Investigating the epidemiology and infection of Chronic bee paralysis virus in European honey bees (*Apis mellifera*)

Theodora Maria Commandeur

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Abstract

Chronic bee paralysis (CBP) is an emerging viral bee disease affecting honey bee (*Apis mellifera*) colonies globally. Until recently, the disease was rare but severe, often leading to colony collapse. However, case numbers are increasing and to prevent further spread it is vital to understand the transmission and infection of the causative agent Chronic bee paralysis virus (CBPV). Several factors mediate viral infection. Laboratory experiments were used to investigate age and gut microbiota following oral exposure to CBPV. Interestingly, the age of bees at the time of exposure impacted susceptibility, with younger bees proving more susceptible to symptomatic infection. DNA sequencing was used to identify the gut microbiota present in symptomatic and asymptomatic cohorts. Variation in susceptibility to CBPV was investigated through comparison of naturally emerged bees and artificially emerged bees as cohorts with differential gut colonisation. The effect of access to beebread post-emergence and prior to oral inoculation of CBPV was also tested to give an indication of the impact of nutrition on susceptibility to infection by CBPV. These data, along with honey bee longevity, were examined using statistical modelling techniques, including survival analysis, competing risks analysis and structural equation modelling to provide a clearer picture of virus infection and transmission routes. Finally, cases of CBP were linked to weather data and mapped across vegetation land cover maps to identify landscape drivers of disease such as forage availability and host density.

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Declaration

The National Bee Unit collected the BeeBase data used in Chapter 5 as part of their colony inspections. In addition, all weather data was collected by the Met Office and vegetation data was collected by the UK Centre for Ecology and Hydrology.

In Chapters 2, 3 and 4, virus crude extract was prepared by Dr Nicola Simcock. In Chapter 2, gut homogenate was prepared by Prof. Giles Budge. In Chapters 2 and 3, lysis buffer was made by Dr Nicola Simcock.

All other work was performed by Theodora Commandeur.

"All models are wrong, but some are useful." - George Box

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List of Acronyms

Organisations & Data

APHA - Animal and Plant Health Agency

LCM - Land cover map

NBU - National Bee Unit

UKCEH - UK Centre for Ecology and Hydrology

Diseases

AFB - American foulbrood

CBP - Chronic bee paralysis

CBPSV - Chronic bee paralysis satellite virus

CBPV - Chronic bee paralysis virus

DWV - Deformed wing virus

EFB - European foulbrood

EID - Emerging infectious disease

IAPV - Israeli acute paralysis virus

Honey bee physiology

AMP - Antimicrobial peptides

hSP - Hypothetical structural protein

Imd - Immune deficiency

Jak/STAT - Janus kinase signal transducer and activator of transcription

nt - Nucleotides

ORF - Open reading frame

PO - Phenol oxidase

pSP - Predicted structural protein

RdRp - RNA-dependent RNA polymerase

Laboratory

DEPC - Diethyl pyrocarbonate

dNTPs - Deoxynucleoside triphosphate

EDTA - Ethylenediamine tetraacetic acid

FAM - Fluorescein amidite

MBGW - Molecular biology grade water

PBS - Phosphate-buffered saline

PCR - Polymerase chain reaction

RNA - Ribonucleic acid

RT-qPCR - Reverse transcription quantitative real-time polymerase chain reaction

TAMRA - Tetramethylrhodamine

TBE - Tris/Borate/EDTA

TE - Tris/EDTA

Modelling

CA - correspondence analysis

CCA - Canonical correspondence analysis

CIF - Cumulative incidence function

DIC - Deviance information criterion

drc - Dose response curve

GLM - Generalised linear model

GLMM - General linear mixed model

INLA - Integrated nested Laplace approximation

MCMC - Markov chain Monte Carlo

NLS - Non-linear least squares

SEM - Structural equation model

SPDE - Stochastic partial differential equations

Chapter 1

Introduction

Insect pollinators are valued for both their natural ecosystem service of pollination, as well as their use as a managed pollination tool by farmers (Gallai et al., 2009). Approximately 75% of crops worldwide benefit from animal pollination, contributing €153 billion in economic value (Gallai et al., 2009; Klein et al., 2007). These pollinators include hymenopterans, lepidopterans and some coleopterans, with the most well-studied being honey bees (*Apis mellifera*). They possess clear adaptations to foraging, including coribiculae (pollen baskets), hairiness and a positive electrostatic charge to attract negatively charged pollen (Vaknin et al., 2000). In the UK, honey bees are responsible for over £150 million in pollination services to crops yearly and also produce £10-£35 million in honey per annum (Brown et al., 2014).

Declines in managed honey bees and other insect pollinators have been reported for a number of years, and emphasis has been placed on investigating potential causes and methods of conservation. The main reasons for decline are exposure to pesticides, lack of forage availability, parasites and pathogens (Vanbergen & Initiative, 2013). Factors such as agricultural intensification and urbanisation contribute to the reduction in natural landscapes where bees typically forage. Agricultural areas tend to be monofloral, with mass production of certain crops based on the time of year, leaving nearby colonies with little option for forage. This has knock-on effects for honey bee health, as studies have found that polyfloral pollen has higher antimicrobial and antioxidant properties and can enhance immunocompetence, making bees less susceptible to certain pathogens (Alaux et al., 2010; Alimoglu et al., 2021). The increase in agriculture for our growing human population also means an increase in pesticide use. Honey bees become exposed during

foraging and have been found to have reduced longevity and immune functioning once exposed to certain pesticides (Brandt et al., 2016; Tosi et al., 2021). This in turn increases their susceptibility to disease.

Honey bees are the subject of a wide variety of co-evolved pathogens and parasites, appearing wherever the honey bee is found (Royce & Rossignol, 1990). Honey bees offer practically ideal conditions for pathogen transmission due to their high density living which causes almost constant close physical contact through grooming, communication and trophallaxis. Pathogens exploit these conditions for transmission, being well adapted to infect individual honey bees and the superorganism they are part of. Honey bees have immune defences at an individual and social scale to protect both themselves and their colonies from pathogens.

It is very likely that disease is promoting the current decline in honey bees, and the rapid spread of pathogens around the world reflects their large impact (Oldroyd, 2007). Understanding the epidemiology and pathology of honey bee diseases is vital for developing methods to control diseases and prevent their spread. The analysis of host-pathogen relationships, disease virulence and progression, and individual impact on the host give an in-depth understanding of disease and allow for well-informed control methods and beekeeping practices that minimise their impact (Genersch et al., 2010).

1.1 Chronic bee paralysis virus

Emerging infectious diseases (EIDs) pose a risk to organisms and are characterised by rapid spread of an existing pathogen or the occurrence of a newly identified pathogen in a population (McArthur, 2019). Chronic bee paralysis (CBP) is an example of an EID affecting honey bees and is increasing in prevalence across England and Wales (Budge et al., 2020). CBP is caused by the aetiological agent Chronic bee paralysis virus (CBPV), a single-stranded RNA bipartite virus which predominantly infects the western honey bee *A. mellifera* (Celle et al., 2008). It has a worldwide distribution and is widespread throughout Britain, likely infecting the majority of colonies (Amiri et al., 2014; Bailey et al., 1983). Infections are often covert and inapparent for long periods, suddenly becoming symptomatic (Ribiere et al., 2008). Colony losses due to CBPV are the most severe during the spring/summer months of the apicultural season when bees are confined and in close contact during periods where they would usually be at their most active. This could be

due to poor weather or the absence or shortage of nectar (Ribiere et al., 2010).

1.1.1 Genomic structure

The CBPV genome sequence was described by Olivier et al. (2008b). The genome is made up of two major RNAs; RNA 1 (3674 nt) which encodes three open reading frames (ORFs) and RNA 2 (2305 nt) which encodes for four ORFs. More recent studies have found that RNA2 encodes for structural proteins expressed by ORFs 2 and 3. The proteins are believed to be hypothetical structural protein (hSP) and predicted structural protein (pSP), which may have roles in the formation of the viral capsid structure. RNA1 encodes non-structural proteins, which require further investigation (Chevin et al., 2015). These proteins were believed to be orphans, however, Kuchibhatla et al. (2014) detected homologs using recently developed methods focusing on profile sequence comparison. They found that ORF1 of RNA1 is homologous to the methyltransferase guanylyltransferase of alphavirus and that ORF3 of RNA2 is a putative virion membrane protein, homologous to various insect and plant viruses. ORF 2 of RNA2 was suggested to be a virion glycoprotein, with similarities found with other viruses within the recently described taxon, Negevirus. Originally, the CBPV genome was described to have three minor RNAs (3a, 3b and 3c), as well as the two major RNAs. These three minor RNAs were found to be identical to the three single-stranded RNA segments of Chronic bee paralysis satellite virus (CBPSV), a serologically unrelated isometric particle to CBPV but one which only replicates in its presence (Overton et al., 1982). It is now suggested that the CBPV minor RNAs and the CBPSV RNAs are actually the same, and have either contaminated or become encapsulated in the CBPV virion (Ribiere et al., 2010).

CBPV is unclassified, but believed to be positioned between the Nodaviridae and Tombusviridae family clusters. This is because of the similarities between the amino acid sequence of ORF 3 on RNA1 and the RNA-dependent RNA polymerases (RdRp) of other single-stranded RNA viruses within these families (Olivier et al., 2008b). CBPV shares other similarities with these families, including a positive single stranded RNA genome, 2 Major RNAs, sub genomic RNAs, and an associated satellite virus. The alphanodaviruses within the family Novaviridae specifically bear resemblances to CBPV, as they are insect viruses which can cause paralysis and also exhibit neurotropism (Ribiere et al., 2010).

1.1.2 Transmission

Diseases and their pathogens can be transferred in a number of ways which are broadly separated into two categories: horizontal transmission, between individuals within a generation, and vertical transmission, between individuals of different generations (Chen, Pettis, et al., 2006). These categories can be further separated when investigating transmission routes both within and between colonies, known as intra- and intercolony transmission. Intracolony horizontal transmission takes place via grooming, communication and trophallaxis, and can also involve using adults as mechanical vectors with covert disease, transmitting pathogens to brood and larvae (Fries & Camazine, 2001). This can take place directly through particles in the air and food, and through venereal transmission. Indirect horizontal transmission involves a vectoral host (Chen, Pettis, et al., 2006). Intercolony horizontal transmission can occur from contaminated food sources used by more than one colony, or through more specific behaviours such as drifting and robbing. Intracolony vertical transmission takes place when a drone or queen passes on pathogens through their reproduction (Fries & Camazine, 2001). There are two types in virus pathologenesis: transovum, where the virus is attached to the surface of the egg, and transovarial, where the virus is passed to the egg intracellularly (Amiri et al., 2018). Intercolony vertical transmission occurs via swarming, which is the separation of the colony (usually in roughly two halves) which forms two new colonies; one led by the original queen which moves to a new location, and one led by a new daughter queen which remains in the original hive. Swarming colonies can bring with them pathogens which will be spread to new brood through reproduction (Fries & Camazine, 2001).

1.1.2.1 Oral transmission

Some modes of transmission of CBPV have been identified, however other possible routes require further investigation. Oral transmission is the process of infection via exposure to a pathogen at the mouth. In bees, this could be done in a number of ways, such as by feeding, trophallaxis, anal grooming and other forms of cleaning. Transmission via feeding has been shown to take place from a bee which was inoculated with CBPV to a healthy bee, however the number of particles transmitted was not enough to cause overt disease (Bailey et al., 1983). However, this method of transmission aids in the spread of the virus throughout the colony due to the passing of nectar between individuals and the feeding of

brood by nurse bees (Ribiere et al., 2010). This highlights the possibility that trophallaxis may be an important method of transmission, which is further reinforced by the finding of CBPV in glandular secretions from the mandibular gland and hypopharyngeal gland, and the presence of CBPV in pollen (Chen, Evans, et al., 2006).

The first controlled experiments investigating the transmission of CBPV appear to be by Burnside (1933), prior to the causative agent of CBP being identified (Bailey et al., 1963). Bees were taken from hives displaying typical paralysis symptoms, and haemolymph was removed from them. Mixed age bees were fed a solution of this haemolymph with sugar syrup, however no symptoms of paralysis were observed, and mortality was the same as that of control bees. This experiment was also done on one day old bees, and Burnside reported that 10-20% of bees showed symptoms of paralysis and had a higher mortality rate than control bees. His findings reveal that while it is possible for CBPV to be transmitted through food, there are unanswered questions regarding susceptibility based on age, and why only a small proportion of bees show symptoms when exposed orally.

Since these initial experiments, other studies have used transmission via feeding to investigate various aspects of CBPV but have not explored the mechanism of oral transmission in detail. Further experiments by Burnside (1945), involving feeding bees the water extract from naturally infected bees with drinking water, caused paralysis symptoms in all bees two weeks after exposure. Burnside went on to mix the virus extract from the bees which died in this experiment with sugar syrup and fed it to healthy bees, which caused the majority to show symptoms after 10 days, and for half of the bees to die three weeks after exposure. These experiments were repeated by Bailey et al. (1963), where virus extract from bees naturally showing symptoms of paralysis was mixed with equal amounts of sugar syrup and fed to bees. They reported that few, or often no bees at all showed signs of paralysis from this form of exposure. Similar experiments were performed by Bailey (1965), where he again observed few bees with symptoms after being fed CBPV in sugar syrup. He observed increased concentrations of CBPV throughout tissues in the body five days after exposure, and by day 12, concentrations were similar to those in bees that were not fed CBPV. Bailey also conducted an experiment where cages were divided by a gauze, with one side containing the food and chronically paralysed bees, and the other side containing healthy bees, a method based on Bailey & Gibbs (1964). Although no symptoms or increased mortality were observed in the healthy bees by day six, the bees appeared to have received or developed up to 10^8 particles of CBPV.

Horvath & Rothenbuhler (1972) investigated the histology of honey bees once infected with CBPV via oral transmission. Only five bees were studied, and were fed a solution of equal amounts of virus extract and sugar for five days. Symptoms or mortality were not mentioned, and the only notable differences histologically were that four out of five virus-fed bees had distended abdomens, which was not seen in any control bees. A study by Kulinčević & Rothenbuhler (1975) used oral transmission to test the susceptibility of different generations of both susceptible and resistant lines of bees. They reported a 20-27% mortality 14 days after exposure in the stock and parental generations. Other studies around this time also reported mortalities of bees fed CBPV. Rinderer & Rothenbuhler (1975) investigated how the behaviour of infected bees and healthy bees in the presence of infected bees may lead to further infection via oral transmission. Healthy bees that attacked infected bees were found to have ingested bee hairs, and bees which were already displaying symptoms had double this amount in their guts. Mixed age bees were fed hairs with virus, virus only, hairs only or syrup only to investigate how hairs impact transmission. The mortality of bees fed hairs or syrup was 7.2% and 7.6% respectively, whereas bees fed virus had a mortality of 37.2% and bees fed both virus and hairs had a much higher mortality of 69.6%. Two experiments by Rinderer & Rothenbuhler (1976) found that feeding 0-1 day old bees virus with an equal amount of syrup for three days caused 46% and 78% mortality, and symptoms of paralysis were observed. While experimental set-ups varied, the method of exposure for both groups of bees was the same. This highlights the vast ranges of mortalities caused by oral transmission of CBPV, both within studies and between them.

1.1.2.2 Fomite transmission

CBPV may also be transmitted via fomites. These are objects which can become contaminated or infected with a pathogen and can spread disease to a host when they come into contact with the object (Moschovis et al., 2021). For honey bees, fomites may be honey bee products such as wax, beebread or pollen and honey, as well as faeces, salivary gland secretions, frames and beekeeping equipment (Alger et al., 2019; Owen, 2017; Ribiere et al., 2007; Shen et al., 2005). CBPV has been detected in pollen, faeces and salivary gland secretions (Ball, 1999; Chen, Evans, et al., 2006; Ribiere et al., 2007). Studies have reported an absence of CBPV in honey and royal jelly (Chen, Evans, et al., 2006). Although these samples tested positive for CBPV, this does not prove that they

are part of a transmission route. The transmission routes require further testing and more studies into where CBPV particles may be harboured is needed.

1.1.2.3 Other modes of transmission

Bailey et al. (1983) found that topical application of CBPV to a cuticle denuded of hairs caused infection, and that in experimental conditions, the virus spread most when bees were in high concentrations and crowded conditions, likely due to the breaking of hairs when bees rub together. This was further evidenced by the decline in colony numbers and decreasing number of cases of CBPV, which was hypothesised to be due to increased foraging activity by the surviving colonies, and therefore less contact between bees (Bailey et al., 1983). Injection of CBPV into honey bees leads to overt infection in about six days, followed by death a few days later. Symptoms which occur from this method of transmission are very similar to those from natural infections (Bailey et al., 1963). Ribiere et al. (2010) detected the presence of CBPV in faeces of naturally and experimentally infected bees, and found that placing healthy bees in cages which previously contained infected bees was enough to cause overt disease. More recently, CBPV was detected in the mite *Varroa destructor* and two species of carnivorous ants, *Camponotus vagus* and *Formica rufa*. It is believed that these species act as reservoirs of the virus, opportunistically infecting honey bees and making them potential vectors of CBPV (Celle et al., 2008).

CBPV can also be transmitted vertically as well as horizontally. It has been detected in bees of all developmental stages, as well as queen bees. It has been shown to spread from queens which were fed contaminated foods, and by injecting queens, drone pupae and adults (Ball et al., 1985; Blanchard et al., 2007). While CBPV has not been detected in the ovaries of queens, an experiment by Chen, Pettis, et al. (2006) found that in a group of queens, 67% showed signs of infection, and 50% of eggs, 17% of larvae and 17% of drones from these queens were also infected with CBPV. It is also possible that the ovaries contain very low levels of CBPV, to allow the infection to be covert and latent, and therefore go undetected.

1.1.3 Symptoms

The symptoms of CBPV can be classified into two groups, Type 1 syndrome and Type 2 syndrome, however both syndromes are caused by the same virus, infect bees with the same quantity of CBPV and can be found within the same colony (Rinderer & Green, 1976). Type 1 syndrome involves paralysis, dislocated and trembling wings, and the inability to fly, leading to large groups of bees crawling on the ground outside their hive (Toplak et al., 2013). Dysentery-like symptoms are also common, induced by the distention and swelling of the crop which causes bloating of the abdomen. This type of syndrome is the most common in Britain, and can lead to colony collapse within a few days of overt infection, leaving behind the queen and a small number of adults. Mounds of dead bees outside the hive entrance are a common sight. Type 2 syndrome is characterised by hairless, greasy, black bees which quickly lose the ability to fly (Bailey, 1976). Symptomatic bees may perform nibbling attacks on other members of the colony, which can lead to them being expelled from the colony due to them being mistaken for robbers (Ribiere et al., 2010; Ribiere et al., 2007).

1.2 Sites of infection and systemic spread within host

Millions of CBPV particles have been observed throughout the honey bee body, with half of those particles found in the head (Bailey & Milne, 1969; Blanchard et al., 2007). CBPV has been detected in most areas of the brain, including the mushroom bodies, the central complex and the optic and antennal lobes, which may account for the symptoms affecting the nervous system caused by the virus (Olivier et al., 2008a). In order to establish the distribution of CBPV within the brain, an in-situ hybridisation method was developed by Olivier et al. (2008a) using CBPV-specific probes to identify specific locations of particles. Prior to this, electron microscopy and histological methods were used, which lead to the finding of CBPV particles in the abdominal and thoracic nerve ganglia, the hypopharyngeal and mandibular glands, and the brain (Lee & Furgala, 1965; Ribiere et al., 2010). CBPV has also been identified in the haemolymph and in the hind-gut epithelium, and was not found in fat and thoracic muscle (Bailey, 1965; Chen, Pettis, et al., 2006; Lee & Furgala, 1965).

The genomic loads of CBPV in these areas vary, with Blanchard et al. (2007) finding

the highest concentration of particles in the head (4.9×10^9), with 6.8×10^5 particles in the brain and 2.5×10^5 particles in the mandibular and hypopharyngeal glands. The abdomen and thorax contained 4.1×10^8 and 2.4×10^9 particles respectively, with the alimentary canal running throughout all body segments containing a mean of 2.1×10^6 particles. Naturally infected bees were found to exhibit slightly lower genomic loads in the haemolymph than experimentally infected bees via topical application to the cuticle denuded of hair, with loads of 4.1×10^9 and 2×10^{10} respectively. However, Blanchard et al. (2007) also reported higher CBPV genomic loads in the organs of naturally infected bees.

Although there is some knowledge of where CBPV is located in the body, the route of infection of the virus once it has entered the body has not yet been established. There are various potential ways in which CBPV could enter the body (Figure 1.1). If the virus entered the bee orally, it may move through the digestive system or enter the mandibular gland. From either of these locations, it is hypothesised that CBPV could enter the haemolymph and move to a number of areas, including the brain, ganglia and the hypopharyngeal gland. Once entering the digestive system faeces would be contaminated with CBPV, meaning excretion would further the spread of the virus (Ribiere et al., 2007). The mandibular gland is connected to the brain via the mandibular nerve, which may serve as a direct route to the brain for CBPV from ingestion (Carreck et al., 2013). Rapid spread of the virus to the nervous system may lead to the onset of paralysis faster than if the virus moves through the digestive system and haemolymph before entering the nervous system. This type of movement may lead to death more swiftly, which would reduce the amount of time an infected individual has to infect others. If CBPV initially moves through the digestive system, potentially inducing dysentery-like symptoms, it may be more beneficial for the virus spread as the bee may live for longer and therefore infect more bees.

If CBPV enters the bee via the mechanical transmission route, the virus will likely immediately invade the haemolymph, from which it can move into any part of the body. Bailey (1965) identified the presence of CBPV in swollen cells in the hind gut epithelium immediately posterior to the Malpighian tubules, which may suggest that this is a replication site for the virus. Replication here could lead to high genomic loads of virus in faeces, aiding in its spread once the bee has defecated or through anal grooming. The presence of the virus in the haemolymph may also assist in mechanical

transmission if it can move out of the haemolymph through the cuticle, infecting other bees and contaminating surfaces. As well as the hind-gut epithelium, the brain and mandibular gland have been suggested as replication sites. The brain has a significantly high concentration of virus particles compared to other body parts of similar sizes, indicating that CBPV may be able to replicate there (Blanchard et al., 2007). Replication in the mandibular gland would be beneficial for transmission via trophallaxis, as this area could act as a reservoir of the virus, easily leaving the body orally or moving throughout the body to continue to infect the individual it is within.

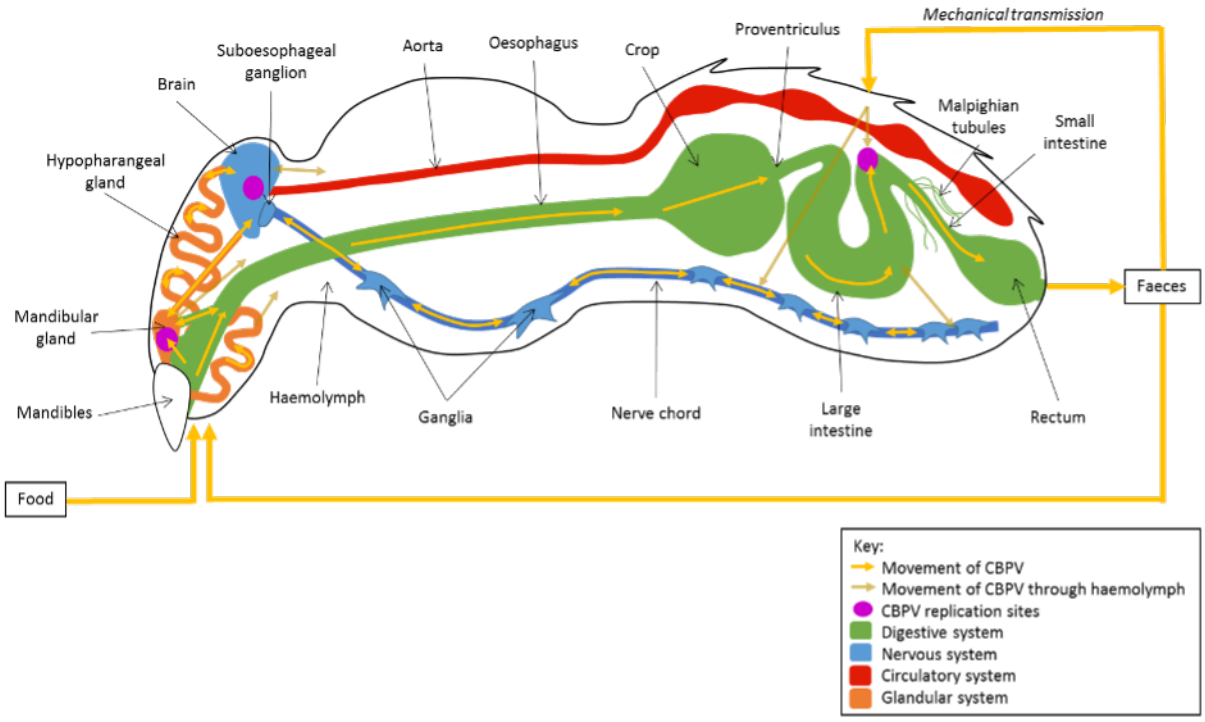


Figure 1.1: A conceptual diagram of a honey bee indicating the infection routes and movement of CBPV within a honey bee.

1.2.1 Treatments

Currently, there are no treatments available for CBPV, however there are a few methods suggested by The National Bee Unit (2017a) to reduce the likelihood of CPBV infections and their severity. CBPV outbreaks are more likely to take place when colonies are overpopulated and crowded, causing bees to be in contact more frequently (Ball, 1999). Larger hives would help to combat this issue, which can be achieved by adding supers or brood boxes. Ensuring that colonies are well spaced out within an area, or removing colonies from highly populated areas reduces transmission via robbing. Frames and supers

should not be transferred between colonies, and any contaminated hive parts should be cleaned or replaced (The National Bee Unit, 2017b). Field evidence in support of these techniques suggests disease management methods are lacking.

1.3 Immune defences

1.3.1 Social immunity

The integral characteristics of social living give rise to increased risk of infection spread and disease outbreak. Honey bees within a colony live in high densities, taking part in continual physical interactions such as trophallaxis, potentially aiding the spread of pathogens and parasites among individuals (Simone et al., 2009). The lack of genetic diversity typical of colonies has been identified as a trait which could lead to disease outbreak, as any successful pathogens will likely be able to infect the entire colony (Cremer et al., 2007; Wilson-Rich et al., 2008). However, many insect groups are equipped with social defence mechanisms and behaviours to prevent infection and disease. The results of the Honey Bee Genome Project found that honey bees possess fewer genes for innate immunity compared to *Drosophila*, have two-thirds fewer gene families and encode fewer proteins involved in their immune pathways (including the Toll, Imd and JAK/STAT pathways) (Evans et al., 2006; Honeybee Genome Sequencing, 2006). One suggested reason for this is that the demand on honey bees' individual immune response is decreased because they rely more heavily on societal defences (Alaux et al., 2010; Evans et al., 2006). These defences were termed as 'social immunity' by Cremer et al. (2007), and are defined as traits which reduce the transmission and intensity of parasites and pathogens at the colony level, involving defences which are behavioural, physiological and organisational in nature.

The first stages of social immunity begin with behavioural defences in the form of general nest hygiene and grooming (Cremer et al., 2018). Both auto-grooming and allo-grooming (the removal of foreign particles including mites from a bee's own or another bee's body) are beneficial to the whole colony by reducing ectoparasites such as mites, and through social immunisation (Evans & Spivak, 2010). This involves low level pathogen exposure through the grooming of parasitised or diseased individuals, preparing the immune system for more successful defence against pathogens (Simone-Finstrom, 2017). If infection

spreads despite this effort, bees may take part in hygienic behaviour where infected or diseased worker brood or workers are expelled from the colony, also known as undertaking. Infected bees have been observed to rapidly take on roles which involve spending more time outside of the colony, and often purposefully die outside of the colony, or once dead are deposited outside foraging ranges by other workers to reduce the risk of disease outbreak (Cremer et al., 2018; Evans et al., 2006; Evans & Spivak, 2010; Natsopoulou et al., 2016). It is important that these behaviours take place immediately after pathogens are detected within the colony, as once infected bees enter a stage where the infection is transmissible, healthy bees taking part in these behaviours are at risk of becoming infected and therefore facilitating infection spread (Evans & Spivak, 2010).

There are many other colony-wide behaviour defences honey bees put in place, such as sealing closed nest entrances, refusing infected individuals entry, and avoiding contaminated areas and food sources (Cremer et al., 2018). Honey bees have been shown to respond to a colony chalkbrood infection in a specific way: by increasing the temperature of the brood nest to inhibit the temperature sensitive chalkbrood fungal spores (Simone-Finstrom, 2017). This is known as 'social fever', which takes place before larvae die from the infection (Starks et al., 2000). While it is known that social fever is induced by the chalkbrood spores, the response is also affected by environmental conditions such as ambient temperature (Simone-Finstrom et al., 2014).

1.3.2 Individual immunity

A honey bee's individual defence against invading pathogens begins with physical and chemical barriers, including the exoskeleton cuticle and the lining of the gut (DeGrandi-Hoffman & Chen, 2015; Evans & Spivak, 2010). If a pathogen succeeds in entering the body and survives, cellular and humoral immune responses are activated. Cellular immunity has been observed through the measurement of haemocytes, which are involved in phagocytosis, nodulation and encapsulation of pathogens (Evans et al., 2006; Ratner & Vinson, 1983; Schmid et al., 2008). Fat body mass can be used as a measurement of the humoral immune response, as it is the site of antimicrobial peptide synthesis (Wilson-Rich et al., 2008). Phenol oxidase (PO) activity has also been used as a measurement of the humoral immune response, but also has roles in the activation of encapsulation and is, therefore, also involved in the cellular immune response (Alaux et al., 2010).

1.3.3 Gut microbiome

Another line of defence is the honey bee gut microbiome. This highly conserved and specialised community is found globally; unusual qualities for an insect species which make it an ideal model system (Hamdi et al., 2011). This community is made up of between eight and 11 species of bacteria, also called phylotypes (Martinson et al., 2011; Moran, 2015). While the exact number and species of bacteria clusters which make up the gut microbiome are disputed, it is widely accepted that five of these species are present in all honey bee workers, making up the core gut microbiome (Raymann & Moran, 2018). They include *Gilliamella apicola*, two species of *Lactobacillus*; Firm5 and Firm4, Bifidobacterium, and *Snograssella alvi* (Bonilla-Rosso & Engel, 2018; Maes et al., 2016). Other species reported to make up the honey bee gut microbiota include *Frischella perrara*, *Bartonella apis*, *Parasaccharibacter apium* and an unnamed Acetobateracae species. Less commonly included species are *Apibacter adventoris* and *Lactobacillus kunkeei* (Zheng et al., 2018). The functions of these bacteria differ, but all are important in the metabolism of carbohydrates and protection against pathogens. Many strains of each species exist, particularly for *G. apicola* and *Lactobacillus* Firm-5, as well as *Lactobacillus* Firm-4 and *Bifidobacterium asteroids* (Raymann & Moran, 2018). These bacteria make up 95% of the gut microbiota; the remaining 5% are likely pathogenic organisms invading at later stages (Cox-Foster et al., 2007; Moran, 2015).

The gut microbiome is established through contact with adult bees or food in the hive following eclosure via methods such as oral trophallaxis and anal grooming, taking between three and five days to fully develop (Powell et al., 2014). Bacteria may also enter the gut via fomite transmission, through the chewing of cell caps during emergence, as well as hive cleaning, auto- and allo-grooming. The first bacteria to colonise the gut are *Lactobacillus* and Acetobacteraceae, and likely are transmitted from a food source as they are present in pollen and nectar (Anderson et al., 2013; Moran, 2015). Bacteria found in the ileum enter the gut exclusively via anal grooming or contact with faeces. These include *S. alvi* and the gammaproteobacteria (Moran, 2015). Despite trophallactic feeding from nurse bees, larvae have little to no bacteria in the gut. This has been suggested to be due to an immune response or antimicrobial agent induced or given by the nurse bees themselves. This also may be related to the digestive system of larvae which is different to that of adults, with a separated gut between the midgut and ileum, which only connects immediately prior to pupation allowing them to excrete for the

first time (Martinson et al., 2011). It is also possible that bacterial cells are present in the larval gut but are in such small quantities at this early stage of development that they are not detectable (Zheng et al., 2018). This feature of recently eclosed honey bees makes them ideal for experimental manipulation in order to identify functional roles of gut microbiota (Bonilla-Rosso & Engel, 2018).

The gut microbiome can be altered by a number of factors, including nutrition, season, age, caste, disease, stress and the use of antibiotics and chemicals (Carding et al., 2015). Depending on the extent of these factors, gut dysbiosis can be caused, where the core gut microbiota collapse and are replaced by species which are usually low in number and frequency in the gut, and therefore important functions do not take place (Maes et al., 2016). If dysbiosis takes place during development, its effects can be life long and cascade into further issues (Stecher, 2015). The microbiota community shifts seasonally, which is likely due to changes in food availability which vary with season. It is therefore possible that extreme weather may impact the gut microbiome, as this will affect food availability and pollen stores, and could lead to gut dysbiosis (Ludvigsen et al., 2015). Bees become more susceptible to pathogen infection when undergoing gut dysbiosis, implying that the gut microbiome is a line of defence against pathogens (Raymann & Moran, 2018). While certain gut bacteria have a specific role in protection against pathogens, the bacterial community as a whole influences this defence (Koch & Schmid-Hempel, 2012).

1.4 Survival analysis

The epidemiology of disease can be informed by analyses which investigate the time taken for individuals to show symptoms of disease or die, and the causes of these symptoms and deaths. This can be done using survival analysis. It is commonly used in the study of human disease, particularly cancer, but has also been applied to diseases impacting a variety of organisms including mammals, insects and plants (Figueroa et al., 2021; Gröhn & Rajala-Schultz, 2000; Tarawneh et al., 2011). Using survival analysis to investigate CBPV and its effect on honey bee mortality allows treatments that may alter susceptibility to the disease to be considered and their effects to be quantified. Survival analysis has been used to investigate other honey bee diseases, including Israeli acute paralysis virus, *Nosema ceranae* and Deformed wing virus (Bhatia et al., 2021; Dosch et al., 2021; Jack et al., 2016).

Survival analysis investigates the time taken to reach an event of interest, and the factors which may affect this. It is used on a wide variety of data, not only that which is related to survival. This analysis is also known as 'time to event' analysis, aiming to estimate when an event of interest will occur for a particular group. The time element of the analysis begins at a specified time, such as the diagnosis of an illness or the beginning of a treatment, and individuals are monitored for an event of interest, also known as failure. While survival analysis is commonly used for the analysis of clinical trials, it can also be applied to engineering scenarios, beginning with the manufacture of an object and monitoring it to see when it fails. Survival time describes the period between the beginning of an observational period or experiment and the event (Liu, 2012; Miller Jr, 1981).

1.4.1 Survival function

Survival analysis uses the survival function to estimate the likelihood of an individual living past a certain time point. It is represented by the formula $S(t) = P(T > t)$, where $S(t)$ is the survival function (or reliability function when referring to manufactured items) and calculates the probability of survival time (T) being greater than the specified time point (t - for example, a predetermined end date of a study) (Kleinbaum & Klein, 2012).

1.4.2 Hazard function

The hazard function involves the estimation of the probability of an individual experiencing an event at a specified time when it has survived up to a certain time, defined as:

$$h(t) = \lim_{t\Delta \to 0} \frac{P(t \leq T < t + \Delta t | T \geq t)}{\Delta t}$$

In this formula, $h(t)$ is the hazard function and $\lim_{t\Delta \to 0}$ gives the instantaneous potential for the event. The numerator is a conditional probability and tells us the likelihood of survival time (T) being within a particular time interval given that the individual has survived until that point. This is represented by $t + \Delta t$, which corresponds to a certain time point where T is greater than or equal to t. The denominator denotes a period of time, and the division gives the hazard rate. This is where, when survival takes place up to time t, we see the instantaneous potential of failure at a given time per unit time (Kleinbaum & Klein, 2012; Liu, 2012).

1.4.3 Censoring

In cases where the event of interest does not take place during the course of the study, censoring occurs. This often happens simply because the experiment was concluded before the event takes place, or an individual was no longer able to take part in the study. In cases where illness is monitored in patients, individuals may recover, or cease participation in the experiment. Any incomplete observations such as these are censored. The beginning of the experiment or the beginning of observational time is referred to as t_0 (Kleinbaum & Klein, 2012).

There are three main types of censoring; right censoring, left censoring and interval censoring. Right-censoring occurs when the event of interest is not observed due to the experiment ending, individual removal from the experiment, or loss of an individual to a cause other than the event of interest during the experiment. This type of censoring can be further categorised into three main groups:

- Type I censoring, which is due to a pre-determined end date of the experiment and results in each observation having a fixed censoring time. Individuals either experience the event within the duration of the experiment, and are therefore not censored, or survive to the end of the experiment. This can be described by $T > C$, where T is survival time and C is the fixed censored time or length of the observation period.
- Random censoring, which differs from type I censoring as the censored time is not fixed. This takes place if the individual had a delayed entry to the study and survives until the fixed endpoint of the study, or an individual is lost or withdraws from the study, regardless of when they entered the study. This can be described by the formula $C_i < T_i$, where C_i is censored time by individual, and T_i is survival time, which is assumed to be unrelated to censored time. If $C_i < T_i$, and $C_i < C$, then the data is randomly censored.
- Type II censoring, where an experiment is scheduled to end when a quota of events of interest have been observed. There is no fixed time, but rather a fixed number of observations. Individuals which have not experienced the event are censored, and their censored survival time is the time from t_0 to the end of the experiment (Liu, 2012; Miller Jr, 1981).

Left censoring occurs when the event of interest has taken place before the experiment

begins or before an individual was recruited into the study. The exact time the event took place is unknown, meaning that the true survival time is less than or equal to the observed survival time. This is especially true when the period of observation represents an entire lifetime (Kleinbaum & Klein, 2012; Klein & Moeschberger, 2006). An example of this would be how long it takes a child to exhibit certain behaviour. The behaviour may have first developed before the study, making that observation left censored (Lougheed et al., 2019).

Interval censoring takes place when the exact time the event of interest takes place is not known, and it is only known to have occurred within a certain period, for example, inspections of machines or equipment which take place at regular periods, or the periodic check-ups of a patient in a clinical trial (Klein & Moeschberger, 2006). This type of censoring can overlap with left and right censoring when the upper or lower bound of the interval is a known value of the true survival time. When in conjunction with left censoring, the lower bound must be 0, and the upper bound be the known upper bound of survival time, and for right censoring, the upper bound must be infinite, and the lower bound be the known lower bound of survival time (Kleinbaum & Klein, 2012).

Left truncation, while not a type of censoring, can affect data in a similar way to censoring. It occurs when there is a delayed entry by individuals to a study and is often due to the selection of individuals with specific traits (Liu, 2012). This can lead to a sampling bias, as individuals which have experienced the event before the study are not included, and individuals which experience the event after the beginning of the study are assumed to not have been at risk of experiencing the event until t_0. In some situations, this can lead to overall observed survival to be higher than true survival, as individuals which did not survive are not recruited into the experiment (Kleinbaum & Klein, 2012).

The use of censored data relies on three assumptions. Censoring should be random, meaning that any individual censored at a particular time should have the same survival risk as all other individuals within the study. Censoring should be independent, meaning that within subgroups, censoring should be random and each individual should have the same survival risk at the point of any one individual being censored. Finally, censoring should be non-informative, meaning censorship times should not determine or affect survival times and vice versa (Kleinbaum & Klein, 2012; Clark et al., 2003; Hosmer Jr et al., 2011).

1.4.4 The Kaplan-Meier method and log-rank test

The Kaplan-Meier method is a non-parametric function which estimates the probability of survival over time, or the survival function, and is used to produce Kaplan-Meier survival curves (see: Kaplan & Meier (1958)). It assumes that if there are competing risks, all of these risks are independent of one another.

The log-rank test compares two or more Kaplan-Meier curves in order to test the null hypothesis. The null hypothesis states that there is no difference in survival and probability of the event of interest between treatments, and therefore the survival curves, will be the same. It is a non-parametric test, which makes no assumptions based on the survival distribution. The results include a chi-squared statistic, which when significant would indicate a significant difference between survival in each treatment (Kleinbaum & Klein, 2012).

Both the Kaplan-Meier curves and the log-rank test have the same assumptions. These are

1. The condition of an individual is not linked to it having been censored or not.
2. The events took place at the time specified.
3. The time at which an individual joins the study does not impact their survival probability (Bland & Altman, 1998).

1.4.5 Cox Proportional-Hazards model

The Cox proportional-hazards model is a regression model. It takes any covariates into account which may affect an individual's condition (see: Cox (1972)).

While the log-rank test is useful for highlighting significant differences in survival, it does not disclose the size of these differences. Cox proportional-hazards models give an effect size for each type of covariate, enabling us to see the weighted effect of each covariate. The model estimates the hazard rate, which is the rate at which the event of interest takes place depending on the associated risk.

The equation for the Cox proportional-hazards model is as follows:

$h(t) = h_0(t) * exp(b_1x_1 + b_2x_2 + ... + b_px_p)$

$h(t)$ is known as the hazard function, which the model calculates. This is the instantaneous potential of failure at a given time per unit time (t). $h_O(t)$ is the baseline hazard; this

represents the hazard for when all the independent variables or covariates (x) equal zero or take their baseline value. The effect size of each covariate is measured by the beta coefficients, b_1, b_2 and b_p, which are estimated from the observed data. A hazard ratio is estimated from $exp(b_i)$, which is a regression coefficient. When the hazard ratio is below one, the predictor aids in survival and can be described as protective. A hazard ratio above 1 means the predictor is linked to decreased survival. If the hazard ratio is 1 or close to it, the predictor has little impact on survival (Bradburn et al., 2003).

The results of the Cox regression using the *survival* package in the statistical programme R gives the coefficients, the hazard ratios (exp(coef)), the standard error of the coefficients (se(coef)), a Wald statistic value (z), p-values (Pr(>|z|)) and upper and lower confidence limits of the hazard ratios (Therneau, 2020). The results of each categorical covariate are relative to the baseline level of the covariate missing from the results. The relative hazard of each covariate is shown by the regression coefficient. A positive coefficient value indicates an increased hazard for a covariate in this category rather than the baseline category, or for a continuous covariate an increased risk as the covariate value increases. The effect size of a covariate is described by the hazard ratio. It can be interpreted as a percentage increase or decrease in hazard. For example, a hazard ratio of 0.47 for a treatment would mean the hazard was decreased by 53%. Hazard ra tios can be visualised using *ggforest* in the *survminer* package in R (Kassambara et al., 2019). The Wald statistic is calculated by dividing the coefficient by its standard error and investigates the difference between the beta coefficient and zero. A significant p-value would tell us that the coefficients of the variable is statistically significant. The output of the model also gives a global significance in the form of three tests: likelihood ratio test, Wald test and the score (log-rank) test. The likelihood test works best for smaller observation sizes, however, all three tests are very similar, and have less variability with a higher number of observations. Significant results for all three tests suggest that the overall model is significant (STHDA, 2020; Therneau & Grambsch, 2000; Therneau, 2020).

There are three types of survival models: all-cause hazard modelling, cause-specific hazard modelling and subdistribution hazard modelling, the latter of which takes competing risks into account. All-cause hazards are those that move an individual from a susceptible state to any other state of interest. The hazards associated with all states are grouped and modelled as one. In survival analysis, this would involve having an event of interest and censoring all individuals which did not experience the event, without knowing the cause

of the event. Cause-specific hazards separate the hazards based on the cause of the event experienced. The cause of the event of interest is taken into account, so that individuals who experience the event, but not due to the cause of interest, are censored and grouped with individuals who may be censored for other reasons, such as survival. This is also a form of Cox model. Subdistribution hazards also separate the causes of the events of interest, but also model these events and take the censoring reason into account. This is known as a Fine-Gray model or competing risks model, and is based on a Cox proportional hazards model, with the addition of the cumulative incidence of events being calculated based on the subdistribution of the hazards (Fine & Gray, 1999; Zhang, 2016).

Competing risks modelling involves individuals with an initial state which then may progress to one of multiple states depending on the possible events in the study. Two types of analysis occur; analysis to the time of event, and analysis of the event that took place (Beyersmann et al., 2011). Competing risk modelling uses a cumulative incidence function (CIF) which calculates the expected proportion of individuals in the study to experience one of the events (Beyersmann et al., 2011). This differs from the Kaplan-Meier approach, as the CIF is based only on risk for each type of event, therefore linking the covariate to its associated risk (Zhang, 2016).

1.4.5.1 Assumptions

A key assumption of the Cox model is that the hazards are proportional and that the hazard ration is independent of time, and therefore, the hazard ratios are constant over time (Kleinbaum & Klein, 2012). This can be tested using the *survival* package in R with the function *cox.zph*, which tests the proportionality of each covariate when interacting with time. This can be visualised by a Schoenfeld residual plot (Therneau, 2020).

Competing risks models follow the same assumptions as the Cox model, however, Beyersmann et al. (2011) discuss that these assumptions are only made for mathematical and interpretation convenience, and that when hazards vary with time, the hazard ratios given are based on a time average, meaning they can still be interpreted (albeit with caution).

1.5 Aims of Thesis

The aims of this thesis are as follows:

1. To identify causes of susceptibility of *A. mellifera* to oral exposure of CBPV (Figure 1.2, *a-c, p, o, s*).

2. Investigate the variation in the gut microbiota of *A. mellifera* and its overall effect on honey bee susceptibility to oral exposure of CBPV and general honey bee health (Figure 1.2, *a-c, p, o, s*).

3. To identify the causes of improved survival in *A. mellifera* when fed beebread, and to investigate its effect on honey bee susceptibility to oral exposure of CBPV (Figure 1.2, *c, d, o, n*).

4. To investigate the drivers of honey bee colony loss in the UK in relation to forage availability, weather and colony management (Figure 1.2, *d-m, r, q*).

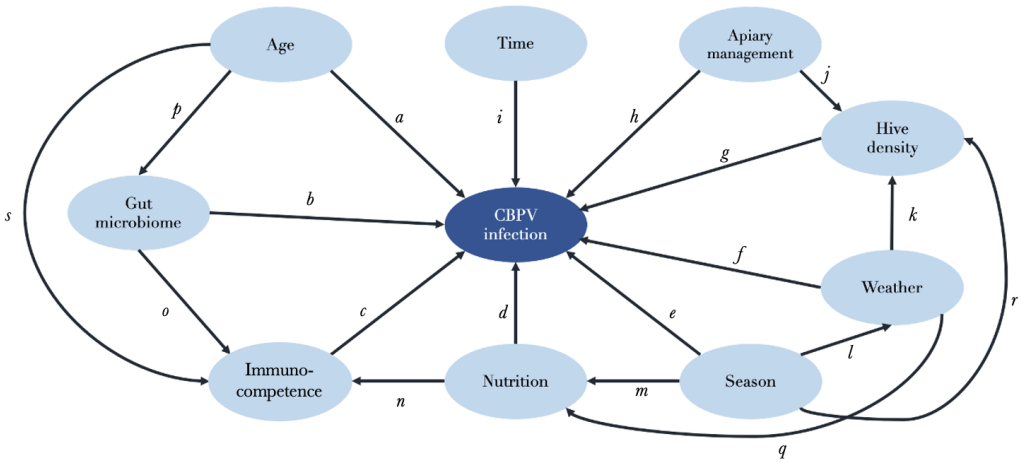


Figure 1.2: A conceptual diagram displaying the interactions between various factors which make up the hypotheses of this thesis.

Chapter 2

Oral transmission and honey bee susceptibility to Chronic bee paralysis virus

2.1 Introduction

Transmission of infectious diseases requires movement of a pathogen from one organism to another. The modes through which transmission is possible are varied, and often rely on the social behaviour and lifestyle of the host (Altizer et al., 2003; Briard & Ezenwa, 2021). Humans are highly social animals that live in groups and their behaviour can therefore impact transmission of pathogens. In humans, human immunodeficiency virus (HIV) is most commonly sexually transmitted through bodily fluids, although it can also be passed from a mother to infant during pregnancy, childbirth, or through breastfeeding (Shaw & Hunter, 2012). Humans are also at risk of infectious respiratory diseases, which are transmitted through airborne droplets, aerosols or direct contact with infected organisms or contaminated surfaces (Pica & Bouvier, 2012). A currently important infectious respiratory disease in humans is COVID-19, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Shi et al., 2020). It is transmitted through droplets and aerosols via sneezing and coughing, as well as through breathing and speech in asymptomatic individuals (Asadi et al., 2020; Salian et al., 2021). To prevent the spread of this disease, restrictions on gatherings and movement were put in place in many countries around the world in 2020.

Honey bees are unusual in the invertebrate world as they are also social organisms. There is an abundance of pathogens which target honey bees, many of which have co-evolved with honey bees themselves (Royce & Rossignol, 1990). Pathogen evolution has taken advantage of the eusocial lifestyle of honey bee colonies, utilising different processes in the behaviour of honey bees to facilitate transmission. Behaviours such as trophallaxis and allogrooming can lead to direct contact and close proximity of individuals. Pathogens can also spread between honey bees of different colonies through robbing behaviour and sharing of food sources (Fries & Camazine, 2001). The way honey bees are managed by beekeepers can also exacerbate transmission between colonies as it influences the way in which individual bees interact/come into contact. Moving frames between colonies and using the same tools to manipulate several hives can lead to the spread of fomites from colony to colony (Mutinelli, 2011).

Chronic bee paralysis (CBP) is an emerging bee disease caused by Chronic bee paralysis virus (CBPV). The disease can cause paralysis, shaking, hairlessness and ultimately leads to death, often resulting in piles of dead bees at the entrance of the hive (Ribiere et al., 2010). In laboratory settings, transmission of CBP has been shown to occur through feeding, injection and topical application (Bailey et al., 1983; Bailey, 1965; Bailey et al., 1963). In the hive there are several potential modes of transmission including trophallaxis, grooming, corpse disposal and hive cleaning. It is also possible that the disease is spread from the queen to offspring via vertical transmission (Chen, Evans, et al., 2006).

Many of the key routes of pathogen transmission involve entry to the honey bee body via the mouth. This is significant as honey bees regularly feed each other, with newly emerged bees receiving their first feeds as an adult from a nurse bee via trophallaxis. While oral transmission has been used to expose honey bees to CBPV experimentally, there has been little research on why responses to oral inoculation differ. Studies involving oral transmission of CBPV often had varied results, with some experiments not seeing any symptoms or mortality in CBPV-fed bees, and other experiments seeing mass die-off from a seemingly similar treatment (Bailey et al., 1963; Rinderer & Rothenbuhler, 1976). While there may be many explanations for these results, it raises an interesting point that honey bees seem to vary in susceptibility to CBPV when exposed *per os*. CBPV appears to be a disease of adult bees only, but we do not know when transmission of CBPV takes place. Many studies which used oral transmission of CBPV do not give the precise age of the bees exposed (Bailey & Gibbs, 1964; Burnside, 1933).

The honey bee has physical and chemical barriers used as the first line of defence against pathogens. The cuticle of a honey bee is made up of two layers; the epicuticle and the protocuticle, which form an exoskeletal barrier preventing entry by exogenous pathogens (Eleftherianos et al., 2022; Stamm et al., 2021). The lining of the gut also serves as a physical barrier. The entirety of the gut is lined with epithelial cells, which not only form a barrier but also produce chitin and proteins, creating the peritrophic membrane in the midgut (Dores Teixeira et al., 2015). This lining allows the midgut to be the main site of digestion and reabsorption (Wang & Granados, 2001). The foregut and hindgut intima have a lining of cuticle instead. Epithelial cells are also where cellular and humoral immune responses are based (Eleftherianos et al., 2022). If a pathogen penetrates these physical and chemical barriers, these responses may be activated. The cellular immune response involves phagocytosis, nodulation and encapsulation of pathogens using haemocytes (Evans et al., 2006; Ratner & Vinson, 1983; Schmid et al., 2008). The humoral response is made up of pathways which synthesise antimicrobial peptides (AMPs), such as the Toll pathway, the IMD pathway and the Jak/STAT pathway (DeGrandi-Hoffman & Chen, 2015; Evans & Spivak, 2010).

As well as the immune responses described above, honey bees also have a highly conserved gut microbiome which may aid in the defence against pathogens (Raymann & Moran, 2018). The gut microbiome is made up of five core bacteria: *Snogressella alvi*, *Gilliamella apicola*, two species of *Lactobacillus*; *Firm5* and *Firm4*, and *Bifidobacterium* (Bonilla-Rosso & Engel, 2018; Maes et al., 2016). Other commonly found species include *Frischella perrara*, *Bartonella apis*, *Parasaccharibacter apium* and an unnamed Acetobateraceae species (Raymann & Moran, 2018). There is evidence that some of these bacteria are involved in immune priming, and a correlation has been identified between the presence of core gut bacteria and the activation of the humoral immune response (Kwong et al., 2017). Studies have also found that when honey bees have gut dysbiosis, they become more susceptible to pathogen infection, indicating that gut bacteria play a role in pathogen defence (Raymann et al., 2017; Raymann & Moran, 2018). While certain gut bacteria have a specific role in protection against pathogens, the bacterial community as a whole influences this defence (Koch & Schmid-Hempel, 2012).

Bees in the pupal stage have guts that are effectively sterile, despite being fed by nurse bees during the larval stage. The gut becomes colonised after emergence, as bacteria are gained through contact with adult bees, mainly through trophallaxis and anal grooming, or

through contact with hive materials (Martinson et al., 2012). Once exposed to bacteria, it can take between three and five days to for bacteria to colonise and the gut microbiome to be established, however, many factors can affect the structure of the established microbial community through the course of a bee's life. These include nutrition, season, age and caste of a bee, disease, stress and the application of antibiotics or chemicals (Raymann & Moran, 2018). The developmental state of the microbiome may impact on the extent to which post-emergent bees are susceptible to CBPV.

