Investigating factors that influence chalkbrood outbreaks in Australia

by John Roberts and Joel Armstrong
April 2021
Investigating factors that influence chalkbrood outbreaks in Australia

by John Roberts and Joel Armstrong

April 2021

AgriFutures Australia Publication No. 21-034
AgriFutures Australia Project No. PRJ-010815
Foreword

The European honey bee (*Apis mellifera*) is one of the most important insects in Australia because it produces honey and provides pollination services. Pollination alone is worth more than $14 billion to the Australian economy and requires about 530,000 hives each year. Unfortunately, honeybees, like other animals used in agriculture, are threatened by several diseases and pathogens. These diseases and pathogens not only impact the hives and the bees, but affect the ability of the honey bee industry to meet pollination needs. The importance of maintaining healthy hives for pollination and honey cannot be underestimated.

Chalkbrood is a brood disease of the honey bee caused by the fungal pathogen *Ascosphaera apis*. It is considered a stress-related disease, with multiple interacting genetic and environmental factors influencing the severity of outbreaks. Chalkbrood disease has become a growing problem for many Australian beekeepers in recent years, but it is unclear what factors are driving current outbreaks.

This report describes the findings of a two-year study into a variety of factors potentially influencing outbreaks of chalkbrood disease in Australian honey bee colonies. This research builds on recent AgriFutures Australia research into this disease and queen mating quality in Australia, and provides important new insights into key influencing factors that can be further investigated and acted upon by industry to reduce the prevalence and severity of chalkbrood disease.

The study found that chalkbrood-infected colonies had lower genetic diversity, measured in queen mating levels, and had lower amounts of stored pollen. Research also showed that some strains of chalkbrood may have higher virulence. These results suggest that increasing queen mating levels and promoting high pollen levels in spring may be effective strategies to reduce the incidence and severity of chalkbrood infection. Breeding for greater larval resistance to chalkbrood with consideration of different strain virulence could also be a valuable complementary strategy for industry.

This report for the AgriFutures Honey Bee & Pollination Program adds to AgriFutures Australia’s diverse range of research publications. It forms part of our Growing Profitability Arena, which aims to enhance the profitability and sustainability of our levied rural industries.

Most of AgriFutures Australia’s publications are available for viewing, free downloading or purchasing online at www.agrifutures.com.au.

**John Smith**  
General Manager, Research  
AgriFutures Australia
Acknowledgments

The authors greatly appreciated the assistance of commercial apiarists James Kershaw, Neil Bingley and Lee Nagle for access to their colonies for sampling. Joel Armstrong (CSIRO) provided great assistance with field work, analysis of bee samples and culturing of chalkbrood isolates. Liz Milla (CSIRO) conducted the pollen metabarcoding analysis. Matthew Morgan and Amy Paten (CSIRO) gave helpful guidance on larval transcriptome and 16S gut microbiome analyses. Jody Gerds (Bee Scientifics) also provided this study with valuable knowledge and insights on chalkbrood disease through her experience with AgriFutures Australia project PRJ-009904.
## Contents

Foreword ............................................................................................................................................... iii
Acknowledgments ................................................................................................................................ iv
Executive summary ............................................................................................................................. vii
Introduction ........................................................................................................................................... 1

### Objectives ........................................................................................................................................... 3

Methodology .......................................................................................................................................... 4

Study design and sampling ................................................................................................................. 4

Genetic factors .................................................................................................................................. 6
  Colony genetic diversity using microsatellite markers ................................................................. 6
  RNA high-throughput sequencing for immune gene analysis ....................................................... 6
  Chalkbrood strain analysis .................................................................................................................. 6

Environmental factors ....................................................................................................................... 7
  Colony resources ................................................................................................................................. 7
  Pollen analyses for species diversity and chalkbrood spore contamination ................................. 8
  Gut microbiome analysis ..................................................................................................................... 8
  Nosema spore levels ............................................................................................................................. 8

