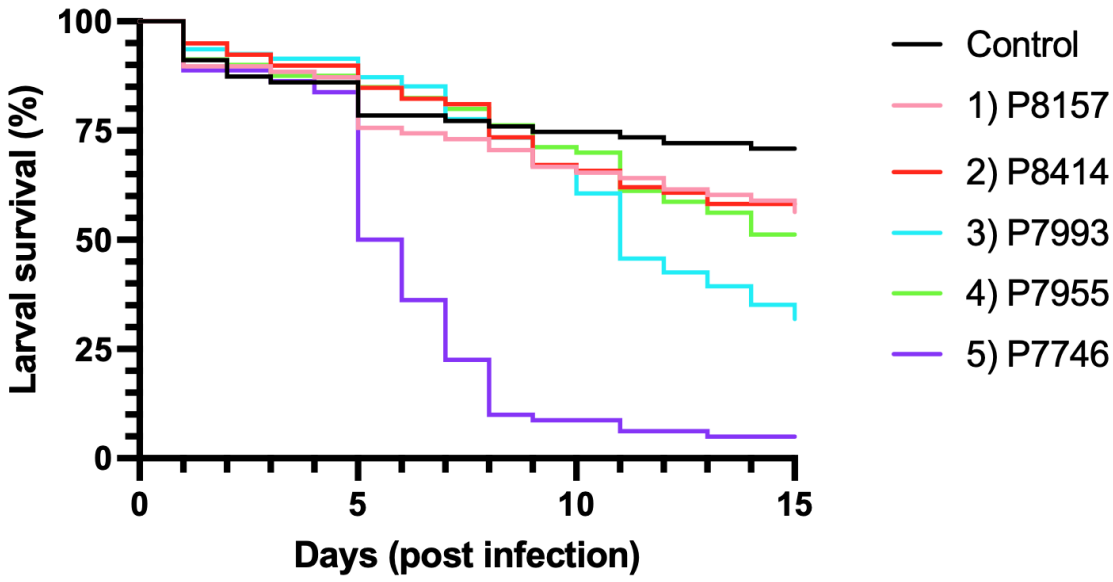

Figure 3.7 Honey bee larvae survival over 15 days post infection with different isolate strains of *M. plutonius*.

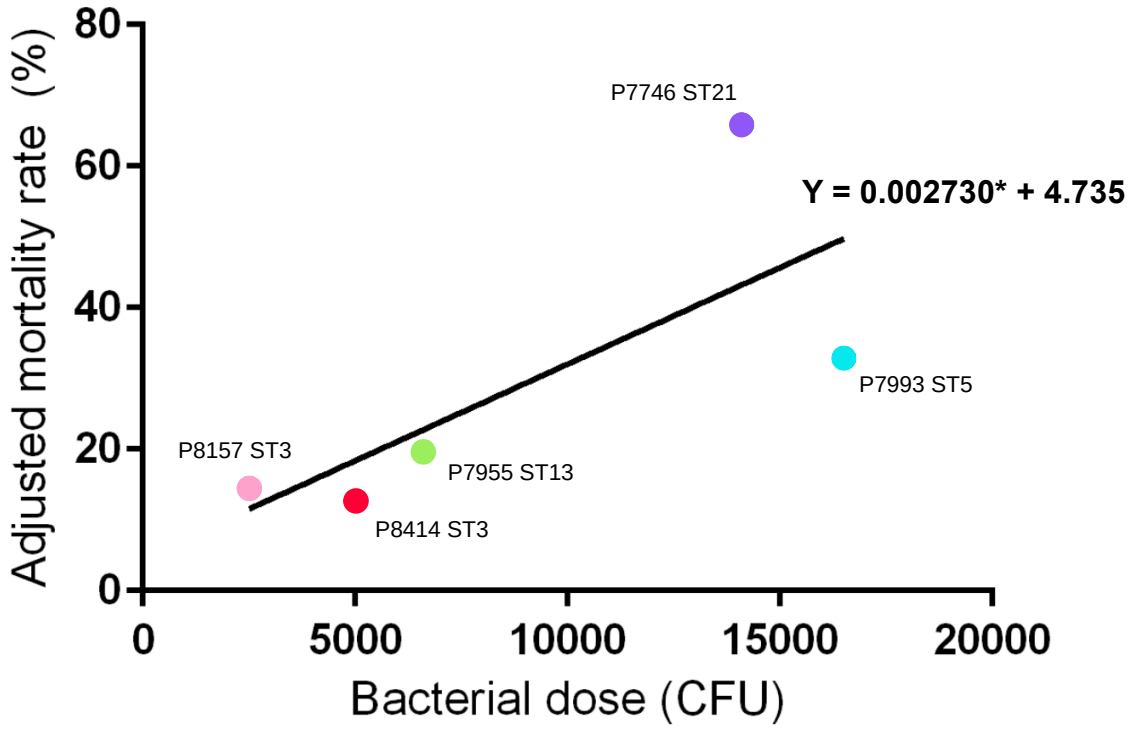

Figure 3.8. Linear regression of differing *M. plutonius* bacterial isolate dose administered on day 0 of experiment 3 compared against control adjusted experiment larval mortality percentage on day 15 post-infection.

Table 3.14. Average weights and standard deviation of seven honey bee larvae in each treatment group day 8 post-infection

Treatment	CC	Larval weight (mg)
Control	N/A	164.8 ± 17.1
P8157 ST3 toxin	3	151.2 ± 32.0
P8414 ST3	3	172.9 ± 21.3
P7993 ST5 toxin	3	168.2 ± 21.4
P7955 ST13 toxin	13	152.8 ± 34.9
P7746 ST21	12	81.1 ± 73.3

Larval weights ranged from an average of 81.1 mg to 172.9 mg on day 8 post infection (Table 3.14). Control group larvae had the lowest standard deviation from the mean, and ST21 isolates the highest, alongside the lowest average weight value.

3.6. Discussion

3.6.1. Context of study

EFB is a destructive and widespread honey bee disease, and a full understanding of the virulence differences of *M. plutonius* isolates is essential to facilitate effective treatment of infected hives. Previous larval studies have attempted to link genetic measures of relatedness, such as strain type or clonal complex, to EFB severity, either in the field (Budge, *et al.,* 2014) or during *in vitro* larval experiments (Arai *et al*., 2012; Nakamura *et al*., 2016; Lewkowski and Erler, 2019). Previous studies have linked the three clonal complexes to virulence: CC13 least, CC3 intermediate and CC12 highest virulence. The most recent study of virulence in larval infection models however did not find an association between strain type and clonal complex and virulence, and instead the presence of a specific putative gene 'melissotoxin A', related to increased mortality (Grossar, *et al*., 2020). If virulence predictions can be based on a gene, strain type or clonal complex of the isolate present in infection, future EFB outbreak isolates could be genotyped, or strain typed, and therefore categorised into clonal complex, which results in the severity of infection being reliably predicted. The most appropriate disease treatment could then be inferred from this information.

This study aimed to further explore the established relationship between ST and CC and how EFB infection progresses in an artificial honey bee larval model. Three experiments were carried out: firstly, to establish if the model was viable and look for preliminary patterns in toxin gene content and severity, secondly, to see if the above mentioned clonal complex categories of virulence always hold

true and finally, the experiments were designed to explore if a lack of virulence has an impact on the spread of specific strain types.

3.6.2. Experiment 1: pilot validation of experimental protocol

The pilot experiment was intended to test if *M. plutonius* UK outbreak isolates could artificially induce EFB infection in honey bee larvae in a new *in vitro* environment. Additionally, there have been previous issues with culturing and diluting *M. plutonius.* Overall, the pilot assay progressed as expected with *M. plutonius* observed microscopically in larval guts at day 7 (with control group remaining clear of infection). By the end of the experiment, the highest overall mortality was in both groups infected with ST5 CC3 (the isolates categorised as semi virulent) and the death curves of these two isolates were significantly different from the control group. The ST13 CC13 isolate (least virulent) was not significantly different from the control group (Figure 3.3.). Analysis of variance of the survival days of each individual larval until day 13 post-infection showed the effect of treatment had no significant effect on time of survival in the experiment suggesting all groups had similar overall mortality when compared. Initially, it appeared that the two isolates that possessed the toxin gene had slightly elevated rates of death day one post-infection, but this trend did not continue for the rest of the experiment (Figure 3.3.). Nonetheless, further exploration of the presence and absence of melissotoxin A was necessary, and the addition of more strains needed to test clonal complex predictions. As an additional observation, the overall mortality of the control larvae was higher in this pilot experiment, and this was likely to be a result of grafting injury due to initial unfamiliarity with the technique.

3.6.3. Experiment 2: Does the toxin gene always matter and does virulence vary within clonal complex?

The second experiment was intended to test if two isolates that belong to the same clonal complex type (CC3 and CC13) but differ in strain type within that clonal complex can vary in virulence level. A CC12 isolate was also included to represent all existing clonal complex types (Table 3.11.). Again, isolates varied in presence or absence of toxin gene. The ST21 CC12 isolate was equally as virulent as the CC3 ST2 and ST5 tested, despite being from different clonal complexes. Only one of these isolates possessed the toxin gene, so therefore would not seem to be the most important factor in larval mortality in this case. In fact, the ST5 CC3 isolate without melissotoxin A had the highest final mortality rate (~88%) in this experiment (Figure 3.5.). ST13 and ST20 both belonged to the least virulent CC13 type, but only ST20 group larval survival did not differ significantly from the control group (Figure 3.5.). Testing using clonal complex groupings (combining the data of ST2&ST5 and ST13&ST20 belonging to the same CC) showed that the groups with highest relative mortality CC3 and C12 did not significantly differ in overall days of larval survival within this experiment. However, distribution values of survival time for both CC3 and CC12 significantly differed from the CC13 group, and the CC13 group did not differ from the control.

Overall, this experiment suggests that the three-tier clonal complex grouping does not always reliably reflect virulence level, and that virulence can vary within a clonal complex. ST13 causing more mortality than ST20, despite both belonging to CC13. In this experiment the CC3 ST5 isolate caused the highest mortality overall when it may have been expected the CC12 ST21 would have

based on predictions from clonal complex type alone, and CC3 and CC12 exhibited statistically similar overall virulence within the survival comparisons of this experiment.

3.6.4. Experiment 3: Further exploration of clonal complex, ST, and virulence

The third experiment was intended to further explore the relationship between clonal complex and virulence, considering it appeared strains belonging to CC13 vary in virulence. ST3 isolates were also introduced, the most common strain type in the UK. The ST21 CC12 isolate was extremely virulent in this experiment, with only 5% of original larvae in the group surviving to pupation, despite not possessing the melissotoxin A gene which was extremely important in a previous larval infection study (Figure 3.7.) (Grossar, *et al.*, 2020). Consistent with Experiment 2, the ST13 CC13 (hypothesised least virulent) isolate treated group survival curve was significantly different from the control group (Figure 3.7.). The ST5 group was significantly different from both ST3 isolate treatment survival, despite all three isolates belonging to CC3, and having observed isolates within CC3 having >99.98% average nucleotide identity to each other in chapter 2 (Figure 2.1.). In contrast, both ST3 CC3 isolates did not kill significantly different numbers of individuals from the control group, which is inconsistent with the previous findings (Figure 3.7.) (Budge, *et al*., 2014; Nakamura, *et al.*, 2016; Lewkowski and Erler, 2019) from the clonal complex alone. However, in Grossar, *et al*., 2020, the single ST3 type tested originating from the UK was deemed ‘avirulent’ in this study.