The aim of this chapter is to undertake experiments to evaluate the extent to which trophallactic interactions influence susceptibility to CBPV based on the bacterial transference that takes place, and how these interactions can alter the gut microbiome. As trophallaxis is a highly heterogeneous encounter, it was controlled experimentally by inoculating bees with a nurse bee gut homogenate. We hypothesise that younger bees which have not taken part in trophallaxis (or seeding experimentally) are more susceptible to infection via individual feeding of CBPV. Over the first four to five days of a honey bee's life post-emergence, the gut microbiota colonise the gut and likely provide protection against pathogens (Powell et al., 2014). This leads to the hypothesis that preventing contact of emerged bees with worker bees may hinder the colonisation of the gut and produce relatively naive bees. The process of emerging by chewing through the cell cap may provide the emerged bee with some bacteria too, however it is believed that this would be a small amount and only pertains to certain bacteria types (Martinson et al., 2012). Susceptibility may also vary with the level of physiological development of the gut, making younger bees more susceptible to infection due to a more permeable gut than older bees. Honey bee development takes 8-10 days after emergence, during which time the cuticle hardens, glands develop, and fat bodies grow, all of which may affect susceptibility to pathogens (Winston, 1987). We aim to test these hypotheses through experiments which quantify the microbiota in relation to emergence and age at the time of exposure to CBPV treatment, both of which we can manipulate using homogenates. Honey bees kept in pots were fed a CBPV homogenate and given access to a gut homogenate, and the effect of these treatments on their symptoms, deaths and gut microbiome were recorded.

2.2 Methods

2.2.1 Gut homogenate preparation

Microbial gut contents were extracted from nurse bees following an adapted method from Motta & Moran (2020). Twenty-four mixed age worker bees were collected from the outside frame of a hive and immobilised by chilling. Guts were removed from bees by placing them on ice blocks and using sterile forceps to gently pull on the stinger to extract the rectum, ileum and ventriculus while keeping the head and thorax in place. The guts formed a homogenate by combining two guts and adding them to a 1.5 ml Eppendorf tube with 250 μl of Phosphate-buffered saline (PBS) solution. The tubes were then vortexed at 3000 g for 10 seconds. The contents of all 12 x 1.5 ml tubes were combined by pipetting into a 30 ml sterilin container, producing approximately 3ml of gut homogenate. The absence of Nosema spores was confirmed through the investigation of 20 μl of homogenate on a slide under magnification using a microscope. The homogenate was then stored in the fridge and used within 48 hours. An equal amount of sucrose solution was added and mixed by inversion prior to use.

2.2.2 Honey bee collection

Honey bees for the following experiments were collected from the roof apiary at Ridley Building 2, Newcastle University. The apiary was checked regularly throughout the year for overall health and treated for Varroa infestations when necessary. Bees were also tested for CBPV and Nosema throughout the year. To obtain young bees, frames of capped brood were removed from the hives and kept in an incubator at 33 °C. To ensure bees were kept as pathogen-free as possible, frames were kept in an incubator in a laboratory used solely for the purpose of collecting emerging bees, and at no point contained CBPV or any materials which came from the laboratory used for the infection of CBPV. The frames were stored individually in plastic boxes (dimensions: 44cm x 26cm x 8cm) where they could stand upright, preventing any part of the comb from touching the box. This also allowed bees which emerged to be contained to their frame. Any bees which emerged prior to the experiment or during periods where they were not observed were returned to their original hive to ensure the age of the bees was known and to keep the amount of time on the frame to a minimum. The examination of the contents of a few cells allowed

the identification of an approximate date of emergence.

2.2.3 Crude extract

To create a CBPV crude extract a method was adapted from the methods listed in Ribiere et al. (2000). Ribiere et al. (2000) inoculated young bees by injecting 1.5 μl of 1.35 x 10^5 CBPV into their heads. Once they died within a matter of days, the head, thorax and abdomen were separated via dissection and stored in a freezer. Eight bees were injected in the heads in the same way as described by Ribiere et al. (2000) and were dissected into heads and abdomens and kept separate. The body parts were ground with a pre-chilled pestle and mortars and 2 ml of 0.9% sodium chloride solution, which was also pre-chilled. This was done until the remains were considered moderately homogenous. The homogenate was collected with a P1000 pipette and then added to a 2 ml Eppendorf tube. The Eppendorfs were then spun in a centrifuge at 4 ^{o}C for 30 minutes at 3000 g. The supernatant of both the head and abdomen samples were collected and added together in a sterile 30 ml sample tube. It was then filtered through a 0.45 μm syringe filter tip and aliquoted into Eppendorf tubes of 400 μl with the intention of use for feeding.

2.2.4 Experimental set-up

The susceptibility to infection was investigated in relation to age at the time of exposure and the microbiota present in the gut. Physiological development and the condition of the gut microbiome vary with age and may impact susceptibility to CBPV. To investigate this, bees were fed a virus or buffer solution either 1) immediately after emergence, 2) one day after emergence, or 3) two days after emergence. Newly emerged bees were used as their age is known and are therefore suitable for studies involving longevity, and they have relatively naive gut microbial communities (Powell et al., 2014; Williams et al., 2013). Gut microbiome naivety was also investigated through seeding bees with nurse bee gut homogenate. These bees are referred to as 'seeded', and while bees which eclosed naturally on a frame without nurse bee contact would have some microbiota present, we theorised that levels would be vastly reduced, so these bees are referred to as naive. Finally, all bees were fed a solution, either a buffer solution used as a negative control, or a virus solution. Feeding a buffer solution ensures that effects caused by being handled during feeding or from the buffer solution itself are known. The overall method is presented in Figure 2.1.

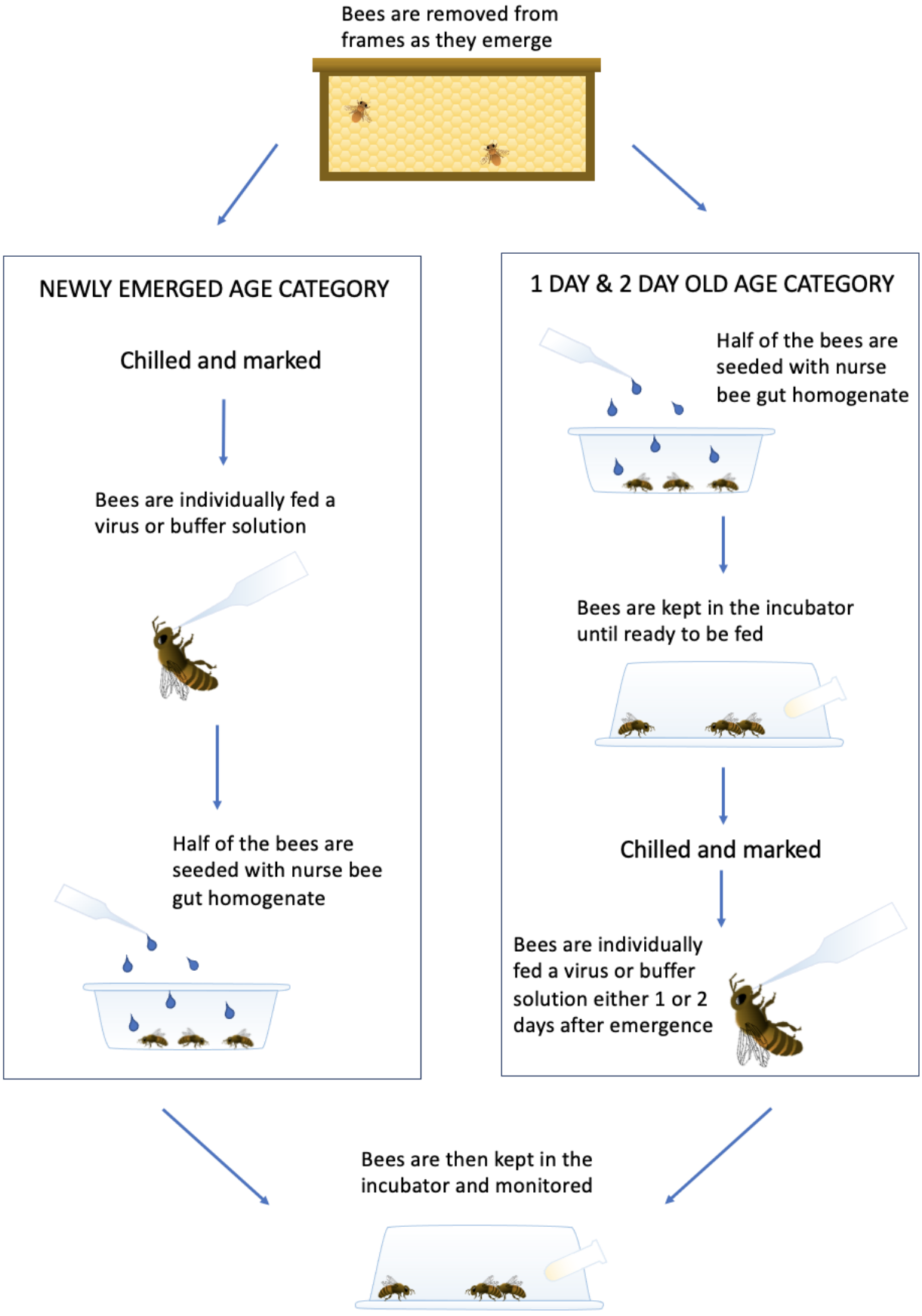


Figure 2.1: The experimental set-up of each treatment.

All bees were removed from the frames immediately after emergence using standardised methods described in Williams et al. (2013). Zero hour old bees were the first to be treated, and were not given access to sucrose syrup so that they had a period of starvation between eclosing and being fed buffer or virus to ensure they would have an apetite and ingest the solution. Bees were immobilised via chilling to avoid agitating them, which could potentially lead to stinging responses. Bees were chilled in the fridge at 4 °C for approximately 15 minutes, or until immobile. In order to identify and track each bee within a pot individually, they were uniquely marked using POSCA paint pens (POSCA, PC 5M, bullet-shaped 1.8-2.5mm) following the mark scheme in Figure 2.2. Half of the total bees were then fed 3 μl of a 1:1 virus and syrup solution, which was fed individually using a 200 μl pipette. This is slightly less than what is recommended by Miranda et al. (2013), however it was used in a higher concentration and matched the quantity of CBPV previously injected into bees in pilot studies. It also proved to be a manageable amount for a bee to eat. The solution was administered by holding the bee in an upright position, gently placing the pipette tip in between the mandibles and gradually expelling. If the liquid formed a bubble outside the mandibles, the pipette tip was moved below the mandibles to encourage the bee to drink the liquid. The bee may also use its proboscis to drink the liquid from the pipette tip. The other half of the bees were fed a buffer solution in the same way, consisting of 0.9% sodium chloride solution in place of virus, as this is the same solution the virus was stored in. In this way, we can see the effect the buffer solution may have on the bees, and separate this from the effect of the virus. Finally, the seeded bees were gently rolled in the homogenate by pot inversion. The bees were then placed into a new clean pot with two 2 ml sucrose sugar feeders and placed in the incubator.

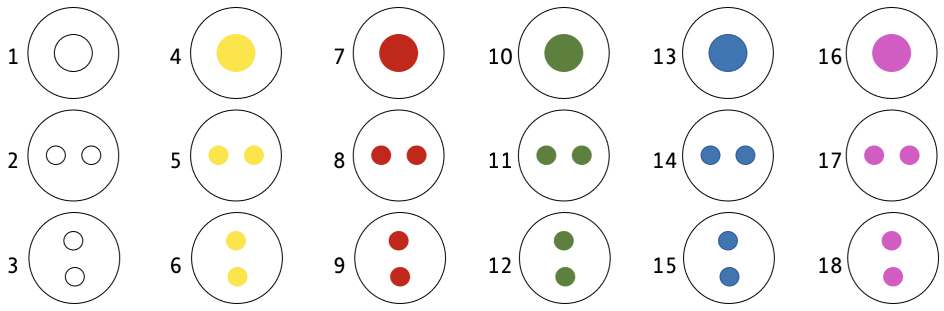


Figure 2.2: The mark scheme used to make bees individually identifiable. Outer black circles represent the thorax where the mark was placed. The marks used are the same as the total number of bees per pot.

Bees in the 24 hour and 48 hour old age categories bees were removed from the frame in the same way as the zero hour old age category, and were placed in pots immediately after emergence with access to sucrose syrup feeders. Half of the bees were seeded using the method described, and then all were placed in the incubator until 24 or 48 hours post eclosure. One hour prior to feeding, their sucrose syrup was removed to ensure they would be hungry enough to ingest the syrup solution. At the 24 or 48 hour mark post eclosure, they were chilled, marked, and once responsive were individually fed either the virus or buffer solution. They were then placed back in the incubator.

Bees were placed in plastic containers (cage dimensions: approx. 0.10 m diameter × 0.04 m height) and given access to two feeders (2 ml Eppendorf tubes with a drilled hole at one end) filled with approximately 2 ml of sucrose syrup (73% w/w; Ambrosia® bee food syrup, Nordzucker AG, Germany). Sucrose solution was used as a carbohydrate source for honey bees because it likely is sterile and uncontaminated, and is commonly used in bee experiments (Forsgren & Fries, 2010; Williams et al., 2013). Bees were kept in a dark incubator at 30 °C and 66% humidity for the duration of the experiment. These specifications are in agreement with suitable methods for honey bee research set out by Williams et al. (2013). In order to reduce bias and minimise the effects of varying conditions within the incubator, pots were assigned a number using a random number generator and were placed in the incubator based on this order.

Bees were collected and treated over two days. The treatments were split into two groups, with one sample of each age category collected and treated each day, and two samples of each age category used in total. Approximately 15 bees per pot were used, with three replicate pots for each of the newly emerged and 1 day old age treatments, and two replicate pots for each of the 2 day old treatments due to constraints on numbers of emerging bees. The required sample size used in honey bee research varies based on the aims of the study, however, Pirk et al. (2013) suggests 15 bees per pot due to the potential for random events which may alter analysis and interpretation of results due to causes unrelated to the pathogen in question. The number of replicates used falls within the range suggested also.

Observations began the day after the experiment was set up. Bees were monitored each day for the duration of the experiment (30 days). Symptoms were recorded (Table 2.1) and dead bees were removed and placed in tubes, and stored initially at -20 °C, before being moved to a -80 °C freezer for molecular analysis. Syrup feeders which were less

than half full were replaced with a fresh 2 ml tube of syrup. Bees which died by day 16 also had their guts removed and stored at -20 °C in preparation for molecular analysis. Bees which died after this point had increased fragility of their guts, making the removal of the guts without destroying them very difficult.

Table 2.1: Symptom groupings used in experiments

Group	Symptom	Description
Lethargy	Lethargic	Bees are slow moving and largely unresponsive
Disorientation	Disorientated	Often change direction, falling from walls and being stuck on back
Partial paralysis	Tongue out	Proboscis stays out of mouth for prolonged periods of time without being used
	Leg paralysis	Some legs are unable to move but bee can still walk albeit with difficulty
	K-wing	Hindwings are perpendicular to body and forewings sit along the body
	Wing shimmering	Wings shake or vibrate
	Shaking	Bees tremble or twitch and have jerky movements
Paralysis	Paralysis	The majority of legs and the body are paralysed, with the bee unable to move or turn itself over

2.2.5 Molecular analysis

To identify which bees became infected with CBPV, all bee and gut samples went through the RNA extraction process and were tested for the presence and quantity of CBPV and honey bee 18s RNA using real-time qPCR. The honey bee 18s result was used as a measure of the effectiveness of the RNA extraction. Using the serial dilution of the CBPV PCR and the 18s PCR, the relative concentration of CBPV and the ratio of CBPV:18s in each bee sample was calculated. We used 16s Amplicon sequencing to identify the bacteria found in the gut samples.

2.2.5.1 RNA Extraction

The following protocol was based on the BOMB.bio protocol and used on individual bee and bee gut samples (Oberacker et al., 2019). Lysis buffer was prepared based on methods described by Boom et al. (1990). In order to make 150 ml of lysis buffer, 120 g of guanidiniun thiocyanate (Guanidine thiocyanate, Apollo Scientific) was dissolved in 100 ml of 0.1 M Tris hydrochloride, pH 6.4 (Fisher Scientific). Once dissolved, 22 ml of 0.2 M Ethylenediaminetetraacetic acid (EDTA, Melford labs) adjusted with NaOH to a pH of 8.0 and 2.6 g of Triton X-100 (Fisher Scientific) were added. Finally, 1500 μl of 1% Antifoam B Emulsion (Sigma) was added and the solution was homogenised.

In order to grind the bee samples, individual bees were added to a 2 ml Precellys o-ring sealed tube containing approximately 250 μl of 2.3 mm zircona grinding beads and 500 μl of lysis buffer. Bees were added to tubes using forceps which were sterilised between use on each sample using a Chemgene solution. Samples were then ground using a Precellys (Bertin Instruments) for three 20 seconds cycles at 6800 g, with a 15 second pause between each cycle to prevent overheating of the sample. A further 500 μl of lysis buffer was added to each sample and before grinding in the Precellys for the same cycles again. Gut samples were not homogenised in the Precellys, and instead 500 μl of lysis buffer was added directly to the 1.5 ml Eppendorf tube they were stored in, and ground with a micropestle. A further 500 μl of lysis buffer was added and the samples were vortexed for approximately 20 seconds.

The debris was pelleted via centrifugation for two minutes at 13000 g. 240 μl of the lysate was collected and added to a 96 well round bottom plate. A plate seal was added and the plates and remaining samples were stored at -80 ^{o}C.

Plates were removed from the freezer and defrosted at room temperature. On each plate, 240 μl of RNase free water was added to one well to create an extraction blank used as a control. Using a multichannel pipette, 300 μl of isopropanol was added to each well and mixed thoroughly by pipetting. Silica-coated magnetic beads were used to bind to RNA present in samples, and so 40 μl of SpeedBead magnetic carboxylate modified particles (Sigma Aldrich, 1:10 diluted from stock in TE buffer) was added to each well and mixed thoroughly by pipetting. The plates were placed on a magnetic stand, and once the beads had settled the supernatant was removed and discarded. The plate was removed from the magnetic stand, and 400 μl of isopropanol was added to each well and mixed by pipetting.

The beads were settled on the magnetic stand again, and the supernatant was discarded. The plate was removed from the stand again, and 400 μl of 80% ethanol was added. The beads were settled on the magnetic stand, and the supernatant was discarded once again. The ethanol wash was repeated a further three times, and on the fourth wash the supernatant was left on, and the plate was stored at -80 °C.

The plates were defrosted at room temperature, and then placed on the magnetic stand so the supernatant could be removed completely. The plate was then dried at 50°C for 30 minutes. Diethyl pyrocarbonate (DEPC) treated water was made using MilliQ (reverse osmosis purified) water, with 1 ml of DEPC added per 100 ml of MilliQ, and mixed thoroughly. The DEPC-mixed water was then left to incubate for 12 hours at 37 °C, and then autoclaved for 15 minutes in order to inactivate any RNases or DNases, and to remove all traces of DEPC. After the incubation of the plate, 44 μl of DEPC treated water was added to elute RNA and mixed by vortex for approximately 10 seconds, followed by centrifugation for 10 seconds, followed by vortexing again for 10 seconds. The magnetic beads were then pelleted on the magnetic stand, and the eluted RNA was transferred to a fresh well. A 1:5 solution of DNase I and 10x Mg^{2+} Reaction buffer (Promega) was made, and 6 μl was added to each well. The plate was then sealed, placed on a plate shaker for two minutes, then added to the centrifuge for approximately 10 seconds, and then placed in the incubator at 37 °C for 30 minutes. Afterwards, the plate was shaken for a further two minutes, and then 5 μl of stop solution (EDTA) was added to each well. The plate was once again sealed and shaken for two minutes, and then added to the incubator at 65 °C for 10 minutes. Plates were then stored at -80 °C. Prior to PCR analysis, the plates were defrosted and diluted to 1:100 with RNase-free water, based on the high Cq of positive samples exposed to CBPV in a similar way. The plates were then stored at -80 °C in preparation for PCR analysis.

2.2.5.2 DNA extraction

DNeasy extraction was performed on bee gut samples. This also involved using a lysis buffer, of which approximately 1 ml was made using 1 ml TE buffer, 12 μl Triton X-100 and 0.02 g lysozyme. The solution was then shaken to ensure the enzyme powder has dissolved.

200 μl of each sample was aliquoted into a labelled tube and spun at 10,000 g for 10 minutes. The supernatant was then removed and transferred to another labelled

Eppendorf and set aside. The pellet was then re-suspended in 200 μl lysis buffer and mixed by pipetting. An extraction blank was created by adding 180 μl of lysis buffer to a fresh tube. The samples were then incubated at 37 °C for 30 minutes. The supernatant that was set aside was then added back into the recently incubated sample tubes and mixed, and then approximately 200 μl removed, leaving a clear pellet. The remaining mixture was then stored at -80 °C. The following protocol was based on a Qiagen DNeasy extraction kit. 200 μl of buffer AL and 24.4 μl of proteinase K were added to the pellet, and the samples were incubated on a hot plate at 56 °C for 30 minutes. The samples were removed from the plate and 224.4 μl of 100% ethanol was added to each tube and mixed thoroughly by vortexing. The entire solution was then transferred to a DNeasy Mini spin column in a 2 ml collection tube, and spun at 800 g for one minute. The column was then transferred to a fresh collection tube and the previous column was discarded. 500 μl of buffer AW1 was added and the tubes were spun again at 800 g for one minute. The column was transferred to a fresh collection tube again and the previous collection tube was discarded. The tubes were spun at 14000 g for three minutes, and then flow-through from the collection tubes was discarded and the column was placed back into the collection tube. The tubes were spun a final time at 14000 g for one minute, and the columns were transferred to a fresh Eppendorf. 50 μl of AE buffer was then added to the tubes, and they were left to incubate at room temperature for one minute. The tubes were then spun a final time at 8000 g for one minute. The columns were discarded and the samples were stored at -80 °C.

2.2.5.3 PCR

Methods for RT-qPCR are as detailed in Budge et al. (2020). Samples were tested for CBPV and 18s using the relative standard curve method of qPCR (Ward et al., 2007). A dual labelled probe for CBPV, CBPV_T (TCAAGAACGAGACCACCGCCAAGTTC), a forward primer, CBPV_F (CGCAAGTACGCCTTGATAAAGAAC) and a reverse primer, CBPV_R (ACTACTAGAAACTCGTCGCTTCG) were designed to the RNA-dependent RNA polymerase gene (Blanchard et al., 2007). *A. mellifera* 18s rRNA was used to normalise the quality of the RNA, using J307465-955F (TGTTTTCCCTGGCCGAAAG), 1016R (CCCCAATCCCTAGCACGAA) and dual labelled probe 975T (CCCGGGTAACCCGCTGAACCTC) (Budge et al., 2020). All fluorogenic probes were modified 3' with TAMRA (tetra-methylcarboxyrhodamine) and

5' with FAM (6- carboxyfluorescein). The real-time reactions were set up in duplicate using hydrolysis probe (TaqMan®) chemistry in 96-well reaction plates using iTaq Universal Probes 1-Step Kit (Bio-rad, Cat no: 1725141), according to the manufacturer's protocols. Each reaction comprised 5 μl iTaq universal probes reaction mix (2×), 0.25 μl iScript advanced reverse transcriptase, 0.4 μl (7.5 μM) of each forward and reverse primer, 0.2 μl (5 μM) probe, 2.75 μl of water and 1 μl of RNA in a final volume of 10 μl. A QuantStudio5 (Quant Studio) was used to conduct the reactions and following published protocols (Blanchard et al., 2007; Ward et al., 2007). In order to identify positive and negative calls and determine the quantification cycle (Cq) for each sample we used QuantStudio5 software using manual threshold determination. Both duplicates were required to have positive CBPV Cq values to be deemed positive.

After each plate had been analysed, a serial dilution was conducted using the sample with the highest Cq for both CBPV and 18s, and was then tested for CBPV and 18s. This was used to calculate a CBPV:18s value for each sample tested.

2.2.5.4 16s Amplicon Sequencing

Gut samples were sequenced to identify the community of gut microbes present. First, a conventional PCR for 16s of the samples was done, using forward primer 16s 341F (5'-**TTTCTGTTGGTGCTGATATTGC**CCTACGGGNGGCWGCAG-3'), and reverse primer 16s 805R(5'-**ACTTGCCTGTCGCTCTATCTTC**GACTACHVGGGT ATCTAATCC-3'), both of which had Minion tags (the portions in bold) (Klindworth et al., 2013). The mastermix used contained 6 μl HF buffer, 0.9 μl deoxynucleoside triphosphate (dNTPs), 0.9 μl forward primer, 0.9 μl reverse primer, 20 μl MBGW and 0.2 μl phusion DNA polymerase per 1 μl of sample. This was distributed on a 96-well plate, and a positive and negative control were added. The plate was run on a BioRad thermocycler at the conditions specified in Klindworth et al. (2013).

The PCR products were assessed using gel electrophoresis. A 1% agarose gel was made up by mixing 200 ml TBE and 2 g agarose in a conical flask, heated in a microwave until the agarose was dissolved. The flask was cooled under running water, and then 15 μl of GelRed was added. This was poured into a plate and left to set. The plate was then placed in a gel tank, and TBE buffer was added to cover the entire plate and fill the wells. To prepare the samples, 5 μl of each was moved into a new plate and 1 μl of loading dye was added. This was mixed by centrifugation, and 5 μl of each sample was loaded onto

the plate. In the first well of each row on the gel plate, 5 μl of a 100 bp HyperLadder™ (Meridian Bioscience, Cat No. BIO-33056) was added. The gel ran for 45 minutes at 100 V. The gel was analysed using a gel dock view to ensure that the sample's bands were at approximately 500 base pairs and that the negative control is clear.

The PCR was then cleaned to remove smaller fragments from the PCR reactions. A bead clean was performed using 20 μl of AMPure beads added to each sample. These samples were then left to incubate at room temperature for five minutes, and then added to a magnetic stand. Once the beads had pelleted, the supernatant was removed and discarded. While still using the magnetic stand, 200 μl of 80% ethanol was added and removed. This was repeated, ensuring that all residual ethanol was removed and the beads were removed from the magnetic stand and left to dry for five minutes. The beads were then re-suspended in 28 μl distilled water and incubated at room temperature for five minutes. The samples were placed on the magnetic stand and once the beads had pelleted, 24 μl of the supernatant was removed and retained.

Using this sample, DNA barcoding was conducted following the PCR barcoding (96) amplicons (SQK-LSK109) protocol (version: PBAC96_9069_v109_revQ_14Aug2019). This protocol was modified for Flongle use in that both the quantities of PCR product and LongAmp mastermix were halved. In addition to this, the denaturation, annealing and extension sections of the amplification were each run for 15 cycles. The resulting products were pooled in equimolar concentration.

The DNA repair and end-prep, adapter ligation and clean-up and the loading of the Flongle flow cell were performed following the Genomic DNA by Ligation (SQK-LSK109) protocol (version: GDE_9063_v109_revAE_14Aug2019).

Nucleotide BLAST (blastn) was used to assign taxonomic sequences against the NCBI Nucleotide Collection. Sequences with a taxon match above 88.9% were considered a match and retained for analysis.

2.2.6 Statistical analysis

All analyses were undertaken in R version 4.2.2 (RCoreTeam, 2022).

2.2.6.1 Generalised linear mixed models

As bees were kept in groups in pots, we expect that there may be an effect of pot. This is due to the varying possibility of transmission of CBPV within different pots depending on the success of inoculation per bee, and the effect the total number of bees has on the bees present, which varies by pot as bees die. Generalised linear mixed models (GLMMs) were used to account for any unmeasured variation in the response that could be attributed to pot by using pot as a random effect, with log base 10 CBPV concentration of each honey bee as the response, using a gaussian distribution with the default link function. Models were fitted with the *glmmTMB* package, which can be used to fit linear and generalised linear mixed models with adaptations to different data, including zero-inflated data (Brooks et al., 2017). When investigating the number of bees that tested positive for CBPV by qPCR, a zero-inflated GLMM was used as 75% of the data were zeros.

2.2.6.2 Survival and Competing risks analyses

Survival analysis was initially performed to assess the risk of death (all-cause hazards) and then repeated to investigate death due to CBPV infection (cause-specific hazards), using the packages *survival* and *survminer* (Kassambara et al., 2019; Therneau, 2020). Kaplan-Meier curves were calculated, comparing the survival rates of each treatment. The curves were then compared using a log-rank test. For all-cause hazards analysis, bees were censored if they did not die within the duration of the experiment (30 days). For cause-specific hazards analysis, bees which did not test positive for CBPV using RT-qPCR were censored. These are both examples of type I censoring; for all-cause hazards, the study was designed to last 30 days, and the only reason for censoring is the event of interest not taking place within this time frame. For cause-specific hazards, we simply analyse the cause of death and incorporate this into the censoring mechanism. In the analysis of survival data using the Kaplan-Meier curve and the log-rank test, individuals which are censored are assumed to have the same likelihood of survival as those who remain in the study (Bland & Altman, 1998).

Cox proportional hazards models were used to investigate the effects of each treatment on the survival in the experimental pots, with treatments as the predictor variables. Models were fitted where death was the event of interest (all-cause hazards), and where death with a positive CBPV qPCR result was the event of interest (cause-specific hazards).

With 546 bee samples and a required power of 0.8, we can predict an effect size of 1.5 when anticipating 50% mortality for all-causes hazard modelling. Of these 546 bees, 378 were fed virus, meaning with an anticipated CBPV-caused mortality of 50%, and a required power of 0.8, we can expect to detect an effect size of 1.75 arising from the CBPV treatment (Therneau & Grambsch, 2000). Effect sizes are presented as hazard ratios.

Assumptions of proportionality in the hazards were assessed by analysing the Schoenfeld residuals for the models and a test of proportionality (Therneau & Grambsch, 2000). Since we had prior evidence that bees might die as a result of factors other than CBPV infection, we used a competing risks analysis to compare treatments under the assumption that any bee that was recorded with CBPV by a PCR test had died due to CBPV infection, and those with a negative PCR test were assumed to have died from other unmeasured causes (for example, the artificial nature of the experimental set-up).

Competing risks analysis was performed using the package *cmprsk* (Gray, 2022). This analysis was used to isolate the risk of death from infection by CBPV and other potential risks introduced due to the experimental set-up. A Fine-Gray subdistribution hazard model was used, which measures the probability of dying due to CBPV infection at a certain time point, if death due to other causes has not taken place (Berger et al., 2020; Fine & Gray, 1999). This analysis was performed on bees in viral treatments only. Bees were grouped into three categories: death due to CBPV (a positive PCR result), death due to the experiment (a negative PCR result), and all bees that survived to the end of the experiment.

The standard way to assess the effect of the grouping variable in survival analysis is the inclusion of frailty, which effectively assesses variation in survival that might be attributed to the grouping variable, which in this case is pot. At each point, the models failed to converge when assessing frailty and therefore frailty was not included. This is likely due to differences in survival between pots of the same treatment, meaning that commonalities between survival curves of each pot cannot be identified, and therefore a mean curve for each treatment could not be generated.

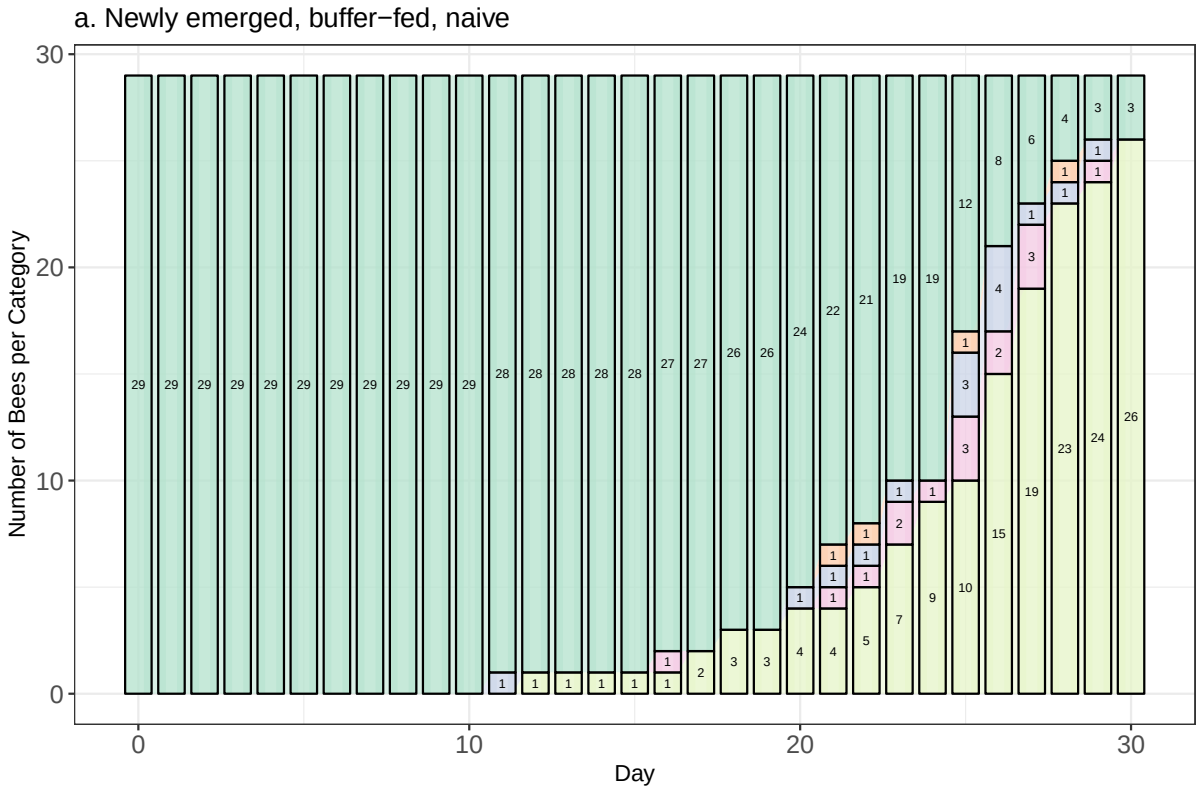
2.2.6.3 Ordination

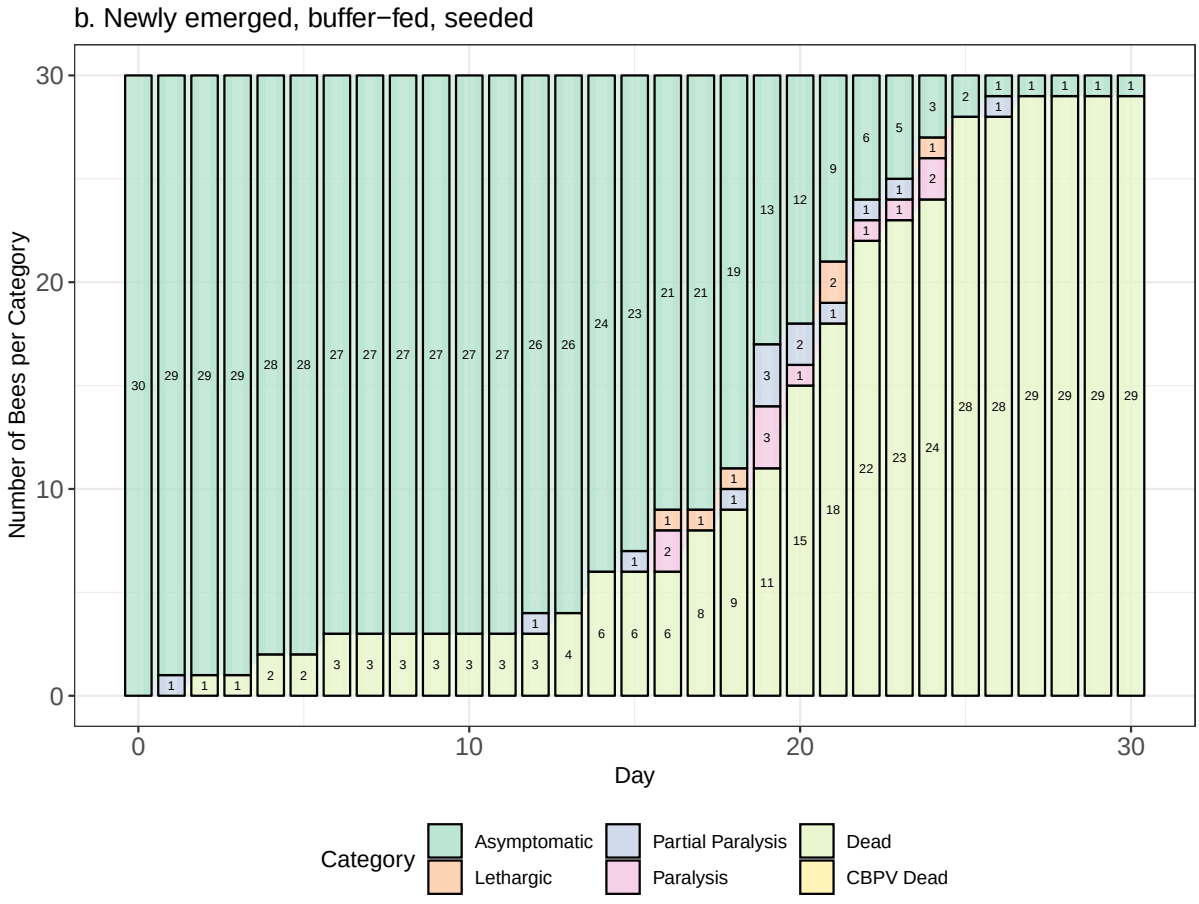
Correspondence Analysis (CA) was used to analyse the bacterial communities identified through DNA sequencing of honey bee gut samples through unconstrained ordination

(Legendre & Legendre, 2012; Ter Braak, 1986). This was done using the *vegan* package (Oksanen et al., 2022). Bacterial species were grouped by genus or family in order to see the overall effect of closely related species. Species which were only observed in one sample were removed from the analysis. The same data were then analysed using Canonical Correspondence analysis (CCA), in order to investigate the effects of age at time of exposure, fed virus or buffer, seeding, time to death and CBPV qPCR result on the bacterial community present in each bee.

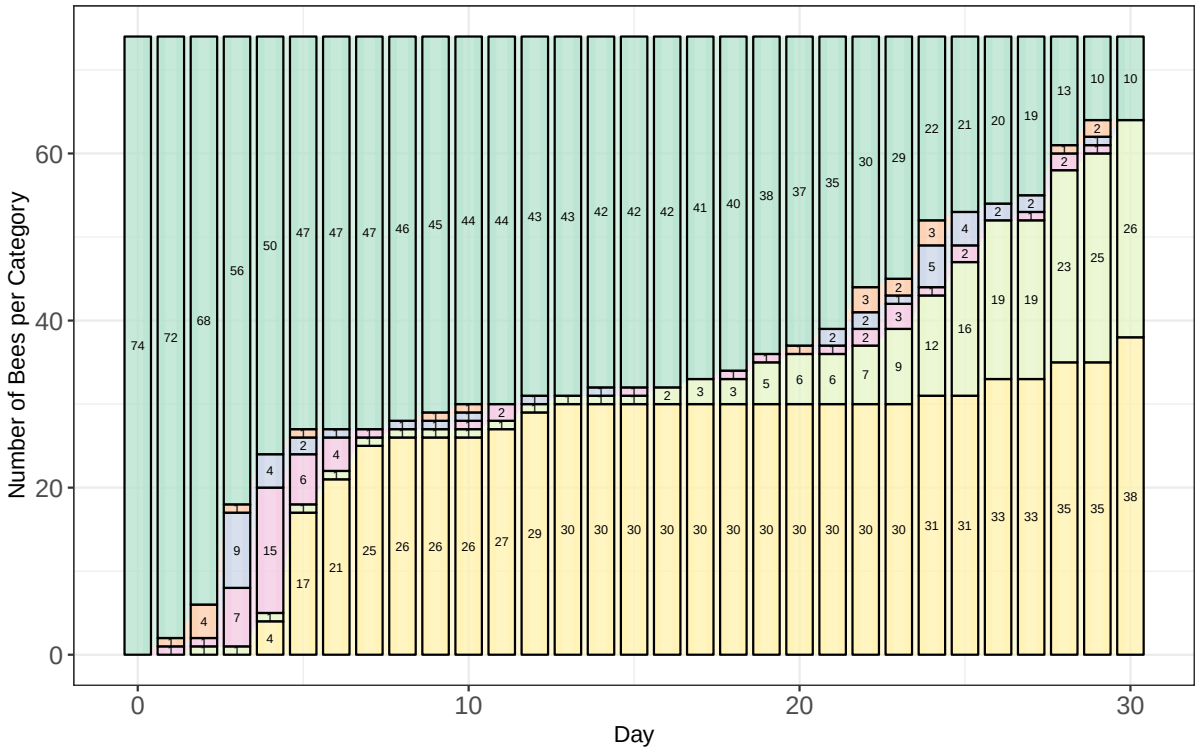
2.3 Results

The newly emerged virus-fed naive and seeded bees both had noticeably higher numbers of symptomatic and dead bees in the first 10 days of the experiment than any other treatments (Figure 2.3c and d). Both of these treatments had bees which showed a range of symptoms. The daily deaths seen in these treatments decreased from days seven to eight, and the mortality rate from this point on remained fairly constant. In all the naive treatments, with the exception of the newly emerged virus-fed treatment, multiple deaths per day did not become a common occurrence until approximately day 15 or later, regardless of whether the bees were fed virus or buffer. All the seeded treatments had a few earlier mortalities, occurring between days one and 10, again regardless of whether the bees were fed virus or buffer. Many of these deaths did not follow an observation of any symptoms. With the exception of the newly emerged virus treatments, there was no obvious difference in the onset of symptoms or mortality between bees fed virus or buffer at different ages. At around day 15, all treatments showed a similar decline in survival, often showing symptoms beforehand.

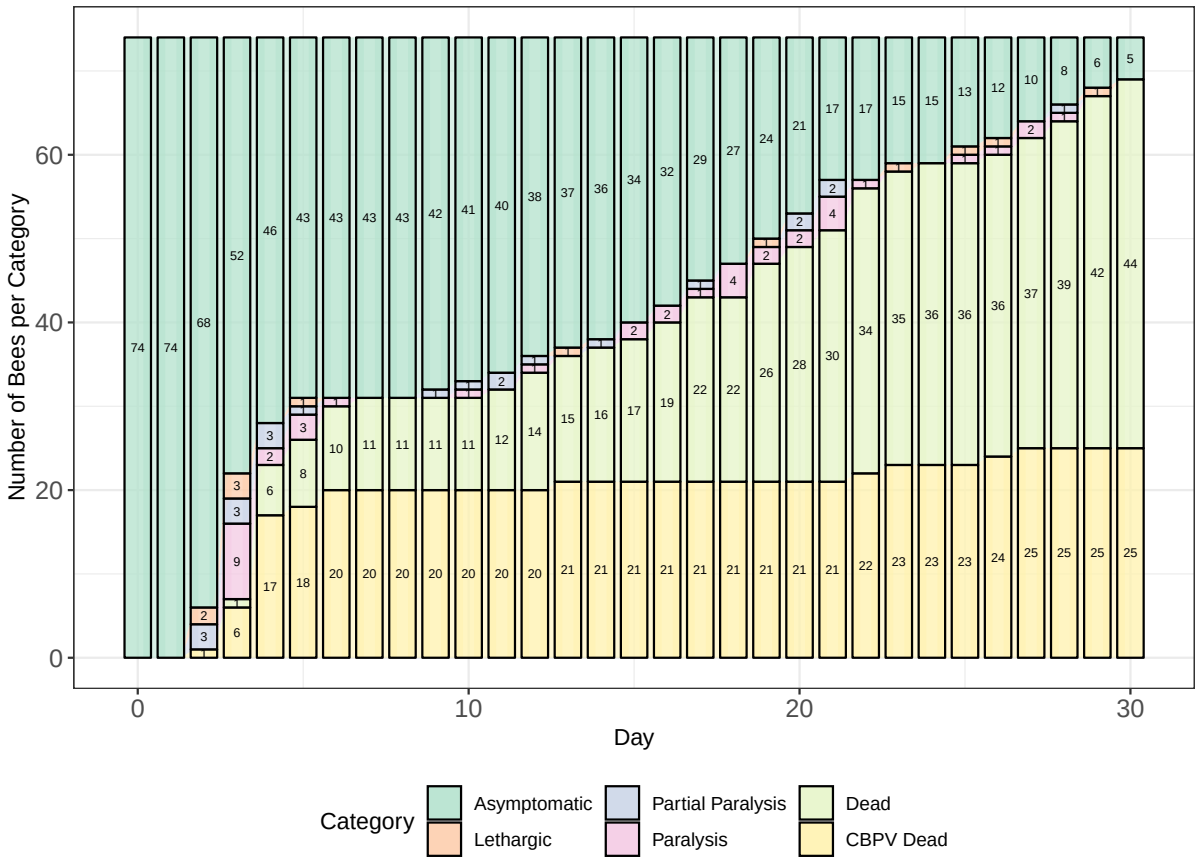

a. Newly emerged, buffer−fed, naive
Number of Bees per Category
Day
0
10
20
30


b. Newly emerged, buffer−fed, seeded
Number of Bees per Category
Day
0
10
20
30
Category
Asymptomatic
Lethargic
Partial Paralysis
Paralysis
Dead
CBPV Dead