Comparison of chalkbrood strain virulence ....................................................................................... 9

### Results ............................................................................................................................................. 10

Genetic factors .................................................................................................................................. 10
  Colony genetic diversity using microsatellite markers ................................................................. 10
  RNA high-throughput sequencing for immune gene analysis ....................................................... 11
  Chalkbrood strain analysis .................................................................................................................. 12

Environmental factors ....................................................................................................................... 13
  Colony resources ................................................................................................................................. 13
  Pollen diversity analysis ...................................................................................................................... 14
  Chalkbrood spore contamination ....................................................................................................... 16
  Gut microbiome analysis ..................................................................................................................... 16
  Nosema spore levels ............................................................................................................................. 18

Multiple logistic regression for chalkbrood factors ......................................................................... 18

Comparison of chalkbrood strain virulence ....................................................................................... 19

### Implications ..................................................................................................................................... 21

### Recommendations ....................................................................................................................... 22

### References ..................................................................................................................................... 23
Tables

Table 1. Microsatellite markers used for genotyping colony workers.................................................... 6
Table 2. Variables included in the multiple logistic regression model for infection status ................. 19
Table 3. Variables included in the multiple logistic regression model for strain virulence................. 20

Figures

Figure 1. Samples were collected from chalkbrood-infected and healthy colonies across five NSW commercial beekeeper apiaries. ................................................................. 4
Figure 2. Colony experiencing a chalkbrood outbreak with infected larvae (mummies) expelled..... 5
Figure 3. Mycelial growth of Ascosphaera apis cultured from infected larvae............................... 7
Figure 4. Frames from the brood box were photographed and area of brood, honey and pollen were estimated using Fiji image analysis software................................................................. 7
Figure 5. Inoculated larvae were uncapped and assessed for infection after five days in the lab...... 9
Figure 6. Total number of patrilines (m) and effective mating frequency (me) of infected (n=17) and healthy (n=16) colonies................................................................................................ 10
Figure 7. Differential gene expression of total larval genes (18,914 honey bee genes) between infected and healthy colonies. Log fold-change expression was not significant for any genes after multiple test correction. Blue lines mark 1 log fold-change................................................................. 11
Figure 8. Gene expression (log counts per million) for key immune genes encoding antimicrobial peptides in larvae from infected and healthy colonies......................................................... 12
Figure 9. Diversity of chalkbrood strains infecting colonies across five NSW apiary sites and the observed number of infected larvae (mummies) in colonies with a single strain or multiple strains present................................................................. 12
Figure 10. Pollen area (cm²) in infected (n=33) and healthy (n=24) colonies. Infected colonies had significantly less pollen (p < 0.05) at Majura and Bimbimbie apiary sites........................................... 13
Figure 11. Pollen area (cm²) in colonies with chronic (n=14), acute (n=11) or low (n=11) chalkbrood infections and healthy (n=21) colonies................................................................. 14
Figure 12. Proportion of ITS2 reads for 32 distinct plant taxa present in the pollen of chalkbrood (n=15) and healthy (n=15) colonies.................................................................................. 15
Figure 13. Species richness of pollen collected by chalkbrood-infected and healthy colonies. ......... 15
Figure 14. Quantification of chalkbrood spores in the stored pollen of chalkbrood-infected (n=28) and healthy colonies (n=15).................................................................................. 16
Figure 15. Relative abundance of core bacterial species dominating the gut microbiome of chalkbrood-infected and healthy colonies................................................................. 17
Figure 16. Observed OTUs (alpha diversity) in the gut microbiomes and relative abundance of non-core (environmental) gut bacteria in chalkbrood-infected and healthy colonies................................. 17
Figure 17. Nosema spore levels in adult bees from chalkbrood-infected and healthy colonies......... 18
Figure 18. Average proportion of larvae that were hygienically removed, pupated or became infected after being inoculated with strain A, B, F or control. Significant difference (p < 0.05) with the control group is marked with an asterisk................................................................. 19
Executive summary

What the report is about

This report describes the findings of a two-year study into a variety of factors potentially influencing outbreaks of chalkbrood disease in Australian honey bee colonies. This research provides important insights into key influencing factors that can be further investigated and acted upon by industry to reduce the prevalence and severity of chalkbrood disease.