3.6.5. Conclusions on using *M. plutonius* strains in laboratory based larval infections

Raising honey bee larvae successfully in the laboratory is technically complex to set up, with high mortality potential due to mechanical damage when grafting, or sub-optimal environmental conditions for growth and survival, causing stress. For these reasons, artificial larval infections may not directly model what may happen in a hive infection. Additionally, there are slight differences between mortality rates of same isolates used in different experiments (Table 3.15.) which may reflect the variation that can occur in an artificial model. Both ST5 and ST13 had comparable doses in experiment 1 and experiment 3, but the mortality on the same day of the experiment was lower in experiment 3, and this may be due to increased grafting practice. The ST21 isolate had a much higher rate of mortality in experiment 3, which may be expected as the dose given in this experiment was higher (Table 3.15).

Overall, individual strains sometimes matched what the clonal complex predicts (ST2 CC3, ST5 CC3, ST13 CC13), but in some cases (ST20 CC13, ST5 CC3 and ST3 CC3) they did not. In experiment 2, analysis of both CC3 and CC12 days survival of individual larvae suggested the two clonal complexes were as virulent as each other. This was also observed in experiment 3, however when CC3 was split further into ST3 and ST5, the groups had significant differences in larval days survival to each other, and the ST3 did not differ from the Control uninfected group. For this reason is useful to further discuss ST3 isolates specifically.

Table 3.15. Comparison of strains used in multiple experiments, including CFU/ml of bacteria administered on day 0 and mortality rate at day 13 post infection.

	Experiment 1		Experiment 2		Experiment 3	
Isolate	CFU/ml	Mort. % day 13	CFU/ml	Mort. % day 13	CFU/ml	Mort. % day 13
P7993 ST5 CC3	2.35×10^4	68			1.65×10^4	53
P7955 ST13 CC13	9.2×10^3	60			6.6×10^3	44
P7746 ST21 CC12			8.4×10^3	84	1.41×10^4	95
Control	**N/A**	**43**	**N/A**	**48**	**N/A**	**28**

3.6.6. ST3: The most common strain in the UK

ST3 isolates are the most common EFB strain type in the UK (Figure 3.9.) with up to 50% of outbreaks identified as this strain type each year. The reason why this strain type is more common is unclear, but it has a relatively good treatment rate of up to 70% success with destruction or shook swarm (Budge, unpublished data).

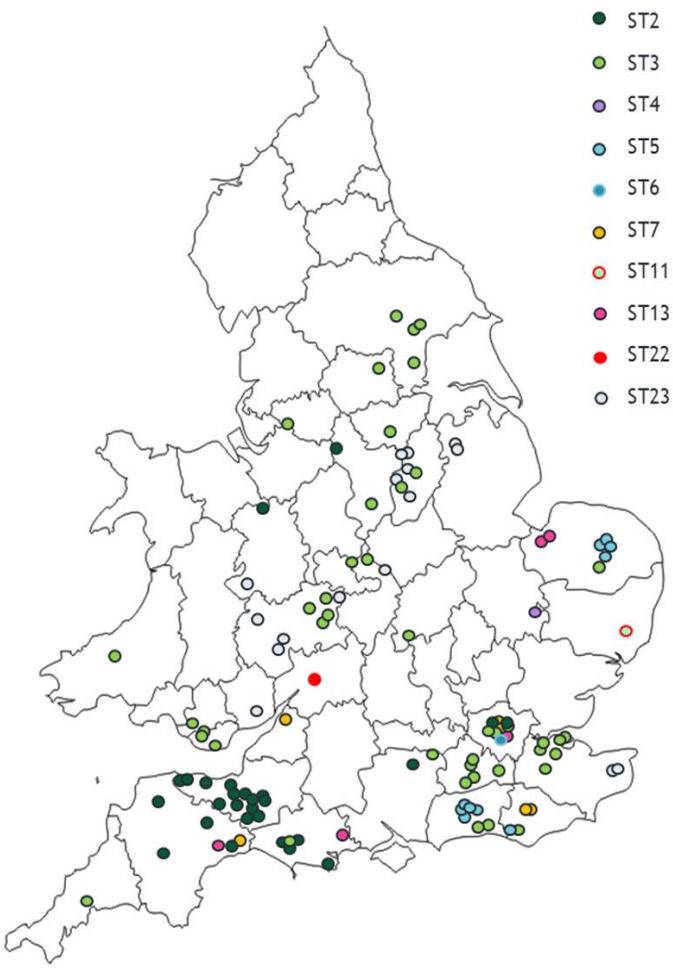

Figure 3.9. Map of the UK showing EFB outbreaks in 2018 (Tomkies & Budge, unpublished).

EFB outbreaks are identified by beekeepers and bee inspectors due to the presence visual symptoms of the disease. A greater number of diseased larvae will increase the likelihood of detections. In experiment 3, almost 50% of larvae infected with an ST3 strain survived and emerged as an adult bee i.e., half of those infected larvae showed little to no symptoms at all. In addition, these infected adult bees which survive to adulthood could also propagate infection. Faeces are an important vector of honey bee disease and have been found to play a part in infection, for example in *Nosema ceranae* (Fries, 2010). Both less

severe infection symptoms, and increased emergence occurrence of infected brood which then associate with young larva as part of their role, may have led to the widespread phylogeography of ST3 in the UK EFB outbreaks.

Another larval study (Grossar, *et al.,* 2020), the most recent artificial larval infection study of *M. plutonius*, used several ST3 isolates. It was also found that several ST3 isolates were avirulent, including an isolate of UK origin (Table 3.16.). This is further evidence that clonal complex type does not fully describe how virulent the infecting *M. plutonius* isolate may be.

Table 3.16. ST3 isolates tested in Grossar, *et al*., 2020, with origin, presence of toxin gene and virulence measured in artificial larval infection experiments.

Isolate code	**Origin**	**Melissotoxin A?**	**Virulence**
UK 36.1	Somerset, UK	No	Avirulent
CH MepS1	Graubünden, Switzerland	No	Avirulent
IT 1.3	Turn, Italy	No	Avirulent
CH 45.1	St. Gallen, Switzerland	No	Low-intermediate
NO 764-5B	Norway	No	Low-intermediate
NO 754-5B	Norway	No	Low-intermediate
CH 49.3	Graubünden, Switzerland	Yes	High

It is also interesting to note that previous field studies determined that many asymptomatic colonies present on diseased apiaries can contain *M. plutonius* (Budge, *et al*., 2010). When it comes to treatment of ST3 in the UK, where many infected individuals may survive to adulthood, it could be prudent to consider treatment of these contact colonies, in addition to those that show symptoms.

3.6.7. The importance of melissotoxin A to *M. plutonius* virulence

Other studies have identified 'melissotoxin A' as a potentially important gene in EFB infection. In Grossar *et al*., 2020, three isolates that were tested in artificial infection experiments in larva that possessed the melissotoxin A gene were found to be highly virulent. The gene, found on a plasmid, was hypothesised to be a good candidate putative marker gene that could inform control measures used in EFB infection (Grossar *et al*., 2020). In these experiments, the presence of melissotoxin A within the genome did not correlate to greater virulence than in strains where it is absent. It has been suggested than repeated sub-culturing of *M. plutonius* isolates in the lab may lead to a loss of the plasmid (Djukic *et al*., 2018) which contributes to the attenuation of the bacteria, however all strains used in this experiment were grown fresh from established strain collections from which the whole genome sequencing in Chapter 2 was achieved. The most virulent strain used in infection in this study, P7746, does not possess the toxin gene at all, and therefore it cannot be concluded that this gene was the only important factor in artificial larval infection studies in these specific experiments.

In experiment 1, for the first ~5 days post infection, the ST5 (toxin) and ST13 (toxin) have a higher mortality rate than the second ST5 (no toxin) and the control group (Figure 3.3.). However, this phenomenon is not observed in the second and third experiments. Theoretically, this gene may be important in some infections in early EFB disease, with high mortality rates of very young larvae before the gut microbiome has fully developed.

In the absence of transcriptomic data, it impossible to say if this gene was expressed at the phenotypic level, simply if the gene is present within the isolate genome, or not. It may be that melissotoxin A expression level can vary amongst strains that possess it or under different environmental conditions. An alternative insect pathogen *B. thuringiensis* (Raymond, *et al.*, 2010), has been shown to become increasingly virulent to the insect host, based on interaction with the gut microbiota. *B. thuringiensis ssp. galleriae*, a subspecies of *B. thuringiensis,* also had variable virulence when tested in wax moth larvae (*G. mellonella*) experiments (Agaisse, *et al*., 1999). Pathogenicity depended on both the presence of a specific toxin (*Cry*) and infectivity level of spores present, leading to either an enhancement or attenuation of strains based on environmental conditions rather than just toxin presence or absence (Agaisse, *et al*., 1999). There may be a similar effect in *M. plutonius* isolates causing the observed virulence variation when the melissotoxin A gene is present. Previous studies testing *M. plutonius* within a larval infection model did not include validation of bacterial expression of this gene (Lewkowski and Erler, 2019; Grossar, *et al*., 2020) but the addition of expression data of melissotoxin A in the future will enhance our understanding of this particular gene in relation to honey bee larval mortality.

3.6.8. Implications for real EFB infections

Ideally, EFB infection treatment and control could be guided by genetic analysis of *M. plutonius* on the whole genome level. However, this is not yet achievable and is beyond the scope of this chapter and thesis. Ideally, a specific set of genes would act as a marker to immediately inform an inspector that the infectious agent will cause severe disease or be easily transmitted between hives. Transcriptomic studies in the future will enhance the understanding of the progression of EFB within an individual larva, however they would not necessarily help with achieving this goal as it would be difficult to easily test not only if a particular gene was present within the genome, but also being expressed, on the time scale in which an inspector would need to decide how to proceed with an identified outbreak. Nonetheless, a complete understanding of *M. plutonius* as a bacterium, from infection to death, can only improve the EFB research field.

Diet composition was altered throughout the course of the experiment to ensure the optimum diet for worker rearing and the avoid the possibility of intercaste honey bees developing (Crailsheim, *et al.,* 2013). Additionally, a larger percentage of water was incorporated into diet A for feeding early larval stages, as smaller volumes were administered (Table 3.7.) and with an increased chance of the food drying out before it was able to be consumed (Schmehl *et al*., 2016). Altering of the diet in this way was also performed in an other larval infection study (Nakamura, *et al.*, 2016). However, it is not certain that the diet compositions used within laboratory rearing methods truly mimics the diet of larvae raised within a hive (Crailsheim, *et al.,* 2013), and further study into

optimal feeding regimes is needed to realise a full representative in vivo laboratory honey bee infection model.