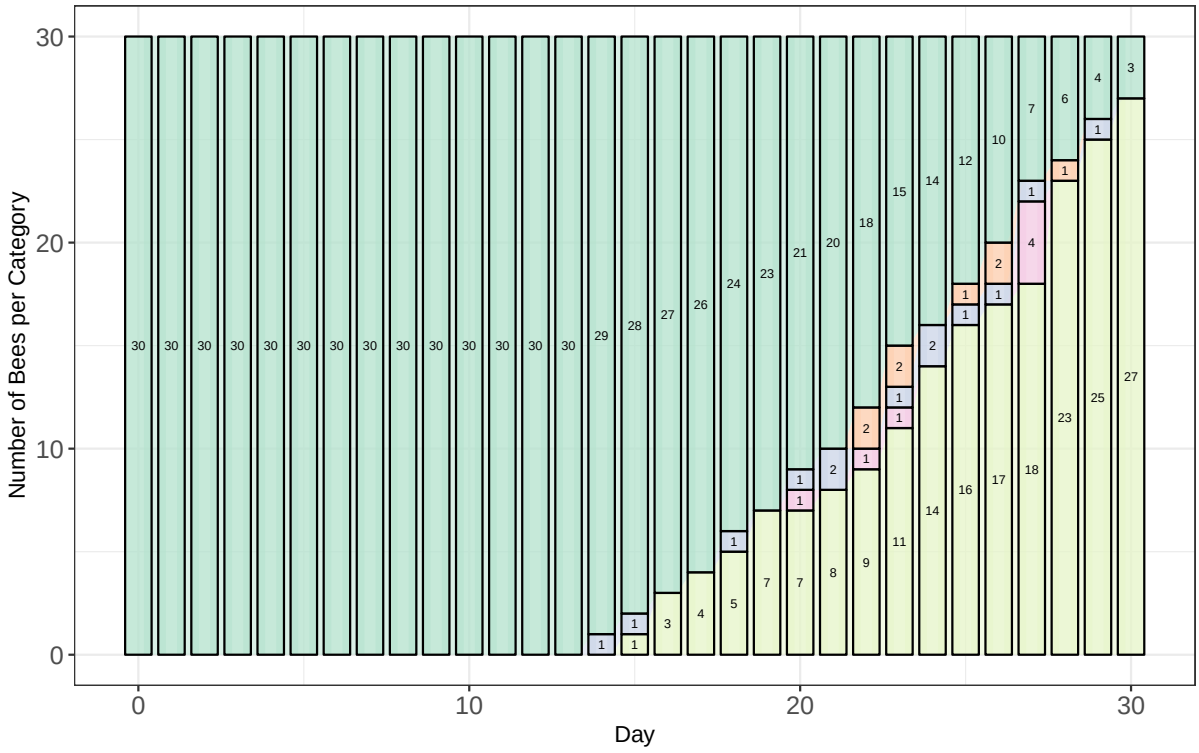
c. Newly emerged, virus−fed, naive



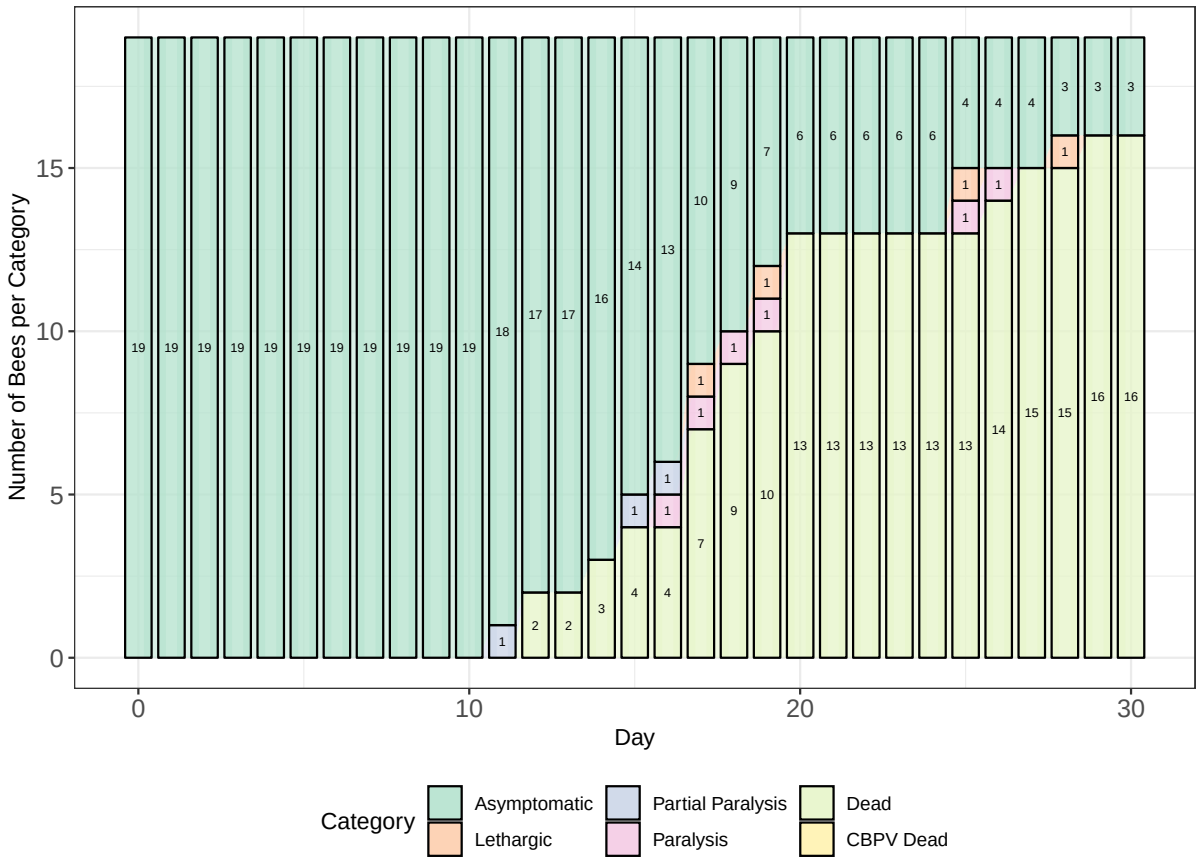
d. Newly emerged, virus−fed, seeded



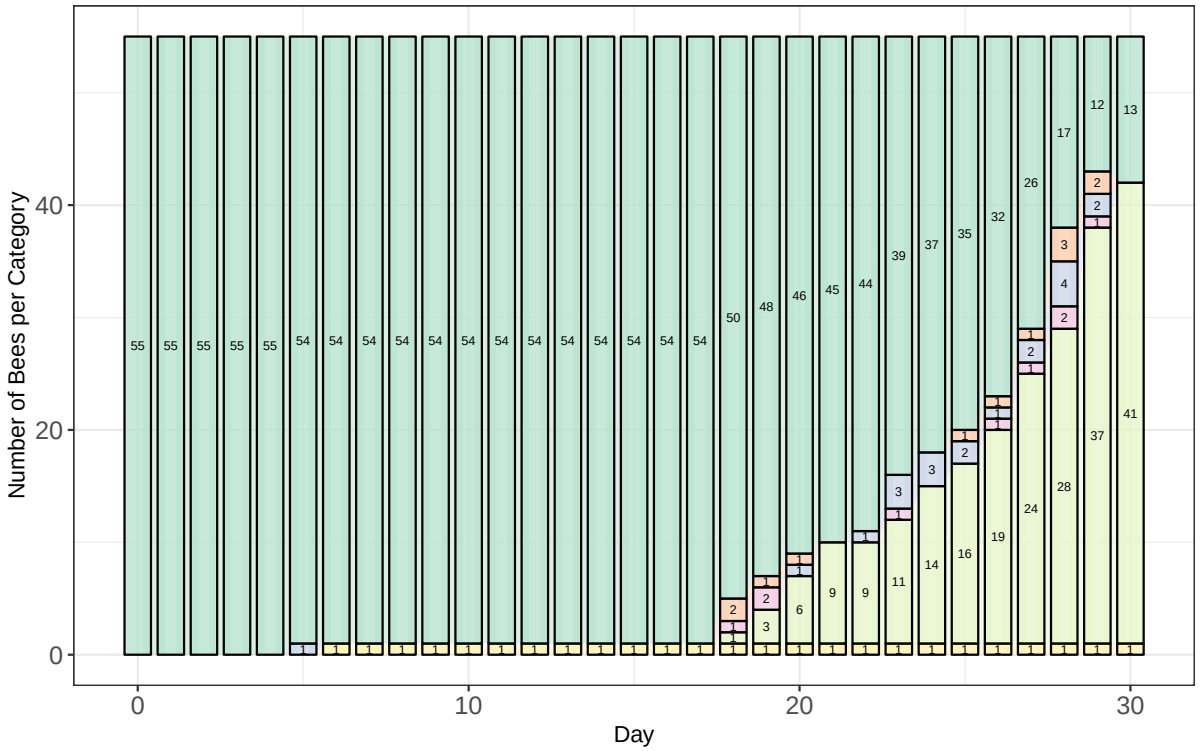
e. 24 hours, buffer−fed, naive



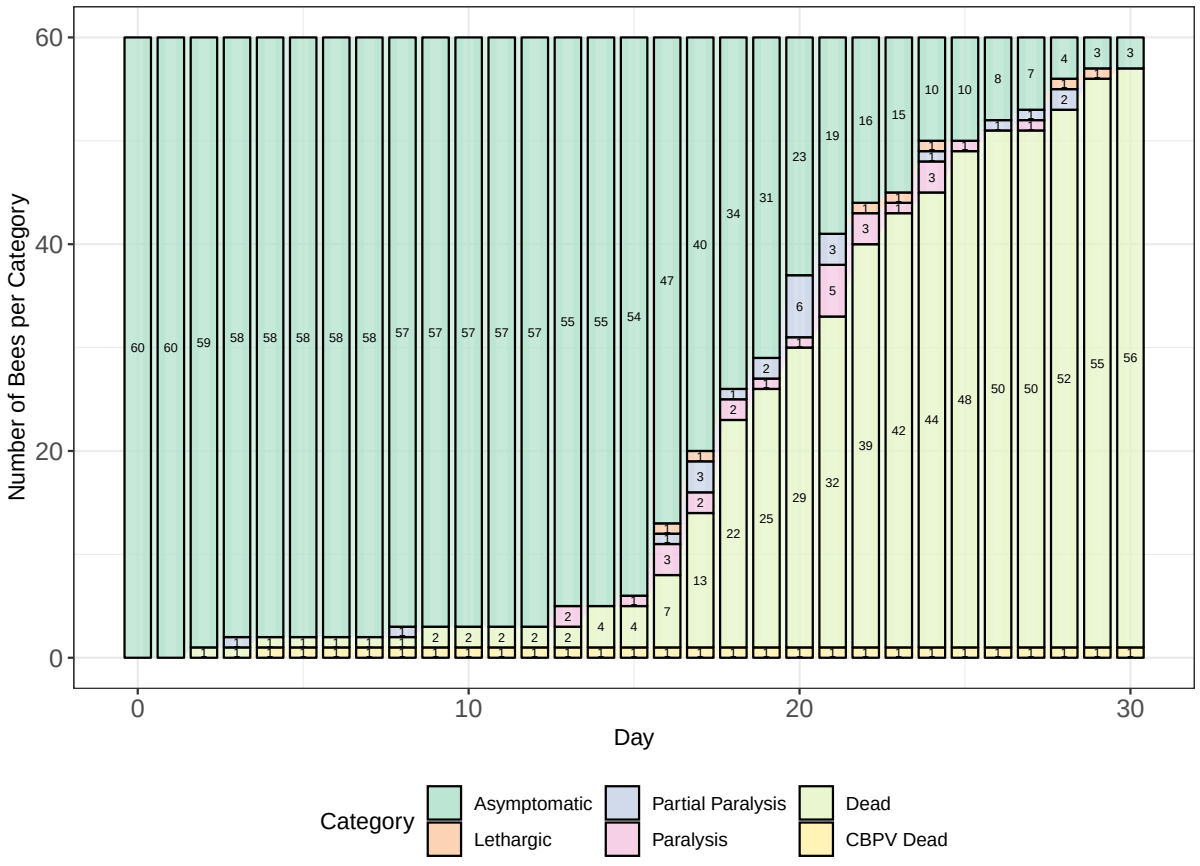
f. 24 hours, buffer−fed, seeded



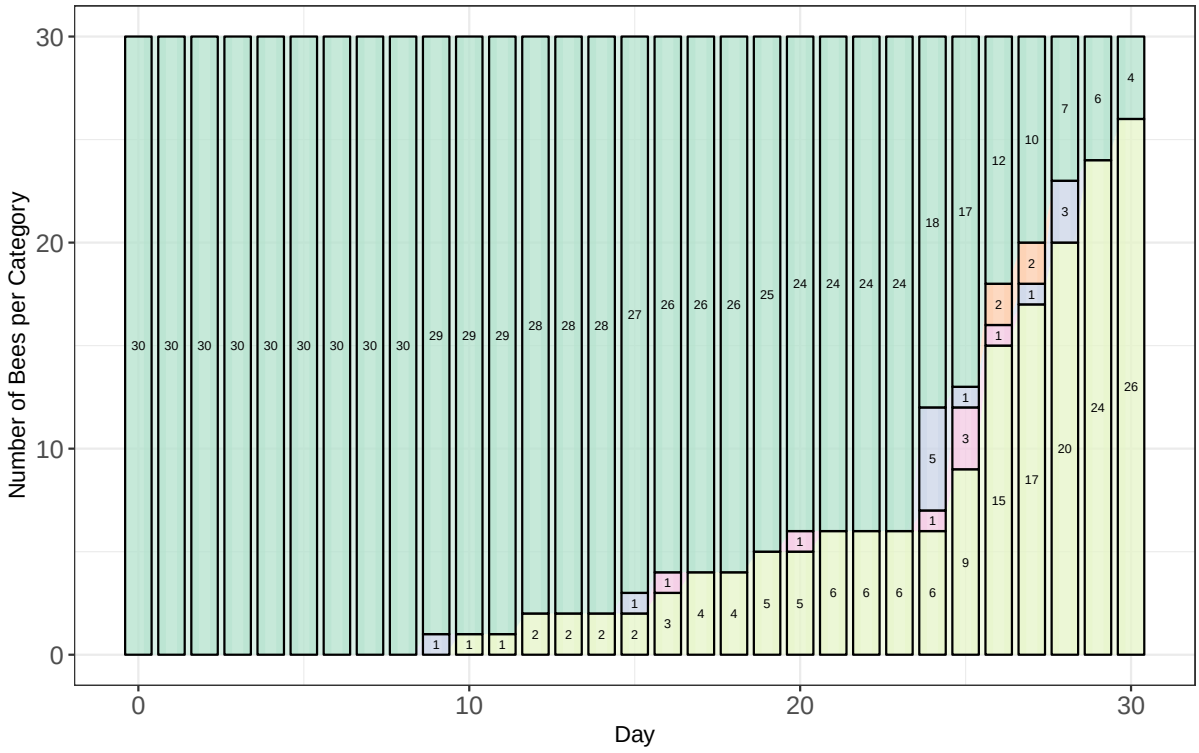
g. 24 hours, virus−fed, naive



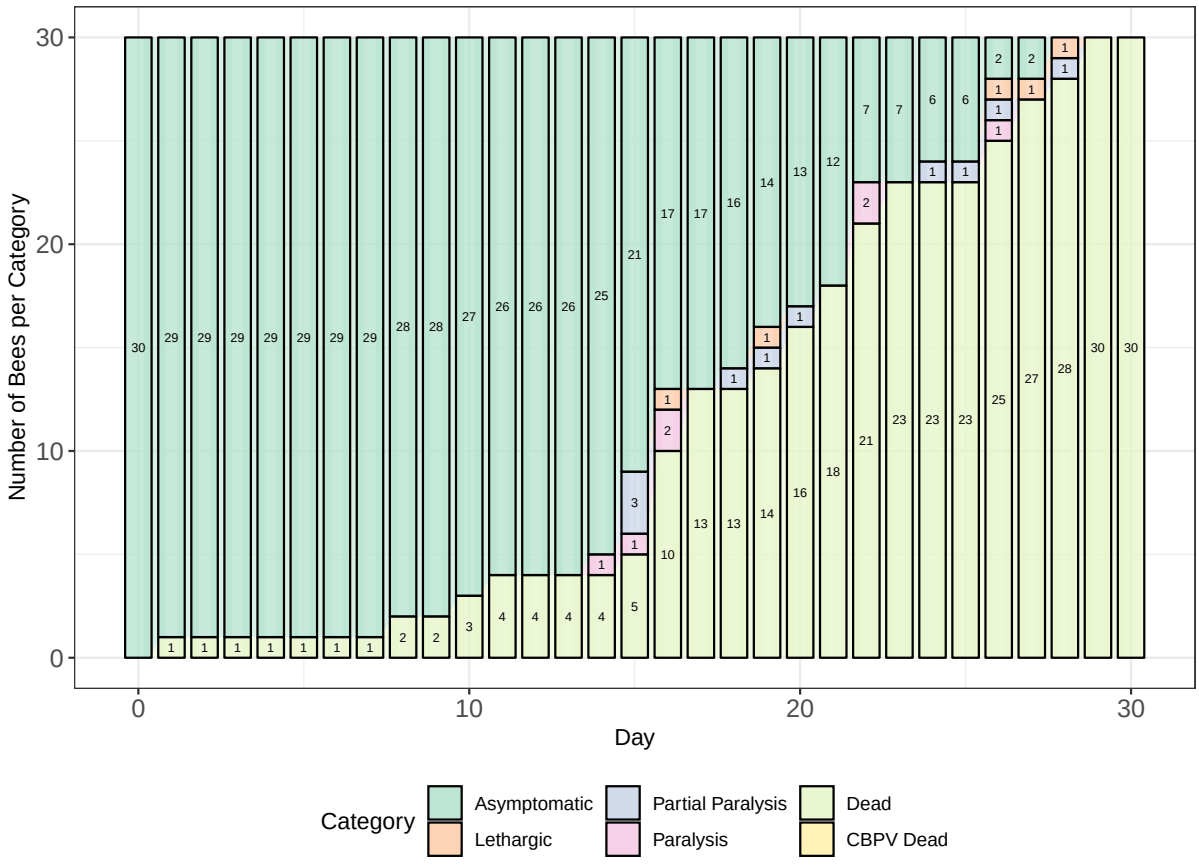
h. 24 hours, virus−fed, seeded



i. 48 hours, buffer−fed, naive



j. 48 hours, buffer−fed, seeded



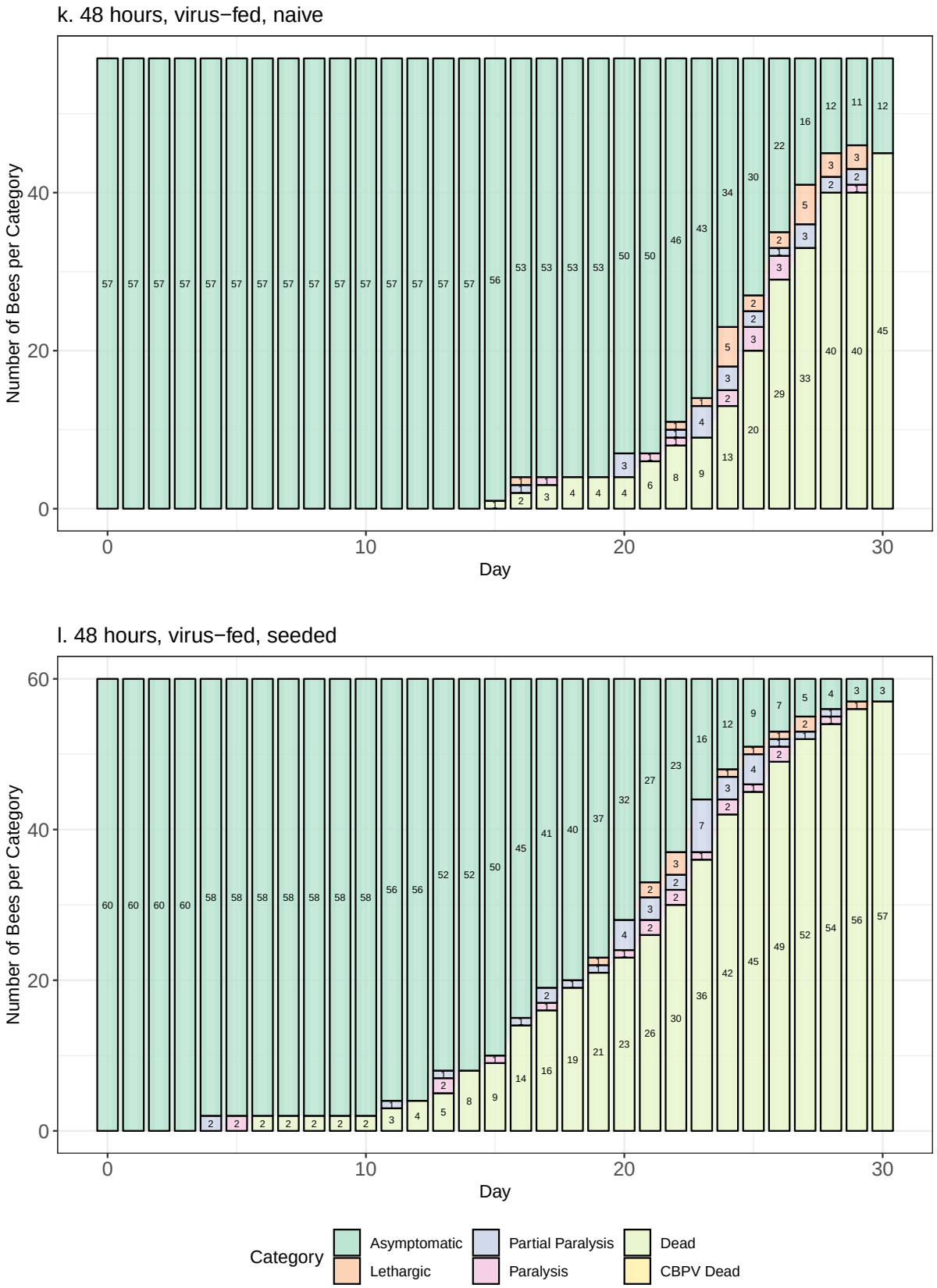


Figure 2.3: The number of bees experiencing lethargy, partial paralysis, paralysis, or that were asymptomatic, dead or dead due to CBP infection each day of the experiment for each treatment.

2.3.1 PCR results

The majority of bees which tested positive for CBPV were in the newly emerged virus-fed treatments (Figure 2.4). In the newly emerged virus-fed naive treatment, 51% of bees tested positive for CBPV, compared to 35% in the paired seeded treatment. One bee from both of the day 1 virus-fed treatments (naive and seeded) also tested positive for CBPV, accounting for 2% of bees in each treatment. All other treatments were negative for CBPV. There were a range of CBPV/18s ratios for each treatment, with newly emerged virus-fed naive having the highest ratios of all treatments. The median ratio was also higher for naive bees compared to seeded bees. The highest ratios were observed in bees which died between days three and 12. No bees which died between days 14 and 21 tested positive for CBPV, and bees that died after day 21 tend to have a lower ratio (Figure 2.4).

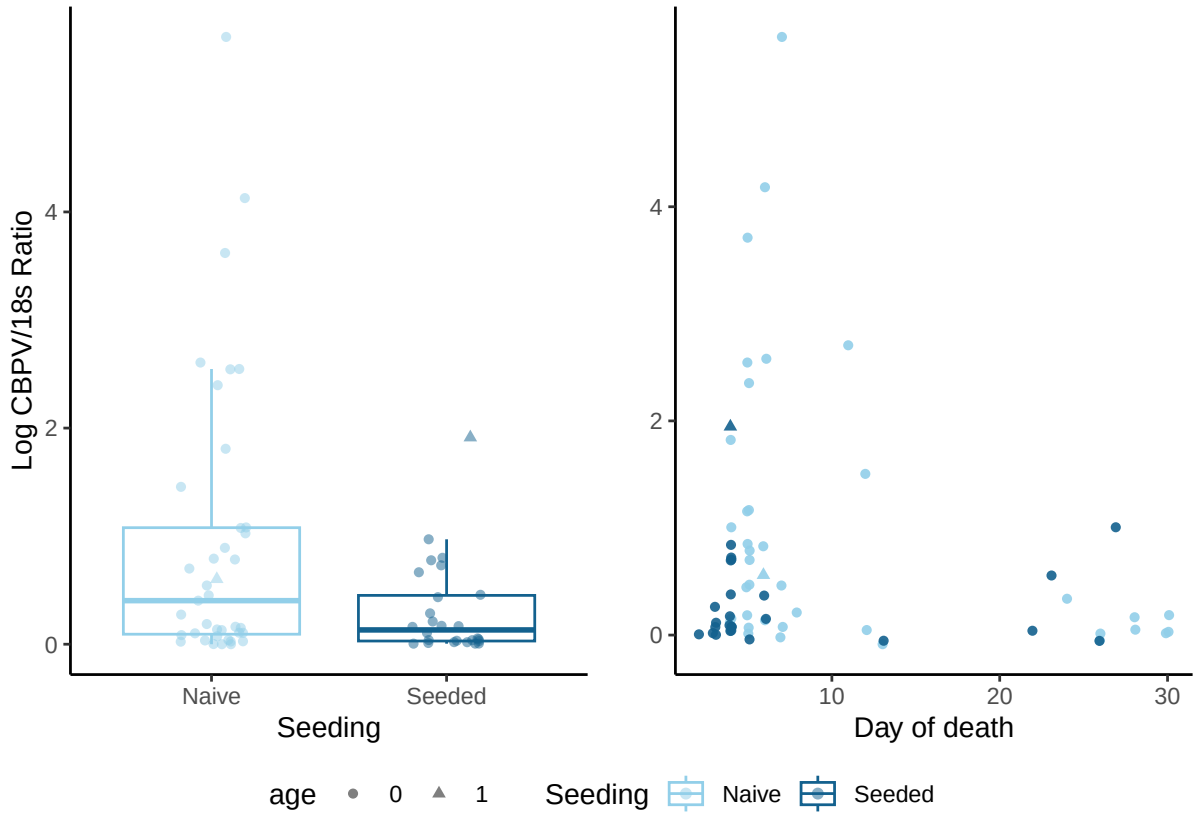


Figure 2.4: (Left) The log base 10 of CBPV/18s RT-qPCR result for each bee grouped by seeding. The centre line represents the median value, the box spans the first and third quartiles. The lower and upper quartiles are represented by the ends of the vertical lines. (Right) The log base 10 of CBPV/18s RT-qPCR result for each bee by the day they died.

The CBPV concentration variable had a high proportion of zeros in the data (Figure 2.5) For this reason, zero-inflated GLMMs were used. The GLMM investigated the impacts of the various treatment groups on the logged relative concentration of CBPV in virus-fed treatments. There was a significant relationship in the conditional model between CBPV concentration and seeding, where the logged CBPV RT-qPCR relative concentration in seeded bees was lower (Table 2.2). In the zero-inflated model, age had a significant positive relationship with CBPV concentration (Table 2.3). Pot as a random effect explained less than 1% of variance in residuals. The results of the normality test found that the residuals did not deviate from a normal distribution (Appendix A, Figure 6.2).

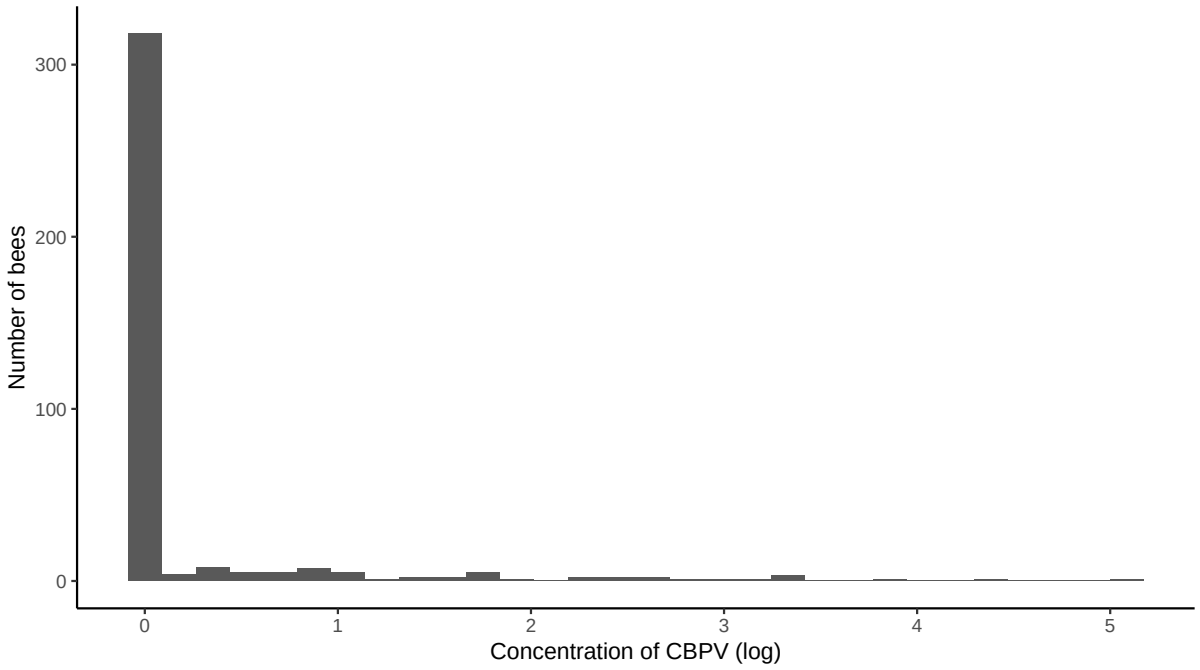


Figure 2.5: The dispersion of CBPV qPCR results of bee samples tested.

Table 2.2: The results from the conditional generalised linear mixed model investigating the relationship between the concentration of CBPV with the age of the bee at the time of feeding and seeding in virus-fed treatments.

Variable	Estimate	Std. Error	z-value	p-value
(Intercept)	0.641	0.851	0.753	0.451
Seeding	-1.026	0.257	-3.989	0.000
Age	0.831	0.806	1.030	0.303

Table 2.3: The results from the zero-inflated generalised linear mixed model investigating the relationship between the concentration of CBPV with the age of the bee at the time of feeding and seeding in virus-fed treatments.

Variable	Estimate	Std. Error	z-value	p-value
(Intercept)	-4.641	0.864	-5.370	0.000
Seeding	0.175	0.466	0.376	0.707
Age	4.254	0.748	5.685	0.000

2.3.2 Survival analysis

2.3.2.1 All-cause hazards

The Kaplan-Meier plots show the probability of survival and the proportion of the population estimated to survive at certain times. A vertical drop in a survival curve represents death of one or more individual bees. We can use the trajectory of the individual bees to make comments about the probability of death for bees in that population. Both eclosed viral treatments had a steep decline in survival probability at days five and six (Figure 2.6). This levelled off, and the decline was very gradual for the rest of the experiment. For the other treatments, there was a slight difference in survival probability between all naive and seeded treatments, with the seeded treatments showing an earlier and steeper decline in survival probability compared to naive treatments (Figures 2.7 and 2.8). Death in most control pots, with the exception of newly emerged, seeded buffer-fed bees, started at approximately day 10. By day 20 at the latest, all treatments declined in a similar manner.

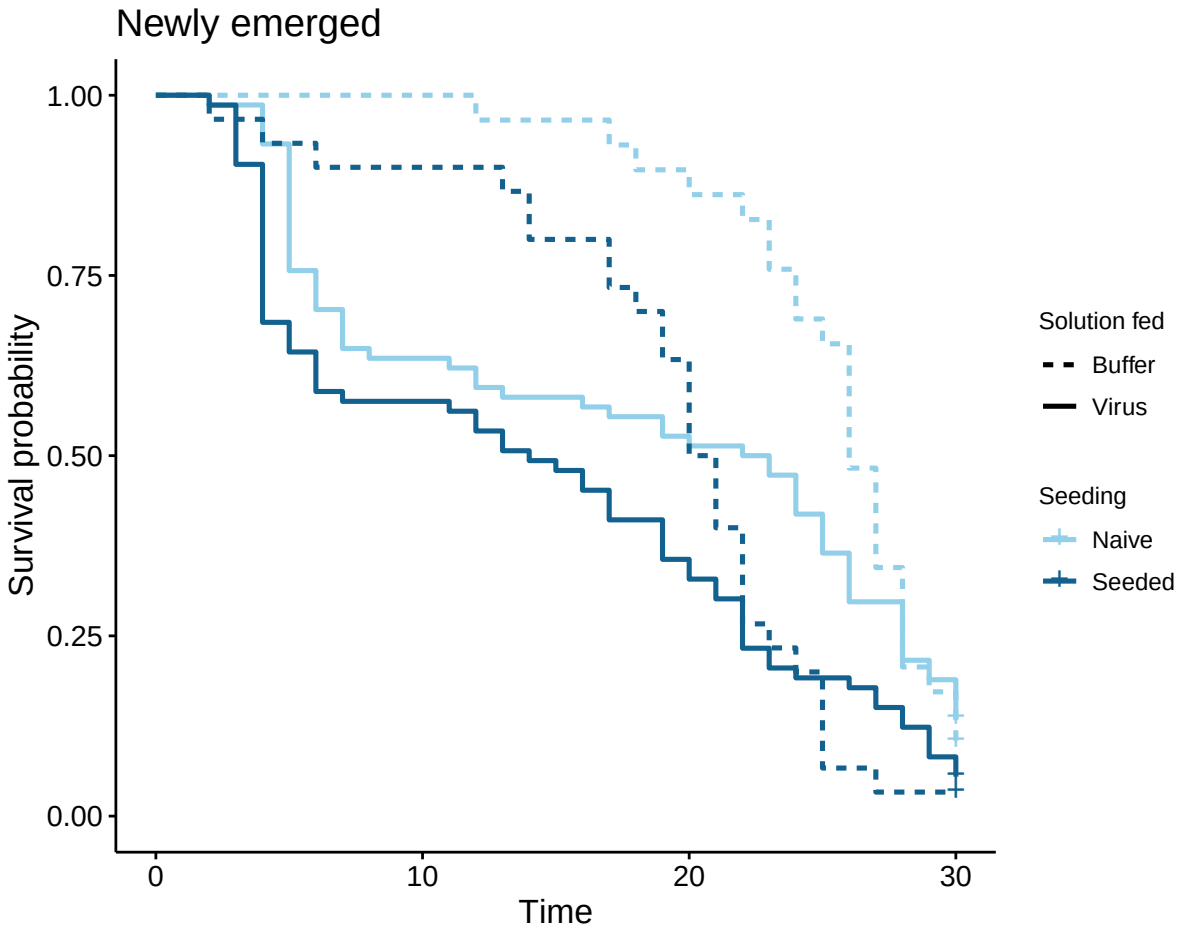


Figure 2.6: The Kaplan-Meier survival curves for bees exposed to virus or buffer when newly emerged, for the duration of the experiment, displaying the probability of survival based on treatment type.

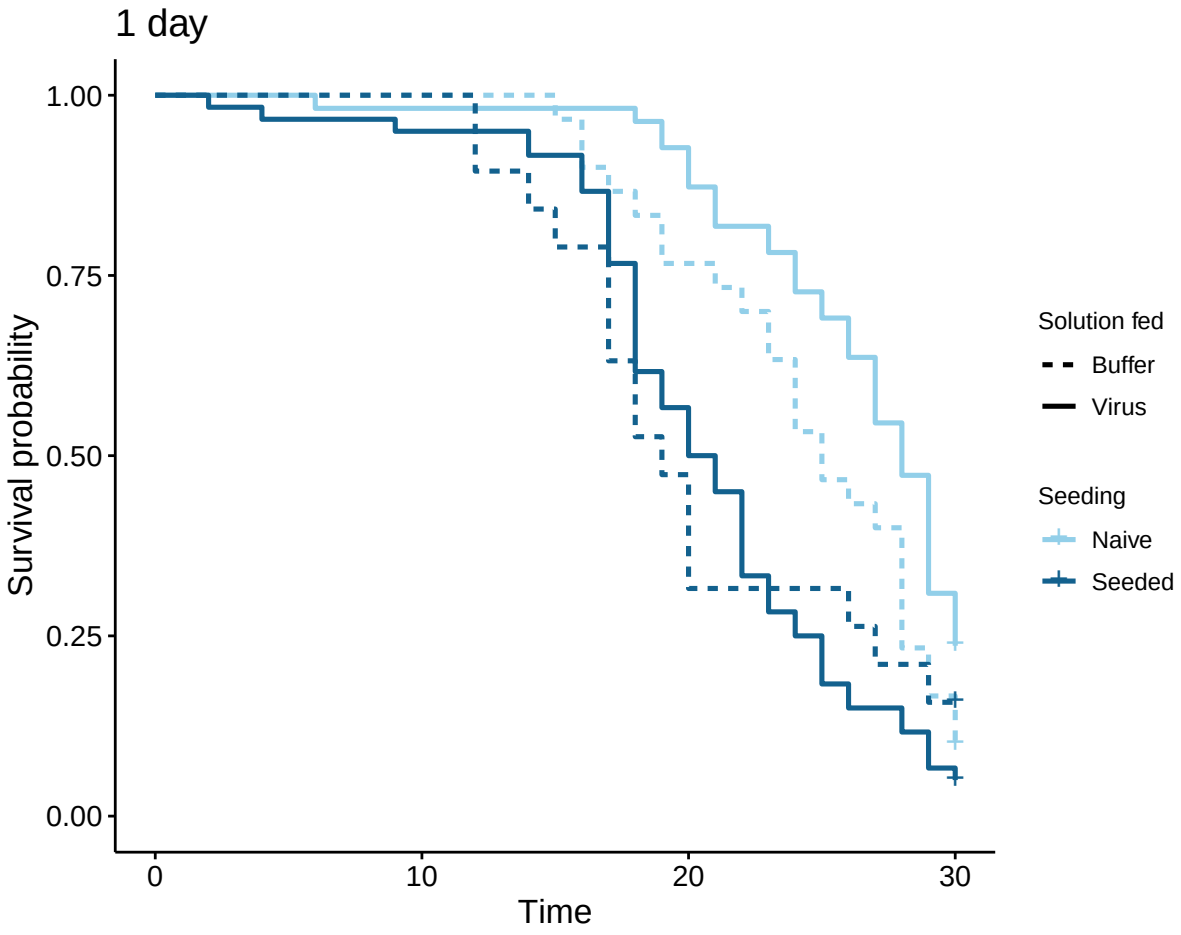


Figure 2.7: The Kaplan-Meier survival curves for bees exposed to virus or buffer at 1 day old, for the duration of the experiment, displaying the probability of survival based on treatment type.

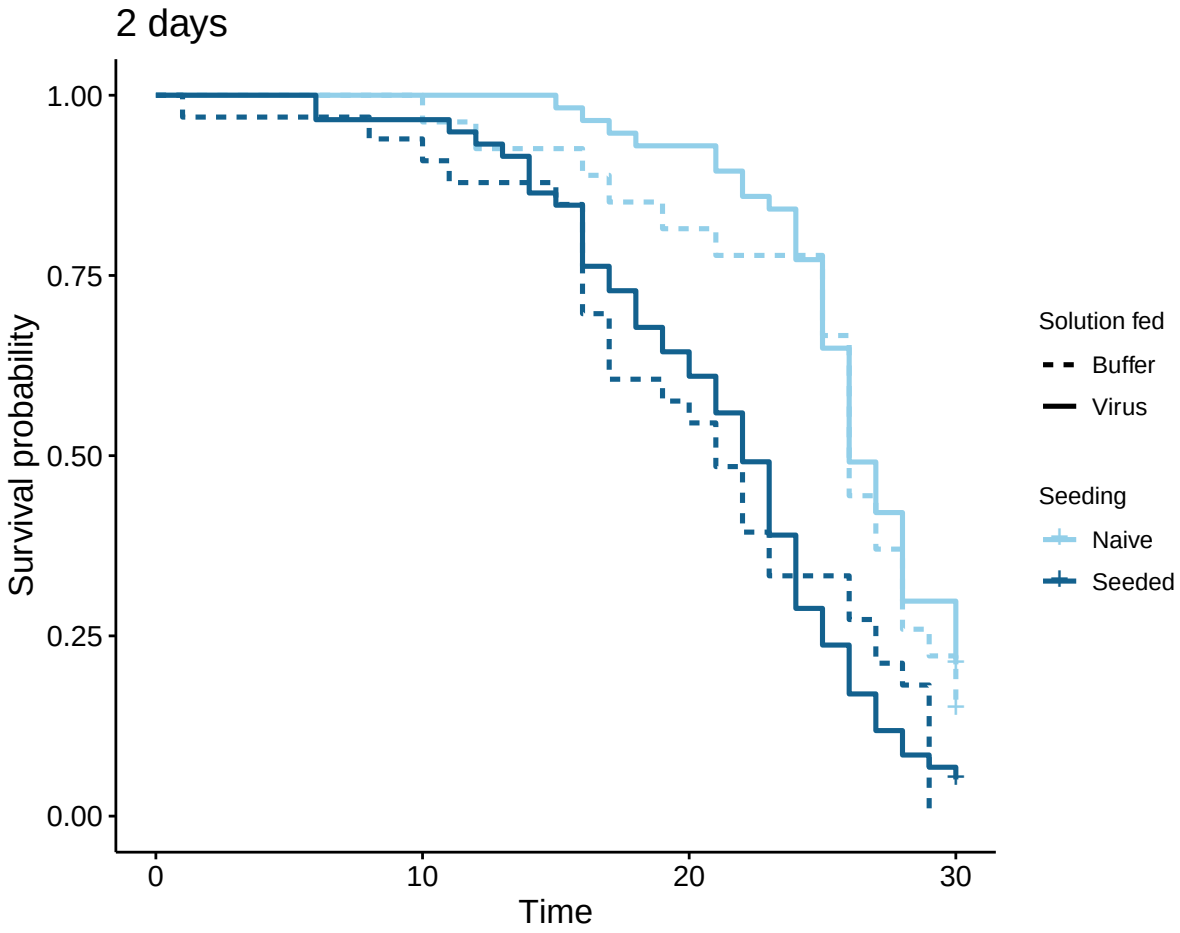


Figure 2.8: The Kaplan-Meier survival curves for bees exposed to virus or buffer at 2 days old, for the duration of the experiment, displaying the probability of survival based on treatment type.

The log-rank test found a significant difference between the survival curves of each treatment (Chi-squared = 91.2, df = 11, $p < 0.01$). The results of the Cox model indicate that seeding significantly increased the risk of death ($z = 7.952$, $p < 0.001$), as did being newly emerged when exposed ($z = 3.301$, $p < 0.001$) (Figure 2.9). There was no significant difference in hazards between bees fed virus or buffer ($z = 0.007$, $p > 0.05$). Additionally, there was no significant difference in hazards associated with being exposed to CBPV at 1 day or 2 days post emergence ($z = 0.146$, $p > 0.05$).

When assessing the proportionality of the hazards over time, all treatments violated the assumptions of the Cox model as the hazards of their treatments varied throughout time (Table 2.4). The hazards for seeding increased throughout the first 18 days of the experiment, whereas the hazards for newly emerged and virus-fed bees decreased over time (Figure 2.10). The hazards associated with 2 day old bees appeared to be relatively constant with time, despite violating the proportional hazards assumption.

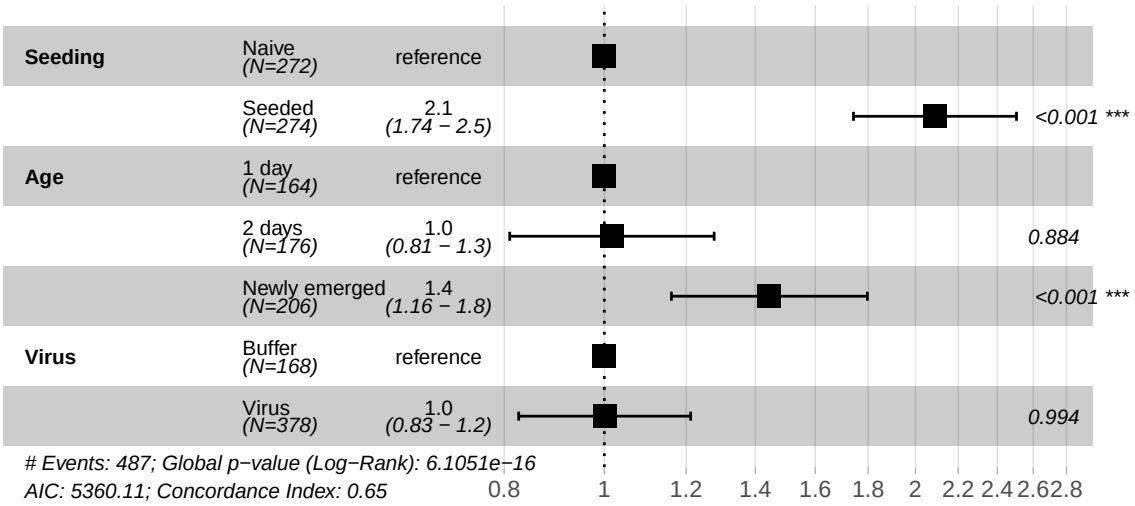


Figure 2.9: The hazard ratios for death in each treatment group from a Cox regression investigating the effects of seeding and age at exposure of honey bees on their survival time with a positive RT-qPCR CBPV result. Scores above one indicate an increased risk of death, and a score below one indicates a decreased risk of death. The p-value for each treatment is also given.

Table 2.4: The results of the proportional hazards assumption test of a Cox regression investigating the effects of seeding and age at exposure of honey bees on their survival time. The proportionality of the hazard associated with each treatment was tested over time. A significant result indicates a violation of the assumption.

Treatment	Chi Squared	df	p-value
Seeded	9.706	1	0.002
2 days	15.619	1	0.000
Newly emerged	31.193	1	0.000
Virus	10.949	1	0.001
GLOBAL	51.692	4	0.000

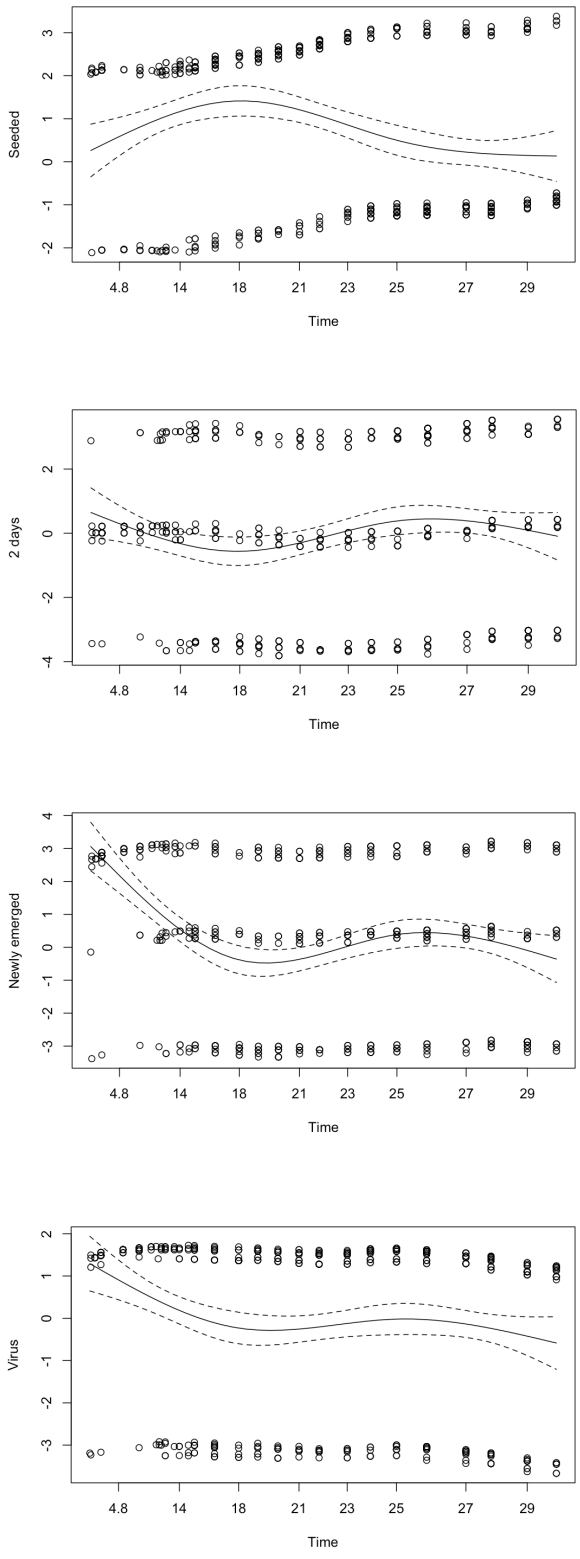


Figure 2.10: Schoenfeld residual plots from a Cox regression investigating the effects of seeding and age at exposure of honey bees on their survival time. Plots examine the proportionality of the hazards of each treatment when interacting with time. The residuals are the observed values minus the expected values for each bee at each time point. The dashed lines are the upper and lower confidence intervals.

2.3.2.2 Cause-specific survival analysis

Survival analysis was conducted on virus treatments, again using death as the event, but using a positive CBPV RT-qPCR result after death as the status. Bees which died but did not test positive for CBPV were censored. Because the 2 day virus treatments had no positive PCR results, they were removed from the analysis. The duration investigated was shortened to 13 days, as this is when the majority of deaths due to CBPV occurred, as shown in Figure 2.4.

The Cox proportional hazards model found that exposure to CBPV as a newly emerged bee had a significant positive effect on the hazard of dying due to CBPV compared to bees exposed when 1 day old ($z = 4.715$, $p < 0.001$) (Figure 2.11). Seeding did not have a significant effect on the hazard of death due to CBPV ($z = -1.135$, $p > 0.05$). The age treatment did not violate the proportional hazards assumption (Chi-squared = 0.439, df = 1, $p > 0.05$) (Table 2.5, Figure 2.12).

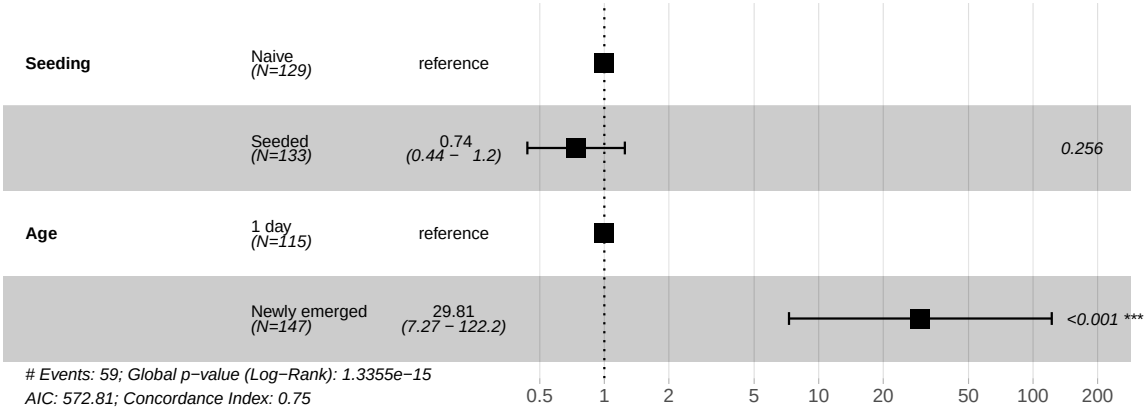


Figure 2.11: The hazard ratios from a Cox regression investigating the effects of seeding and age at exposure of honey bees on their survival time with a positive RT-qPCR CBPV result. Scores above one indicate an increased risk of death, and a score below one indicates a decreased risk of death. The p-value for each treatment is also given.

Table 2.5: The results of the proportional hazards assumption test for a Cox regression investigating the effects of seeding and age at exposure of honey bees on their survival time with a positive RT-qPCR CBPV result. The proportionality of each treatment was tested over time. A significant result indicates a violation of the assumption.

Treatment	chisq	df	p-value
Seeded	10.564	1	0.001
Newly Emerged	0.439	1	0.508
GLOBAL	10.937	2	0.004

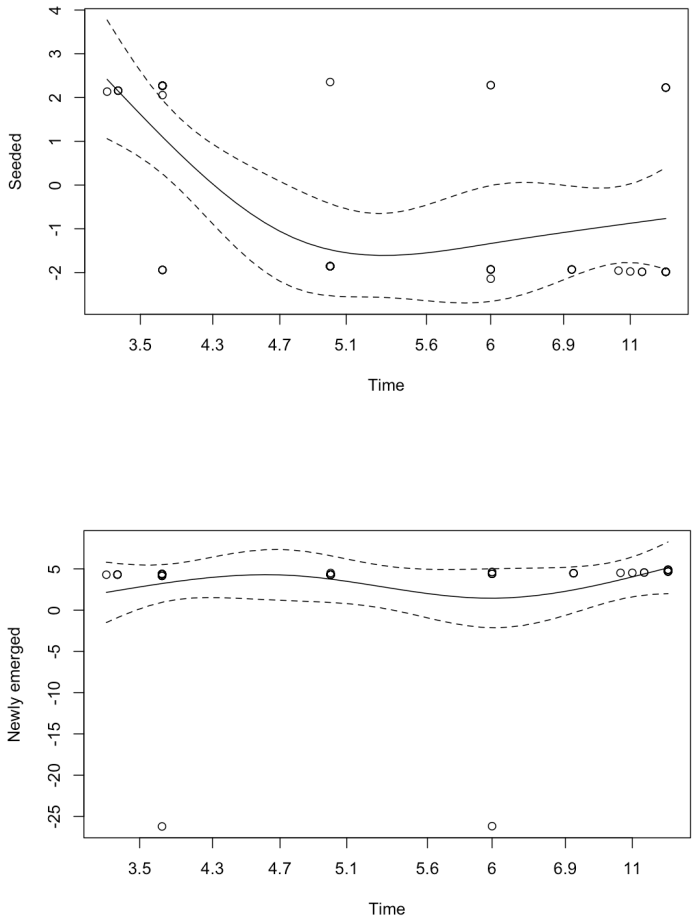


Figure 2.12: Schoenfeld residual plots from a Cox regression investigating the effects of seeding and age at exposure of honey bees on their survival time with a positive RT-qPCR CBPV result. Plots examine the proportionality of the hazards of each treatment when interacting with time. The residuals are the observed covariate values minus the expected covariate values for each bee at each time point. The dashed lines are the upper and lower confidence limits for the coefficients of the covariates.

2.3.3 Competing risks analysis

The competing risk regression analysis showed that age significantly impacted the time of death due to CBPV infection ($z = -5.083$, $p < 0.01$). Seeding did not significantly impact the time of death due to CBPV infection ($z = -1.248$, $p > 0.05$).

Cumulative incidence analysis was conducted on the virus treatments. This compared the subdistribution hazard for each event and found that the events of death ($t = 20.194$, $p<0.05$) and death due to CBPV ($t = 64.285$, $p<0.05$) were significant compared to survival among treatments ($t = 2.307$, $p>0.05$). This is shown in Figure 2.13. Newly emerged, virus-fed bees, both naive and seeded, had significant deaths due to CBPV infection between days three and six. Approximately 50% of deaths in the naive treatment were attributed to CBPV, compared to approximately 30% of deaths in the seeded treatment. Both 1 day virus-fed treatments had 2% of deaths attributed to CBPV. The other virus treatments had significant deaths due to other causes by approximately day 16, and no deaths caused by CBPV.

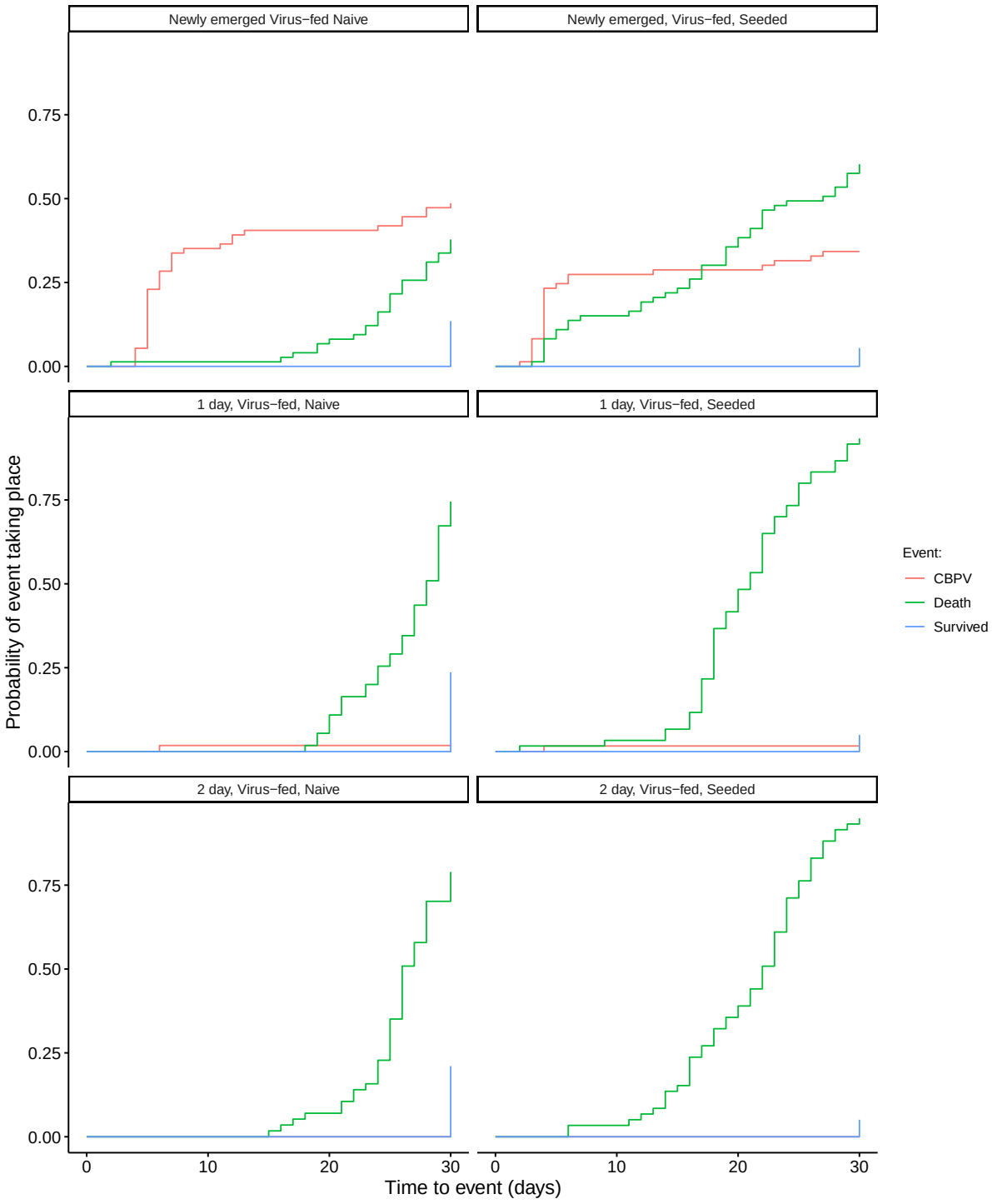


Figure 2.13: The plotted cumulative incidence for death by CBPV (red), death by other causes (green) and survival (blue) when investigating the effects of seeding and age at exposure on honey bee survival. This shows the estimated marginal probability of one of these events occurring on each day of the experiment.

2.3.4 Bacteria analysis

The guts of all bees which died prior to day 16 were analysed with 16s Amplicon DNA Sequencing. The total number of supporting reads per bee gut sample was between 374 and 1706, with an average of 828 reads per sample. There was no noticeable difference in the number of supporting reads between samples of different treatments (Figure 2.14). The species identified and the categories they were grouped into are shown in Table 2.6. Both seeded and naive groups had similar species diversity overall, the only difference being the presence of *Mesorhizobium terrae* and *Agrobacterium rhizogenes* in naive bees only (Figure 2.15). The number of gut samples which had any of the bacteria listed is similar between groups, however the number of supporting reads differs. Seeded bees had a higher quantity of reads for *Gilliamella apicola*, *Frischella*, *Bartonella*, Acetobacteraceae, *Lactobacillus*, and *Snodgrassella alvi*. The majority of reads for naive bees were for *M. terrae*.

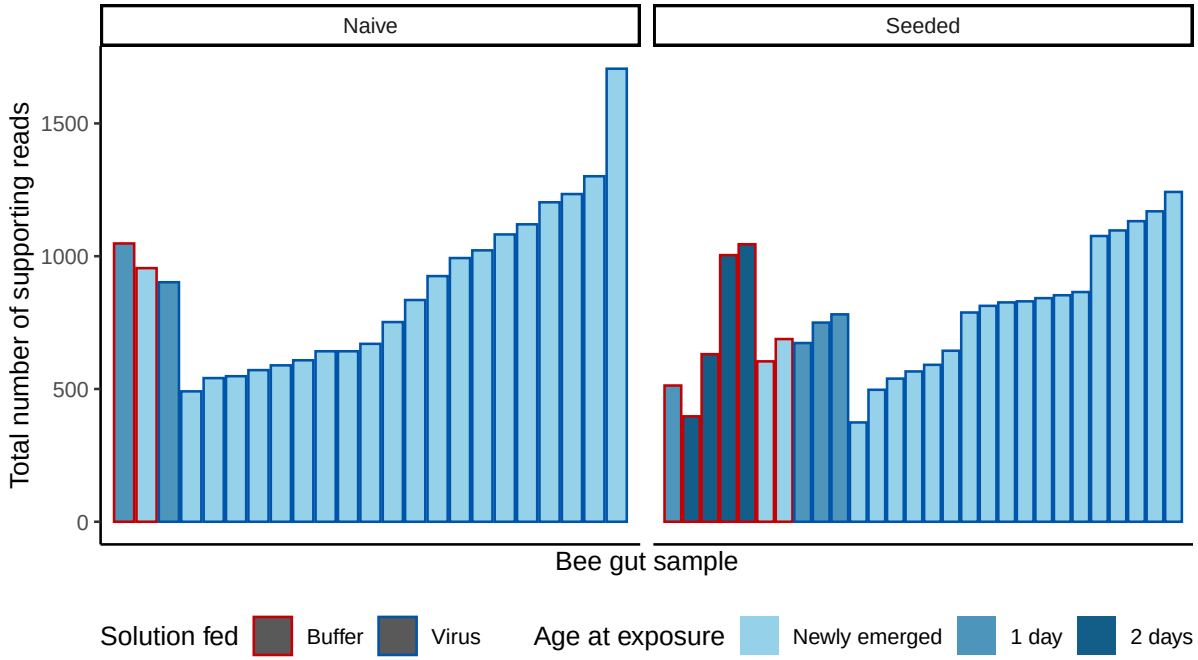


Figure 2.14: The number of supporting reads per individual bee gut sample, grouped by the three treatment categories: age at time of exposure, fed buffer or CBPV and seeding.

Table 2.6: Bacterial species identified in honey bee guts as a result of DNA metabarcoding. Bacteria are grouped by family or genus.