Who is the report targeted at?

This report is targeted at the Australian beekeeping industry and research community to increase understanding of chalkbrood disease in the Australian context and facilitate improved disease management.

Where are the relevant industries located in Australia?

The Australian honey bee industry is represented across all states and territories. Through managing Apis mellifera, this industry supplies honey and other bee products, including queens and packaged bees, for domestic use and international export markets. The industry has an estimated gross value of production of $98 million annually (ABARES, 2016) and provides paid pollination services of significant value to the horticultural and agricultural sectors.

This research will benefit the whole honey bee industry, as chalkbrood disease is common in all regions except for the Northern Territory.

Background

Chalkbrood is a brood disease of the honey bee, Apis mellifera, caused by the fungal pathogen Ascosphaera apis. It is considered a stress-related disease, with multiple interacting genetic and environmental factors influencing the severity of outbreaks. Chalkbrood disease has become a growing problem for many Australian beekeepers in recent years, but it is unclear what factors are driving current outbreaks.

Genetic factors that influence chalkbrood outbreaks include the larval immune response, social immunity behaviours (e.g. hygienic behaviour), and the virulence of chalkbrood strains. Requeening is common strategy to overcome chalkbrood infections, with the idea being that new queens will have better innate larval immunity or hygienic behaviour, but this is unproven.

Overseas research has shown important variation in larval immunity to chalkbrood, with complex strain-specific interactions affecting virulence. Recent AgriFutures Australia-supported research has focused on hygienic behaviour (Gerds 2020), but innate larval immunity to infection has received relatively little attention in Australia, despite it being an essential factor in preventing chalkbrood outbreaks. Gerds (Gerds 2020, Gerds et al. 2021) did identify multiple genetic strains of chalkbrood in Australia, but a comparison of some strains did not find virulence differences, and therefore it is still unclear how this diversity influenced current chalkbrood infections.

It is also well-established that colonies with higher genetic diversity have reduced incidence of chalkbrood. The underlying mechanisms behind this are not fully understood, but higher colony genetic diversity may allow a more stable larval immune response to infection. Colony genetic diversity is a direct consequence of the number of mates of the queen, hence insufficient queen mating can increase colony susceptibility to infection. Other recent AgriFutures Australia research (Oldroyd and Chapman 2018, Chapman et al. 2019) identified evidence of under-mating in Australian queens, particularly in autumn, which could contribute to disease susceptibility.
Environmental factors that influence chalkbrood outbreaks relate to the climatic, nutritional, chemical, pathogen and hive management stressors that colonies are exposed to and which interact to exacerbate infections. The importance of nutritional resilience as a general strategy in disease management has also become increasingly understood in recent years. Higher pollen diversity and quality can improve bee immunity and survival, and the gut microbial community (i.e. microbiome) of bees may also influence disease resistance. Investigating the link between honey bee nutrition, gut microbiome and chalkbrood resistance in Australia is a valuable area of research with broader implications for bee health.

Aims/objectives

This study investigated several genetic and environmental factors that may influence chalkbrood infections, and aimed to identify key factors involved with current disease outbreaks.

The specific project objectives were:
1. Examine factors influencing the development of chalkbrood infections in Australian colonies
2. Examine factors affecting within-apiary variability of chalkbrood infection

Methods used

Colonies from several apiaries in southern NSW that experienced chalkbrood infections in spring/summer 2018 were examined, and genetic and environmental factors that could influence the health of colonies investigated. For healthy and infected colonies, key genetic factors (colony genetic diversity, baseline immunity and chalkbrood strain diversity) and environmental factors (food stores, pollen diversity, gut microbiome, background spore contamination and Nosema spore levels) were compared. In a separate experiment, in-hive larval inoculations with three isolated chalkbrood strains (A, B and F) were conducted to compare strain virulence and investigate whether past infection history influenced chalkbrood susceptibility.