It has been previously suggested that larvae may starve to death due to *M. plutonius* absorbing the food source or nutrients instead of the bee (Forsgren, 2010). In this experiment it did not appear that the larvae would have starved to death due to lack of food taken in, as there was uneaten food in cells during the larval experiments, with infected larvae often being much smaller than their control counterparts (Figure 3.10). Larval weight measurements (Table 3.14) showed many of the treatment groups had similar average weights to the control group (except for ST21), despite higher mortality rates in some groups relative to the control (Figure 3.7.). Further quantification and observational experiments could have been carried out to expand on this acknowledged growth rate variation, and this laboratory model could have improved overall understanding of how *M. plutonius* infected larvae develop in a hive once infected.

It is possible that the bacterium lining or filling the gut cavity, means the larvae either feel full or cannot further absorb any food given, or there is damage to tissues prevents absorption efficiency. By understanding the mechanism in which the larva is killed by the bacterial infection, we can more easily unpick the genes involved in pathogenesis, and vice versa.

Figure 3.10. Image from experiment 3 larval infection. Clear differences in size of larvae can be observed between control group and some larvae in the treatment groups (1-5).

3.6.9. Improvements to experimental design of larval rearing and EFB infection models

Although starting doses of *M. plutonius* did not significantly predict day 15 post-infection mortality percentages, there was still a positive relationship in two out of three experiments. It was observed by the author during culturing of *M. plutonius* that growth rate of different isolates varied, although this was not quantified experimentally. This variability was also observed in another study, where some bacterial isolates rapidly grew to high densities *in vitro*, with others growing much slower and never reaching the same final bacterial numbers within the specified time frame (Grossar, *et al*., 2020). It was however not found to influence relative virulence of the strains, with strains of high final density still shown to be of low virulence within larval infection experiments (Grossar, *et al*., 2020). Additionally, another larval infection study also found that their starting

doses administered of *M. plutonius* significantly differed within their experiment (Lewkowski & Erler, 2019).

It would therefore be useful for future work to develop an accurate protocol to quantify *M. plutonius* bacterial cell numbers within a liquid culture, to facilitate accurate and comparable dosage to be mixed into larval food. Microscopic methods, such as using a bacterial counting chamber, could have been employed to help with greater accuracy, although this would improve the overall estimate given on the day of infection, as growing up doses on agar plates post-infection to check viable cell colony numbers is a way to be sure the bacteria observed visually are still alive within the culture. Additional bacterial cell count measurements could have been taken from the larvae or adult bees post-mortem, to estimate bacterial loads of *M. plutonius* at later experimental time points. This type of end point validation would need to incorporate PCR methods to ensure colonies grown were the species of interest, as it is likely other gut microbiota would be able to also grow anaerobically using the M110 agar media.

The bacterial doses administered for the three experiments was at the magnitude of between 10^3-10^4 CFU of *M. plutonius,* quantified by culturing of the food inoculum upon agar plates. In other larval infection studies, administered doses around 10^5-10^6 CFU of *M. plutonius* to larva at the start of the experiment (Takamatsu, *et al.,* 2016; Lewkowski & Erler, 2019; Grossar, et al., 2020) or even higher at up to 10^7 (Nakamura *et al*., 2016). The doses therefore used within this thesis were on the lower end of the spectrum in comparison with other studies, although this was not intentional and instead an

artifact of the variable nature of *M. plutonius* growth *in vitro* detailed above. However, as shown by regression analysis, within experiment doses did not influence mortality percentage on day 13/15 post-infection, and therefore conclusions can still be drawn from the data generated here. It is important to compare *in vivo* experiments under differing conditions, for example performed in different laboratories, by different experimenters, with caution due to the nature of variability present in this type of assay.

It is highly desirable to keep any independent variables constant, such as starting bacterial inoculum, to both allow extrapolation to genuine EFB infections, and to move forward knowledge in a collaborative way by building on previous experimental results. It is therefore optimal to try and standardise microbiological methods of *M. plutonius*, and this should be a goal of researchers continuing work within this field. It would be ideal to be able to test statistically and draw conclusions on statistical significance of both ST and CC upon end point mortality rates for these experiments presented in this thesis.

Unfortunately, only a small number of isolates have been tested here, for example only one single isolate from CC12, one isolate from ST20 and with several isolates tested repeatedly. Additionally, replication of at least three, but preferably five, tests of the same strains in independent larval challenge experiments would allow statistical methods to be performed to analyse end point mortality rates for each treatment. As the experiment 1 pilot was halted earlier than the second and third experiments, there is unfortunately a lack of pupation data and emergence day for the pilot study.

For the reasons outlined, it is difficult to both analyse and make meaningful conclusions about ST and CC and virulence in the wider context, and instead we can only fully discuss *M. plutonius* isolate strain virulence and survival analysis results within this chapter in relation to the others also tested in that single experimental time course.

3.6.10. Further experiments to identify genes important in infection

These larval studies have identified that previous classifications of clonal complex and virulence may not be accurately predicting the virulence of all the isolates from within each group. The experiments performed indicate that it is possible for individual isolates from within the same clonal complex to cause differing mortality of honey bees using this larval bioassay within an artificial laboratory infection. However, no specific genes responsible for the observed mortality differences have been discovered within this thesis, and this is due to the lack of comparative WGS approach. The genomic presence of melissotoxin A gene did not seem to correlate with overall disease severity of any of the isolates tested in this study but has been found to be potentially important previously (Grossar *et al*., 2020). Individual proteins and genomic regions that may be important in infection in the gut environment have been tested using knock-out strains in larval experiments (Nakamura, *et al*., 2021). A similar approach could be taken by engineering melissotoxin A knockout strains, to see if the wild-type isolate has a higher mortality in *vivo* compared to the mutant strain, thereby confirming this specific gene does indeed cause higher death rates, albeit in artificial infection experiments.

Importantly, there is a high probability that the differential expression of specific genes that predict how virulent an isolate is, not merely presence or absence. Random larvae were frozen from all treatment groups at timepoints in experiment 2 and 3 and could be tested using qPCR to see if the isolates that were particularly virulent (e.g. ST21) show over-expression of any of the potential genes of interest (Appendix 3). An unbiased transcriptomic study, with the aim of identifying which genes are up regulated within honey bee larval tissues over the time course of an EFB infection, would be a conclusive and invaluable next step to understanding the pathogenesis of the bacterium *M. plutonius.*

3.6.11. Conclusions

Genomic studies identify the presence or absence of genes that may be important in infection, but these are putative and hypothetical. Recently it has been hypothesised the presence or absence of specific genes, for example the melissotoxin A gene, may be the key to understanding why some EFB outbreaks are deadly, and others are not. Several of the isolates tested in this chapter did not cause larval mortality at levels that may be predicted for their clonal complex type or possession of the toxin gene, although doses administered in these experiments were lower than in previous studies. An *M. plutonius* isolate ST21, belonging to the most virulent clonal complex type 12, was virulent in infections despite not having the melissotoxin A gene within the bacterial genome. The most common strain type of *M. plutonius* in the UK, ST3, was relatively avirulent when tested in the laboratory using this model despite belonging to CC3, an intermediately virulent clonal complex type. Testing isolates of *M. plutonius* in artificial larval infection experiments may illuminate

virulence differences both within and amongst clonal complex groups, the currently accepted predictor of EFB disease severity, however caution should be taken when making wider conclusions. Further gene expression studies are required alongside larval infection experiments to validate which putative genes are utilised by *M. plutonius* in real honey bee larval EFB infections.

4. Testing the susceptibility of *Melissococcus plutonius* strains to oxytetracycline

4.1. Abstract

European foulbrood (EFB) infection in a hive has three treatment options, shook swarm, antibiotics, and destruction. The antibiotic used for EFB treatment in the United Kingdom is oxytetracycline (OTC), and no specific *M. plutonius* resistance genotype has been identified thus far. Anecdotally, treatment success of EFB with OTC can vary by strain type present in the infection. Sixteen isolates of *M. plutonius* were tested for OTC resistance *in vitro* by using minimum inhibitory concentration (MIC) assays. Isolates had varying MICs among and within ST, and several isolates tested classified as resistant to OTC. All ST5 isolates tested exhibited some level of *in vitro* resistance. Whole genome sequencing also identified shared resistance genes that were universal in the isolates tested. Future large-scale screening of *M. plutonius* isolates for OTC resistance may help eliminate the use of ineffective antibiotic treatment in outbreaks, and therefore keep OTC as an alternative treatment type for susceptible infections in future.

4.2. Introduction

4.2.1. Tetracycline antibiotics: history, mode of action and applications of OTC

Tetracyclines were first discovered in the 1940s and were found to be effective against both Gram-positive and Gram-negative bacteria, as well as other micro-organisms and parasites (Nelson & Levy, 2011). Tetracyclines inhibit protein synthesis and therefore the growth and multiplication of the microorganism (Chopra & Roberts, 2001), rather than killing outright. *Streptomyces rimosus* bacteria naturally produce oxytetracycline (5-hydroxy-TC, OTC) (Zygmunt, 1961) and this biosynthesised product has both medical and veterinary applications.

OTC may be utilised in treating human conditions, including acne (Gibson *et al*., 1982) and wound infections (Carter, *et al*., 1966). The use of OTC is particularly useful if a patient is allergic to other widely used antibiotics such as penicillin, but preferential use has declined sharply in recent years due to increasing widespread tetracycline resistance of human pathogenic bacteria (Eliopoulos & Roberts, 2003). OTC is also used on livestock around the world, often under the Terramycin brand (Finlay, *et al*., 1950), both prophylactically and to treat specific disease in animals including cattle (Coetzee, *et al*., 2005), poultry (Jones & Ricke, 2003), fish (Kerry, *et al*., 1994) and honey bees (Mckee, *et al*., 2003).

4.2.2. Bacterial resistance to tetracyclines

Tetracycline antibiotics are utilised against a wide range of bacterial species due to their low toxicity, and it is therefore inevitable that resistance mechanisms have developed and propagated in bacterial populations (Levy, 1998). There are several mechanisms by which tetracycline resistance can be conferred, such as energy-dependent efflux pumps, the production of ribosomal protection proteins (RPPs) or enzymatic inactivation of the antibiotic (Grossman, 2016). Efflux resistance genes are often found on plasmids, particularly in the species *Streptococcus* (Poole, 2007). Additionally, attenuation of growth in response to tetracyclines has been observed in Gram-positive bacteria, whereby a reduced growth speed allowed bacterial populations to survive in the presence of antibiotics (Speer *et al*., 1992).