Group	Organism
Acetobacteraceae	Commensalibacter sp
Acetobacteraceae	Acetobacteraceae sp
Agrobacterium	*Agrobacterium rhizogenes*
Bartonella	*Bartonella apis*
Frischella	*Frischella perrara*
Gilliamella	*Gilliamella apicola*
Lactobacillus	*Lactobacillus apis*
Lactobacillus	*Lactobacillus sp*
Lactobacillus	*Lactobacillus helsingborgensis*
Lactobacillus	*Lactobacillus melliventris*
Lactobacillus	*Lactobacillus kimbladii*
Mesorhizobium	*Mesorhizobium terrae*
Snodgrassella	*Snodgrassella alvi*
Uncultured	Uncultured bacterium

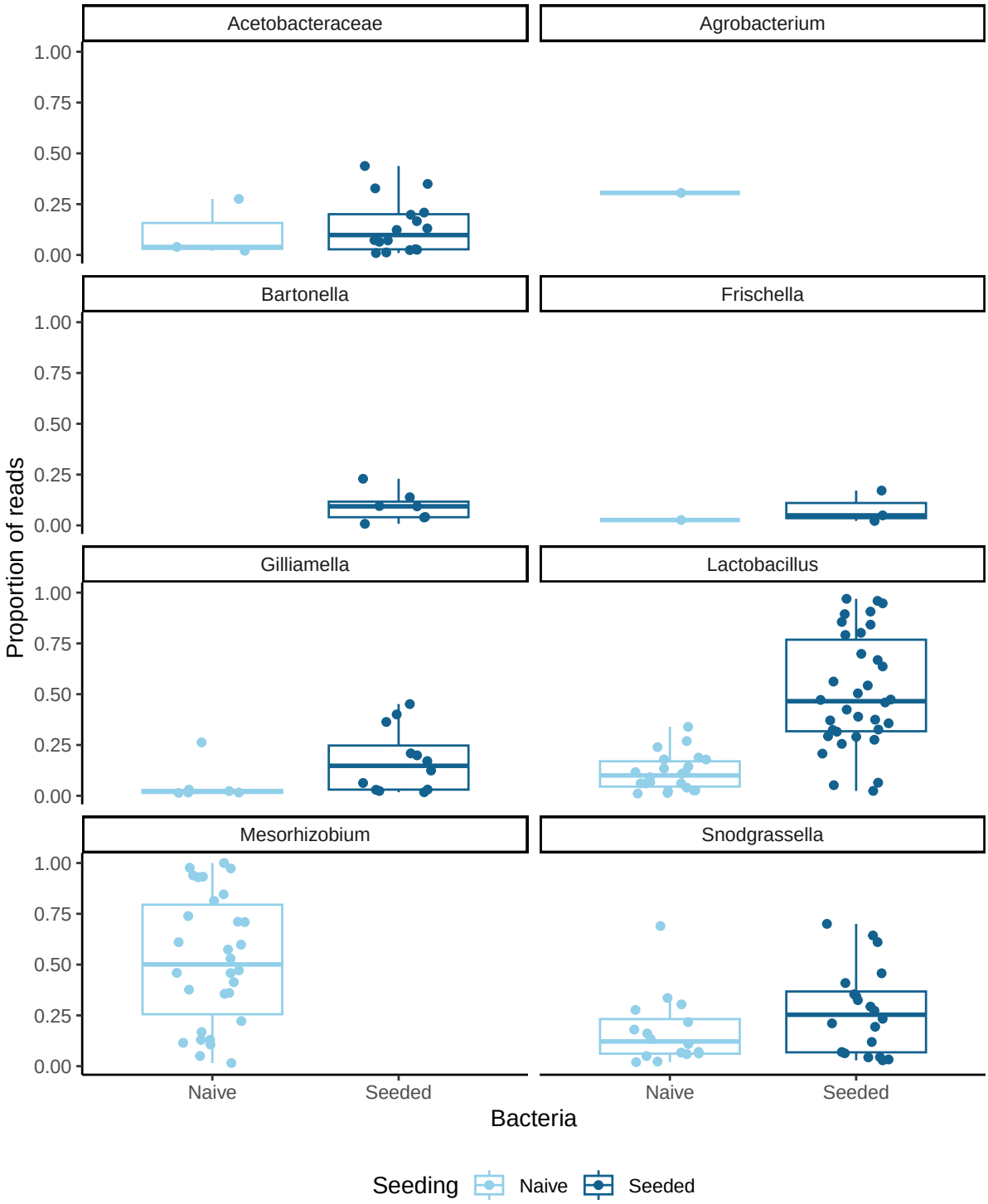


Figure 2.15: The proportion of reads for each bacterial group identified in all naive (light blue) or seeded (dark blue) bee guts. Points represent the proportion of reads of the bacterial group for an individual bee gut. The centre line represents the median value, the box spans the first and third quartiles. The lower and upper quartiles are represented by the ends of the vertical lines.

The results of the CA found that 31.37% of the variation of each bee's gut bacteria composition was explained by axis one, and 25.02% of the variance was explained by axis two, totalling 56.39% of variance explained by both axes. The spread of both bee samples and their gut bacteria on these axes are shown in Figure 2.16. *Mesorhizobium* and *Snodgrassella* are associated with naive bees, which have lower scores on both axes. *Bartonella*, *Acetobacteraceae*, *Commensilabacter*, *Agrobacterium*, *Gilliamella* and *Lactobacillus* are associated with seeded bees.

Generally, seeded bees have a higher score on the CA1 and CA2 axes when compared to naive bees. There is some overlap between both groups on both axes, however, the median score for each group differs clearly (Figure 2.17). There was no clear difference in CA1 and CA2 axes scores between bees which were exposed to virus at different ages. However, there were much fewer bees included in the analysis that were exposed at days 1 and 2 compared to those exposed when they were newly emerged.

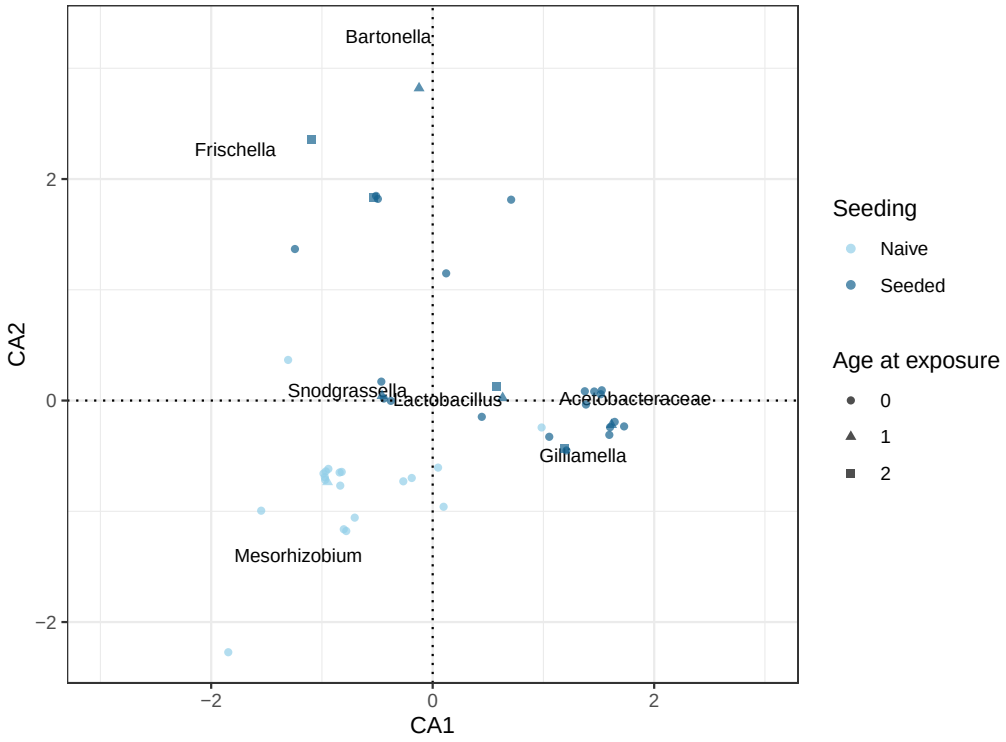


Figure 2.16: The distribution of individual bees and gut bacteria on axes 1 and 2 of a correspondence analysis ordination.

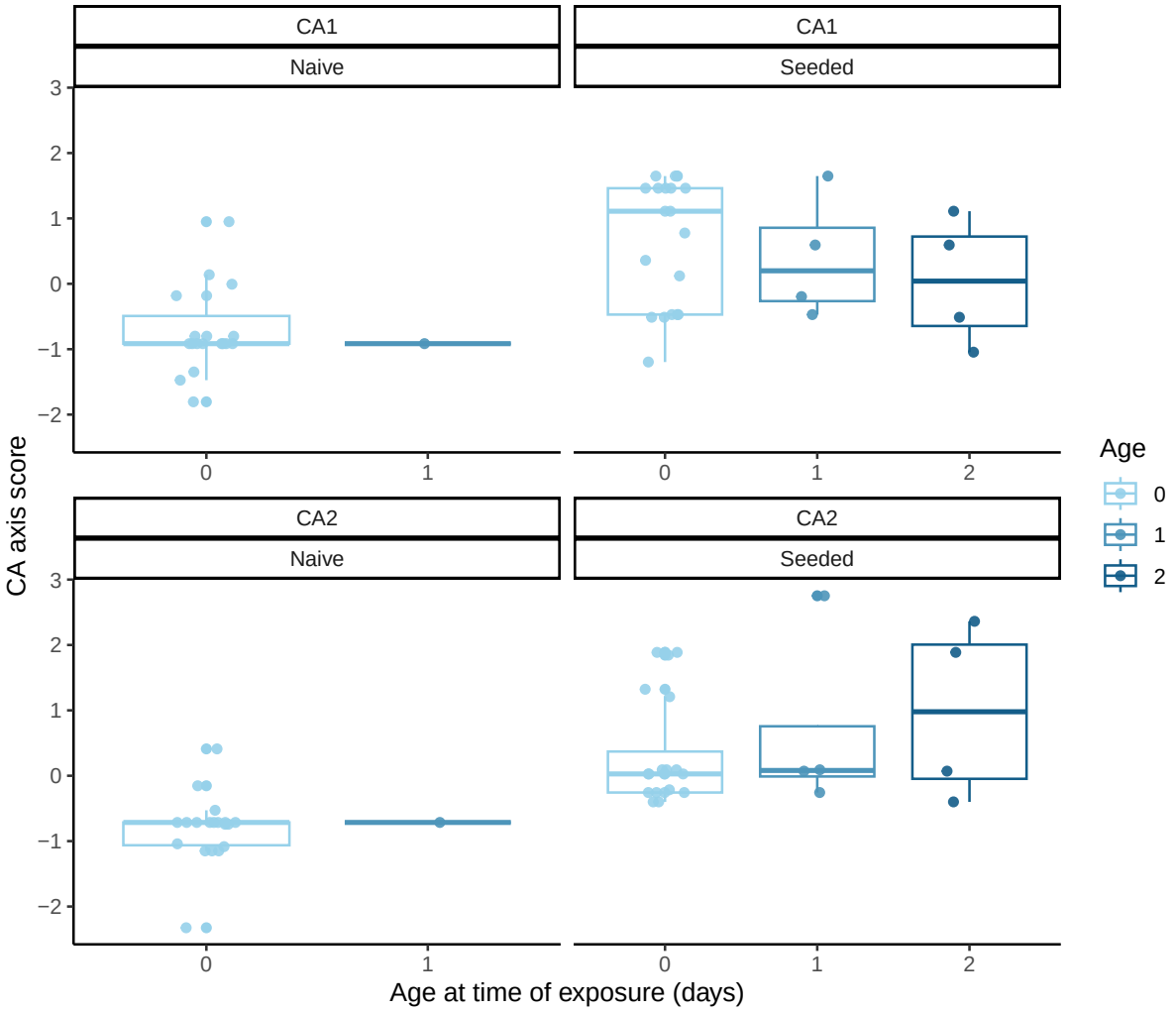


Figure 2.17: The CA1 and CA2 axes scores for bee bacterial communities grouped by age at the time of exposure to CBPV. The central line is the median score, the outer box represents the inter-quartile range, and the vertical lines reach the largest and smallest points within 1.5 times the interquartile range (if any are present within this range).

The results of constrained ordination using CCA found that CA1 explained 20.52% of the variation and CA2 explained 5.21% of the variation, totalling 25.73% of variation explained overall. Axis 1 was significant in explaining variation of the model, however axis 2 was not (CCA1: chi-squared = 0.389, df = 1, $p < 0.001$, CCA2: chi-square = 0.194, df= 1 , $p > 0.05$). Figure 2.18 shows that age at the time of exposure and a higher concentration of CBPV are negatively correlated. Age is associated with a higher score on CCA2 and CBPV is associated with a lower CCA2 score. Bees fed buffer are associated with a higher CCA2 score than bees fed virus, and there is a negative correlation between CBPV concentration and being fed buffer. Being fed virus and a higher concentration of CBPV are positively correlated. Seeded and naive samples occupy a similar area on CCA2, however seeding is associated with a lower CCA1 score. Anova tests on the effect of the explanatory variables on the axis scores found that both seeding and being fed virus had a significant effect on the CCA1 and CCA2 axis scores (seeding: F = 11.53, p = 0.001, fed virus: F = 3.23, p = 0.009) (Table 2.7).

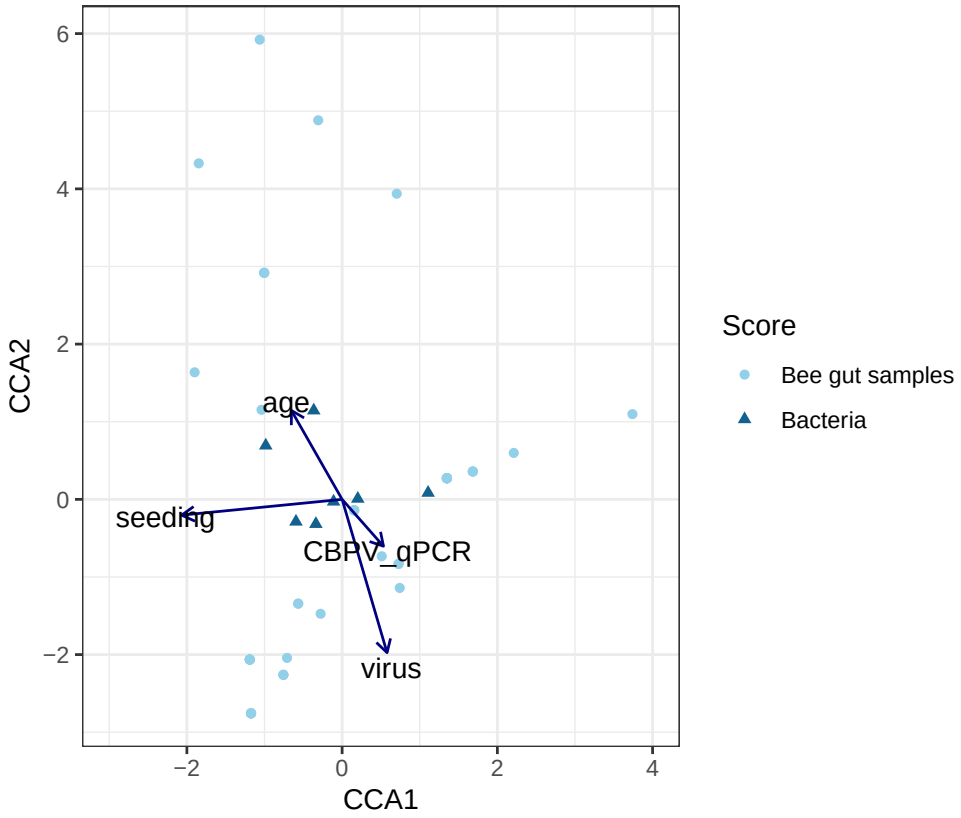


Figure 2.18: The CCA axis scores of bee gut sample bacterial communities and the bacteria identified, constrained by age at time of exposure, virus or buffer-fed, CBPV qPCR result and seeding.

Table 2.7: The results of the constrained ordination of gut bacteria with treatment categories. (Top) The results when all variables are included. (Bottom) The results of stepwise selection of variables.

Variable	Df	ChiSquare	F	p-value
Age	1	0.013	0.548	0.754
Virus	1	0.056	2.326	0.041
Seeding	1	0.261	10.937	0.001
CBPV Concentration	1	0.034	1.430	0.197
Residual	43	1.027		

Variable	Df	ChiSquare	F	p-value
Virus	1	0.077	3.234	0.013
Seeding	1	0.275	11.527	0.001
Residual	45	1.074		

2.4 Discussion

The results of the survival analysis (Section 2.3.2) and competing risks analysis (Section 2.3.3) indicate that age is a key driver of susceptibility to CBPV. Bees which were exposed to CBPV when they were newly emerged were more susceptible than those exposed at 1 or 2 days post emergence. No bees in the 2 day age category became infected with CBPV, and only two bees became infected in the 1 day age category, indicating that susceptibility changes with age. Age was also a significant predictor of CBPV concentration in the zero-inflated GLMM, because as age increased the likelihood of having a lower CBPV concentration increased (Section 2.3.1, Table 2.3). As naive bees had gut bacteria present at the time they died, it is likely that age is the main driver of oral susceptibility, rather than the state of the gut microbiome (which also changes with age). It is possible, however, that the gut was not fully colonised by the bacteria present, and this still had an impact on susceptibility (Raymann & Moran, 2018). We also found that many bees in the newly emerged treatments survived past the initial two week phase where many bees became symptomatic and died. This could be based on intake of the virus, due to the possibility that the feeding method used was not uniform in ensuring bees ingested the total amount of virus. When feeding bees, there were visual differences in intake: some bees clearly drank the entire droplet of liquid, whereas others resisted, making it harder to see if the entire droplet was ingested. However, due to the fact that some bees in the 1 day age category became infected, it is likely that there are other factors which impact susceptibility that were not monitored in this experiment.

These conclusions are derived from survival analysis, which assumes that the hazard of death is proportional over time (Therneau & Grambsch, 2000). The nature of this experiment means that the hazard of death is not proportional over time; the experiment lasts for almost the length of the honey bee's life, so it is expected that most bees will have died by the end of the experiment, regardless of treatment. With the addition of virus, death is likely to occur closer to the time of exposure, so more death is likely to take place at the beginning of the experiment. We also see a short period of resilience in bees exposed to virus, which take a few days to show symptoms and die. Investigating which variables violate the assumption is interesting, as that means these variables either accelerate or decelerate death. Seeding, age at exposure and being fed virus all violated the proportional hazards assumption in relation to death from any cause, meaning all of these variables impacted the hazard of death through time (Section 2.3.2.1). The

virus-fed variable and 2 day old factor level were not significant in the Cox model. This is likely due the number of bees which did not become infected with CBPV and die early in the experiment. When survival analysis was repeated using virus treatments only and death with a positive CBPV result which occurred before day 13, only the newly emerged treatment significantly affected death due to CBPV and did not violate the proportional hazards assumption (Section 2.3.2.2). This is because death due to CBPV was mainly observed in this age category, and many bees died due to this cause throughout the initial 12 days of the experiment, so hazards were proportional through time.

Survival analysis found that seeding impacted death in all treatments, but not death caused by CBPV, indicating that seeding had a negative effect on the survival of honey bees. This could be due to a number of reasons. While 35% of newly emerged seeded bees tested positive for CBPV, it is possible that these bees did not die from CBPV but rather the seeding treatment. However, in order to test positive for CBPV after death, some replication likely took place. It is possible that along with gut bacteria, pathogens were present in the gut homogenate which infected the bees, leading to death. Seeding may have caused gut dysbiosis or led to an unstable bacterial community, causing bees to become more prone to infection from pathogens introduced through other sources, such as the virus solution or equipment in the laboratory (Anderson & Ricigliano, 2017). Finally, the administration of the gut homogenate may have caused increased mortality in seeded treatments. Overall, seeded bees which tested positive for CBPV had lower concentrations of the virus than naive bees which tested positive (Section 2.3.1, Figure 2.4). This was also highlighted in the zero-inflated GLMM on CBPV concentration, as seeding was associated with lower CBPV concentrations. This indicates that the bees may have been weaker, and therefore succumbed to a lower concentration of CBPV, or that another pathogen was the true cause of their death, and the bees died before CBPV was able to replicate to the level seen in some naive bees.

All of the core members of the honey bee gut microbiota were identified in the honey bee guts tested, with the exception of *Bifidobacterium* and the Firm4 group within the *Lactobacillus* genus. The gut microbiota is established through contact with adult bees or food in the hive following eclosure. It can take between three and five days for the microbiota to fully develop, which involves oral trophallaxis and anal grooming. The first bacteria to colonise the gut are *Lactobacillus* and Acetobacteraceae. Both are found in pollen and nectar and therefore likely come from a food source. Bacteria found in the

ileum enter the gut exclusively via anal grooming or contact with faeces. These include *Snodgrassella alvi* and the gammaproteobacteria (Moran, 2015).

Lactobacillus species are found in the rectum of the honey bee. They are members of the core microbiome and consist of two groups: Firm4 and Firm5, named due to *Lactobacillus* making up a large number of bacteria in the Firmicutes phylum. Firm5 are the most abundant of all core bacteria and consist of *Lactobacillus helsingborgensis*, *Lactobacillus melliventris* and *Lactobacillus kimbladii*, all of which were identified in this experiment (Bonilla-Rosso & Engel, 2018). Firm4 consists of *Lactobacillus mellifer* and *Lactobacillus melis*, neither of which were observed in this experiment. *Lactobacillus* species have roles in carbohydrate degradation and nutrient absorption, as well as protection from pathogens through induction of immune responses (Dong et al., 2020; Evans & Lopez, 2004; Hyrsl et al., 2017; Raymann & Moran, 2018).

Snodgrassella alvi is one of the five core gut microbiota, and can be found in every honey bee colony across the globe (Raymann & Moran, 2018). It is a highly specialised bacterium and is rarely found outside the honey bee gut. It is primarily located in the honey bee hindgut, specifically the ileum wall (Moran, 2015). *S. alvi* forms a layer in the ileum, which may provide protection against pathogens (Maes et al., 2016). There is also evidence that *S. alvi* may be involved in immune priming, as more AMPs were found in bees which were exposed to *Escheresha coli* and inoculated with *S. alvi* than bees with no gut microbiome (Kwong et al., 2017). Zheng et al. (2017) found that *S. alvi* is also responsible for maintaining anoxic conditions in the honey bee gut, which provides the conditions needed for other bacteria to perform metabolism.

Gilliamella apicola is another core gut bacterium, and is also found in the ileum, where it forms a layer above *S. alvi*. *G. apicola* has roles in digestion and metabolism and has been found to utilise carbohydrates that bees cannot digest and sugars which would otherwise be harmful to the bee (Engel et al., 2012; Zheng et al., 2016).

Frischella perrara is a widespread honey bee gut bacterium and a close relative of *G. apicola* (Raymann & Moran, 2018). While it is less abundant and less widely distributed than core gut bacteria, it colonises a specific region of the gut, the pylorus (Dong et al., 2020). A correlation has been found between the presence of a scab in this region, caused by the melanisation immune response, and the presence of *F. perrara* in honey bees (Engel et al., 2015; Raymann & Moran, 2018). This indicates that *F. perrara* is involved in the immune response in honey bees and may protect them against pathogens.

Species from the Acetobacteraceae family were identified in the honey bee gut. There are two types found in the honey bee gut: Alpha2.1 and Alpha2.2. Alpha2.1 is found in the honey bee gut only and specialises in metabolism. This group is also known as *Commensalibacter sp.*. The Alpha2.2 group is present in the honey bee diet, hypopharyngeal glands and larvae (Moran, 2015). Bonilla-Rosso et al. (2019) speculated that this group is involved in fast resource utilisation, whereas Alpha2.1 has a broader metabolic range.

Bartonella apis is a widespread but non-core member of the worker honey bee microbiome (Raymann & Moran, 2018). Generally, it is less abundant than core microbiota, and reaches a stable relative abundance at 12 days post emergence (Dong et al., 2020). It is an Alpha-1 species of Alphaproteobacteria, a group which has only been found in the honey bee gut (Kešnerová et al., 2016). Many *Bartonella* species are mammalian pathogens, infecting both wild and domestic animals which may act as a reservoir of the pathogen and lead to human infection (Breitschwerdt & Kordick, 2000). However, a study by Cornman et al. (2012) found *B. apis* to be more abundant in healthy colonies when compared to those which had collapsed. This may suggest that *B. apis* is beneficial to colony health (Raymann & Moran, 2018).

Two species of bacteria not commonly found in honey bees were also identified: *Mesorhizobium terrae* and *Agrobacterium rhizogenes*. Both of these bacteria are plant or soil-based, and therefore may have been brought into the hive by foraging bees (McFrederick et al., 2012). Generally, some soil bacteria are known to be antimicrobial for plant roots, and may have a similar effect in honey bees (Kim et al., 1997; Piccini et al., 2004). However, some soil bacteria are pathogenic and therefore may negatively impact honey bee health. *A. rhizogenes* is an infectious agent for hairy root disease in plants, and is closely related to *A. tumefaciens*, which causes crown gall disease (Veena & Taylor, 2007). This bacterium has been identified in the honey bee gut, however its effect on honey bees is unknown (Engel et al., 2012). The effect of *M. terrae* on honey bee health is also unknown, however, *Mesorhizobium sp.* have been found in Japanese honey bees (*Apis cerana japonica*) previously (Yoshiyama & Kimura, 2009).

We expected that seeding would introduce an abundance of bacteria to the gut in a manner similar to trophallaxis. These bacteria would then colonise and potentially compete with one another for their niche in the gut (Engel et al., 2012). We also expected naive bees to have a less diverse and well-colonised gut than seeded bees, only gaining bacteria from wax

caps and potentially through the virus solution (Schwarz et al., 2016). We instead found that both groups had a similar bacterial species diversity, with the only clear difference between groups being the presence of *M. terrae* and *A. rhizogenes* in naive bees. This implies that naive bees have gained gut microbiota through the chewing of the wax caps of their cell during emergence.

M. terrae was the only bacterium observed with a strong correlation to naive bees. Honey bees spend the first days post emergence taking part in social interactions with other bees and hive cleaning, leading to the colonisation of bacteria in their guts (Moran, 2015). As a soil bacterium, it is possible that *M. terrae* would be one of the first bacteria a newly emerged bee would pick up, as it likely is spread throughout the hive by foragers. This bacterium was not present in seeded bees, which may be due to the competition which takes place between microorganisms in the gut (Engel et al., 2012). Seeded bees would have had a larger variety and abundance of bacteria in their guts, which may have outcompeted non-specialised bacteria such as *M. terrae*.

Bacteria associated with a seeded gut include *Bartonella* and Acetobacteraceae species, which were likely highly abundant in the gut homogenate used to inoculate the bees. *Lactobacillus* species and *S. alvi* were common in both naive and seeded bee guts. *F. perrara* and *G. apicola* were also present in both groups, albeit less common. We would expect to find these bacteria in seeded bees, as these bacteria are found in most adult honey bees and were therefore likely present in the gut homogenate used to seed the bees. However, as they are also present in naive bees which did not receive this treatment, these bacteria must have also colonised the gut from a different source.

Despite the similarities between communities in the naive and seeded groups, seeding did have a significant impact on the CCA1 and CCA2 axis scores of the constrained ordination. This may be based on the presence of *M. terrae* and *A. rhizogenes* in naive bees, or differences in the abundance of bacteria among bees in each group, as Acetobacteraceae and *Bartonella* species were more prevalent in seeded guts. The feeding of virus or buffer also had a significant impact on the axis scores, indicating that bacteria may have been introduced to bees through these methods, or that these solutions disrupted the gut bacteria and altered the bacterial communities. This could cause an increased susceptibility to CBPV, as gut microbiota have a role in pathogen defence, as previously discussed.

2.5 Constraints

There are weakness to the experimental design. To investigate differences in susceptibility based on variation in the development of the gut microbiome, seeding was used. Seeding aimed to mimic the effects of trophallaxis through uniform application of nurse bee gut homogenate, which could then be compared to naive bees which had not been seeded or taken part in trophallaxis with nurse bees. However, through chewing the caps of their cells during emergence, all bees in the experiment became exposed to bacteria which may be very similar to the bacteria gained through trophallaxis. Due to this, the effect of trophallactic interactions on the gut microbiome has not been conclusively measured in this experiment.

In addition, not all bee guts were tested in this experiment due to difficulty in removing bee guts later in the experiment. This means that bees which survived until later in the experiment may have had an established or different microbiome to the bees tested, which may have led to their extended survival.

2.6 Conclusions

Honey bees exposed to CBPV when newly emerged are more likely to become infected than those exposed one to two days after emergence. For bees exposed to CBPV at days one or two, seeding had no effect on the likelihood of testing positive for CBPV. There were differences in the number of bees exposed when newly emerged which tested positive for CBPV, whether seeded or naive, however the results of seeding are ambiguous and therefore the hypotheses cannot be answered definitively. The seeding treatment was problematic for two reasons. Firstly, seeding seemed to cause increased mortality in all treatments, but had a large effect on newly emerged bees, indicating the possibility that other pathogens were introduced to bees through this treatment. Secondly, gut bacteria were likely also introduced during emergence through the chewing of cell caps, which may have masked the effects seeding on the gut microbiome. To know if fomite transmission has played a part in susceptibility, experiments would need to be undertaken where bees are emerged in a completely sterile environment, and the gut communities can be compared. As these results indicate an increased oral susceptibility to CBPV in younger bees, the ages of bees that die in naturally infected hives should be investigated. If many young

bees die in CBPV-infected hives, this would indicate that oral transmission is likely the cause.

Chapter 3

Investigating the variation in susceptibility of bees to infection by Chronic bee paralysis virus

3.1 Introduction

The gut microbiome is the community of microorganisms living in the intestines of many animals. The microbial community provides functions in metabolism, physiology, immunity and development which vary between organisms. Gut microbiota prime the gut for the mechanisms underlying these processes, unlike pathogens which can cause dysbiosis (Martinson et al., 2011). The microorganisms which make up this community vary in their diversity and abundance, depending on a multitude of factors including host species, diet and habitat. In humans, the gut microbiome is made up of trillions of microbes belonging to more than 50 bacterial phyla (Bull & Plummer, 2014; Wilson et al., 2020). The quantity of bacteria is approximately 1:1 with the cells of the human body, making the gasto-intestinal tract one of the densest microbial environments (Rinninella et al., 2019; Sender et al., 2016). Associations have been found between conditions such as inflammatory bowel disease, irritable bowel syndrome, obesity, type 2 diabetes and gut dysbiosis, where the usual gut microbiota becomes disrupted and unstable (Bull & Plummer, 2014; Cho & Blaser, 2012). The composition of the gut microbiota is affected by many conditions, including age, diet, stress and pathogen exposure (Raymann & Moran, 2018).

The honey bee gut microbiome is a unique community in both its specialisation and consistency, and has a relatively low diversity in comparison to other animals, making it an ideal model system for the study of microbiota (Hamdi et al., 2011). There are eight bacterial phylotypes which make up 95% of bacteria found in *Apis mellifera* adults (Moran et al., 2012). The most common taxa are *Snograssella alvi*, Bifidobacterium, *Gilliamella apicola* and two species of *Lactobacillus*; Firm5 and Firm4 (Bonilla-Rosso & Engel, 2018; Maes et al., 2016). A few other bacteria are also common in the gut, which tends to have between eight and 11 species of bacteria. These include *Frischella perrara*, *Bartonella apis*, *Parasaccharibacter apium* and an Acetobateraceae species (Zheng et al., 2018). These bacteria are gained through contact with adult bees, food and the hive itself. Newly emerged bees, which are almost completely bacteria-free, engage in trophallaxis and anal grooming which facilitates the transfer of bacteria from worker bees to young bees (Powell et al., 2014). Bacteria can also be transmitted via fomites, specifically through the chewing of cell caps during emergence, hive cleaning and auto- and allo-grooming. Core gut bacteria such as *Lactobacillus* and Acetobacteraceae have been found in both pollen and nectar, suggesting that these bacteria colonise following contact with the food stores brought into the hive by forager bees (Anderson et al., 2013; Moran, 2015). A multitude of factors can affect the bee gut microbiome and cause gut dysbiosis. These include nutrition, season, age, caste, disease, stress and the use of antibiotics and chemicals (Carding et al., 2015). Honey bees undergoing gut dysbiosis have been found to be more susceptible to pathogen infection, indicating that the gut microbiome plays a role in pathogen defence (Raymann & Moran, 2018). Equally, disease can also disrupt the gut microbiome (Maes et al., 2016).

During honey bee development, the bacteria present in the gut vary in both type and quantity (Martinson et al., 2012; Zheng et al., 2018). During the larval stage, bacteria are gained through feeding from nurse bees (Martinson et al., 2012). These bacteria are often those found in adult bees, but can be in such small quantities that they are undetectable via PCR. Once the cells are capped, larvae go through ecdysis where the tracheae, foregut and hindgut intima are shed with the exoskeleton, and any bacteria present from the larval stage are lost (Jay, 1963). Prior to emerging, pupae are almost completely bacteria-free and can remain this way if removed from the frame prior to natural emergence (Gilliam, 1971). Newly emerged bees are often almost completely free of gut bacteria, although they may gain some bacteria during emergence when they chew

through the cap of their cell (Schwarz et al., 2016). The gut microbiome is usually fully established between three and five days after emergence (Powell et al., 2014).

In addition to the colonisation of the gut, the adult honey bee also undergoes physiological changes during their development, including the hardening of the cuticle and development of glands, which may affect their susceptibility to pathogens (Winston, 1987). Some physiological changes of honey bees occur in parallel with a change in role (Elekonich & Roberts, 2005). As bees become older they switch from in-hive tasks to those outside the hive such as foraging, which requires a lower body mass, higher glycogen content and an increase in juvenile hormone, which lead to increased metabolic and flight capacity (Harrison, 1986; Harrison & Fewell, 2002; Jassim et al., 2000; Winston, 1987). Honey bees undergoing this task change also undergo a change in diet, and therefore must produce enzymes that digest nectar rather than beebread (Ohashi et al., 1999). Immunity of honey bees also changes with age. A study by Lin et al. (2022) found an increase in the expression of immune genes in 25 day old bees compared to five day old bees reared in an incubator, indicating increased innate immunity in older bees.

Chronic bee paralysis (CBP) is an emerging bee disease with a worldwide distribution. It is caused by chronic bee paralysis virus (CBPV) and produces trembling, paralysed and hairless honey bees, often leading to full colony collapse (Ribiere et al., 2010). Transmission of CBPV in a laboratory setting has been shown to occur through feeding, injection and topical application (Bailey et al., 1983; Bailey, 1965; Bailey et al., 1963). Bees injected with CBPV tend to show symptoms within six days of inoculation, and death is observed shortly afterwards. Symptoms are consistent with those seen in naturally infected colonies (Bailey et al., 1963; Ribiere et al., 2010). Although the exact modes of transmission which occur naturally are unknown, it is likely that CBPV is transmitted via trophallaxis, hive cleaning, grooming and corpse disposal. A study by Chen, Evans, et al. (2006) also found that CBPV can be spread from infected queen bees to their offspring. Despite the prevalence of this disease, the ways in which to prevent its spread are mainly unknown.

Previously, we have investigated the effects of trophallaxis and fomite transmission of gut microbiota. In this chapter we will focus on fomite transmission of gut bacteria and the effects this may have on the susceptibility of honey bees to CBPV. We hypothesise that chewing the caps of cells during emergence will lead to a more diverse or abundant gut microbiota than in bees which do not chew through cell caps. We also hypothesise that

susceptibility is influenced by the condition of the gut. This includes the presence of individual core bacteria, the quantity of bacteria and the composition of the communities overall. We expect that naturally emerged bees or pre-eclosed bees have different gut microbial communities, which may affect their susceptibility to CBPV. The age of a bee at the time of exposure to CBPV will likely also affect its susceptibility to the virus. We hypothesise that this could be due to the colonisation of the honey bee gut over time, or the physiological development of the bee. These ideas can be summarised in a conceptual model (Figure 3.1). We used Structural Equation Models (SEMs) to analyse the hypothesised direct and indirect relationships.

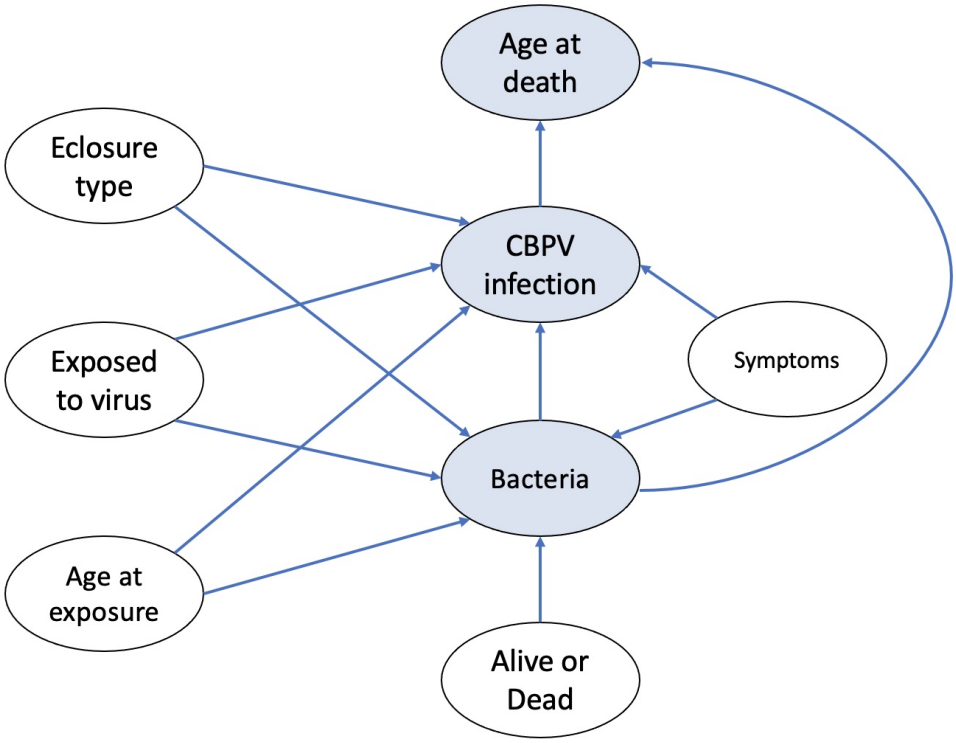


Figure 3.1: A conceptual model of the factors impacting CBPV infection and bacterial communities in the honey bee gut. Blue nodes are response variables.

The aim of this study is to investigate the relationships between the factors shown in Figure 3.1 in order to identify the key drivers of honey bee susceptibility to CBPV infection. We need to collect experimental data with which to address the following aims/phenomena:

i. Assess CBPV infection rates of artificially and naturally emerged bees.

ii. Investigate the difference in key gut microbiome species between naturally emerged and pre-eclosed bees that have been artificially emerged.

iii. Determine if gut bacteria or overall bacterial quantity influences the likelihood of CBPV infection.

iv. Determine if the age at the time of exposure to CBPV affects the gut bacteria that develop or CBPV infection rates.

v. Assess how gut bacteria or CBPV infection impacts the time to death.

vi. Test if symptomatic individuals are more likely to test positive for CBPV and if symptoms affect the gut microbiome.

3.2 Methods

To challenge these hypotheses, an experiment was conducted to investigate the variables shown in Figure 3.1, using eclosure type, exposure to virus and age at exposure as treatments, and measuring CBPV infection, bacterial 16s level and types of bacteria in the bees. This required gnotobiotic bees which are generated through the artificial emergence of pre-eclosed bees. To identify the best way to create this resource, we tested the bacteria levels of bees emerged in various ways using 16s RT-qPCR.

3.2.1 Resource testing: Creating gnotobiotic bees using artificial emergence

Brood were collected from the Ridley building roof apiary and placed in containers (dimensions: 44 cm x 26 cm x 8 cm), and were kept in a dark incubator at 33 °C. Honey bees which emerged overnight were removed from the experiment as their exact age was unknown and the frames were then monitored for emerging bees. Bees were taken at three different stages: naturally emerged, artificially emerged by removing the caps of pre-eclosed bees (fully formed adults that have yet to begin chewing through the cap), and black-eyed pupae. Naturally emerged bees are those which had emerged themselves and were removed from the frame as soon as they were noticed moving around the frame. Artificially emerged bees were moving and hairy in appearance and were uncapped with forceps and removed from their cells. Care was taken to avoid removing bees which had already begun to chew through their cells. Black-eyed pupae showed signs of melanisation, having grey bodies and dark or black antennae. Pupae which seemed very active were deemed too old for this category.

Approximately 150 black-eyed pupae were removed from cells and placed on a tray lined with dry filter paper in a box, containing a salt solution made from 83 ml distilled water and 160 g salt to maintain 75% relative humidity. Bees were removed from the tray when they were able to walk and were marked with a single colour referencing the day they emerged using POSCA paint pens as described in Chapter 2 Section 2.2.4. Emerged bees were then placed in pots and kept inside a box with a salt solution for one day.

Bees were kept in pots in groups of 20. Five pots for each age group were used. On the day of set-up, only four pots of naturally emerged bees were collected. The fifth was collected

one day later, meaning that individuals in this pot were one day behind compared to the other bees in that treatment.

To ensure that the syrup fed to these bees was completely sterile, it was made in the laboratory using 50% distilled water and 50% sugar, and was heated for approximately two minutes. Each pot had two 2 ml Eppendorf tubes containing the syrup, which were replaced when they were empty. A feeder was also placed in the tray for any emerged black-eyed pupae.

Bees were monitored every day for the duration of their lives, and any symptoms, irregularities and deaths were noted to attempt to ensure bees were healthy. Dead bees were removed from the pots and if possible, their guts were dissected in preparation for bacterial 16s RT-qPCR testing, although an increase in the fragility of the guts as bees age meant this was hard to achieve in older bees. To see how levels of bacteria changed over time in uninfected bees, five individuals (one from each pot) were sacrificed each day, from day zero to day four and their guts dissected. For emerged black-eyed pupae, day zero is considered to be the day the bees emerged (when they are hairy and can walk around). A sample of black-eyed pupae were also taken the day before day zero (one day before they were classed as 'emerged'). Extracted guts and dead bees were stored at -80 ^{o}C for molecular analysis.

3.2.2 Identifying the cause of susceptibility to CBPV in young bees

3.2.2.1 Virus preparation

A CBPV crude extract used to inoculate bees was created using the method described in Chapter 2 Section 2.2.3.

3.2.2.2 Experimental set-up

Two frames of brood were collected from the Ridley roof apiary and kept in plastic boxes in an incubator at 30 ^{o}C and approximately 70% humidity. Two types of bees were collected; naturally emerged, which were bees which emerged themselves and were removed from the frame between 0-1 hour post-emergence, and artificially emerged, which were hairy, moving bees uncapped using forceps before they began to chew through the cap of their

cell. Bees were kept in pots of approximately 15 bees. All bees were marked individually shortly after emergence using POSCA paint pens for individual identification, as described in Chapter 2 Section 2.2.4. Bees were either fed CBPV or buffer, which was done between 3-7 hours post-emergence, or 24-26 hours post-emergence. The virus solution contained 50% CBPV extract in 0.9% sodium chloride buffer and 50% sucrose syrup, and the buffer solution contained 50% of the 0.9% sodium chloride buffer and 50% sucrose syrup. Bees were fed 3 μl of either solution, as described in Chapter 2 Section 2.2.4. To ensure the syrup was not contaminated with bacteria, sucrose syrup was made from 50% autoclaved distilled water and 50% sugar, as described by Williams et al. (2013). Replicates were set-up evenly over two days in order to collect enough bees. One replicate consisted of two buffer-fed pots and three virus-fed pots per emergence type and feeding type. Each treatment also had its own sacrificial pot. These bees were treated in the same way, and whenever a bee from a treatment pot showed symptoms or died and was removed, it was matched with at least one asymptomatic bee in the sacrificial pot, which are referred to as asymptomatic removed bees.

3.2.2.3 Data collection

Observations began the day after the trials were set-up and followed the method described in Chapter 2 Section 2.2.4.

3.2.3 Molecular analyses

Bee samples went through the grinding and RNA extraction process described in Chapter 2 Section 2.2.5.1, before being tested for CBPV via RT-qPCR. Gut samples were also ground and went through the DNA extraction process. They were then tested for bacterial 16s, *Lactobacillus*, *Snodgrassella alvi*, *Gilliamella apicola* and *Frischella perrara* through RT-qPCR which was then quantified as described in Chapter 2 Section 2.2.5.3. The sequences used for the probes, forward primers and reverse primers are shown in Table 3.1. Similarly, gut samples from the resource testing went through the grinding and DNA extraction method described in Chapter 2 Section 2.2.5.2. These samples were then tested for 16s using the same RT-qPCR method.

Table 3.1: The sequences used for the probes and forward and reverse primers for each of the bacteria tested for, and 16s. *Indicates assay is from Budge et al. (2016)

Bacteria	Type	Sequence
Gilliamella	Forward	CCCTTACGACCAGGGCTACA
	Reverse	TACTTTATGAGGTCCGCTTGCTCT
	Probe	AATGGCGTATACAAAGGGAGGCGACCTC
Frischella	Forward	CCTTACGACCAGGGCTACACA
	Reverse	TTAGGAGGTCCGCTCCAGC
	Probe	CTACAATGGCGTATACAAAGGGAAGCGAAGGT
Snodgrassella	Forward	CAATCTCAGAAAGCCGATCGTA
	Reverse	GCGATTACTAGCGATTCCGAC
	Probe	TCCGGATTGCACTCTGCAACTCGAG
*Lactobacillus**	Forward	GAATAACCTACCTCAAAGTCTGGGATA
	Reverse	TGCACCGCGAATCCAT
	Probe	AAAAGCTGCGTTTGCAGCGCTTTAA
16s	Forward	TGGAGCATGTGGTTTAATTCGA
	Reverse	ATGCAACGCGAAGAACCTTAC
	Probe	CACGAGCTGACGACATCCATGCA

3.2.4 Statistical analyses

All analyses were undertaken in R version 4.2.2 (RCoreTeam, 2022).

3.2.4.1 Survival and Competing risks analyses

Bees are highly social animals living in colonies and have complex social interactions involving food, food provision and general hive maintenance. Replicating this in an experimental system is impossible. We have to use small pot experiments for replication in which a restricted number of bees are kept in an artificial environment. It is therefore likely that both the experimental set-up and any imposed interventions/treatments could lead to the outcomes of interest, in this case paralysis symptoms and death. This is a case of competing risks where the experimental design and the treatments could both mask the impacts of each other. In order to evaluate the impacts of the experimental protocol on death outcomes we used control pots and monitored honey bee deaths with no treatment intervention. The results of the previous chapter showed that the bees would gradually decline as the time increased since they were last in a normal hive. This appears to be at approximately day 13, but here we used a more formal quantification based on this experiment. Preliminary investigations showed that bees were resilient in the sense that none died for several days after the set-up of the controls. This implies that bees show some initial resilience to the imposition of being put in the experimental protocols. In order to quantify this resilience, we fitted asymptotic regressions models of the number of deaths in relation to time since the initiation of the experimental set-up. We then used the models to predict the point at which this resilience started to decline and bees died. We used this point as a cut-off in investigating the impacts of different disease treatments in subsequent experiments, thus separating the competing risks of experimental design and disease. We used non-linear least squares (NLS) modelling with the *nlme* package to do this, which identified a threshold of nine days (model results are shown in Appendix B.). A three parameter self-starting log-logistic model was used based on the following equation:

$$f(x) = c + \frac{1 - c}{1 + exp(b(log(x) - log(e)))}$$

Survival analysis was used to identify how risk of symptoms or death due to CBP infection varies between bees exposed to CBPV at different ages, and in bees emerged naturally or artificially. The packages *survival* and *survminer* were used to create Kaplan-Meier curves and perform a log-rank test to compare the survival curves in each treatment (Kassambara et al., 2019; Therneau, 2020). Survival analysis was initially conducted on

all treatments using death or paralysis symptoms as the event (all-cause hazards). Bees which were in sacrificial pots and were removed when asymptomatic were excluded from the analysis. This analysis included 518 bees, which with a required power of 0.8 we can predict an effect size of 1.5 when anticipating 50% mortality (Therneau & Grambsch, 2000). The effects of these treatments on risk of symptoms or death were then investigated using Cox proportional hazards models. Effect sizes were presented as hazard ratios. The proportional hazards assumption was tested using proportionality tests and analysis of the Cox model's Schoenfeld residuals (Therneau & Grambsch, 2000).

To assess the effect of pot as a grouping variable, frailty was initially included in the model. However, the models failed to converge and therefore, frailty was not included, as discussed in Chapter 2 Section 2.2.6.2.

As symptoms and death can be caused by factors other than CBPV (as shown in Chapter 2), competing risks analysis was conducted on the viral treatments using the package *cmprsk* (Gray, 2022). This analysis only involved the bees which died within the first eight days, based on the results of the NLS model, and any bees which were still alive and symptom-free on day eight were censored. A Fine-Gray subdistribution hazard model was used, which measures the probability of symptoms or death due to CBPV infection at each time point, if symptoms or death due to other causes have not taken place (Berger et al., 2020; Fine & Gray, 1999). This separates the risk of symptoms or death due to CBPV from the risk of symptoms or death caused by the experimental set-up.

3.2.4.2 Structural equation modelling

Structural equation models (SEMs) were used to analyse the relationships between the various treatments and PCR results for CBPV, 16s, *S. alvi*, *G. apicola*, *F. perrara* and *Lactobacillus*, as we hypothesised that there would be indirect effects between CBPV concentration and bacteria concentration. The model was based on the conceptual model shown in Figure 3.1, adapted into the model shown in Figure 3.2, and challenged with experimental data. A grouped SEM using the *lavaan* package was performed, where the group was each of the four bacteria (Rosseel, 2012). This allowed the individual direct and indirect impacts of the different bacterial species to be compared. A separate SEM was conducted for 16s to assess the effect of the overall quantity of bacteria in the gut. As SEMs assume data is multivariate normal, the relationship between each response and predictor variable was tested prior to the SEM using generalised linear

models (GLMs) (shown in Appendix C), which revealed that the residuals of these models were not normally distributed (Appendix C, Figure 6.4). Therefore, the SEMs were conducted using bootstrapping with 1000 runs each following the rationale of Manly (1997). The model ran with subsamples of the original data and created parameter estimates for each run of the SEM. This assumes that repeated runs of this magnitude will generate distributions for each parameter estimate which will approximate to normal. Thus, the confidence intervals on the means of each of the parameter estimates can be used to quantify the extent to which the estimates are different from zero (i.e. no effect on the individual SEM pathway).

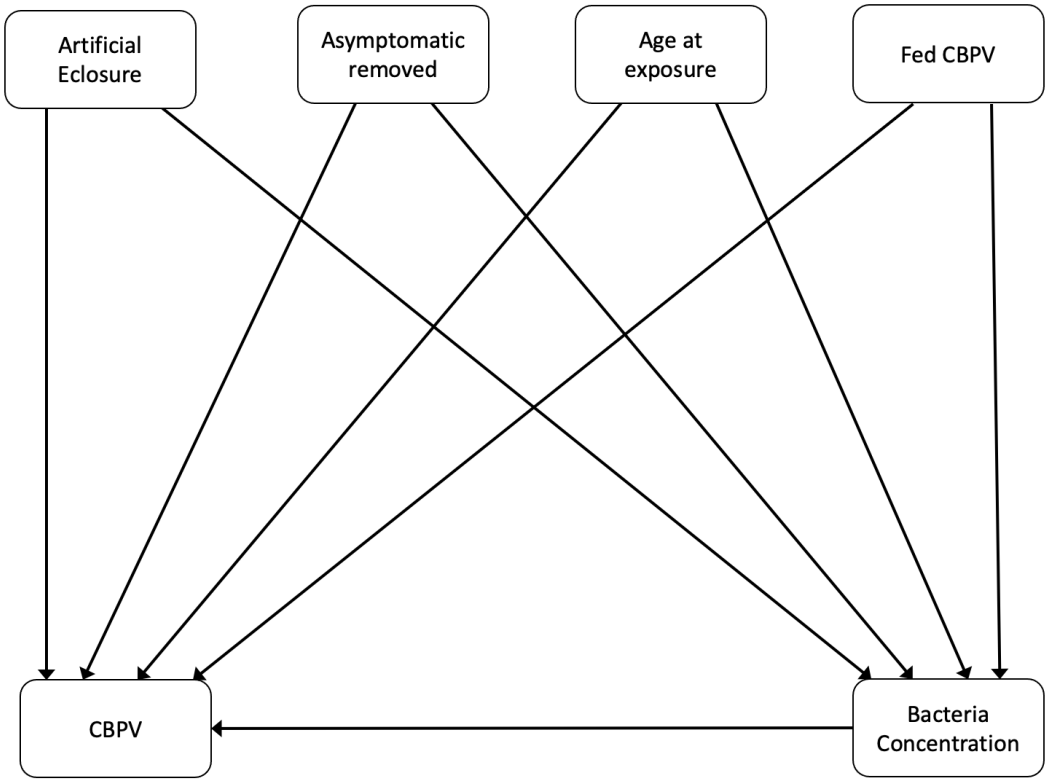


Figure 3.2: A conceptual model of the factors impacting CBPV infection and bacterial communities in the honey bee gut.

3.2.4.3 Generalised linear models

Generalised linear models (GLMs) were used to investigate the effect of day of removal from the experiment on the qPCR results. Models were fitted using a Gaussian error structure.

3.3 Results

3.3.1 Resource testing: Creating gnotobiotic bees using artificial emergence

The results of the 16s RT-qPCR analysis of the time series of differently emerged bees found two groupings of Cq readings; one group between 24-28 Cq, which included the extraction buffers, and the other between 15-18 Cq, indicating bacterial levels that were 100 times higher. Our 16s assay identifies a wide range of bacteria, including those that may be found in the laboratory environment and were introduced during DNA extraction and PCR set-up. As such, a conservative threshold was used to identify true 16s PCR positives with samples with a Cq below 26 considered positive, and those above considered negative. None of the emergence types led to bees which all tested negative for 16s, and uncapped adults had the lowest number of positive bees overall (Figure 3.3). There was also very little difference in survival between bees from each type of emergence (Figure 3.4).

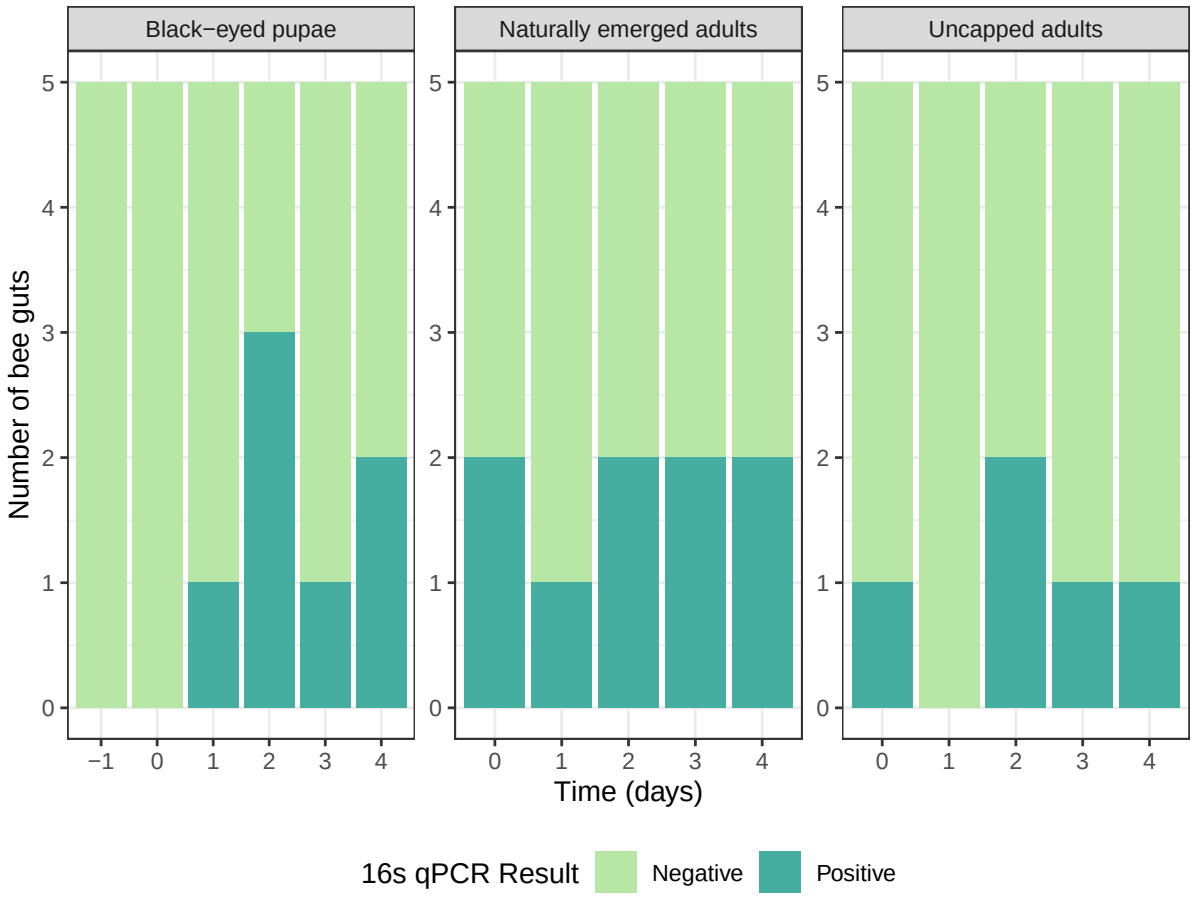


Figure 3.3: The number of individuals from each type of eclosure group with a positive 16s qPCR result each day (<Cq 26). Eclosure groups were naturally emerged, uncapped adult bees and black-eyed pupae.