Results/key findings

This study showed several key factors had a significant influence on chalkbrood infections in our study colonies. Colony genetic diversity, levels of pollen stores and difference in virulence between chalkbrood strains were each found to be important and likely interacted to influence colony infection.

It was found that infected colonies generally had fewer patrilines \( (m) \) and a lower effective mating frequency \( (m_e) \) than healthy colonies. Infected colonies had an average of 12.24 patrilines and healthy colonies an average of 14.19 patrilines \( (t_{31} = 1.86, p = 0.072) \). This difference was even more pronounced for \( m_e \), with an average of 13.08 and 20.72 for infected and healthy colonies, respectively \( (t_{31} = 2.397, p = 0.023) \). Using the average \( m_e \) of 13 for the infected hives as a threshold, 71% of infected colonies had \( m_e < 13 \) but only 25% of healthy colonies had \( m_e < 13 \). However, infected colonies still had estimates of \( m_e > 7 \), which is considered sufficiently mated for colony survival and productivity – but this may underestimate the genetic diversity needed to improve disease resilience.

Image analysis of brood box frames found that chalkbrood-infected colonies had less pollen stores than healthy colonies \( (F_{1,47} = 11.22, p = 0.002) \), but there was no significant difference in the amount of brood or honey. Comparison of pollen stores between healthy colonies and colonies with chronic, acute or low infections showed an overall significant effect \( (F_{3,53} = 5.76, p = 0.002) \) and a significant linear trend of higher pollen levels in healthier colonies \( (F_{1,53} = 16.54, p < 0.0002) \). Higher pollen levels provide more nutrition for the developing brood and help with resilience against disease, suggesting colonies with insufficient pollen stores may be at greater risk of severe chalkbrood infection.
A multiple logistic regression ($\chi^2 = 12.25, p = 0.002$, Nagelkerke $R^2 = 0.41$) had mating number ($m$), and pollen area (per 1,000 cm$^2$) as significant predictors of a colony’s chalkbrood infection status. The odds ratio for mating number was 1.49, indicating that a colony was 49% more likely to be healthy with each additional drone father. This suggests that a colony with $m = 14$ is 16 times more likely to be healthy than a colony with the minimal $m = 7$. The odds ratio for pollen area was 3.31, indicating that a colony is three times more likely to be healthy with each 1,000 cm$^2$ of pollen, which is roughly half a frame of pollen.

There was also a high overall predictive power of 75.76%, with the model correctly predicting 68.75% and 82.35% of infected and healthy samples, respectively. Based on this model, a colony with $m = 14$ and 2,000 cm$^2$ of pollen (i.e. one full frame) has a predicted 18% chance of becoming infected. Whereas, if this colony has only $m = 7$ the probability of being infected increases to 79%, and if the colony has $m = 14$ but very little pollen the probability of being infected is 58%.

Genotyping of chalkbrood isolates found that strain A was the dominant strain infecting colonies across apiary sites studied, with strains B, F and G at less than 20% prevalence. Strain diversity was also significantly higher in colonies with higher chalkbrood levels ($t_{28} = 2.12, p = 0.043$).

The inoculation experiment identified significant differences in larval susceptibility to strains A, B and F. The results showed significantly more larvae were removed when inoculated with strain B ($p = 0.010$) and strain F ($p = 0.003$) compared with the control, and there was a significant overall trend for increasing larval removal between strains A to B to F ($F_{1,28} = 9.770, p = 0.004$). There was a similar non-significant trend for increasing larval infection between strains ($F_{1,28} = 3.519, p = 0.07$) but no strain was significantly different to the control larvae. Significant relationship between larval susceptibility and past infection history was not found.