4.2.3. Testing resistance of bacteria to antibiotics: Minimum Inhibitory Concentration (MIC) assays

The minimum inhibitory concentration (MIC) of any chemical is the lowest concentration at which growth of a microorganism is prevented (Andrews, 2001). MICs are used widely in microbiology and in clinical settings to test for susceptibility to specific antibiotics, for example in hospitals to avoid treatment with unsuitable drugs. Isolates can be classified as susceptible, intermediate, or resistant and the specific antibiotic concentration determining these definitions are known as breakpoints. These breakpoints are implemented by the Clinical and Laboratory Standards Institute (CLSI) with guidelines to follow when performing MIC assays (CSLI, 2007). Breakpoints are dependent on both the bacteria (species or genus) and the antibiotic to be tested. The breakpoints for

Enterococcus species and Tetracycline are ≤4 susceptible, =8 intermediate and ≥16 resistant (CSLI, 2007).

4.2.4. Treating European foulbrood disease with OTC

OTC has been used as a treatment option for EFB since the 1960s in the UK, after it was shown to be effective in reducing hive infection (Katznelson, *et al*., 1952). It has also been used prophylactically in other countries such as the United States, despite the fact it was feared that widespread resistance may begin to emerge (Levy & Marshall, 2013). *Paenibacillus larvae* the bacterial cause of American foulbrood (AFB) has also shown plasmid-encoded resistance to OTC (Murray & Aronstein, 2006; Alippi, *et al*., 2007). More recently, this practise was halted in 2017 by amendments to legislation by the US Food and Drug Administration (FDA, 1996), giving reduced access to OTC by prohibiting over the counter access (FDA, 2017).

4.2.5. Previous studies of *M. plutonius* and OTC

More than 15 years ago, a study investigated OTC MICs of more than 50 UK *M. plutonius* isolates (Waite, *et al*., 2003b), but as only few isolates had a MIC value greater than 4 on average (Figure 4.1.), resistance to OTC was concluded to be absent.

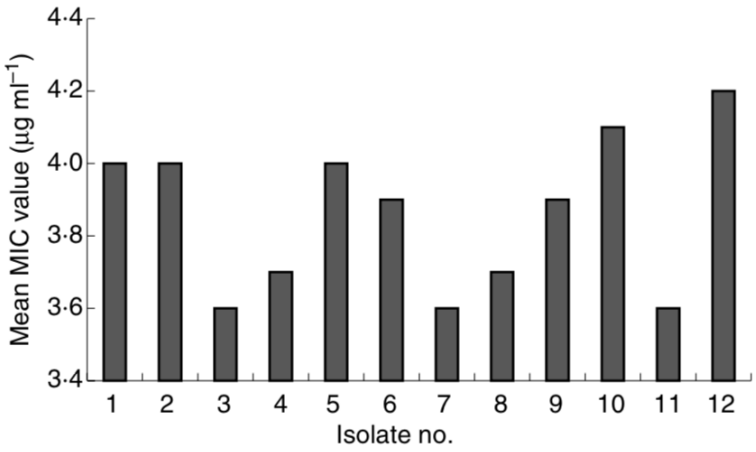

Figure 4.1. Mean minimum inhibitory concentration (MIC) values for selected isolates of *M. plutonius* (from Waite *et al*., 2003).

Further experiments during the early 2000s using larvae from OTC treated hives showed the concentration of antibiotic maintained within the gut environment was above the hypothesised MIC for *M. plutonius* (Mckee, *et al*., 2003), and therefore antibiotics have remained suitable for EFB treatment.

4. 2. 7. Improving knowledge of EFB treatment success with OTC

Since this preliminary antibiotic resistance study in 2003, the classification and characterisation of *M. plutonius* has vastly improved with MLST typing, as previously discussed in Chapter 1 (Haynes, *et al*., 2013). An analysis of treatment failure in apiaries containing ST3 or ST5, conducted in 2017 (Budge, unpublished), suggested that 92% of ST5 cases treated with OTC reoccurred (32/38), compared with only 43% of ST3 cases (33/58). This data could suggest that ST5 isolates may have higher disease reoccurrence when treated with OTC.

4.3. Aim of study

This study investigates whether OTC MICs significantly differ among *M. plutonius* isolates, and importantly if ST is a reliable predictor of bacterial antibiotic resistance. The ability to infer *M. plutonius* antibiotic susceptibility using genetic typing or genomic sequencing would greatly improve treatment success of EFB by removing the use of OTC in disease outbreaks where a resistant isolate is present.

4.4. Materials and Methods:

4.4.1. Selecting and culturing *M. plutonius* isolates

Sixteen isolates of varying ST and UK disease outbreak origin (year 2010-2012) were selected for resistance testing (Table 1) from the isolates sequenced previously (Chapter 2). The culturing method of *M. plutonius* is previously described (Chapter 2). Bacterial absorbance (OD600) readings were taken from isolate cultures after seven days and then *M. plutonius* cultures were diluted to comparable densities (approx. 0.1 OD_{600}) (Table 4.2.) to use in the experiment as suggested by the standard protocol. The standard MIC protocol suggests diluting bacteria to the McFarland standard of 0.5, which should have an OD600 value between about 0.08 nm and 0.1 nm. The cultures used to inoculate broth were diluted further and then spread onto solid M110 agar plates to verify that bacterial doses were comparable (Table 4.2.).

4.4.2. Identification of M. plutonius OTC resistance genes

All isolates in this study were annotated and then checked for the presence of known oxytetracycline and tetracycline resistance gene regions, as well as

other resistance type genes (Chapter 2). *M. plutonius* isolates were also screened against the Comprehensive Antibiotic Resistance Database (CARD) (https://card.mcmaster.ca/) using ResFinder (Bortolaia, *et al*. 2020) to verify any additional resistance genes that may be present in each isolate.

Table 4.1. Theoretical putative antibiotic resistance protein regions present in *M. plutonius* genomes.

RAST annotation of protein region	**Theoretical function**	**Locus tag in Reference ATCC35311**
Aggregation promotion factor	Increased biofilm formation	MPTP_1692 MPTP_1718
Beta-lactamase	Inactivation of beta-lactam antibiotics	MPTP_1048
Multidrug resistance efflux pump protein (PmrA)	Efflux pump	MPTP_1344
Regulatory protein, TetR	Tetracycline repressor protein	Not found
Transcriptional regulator, TetR family	Tetracycline repressor protein	MPTP_0694 MPTP_0793
VanS/VanR	Vancomycin resistance	Not found
Zn-dependent hydrolase (beta-lactamase superfamily)	Enzyme inactivation of beta-lactam antibiotics	MPTP_1308

4.4.3. Minimum inhibitory concentration determination

MICs were determined by a standard 2-fold serial broth microdilution method using the Clinical and Laboratory Standards Institute (CLSI) standard guidelines. Oxytetracycline (Sigma-Aldrich) was prepared first as a stock concentration (5 mg/ml) and then aliquoted to vials of M110 media at the appropriate concentrations.

Sterile 48 well plates were filled with liquid M110 media and oxytetracycline in the format shown (Figure 4.2), and *M. plutonius* isolates tested in triplicate. Experimental well plates were incubated at 37°C anaerobically for 10 days, then inspected for growth by both eye and by a spectrophotometer. MICs for isolates were determined by the lowest concentration of OTC in which *M. plutonius* growth was inhibited (CLSI standard protocol).

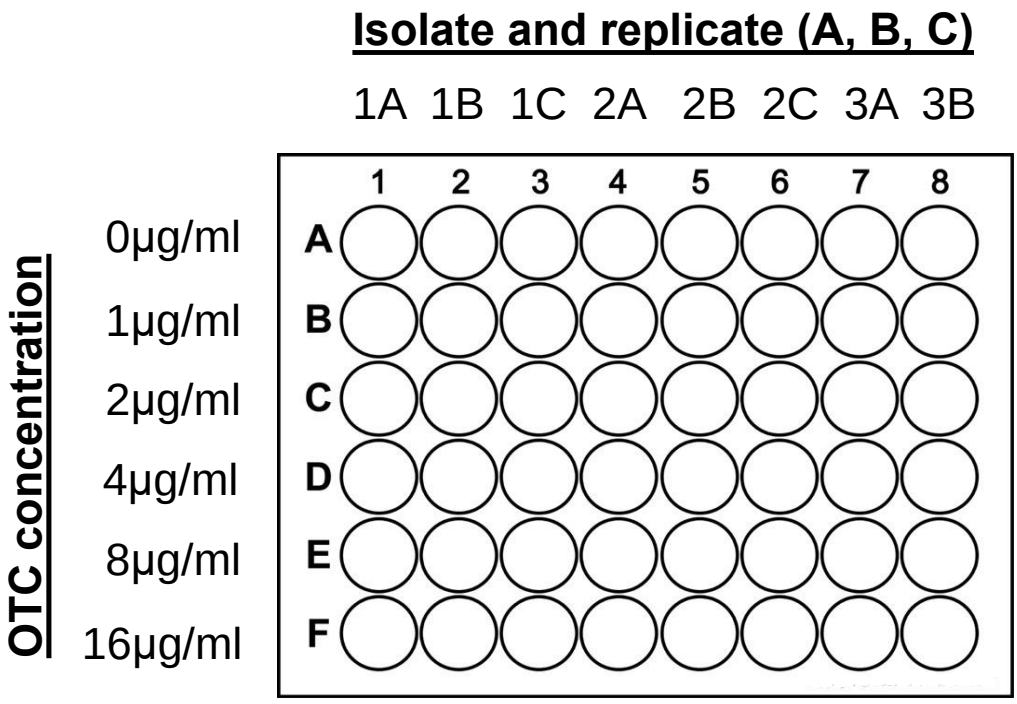

Figure 4.2. Example well plate structure used to test *M. plutonius* isolate OTC resistance

Table 4.2. Metadata of *M. plutonius* isolates used in MIC study with absorbance reading at day 7 and CFU/ml observed when grown on agar plates post-assay. *No data as diluted isolates did not grow on agar plates post-experiment to allow CFU quantification.

				OD_{600} of *M. plutonius* isolate		
ID	**ST**	**CC**	**Origin**	**7 days**	**After dilution**	**CFU/ml**
8176	2	3	Dorset	0.225	0.082	6.5×10^3
8282	2	3	Cornwall	0.724	0.112	4.8×10^4
8289	2	3	Somerset	0.224	0.081	3.7×10^4
8450	2	3	Oxfordshire	0.208	0.081	2.3×10^4
8284	3	3	Berkshire	1.36	0.078	No data*
8257	3	3	Suffolk	2.047	0.084	3.3×10^4
7606	5	3	Essex	1.827	0.085	1.6×10^4
7715	5	3	N. Yorkshire	3.553	0.113	1.7×10^4
7970	5	3	Suffolk	2.06	0.089	5.2×10^4
8325	5	3	Norfolk	2.647	0.093	3.8×10^4
7531	7	3	Devon	3.553	0.11	1.7×10^4
7928	7	3	Suffolk	1.94	0.093	1.9×10^4
7534	8	13	N. Yorkshire	2.353	0.098	5.4×10^4
7641	13	13	Norfolk	1.9	0.088	2.4×10^4
7955	13	13	Norfolk	1.96	0.09	4.8×10^4
7746	21	12	Mid Glamorgan	1.513	0.077	1.6×10^4

4.5. Results

4.5.1. Minimum inhibitory concentration of isolates

Isolates varied in their MIC values, both amongst and within ST (Figure 4.3.). All isolates had comparable starting bacterial doses (Table 4.2.), apart from P8284 (ST3), which did not grow post-experiment when plated onto M110 agar.