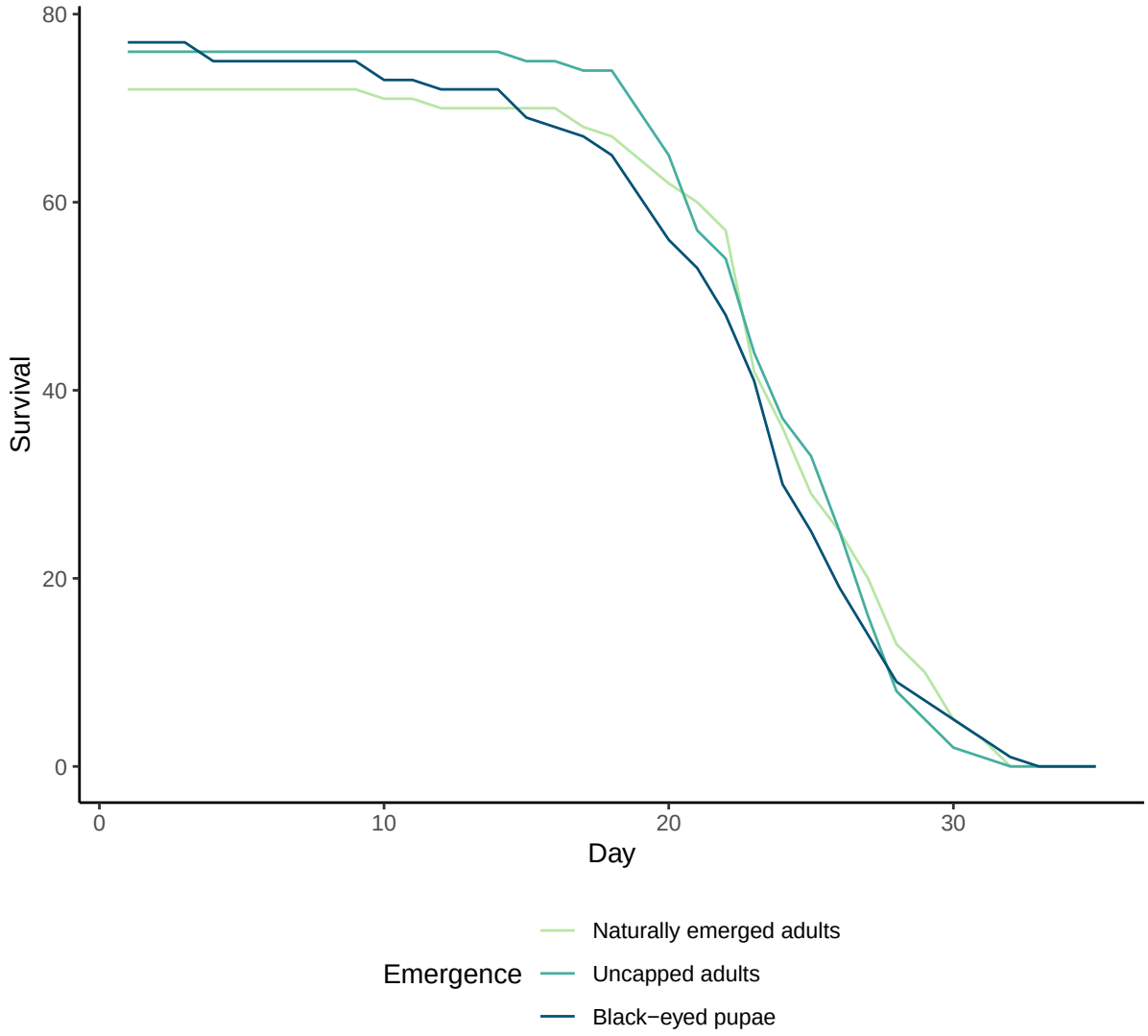
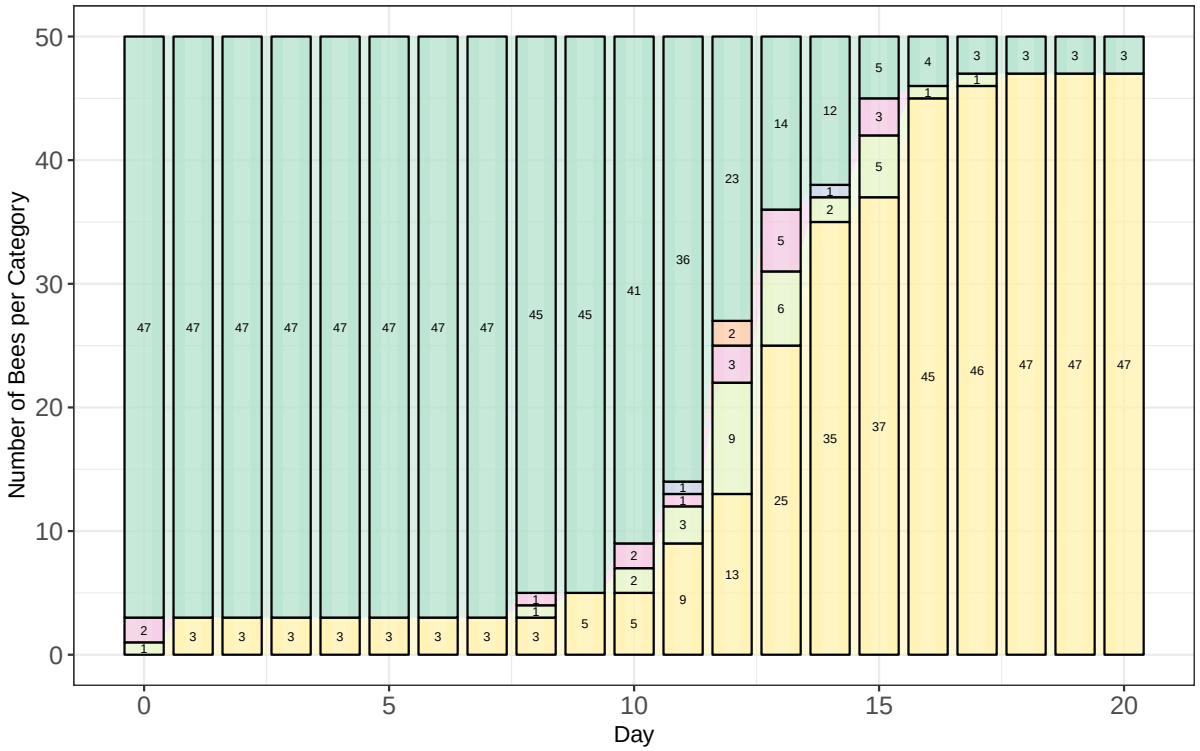


Figure 3.4: The number of surviving bees based on the way they were emerged. Bees were either taken as black-eyed pupae, uncapped when they were adults, or allowed to emerge naturally.

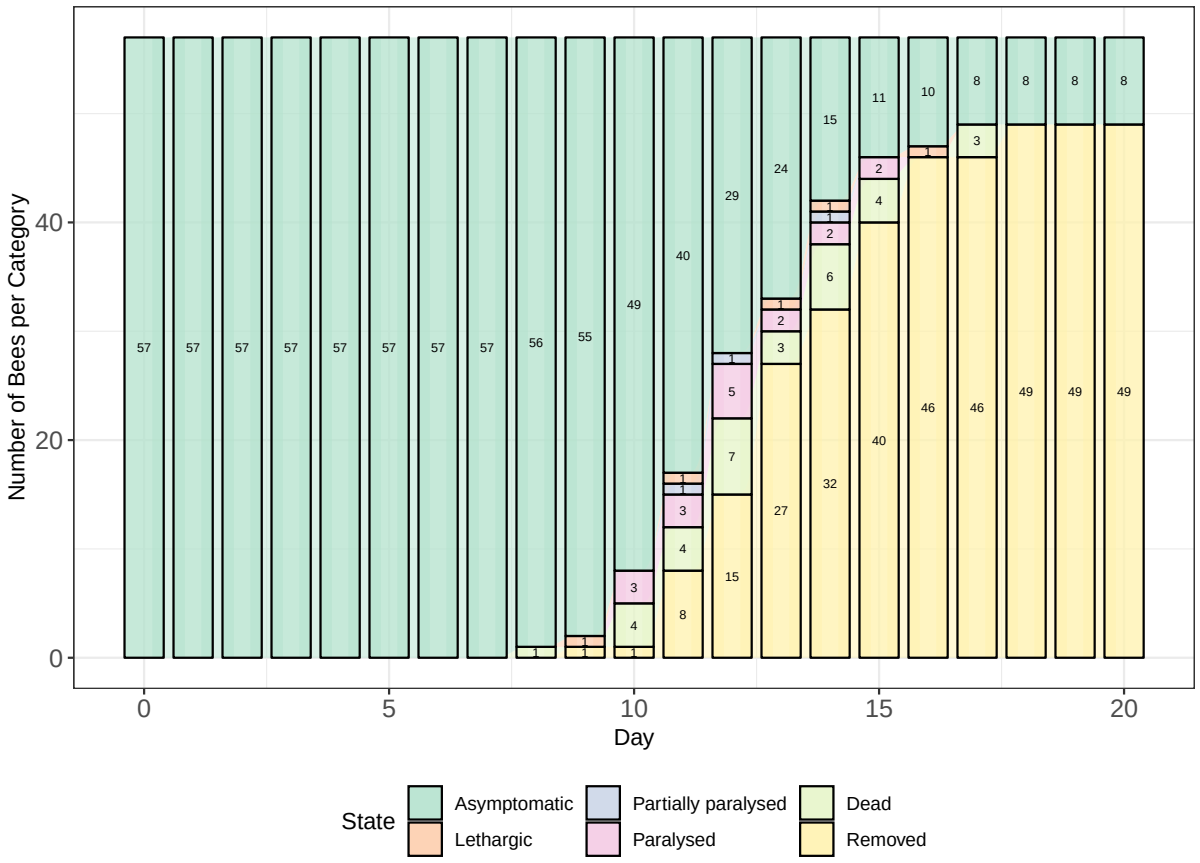
3.3.2 Identifying the cause of susceptibility to CBPV in young bees

Artificially emerged bees fed virus on day 0 had a much higher proportion of symptomatic and dead bees than any other treatment by day 10, with 80% of bees showing symptoms or dying by this point (Figure 3.5). Both of the artificially emerged buffer-fed treatments had relatively low numbers of symptomatic and dead bees by day 10, ranging from 14-18%. There were very similar numbers of symptomatic and dead bees seen in the artificially emerged virus-fed on day 1 treatment and the naturally emerged virus-fed on day 0 and 1 treatments, with all treatments having between 33-38% of bees symptomatic or dead by day 10. The naturally emerged buffer-fed on day 1 treatment had a higher symptomatic and mortality rate than expected, with 28% of bees showing symptoms or dying by day 10. However, no bees were symptomatic or died until day 8, which is similar to the other buffer treatments, which also had very few or no dead or symptomatic bees up until this point.

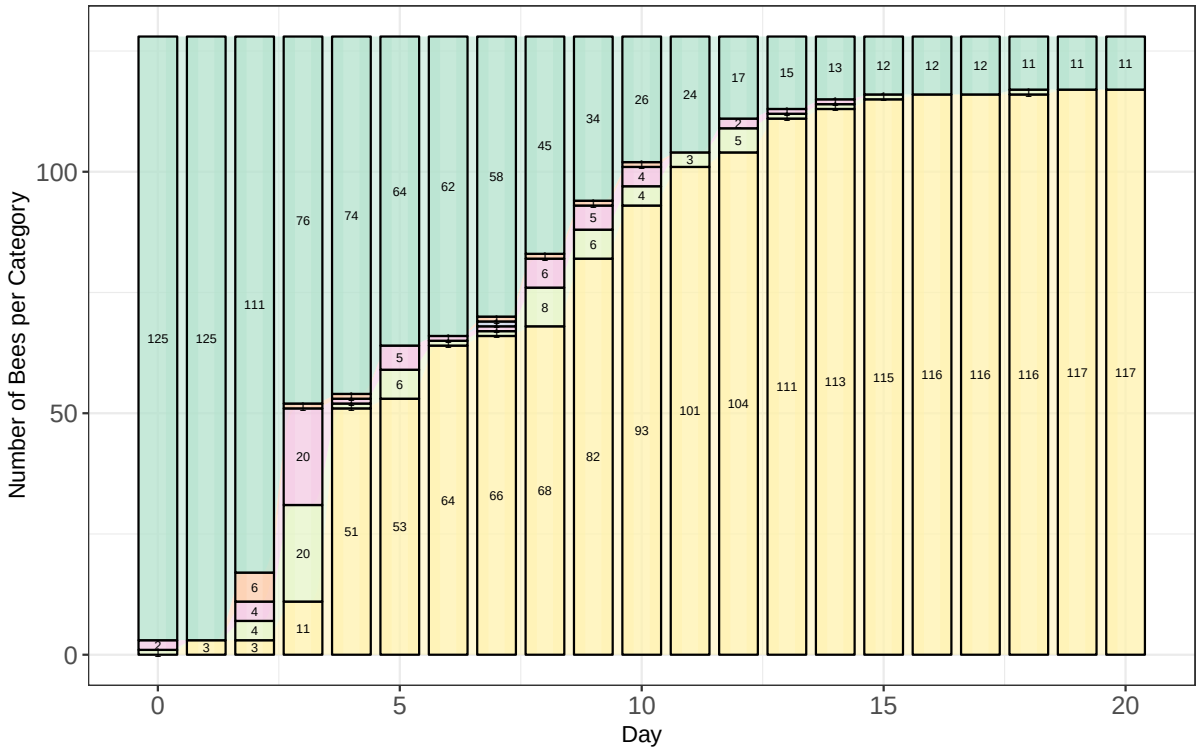
a. Artificially emerged, buffer−fed, day 0



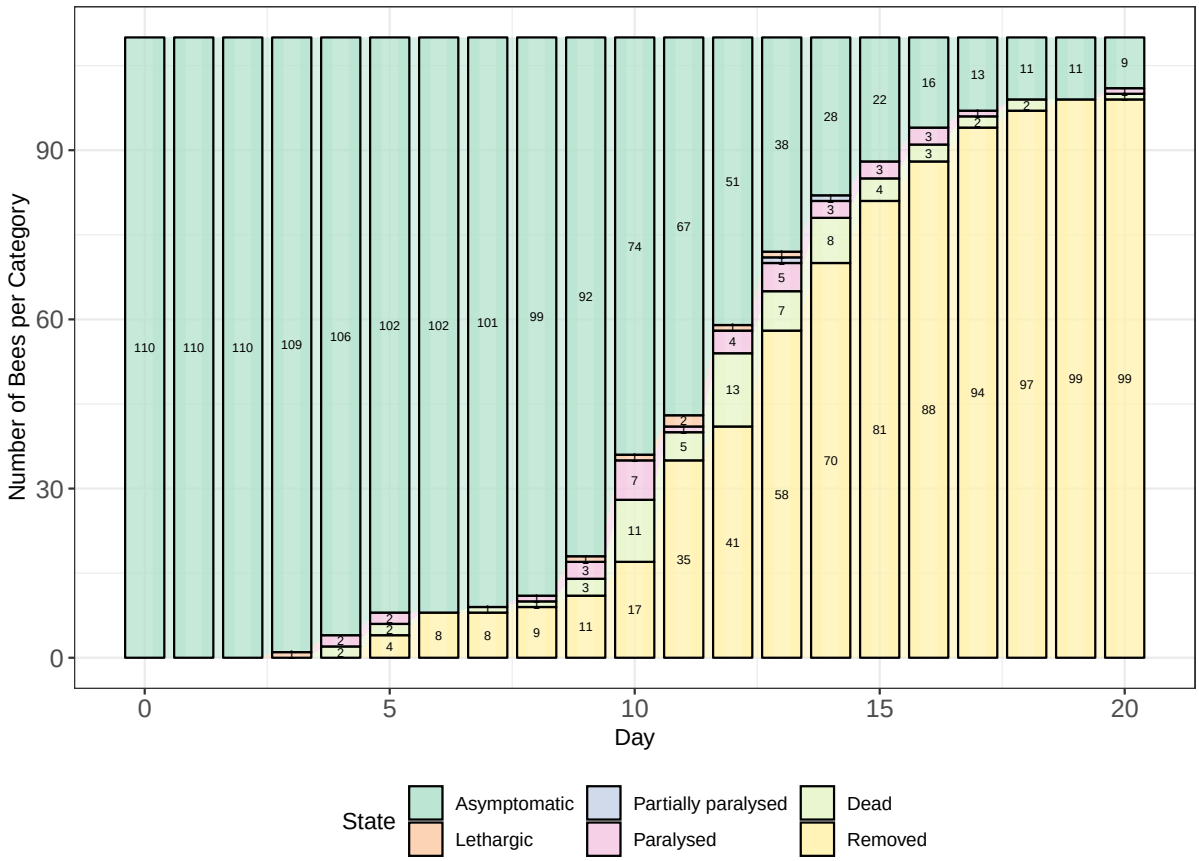
b. Artificially emerged, buffer−fed, day 1



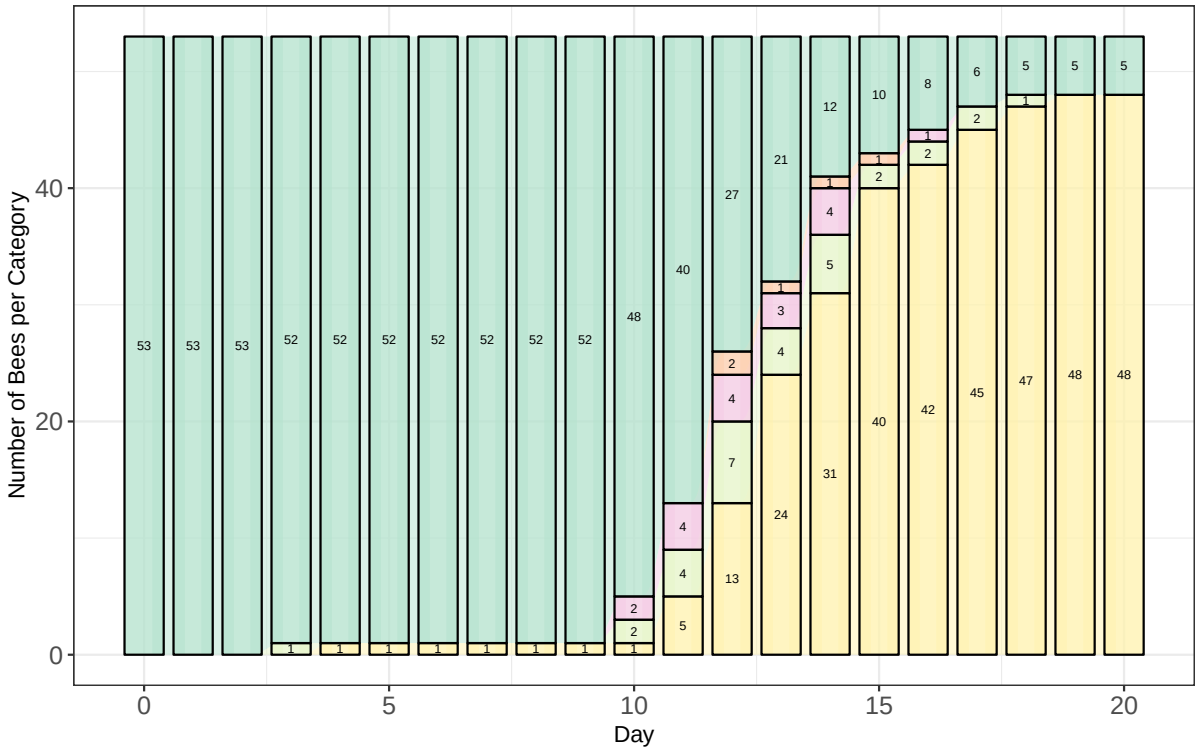
c. Artificially emerged, virus−fed, day 0



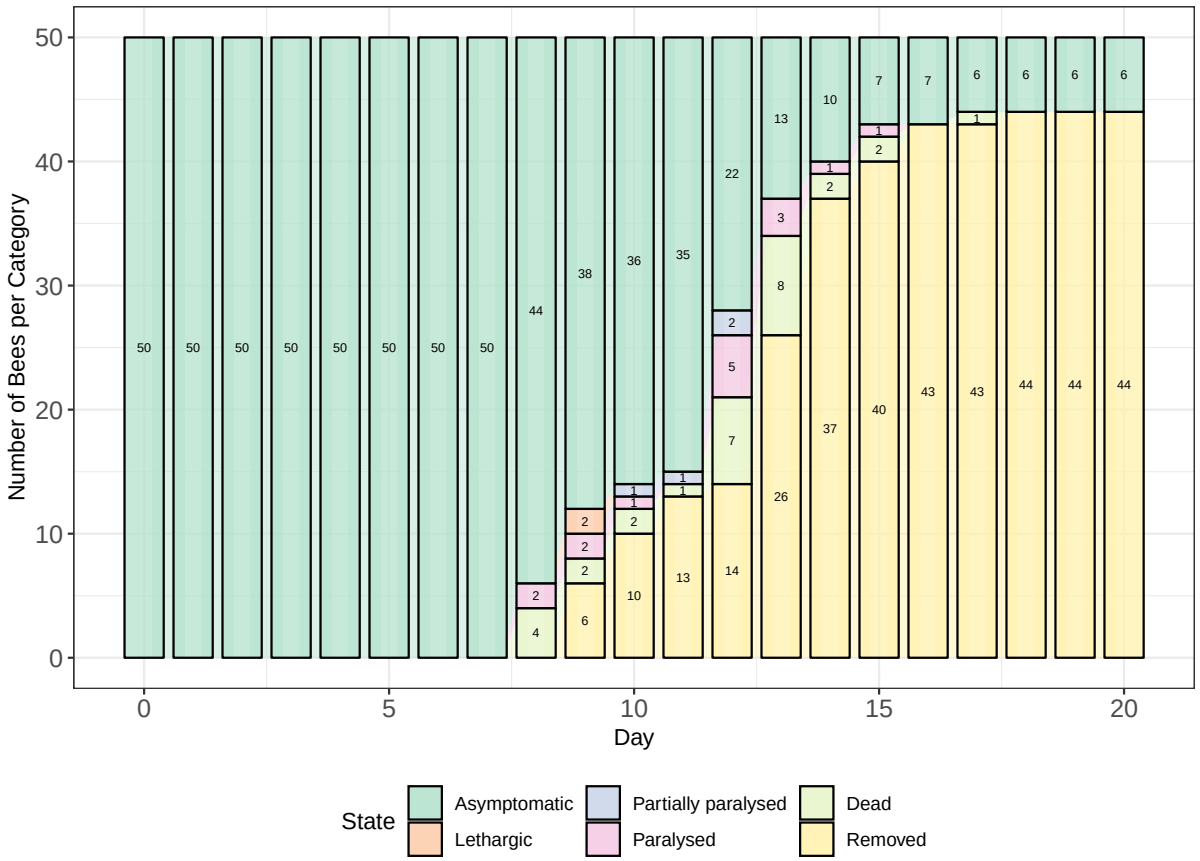
d. Artificially emerged, virus−fed, day 1



e. Naturally emerged, buffer−fed, day 0



f. Naturally emerged, buffer−fed, day 1



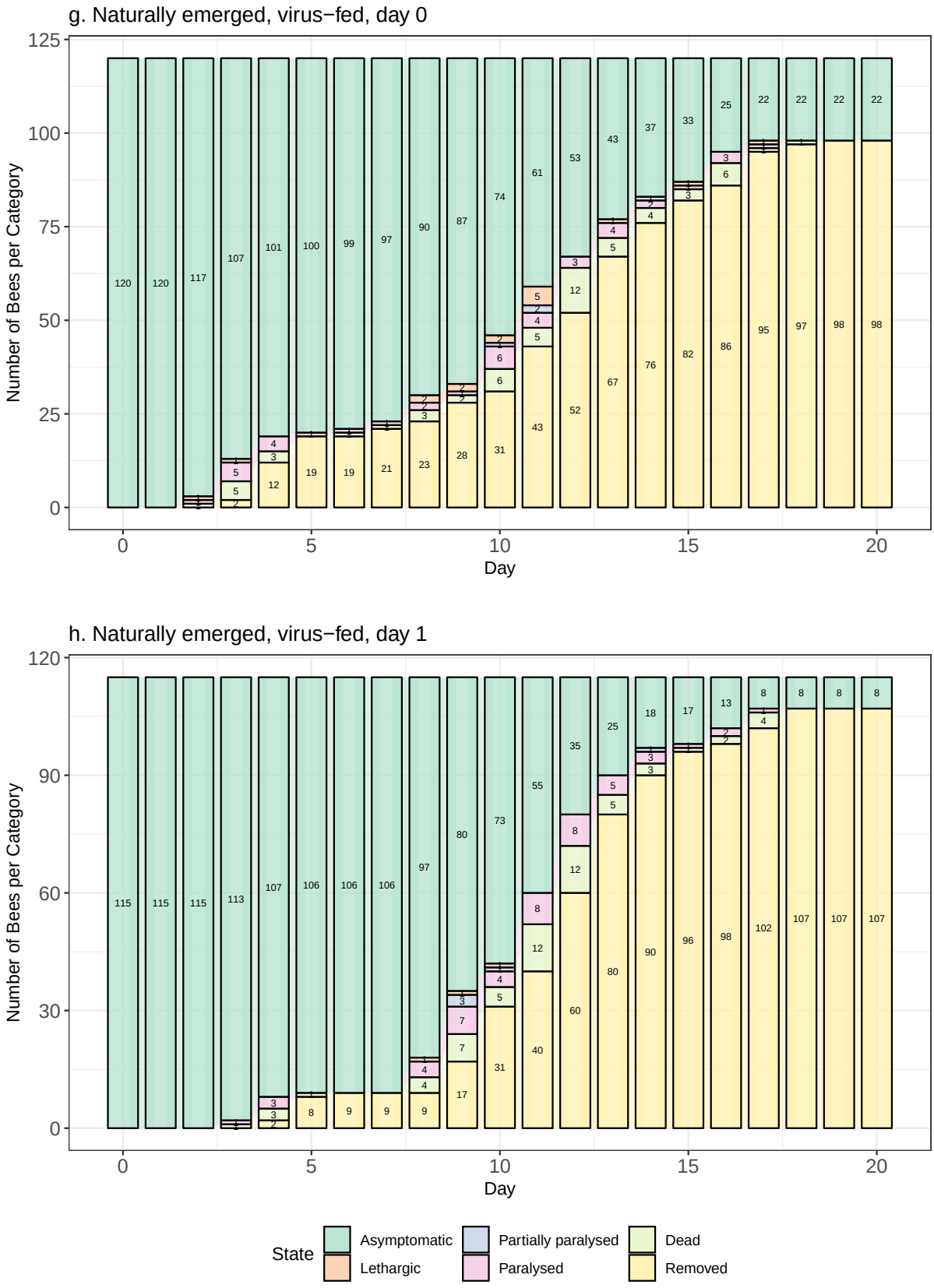


Figure 3.5: The number of lethargic, partially paralysed, paralysed, asymptomatic or dead bees each day of the experiment for each treatment. Bees which were paralysed or dead were removed from the experiment and are subsequently shown as removed.

3.3.3 PCR Results

The results of the CBPV RT-qPCR found low levels of CBPV in the extraction buffers of each plate. The lowest Cq value from these buffers was used as a threshold, where any samples with Cqs above this were considered negative. The number of positive and negative samples in each treatment is displayed in Figure 3.6. Using this method, all the samples which tested positive for CBPV were in the virus-fed treatments, with the exception of one sample in the naturally emerged buffer-fed on day 0 treatment. There were also samples in the virus-fed treatments which tested negative for CBPV as a result of the threshold used. All the positive samples come from bees which were removed from the experiment on day six or earlier.

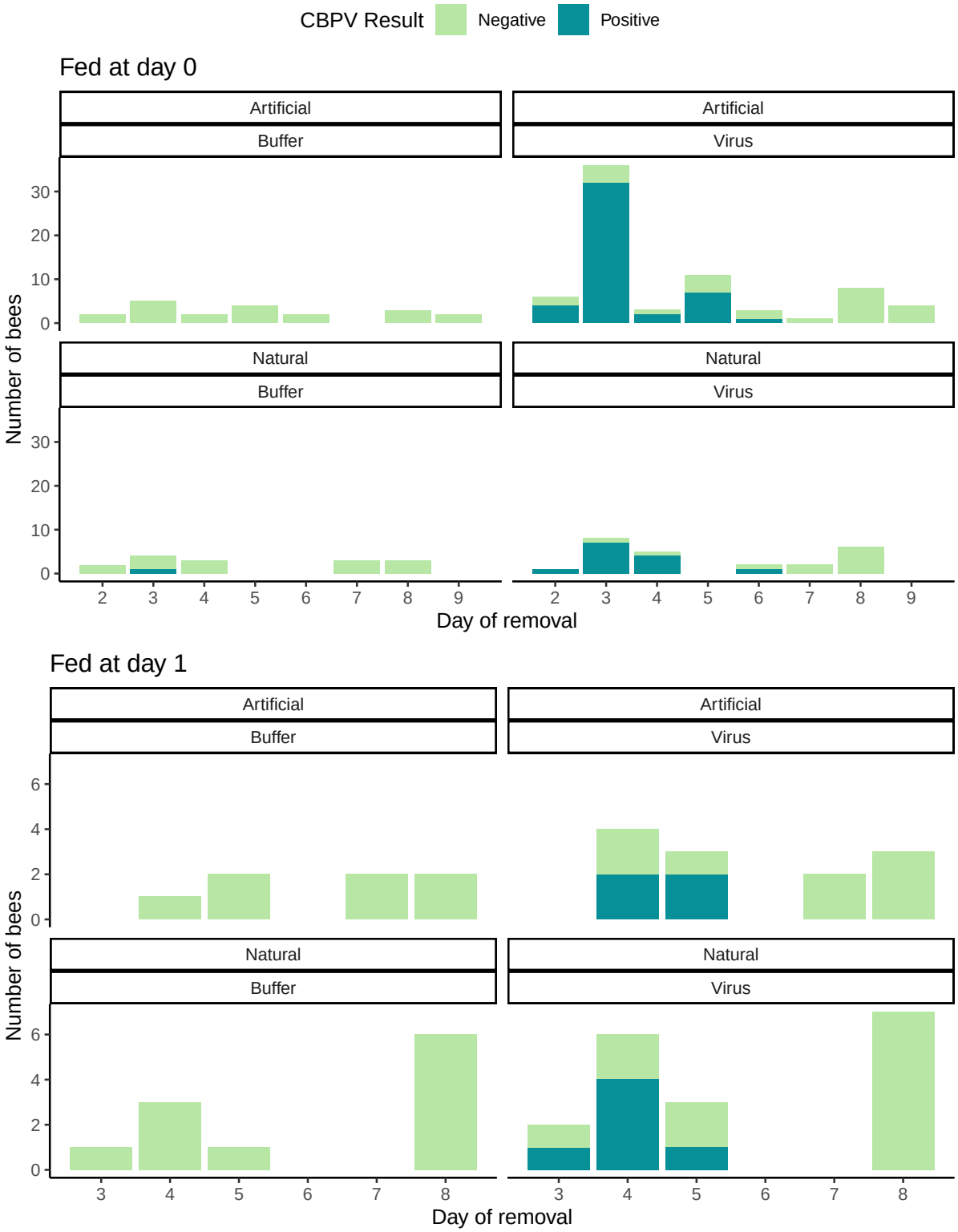


Figure 3.6: The number of bees which tested positive or negative for CBPV per treatment by date of removal

The bacteria identified in the bee guts varied greatly in both type and quantity between bees of the same treatment (Figure 3.7). One exception to this is *Lactobacillus*, which was only observed in naturally emerged bees, but still varied in quantity between bees. *S. alvi*, *F. perarra* and *G. apicola* were rarely identified. The bacterial 16s concentration was generally higher in naturally emerged bees compared to artificially emerged bees, irrespective of age or virus treatment.

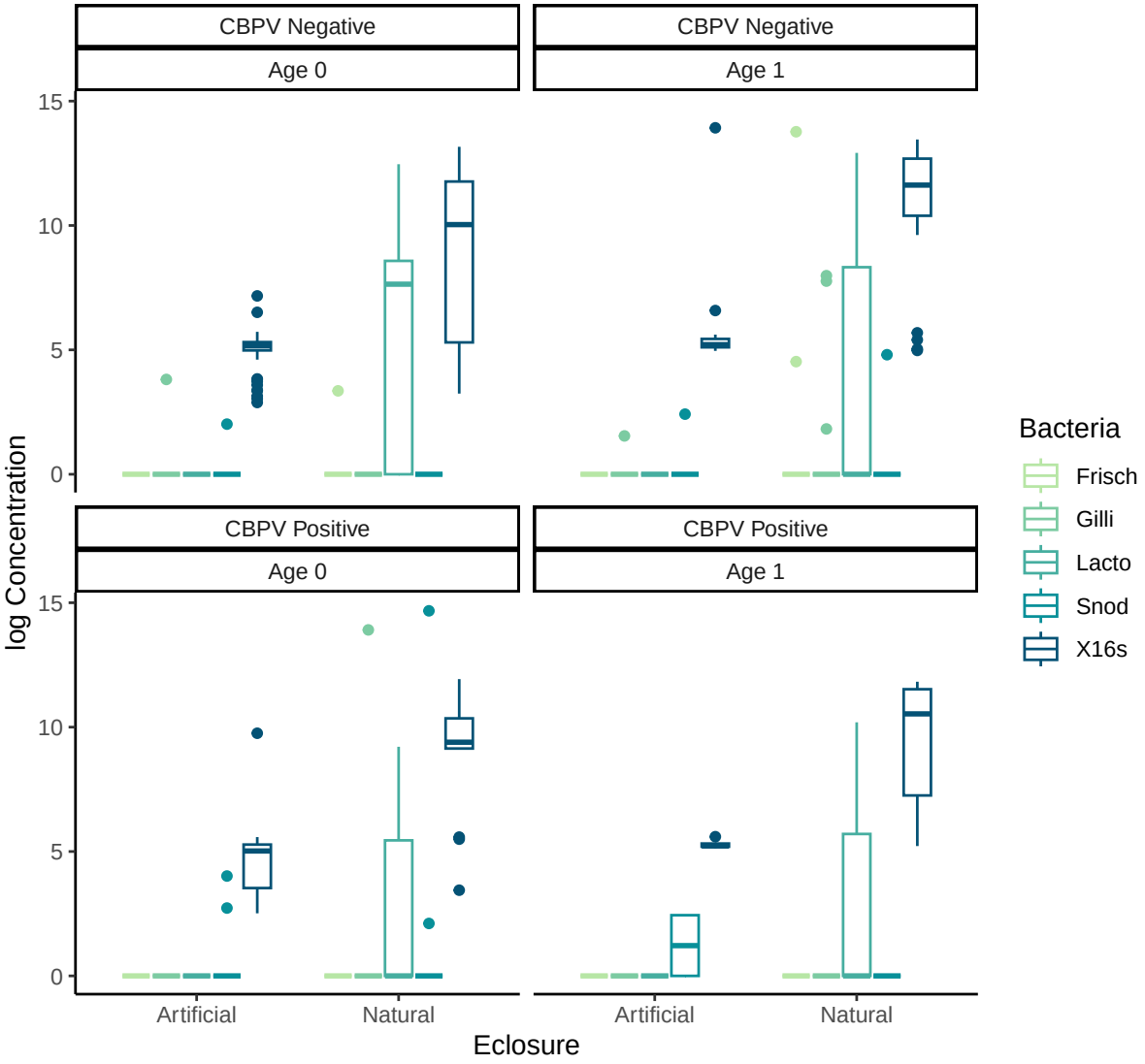


Figure 3.7: The log base 10 concentrations of each type of bacteria or 16s RT-qPCR result, grouped by age of the bee at the time of exposure (in days), a positive or negative CBPV result, and the type of eclosure. The central horizontal line represents the median value, the box joins the first and third quartile. The lower and upper quartiles are represented by the horizontal lines at each end of the box. Points are outliers.

3.3.4 Survival Analysis

3.3.4.1 All-cause hazards

There was a noticeable difference in survival between artificially emerged bees fed virus at day 0 and all other treatments (Figures 3.8 and 3.9). By day three, the survival probability for artificially emerged virus-fed bees was approximately 0.55 and dropped to approximately 0.4 at day 5. Naturally emerged bees fed virus at zero days old also had an initial decline in survival probability at day three, plateauing at approximately 0.80 before gradually declining at approximately day eight. All other treatments also saw a gradual decline in survival probability from this point onwards.

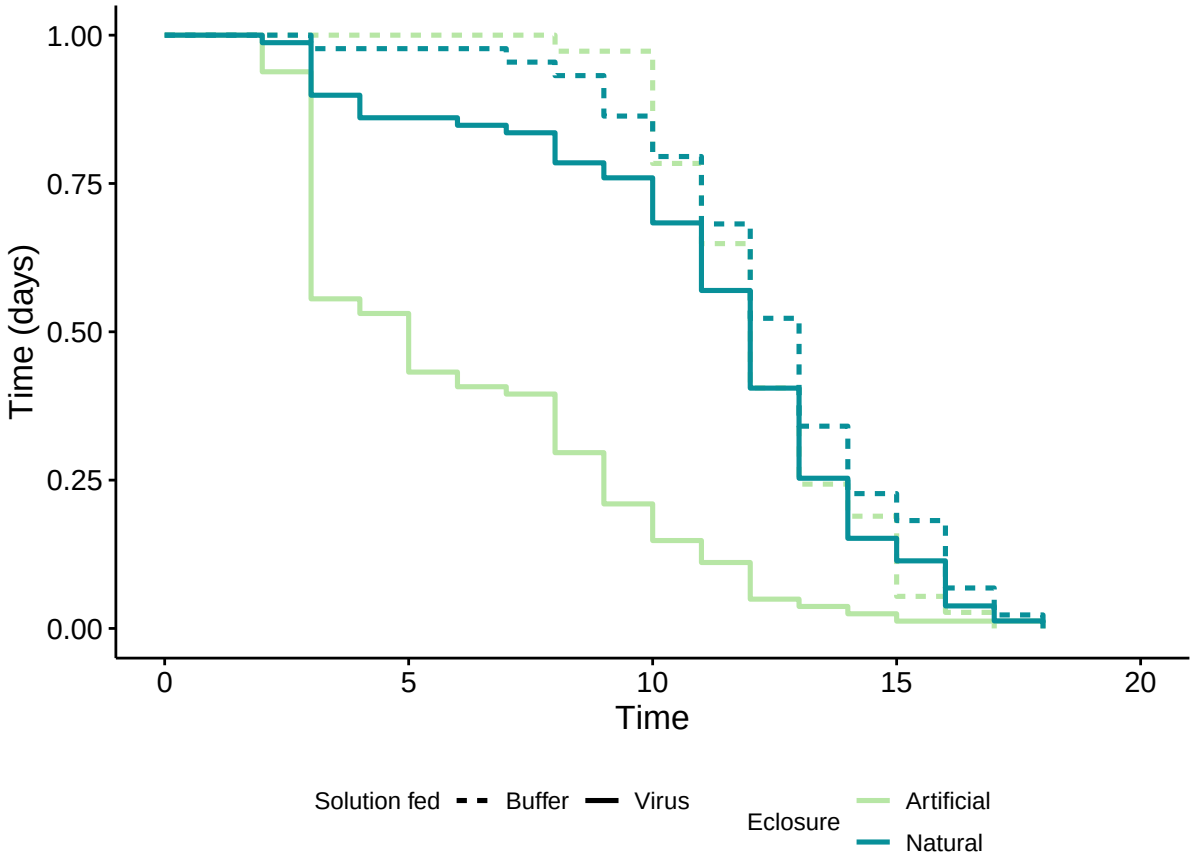


Figure 3.8: The Kaplan-Meier survival curves for bees exposed to virus or buffer when newly emerged (at 0 days old), for the duration of the experiment, displaying the probability of survival based on treatment type.

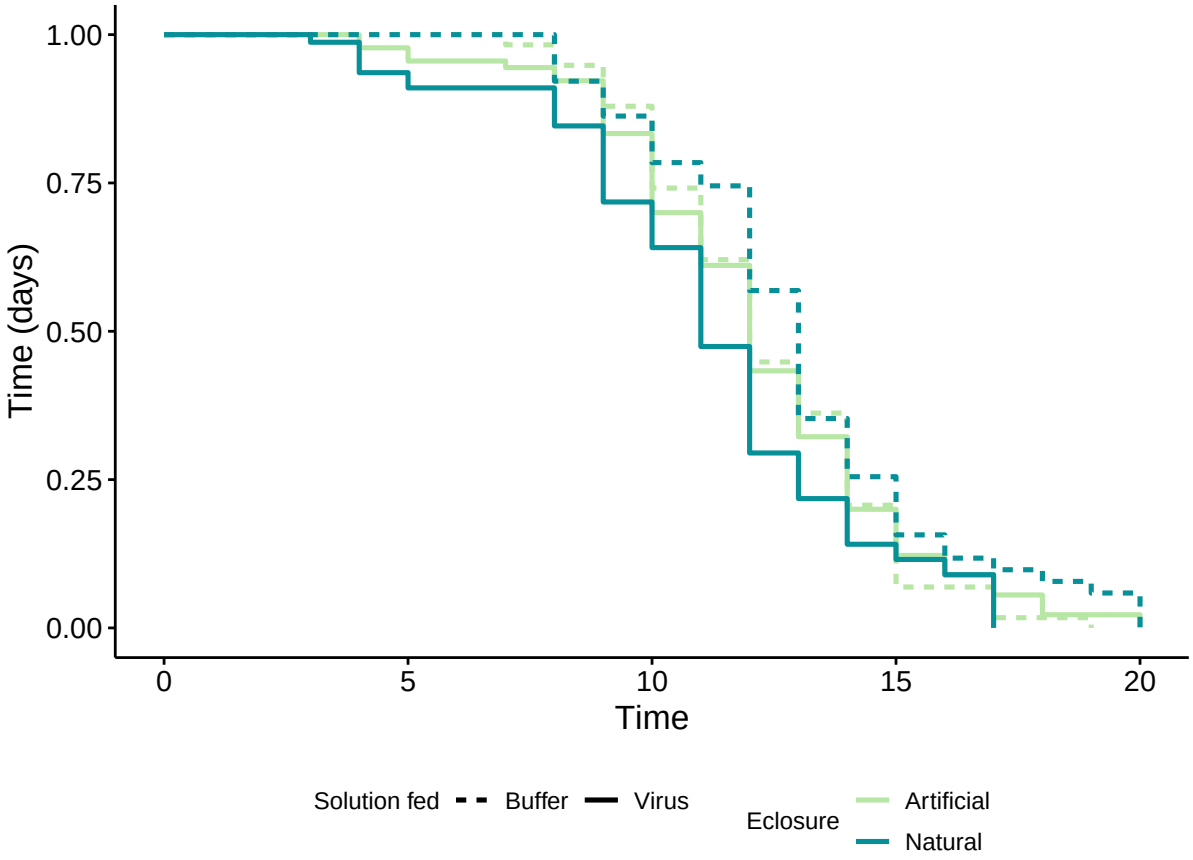


Figure 3.9: The Kaplan-Meier survival curves for bees exposed to virus or buffer when 1 day old, for the duration of the experiment, displaying the probability of survival based on treatment type.

The log-rank test found a significant difference between the survival curves of these treatment (Chisq = 169, df = 7, $p < 0.001$). The Cox proportional hazards model found that virus inoculation ($z = 4.35$, $p < 0.001$), artificial eclosure ($z = 3.724$, $p < 0.001$) and exposure at day 0 ($z = 5.207$, $p < 0.01$) significantly increased the risk of death (Figure 3.10).

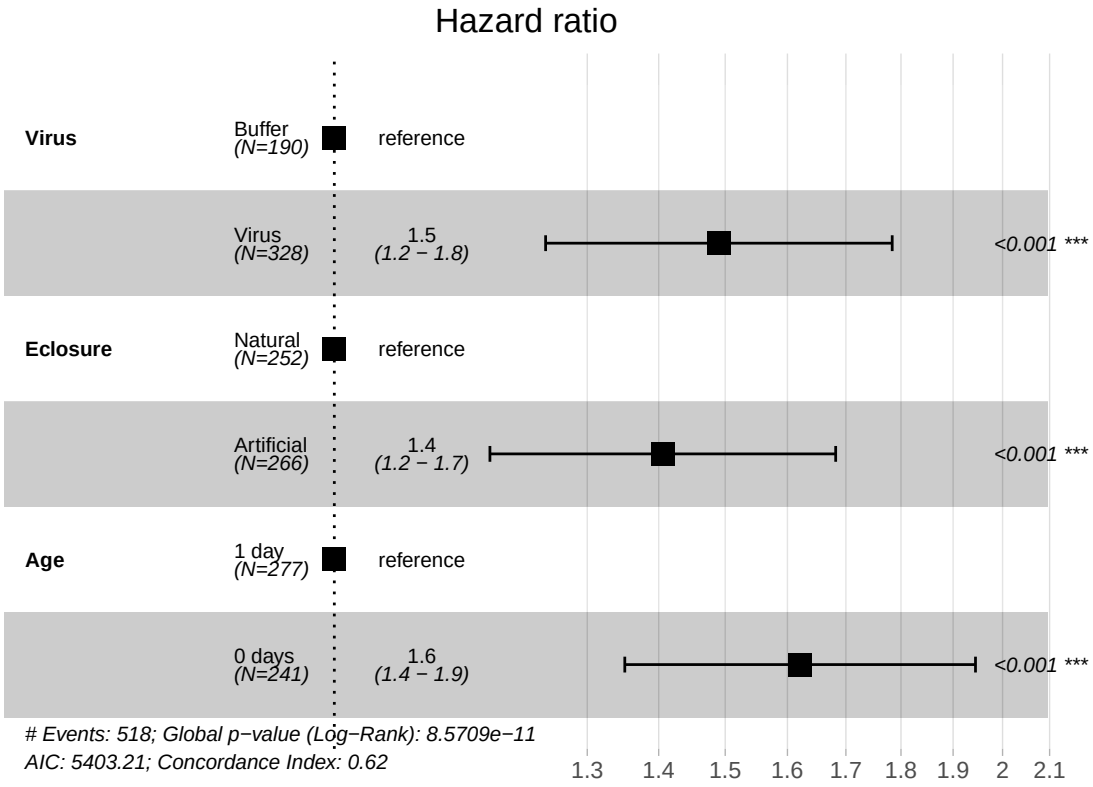


Figure 3.10: The hazard ratio of death in each treatment type; virus inoculation, artificial eclosure and age at time of exposure. Scores above one indicate an increased risk of death, and a score below one indicates a decreased risk of death. The p-value for each treatment is also given.

The proportional hazards assumption of the Cox model was tested. Virus inoculation, artificial emergence and age at exposure violated the assumption (Table 3.2). The hazards related to virus inoculation, artificial emergence and exposure to virus at day 0 decreased in the first 10 days of the experiment, indicating that the hazards associated with these treatments were higher in the first three to four days of the experiment, and fewer deaths due to CBP took place after this point (Figure 3.11).

Table 3.2: The results of the proportional hazards assumption test of a Cox regression investigating the effect of expsure to virus, age at exposure and eclosure type of honey bees on survival time. The proportionality of the hazard of each treatment was tested over time. A significant result indicates a violation of the assumption.

Treatment	Chi Squared	df	p-value
Virus	25.385	1	0.000
Artificial eclosure	7.196	1	0.007
Fed at 0 days	6.455	1	0.011
GLOBAL	41.487	3	0.000

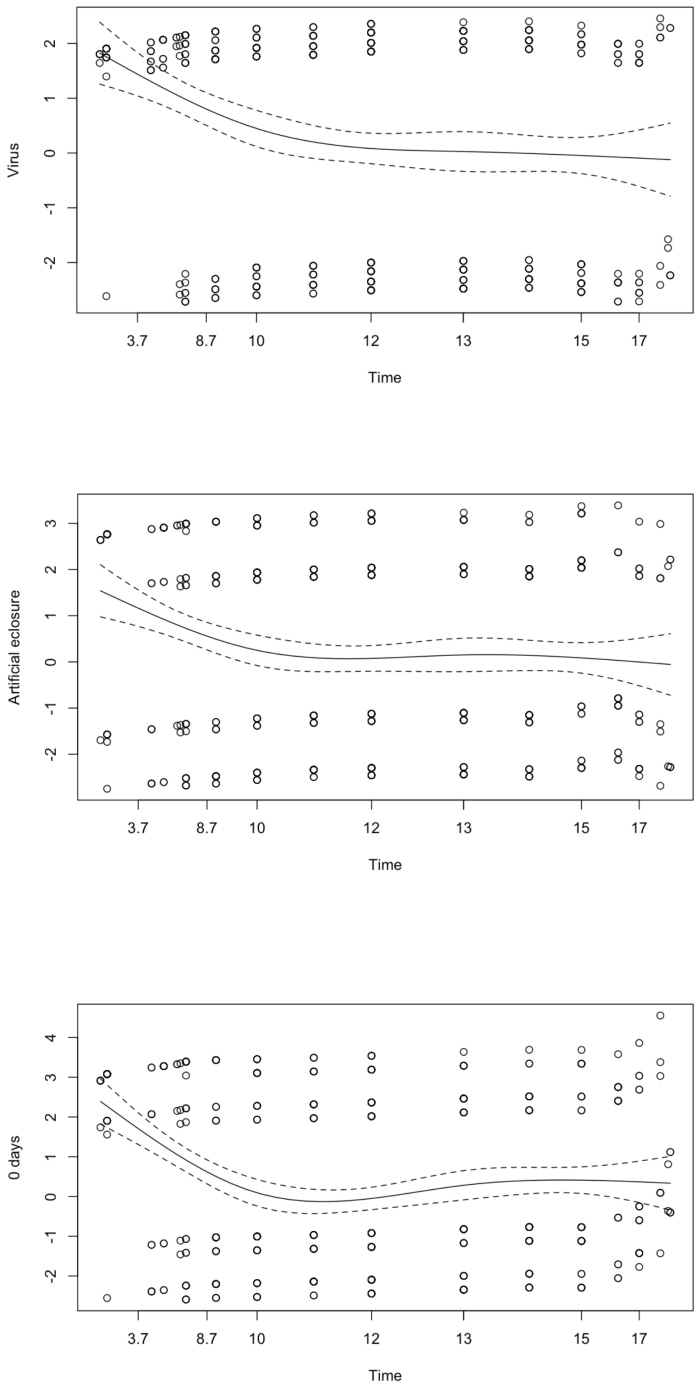


Figure 3.11: Schoenfeld residual plots from a Cox regression investigating the effects of exposure to virus, age at exposure and eclosure type of honey bees on survival time. Plots examine the proportionality of the hazards of each treatment when interacting with time. The residuals are the observed covariate values minus the expected covariate values for each bee at each time point. The dashed lines are the upper and lower confidence limits for the coefficients of the covariates.

3.3.4.2 Competing risks analysis

Competing risks analysis was conducted on all virus treatments with bees that died within the first eight days of the experiment, based on the results of the NLS threshold analysis (Appendix B). Age of the bee at the time of exposure and type of eclosure were both found to be significant in causing death or paralysis symptoms due to infection by CBPV (Table 3.3).

Table 3.3: The results of the competing risks analysis on all virus-fed treatments, using age at exposure (newly emerged or one day old) and eclosure type as covariates.

Covariates	Coef	z-value	p-value
Age	-1.997065	-5.994945	0
Eclosure	1.105988	4.109774	0

There were deaths caused by CBPV in each virus treatment (Figure 3.12). For artificially emerged bees fed virus after emergence, 60% of the bees died due to CBPV, compared to approximately 20% in naturally emerged bees of the same treatment. Much fewer deaths were caused by CBPV in bees fed virus 24 hours after emergence. All treatments had at least one death caused by CBPV, however there were most in the artificially emerged bees fed virus after emergence.