4.5.2. *M. plutonius* isolates susceptible to OTC

Seven isolates in total were susceptible to OTC in this study (MIC value ≤4). These isolates include one that CFU/ml info is unknown for, due to no growth on agar plates used for dose checking (Figure 4.3.). Both ST13 isolates tested were susceptible, showing no resistance to OTC. There are three ST2 isolates that fall into the susceptible category but one that is resistant (Figure 4.3.). ST7 has one susceptible isolate and one that is resistant, as does ST3.

4.5.3. *M. plutonius* isolates intermediately resistant to OTC

Three isolates tested were intermediately resistant to OTC (MIC value =8), two isolates ST5 and one ST21 (Figure 4.3.).

4.5.4. *M. plutonius* isolates resistant to OTC

Six isolates in total were resistant to OTC (MIC value = 16). These isolates belonged to 5 different strain types, 2, 3, 5, 7, and 8 (Figure 4.3.). Out of four ST5 isolates tested, two of them were resistant to OTC. No isolate had an MIC value >16.

			Average OD600 at OTC concentration (µg/ml)						Starting concentration*	
Protect	**ST**	**Origin**	**0**	**1**	**2**	**4**	**8**	**16**	**CFU/ml**	**OTC MIC**
8282	2	Cornwall	0.67	0.105					4.8×10^4	2
8450	2	Oxfordshire	0.93	0.388					2.3×10^4	2
7641	13	Norfolk	0.404	0.115					2.4×10^4	2
8176	2	Dorset	0.906	0.496	0.317				6.5×10^3	4
8284	3	Berkshire	0.919	0.813	0.659				No data	4
7928	7	Suffolk	1.297	0.177	0.169				1.9×10^4	4
7955	13	Norfolk	0.898	0.177	0.144				4.8×10^4	4
7606	5	Essex	0.276	0.342	0.323	0.331			1.6×10^4	8
7715	5	N. Yorkshire	1.608	0.8	0.704	0.45			1.7×10^4	8
7746	21	Mid Glamorgan	0.838	0.8	0.704	0.385			1.6×10^4	8
8289	2	Somerset	1	0.171	0.198	0.272	0.299		3.7×10^4	16
7970	5	Suffolk	1.086	0.266	0.246	0.279	0.13		5.2×10^4	16
7531	7	Devon	1.273	0.699	0.605	0.503	0.404		1.7×10^4	16
7534	8	N. Yorkshire	0.802	0.525	0.538	0.414	0.206		5.4×10^4	16
8325	5	Norfolk	0.699	0.395	0.442	0.386	0.384		3.8×10^4	16
8257	3	Suffolk	0.427	0.228	0.265	0.376	0.316		3.3×10^4	16

Figure 4.3. MIC values (lowest concentration of OTC in which *M. plutonius* growth was inhibited) of M. plutonius isolates, with detailed average bacterial density information. Average taken between the three replicates of the same antibiotic concentration. Grey cells had no bacterial growth (OD≤0.1(600)). *Starting concentration based upon plated bacterial counts from culture added to experimental wells.

4.6. Discussion

4.6.1. Context of study

An understanding of bacterial antibiotic resistance is very important to disease treatment and prevention. The last study of OTC resistance of *M. plutonius* was over 15 years ago, with no resistant isolates found (Waite, *et al*., 2003b). Antibiotic resistance can develop rapidly in bacteria (Holden, *et al*., 2004; Hermsen, 2012), particularly when used in agricultural environments (Heuer, *et al*., 2011). Limiting antibiotic usage in disease outbreaks where is likely to be ineffective is a major aim in the prevention of antimicrobial resistance development. In addition, antibiotic traces can commonly be found in honey (Bargańska, *et al*., 2011; Al-Waili, *et al*., 2012) and this can pose a problem for both individual beekeepers and the honey industry, as food industry legislations are strict. Eliminating OTC as an EFB treatment option in certain cases will further avoid this contamination issue.

4.6.2. MIC assay: are all *M. plutonius* isolates still susceptible to OTC?

This study did not identify specific genes to explain phenotypic differences in OTC resistance due to a lack of both comparative genomics or transcriptomic approaches, so it is still unclear what may be causing both the susceptibility variation amongst isolates when tested *in vitro*, and as observed in the initial metadata study of treatment success (Budge, unpublished), with ST5 isolates causing more persistent EFB infections when treated with antibiotics. All four ST5 isolates tested in this study showed some level of resistance to OTC (Figure 4.3). Other isolates showed high level of antibiotic resistance that belonged to sequence types 2, 3, 7, and 8. However, another ST3 isolate was still susceptible, suggesting that there may be variation on a ST level for some

bacterial isolates. These two ST3 isolates, differing in OTC resistance, separated phylogenetically in chapter 2 (Figure 2.2). Additionally, only one ST8 isolate was tested in this study so it is hard to extrapolate for this strain type. Overall, this study suggests that OTC may not be suitable for treatment in over half of cases of EFB (Figure 4.3.), and therefore the sharp decline in OTC use as a routine treatment in the UK (Figure 1.4.) is a wise decision.

4. 6. 3. Improvements to experimental design

Several improvements could have been made to this experimental assay. Unfortunately, one used in this study (ST3, Figure 4.3.) did not grow when trying to estimate the CFU/ml of isolates, and therefore the dose at the beginning of the experiment is not known. Nonetheless, this isolate was not resistant, but some growth did occur in the absence of antibiotics (Figure 4.3.) Additionally, using the McFarland standard 0.5, an average 0.1 OD600 value should correspond to approximately $1.5x10^8$ cells/ml (Franklin *et al*., 2012). For *M. plutonius*, this is not the case (Table 4.2) with and therefore a lower dilution factor would have been more appropriate in future for this specific bacterium, greater than 0.1 OD600 nm. Despite the higher dilution, it is likely the isolates would have been added to the antibiotic assay at a lower, rather than higher concentration than the standard MIC assay suggests, and therefore any antibiotic resistance shown is still valid.

A large shortcoming of this MIC experiment is the lack of isolates for some strain types. As previously mentioned, *M. plutonius* is often fastidious to grow, and the rate at which individual isolates reach high optical densities wildly varies (Table 4.2.). Because of this, it has been challenging to fully represent all strain

types and include multiple isolates of the same strain type for meaningful comparison. This could be overcome by isolating fresh *M. plutonius* isolates from newer EFB outbreaks to “fill in the gaps” of the NBU and Fera Science Ltd.’s *M. plutonius* Protect isolate collection.

Lastly, an importantly, both a fully comprehensive comparative study of the genes that may be important for antibiotic resistance in *M. plutonius* would be more appropriate than the methods used in this thesis to help to identify the key genes for the phenotypic differences shown amongst *M. plutonius* isolates (Figure. 4.3.). This could be further complemented by transcriptomic study, whereby the *M. plutonius* isolates were grown in the laboratory in the presence of OTC and genes showing higher and low gene expression determined. (Suzuki, *et al*., 2014). The approach taken here, looking for the presence of previously identified genes, would not be helpful if the key genes involved are novel or unannotated CDS regions but are in fact be the key to unpicking OTC resistance in *M. plutonius*. Isolates that are resistant have been identified, but the reason *why* these strains have lost susceptibility is completely unknown.

4. 6. 4. Antibiotics in the environment

Antibiotics are universally present in the environment (Larsson, 2014), both naturally in the soil produced by bacteria themselves and because of human applications, such as farming and agriculture (Van de Bogaard, *et al.,* 2001; Zhi, *et al*., 2013). Recent literature suggests that high antibiotic resistance levels can develop at these low background environmental levels (Gullberg, *et al*., 2011). This is largely due to small cumulative mutations in genes that are otherwise housekeeping genes, and therefore have little or no cost to the bacteria

(Wistrand, *et al*., 2018). These genes have also been shown to have an epistatic effect, with more antibiotic resistance conferred by additive multiple small mutations than would be expected by the presence of each individual mutation alone (Wistrand, *et al*., 2018).

Problematically, they are also difficult to identify using whole genome sequencing as they do not annotate as classic resistance genes. It may be that some of the *M. plutonius* isolates that show high persistence when treated with OTC have developed some resistance through this method. It is worth noting that a lot of ST5 isolates studied originate in either Norfolk or Suffolk, areas traditionally associated with an increase in farming activity. This may have increased the amount of excess antibiotics present in the soils and consequently, the exposure levels of crops and other plants in the environment where the bees forage. If it were possible to link resistance of *M. plutonius* isolates to historical excess antibiotic use in the area, this would inform predictions of where OTC resistance might develop in the future if similar farming practices were adopted in a new area.

4. 6. 5. Biofilm formation of *M. plutonius*

Biofilms are populations of microorganisms that are encapsulated by a secreted extracellular slime. These polysaccharide matrices protect the bacteria from substances such as antimicrobial compounds and attack from the host immune system (Dunne, 2002). Aggregation promoting factor genes are essential to the formation of biofilms, and the whole genome sequencing of *M. plutonius* has identified that isolates vary in copies of this gene (Chapter 2). Variation in bacterial ability to resist treatment by OTC may be affected by the presence or

absence of these genes. Microtiter dish biofilm formation assays (O'Toole, 2011), using crystal violet to stain fixed bacteria, may be useful to quantify the ability of *M. plutonius* isolates to form biofilms, and this would be a useful further study.

4. 6. 6. Phage therapy – an alternative to antibiotics?

The term 'phage therapy' refers to the use of bacteriophages, a form of virus, as a means of controlling pathogenic bacterial populations by infecting susceptible bacteria, and therefore reducing overall numbers (Lin *et al*., 2017). Phage therapy has already been explored in mouse models with the aim of treating several clinical bacterial infections, for example *P. aeruginosa* (Watanabe *et al*., 2007), *C. difficile* (Ramesh *et al*., 1999) and *E. coli* (Chibani-Chennoufi *et al*., 2004). Additionally, human trials have been undertaken in several institutes in Eastern Europe, for example the Eliava Institute in Poland (Międzybrodzki et al., 2021), although currently there are no approved phage therapy solutions for use in humans.

Outside of human medicine, there is significant phage therapy research being trialled in agriculture, known instead as 'biocontrol' treatments, to control foodborne pathogens such as *E. coli* and *Salmonella* (Goodridge & Bisha, 2011). A recent study of the potential of *P. larvae* bacteriophage as a biocontrol agent against AFB disease, showed protection for phage treated hives that were at risk of natural infection (Brady, et al., 2017). Using viruses seems intuitively dangerous, however as they are often species specific (Koskella & Meaden, 2013), they may be ideal for the purpose of controlling animal diseases such as EFB.