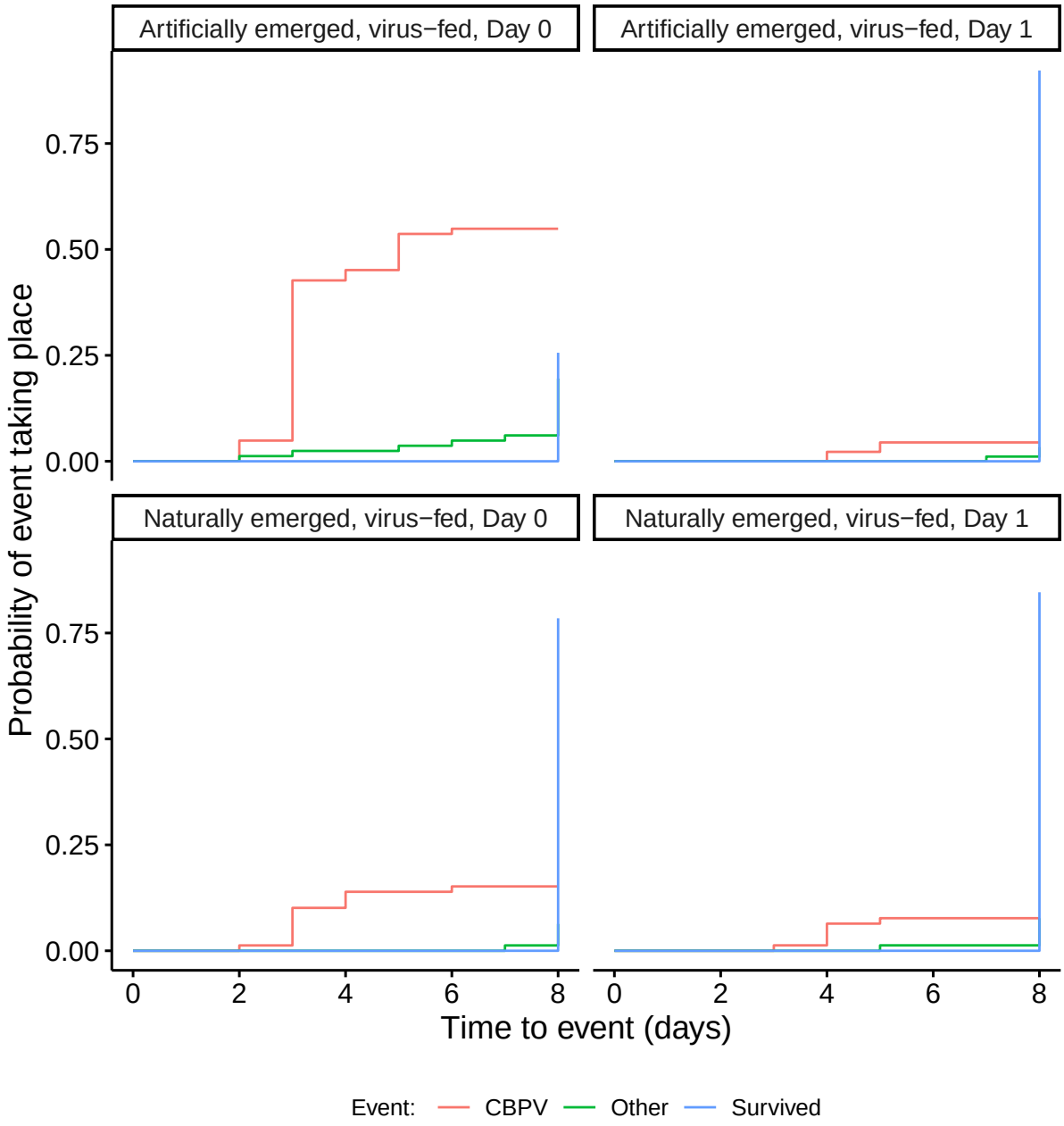


Figure 3.12: The plotted cumulative incidence for death by CBPV (red), death by other causes (green) and survival (blue) when investigating the effects of eclosure type and age at exposure on honey bee survival. This shows the estimated marginal probability of one of these events occurring on each day of the experiment.

3.3.5 Structural equation modelling

3.3.5.1 Individual bacteria

In each SEM, the CBPV qPCR result had a significant relationship with the virus treatment, age of the bee at time of exposure and whether the bee was symptomatic or asymptomatic at time of removal (Appendix D, Tables 6.4, 6.5, 6.6, 6.7). Eclosure had no significant effect on CBPV qPCR result. The concentration of each bacterium did not impact the CBPV qPCR result. For *G. apicola*, *F. perrara* and *S. alvi*, there was no significant relationship between their concentrations and any of the variables included. The output of these models are shown in Appendix D. The concentration of *Lactobacillus* was significantly impacted by eclosure type, with artificially emerged bees having lower concentrations (Figure 3.13, Appendix D Table 6.7). There was also a significant relationship between *Lactobacillus* concentration and virus treatment and removal, with both of these variables being associated with an increase in concentration.

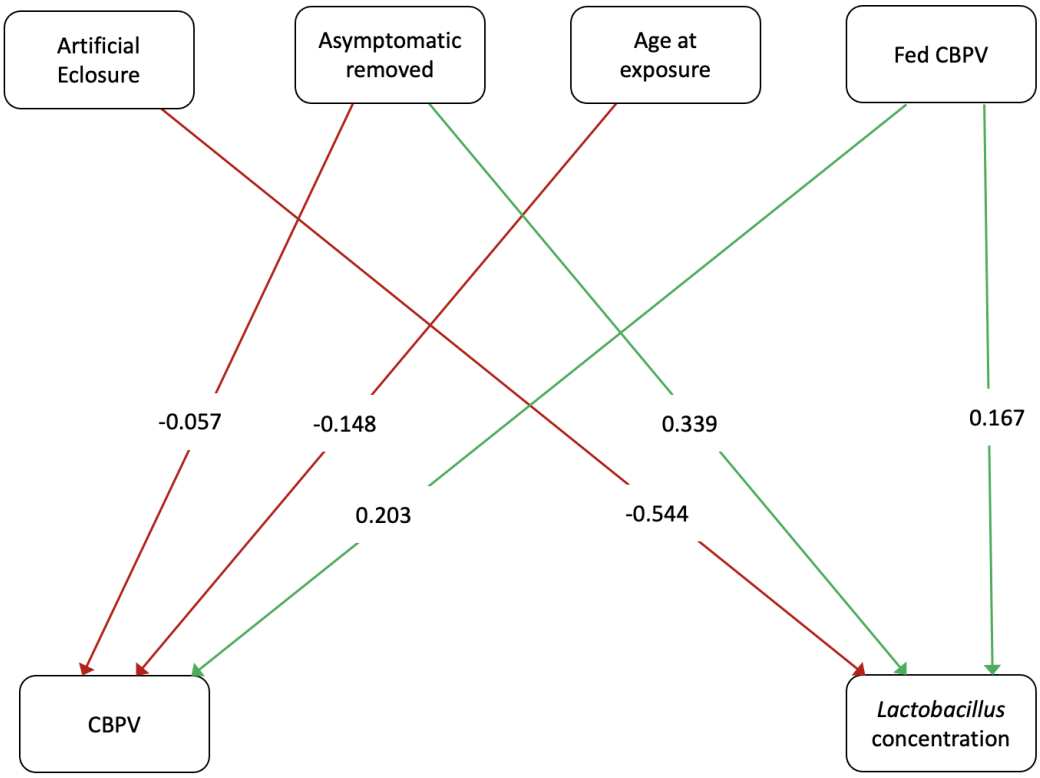


Figure 3.13: The results of a structural equation model investigating the impact of the treatments (artificial eclosure, virus, age exposure to virus, and symptomatic or removed) on CBPV infection and Lactobacillus concentration, and the effect of Lactobacillus concentration on CBPV infection. Green lines indicate a positive effect and red lines indicate a negative effect. Only significant relationships are shown. Values are the standardised observed variables.

3.3.5.2 16s qPCR

Artificial eclosure had a significant negative impact on bacterial 16s concentration, with artificially emerged bees having lower bacterial 16s concentrations (Figure 3.14). There was also a significant relationship between bacterial 16s concentration and age at exposure, with bees that were fed 1 day post-emergence having higher bacterial 16s concentrations. Bees which were removed from the experiment when symptomless had higher bacterial 16s concentrations than those which were symptomatic.

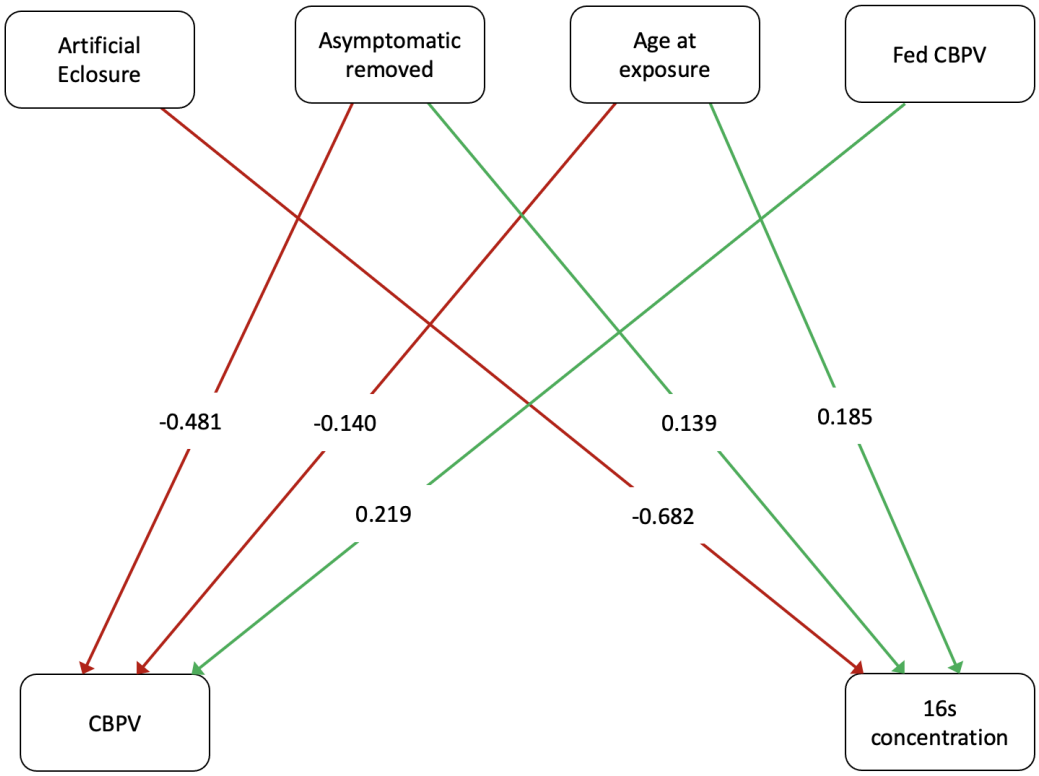


Figure 3.14: The results of a structural equation model investigating the impact of the treatments (artificial eclosure, virus, age at exposure to virus, and symptomatic or removed) on CBPV infection and 16s concentration, and the effect of 16s concentration on CBPV infection. Green lines indicate a positive effect and red lines indicate a negative effect. Only significant relationships are shown. Values are the standardised observed variables.

3.3.6 Day of symptoms/death GLM

The results of the GLM found that there was no significant relationship between any of the gut bacteria or bacterial 16s and the day of symptoms/death (Table 3.4). Both age at exposure and CBPV qPCR result had a significant relationship with day. Bees exposed at day 0 were more likely to show symptoms or die sooner than those exposed at day 1. A positive CBPV qPCR result meant that bees were more likely to die or show symptoms sooner. A normality assessment of the residuals for the model is shown in Appendix E.

Table 3.4: The results from a generalised linear model investigating the relationship between day of symptoms/death with eclosure type, virus treatment, age at exposure, and concentration of F. perrara, G. apicola, Lactobacillus, S.alvi or 16s.

Variable	t-value	p-value
Natural Emergence	-0.197	0.844
Virus	1.699	0.092
Age	3.087	0.003
CBPV	-11.407	0.000
F. perrara	1.080	0.283
G. apicola	0.566	0.573
S. alvi	-0.265	0.792
Lactobacillus	-0.583	0.561
16s	0.943	0.348

3.3.7 Summary

Here we summarise the key results.

1. There is little difference in bacterial concentration or survival in bees emerged as black-eyed pupae, uncapped adults or naturally emerged bees when tested within the first five days post-emergence (Section 3.3.1).

2. Artificially emerged bees fed virus on day 0 are more susceptible to CBPV infection compared to naturally emerged bees fed on day zero (Section 3.3.4.2.

3. Bees fed CBPV one day after emergence have a much lower susceptibility to CBPV infection compared to bees fed on day zero, regardless of emergence type (Section 3.3.4.2).

4. There is no significant relationship between 16s quantity or the quantity of *Lactobacillus*, *S. alvi*, *G. apicola* or *F. perrara* and a positive CBPV result (Section 3.3.5.1).

5. *Lactobacillus* was only found in naturally emerged bees (Section 3.3.3).

6. The amount of bacterial 16s was lower in artificially emerged bees, but the lower bacterial 16s levels were not an indirect driver of CBPV levels/infection (Section 3.3.5.2).

3.4 Discussion

We found very little difference in the 16s results between bees raised in a sterile environment from black-eyed pupae, uncapped adult bees and naturally emerged bees (Section 3.3.1). More naturally emerged bees tested positive for bacterial 16s, however none of the emergence types led to all bees being negative for 16s. There is the possibility that stages of the experimental set-up introduced bacteria despite efforts to maintain a sterile environment. It is commonly reported that bees removed from frames as pupae and kept in a sterile environment can emerge into bacteria-free bees (Zheng et al., 2018). However, studies which use this method often produce bees with low quantities of 16s and core gut bacteria (Motta et al., 2018; Powell et al., 2014). We also know that the bacterial 16s result does not tell us about the bacteria making up these communities, and therefore there may be differences in the types of bacteria present between the bees in each treatment. The bacteria present likely do not represent the core gut microbiota found in adult bees, as this takes between three and five days to establish, and many of the samples tested were positive on day one (Powell et al., 2014). There was also very little difference in survival between these groups. Uncapped bees were chosen to be used as gnotobiotic bees in the main experiment, as this group had few bees which tested positive for bacterial 16s and the method of emergence was relatively noninvasive while still reducing transferal of bacteria.

The method of emergence and age at the time of exposure to CBPV have been identified as key drivers of susceptibility to CBPV through survival analysis and competing risks models (Sections 3.3.4.1 and 3.3.4.2). Almost 55% of artificially emerged bees fed virus at day zero showed symptoms or died by day eight of the experiment, in comparison to approximately 15% in the naturally emerged treatment. For both emergence types, bees fed at day 1 had between 4-8% probability of death or symptoms. These results indicate an increased susceptibility when emergence was a near-sterile event, and when bees were exposed to CBPV within seven hours of emergence.

In many organisms, immunity changes with age. This is especially true for honey bees, as the increase in juvenile hormone when they transition from nurses to foragers is accompanied by a decrease in haemocytes responsible for many innate immune functions (Amdam et al., 2005). Our results found that susceptibility to CBPV decreases with age at the time of exposure to CBPV, when investigating the first day of a honey bee's life

post-emergence. This aligns with the risk of pathogen infection associated with a honey bee's life stage, as younger bees which stay in the hive are less likely to come into contact with pathogens than forager bees sharing food sources with bees from other hives. Studies have shown that the quantity of antimicrobial peptides increases with age, which are produced with the humoral immune response (Amdam et al., 2005; Danihlík et al., 2015; Laughton et al., 2011). PO, which is involved in both the humoral and cellular immune response, also increases with honey bee development (Alaux et al., 2010; Laughton et al., 2011; Wilson-Rich et al., 2008). However, the immune response to CBPV in honey bees is unknown. A study investigating Acute bee paralysis virus found no humoral immune response, nodulation or increased AMP production in infected individuals (Azzami et al., 2012). This raises the question of which parts of the honey bee immune system are relevant to CBPV infections, and how do they interact with susceptibility? Interestingly, Bull et al. (2012) found that younger honey bees were more susceptible to infection of *M. anisopliae* than older forager bees, but that younger bees exhibited a greater immune response to the fungus than older bees, suggesting that the relationship between susceptibility and immune response are not as clear as was hypothesised.

Other studies have found similar results when investigating the gut microbiome and other pathogens. When infected with deformed wing virus, bees with an experimentally established gut microbiome have a higher viral tolerance and survival probability than bees without a gut microbiome (Dosch et al., 2021). Motta et al. (2018) found an increased susceptibility to *Serratia marcescens* in honey bees almost free of gut bacteria compared to those with a natural gut microbiome. Studies have also shown that the presence of a gut microbiome increases the up-regulation of certain AMPs (Li et al., 2017). Specific gut bacteria also serve specific roles in immune function. The addition of *S. alvi* or *Lactobacillus* alone can result in an increase in AMPs (Evans & Lopez, 2004; Kwong et al., 2017). The presence of *F. perarra* in the honey bee gut induces melanisation (a type of cellular immunity) causing a scab to form around the pylorus where *F. perarra* becomes localised (Engel et al., 2015; Zheng et al., 2018). The presence of *Lactobacillus* in the bee gut can have a probiotic effect, as it also improves immune function, and when administered, up-regulates the expression.

Further analyses to investigate how different emergence methods affect the microbiome found that artificial emergence led to a lower bacterial 16s concentration in honey bees than natural emergence. This indicates that while bacteria were gained through both

methods of emergence, natural emergence may involve the transferal of bacteria from the cell cap to the bee as hypothesised. Although our aim was to create completely bacteria-free bees through artificial emergence, it is also possible that bacteria were gained throughout the experiment and during the analysis of samples. However, low levels of bacteria are common in studies involving gnotobiotic bees (Motta et al., 2018; Powell et al., 2014). Testing for individual core bacteria found that only naturally emerged bees had *Lactobacillus* communities, as none were found in artificially emerged bees. *S. alvi*, *G. apicola* and *F. perarra* were not consistently identified in any treatments and were absent in the majority of samples. This is likely due to the lack of interactions with nurse bees or other workers and the hive environment itself. We identified no differences in the presence or quantity of bacteria between samples which tested positive or negative for CBPV.

The results of the SEMs found no relationship between CBPV concentration and *S. alvi*, *G. apicola* or *F. perarra*. These bacteria may not play a role in susceptibility to CBPV in honey bees, or may have been too sparse or not part of a balanced community to function appropriately. We found that *Lactobacillus* was driven by the treatments in the experiment, being more common in bees fed virus and those removed while asymptomatic. As the virus crude extract was filtered, it is unlikely that *Lactobacillus* was introduced with the virus, but possible. Interactions between CBPV and the gut microbiome may allow *Lactobacillus* to establish and dominate the gut microbiome. *Lactobacillus* also had a negative relationship with artificial emergence, and was therefore positively associated with naturally emerging bees. This suggests that *Lactobacillus* is present in cell caps, and that the act of chewing through a cap is enough for the bacteria to establish. We also found that 16s levels were higher in bees which were removed when asymptomatic. This may mean that bees which are infected with CBPV rapidly lose bacteria once symptomatic, which may facilitate their demise. The age of a bee at the time of exposure to CBPV is also positively associated with bacterial 16s. This is likely because many bees which are exposed at a younger age die before bacterial communities can establish. Artificial eclosure had a significant negative relationship with 16s, which indicates that the sterile emergence of these bees was successful in significantly reducing their bacterial communities. No relationship was found between CBPV infection and *Lactobacillus* or 16s concentration. While this initally suggests that the gut microbiome does not play a role in susceptibility of honey bees to CBPV, it does not take into account the full community of bacteria

and the effect this may be having. The presence of individual bacteria and the overall bacterial load do not have an effect on CBPV susceptibility, however the gut microbiome as a whole may play a role.

Pot effect not included in the SEM, as this is a conservative method and means that all potential error is included in the model. There may be interactions between the pot which a bee is in and its likelihood of gaining bacteria, as bacteria may be spread within the pot via trophallaxis and grooming (Powell et al., 2014). By not removing the pot effect, we are ensuring that these instances are included in our models.

3.5 Conclusions

The key driver of honey bee susceptibility to CBPV in these experiments is age. There is a window of time before a bee reaches 24 hours post-emergence where their physiological condition makes them more likely to become infected with CBPV when fed orally. The condition of the gut microbiome also plays a role, as naturally emerged bees are less susceptible in comparison to artificially emerged bees. This is likely due to the overall bacterial community and its establishment rather than one of the bacterial species tested for. The consequences of these results may mean that newly emerged bees are very likely to become infected with CBPV if fed by a nurse bee who is infected, which would lead to large numbers of young bees dying within days of emergence. This aligns with the observation that CBP often leads to colony collapse, with large piles of dead bees lying outside the hive (Ribiere et al., 2010).

Chapter 4

The effect of nutrition post-emergence on susceptibility to CBPV

4.1 Introduction

Honey bees (*Apis mellifera*) are important pollinators which are heavily relied upon worldwide for their ecosystem services. Their populations have been in decline for a number of years due to a range of threats such as pathogen and parasite spread, agricultural intensification and pesticide use (Blacquiere et al., 2012; Potts et al., 2016; Vanbergen & Initiative, 2013). These stressors can interact, having knock-on effects such as reduced forage availability and decreases in individual immunocompetence (Brandt et al., 2016). These factors ultimately increase susceptibility to disease, which is a leading cause of honey bee decline (Raymann & Moran, 2018; O'Neal et al., 2018).

One of the diseases threatening honey bees is Chronic bee paralysis (CBP), an emerging disease which can lead to the full collapse of colonies (Ball, 1999; Budge et al., 2020). Symptoms of the disease include paralysis, trembling, hair loss and clustering outside the hive (Bailey et al., 1983). The causative agent is Chronic bee paralysis virus (CBPV), an unclassified RNA bipartite virus (Bailey et al., 1963; Olivier et al., 2008b). Cases of CBP are thought to be linked to confinement of bees during unseasonable weather, which is particularly problematic for high density colonies (Ball & Bailey, 1997). Spread of the virus is facilitated through close contact which is accentuated in these circumstances.

Confinement in the hive can also lead to depletion of pollen stores, which in turn can lead to pollen dearth and the consumption of old pollen. At a colony level, pollen dearth can cause larval cannibalism and shorten worker lifespans, leading to colony collapse (Haydak, 1935; Knox et al., 1971; Schmickl & Crailsheim, 2001). At an individual level, the consumption of old pollen can cause gut dysbiosis, which may in turn make bees more susceptible to pathogens (Maes et al., 2016).

Beebread is the main source of nutrition for newly emerged bees, which is produced from a combination of pollen and honey. Forager bees bring pollen to the hive and use regurgitated nectar to bind the pollen together, forming a pellet (Didaras et al., 2020). Nurse bees add glandular secretions to the pellet and a layer of honey for preservation, and over time it ferments (Gilliam, 1997). Bacteria, fungi and yeasts are added to the beebread during this process, and lactic acid bacteria in particular aids in its preservation (Haydak, 1958). Beebread is then fed to larvae and newly emerged bees by nurse bees via trophallaxis.

Protein can affect bees at many levels, both for the colony as a whole (as previously discussed) and on an individual level. Pollen can affect bees' immune responses as well as their overall physiology. Pollen diets can increase hypopharyngeal gland size and longevity (DeGrandi-Hoffman & Chen, 2015; Knox et al., 1971). Links have been identified between pollen consumption and increased gluco-oxidase activity and Phenol oxidase (PO)activity, both of which are part of the immune innate response (Alaux et al., 2010). Pollen has been shown to reduce susceptibility to disease, such as Israeli acute paralysis virus (IAPV) (Dolezal & Toth, 2018). There is also evidence that pollen can reduce viral loads in bees infected with Deformed wing virus (DWV) (DeGrandi-Hoffman & Chen, 2015).

Honey bee food can also be a source of gut bacteria. There is evidence that diet can affect the gut microbiome, particularly when bees begin foraging and their diet changes (Ludvigsen et al., 2015). The glandular secretions and honey that are added to pollen to create beebread contain bacteria which aid in its fermentation and preservation (Haydak, 1958; Powell et al., 2014). A wide range of bacteria have been identified from beebread samples, including the core bacteria making up a honey bee gut microbiome (Donkersley et al., 2018). This suggests that beebread is a source of bacteria for newly emerged bees and helps in the colonisation of the gut. Gut microbiota play a role in overall health in honey bees, contributing to honey bee physiology which impacts immune function and susceptibility (Raymann & Moran, 2018). The gut microbiome has roles in immune

priming which can lead to an increase in the amount of antimicrobial peptides, an important part of the immune response (Emery et al., 2017).

The main aims of this study are to investigate the possible effects of beebread on susceptibility to CBP. We then aim to separate the effects of protein and gut bacteria to identify the true cause of any variation in susceptibility. We hypothesise that protein in the form of beebread will increase honey bee longevity and reduce susceptibility to CBP. We aim to investigate the cause of this reduced susceptibility, using untreated beebread as a protein plus bacteria treatment, and heated beebread as a protein-only treatment. This will allow us to identify if the effect of beebread is due to its nutritional value or the beneficial bacteria which can be gained through its consumption. We also hypothesise that the gut microbiota communities will change over time as more bacteria colonise the gut.

4.2 Methods

Two experiments were conducted to challenge these hypotheses. The first simply sought to examine survival of honey bees exposed to CBPV orally in response to the presence and absence of beebread. The second used different forms of beebread to investigate what aspect of beebread may alter susceptibility: the nutritional component of the food or the addition of beneficial bacteria. To separate these two possibilities, beebread was fed in two forms: untreated and heated beebread. The heated beebread would still offer the beneficial protein but would theoretically not contain any beneficial gut microbiota.

4.2.1 Experiment 1. Beebread Presence/Absence - Investigating the impact of access to beebread on susceptibility to CBPV when exposed orally

A frame of pollen and a frame of emerging bees were collected from the Ridley roof apiary. The brood frame was stored in a box and in an incubator at 33 °C. Beebread was removed from the frame using a hive tool and placed in a cut section of 1000 μl pipette tips (total weight approximately 0.7 g). All beebread was stored at -20 °C.

Bees were collected by uncapping cells with sterile forceps and removing bees which were moving and hairy (as described in Chapter 3). Bees were placed in pots of 15 with access to sugar syrup (50% sugar, 50% distilled water). The bee bread was removed from the freezer and weighed before being added to the pots within one hour of the bees emerging. Approximately four hours after emergence and three hours after the beebread was added, bees were individually fed a 3 μl solution of 50% sodium chloride buffer or CBPV, with 50% sugar syrup. Four treatments were used in total: fed buffer, fed buffer with beebread, fed virus, and fed virus with beebread.

4.2.2 Experiment 2. Beebread Types - Investigating the cause of reduced susceptibility to CBPV of honey bees with access to beebread

A frame of pollen was removed from the Ridley roof apiary as described in Section 4.2.1. The beebread feeders were stored at -20 °C. Half of the beebread samples were heated

to 70 °C for 2 hours to kill bacteria present in the beebread, a temperature which is commonly used for high-temperature short-time pasteurisation and therefore adequate to prevent bacterial recovery (Holsinger et al., 1997). All pollen was placed in the freezer afterwards and stored at -20 °C. Frames of emerging brood were collected from the Ridley roof apiary and kept in a box in an incubator at 33 °C.

Bees were removed from the frames and treated exactly as explained in Section 4.2.1. Bees were either not given access to beebread or given access to untreated beebread or heated beebread. All feeders were replaced on day 7.

Bees were observed for paralysis symptoms each day for 24 days in the first experiment, and 10 days in the second experiment. Paralysed or dead bees were removed with sterile forceps and stored at -80 °C. Methods were based on those in Chapter 2.

4.2.3 Statistical methods

All analyses were undertaken in R version 4.2.2 (RCoreTeam, 2022).

4.2.3.1 Survival analysis

Survival analysis was performed to assess the risk of death due to CBPV infection under the different beebread treatments for both experiments, using the packages *survival* and *survminer* (Kassambara et al., 2019; Therneau, 2020). Kaplan-Meier curves were calculated, comparing the survival probability of each treatment. A vertical drop in a survival curve represents death or one or more individual bees. We can use the trajectory of the individual bees to make comments about the probability of survival in the whole population. The curves were then compared using a log-rank test. Bees were censored using type I censoring, where they were censored if they did not die within the selected time frame of the experiment. In the analysis of survival data using the Kaplan-Meier curve and the log-rank test, individuals which are censored are labelled as having an increased risk of death at the point of censoring only (Bland & Altman, 1998). Experiment 1 involved 180 bees. With an assumed mortality of 50% and a required power of 0.8, we can predict an effect size of 2. Experiment 2 had 369 bees, which with a required power of 0.8 and an assumed mortality of 50% means we can detect an effect size of 1.75 (Therneau & Grambsch, 2000). A Cox proportional hazards model was then performed, using time

of death as the event. Effect sizes were presented as hazard ratios. The proportional hazards model assumptions were tested and visualised using Schoenfeld residual plots.

Frailty was initially included in the model to assess the effect of pot as a grouping variable. However, the models failed to converge and therefore, frailty was not included, as discussed in Chapter 2 Section 2.2.6.2.

4.2.3.2 Dose-response analysis

Dose-response analysis was used to investigate differences in survival curves in the second experiment using the package drc in R (Ritz et al., 2015). A three parameter log-logistic model was fitted to investigate the decline in number of bees over time (Finney, 1971). The estimate of the slope and the midpoint of the curve (ED50) were compared between treatments to further analyse mortality, identifying the time points in which 50% of all individuals which showed symptoms or died were removed from the experiment, and how steep or gradual the decline of bees was in each treatment.

4.3 Results

4.3.1 Experiment 1. Beebread Presence/Absence - Investigating the impact of access to beebread on susceptibility to CBPV when exposed orally

4.3.1.1 Survival analysis

During our study period, all bees fed buffer and given access to beebread survived (Figure 4.1). Bees fed buffer without access to beebread began to die on day 9, and their survival probability declined gradually from this point onwards. Both treatments involving virus saw deaths begin on day two, and their survival probabilities remained similar until approximately day five, where the survival probability of bees fed beebread remained constant at approximately 45%. All bees fed virus without access to beebread died by day 10.

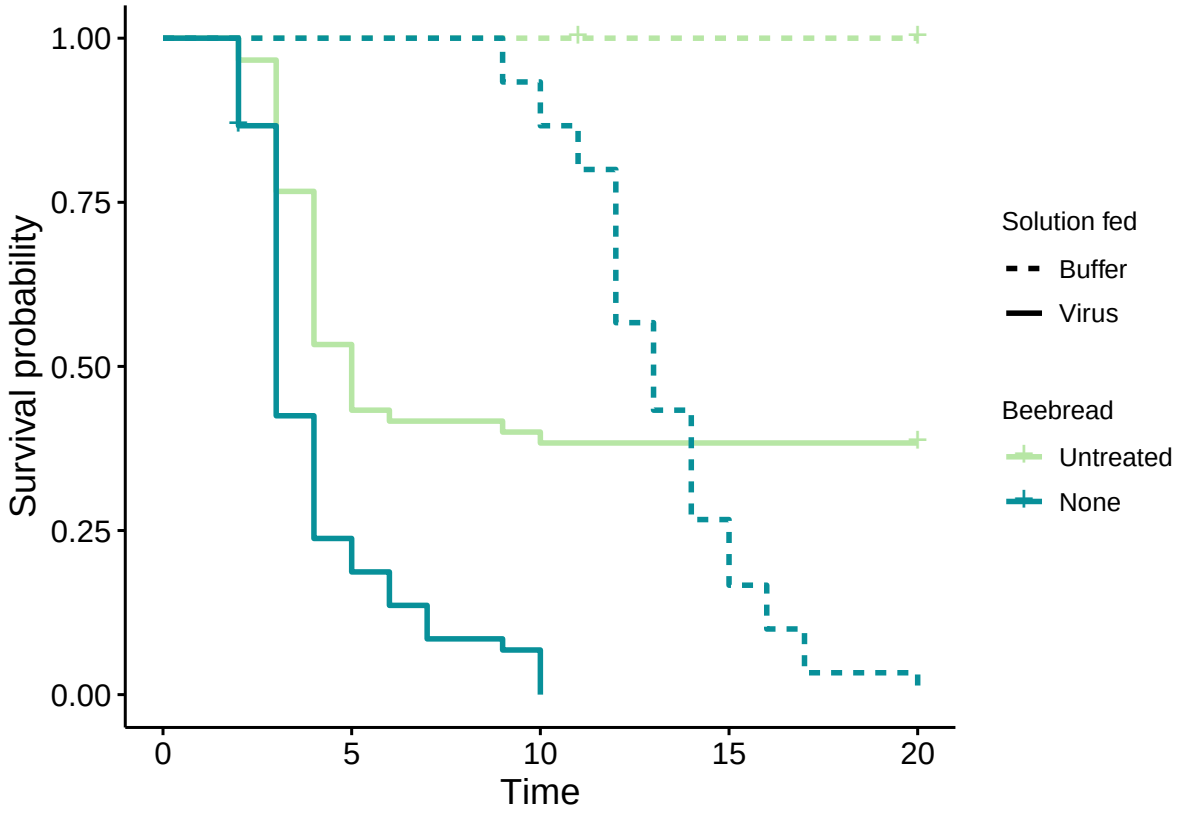


Figure 4.1: The Kaplan-Meier survival curves for the first 20 days of the Beebread Presence/Absence experiment for each treatment.

We found a significant difference between survival curves using the log rank test (Chisq = 129, df = 3, $p < 0.001$). The results of the Cox model found that the feeding of virus significantly increased the hazard of death compared to the feeding of buffer ($z = 7.84$, $p < 0.001$). Having access to untreated beebread significantly reduced the hazard of death compared to bees which did not have access to beebread ($z = -8.533$, $p < 0.001$) (Figure 4.2).

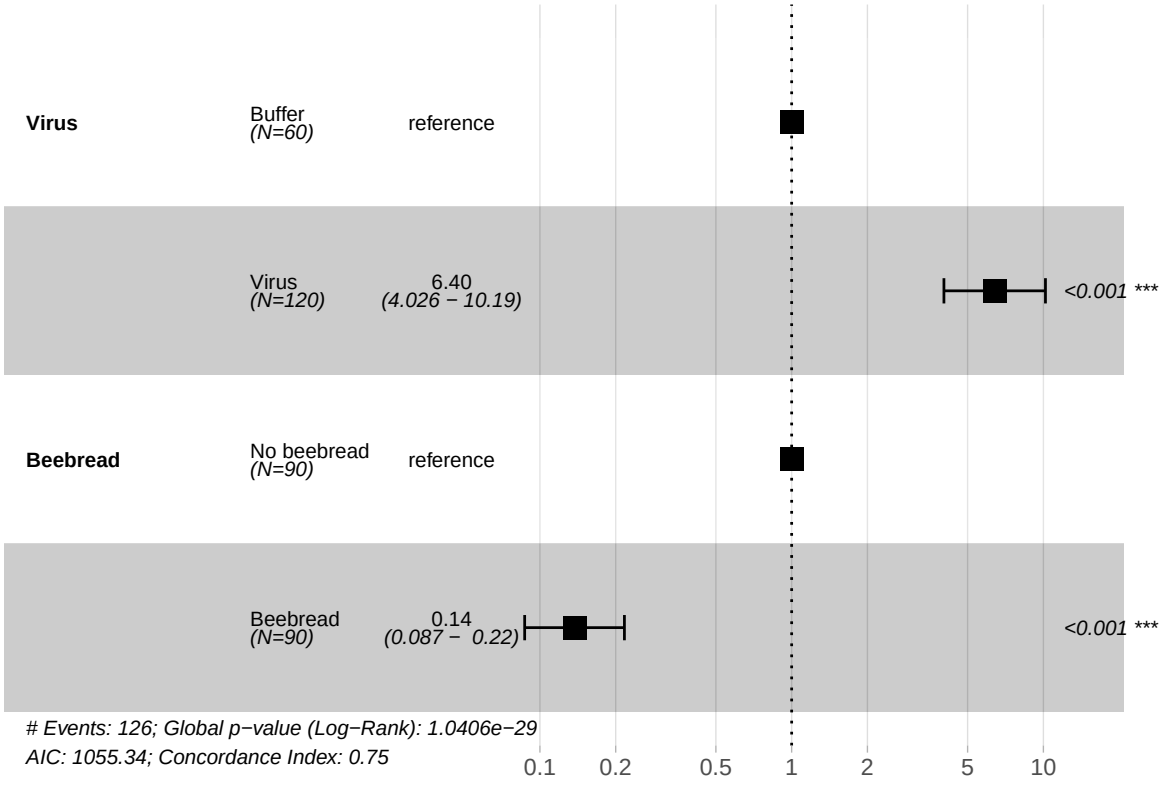


Figure 4.2: The hazard ratios of each treatment group in the Beebread Presence/Absence experiment. Hazards are compared to treatments where bees were fed buffer and not given access to beebread.

The assumption of the hazard remaining proportional over time was violated by treatments with virus-fed bees or beebread present (Table 4.1). This is visualised in Figure 4.3, where we can see that the hazards associated with virus were higher for the first 5 days of the experiment, and then declined. The hazards associated with beebread initially increased and also began to decline at day 4.

Table 4.1: The results of the proportional hazards assumption test of a Cox regression. The proportionality of each treatment was tested over time. A significant result indicates a violation of the assumption.

Treatment	Chi Squared	df	p-value
Virus	32.556	1	0
Beebread	65.184	1	0
GLOBAL	73.615	2	0

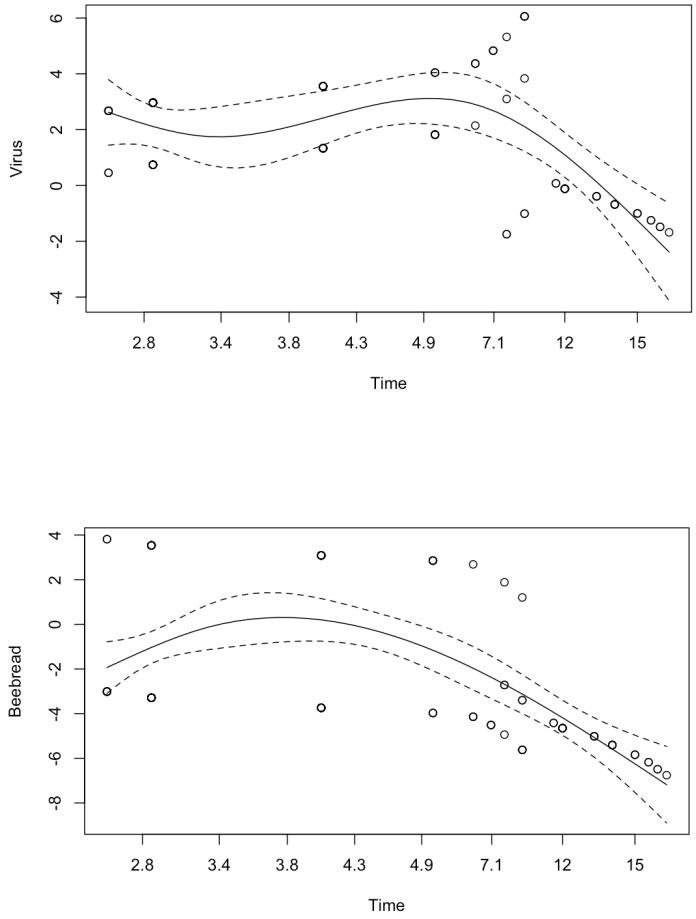


Figure 4.3: Schoenfeld residual plots for each treatment group of the Beebread Presence/Absence experiment showing the change in hazard over time in days.

4.3.2 Experiment 2. Beebread Types - Investigating the cause of reduced susceptibility to CBPV of honey bees with access to beebread

4.3.2.1 Survival analysis

Very few or no deaths were observed in any of the buffer-fed bees for the duration of the experiment. Deaths in the virus treatments began at day three. By day 10, the survival probability for the virus control and virus untreated beebread treatment was around 0.75, compared to approximately 0.5 in the virus heated beebread treatment (Figure 4.4).

A log-rank test was conducted to investigate the differences in the survival curves. Survival curves were found to be significantly different from one another (Chisq = 56.6, df = 5, p < 0.01).

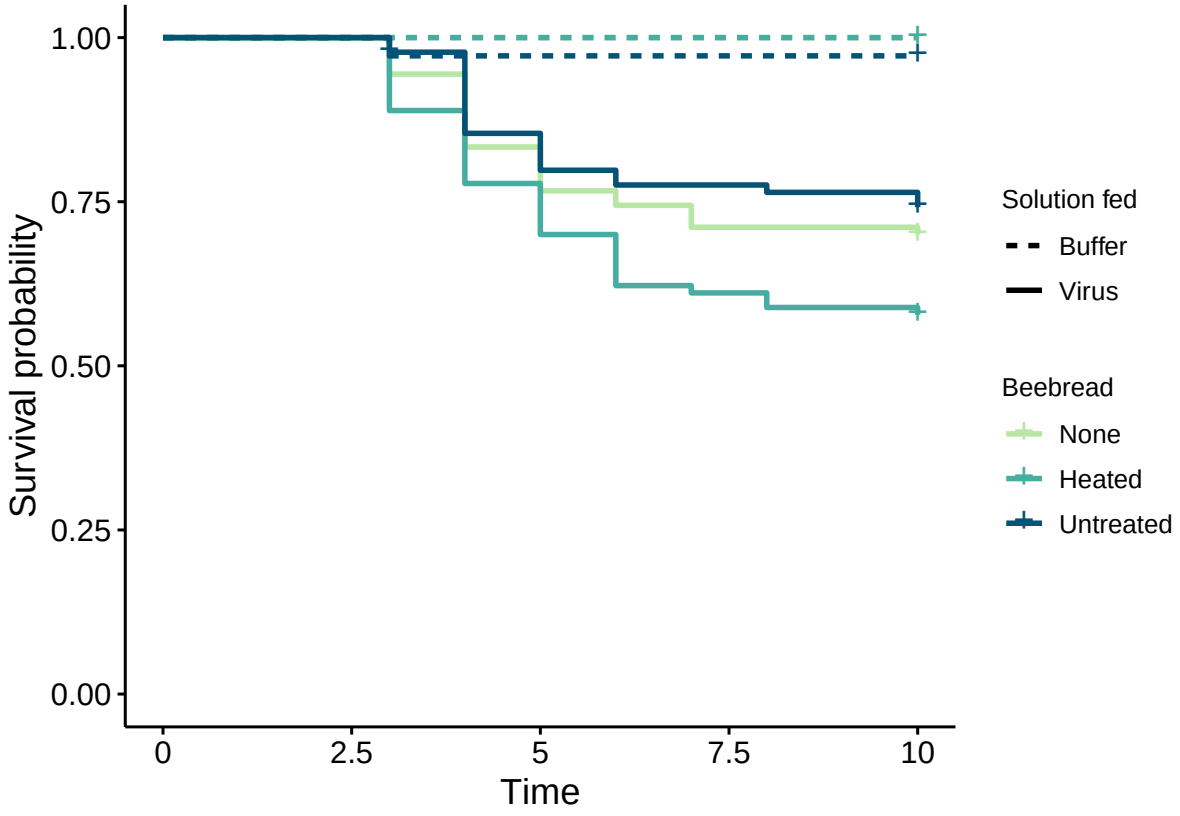


Figure 4.4: The Kaplan-Meier survival curves for the first 10 days of the Beebread Types experiment for each treatment.

The Cox proportional hazards model found that virus significantly increased the hazard of death ($z = 3.872$, $p < 0.001$), but that there was no significant difference in hazard between bees without access to beebread and bees with access to beebread, regardless of the type of beebread (Heated beebread: $z = 1.696$, $p > 0.05$, Untreated beebread: $z = -0.489$, $p > 0.05$) (Figure 4.5). None of the treatments violated the proportional hazards assumptions and hazards remained relatively proportional with time (Table 4.2, Figure 4.6).

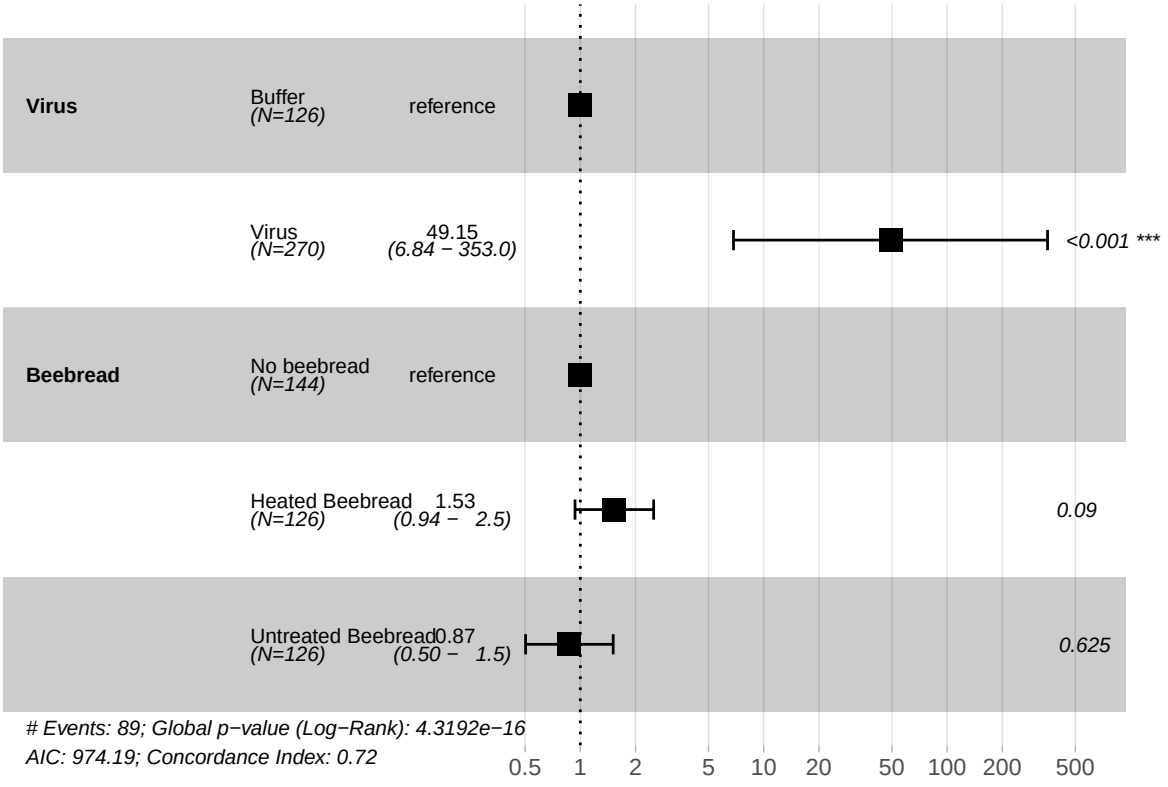


Figure 4.5: The hazard ratios of each treatment group in the Beebread Types experiment. Hazards are compared to treatments where bees were fed buffer and not given access to beebread.

Table 4.2: The results of the proportional hazards assumption test of a Cox regression. The proportionality of the hazard of each treatment was tested over time. A significant result indicates a violation of the assumption.

Treatment	Chi Squared	df	p-value
Virus	1.789	1	0.181
Heated Beebread	0.073	1	0.788
Untreated beebread	0.022	1	0.883
GLOBAL	1.858	3	0.602

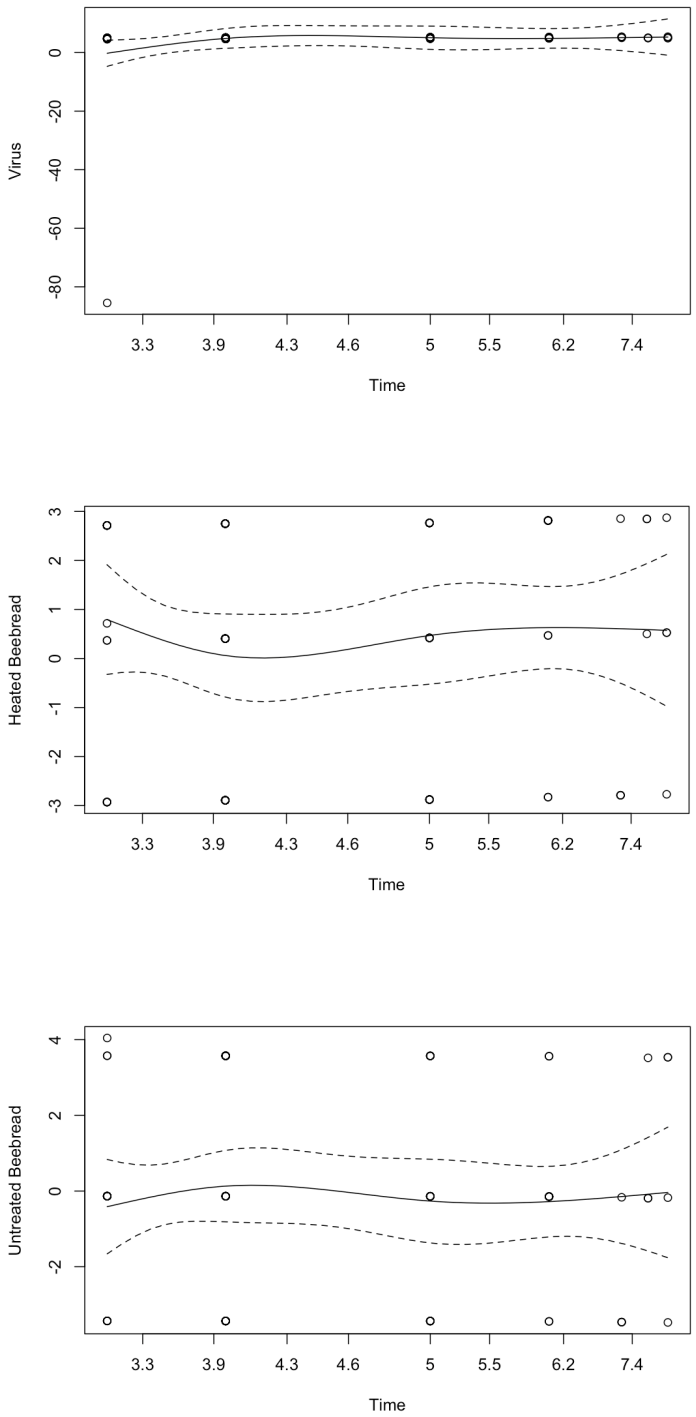


Figure 4.6: Schoenfeld residual plots for each treatment of the Beebread Types experiment showing the change in hazard over time in days.

4.3.3 Dose response curves

The results of the survival analysis indicate a period of resilience in honey bees, to succumbing to CBP or to the effects of the experimental design. In this period, honey bees can become infected with CBPV and take a few days before virus concentration reaches a level where the honey bee is severely affected, either showing paralysis symptoms or dying. Similarly, bees which are not infected will also have a resilience to the experimental design before its effects become apparent. Dose-response analysis was conducted to analyse differences in the midpoint and slope of survival curves for both experiments (see Figures 4.7 and 4.9). Estimates for each treatment within each experiment were compared. For the beebread presence/absence experiment, both the ED50 and the slope estimates are higher for the virus untreated beebread treatment compared to the virus treatment without access to beebread, indicating that with access to beebread, virus-infected bees will die just as quickly as those without beebread, but fewer bees will die overall. For the beebread types experiment, the ED50 estimates were similar for all treatments, and both virus-fed beebread treatments had estimates which overlapped with the estimate for the virus treatment without beebread access. The slope estimates varied, with heated beebread having the lowest slope estimate, followed by the virus treatment without beebread access, and the virus treatment with untreated beebread having the highest estimate for the slope. This suggests that bees in the heated beebread treatment died faster than bees in the other treatments. All estimates were significantly different from zero (Tables 4.3 and 4.4). The curves estimated are presented in Figures 4.8 and 4.10.

Table 4.3: The estimates for the ED50 and slope from dose response curves for each virus treatment in the Beebread Presence/Absence experiment.

Type	Treatment	Estimate	Std. Error	t-value	p-value
Slope	Virus No Beebread	4.112	0.469	8.769	0
Slope	Virus Untreated Beebread	5.994	1.057	5.672	0
ED50	Virus No Beebread	2.764	0.078	35.403	0
ED50	Virus Untreated Beebread	3.234	0.103	31.286	0

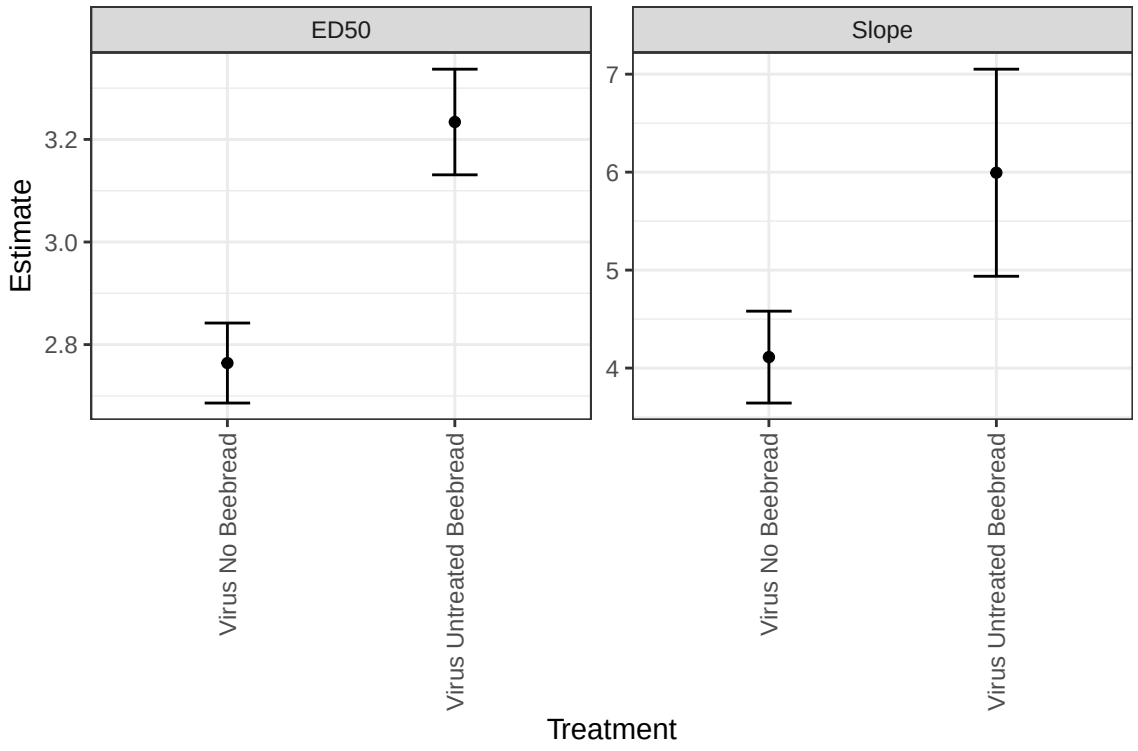


Figure 4.7: The estimates for the ED50 and slope from dose response curves for each virus treatment in the Beebread Presence/Absence experiment. Lines represent the positive and negative standard errors.