M. plutonius isolates contain several different types of bacteriophages within their genomes (Chapter 2), with isolates of the same strain type usually possessing the same phage (Table 2.4.). Further work is needed to firstly isolate these newly discovered phage types in culture, and secondly to test if isolated phage can infect and effectively lyse *M. plutonius* from differing strain types or clonal complexes. It would be particularly useful if one of the phage types present in the CC13 isolates, which cause significantly less disease in real EFB infections (Budge, *et al*., 2014; Lewkowski & Erler, 2019), could be used as a biocontrol for the more common CC3, or more severe CC12 isolate infections. If this can be achieved, and there are no mortality side effects to administering bacteriophage to honey bee larva in *in vivo* infections, phage therapy may be a viable option to control EFB infection.

4. 7. Conclusions

Metadata suggests that OTC is less effective in treating ST5 *M. plutonius* isolates, with more common disease reoccurrence in this strain type (Budge, unpublished). This OTC resistance phenotype was confirmed *in vitro*, using MIC assays, with all ST5 *M. plutonius* isolates tested possessing some resistance level. However, there were additional strain types that also showed high levels of resistance to OTC in *in vitro* infection (Figure 4.3.). A comparative genomics approach would be more appropriate than the methods used to identify candidate genes of interest, to better determine if the any antibiotic resistance genes found in *M. plutonius* are varied in sequence or expressed differentially. If MIC values vary by ST, it may be that other factors are facilitating *M. plutonius* oxytetracycline resistance in the UK, such as the increased expression of

biofilm forming genes, resistant gut microbiome communities and beehive proximity to industrial or farming use of antibiotics.

5. General Discussion

5.1. Context of this thesis

European foulbrood (EFB) is an important pathogen of honey bees, and is the most common bacterial infection found in managed hives globally (Forsgren, 2010). A thorough understanding of all aspects of EFB, and the causative bacteria *M. plutonius*, is essential to improve treatment strategy and disease control in the future. At the outset of this PhD project there were no comparative genomic studies of *M. plutonius*, despite reference strains published (Okumura, *et al*., 2011), with little understanding of the genetic basis of this bacterium outside multi-locus sequence typing (MLST) (Haynes, *et al.*, 2013). Additionally, very few larval infection studies have been published, largely due to the difficulty of assay, specifically the difficulty of rearing honey bee larvae successfully in a laboratory environment. Anecdotal evidence (Budge, unpublished) also suggested that oxytetracycline (OTC) treatment success may be lower when treating a specific strain type of *M. plutonius*, despite previous research showing no evidence for OTC resistance when strains were tested *in vitro*, and a reduction in this treatment type over recent years. Therefore, the main aims of this thesis were to: improve the genetic understanding of *M. plutonius*, using whole genome sequencing, explore the link between bacterial genotype and pathogenic phenotype in artificial honey bee larval infections and lastly, test the effectiveness of a treatment strategy of EFB, OTC.

5.2. Improving the genetic understanding of *M. plutonius* virulence using whole genome sequencing

At the start of this PhD project in 2016, knowledge of *M. plutonius* as an organism was relatively limited, particularly the genetics, with the first comparative genomics study only being published two years later (Djukic, *et al*., 2018). Comparative genomic studies of bacterial isolates within a species are important for several reasons. Firstly, it allows the differentiation of individual strains, in a way that differs from the MLST typing, used more widely for tracing movement of disease, rather than exploring and predicting disease severity. Secondly, it allows the identification of genes that are both ubiquitous and rare in a species, giving candidates to be further explored experimentally. And lastly, comparative genomics studies can give a foundation to quantitative experiments, such as transcriptomics or genetic editing, to show which genes definitively contribute to pathogenic virulence. There are also other specific comparative methods, such as genome-wide association studies (GWAS) studies can be used to examine how genes present in a group of organisms can relate to phenotypic differences observed (Sheppard, *et al*., 2013;) that are useful in large isolate datasets. Although sequencing a bacterial genome is now relatively easy to do, to fully utilise the nucleotide sequences generated requires access to more advanced bioinformatic tools and methods that use the Unix command-line, a skill that was not able to be pursued within this project's timescale and has therefore led to limitations in Chapter 2's methods and results.

However, the whole genome sequencing and analysis that was performed did find as expected from the previous comparative study (Djukic, *et al*., 2018), that *M. plutonius* isolates are indeed genetically very similar at the genome level

with a large amount of shared core genes (Figure 2.5., Table 2.7) and at least 99.08% Average Nucleotide Identity amongst all isolates (Figure 2.1.). Genetic regions coding for putative protein regions described in this paper (Djukic, *et al*., 2018) were also found here, and the phage sequence content of genomes was estimated (Table 2.5.). Presence of a putative gene, melissotoxin A, a toxin gene with a similarity to epilosin toxin gene (Djukic, *et al*., 2018; Grossar, *et al*., 2020), varied even amongst isolates of the same strain type (Table 2.4.). Phylogenetic analysis of core SNPs of some UK isolates resulted in ST3 isolates being split into two clades (Figure 2.2., Figure 2.3.), suggesting the current differentiation of isolates may lack sufficient depth. As mentioned above, a more comparative approach to identifying genes that of interest in an unbiased way would have strengthened any findings within this thesis.

There are also still some limitations to the microbiological portion of sequencing of *M. plutonius* isolates. Out of over 120 bacterial isolates cultured from the Fera Science Ltd. strain collection, only 46 were newly sequenced during this PhD. This was due to a multitude of reasons, including contamination detected in the strain collection when cultured, insufficient quantity or quality of DNA extracted, and several sequencing failures. If some *M. plutonius* isolates are hard to sample and culture under laboratory conditions, there may only be a biased sample of the *M. plutonius* genome diversity currently available to study. The media that *M. plutonius* grows in, M110, contains a supplement of potassium for growth (Forsgren, *et al*., 2013), with cysteine hydrochloride added immediately prior to inoculation of broth or pouring of agar plates. This is designed to inhibit the growth of other organisms, due to *M. plutonius* isolates growing relatively slowly. It may be that this growth method is not optimum for

growing all *M. plutonius* bacterial isolates in the lab. Additionally, isolates previously sequenced that would have been of interest to compare, for example ST21 isolates, could not be used in some aspects of analysis due to bioinformatic constraints (Seemann, *et al*.).

Overall, whole genome approaches are the future of *M. plutonius* research. Strain typing using MLST is extremely useful for differentiating *M. plutonius* isolates. However, the current typing scheme may not group isolates at the depth required when trying to establish which genes are important in EFB disease dynamics. Genetic sequencing and bioinformatics analysis is changing and improving rapidly, and therefore using a whole genome approach will be an improvement on the current MLST scheme which uses SNP differences in just four housekeeping genes (Maiden, *et al*, 2013).

5.3. Understanding the virulence of *M. plutonius* using larval infection models

Testing the virulence of *M. plutonius* strains using laboratory reared honey bee larvae revealed that clonal complex, previously used to categorise isolates into a virulence hierarchy (Budge, *et al*., 2014; Nakamura, *et al*., 2016; Lewkowski & Erler, 2019), may not always predict the resultant larval mortality rate in artificial infection. The same was the case for possession of the melissotoxin A gene, despite previous research finding the presence of this gene important for virulence (Grossar., *et al*., 2020).

Clonal complex 12 strains are known as a highly virulent type of *M. plutonius* isolates (Budge, *et al*., 2014, Nakamura, *et al*., 2016). This was seen in Chapter

3 when tested in larval infection models, with a 95% mortality rate of larvae in experiment 3 (Figure 3.7.). However, this strain type is extremely rare in the UK, with only two isolates (7746 and 7892, both ST21) belonging to CC12, that were available for sequencing from the Fera Science Ltd*. M. plutonius* strain collection. These isolates were also obtained from samples belonging to the same beekeeper in multiple years (Haynes, 2013). The single CC12 isolate (P7746) which was tested in larval infection did not have the melissotoxin A gene present as mentioned above but was still virulent regardless of this (Figure 3.5, Figure 3.7.). This suggests there may be alternative virulence genes and pathways important in EFB infection left to discover. Neither of the two most recent larval infection studies (Lewkowski and Erler, 2019; Grossar, *et al.,* 2020) tested any isolates belonging to CC12, only CC3 and CC13, as this type remains relatively rare.

During larval infection, isolates infected with ST21 showed a delayed development in relation to the control, despite having an excess of food available. Death from *M. plutonius* is more likely in younger, smaller larvae in natural EFB infection (Forsgren, 2010). This may be one reason that ST21 was so deadly when tested, despite not possessing the toxin gene, because of the reduction in larval development rate caused by this isolate. This has also been observed in AFB (Genersch, 2010a), caused by another bacteria *Paenibacillus larvae*, when an infected honey bee larvae infected survived to pupation against the odds but was significantly less developed after the same amount of time.

Using previous clonal complex virulence classifications, ST3 isolates, the most common type in the UK, should be intermediately virulent in larval infections (R

Budge, *et al*., 2014; Nakamura, *et al*., 2016; Lewkowski and Erler, 2019). In Grossar, *et al.,* 2020, seven ST3 isolates tested in larval infection assays had differing virulence based on melissotoxin A presence. Two of these ST3 isolates from the same origin, Graubünden, Switzerland, were tested and one was found to be avirulent and the other highly virulent (Table 3.16; Grossar, *et al.,* 2020). When tested in larval infection experiments (Chapter 3) two UK isolates were avirulent, with the mortality of both groups not significantly different from the control group (Figure 3.7.). This suggests high variability of how virulent ST3 *M. plutonius* strains are when tested in a larval infection model, and that the intermediate level of virulence currently assigned to CC3 isolates may be due in part due to being an average measure of polar extremes.

Artificial infection of laboratory reared honey bee larvae is one mode of testing the virulence of individual isolates. However, there are some weaknesses to this approach, other than just the weaknesses to the chapter in this thesis. Recently, it was found that the grafting of honey bee larvae negatively affects developmental times of brood (Vázquez & Farina, 2020), and therefore increases the chance of mortality, even if no disease is introduced. This may be one of the reasons for variable mortality for the same strains in different experiments (Table 3.15), and relatively high control mortality, particularly in experiment 1 (Figure 3.3): the pilot. Nevertheless, infecting whole colonies of honey bees with EFB is both impractical and unethical, so artificial rearing methods in the laboratory are likely to remain a method of practically exploring *M. plutonius* isolate virulence differences, particularly with the increased availability of theoretical genomic data to test empirically.

5.4. Using both genomic analyses and phenotypic experiments to improve the understanding of real EFB infections

Identifying putative genes that may be important in EFB infection is only the first step in understanding *M. plutonius* pathogenesis. The second step is to see if theoretical genes are important in EFB infection using larval infection models. The goal of combining these two approaches is to gain a better understanding of how an EFB infection may progress in real honey bee colony infections, based on both isolate genetic content and experimental infection data.