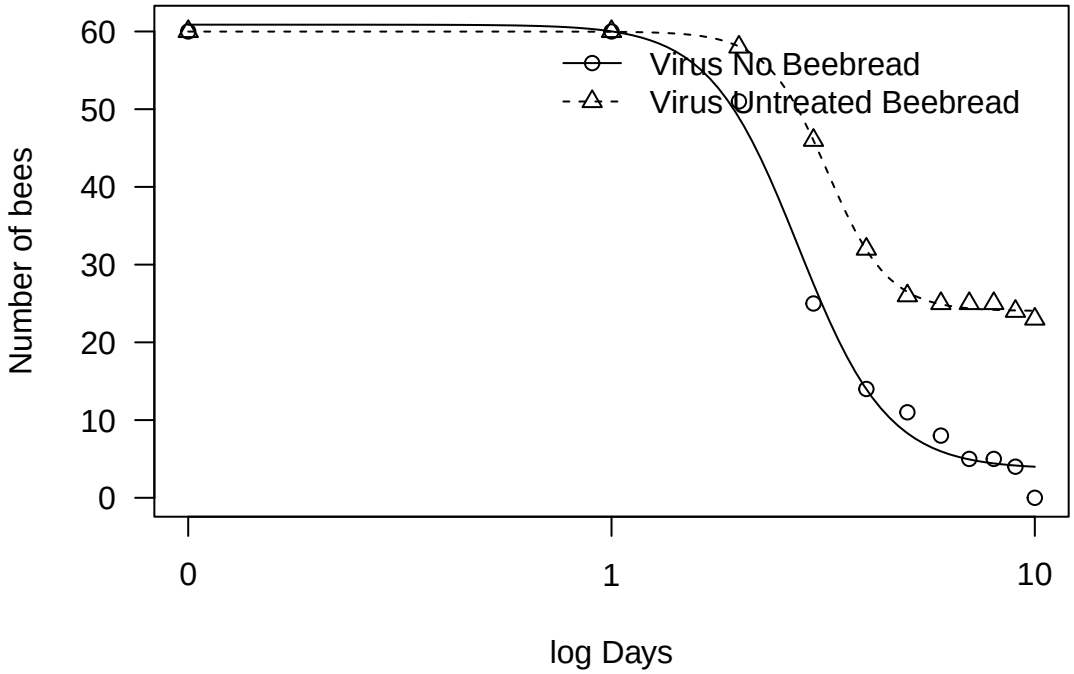


Figure 4.8: The dose response curves for each treatment, showing the decline in the number of bees per treatment throughout the first ten days (on a log base 10 scale) of the Beebread Presence/Absence experiment.

Table 4.4: The estimates for the ED50 and slope from dose response curves for each virus treatment in the Beebread Types experiment.

Type	Treatment	Estimate	Std. Error	t value	p-value
Slope	Virus No Beebread	5.411	0.556	9.730	0
Slope	Virus Heated Beebread	4.214	0.289	14.591	0
Slope	Virus Untreated Beebread	7.686	1.022	7.519	0
ED50	Virus No Beebread	3.839	0.081	47.320	0
ED50	Virus Heated Beebread	3.911	0.074	52.902	0
ED50	Virus Untreated Beebread	3.731	0.073	51.175	0

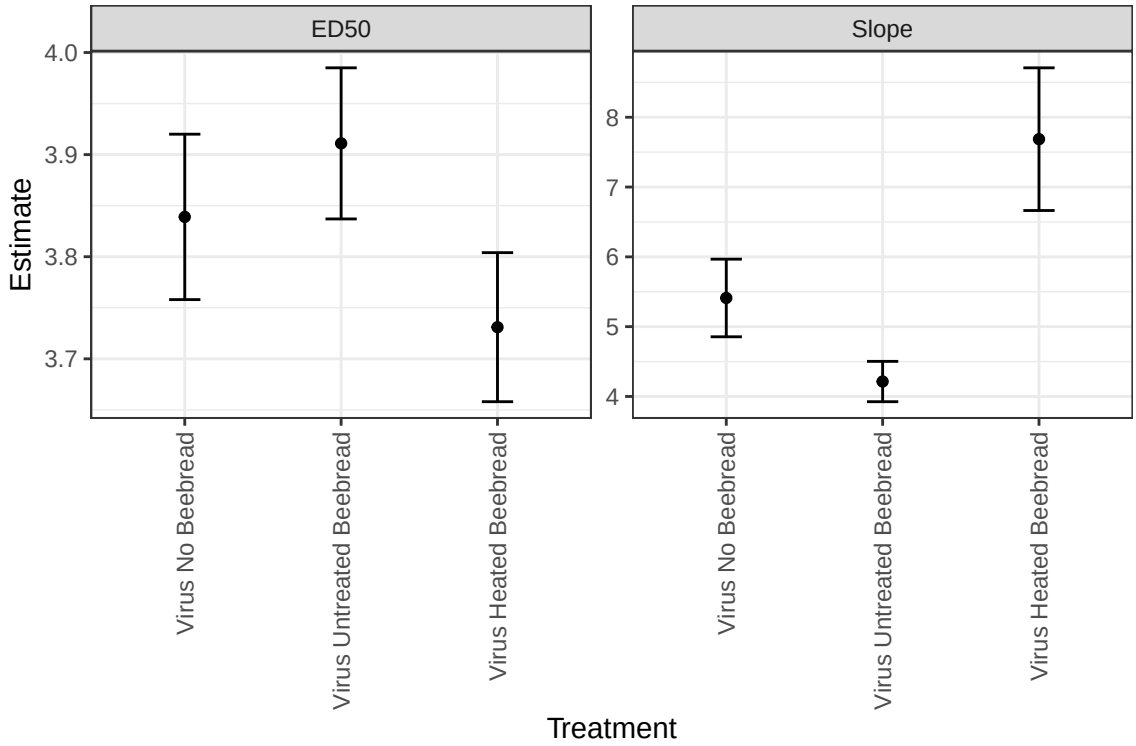


Figure 4.9: The estimates for the ED50 and slope from dose response curves for each virus treatment in the Beebread Types experiment. Lines represent the positive and negative standard errors.

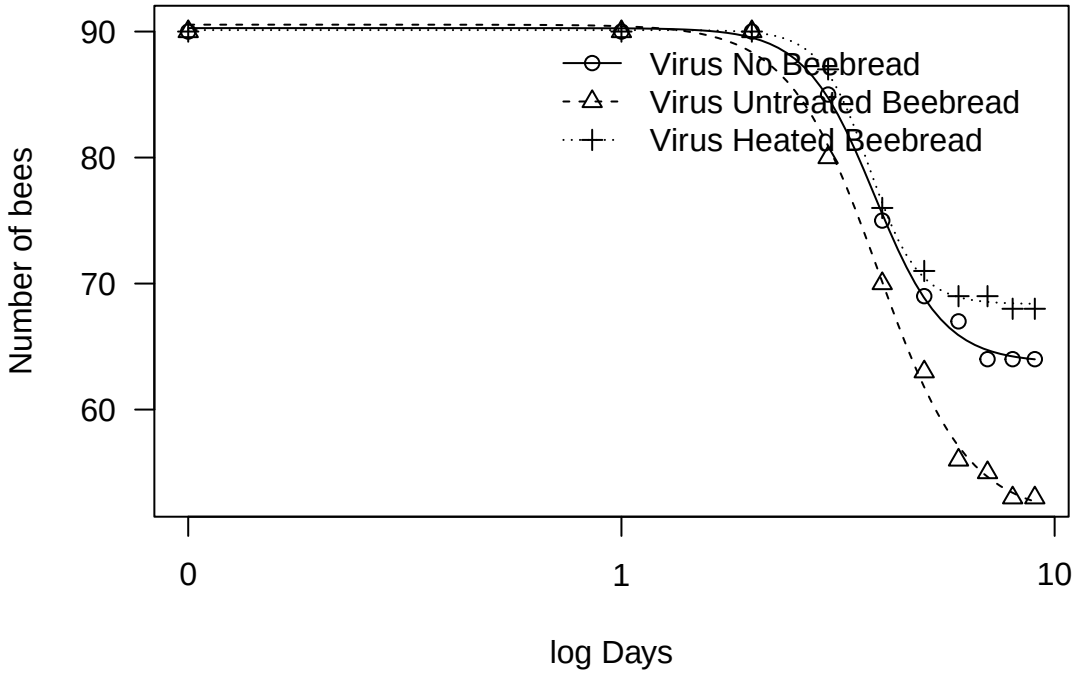


Figure 4.10: The dose response curves for each treatment, showing the decline in the number of bees per treatment throughout the first ten days of the Beebread Types experiment on a log base 10 scale

4.4 Discussion

The results of our initial experiment indicate the positive effect of beebread on honey bee survival and the reduction in susceptibility to CBP (Section 4.3.1). The addition of beebread led to an increase in survival probability of 45% and increased longevity in virus-fed bees. We saw that the risk associated with virus increased at approximately day two, when bees began to show symptoms and die, and decreased by approximately day seven, by which point most bees had already died, likely due to the virus (Figure 4.1). The risk associated with beebread also showed an initial increase followed by a decline, likely reflecting the virus-fed bees with access to beebread which died initially. Similar results have been discovered in honey bees in response to other diseases, such as IAPV, DWV and *Bacillus larvae* (DeGrandi-Hoffman & Chen, 2015; Dolezal et al., 2019; Rinderer et al., 1974). As well as fewer bees dying in the virus-fed beebread treatment in comparison to virus-fed without beebread, no bees died in the buffer-fed beebread treatment. This suggests that access to beebread not only reduces susceptibility to CBPV, but also makes bees more resilient to the experimental set-up, as all buffer-fed bees without access to beebread died by day 20. In the hive, newly emerged bees would eat a combination of pollen, honey and nectar. Haydak (1970) reported that young bees do not begin to grow until they eat pollen, and this source of protein promotes growth of fat bodies and the internal organs, including the hypopharyngeal glands. We can therefore assume that without a source of protein, honey bees do not develop naturally, which would likely affect their susceptibility and longevity in a variety of ways.

The results of our second experiment did not show a clear difference between virus-fed treatments with and without beebread (Section 4.3.2). This could be due to a number of factors, including the experimental length, which was 10 days due to time constraints, in comparison to the earlier experiment which was 20 days. While our first experiment showed differences between treatments within 10 days, it is difficult to study longevity over such a short time period. It is possible that had the experiment run for longer, differences in survival may have been observed. We must also take into account the time in the season these experiments took place. Our first experiment ran in July, and the second ran at the end of August. We know that honey bees are less susceptible to CBP in the winter months, likely due to a combination of the change in physiology of winter bees and the change in behaviour (less foraging and reproduction). Winter bees have a much longer lifespan than summer bees due to their increased fat bodies and enlarged

hypopharyngeal glands, which may reduce susceptibility to disease (Fluri & Bogdanov, 1987; Fluri et al., 1982). Studies have shown that the production of winter bees begins in late August, with some bees emerging at this time surviving until the following May (Mattila et al., 2001).

The Kaplan-Meier survival curves revealed a resilience period in both experiments, where the effects of the virus or negative impacts of the experimental set-up did not affect mortality. In both experiments, bees in the virus-fed treatments began to die on day three, and buffer-fed bees from the Beebread Presence/Absence experiment began to die on day nine (Figures 4.1 and 4.4). Through the use of dose response curves, the differences in survival curves irrespective of this resilience can be investigated. The differences in the ED50 and slope between virus treatments with and without beebread access in the Beebread Presence/Absence experiment suggests that beebread reduces the susceptibility of honey bees to CBP, as the treatment with access to beebread reached the midpoint of the curve later than the treatment without beebread, as fewer bees died in this treatment overall (Figure 4.7). The same analysis on the Beebread Types experiment found no differences between the ED50 of the virus only treatment and either of the beebread treatments, further evidencing the likelihood that beebread had no effect on susceptibility in this experiment (Figure 4.9). The slope estimates for these treatments did vary however, with virus-fed untreated beebread having a steeper slope, followed by virus-fed without beebread, and the virus-fed heated beebread treatment having the least steep slope of all treatments. This is because symptoms/deaths which occurred in the virus-fed heated beebread treatment occurred over a longer period, whereas the majority of symptoms/deaths observed in the virus-fed untreated beebread treatment took place between days three and five (Figure 4.4). This method demonstrates a more in-depth technique to analyse survival data which is particularly useful when the cause of death cannot be determined, and only all-cause hazards can be analysed.

The results of our analyses did not identify any differences in survival between bees with access to untreated or heated beebread (Section 4.3.2.1). This means that we cannot definitively refute the hypothesis that the gut microbiome alone affects the susceptibility of honey bees to CBP. Emphasis needs to be placed on the bacterial benefit of pollen in future experiments to identify its possible effects.

Our experiments investigated the effect of beebread consumption in honey bees after emergence, however, protein is important during development also. Larvae receive protein

in two forms: directly from pollen, and processed pollen, which is developed into larval food from hypopharyngeal gland secretions (Brodschneider & Crailsheim, 2010). A lack of protein during larval development has been found to reduce lifespan and body and wing size in bees. This brings up an interesting question of how and when a lack of protein throughout a honey bee's development would affect honey bee susceptibility most severely. As all of our bees came from the same colony for each experiment, we can assume that all bees had the same access to pollen and were fed in a similar way. However, we cannot say how differences in pollen stores between the experiments may have affected overall susceptibility to disease, which could be another reason for the differences we observed between experiments.

We must also consider the length of time in which honey bees would need access to beebread prior to exposure to CBPV in order for bacteria to colonise and provide them with a form of defence against infection. It is possible that bacteria in the beebread colonised the bee gut, but that infection of CBPV took place faster and therefore the bacteria did not have an effect. Our previous studies have shown that bees are most susceptible to CBPV when fed between 0-7 hours post emergence, which limited the amount of time bees could be exposed to beebread prior to the oral inoculation of CBPV.

The bees used in this experiment were not tested for CBPV via RT-qPCR. We can therefore not definitively say that the cause of death in our experiments is due to infection by the virus. However, based on experiments in Chapters 2 and 3, it is likely to be the cause due to the time between exposure and symptoms or death, and the types of symptoms observed, which were typical paralysis symptoms. Further analysis of these samples would be beneficial for conclusive results. In addition, testing of the bee guts and beebread samples used would reveal the bacterial communities the bees are exposed to and those that colonise the gut from beebread consumption. Differences could then be identified between the gut microbiome of bees without access to beebread and those with access to beebread, as a potential cause of differences in susceptibility to CBP.

4.5 Conclusions

Our results suggest that honey bee susceptibility to CBP can be reduced by access to beebread and that beebread can increase worker longevity in laboratory experiments. The exact cause of the reduced susceptibility to CBP remains unclear and requires further

examination to disentangle the nutritional benefit of beebread from its source of gut bacteria. These results also highlight important factors to bear in mind when conducting tests on honey bees in laboratories, including the effect of lack of protein and the time of year experiments take place.

Chapter 5

Investigating the spatial and temporal drivers of Chronic bee paralysis in Southern England

5.1 Introduction

Honey bees play a vital role in pollination and human nutrition. In the UK, 20% of crops are pollinated by insects, estimated to be valued at £430 million in 2007, with £150 million being provided by honey bees (Breeze et al., 2011; Brown et al., 2014; Vanbergen et al., 2014). Various threats are faced by honey bees, including disease, climate change, urbanisation and land-use intensification (Vanbergen & Initiative, 2013). These factors are contributing to pollinator decline globally, including in the UK (Potts et al., 2010).

There are a variety of diseases which honey bees face and their social and high density living offers practically ideal conditions for parasite transmission due to almost constant close physical contact. Pathogens co-evolve with honey bees to exploit behaviours which promote transmission and have appeared wherever the honey bee has been introduced (Royce & Rossignol, 1990). One of the diseases facing honey bees is Chronic bee paralysis (CBP), an emerging bee disease causing widespread colony losses. It is found worldwide and is becoming more prominent in many countries including the UK, where records of CBP symptoms during colony inspections have been rising since 2007 (Figure 5.1). The causative agent of CBP is Chronic bee paralysis virus (CBPV), an unclassified RNA virus (Olivier et al., 2008b). Overt infection causes characteristic paralysis or trembling and

hair loss in bees, leading to death within approximately 10 days of infection (Bailey et al., 1963). This often results in colony collapse, with piles of dead bees appearing outside the hive (Ribiere et al., 2010).

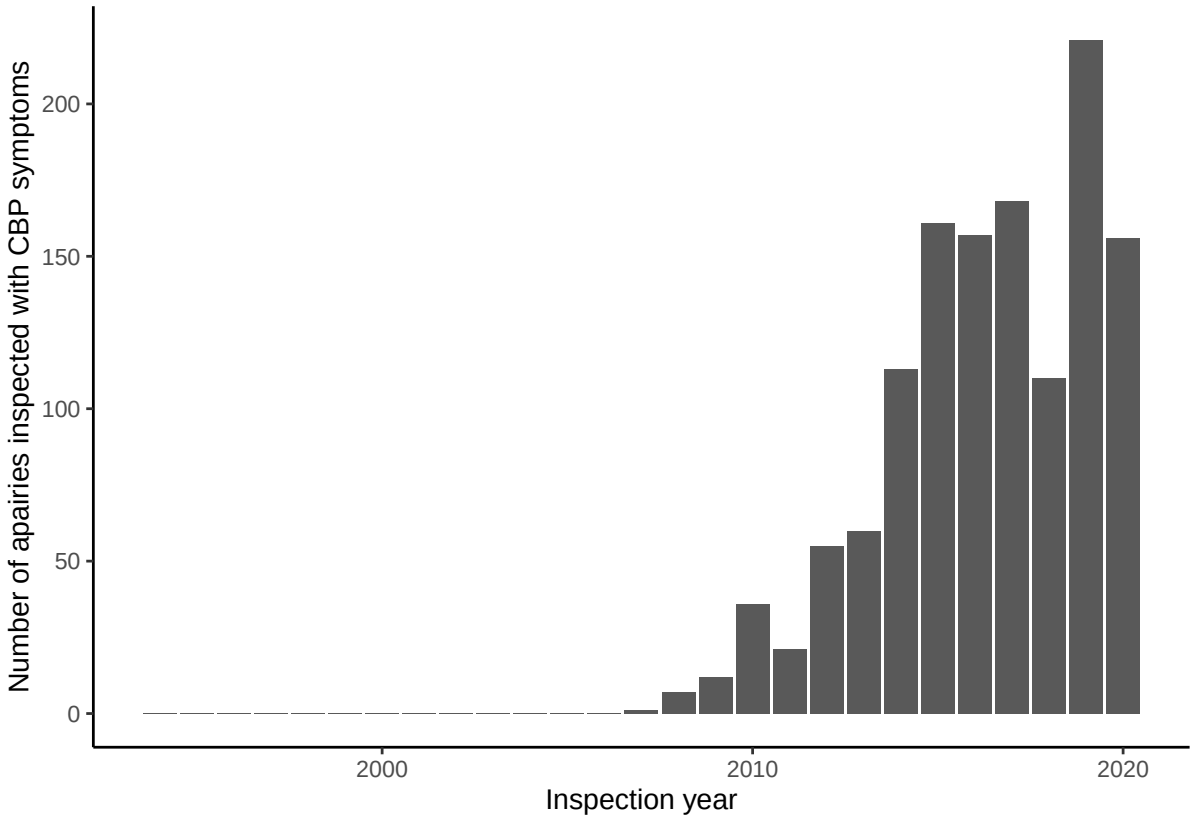


Figure 5.1: The number of apiaries displaying symptoms of Chronic bee paralysis (CBP) in the UK between 1983 and 2020. Records are taken from apiary inspections carried out by bee inspectors. Data from Budge et al. (2020).

Honey bees are central place foragers, collecting nectar and pollen from areas surrounding the hive (Bell, 1990). Bees are not territorial in the mammalian sense as foraging areas may overlap, which can lead to interactions between bees from different hives. This presents an opportunity for disease transmission, either through direct contact or via fomites present at foraging sites (Koch et al., 2017). The land over which honey bees forage differs in terms of the vegetation present and therefore the extent of floral and nectar resources (Couvillon et al., 2015). Foraging distance can also be affected by month, season and disease status (Couvillon et al., 2015; Koch et al., 2017; Van Der Steen, 2015). There is evidence that nutrition and diet affect honey bee health and susceptibility to disease. Links have been found between colony loss and the amount of open land proportionate to the amount of developed land, finding an increased number of cases of the syndrome known as Colony Collapse Disorder in areas with less open land (Naug, 2009). Polyfloral diets are preferred by honey bees, and can improve immune functioning and longevity in

adult bees, in comparison to monofloral diets (Alaux et al., 2010; Schmidt, 1984; Schmidt et al., 1987). We therefore expect that apiaries with less diverse forage availability may be more susceptible to disease.

In addition, the UK climate varies geographically because of its proximity to the Atlantic from which much of the prevailing weather comes. Thus, we might anticipate weather and habitat in the landscape will vary geographically and this might impact on bee foraging success and also contact between bees. This indicates that landscape and weather may influence disease transmission and if we are to evaluate likely transmission, we need to adjust for the impacts of these drivers on honey bee disease. Weather events such as rainfall, high winds and hot temperatures can affect bees in various ways and often restrict bees to the hive, preventing them from foraging (Clarke & Robert, 2018; Rowland et al., 2021). Extended periods of these weather events could lead to depletion of pollen stores, as colonies tend to maintain small quantities of pollen stores (Schmickl & Crailsheim, 2001, 2002). A lack of pollen leads to utilisation of body protein supply, cannibalisation of brood, decreased longevity of workers and a cessation of brood production (Brodschneider & Crailsheim, 2010; Haydak, 1935; Knox et al., 1971). This reduces colony productivity and can lead to colony collapse (Neupane & Thapa, 2005). The restriction of bees to the hive can lead to crowded conditions, which may increase the likelihood of disease transmission (Bailey et al., 1983). Studies have found that low humidity can lead to reduced survival and a decrease in body water content and body weight, which can be indicators of individual bee health (Abou-Shaara et al., 2012; Atmowidjojo et al., 1997). We therefore hypothesise that weather will impact overall colony health and susceptibility to disease.

The incidence of bee disease may also be spatially correlated because of the link between habitat and weather, and the interaction between honey bees in hives which are closer together, causing clustering of infection events (Basáñez et al., 2004). It is also likely that bee disease is temporally correlated, as CBP is a transmissible disease and transmission events occur when bees from an infected hive lead to infection arising at other hives at future time points. The timing for which this would occur will depend on disease latency and detectability.

When events are spatially or temporally correlated assumptions of many statistical modelling approaches are violated. In practice this means that each sample cannot be considered independent of others and estimates of the significance of parameter

values will be biased (usually upwards when assuming local spread) so that significance appears to be greater than it is in reality. This means that off-diagonal terms must be included in the covariance matrix which represent the outcome-driver relationship to account for the relatedness between samples. Mixed effect models and generalised least squares modelling approaches which seek to model the relationship between response and putative drivers can accommodate either spatial or temporal autocorrelation by explicitly modelling these off-diagonal covariances. However, if there is both spatial and temporal autocorrelation then this adjustment cannot be made because it is not possible to distinguish between the two forms of correlation. In this case recourse has to be made to Bayesian approaches using the integrated nested Laplace approximation (INLA) methods, which offer a more computationally efficient and powerful alternative to Markov-Chain Monte Carlo (MCMC) approaches (Blangiardo & Cameletti, 2015). INLA uses a deterministic approach based on latent Gaussian models, which can be used to model sample dependence and make predictions (Blangiardo & Cameletti, 2015; Sigrist, 2022). The algorithm was introduced by Rue et al. (2009) and can be used to calculate accurate estimates of marginal posteriors over short periods of time.

Here, we used stochastic partial differential equations (SPDE) point-based models to investigate the spatial and temporal patterns of CBP infection in UK apiaries and the effect of environmental, meteorological and management factors on the spread of this disease (Blangiardo & Cameletti, 2015). SPDE models generate a continuous spatial process (in this case of risk) on the basis of point estimates of that surface and measures of putative risk factors at these points (Lindgren et al., 2011). The apiaries are points on the surface that are then related to the drivers measured at these points which are spatially linked using a mesh. The latter stage of this process quantifies the spatial dispersion and relatedness of the points.

5.2 Methods

5.2.1 Study Area

The South of England was chosen as a study area because a high number of cases of CBPV were recorded in this region between 2017 and 2020 on BeeBase, a database recording all apiary visits conducted by inspectors from the National Bee Unit (NBU) of the Animal and Plant Health Agency (APHA) in England and Wales, between 1994-2020 (Brown et al., 2014). The area also has clear borders of coastline around the western, southern and eastern perimeter. The Northern border was based on county borders. The counties investigated are displayed in Figure 5.2.

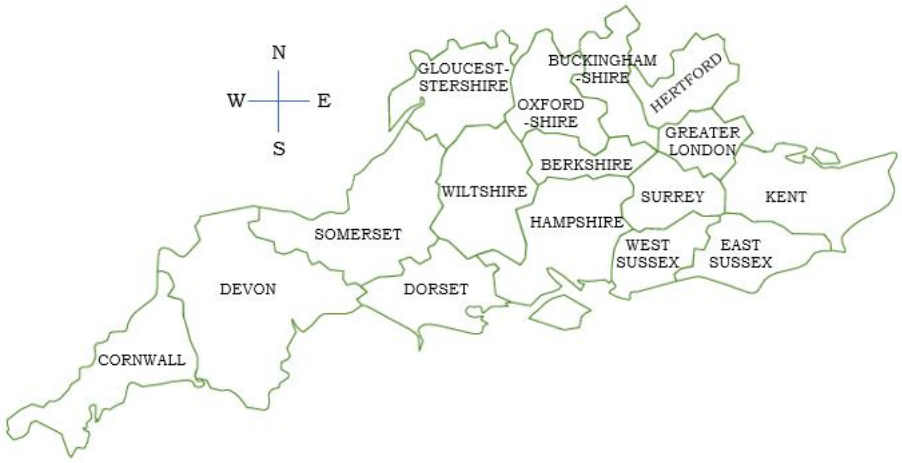


Figure 5.2: A map of the Southern counties of the United Kingdom included in these analyses.

5.2.2 Data

5.2.2.1 Apiary data

Data on honey bee health were obtained from apiary inspection records on BeeBase. Inspections can be randomly assigned to apiaries or may take place due to a request from the beekeeper themselves. Other reasons include a new beekeeper, imported queens or signs of a statutory notifiable disease, such as European Foulbrood (EFB) and American Foulbrood (AFB). Inspectors are involved in the control of these diseases and can conduct lateral flow immunoassays to detect AFB and EFB (Brown et al., 2014). Other information recorded from inspections includes symptoms of disease, the number and

types of hives present in the apiary, the number of colonies owned by the beekeeper and whether queens have been imported and where from (Budge et al., 2020). Inspectors also record the number of bees and brood per frame of the hive, to give an estimation of the total population.

BeeBase contains records of approximately 6000 apiary inspections per year, most of which take place between April and September each year (Brown et al., 2014). It, however, does not necessarily contain information of every hive in England and Wales, as honey bee hives do not have to be registered in the UK. Information on number of hives and/or apiaries and the density of apiaries in the UK is based on apiaries which have been inspected or registered only.

5.2.2.2 Vegetation

As a measurement of habitat quality for foraging, vegetation data for the landscape surrounding apiaries were obtained from the UK Centre for Ecology and Hydrology (UKCEH). The 2017, 2018, 2019 and 2020 Land Cover Map (LCM) datasets were used (Morton et al., 2020b, 2020c, 2020a, 2021). The data are digital and were imported into R.

The vegetation data are split into areas of land parcels varying in size and shape. LCM data for 2017 to 2019 are made up of 20 m^2 pixels, each of which correspond to a vegetation type. For LCM 2020, however, these pixels are 10 m^2. Land parcels in LCM data often contain more than one vegetation type, and the number of pixels per vegetation type is provided. The size of these land parcels was calculated using the function st_area from the sf package. This was converted to a number of 20 m^2 pixel squares or 10 m^2 for 2020 data. The 2017, 2018 and 2019 vegetation data were then all scaled to 10 m^2. The land types included in the data sets are shown in Table 5.1.

To collate data for individual sample points, a buffer of 1 km was placed around each apiary (per inspection) and land parcels from the vegetation data which were found within the buffer were collected using the sf package in R (Pebesma, 2018). Per apiary inspection, there were a total number of pixels per vegetation type present. We then calculated the size of the area of overlap between each land parcel and the apiary buffer area to gain a more precise estimate for the quantity of vegetation in each buffer. This was used to scale the number of pixels per vegetation type to the size of the land parcel that overlapped

with the buffer. The vegetation data were logged (log base 10) as the values vary in scale, followed by analysis using multivariate analysis techniques with the vegan package in R (Oksanen et al., 2022). To avoid the unit sum constraint imposed by analyses of areas the vegetation data were subjected to correspondence analysis (CA) to provide two axes representing the major trends in variation in the vegetation (Legendre & Legendre, 2012; Ter Braak, 1986). Axes were evenly split into categories and apiaries were assigned a category for each axis based on their scores (Appendix F, Table 6.9). We investigated the relationship between the axes and CBPV using SPDE models (further explanation found in Section 5.2.3).

All vegetation groups that do not include forage usable by a honey bee were grouped in the analyses and are referred to collectively as unusable habitat. These include habitats lacking flowering plants, such as rock, sediment, marsh, water and urban areas, and habitats whose typical plants do not use insect pollination, which include coniferous woodland, arable land, improved grassland and acid grassland (Gabriel & Tscharntke, 2007; Provan et al., 2008).

Table 5.1: The categories of vegetation measured in the Land Cover Map data sets. Descriptions are taken from the Land Cover Map product documentation.

Type	Description
Deciduous woodland	Areas dominated with trees over 5m high with over 20% tree cover, and scrub less than 5m high with over 30% cover
Coniferous woodland	Semi-natural stands and plantations with over 20% tree cover with trees greater than 5m high
Arable	Crops, perennial crops and ploughed land
Improved grassland	Regularly cut and intensely managed grassland in arable areas dominated by *Lolium* spp. and white clover
Neutral grassland	Based on botanical classification and includes some semi-natural grasslands where soil pH is between 4.5 and 6.5
Calcareous grassland	Dominated by grasses and herbs on shallow, well-drained soils with a soil pH above 5
Acid grassland	Grasses and herbs on lime deficient soils, dominated by *Molina caerula*
Fen	Fens, fen meadows, rush pasture, swamp, flushes and springs
Heather	Greater than 25% of heather cover
Heather grassland	Less than 25% of heather cover
Bog	Ericaceous, herbaceous and mossy swards in areas with a peat depth >0.5m
Inland rock	Natural and artificial exposed rock surfaces >0.25ha
Saltwater	Seawater in tidal rivers, coastal regions and intertidal regions
Freshwater	Standing open water, canals, rivers and streams

Type	Description
Supralittoral rock	Vertical rock, boulders, gullies and ledges occurring above the high water mark
Supralittoral sediment	Sand dunes, shingle beaches and machair
Littoral rock	Rocky coastline in the maritime zone in wave sheltered conditions
Littoral sediment	Beaches, sandflats and intertidal mudflats
Saltmarsh	Coastal vegetation/wetlands
Urban	Dense urban areas including towns, city centres, docks, car parks and industrial estates
Suburban	A mixture of urban and vegetation signatures such as gardens and parks

5.2.2.3 Weather

To investigate the impact of weather on colony health, meteorological data were collated from the Centre for Environmental Data Analysis Had-UK dataset (Hollis et al., 2018). This included average hourly wind speed (knots), monthly mean air temperature (°C), average hourly humidity over a month (%), daily rainfall amount (mm) and duration of sunshine during the month (hours). The measurement used was an average of each of these variables over the three months prior to the inspection, as seen in Rowland et al. (2021). This time period was chosen to account for the weather at the time of the inspection and prior to the inspection which may have affected newly emerged bees during development or forager bees during foraging (Rowland et al., 2021). The weather data were at a 1 km^2 scale. The 1 km grid square for each apiary was matched to the weather data for the same location.

5.2.3 Spatial analysis

Data on apiaries were collected from BeeBase and limited to apiary inspections which were requested by beekeepers between 2017-2020. Only using apiaries where inspections were requested increases the likelihood of severe infections of CBPV, which should ensure that symptomatic colonies were likely to be correctly identified in having CBP. Between 2017 and 2020, there were approximately 1,700 apiary inspections requested by beekeepers, of which 143 had symptoms of CBP. Other information about the inspector, beekeeper and apiary were also collected if pertinent to colony health and symptom identification.

The experience of the beekeeper responsible for the apiary was included as a predictor of disease presence or detection and was based on the number of colonies owned by the beekeeper. Professional beekeepers were identified by owning 40 or more colonies (Budge et al., 2020). A link has been identified between professional beekeepers and CBP cases, potentially due to the higher density of colonies in apiaries run by professionals (Budge et al., 2020). Newer beekeepers have less experience of beekeeping and may be more likely to miss signs of poor colony health or cause stress in colonies which may make them more susceptible to disease. The number of colonies owned by the beekeeper was also included as a predictor variable as another indicator of expertise, and we also hypothesise that beekeepers with more colonies may see spread of CBP between apiaries, and are therefore more likely to have cases of the disease (Brown et al., 2014). This also applies

to the number of colonies in the apiary, as more colonies means more opportunities for transmission. We also included inspector experience as a predictor variable, as although inspectors all receive training to examine honey bee health and diagnose disease, precision and early detection of disease may improve with experience (Brown et al., 2014). We hypothesise that density of apiaries will increase the likelihood of CBPV infection, as honey bees from apiaries which are closer together likely share foraging sources or engage in robbing which increases the chances of transmission (Durrer & Schmid-Hempel, 1994; Fries & Camazine, 2001). Density of apiaries was calculated using inspection data and colony registration data from the NBU. The number of intersects of apiary buffers was calculated to give the number of apiaries which occur in a 2 km radius.

As CBP is an emerging disease, we were interested to know how the number of cases changed over time (Budge et al., 2020). Time of the inspection based on month was used as a predictor variable. This was calculated by sequentially numbering inspection month from the first month inspected in 2017 to the final month inspected in 2019. Month of inspection was investigated using harmonic transformations to account for the increased number of inspections between March and September while still including seasonality as a covariate. This was calculated using the sine and cosine of continuous time, which adjusts for the effect of season in relation to other covariates.

The response variable used was symptoms of CBP and was assumed to have a binomial distribution. This involved the inspector observing symptoms of the disease and recording these observations. Inspectors may not formally identify the disease but make notes of the symptoms observed. Colony inspections which noted “paralysis”, “shivering”, “quivering”, “trembling”, “black”, “shiny”, “crawling”, “many dead bees” or “k-wing” were considered to be symptoms of CBP as they are characteristic symptoms of the disease, as identified by Budge et al. (2020).

To analyse these data, we used point based stochastic partial differential equation (SPDE) models implemented into the INLA library in R using version 21.11.22 and default priors (Lindgren et al., 2011). The INLA approach creates a probability surface of disease with point estimates using SPDEs (Blangiardo & Cameletti, 2015). When modelling cases of disease, the locations of these cases are pertinent to the likelihood of spread. We hypothesise that apiaries close to apiaries with CBP cases are more likely to become infected. Therefore, our analysis must take the spatial dependency of apiaries into account. SPDE models account for the spatial clustering and dependency of disease while

allowing us to analyse the effects of the predictor variables. Estimates of the effects of covariates on cases of CBP were produced by the models. Any estimates with a confidence interval that overlaped with zero were considered to be nonsignificant. Nonsignificant covariates were removed one at a time, and the deviance information criterion (DIC) values were compared. If the DIC value decreased with the removal of a nonsignificant covariate, the covariate was permanently removed from the model. This process continued until only significant covariates were remaining. The resulting INLA models were compared using DIC values, where a lower value indicates a better model fit.

After identifying the best model, the significant covariates with and without the spatial field were used to predict areas of risk of CBP infection for the whole region. We used the data from 2017, 2018 and 2019 in order to predict the probability of CBP in all apiaries inspected in 2020. The risk of CBP from each model was compared to the actual CBP cases observed in 2020 using a Procrustes test with the vegan package (Oksanen et al., 2022). Procrustes analysis compares the spatial patterns of data, and was used to investigate the similarities between the predicted and observed data. It finds the optimum rotation (in this case in space) and scaling of distance to optimise the fit between two spatial data sets, whilst maintaining the relative positions of events in 2D space. This was used in an attempt to overcome the problems of comparing patterns in space which may differ only mildly in either the x or y direction, but which would otherwise appear as completely dissimilar if compared on a point-by-point basis. To compare visualisations of the risk in areas surrounding apiaries with CBP symptoms, linear interpolation was performed using the akima package (Akima et al., 2022).

All analyses were undertaken in R version 4.2.2 (RCoreTeam, 2022).

5.2.3.1 Predictor variables

A summary of the predictor variables included are shown in Table 5.2.

Table 5.2: The predictor variables used in the INLA models, including how they were calculated and where the data was sourced from.

Variable	Calculation	Source
Humidity	Average monthly humidity for three months prior to inspection	Centre for Environmental Data Analysis Had-UK
Temperature	Average monthly air temperature for three months prior to inspection	Centre for Environmental Data Analysis Had-UK
Wind	Average hourly wind speed for three months prior to inspection	Centre for Environmental Data Analysis Had-UK
Rain	Average daily rainfall for three months prior to inspection	Centre for Environmental Data Analysis Had-UK
Sun	Average sunshine duration for three months prior to inspection	Centre for Environmental Data Analysis Had-UK
Vegetation axis 1	Axis 1 of the correspondence analysis of the vegetation data	Centre for Environment and Hydrology
Vegetation axis 2	Axis 2 of the correspondence analysis of the vegetation data	Centre for Environment and Hydrology

Variable	Calculation	Source
Professional beekeeper	Beekeepers with 40 or more colonies	BeeBase
Inspector experience	The number of apiary inspections conducted by the bee inspector	BeeBase
Number of colonies in apiary	The total number of colonies present in each apiary	BeeBase
Number of colonies owned by beekeeper	The total number of colonies owned by this beekeeper across all their apiaries.	BeeBase
Density	The number of apiaries whose 1km radius intersects with the 1km radius of a particular apiary	BeeBase
Continuous time	Each month of inspection is numbered sequentially from the beginning of the study period (March 2017) to the end (November 2020).	BeeBase
Cosine	Cosine harmonic regression of month	BeeBase
Sine	Sine harmonic regression of month	BeeBase

5.3 Results

5.3.1 Correspondence analysis

The correspondence analysis (CA) of the vegetation data found that axis 1 (CA1) captured 22.8% of the variance in the data, and axis 2 (CA2) captured 18.8% of variance in the data, totalling 41.6% overall. A high CA1 score reflects a habitat with more heather and heather grassland, while a low CA1 score is associated with neutral grassland and fen habitats. CA2 mainly reflects a difference in grassland types, with acid grassland being associated with a high CA2 score and calcareous grassland being associated with a low CA2 score (Figure 5.3). There were no clear trends in apiaries with paralysis and their axis scores, and no differences between years.

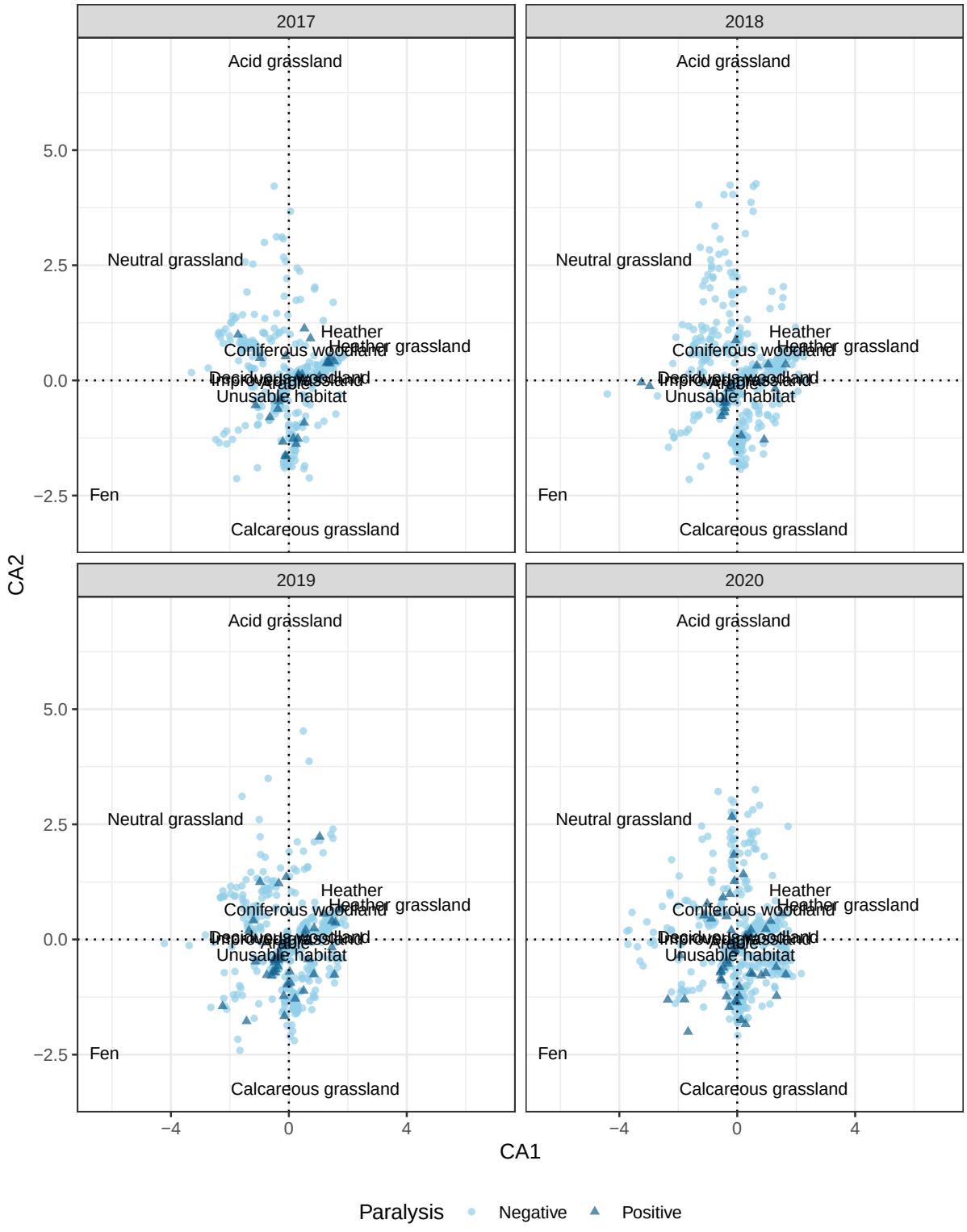


Figure 5.3: The correspondence analysis scores for axis 1 and 2 of apiaries with and without CBPV in relation to the amount of various vegetation types they had access to.

5.3.2 SPDE Models

The result of the initial SPDE model with all covariates found a significant relationship between CBP symptoms and inspector experience and humidity (Figure 5.4). The risk of CBP increased with lower humidities and reduced inspector experience. The effect size of humidity was larger than that of inspector experience, and also had much larger confidence limits. When the spatial field was added to this model, inspector experience remained significant and the estimate was similar. Humidity became nonsignificant and was removed from the model. A table of results for each model including the 2.5% and 97.5% confidence limits can be found in Appendix G. We found that the model including all significant fixed effects (humidity and inspector experience) without the spatial field had an DIC value of 667.47, and the model with the spatial field plus significant fixed effects (inspector experience) had a DIC of 660.46, indicating that there was spatial dependency in the outcomes.

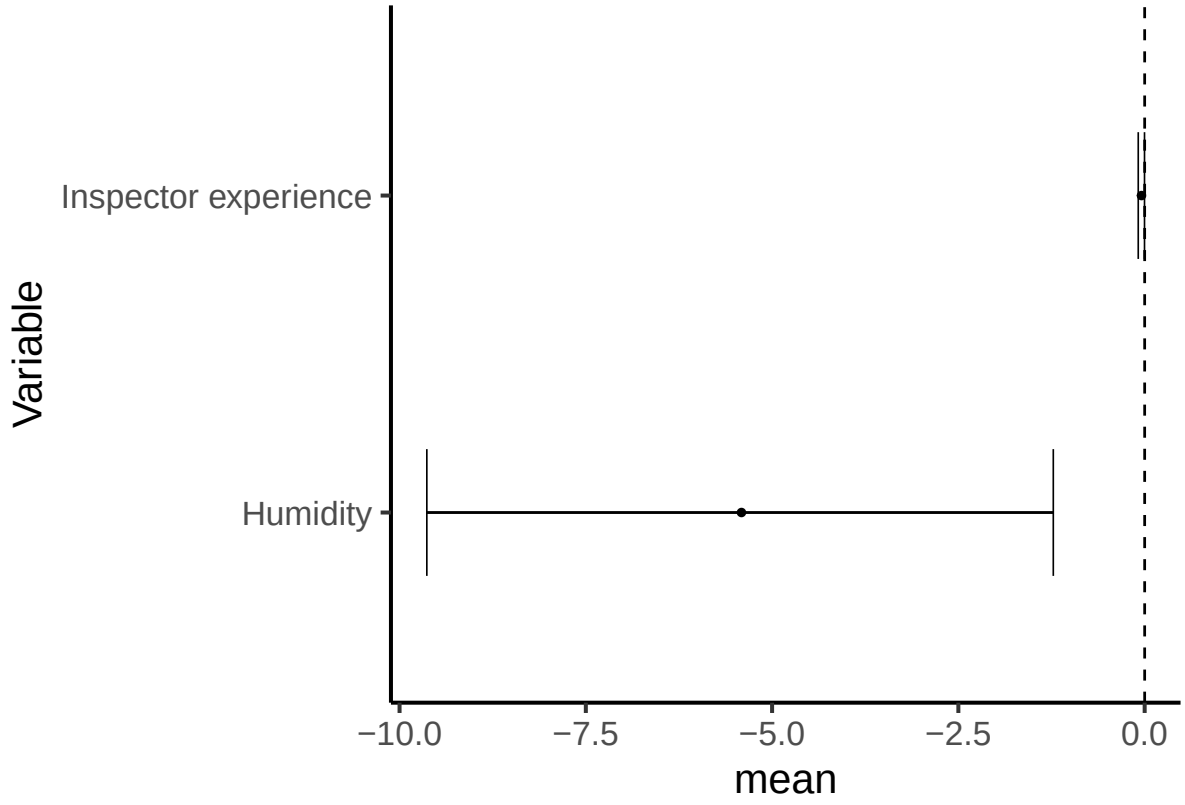


Figure 5.4: The fixed effects from the INLA model without spatial field. Points represent the mean, and bars and whiskers represent the 2.5% and 97.5% confidence limits. Only significant variables (where the interval does not encompass 0) are shown.

As humidity was a significant covariate prior to the addition of the spatial field, a separate SPDE model was conducted using humidity as a response variable and the DIC was recorded with and without the spatial field included. We found that the humidity model including the spatial field had a lower DIC (7638.10 in comparison to 15673.76 without the spatial field), which suggests that there is spatial autocorrelation in the humidity variable, meaning that the effect of humidity is being masked by the spatial field when included in the model.

DIC values for all models run are shown in Table 5.3. The model with the lowest DIC value included the spatial field and the significant covariate inspector experience.

Table 5.3: The DIC values for each INLA model for CBPV and the covariates used.

Covariates	DIC values
Intercept	674.8148
Intercept + all fixed effects	837.9051
Intercept + humidity + inspector experience	667.4651
Intercept + spatial field	663.2068
Intercept + spatial field + inspector experience	660.4622

5.3.3 Prediction

Two models were chosen to make predictions of spatial risk for 2020 based on their DIC values and the type of covariates involved. These were significant fixed effects (humidity and inspector experience) and spatial field plus significant fixed effects (inspector experience).

When comparing the results of the prediction maps with fixed effects only and spatial field with fixed effects, we can see that the majority of risk identified with the spatial field is fairly evenly spread with a few areas reaching risks of approximately 10% (Figure 5.5). In comparison, the prediction without the spatial field identified a hotspot, where

the probability reached approximately 20% on the border between Greater London and Buckinghamshire, as well as other areas with lower probabilities. This model also predicted a higher probability of CBP for larger areas in the centre and east of the region, in comparison to the west. The actual cases of CBP in 2020 were visualised as risk in Figure 5.6. The risk identified in both prediction maps does not clearly mirror the actual risk observed, however, some areas of risk prediction are similar to the actual risk observed.

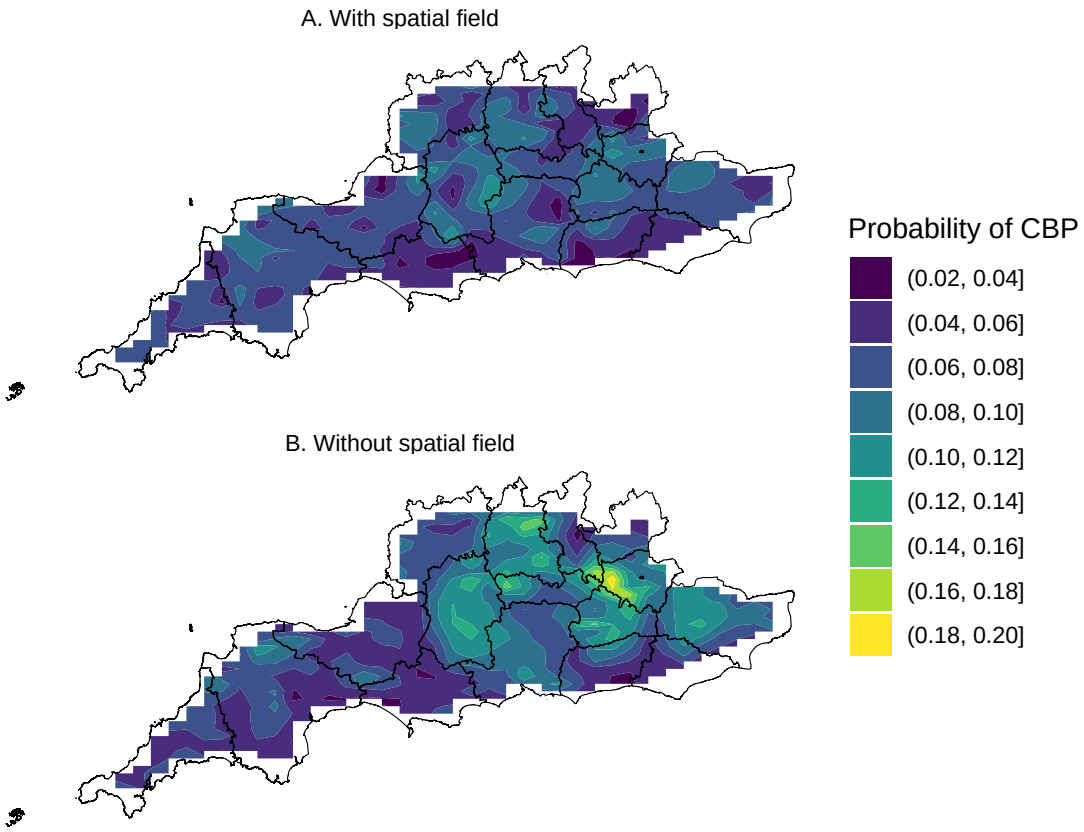


Figure 5.5: The INLA prediction of Chronic bee paralysis (CBP) cases in Southern UK in 2020. (A) Prediction used the spatial field and 2017-2019 call out data on inspector experience. (B) Prediction used 2017-2019 call out data on inspector experience and humidity data. A lighter colour shows a higher probability of CBP in that area.

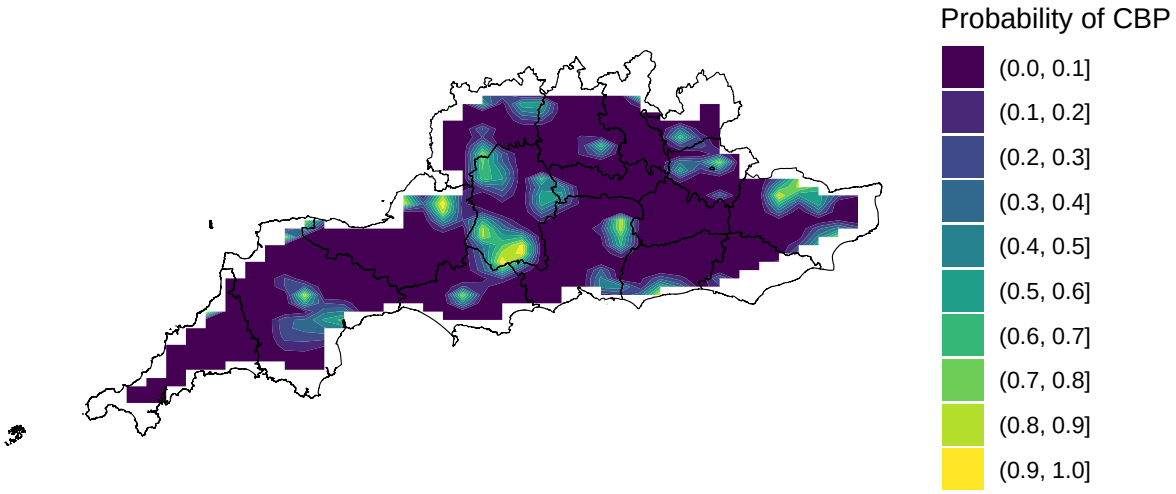


Figure 5.6: The actual risk of Chronic bee paralysis (CBP) identified during call out inspections at apiaries in Southern UK in 2020. A lighter colour shows a higher risk of CBP in that area.

The predicted risk of CBP from both models was compared to the actual locations of apiaries with and without CBP in 2020 (Figure 5.7). There was little difference in the distribution of the probability of CBP at apiaries which did or did not have symptoms for CBP. The model without spatial field predicted higher probability overall when compared to the model with spatial field, however, this result did not appear to be correlated to apiaries with CBP. The results of a Procrustes test found that both methods of CBP risk prediction were significantly correlated to the actual risk observed in 2020 (Table 5.4).

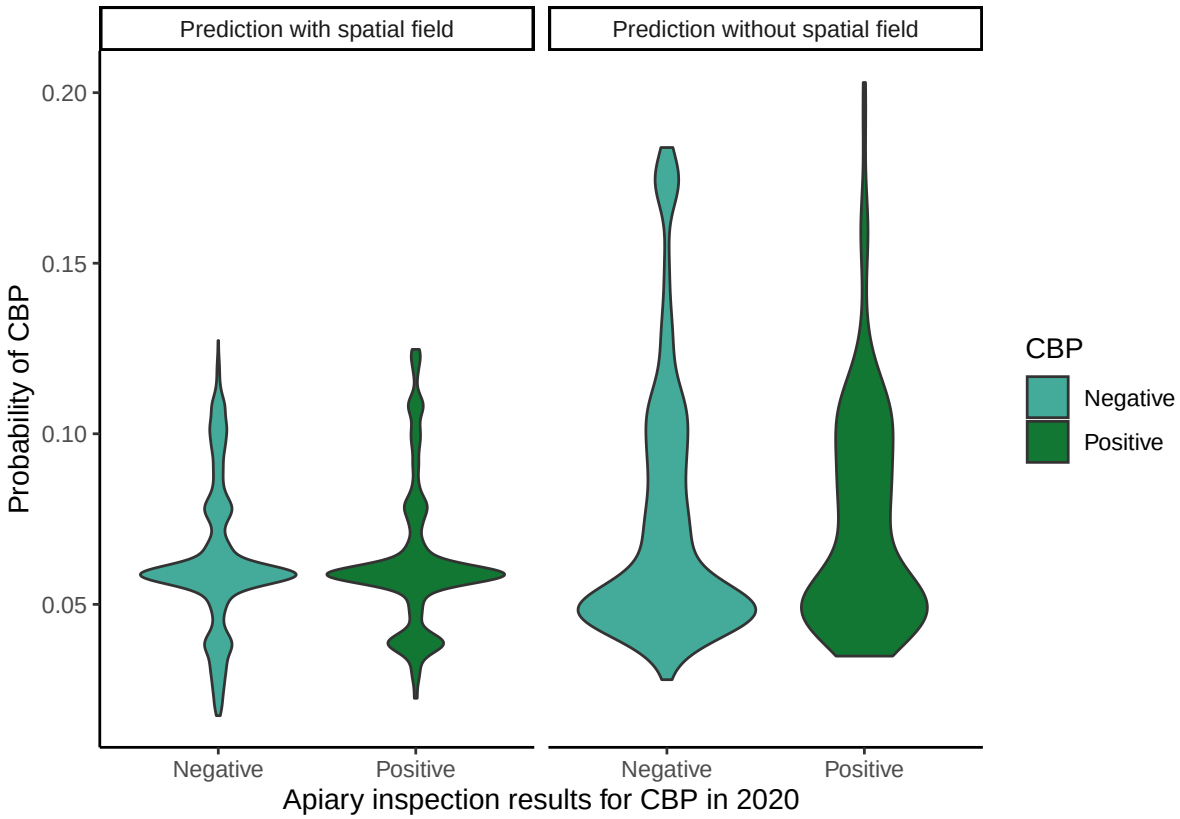


Figure 5.7: The distribution of the predicted probability of Chronic bee paralysis (CBP) from models with and without the spatial field at actual locations of apiaries with and without cases of CBP in 2020.

Table 5.4: The results of the Procrustes test of significance between the risk calculated from the observed cases of CBP for 2020 and the probability of CBP predicted by both prediction models.

Model type	Procrustes sum of least squares	Signficance
Prediction with spatial field	8.881340e-12	0.001
Prediction without spatial field	8.928414e-12	0.001

5.4 Discussion

CBP incidence increased with lower humidity. In the UK, humitidy is generally lowest during the spring and summer months, which coincides with a higher number of cases of CBP. As previously mentioned, laboratory experiments have found that low humidity can cause a decrease in body water content and body weight as well as lower survival in bees (Abou-Shaara et al., 2012; Atmowidjojo et al., 1997). These factors may cause an increased susceptibility to disease, leading to higher rates of CBP spread in periods of low humidity. While this is an interesting result for future predictions, especially in relation to climate change, it has little utility at present as it requires a previous three month mean to identify disease, which in practice would mean the disease was already present at the time a prediction could be made. No other weather variables were significant in our models. While the models suggest that other weather variables did not impact CBP prevalence in our study area during the time period, it is also possible that certain combinations of weather impact bees, and that investigating weather types individually does not allow us to identify the overall effect of weather. Many types of weather may cause confinement to the hive, such as rainfall, high winds and extreme temperatures, which is believed to be a risk factor for CBP (Ball & Bailey, 1997). It is also possible that shorter or longer periods of certain types of weather may have an effect on CBP infection, and these periods were not identified in our three month average of weather data.

We found that reduced inspector experience was linked to cases of CBP. This may be due to the nature of inspection requests. Inspectors have a choice in how they respond to these requests; they can either visit the apiary and perform an inspection or provide a diagnosis based on a description of symptoms provided by the beekeeper over the telephone. In the cases where an inspector does not visit the apiary, this potential diagnosis of CBP is not recorded in BeeBase as no apiary inspection was conducted. More experienced inspectors may be more likely to give advice over the phone, based on their knowledge of honey bee disease. It may be even less likely that an experienced inspector would visit an apiary with a suspected case of CBP, knowing that there is no treatment available. Newer inspectors with less experience may be more likely to visit apiaries before providing a diagnosis, and may be more diligent in providing descriptions of the symptoms they observe, which is how CBP cases are identified in BeeBase. Additionally, inspectors undergo training before taking part in apiary inspections, which may mean they are more aware of the symptoms of CBP as they have received training more recently.

Our models did not identify a significant relationship between CBP cases and time. This may be due to the length of the time period studied, as identifying trends within a three year time scale may be challenging. Budge et al. (2020) found an increase in CBP by year overall between 2007 and 2017, but the increase was not constant, which is especially true for 2017 to 2020 (Figure 5.1).

We found no significant relationships between CBP and apiary density, number of colonies per apiary, number of colonies owned by the apiary beekeeper or being a professional beekeeper. High densities of colonies and apiaries can lead to low pollen in that area, which can reduce immunocompetence and therefore overall colony health (Alaux et al., 2010). As this has not been highlighted by our models, it may be that the regions analysed are not restricted by pollen at the current stocking rates. However, overall, this may be due to the fact that CBP is an emerging disease, and that the conditions of apiaries are less important than the distance to an infected apiary when it comes to disease spread at this scale. For example, the number of colonies in an apiary will have no impact on disease spread if the disease is not already present. The sporadic nature of CBP infections at this stage in the endemic/epidemic status of disease may mean that these factors are not impacting disease spread on a large scale. There were also no significant relationships between the CA axes scores and CBP occurrence. Although there is evidence that pollen can reduce susceptibility to disease, it is likely that it would not completely prevent infection and transmission of a pathogen at a hive level.

Spatial autocorrelation was identified in CBPV disease spread. Infectious diseases can be transmitted in a multitude of ways, on both a small and large scale. In honey bees, disease can be spread between colonies within an apiary or between apiaries that are in close proximity via fomites during foraging, robbing behaviour and through queens mating (Chen, Evans, et al., 2006; Durrer & Schmid-Hempel, 1994; Fries & Camazine, 2001). Spatial autocorrelation in our data indicates that CBP may be spreading through these methods. Emerging disease spread is often characterised by a main focal point from which the disease gradually disseminates, however this clearly is not the case for the emergence of CBP in Southern UK (Morse, 1995). We do, however, see a similar pattern on a small scale, with disease occurring in multiple apiaries in certain areas. This is evident in Figure 5.6, which shows the risk of CBP in Southern England in 2020. There are multiple areas with a higher concentration of CBP cases, but no clear spread between these areas, which was also identified in the national spread of CBP by Budge et al.

(2020).

We also investigated the interaction between spatial autocorrelation in our data and the predictor weather variables, as spatial dependence of weather variables may obscure or replace the apparent spatial dependence in the response. As weather varies based on location due to factors such as altitude, latitude and where prevailing weather comes from, it is likely that weather in our data will vary based on location. We identified spatial dependence in the humidity variable. For this reason, two models were used in the prediction: significant fixed effects without a spatial field and significant fixed effects (without weather variables) with a spatial field.

The prediction models identified some areas where risk levels were similar to those observed in the 2020 data. This is mainly true for the models which included inspector experience and humidity, where areas of higher risk were often similar in location to the real data. Despite this, none of the key hotspots for disease risk observed were identified in the models as being areas with more severe risk. The results of both models found a significant correlation with the actual cases of CBP observed in apiaries. This is likely due to the zero inflation in the observed data and the low levels of risk predicted in both models, rather than the prediction models also identifying the areas of high risk. In addition, diseases can often jump in their spread and do not naturally follow a simple diffusion pathway. This is often the case with livestock disease, where human movements of animals leads to unusual patterns of spread (Shirley & Rushton, 2005). In honey bees, nucleus colonies are often moved around the country and queens are regularly imported from abroad, which may explain how human involvement aids in the transmission of CBP. While the prediction models may give some indication of factors that contribute to disease risk, it is unlikely that the model could be used to successfully predict disease because of these circumstances.

Data were limited to requested apiary inspections as we hypothesised that these apiaries would have more severe disease, and would therefore give a clearer signal as to what drives CBP. This bears the assumption that all beekeepers would request an inspection upon observing a similar level of disease. It is likely that beekeepers who are more experienced may not request inspections and deal with the disease themselves, as evidenced by Budge et al. (2020) who found that professional beekeepers were much less likely to request a bee inspection, despite being more likely to have diseased colonies. Less experienced beekeepers may not notice symptoms as early as more experienced beekeepers. Ball (1999)

noted that samples of bees which tested positive for CBPV often came from colonies which beekeepers considered to be healthy. Due to this heterogeneity in the ability to detect disease, temporal trends in disease spread will be harder to identify. We must also consider what a beekeeper does with their hive once they discover it is diseased. There is a lack of established husbandry advice for what to do with infected colonies, which may lead to beekeepers resorting to the destruction of infected hives. This would likely end any potential transmission from this site, impacting both the temporal and spatial trend in disease spread.

5.5 Conclusions

Our key findings are that CBP is more prevalent with decreased humidity, indicating that weather events can affect bee behaviour, which in turn increases disease spread. Additionally, CBP is linked with less experienced inspectors, which may highlight an issue with the detectability of the disease. We identified spatial autocorrelation in our models, which is to be expected as disease tends to spread based on spatial closeness and contact. Our models identified difficulties in modelling this emerging disease, as disease occurrence can be sporadic and influenced by human movements, and therefore will not follow a natural trend in spread. It is likely that these factors affect the spread of disease more than environmental variables which may influence honey bee health and susceptibility, but have no effect if the disease is not present.

Chapter 6

Discussion

The aim of this research was to investigate the epidemiology of Chronic bee paralysis (CBP), an emerging bee disease caused by the pathogen Chronic bee paralysis virus (CBPV) which has been increasing in prevalence in the UK over the last 10-15 years. A combination of molecular diagnostics, laboratory experimentation and modelling was used to investigate the underlying processes associated with the transmission and progression of disease. The laboratory experiments sought to investigate the mechanisms by which the virus is transmitted between bees, the aetiology of disease and the outcomes of infection. Event-based approaches (survival analysis) were used to investigate how symptoms or death as outcomes were related to feeding behaviour and sources of infection. The research was then expanded to the field-scale to investigate the spread of disease and the likely drivers of disease using spatio-temporal statistical modelling approaches based on stochastic partial differential equation approaches.

Transmission of CBPV was studied in the form of small pot experiments to investigate oral exposure to the virus. The technique used to inoculate bees most closely resembled what we assumed to occur in a hive as trophallaxis and demonstrated how transmission might occur from an infected nurse bee to a newly emerged bee receiving its first feed. Transmission only appeared to take place in bees up to one day after emergence, suggesting that transmission by trophallaxis only occurs in a narrow window of adulthood (see Chapters 2 and 3). The question arises as how realistic the experimental set-up was in regards to disease transmission. Oral exposure under hive conditions could take place via many routes such as both allo- and auto-grooming, the cleaning of cells, feeding on hive materials and the removal of corpses from the hive. The role of nutrition in determining

disease transmission was addressed to investigate the hypothesis that poor nutrition might alter susceptibility to transmission (see Chapter 4). During periods of unseasonal weather where honey bees are confined to the hive when they typically would be foraging, food stores can be used up quickly, resulting in a pollen dearth. This could mean that bees which emerge during these periods will not be able to consume the beebread that is required for healthy development and growth, which may alter their susceptibility to disease. The results of the experiments presented in this thesis suggested that under laboratory conditions, transmission of disease only occurs in the first day after adult emergence, and that newly emerged bees in particular were most susceptible to infection (Chapter 2, Section 2.3.3). Further investigation revealed that this susceptibility was based on the age of the bee at the time of exposure to CBPV, and the gut microbiome of the bee seemed to have no effect (Chapter 3, Section 3.3.4.2). Additionally, laboratory results suggested the consumption of beebread after emergence may reduce susceptibility to CBPV (Chapter 4, Section 4.3.1.1).

6.1 Significance of results

What is the significance of these results for expanding our understanding of epidemiology and aetiology of CBP? We need to assess the extent to which the results relate to the reality of disease transmission in a hive setting, and consider the limitations of the experimental set-up and their impacts on the subsequent modelling analyses used. These analyses showed that the experimental set-up had a negative impact on bees in that bees kept in small pots tended to show increased mortality after 10 days, even in control treatments without disease. Studies have reported that summer bees tend to live for between 15-38 days (Winston, 1987). Even when it is attempted to make the conditions bees are kept in as close their natural habitat as possible, the requirement for sterile equipment, lack of contact between bees of different ages/conditions and a food source invariable in its composition limit how authentic their living conditions within a laboratory can be. Due to these factors, honey bees in our experiments are lacking proper nutrition, forms of social contact, many types of hive-related tasks and flight. Nutrition and social contact have direct impacts on health, as honey bees require certain foods for growth and development, and social contacts allow the transmission of gut bacteria which have been shown to be involved in immunocompetence (Haydak, 1970; Powell et al., 2014).

The inability to perform certain behaviours under experimental conditions may influence temperament, causing bees to become agitated and stressed. Studies have found that when colonies are under stress individuals may respond through precocious foraging and therefore accelerate through the temporal caste structure, which is usually age-related (Perry et al., 2015; Woyciechowski & Moroń, 2009; Winston, 1987). It is possible that stress caused by an experimental set-up could induce a change in behaviour, such as a drive to forage, which ultimately the bee cannot carry out. However, how this affects honey bee physiology is not known.

The independence of samples must also be considered. There are many ways in which bees may be affected by the group they are housed with. In pathogen research, we may see increased or decreased transmission within different replicates, based on the number of individuals that were successfully inoculated initially. This may lead to variations in the number of bees dying between pots. In our experiments, bees which displayed symptoms or died were removed from pots. This meant that the number of bees remaining varied by pot and was dependent on the conditions of individuals within the pot. Studies have shown that the physiology of bees is affected by group size and that bees kept in small groups experience a decrease in fat body mass (Jones et al., 2018). In experiments which involve measuring survival and that last until all bees are dead, a decline in the number of bees over time would likely be observed. The deaths may be accelerated in pots where only a few bees remain. This can be examined in the Schoenfeld residual plots which investigated the change in hazards through time. Cox models assume that hazards are proportional and stay constant with time, however, in most cases they were not constant in these experiments. The Schoenfeld residuals demonstrated that hazards did change with time in many of the survival experiments (Chapter 2, Section 2.3.2.2 & Chapter 3, Section 3.3.4.1). These results allow the risk of each treatment for survival to be quantified over time and reveal when the risk is most apparent. The biological processes that led to a change in survival and the failure of the proportionality assumptions are relatively easy to explain. The Cox model was developed to investigate differences in survival and has been most frequently used in medical settings where each individual modelled is considered independent of the others in the trial (Faradmal et al., 2012). In a drug trial, one might anticipate that the drug would simply alter the hazard of dying for each individual as an independent entity of life (i.e. subject to an average population level of risk) and that this would be reflected in the gradients of the Cox proportional hazards

model. In this example, the hazard would ideally be lower under the drug treatment, with fewer individuals dying per unit time. In an infectious disease scenario, however, once transmission has taken place, the risk of ongoing transmission to other uninfected bees has to rise as there are more opportunities for transmission between infectious and susceptible individuals; thus, the risk should increase as time passes. We might therefore hypothesise that the proportionality assumption has been violated and we would expect to see increases in the hazards in a disease treatment as time passes. This is not something that was typically observed in our results, as generally we saw an increase or decrease in the hazard within the first 10 days of the experiment, and then a stabilisation where the hazard remained fairly constant. This, however, was affected by the length of the experiment or period that was being tested; a shorter experiment may not yield these results, as it would end before further transmission could take place. Furthermore, in all of our experiments the majority of deaths took place at the beginning of the experiment, particularly in truncated experiments, meaning the hazard associated with death was often greater in this period of the experiment. Other risk factors are also not considered in Cox models, and if present, these could influence the hazard of death at the same time as the risk of interest (in this case the CBPV infection), leading to a competing risks scenario. A bee can only die once, and it is important to identify the cause of death. Furthermore, there is no reason why the competing risk should have the same hazard at the same point during the exposures to each of the multiple risks.

To overcome some of these issues, we explored a range of analytical techniques. Firstly, where possible bees were tested for CBPV using RT-qPCR to confirm that symptoms displayed and deaths were attributable to the disease rather than the effects of the experimental design. This allowed the use of cause-specific hazards and competing risks analysis, rather than all-cause hazards to analyse the event of interest, ensuring that the events investigated were caused by CBPV infection. Through these analyses and threshold models, experiments were shortened to time periods where the effects of CBPV would likely be present, rather than analysing experiments from their set-up until all bees were dead, which, when investigating diseases which cause rapid onset of symptoms and death, is not particularly useful. Each pot was considered as one experimental unit and as many replicates were used as possible.

Differences in the infectivity of CBPV between experiments were identified, firstly in Chapter 3, where we saw an increase in the number of infected bees which were exposed

to CBPV at 24 hours after emergence compared to those of the same treatment in Chapter 2. We also saw a difference in susceptibility in Chapter 4, where the initial experiment identified a difference in the number of symptomatic bees between those that had access to beebread and those that did not, however this was not observed in the second experiment in that chapter. There are a few possible explanations for differences in susceptibility between experiments. Firstly, we believe that the time of year the experiment took place has important impacts on the susceptibility of honey bees to disease and other risk factors. Studies have shown that bees which emerge in late August show increased longevity and therefore are considered to be winter bees. Winter bees are different physiologically to summer bees in that they have enlarged fat bodies and hypopharyngeal glands, which likely impacts their susceptibility and longevity (Fluri & Bogdanov, 1987; Fluri et al., 1982). Therefore, experiments which take place in the late summer will likely yield different results to those held in the early summer. This also aligns with common observations of CBP taking place in spring and summer months (Ribiere et al., 2010). At a technical level, we also may see differences in infectivity of virus solutions used in the experiments. These were made in the same way each year and stored at -20 °C, but length of storage and the viral load of bees used to create the crude extract may have impacted its effectiveness as a source of an infectious dose.

CBP is often characterised as causing mass die-off events where whole colonies collapse. As these laboratory results suggest that only newly emerged bees are susceptible to CBPV, the mechanisms by which colony collapse could take place from this source of infection need to be considered. Is it likely that colony collapse could arise from transmission that only occurs in the one day period after adult bees emerge? Historical data from BeeBase were used to establish how brood and adult bee populations vary in an average colony through the year. Based on these data, brood production is at its highest at approximately week 19, which is early to mid-May (Figure 6.1). Both bee and brood numbers decrease after this, which may be due to swarming as well as population declines. At this point, there is an average of 30,000 brood, meaning that over a three week period (the time taken for a honey bee to develop from an egg) we can expect 30,000 bees to emerge. This would equal approximately 1,428 bees emerging each day during May. This aligns with figures in the literature, which state that queen bees can lay between 1,000 and 3,300 eggs per day (Avni et al., 2014; Khoury et al., 2013). Although some of these will be subject to mortality during development, egg mortality is considered to be at around 10%, and

therefore has a small effect on the overall number of emerging bees per day (Winston, 1987). Figure 6.1 shows that honey bee colonies often contain 20,000 bees on average, but this figure can be as high as 60,000, meaning newly emerged bees below one day old can make up between 1.7% and 16.5% of the colony (based on the lowest number of emerging brood in the largest colony, and the highest number of emerging brood in the smallest colony) depending on its size and brood production at that time. As newly emerged bees take on nursing roles within the first few days after emergence, it is clear how CBPV may be passed through continually emerging bees (Winston, 1987). The number of observed paralysis cases in colonies increases overall until week 23, where it stabilises before decreasing towards the end of the year. This aligns with the trend in population size and brood production, which steadily increases until week 19, indicating a possible relationship between brood production and CBP occurrence. The difference between the amount of brood and the number of bees was used as a predictor of risk of CBPV infection. This was compared to the fitted values for the proportion of inspected colonies with CBP per week using a general linear model (GLM) with a Gaussian distribution and an identity link function in R version 4.2.2 (RCoreTeam, 2022). There was a significant relationship between high amounts of brood and CBP infection, when brood amount was lagged by three weeks (t-value = 2.538, p-value = 0.017). This time-frame accounts for the development period of brood, which takes three weeks from egg laying to emergence, and the length of time it takes for bees to become exposed to CBPV after emergence and become symptomatic and die.

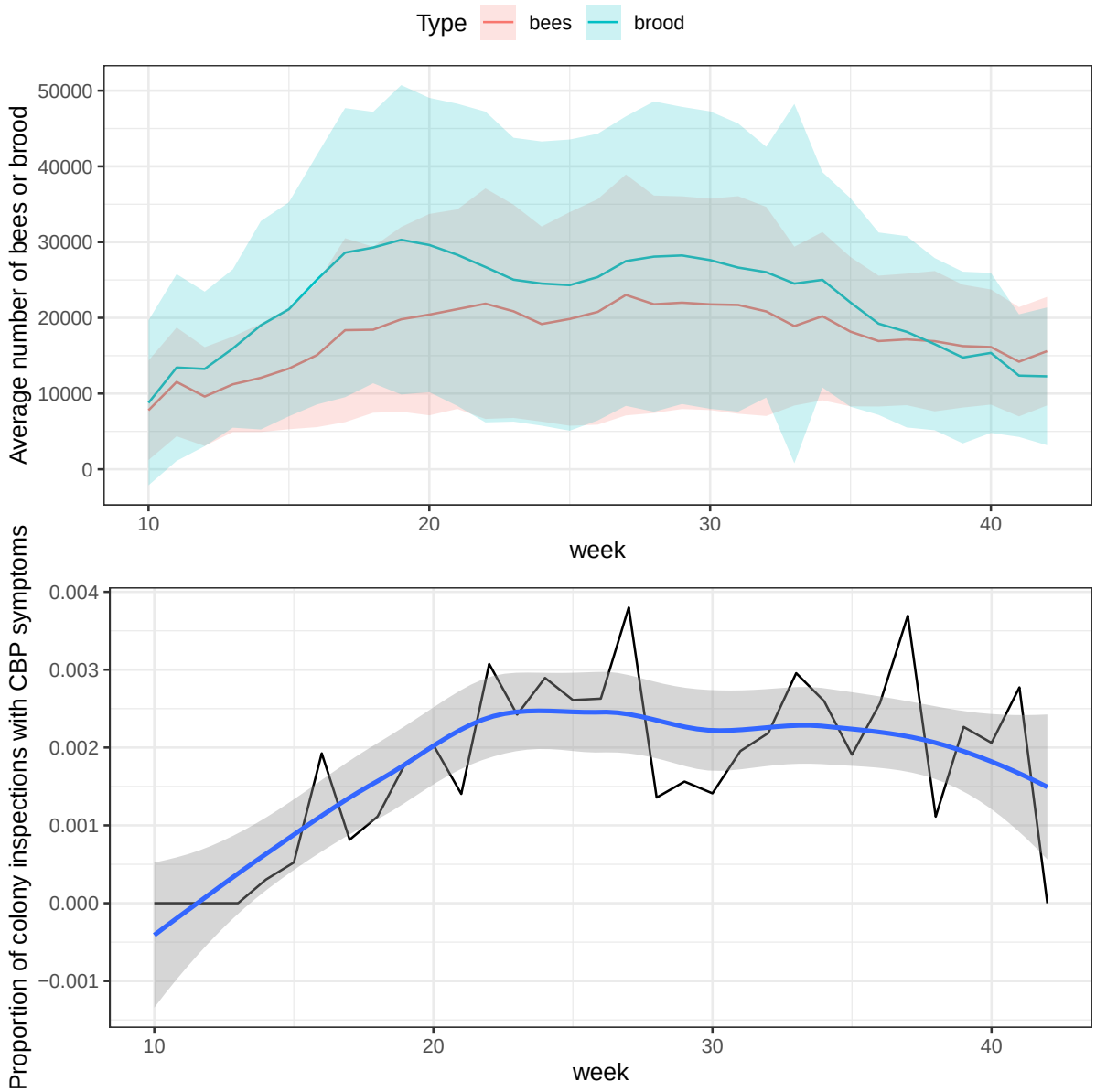


Figure 6.1: (Top) The average number of bees and brood in a colony per week of the year based on colony inspection data from BeeBase. The shaded areas are the standard deviations. Brood is defined as eggs, larvae or capped brood of any developmental stage. (Bottom) The number of cases of Chronic bee paralysis per week of the year from colony inspection data from BeeBase. The blue line shows the fitted values from a linear regression and the grey areas are the confidence intervals for the proportion of colonies with CBP.

6.2 Other modes of tranmission

These results suggest that the oral susceptibility of newly emerged bees to CBP in part explains the mass die-off seen in colonies infected with CBPV, but that other transmission methods may exist that affect older bees. There are other modes of transmission which have not been possible to explore in this thesis, which require attention in future studies. It is believed that the virus can be transmitted when friction between bees causes the removal of hairs on the cuticle. Contact with an infected bee or forms of grooming may then allow the virus to enter through the cuticle and infect the bee. Tests by Bailey et al. (1983) found that transmission between bees injected with CBPV and healthy bees was increased in pots with higher densities of bees, and found that topical application of CBPV on bees which had been shaved led to infection. Additionally, CBPV may be transmitted vertically by queens and has been identified in eggs and larvae (Blanchard et al., 2007; Chen, Pettis, et al., 2006). Faeces from infected bees and infected corpses may be reservoirs for the virus. Honey bees clean faeces from inside the hive and also remove corpses, and may become infected through these contacts. CBPV has been detected in the faeces of infected bees, and healthy bees have become infected through contact with contaminated faeces under laboratory conditions (Ribiere et al., 2007). Additionally, bees which have died from CBP have high quantities of virus particles in their bodies after death (Bailey & Milne, 1969; Blanchard et al., 2007). These results highlight the possibility that bees may be susceptible to infection based on the method of transmission or condition of the queen, and therefore, our results that younger bees are more susceptible to CBPV only tell part of the story as to how whole colonies can collapse from the disease. However, oral transmission may lead to other transmission pathways. Symptomatic and dead bees are removed by undertaker bees, which carry them out of the hive entrance. The piles of dead bees which are found outside hives due to CBPV infections are often completely hairless, which may be directly caused by infection, friction between bees, or hairs are chewed off during removal. These hairs are likely then ingested by the undertaker bees and may cause infection. Experiments by Rinderer & Rothenbuhler (1975) tested how the ingestion of hairs alters oral susceptibility, and found that bees which consumed hairs had a higher mortality rate, likely due to perforation of the gut.

The analyses in this project then shifted in focus to a larger scale in Chapter 5, and concentrated on the potential role of environmental and management factors in disease. The environmental conditions a hive experiences will likely affect the health of the colony

and therefore its susceptibility to disease. Factors such as weather, food availability and density have direct impacts on health and disease spread. The likelihood of disease being identified by beekeepers must also be considered, as well as their level of expertise and the number of colonies they are responsible for. Additionally, the fact that CBP is an emerging disease with a sporadic spread requires consideration, as transmission of disease may be mainly based on contact between infected individuals or materials, making external factors impacting susceptibility hard to identify. The results of the spatial statistical analyses suggested that CBP occurrence is associated with periods of low humidity and requested inspections which were performed by bee inspectors with less experience. This suggests that disease spread can be impacted by weather, and weather may have direct and indirect effects on colonies, such as pollen availability and accessibility. Cases were also spatially autocorrelated, indicating that areas with CBP are more likely to have more infections. This suggests that CBPV can be transmitted between hives, which may take place through a number of mechanisms. Bees from different colonies may spread disease through the use of the same floral resources during foraging, either through direct interactions with other bees or fomites on the flowers themselves (Koch et al., 2017). Bees can also partake in robbing behaviour and enter other colonies to steal food resources (Willingham et al., 2000). Intruders are often attacked by guard bees, which gives rise to the possibility of pathogen transmission between them (Nouvian et al., 2016). Bees from different hives also interact through mating, where a queen from one hive will go on flights to mate with drones from other hives, an interaction which may lead to transmission (Chen, Evans, et al., 2006). Additionally, pathogens may be spread between hives by beekeepers using the same tools and equipment on multiple hives, which may be fomites of disease (Owen, 2017).

6.3 CBP Mitigations

As the results from Chapters 2 and 3 indicate a heightened susceptibility to CBPV in newly emerged bees, it seems particularly important to prevent emerging bees from coming into contact with infected individuals. This could be done by placing capped brood in a separate brood box equipped with efficient food stores and allowing brood to emerge there. Once approximately 24-48 hours old, an age where we know bees are less susceptible to the oral exposure of CBPV, the bees could be added back to the hive and allowed to

mix with older bees. Ideally, this would prevent infections and deaths of lots of young bees. We also know that one of the first roles bees take on after emergence is nursing, which involves feeding emerging bees (Winston, 1987). If nurse bees are infected with CBPV, we can hypothesise that it is likely they would transmit the virus to emerging bees through engaging in trophallaxis with them. Avoiding this interaction could prevent a cascading effect of infections between newly emerged bees moving into nursing roles. This mitigation strategy would be relatively straightforward for amateur beekeepers with a few colonies to implement, however, for professional beekeepers with over 40 colonies, it would be very time-consuming. Additionally, pollen supplementation for colonies during times of peak reproduction or poor weather could improve the health of the colony overall and reduce susceptibility to disease.

6.4 Conclusions

The results of this thesis suggest that age is a key factor in oral susceptibility to CBP, and that this susceptibility may be reduced with sufficient access to pollen. This work highlights the necessity of research focusing on other modes of transmission to understand fully how hives can collapse rapidly from CBPV infection. This would enable informed mitigation strategies to be recommended to beekeepers to further prevent transmission and risk of disease. Additionally, the limitations of pot studies highlighted in this thesis indicate a need for other approaches to investigating transmission in honey bees. Our analyses investigating the spread of CBPV throughout Southern England supported the evidence that CBPV is an emerging disease, with its prevalence increasing over time. Other factors including humidity, inspections and spatial factors also impacted the prevalence of disease. There is a clear need for further research which focuses on the role of other putative mechanisms impacting disease transmission. The complexity of bee sociality and bee life histories suggest that this might be difficult technically, however, identifying this need is a step towards finding a solution.

Appendix A.

Chapter 2: Normality test for the zero-inflated GLMM shown in Table 2.2

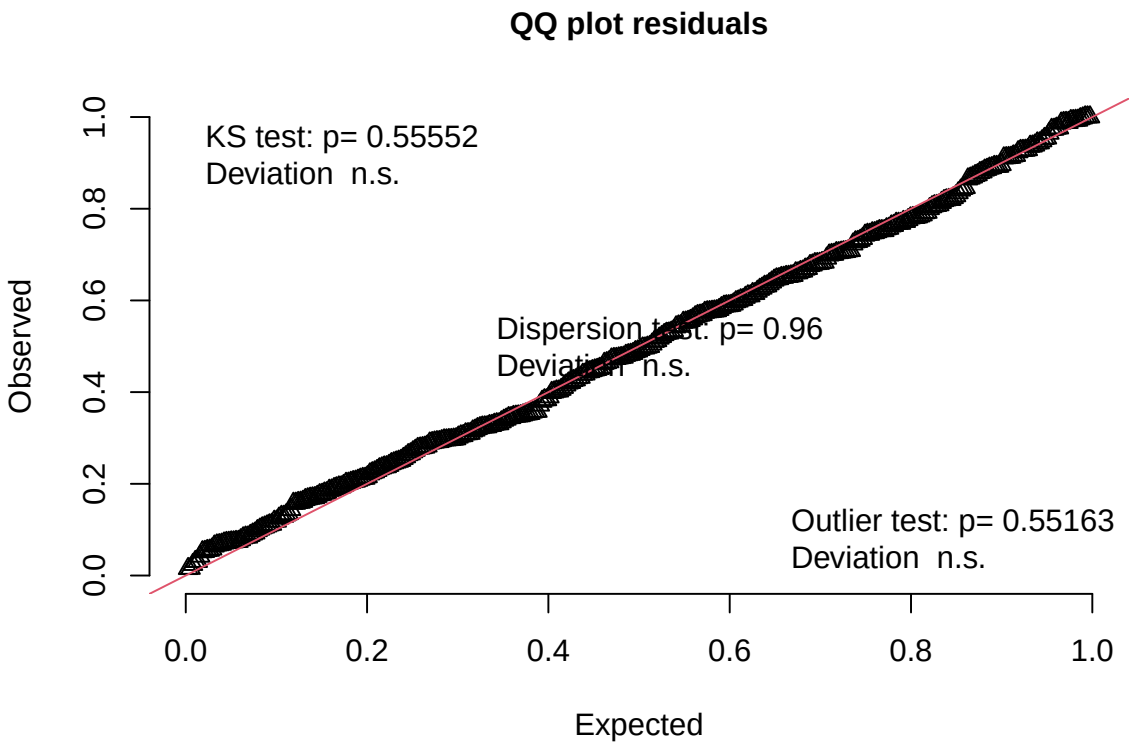


Figure 6.2: Normality tests for the zero-inflated GLMM investigating the effect of seeding and exposure to Chronic bee paralysis virus (CBPV) on the log base 10 concentration of CBPV.

Appendix B.

Chapter 3: Threshold models

The output of the NLS model was used to predict mortality at day 9 (Table 6.1). Based on the prediction model, 95% of bees were still alive at day 9.

Table 6.1: The output of the non-linear least squares model of the survival of susceptible bees which were fed buffer.

Type	Estimate	Std. Error	t value	p-value
Asymptote	210.111	3.758	55.913	0
x value at curve midpoint	12.245	0.172	71.377	0
Scale	-1.676	0.146	-11.452	0

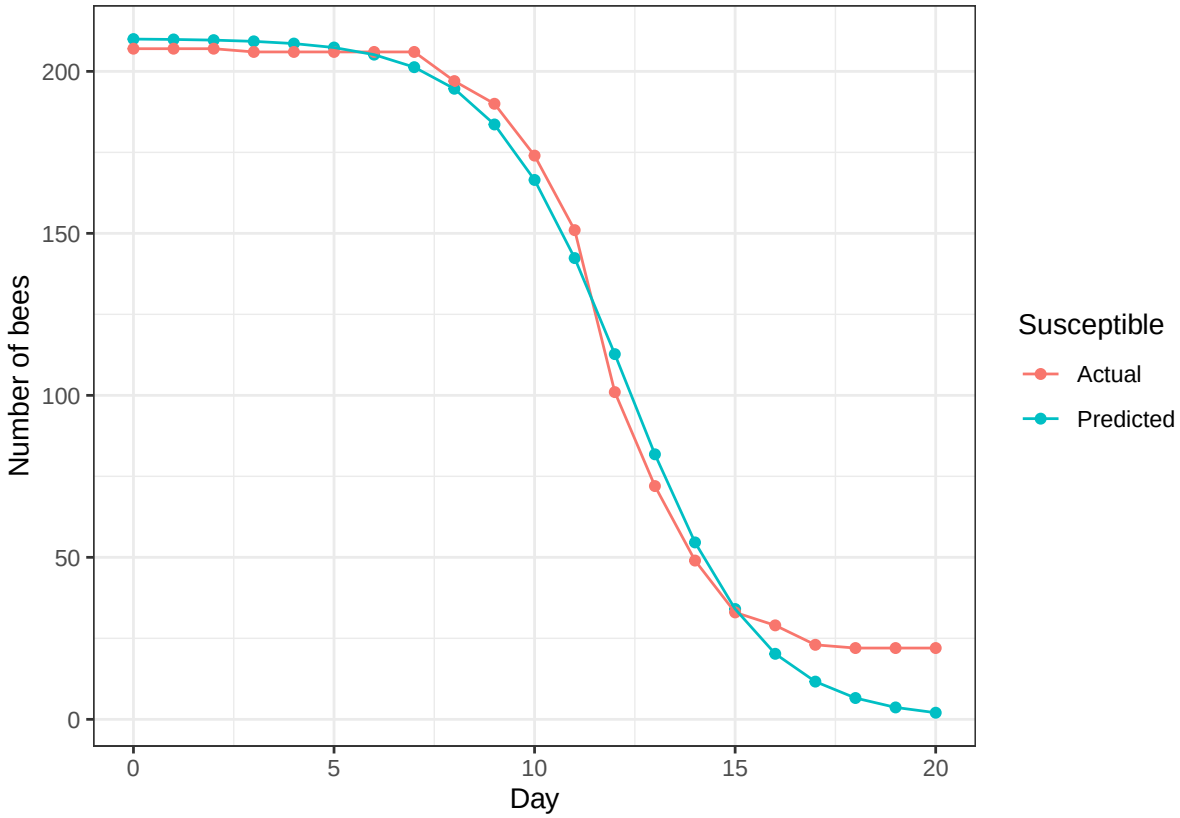


Figure 6.3: The survival of all bees in buffer-fed treatment for the duration of the experiment.

Appendix C.

Chapter 3: Generalised linear models

Table 6.2: The results from a generalised linear model with a binomial distribution investigating the relationship between a positive CBPV RT-qPCR result with the virus treatment, the age of the bee at time of exposure, the type of eclosure, whether a bee was symptomatic or healthy at time of removal, and the concentration of 16s, F. perrara, G. apicola, Lactobacillus or S. alvi from the community of these bacteria

Response	Variable	z-value	p-value
CBPV Result	Natural emergence	-2.019	0.043
CBPV Result	Virus	4.060	0.000
CBPV Result	Age	-3.016	0.003
CBPV Result	Removed	-5.141	0.000
CBPV Result	16s	-2.636	0.008
CBPV Result	F. perrara	-0.013	0.990
CBPV Result	G. apicola	-0.047	0.963
CBPV Result	S. alvi	1.352	0.177
CBPV Result	Lactobacillus	-2.137	0.033

Table 6.3: The results from a generalised linear model with a gaussian distribution investigating the relationship between all variables, including; qPCR result with the virus treatment, the age of the bee at time of exposure, the type of eclosure, whether a bee was symptomatic or healthy at time of removal, and the concentration of bacterial 16s, F. perrara, G. apicola, Lactobacillus or S. alvi from the community of these bacteria.

Response	Variable	t value	p-value
16s	Natural emergence	14.263	0.000
16s	Virus	-1.118	0.265
16s	Age	5.321	0.000
16s	Removed	3.112	0.002
F. perrara	Natural emergence	1.887	0.061
F. perrara	Virus	-2.255	0.025
F. perrara	Age	1.892	0.060
F. perrara	Removed	1.476	0.142
G. apicola	Natural emergence	2.005	0.046
G. apicola	Virus	-1.019	0.309
G. apicola	Age	1.121	0.264
G. apicola	Removed	0.279	0.781
S. alvi	Natural emergence	0.904	0.367
S. alvi	Virus	0.843	0.400

Response	Variable	t value	p-value
S. alvi	Age	0.249	0.804
S. alvi	Removed	-0.839	0.402
Lactobacillus	Natural emergence	8.610	0.000
Lactobacillus	Virus	-1.167	0.245
Lactobacillus	Age	1.663	0.098
Lactobacillus	Removed	4.957	0.000

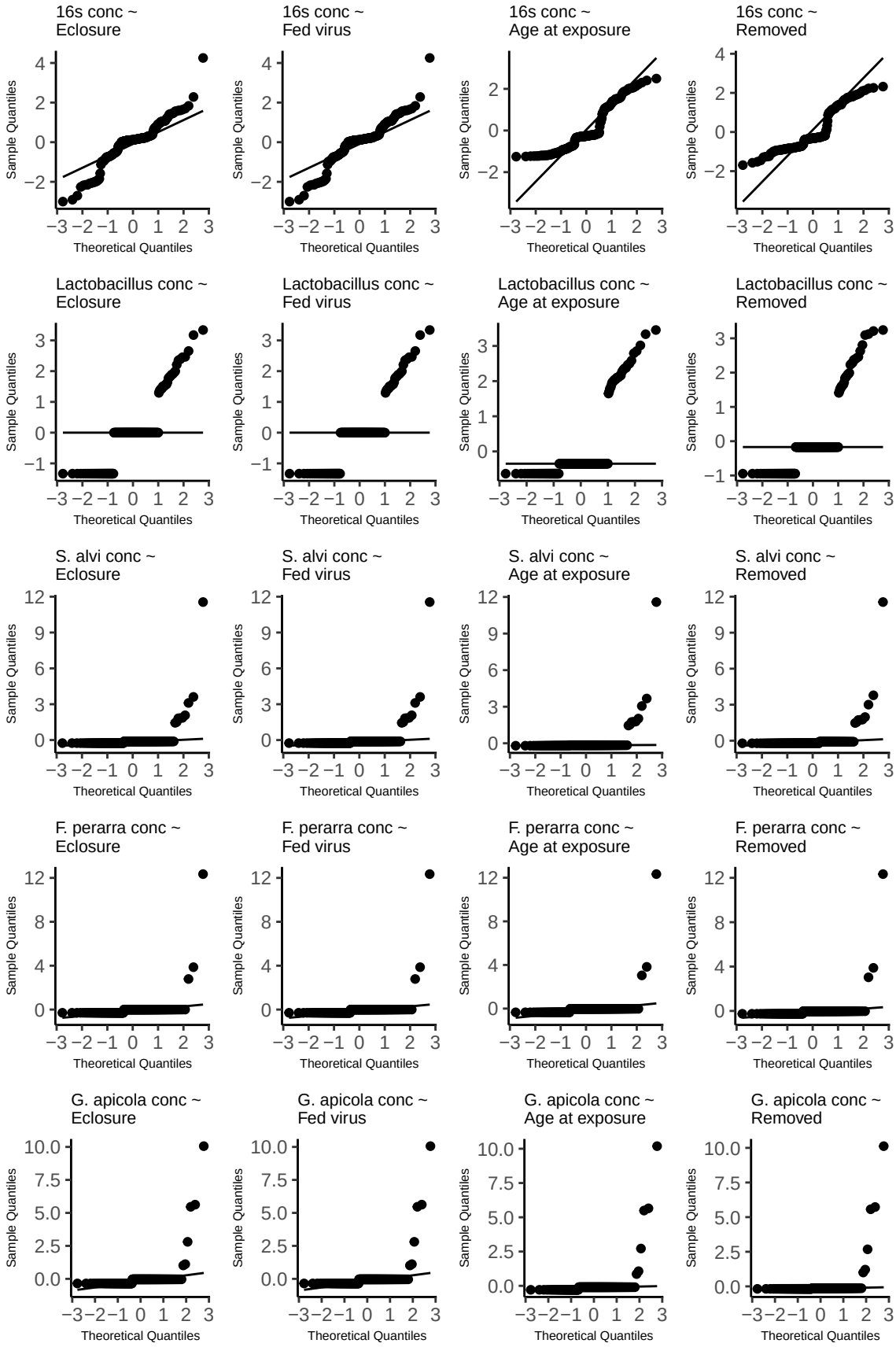


Figure 6.4: Normality assessments for the generalised linear models with a gaussian distribution investigating the relationship between all variables, including; qPCR result with the virus treatment, the age of the bee at time of exposure, the type of eclosure, whether a bee was symptomatic or healthy at time of removal, and the concentration of bacterial 16s, F. perrara, G. apicola, Lactobacillus or S. alvi from the community of these bacteria.

Appendix D.

Chapter 3: Structural equation models

Table 6.4: The output of the Structural equation model investigating the relationship between CBPV RT-qPCR concentration and F. perrara RT-qPCR concentration.

Response	Predictor	Estimate	Standard error	z-value	p-value	Standardised observed and latent
cbpv	Eclosure	0.038	0.056	0.677	0.499	0.037
cbpv	Virus	0.230	0.056	4.113	0.000	0.216
cbpv	Age	-0.166	0.064	-2.589	0.010	-0.152
cbpv	Removed	-0.483	0.061	-7.955	0.000	-0.488
cbpv	F. perrara	0.010	0.047	0.213	0.831	0.022
F. perrara	Eclosure	-0.213	0.132	-1.605	0.108	-0.094
F. perrara	Virus	-0.339	0.211	-1.609	0.108	-0.141
F. perrara	Age	0.244	0.232	1.050	0.294	0.098
F. perrara	Removed	0.005	0.125	0.038	0.970	0.002

Table 6.5: The output of the Structural equation model investigating the relationship between CBPV RT-qPCR concentration and G. apicola RT-qPCR concentration.

Response	Predictor	Estimate	Standard error	z-value	p-value	Standard observed and latent
cbpv	Eclosure	0.042	0.057	0.731	0.465	0.042
cbpv	Virus	0.230	0.054	4.213	0.000	0.216
cbpv	Age	-0.166	0.064	-2.611	0.009	-0.152
cbpv	Removed	-0.483	0.057	-8.476	0.000	-0.487
cbpv	G. apicola	0.018	0.055	0.327	0.744	0.050
G. apicola	Eclosure	-0.363	0.287	-1.263	0.206	-0.130
G. apicola	Virus	-0.175	0.182	-0.957	0.339	-0.059
G. apicola	Age	0.130	0.247	0.528	0.598	0.043
G. apicola	Removed	-0.031	0.140	-0.222	0.824	-0.011

Table 6.6: The output of the Structural equation model investigating the relationship between CBPV RT-qPCR concentration and S. alvi RT-qPCR concentration.

Response	Predictor	Estimate	Standard error	z-value	p-value	Standard observed and latent
cbpv	Eclosure	0.042	0.059	0.704	0.481	0.041
cbpv	Virus	0.223	0.057	3.944	0.000	0.210
cbpv	Age	-0.165	0.061	-2.704	0.007	-0.150
cbpv	Removed	-0.478	0.060	-7.959	0.000	-0.483
cbpv	S. alvi	0.031	0.030	1.058	0.290	0.080
S. alvi	Eclosure	-0.198	0.289	-0.684	0.494	-0.077
S. alvi	Virus	0.109	0.120	0.905	0.365	0.040
S. alvi	Age	0.025	0.219	0.114	0.909	0.009
S. alvi	Removed	-0.165	0.141	-1.167	0.243	-0.065

Table 6.7: The output of the Structural equation model investigating the relationship between CBPV RT-qPCR concentration and Lactobacillus RT-qPCR concentration.

Response	Predictor	Estimate	Standard error	z-value	p-value	Standard observed and latent
cbpv	Eclosure	0.067	0.076	0.881	0.379	0.066
cbpv	Virus	0.216	0.056	3.852	0.000	0.203
cbpv	Age	-0.162	0.062	-2.595	0.009	-0.148
cbpv	Removed	-0.503	0.064	-7.806	0.000	-0.507
cbpv	Lactobacillus	0.008	0.010	0.888	0.375	0.057
Lactobacillus	Eclosure	-3.697	0.508	-7.271	0.000	-0.544
Lactobacillus	Virus	1.200	0.528	2.275	0.023	0.167
Lactobacillus	Age	-0.272	0.558	-0.488	0.625	-0.037
Lactobacillus	Removed	2.272	0.456	4.979	0.000	0.339

Table 6.8: The output of the Structural equation model investigating the relationship between CBPV RT-qPCR concentration and 16s RT-qPCR concentration.

Response	Predictor	Estimate	Standard error	z-value	p-value	Standard observed and latent
cbpv	Eclosure	-0.001	0.086	-0.010	0.992	-0.001
cbpv	Virus	0.232	0.058	3.994	0.000	0.219
cbpv	Age	-0.153	0.071	-2.146	0.032	-0.140
cbpv	Removed	-0.476	0.063	-7.536	0.000	-0.481
cbpv	16s	-0.008	0.014	-0.601	0.548	-0.053
16s	Eclosure	-4.361	0.376	-11.603	0.000	-0.682
16s	Virus	0.732	0.422	1.733	0.083	0.108
16s	Age	1.290	0.416	3.104	0.002	0.185
16s	Removed	0.873	0.381	2.291	0.022	0.139

Appendix E.

Chapter 3: Normality assessment for day of death/symptoms GLM

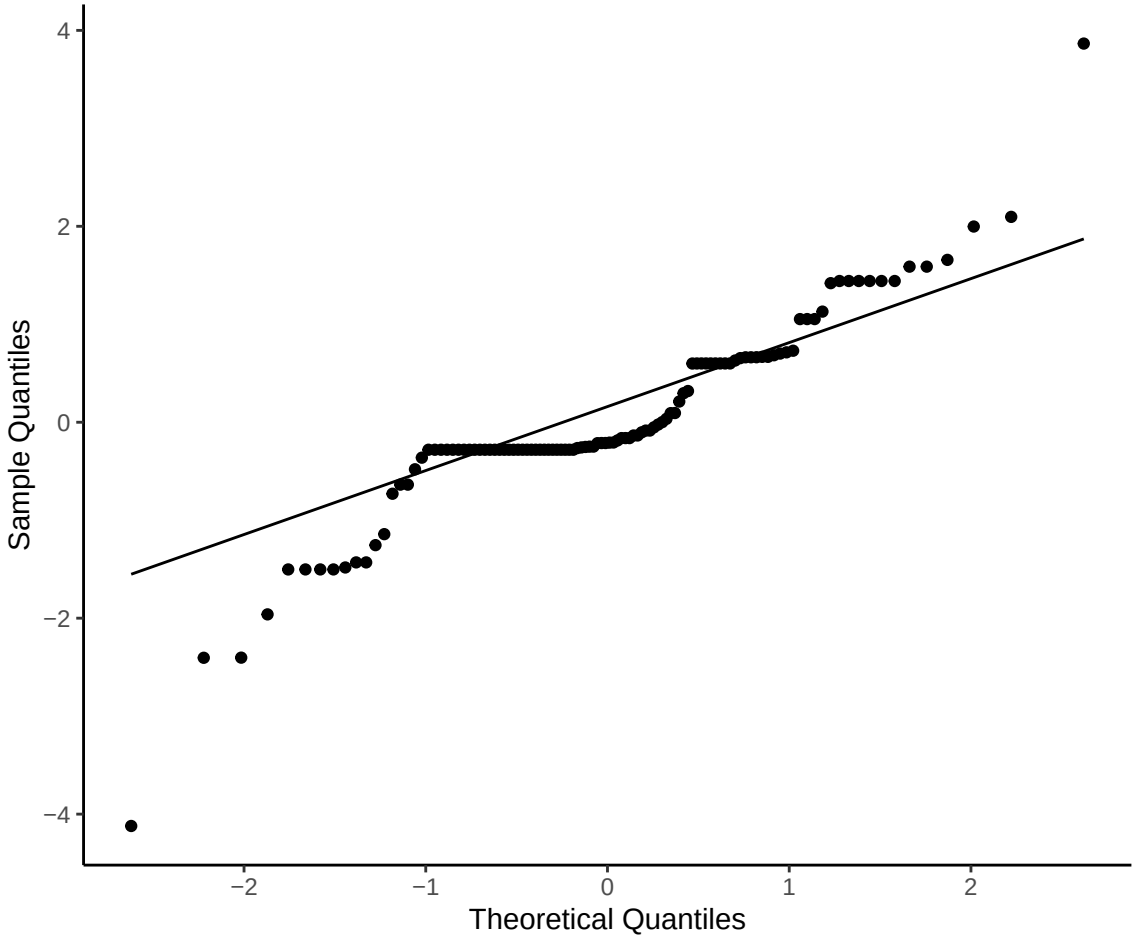


Figure 6.5: The distribution of residuals from the model exploring the relationship between treatments, each bacteria type (*F. perrara*, *G. apicola*, *S. alvi* and *Lactobacillus*) and 16s concentration with the day of removal from the experiment.

Appendix F.

Chapter 5: Vegetation categories

Table 6.9: The categories and the scores they represent for each correspondance analysis axis.

Vegetation Axis	Category	Minimum Axis Score	Maximum Axis Score
1	1		-3
	2	-3	-2
	3	-2	-1
	4	-1	0
	5	0	1
	6	1	2
	7	2	
2	1		-2
	2	-2	-1
	3	-1	0
	4	0	1
	5	1	2
	6	2	3

Vegetation Axis	Category	Minimum Axis Score	Maximum Axis Score
	7	3	4
	8	4	

Appendix G.

Chapter 5: Full R-INLA model outputs

Table 6.10: The results of the R-INLA model for CBPV with all covariates prior to the removal of non-significant covariates and improvement of the model using DIC values. Significant covariates are those where the confidence interval does not include zero.

Covariate	mean	sd	2.5%	97.5%
Intercept	-28.778	13.565	-58.192	-5.060
Humidity	-11.503	4.124	-19.655	-3.470
Sun	-0.662	0.466	-1.590	0.239
Rain	0.602	0.511	-0.406	1.598
Temperature	-11.000	12.465	-35.538	13.386
Wind	5.885	14.452	-22.856	33.864
Professional beekeeper	-0.291	0.901	-2.203	1.331
Inspector experience	-0.047	0.022	-0.093	-0.005
Density	0.010	0.012	-0.016	0.031
Continuous time	0.008	0.013	-0.017	0.033
Sine	-0.516	0.615	-1.740	0.673

Covariate	mean	sd	2.5%	97.5%
Cosine	-1.468	0.737	-2.938	-0.046
No. colonies in apiary	0.001	0.003	-0.006	0.006
No. colonies owned	-0.003	0.007	-0.016	0.009
Vegetation Axis 1 Cat. 1	8.704	11.420	-13.456	31.365
Vegetation Axis 1 Cat. 2	6.921	11.390	-15.178	29.528
Vegetation Axis 1 Cat. 3	6.787	11.379	-15.288	29.373
Vegetation Axis 1 Cat. 4	7.588	11.373	-14.475	30.164
Vegetation Axis 1 Cat. 5	7.284	11.373	-14.779	29.860
Vegetation Axis 1 Cat. 6	7.506	11.374	-14.559	30.084
Vegetation Axis 1 Cat. 7	-39.669	16.635	-78.479	-14.946
Vegetation Axis 2 Cat. 2	15.697	7.624	4.333	33.470
Vegetation Axis 2 Cat. 3	15.635	7.622	4.274	33.403
Vegetation Axis 2 Cat. 4	15.263	7.623	3.901	33.032
Vegetation Axis 2 Cat. 5	15.312	7.632	3.933	33.103
Vegetation Axis 2 Cat. 6	14.286	7.681	2.818	32.182
Vegetation Axis 2 Cat. 7	-5.307	18.141	-48.055	20.578
Vegetation Axis 2 Cat. 8	-5.261	18.582	-49.060	21.222

Table 6.11: The results of the R-INLA model for CBPV with all signifcant covariates prior to the addition of the spatial field. Significant covariates are those where the confidence interval does not include zero.

Covariate	mean	sd	2.5%	97.5%
Intercept	1.833	1.639	-1.383	5.048
Humidity	-5.411	2.142	-9.634	-1.226
Inspector experience	-0.042	0.021	-0.086	-0.002

Table 6.12: The results of the spatial R-INLA model for CBPV with all covariates that were significant in the prior models. Significant covariates are those where the confidence interval does not include zero.

Covariate	mean	sd	2.5%	97.5%
Intercept	0.222	6.352	-14.635	13.707
Inspector experience	-0.044	0.022	-0.088	-0.002
Humidity	-3.504	2.711	-8.703	1.857

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