Chi-squared analysis of the relationship between treatment type (OTC, shook swarm or destruction) used at the time of the EFB outbreak, as a proxy for symptom severity, and whether the melissotoxin A gene was present in each strain genome was performed (Table 2.6.). The result of this analysis suggested that possession of the melissotoxin A gene was linked to attempted treatment rather than destruction of colonies. In the larval infection studies (Chapter 3), the isolate causing the highest mortality in experiment 2 (ST5 P7613, Figure 3.5.) was treated with OTC in the field (Table 3.11.). In experiment 3, one of the ST3 isolates (P8414) was destroyed in the field (Table 3.13.), despite having a low mortality rate in artificial larval infection (Figure 3.7.). Other studies of *P. larvae* have supported the hypothesis that bacterial isolates that are less virulent at the individual level are highly virulent at the colony level, and vice versa (Genersch, *et al*., 2005). This is due to the slowed removal of larvae that have lower doses or lesser disease symptoms, leading to increased spread within the hive. This could be one explanation for EFB outbreaks caused by *M.*

plutonius isolates, that are theoretically more virulent, being treated rather than destroyed, and vice versa, as diseased larvae are quickly removed from the hive by worker bees, and therefore the colony does not look as severely affected to the bee inspector. This mechanism could also account for the widespread phylogeography of ST3 *M. plutonius* strains in the UK, both of which were avirulent in larval infection, and the rarity of isolates belonging to CC12 which caused great mortality.

However, using honey bee colony infection data, in this case the treatment used upon the EFB outbreak at the time, has limitations. Treatment choice is decided by the bee inspector, and may therefore be subjective, dependent on individual assessment of disease severity. Additionally, small colonies will be destroyed routinely regardless of hive symptom severity due to the extremely small chance of survival from EFB. These examples, using both genomic features and larval infection data, support either a hypothesis that *M. plutonius* isolates that are particularly virulent at the individual level are less so at the colony level, and vice versa, or that bee inspector treatment choice is too subjective to meaningfully use as a proxy for EFB severity.

5.5. Antibiotic resistance of *M. plutonius* isolates

Metadata (Budge, unpublished) suggests that ST5 isolates treated with OTC have either a high treatment failure or high possibility of EFB reoccurrence. The only published study (Mckee, *et al*., 2003) found no evidence of OTC resistance of *M. plutonius*, but this predates strain typing methods (Haynes, *et al.,* 2013) and was undertaken around 20 years ago. Genes present in strains were identical despite some *M. plutonius* isolates in this thesis study being shown as

resistant to OTC (MIC=16). This is despite OTC falling greatly out of favour as a treatment type in the UK by bee inspectors (K. Stainton, pers. comm.) If OTC is rarely used now, it may not be considered of high importance that resistance is emerging in multiple *M. plutonius* isolates. However, OTC is still widely used in other countries, including the US (Raybroeck, *et al*., 2012). With the global movement of bee products, such as honey and beeswax, and honey bee queens themselves, it is proactive to understand the fundamental antibiotic resistance that may exist in any bacterial species. Additionally, if shook swarm success ever dwindles in the UK, OTC is the only viable alternative to destruction at present. A previous study showed a combination treatment is relatively successful in the field (Waite, *et al*., 2003a), so having this option remaining would be preferable.

5.6. Next steps for EFB control and treatment

EFB outbreaks have increased in the UK the past five years (Figure 5.1.), from 426 colonies affected in 2016, to 841 colonies in 2021 thus far (https://www.nationalbeeunit.com/). It would therefore be sensible to focus on reducing the transmission of EFB if possible. When tested in larval infection experiments, two ST3 isolates were avirulent, and many infected larvae survived to emerge as adult bees (Table 3.12.). A previous study of EFB in the UK showed that *M. plutonius* can be present in asymptomatic hives (Budge, *et al*., 2010). Adult bees spread disease horizontally, via swarming and robbing honey from other hives (Forsgren, 2010), particularly hives which may be weaker due to pathogenic infection or infestation. Adult bees also spread disease vertically in the hive, passing disease to larvae via food and faecal matter (Forsgren, 2010). It is still unclear if the honey bee larvae that survive

infection with EFB still contain viable bacterial cells. If this is the case, it could be more problematic for the colony when compared to larvae quickly dying or being ejected from the hive due to a severe *M. plutonius* infection.

Shook swarm, the preferred treatment type in the UK alongside destruction, relies on reducing the bacterial load of a hive by moving the adults to new frames and destroying the *M. plutonius* infected larvae. If adults that survive from ST3 infection also harbour *M. plutonius*, for example as part of their microbiota, then this method of treatment is unlikely to work consistently as a new brood will quickly become reinfected by asymptomatic adult bee carriers. This would mean that having a less virulent strain is worse for the colony, and we might consider monitoring closely any hives where ST3 isolates are present. This is particularly prudent considering ST3 strains can clearly transmit well, due to becoming the most common in the UK (Figure 3.9.). EFB strains are routinely typed at Fera Science Ltd. so facilitating the whole genome sequencing ST3 isolates from outbreaks would be relatively simple.

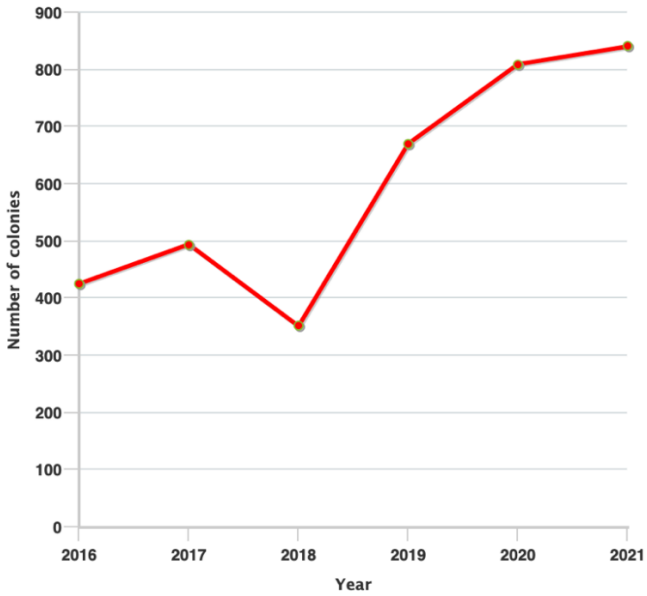

Figure 5.1. Graph of number of EFB outbreaks in colonies in the UK in the past 5 years, and during the first half of 2021 (https://www.nationalbeeunit.com/)

Strain typing of isolates using MLST is an extremely useful tool for tracing disease outbreaks of relatively rare isolates. However, in the UK, some strain types (2, 3, 5) are particularly common and therefore typing is less informative in this case. This is because geographically close cases of the same ST could be either be linked, or instead possess the same strain type just by chance because of the relative abundance of this strain type. Core-genome MLST (cg-MLST) relies on the same principle of MLST, using SNPs to differentiate isolates, but instead uses many genes from the whole bacterial genome. The availability of more than 50 fully sequenced *M. plutonius* isolates from this thesis would allow the creation of a cg-MLST for this bacterium, and therefore give higher resolution of the transmission routes of EFB throughout the UK, and worldwide.

5.7. The future of EFB research

M. plutonius studies have focused on comparative genomics (Djukic, et el., 2018), field studies (Budge, *et al*., 2014) or larval infection studies (Nakamura, *et al.,* 2016; Lewkowski & Erler, 2018; Grossar, *et al,* 2020). Gene expression studies are an important way to discover which genes are being used by a pathogenic organism at different infection time points. Using RT-qPCR methods, which sets of genes are both up regulated and down regulated by bacteria can be studied (Rocha, *et al*., 2020). This would be relevant for studies of *M. plutonius* whilst inside the honey bee larvae, and would potentially indicate a definitive mechanism by which it kills its host, or instead assimilates in the gut microbiota for less virulent strains. It is also important to look at infection in time stages, as different genes may be important at different levels of the larval development. Some primers were designed towards the end of this

project for the expression testing candidate regions of interest on larvae from differing time points of all experiments. One study has already included measuring the expression of melissotoxin in larval tissue (Djukic, *et al*., 2018) and found it to be expressed in *vivo*.

Alternatively, genetic manipulation of *M. plutonius*, through modification and inactivation of genes of theorised importance in pathogenesis, may allow establishment of a functional genomics pipeline. By using a genetic knock-out method (Doudna & Charpentier, 2014) and creating mutant *M. plutonius* strains that lack functional candidate virulence genes, direct gene effect upon larval mortality can be tested simultaneously against the unattenuated strain in larval infection experiments. This type of knock-out approach has already been successfully employed (as mentioned earlier in this thesis) into a study into peritrophic matrix-degrading proteins of *M. plutonius*, whereby the proteins were found to not influence the ability for strains to be virulent in larval infection studies (Nakamura, *et al*., 2021). However, the research in this thesis suggests the possibility that there are multiple types of genes that are important in EFB pathogenesis, not just melissotoxin A for example, so we may be unlikely to find a ‘silver bullet’ for halting or tracking the variable virulence of *M. plutonius* isolates within the field outbreaks of EFB. It would however make sense for the next step to be manipulating the expression of this toxin, as it was found previously to previously correlate with virulence in vivo (Grossar, *et al.,* 2020).

Nanopore sequencing is a rapidly emerging sequencing technology (Kono & Arakawa), which allows DNA or RNA to be sequenced independent of PCR or culturing methods. A portable field kit has been developed

(https://store.nanoporetech.com/uk/field-sequencing-kit.html) that allows relatively low-cost reactions to be performed in even remote locations (Gowers, *et al*., 2019). EFB diagnosis confirmation from field to lab may take days, but on-site testing for infection would shorten time to treatment and therefore risk of further onward EFB transmission. It would likely require dedicated field scientists to carry out the reactions, but the National Bee Unit could potentially recruit technicians to work alongside bee inspectors to achieve this. The sequencing data generated by the MinION can also be analysed at another central location. This technology has already been used on a mosquito species, *Culex cedecei*, to look for the presence, and if found, strain type of Venezuelan Equine Encephalitis Virus that the insect harbours (Russell, *et al*., 2018). At present, at around £500 for six reactions, it would be unlikely to be financially viable to introduce UK-wise. However, in the next 10 years this cost is likely to fall as it has for Illumina sequencing (Loman, *et al*., 2012). In addition, considering the recent increase in EFB cases in the UK, it may become financially viable considering the economic cost of honey bee loss (Gallai, *et al.*, 2009), if this upward trend in disease outbreaks continues. If we can ascertain the strain type of *M. plutonius* or whether a virulence gene is present in a colony in real time, this could rapidly inform inspectors of the scale of risk the EFB infection poses and allow destruction to occur more rapidly where necessary to contain the disease.

Finally, the discovery of different bacteriophage types in *M. plutonius* needs further research. In the UK it is very rare to encounter more than one ST of *M. plutonius* in the same hive or colony (G. Budge, pers. comm.), and it could be that this single strain type dominance is mediated by phage presence. This has been observed in hospital environments, where bacteriophage abundance has

allowed specific clinical strains of MRSA to flourish (Cox, *et al*., 1995). It also gives the potential for phage therapy to be an effective treatment if there is a virulence hierarchy of specific phage types (Wagner & Waldor, 2002), as can be observed in many other bacteria, including *Pseudomonas aeruginosa* (Inglis *et al*., 2009), *Salmonella* (Figueroa-Bossi, *et al*., 2001) and as previously discussed, *P. larvae* (Beims, *et al*., 2015). As of now, there are no published studies on elucidating phage from *M. plutonius* in the lab, although it was attempted at Fera Science Ltd. around a decade ago with no success.

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6. Thesis conclusions

There are many questions left to answer about the genetics and virulence of EFB. This thesis has explored aspects of the causative bacteria *M. plutonius.* This includes exploring the bacterial genes and features using whole genome sequencing, the virulence levels of several isolates in artificial larval infections and the effectiveness of OTC at limiting growth *in vitro*. Current classifications of isolates, using MLST typing, does not seem to categorise isolates at sufficient depth, and therefore linked clonal complex-based classifications of virulence may need to be adapted in the future. *M. plutonius* virulence should be explored at both the individual level and the colony level. Treatment success of EFB outbreaks should continue to be monitored closely in the future, and the development of a cg-MLST scheme would improve the tracing of less deadly, but more transmissible strains, such as the common ST3 in the UK. Further methods such as transcriptomic based experiments to identify differentially expressed genes and creating knockout isolates to use in larval infection experiments are key areas of *M. plutonius* research. A comprehensive understanding of the disease European foulbrood is a key step towards improving the collective health of the honey bee in the future.

7. Appendices

7.1. Appendix 1: Table of annotations of putative genome regions of interest encoding proteins found in *M. plutonius* genomes using RAST (Aziz, *et al*., 2008).

Genomic region annotation by RAST
ABC-type antimicrobial peptide transport system
ABC-type multidrug/protein/lipid transport system
Aggregation promoting factor (1) APF
Aggregation promoting factor (2) APF
Aggregation promoting factor (3) APF
Aggregation promoting factor (4) APF
Aggregation promoting factor (42) APF
Arginine-ornithine antiporter (ArcD)
Bacitracin export permease protein BceB
Beta-lactamase
Beta-lactamase C and other penicillin binding proteins
Cell division protein FtsI (peptidoglycan syntetase)
Cell wall surface anchor family protein
Cell wall-binding protein
Chitin binding protein, CBP
Chloramphenicol acetyltransferase
Chromosome (plasmid) partitioning protein parA
Colicin V production
Drug resistance transporter EmrB/QacA
Ethidium bromide-methyl viologen resistance protein EmrE
Fibrinogen/Fibronectin binding protein
Lantibiotic ABC transporter
Lantibiotic transport ATP-binding protein spaF/mutF
Lipid A export ATP-binding permease protein MsbA
Metal-dependent hydrolases or the beta-lactamase superfamily
Methionine ABC transporter ATP-binding protein
Mobile element protein
Mosquitocidal toxin
Multidrug resistance efflux pump PmrA
Multimodular transpeptidase-transglycosylase
Predicted cell-wall-anchored protein SasA
Putative toxin component near putative ESAT-related proteins
Putative superinfection immunity protein
Regulatory protein TetR
S1 RNA binding domain
Secreted Endo-beta-N-acetylglucosaminidase (EndoS)
Transcriptional regulator, TetR
Transcriptiopnal regulator of fatty acid biosynthesis FabT
Transcriptiopnal regulator of rhamnose utilization, AraC
Vancomycin response regulator VanR
Zn-dependent hydrolase (beta-lactamase superfamily)

7.2. Appendix 2: Table of genome assembly metrics generated by Nullarbor (Seemann, *et al*.) used in section 2.4.10. Further bioinformatics analysis.

Study ID	Isolate identifier	ST	Assembled size (bp)	Contigs	N50	Comments
1	P7707	3	2064845	18	292401	
3	P7821	3	2063684	9	405950	
5	P8157	3	2060634	29	406377	
10	P8261	3	2065320	13	325941	
11	P8279	3	2062412	29	292515	
13	P8284	3	2064210	16	204915	
15	P8348	3	2065880	11	405814	
17	P7715	5	2048161	19	209015	
19	P7917	5	**3925669**	**1419**	47480	3.38% *S. aureus*
20	P7970	5	2065299	14	325941	
21	P7810	5	2064892	10	405882	
22	P8234	5	2065524	26	325940	
23	P8322	5	2046861	10	325940	
28	P8325	5	2047379	12	291789	
29	P8107	5	**4386252**	**627**	16494	4.96% *S. epidermis*
30	P8257	5	2064990	9	405858	
36	P8414	3	2045289	26	200911	
38	P8185	3	**2117387**	**159**	154295	1.56% *S. pyogenes*
46	P8289	2	2066525	13	325939	
47	P8456	2	2065072	12	325940	
48	P7516	3	2046476	9	404635	
49	P7523	3	2047536	9	404665	
51	P7915	3	2062281	21	292233	
53	P8527	3	2064103	15	405971	
55	P7606	5	2046990	8	404123	
58	P7595	6	2046827	9	404222	
60	P7511	7	2003771	13	325940	
62	P7531	7	2021915	15	404735	
63	P7928	7	2021486	21	325941	
64	P8265	7	2021556	12	325940	
66	P7534	8	2039581	10	440992	
67	P7604	8	2036743	11	328537	
71	P7641	13	2077592	13	326222	
73	P7611	11	2056572	21	326193	
75	P7935	11	2054787	29	325940	
81	P8423	13	2075093	34	170399	
84	P8251	22	2069773	19	325940	
85	P8371	22	2059758	20	204913	
86	P8115	23	2063623	27	325940	
90	P8518	23	2068760	18	326195	
91	P8081	7	2023457	14	404646	
106	P7993	5	2065169	12	325940	
113	P7613	5	2045345	12	325940	
116	P8070	5	2056484	16	292402	
118	P8305	5	2063648	13	325940	
119	P7596	13	2095397	24	292697	

7.3. Appendix 3: Table of primers designed for specific *M. plutonius* gene regions of interest to test levels of expression (candidate gene region of interest = red, housekeeping genes = black)

Gene	F	Sequence (5->3)	R	Sequence (rev complement) 5'->3'	Size (bp)
1) Aggregation promoting factor 4	AGF1	GATTCAGCTGTAGCTACACC	AGR1	GGCGCACTTGTTTGAGCTG	100
	AGF2	AAGCACCAGCTCAAACAAGTG	AGR2	GATTCTCTTTGTGCAATCCATTC	70
	AGF3	GGGAAATACATAGGTCGATATC	AGR3	CTTGGTTTGCAGGTGAATAATC	70
2) Chitin binding protein	CBF1	GCTGGTAATTATGAAGTGTCTG	CBR1	CTGACCAACCAAAGCCATTG	90
	CBF2	CACAAGTTCATAAGACAAATCGC	CBR2	GGATAGCCATCTACACCAATG	110
	CBF3	GACTTTGGAAGATATGGATGTC	CBR3	GGAACAGTGATTTTATGAACAGG	90
3) ESAT-related toxin	ETF1	CCTAATGTAACCTATCAGACAG	ETR1	ATGAGGTAGACGATCGGAAC	120
	ETF2	GGAGGTTCACCACGCTTG	ETR2	CCCAGGTGTTCTCAAGCTG	80
	ETF3	TTGTTTCACAGGCATCACATG	ETR3	CTGTAAAAGCTGTAGGTCTTG	120
4) Fibronectin/fibrinogen-binding protein	FPF1	ACAACTTTCTCTAGCTGATCC	FPR1	CATTCAATCGAAAGGCCAATTC	100
	FPF2	TTATGCAGCAAATACCTAGAGG	FPR2	CTGATTTGGAGAAAGTGCAGG	100
	FPF3	GGAATCTGTTCTCGCTCAAC	FPR3	CTTGTTCAAGCAATTCCTCAC	80
5) Mosquitocidal toxin	MTXF1	AGATGGTACTCCTAATACCTC	MTXR1	CTTGCAGTATTATATGTTTGGTC	120
	MTXF2	ATTGATGGAAATGAAGTGACAGG	MTXR2	TGCCGGAACTTTAACAGGTTG	120
	MTXF3	ACCTGTTAAAGTTCCGGCAC	MTXR3	TGTTCTAGAGAAACAGTCACC	60
6) Siderophore production	SPF1	ATGAGAACACTACAAGTTGAGG	SPR1	TGAAGTTTGACGACCTCTGC	140
	SPF2	AGGTGGTTTATGTGTGCTGG	SPR2	CTTGATCCCACCATAAAGAAG	90
	SPF3	GTCCTTCTTTATGGTGGGATC	SPR3	CCAAAGAGCCAACAGATAAATG	100
7) T1SS	TSF1	CACGCTGGACGTCAGATG	TSR1	GTTGATGCTTCGGTTATAACAAG	80
	TSF2	TGATGGCTTAGGACTTGTGG	TSR2	GTTAAAGCTTGGACAGCAGC	70
	TSF3	ACCAGGAGATGCCTTACAAG	TSR3	CCGTCGGATCTGTTGGTG	90
8) Predicted tyrosine transporter	TTF1	GGTAGCTTCTTGGATCTTGTC	TTR1	CACCAACAATAGCACATACAAG	120
	TTF2	ATGCTATGACTGCTGGTGC	TTR2	GTTGTGTCAGACAACGGTG	70
	TTF3	CATTAAACGAATTCCGGCTATC	TTR3	CGTTCATCAATGTATCAGCTAC	120
A) ffh	FHF1	TCGTCAGGGAATAGCTCTAG	FHR1	GACGTTTGCATATTGATGAAGC	80
	FHF2	TGCCATGACAGGACAAGATG	FHR2	CACTCCTGTAATGCCTAATTG	70
	FHF3	GCGAGAAGCTAACAGACTTAG	FHR3	CAATTAAAGTCAGCATGTCACC	90
B) proC	PRF1	GAAGATTGCCAGATGATCATTC	PRR1	AGCAGCTACCGAAAGAATTGG	120
	PRF2	TAGCAGGCTGTGGACCAG	PRR2	CCCTTCATCATTTGAGCTGC	120
	PRF3	TGCAGCTCAAATGATGAAGG	PRR3	CTTTCAATTCACCGGGATGC	70
C) recA	RCF1	TTGGTATCTAGTGGTGCAATTG	RCR1	GGCGCCCATTTCTCCATC	90
	RCF2	TATGCAACAGTTCGTTTGGAAG	RCR2	GTACGATTACCGACAATATC	70
	RCF3	GCGCATGGTATAGTTACAATG	RCR3	ATACCAAAGGCCTTACGCAC	120

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