Odds ratios from a significant multiple logistic regression model ($\chi^2 = 211.3, p < 0.001$) indicated that larvae are 2.1, 3.0 and 3.6 more likely to become infected following inoculation with strain A, B and F, respectively. Overall, our results suggest important differences in the virulence of three chalkbrood strains circulating in Australian honey bee colonies, with evidence that strains B and F are more virulent than strain A, and strain F may also be more virulent that strain B.

**Implications for relevant stakeholders**

Our findings add to extensive evidence that shows the benefits of higher effective mating to colonies. The mechanism for how genetic diversity supports disease resilience is not fully understood but can still be acted upon by industry to manage chalkbrood. Colonies with an effective mating level $> 7$ have been considered adequate, but this threshold is to ensure colony survival. It was recently found that Australian commercial queens had an average $m_e$ of about 10, with one-third of autumn-produced queens with an $m_e < 7$. Our results suggest that higher effective mating levels may be needed to develop chalkbrood resilience in hives.

The study also found infected hives had lower pollen stores, which suggests that colony nutrition is a significant factor in infection. Genetic diversity could also affect stored pollen levels, as higher $m_e$ is linked to increased pollen foraging, which in turn has a positive nutritional effect on larvae and their disease resilience.

This knowledge could be used by beekeepers in several ways. The most practical strategy to implement is prophylactic pollen supplementation in spring, however the logistic regression model indicated that a well-fed colony still had a high chance of chalkbrood infection if the mating level was not high. Therefore, a likely more effective strategy is having a greater focus on increasing the average mating levels of commercial queens.

It is clear that infection severity is influenced by the genetics of both the colony and chalkbrood strains. However, further work is needed to better understand the impact of strains B and F on current outbreaks in Australia. Knowing that there is variation in larval resistance to chalkbrood, there is
opportunity for industry to undertake selective breeding for this trait, noting that resistance mechanisms may differ between strains. There is also likely an important interaction with colony genetic diversity, as colonies with higher mating levels will have a greater diversity of larval genotypes with potential resistance to chalkbrood strains. Breeding for chalkbrood resistance could even be achieved indirectly by selecting bees with high pollen foraging rates for colonies. This would improve colony nutrition and thus resilience to pathogens more generally.

Recommendations

This study found queen mating levels and colony pollen levels to be key factors in chalkbrood infection. The research showed virulence differences between chalkbrood strains likely influence disease levels. The following recommendations will help industry decision-makers, researchers and beekeepers improve understanding and management of chalkbrood outbreaks in Australia. They are:

1. Strategies to assist beekeepers to increase queen mating levels should be further developed and promoted. Oldroyd and Chapman (2018) suggested focusing on management practices to increase drone production, particularly in autumn. This could include feeding supplementary protein to encourage drone production and retention. Beekeepers should also adopt artificial insemination to increase queen mating levels and colony genetic diversity. The health benefits of higher genetic diversity are clear and warrant greater industry attention.

2. Beekeepers who purchase queens have no control of mating levels. However, managing the nutrition of colonies to maintain high pollen levels could be an effective strategy to prevent chalkbrood. Supplementary feeding to promote pollen foraging or providing supplementary protein are practical strategies that could build larval resilience to disease.

3. Further research is needed to explicitly test the impact of queen mating levels and colony pollen levels on chalkbrood. Determining thresholds for these factors to lower the risk of infection would be valuable for beekeepers. The current average $m_0 = 10$ for Australian commercial queens (Oldroyd and Chapman 2018) is potentially too low. It is also unknown how effective various protein supplements are to achieve a desired nutrition threshold.

4. Innovative genetic testing technologies and hive monitoring equipment should be further explored to develop rapid screening tools that can detect queen mating levels and pollen foraging activity. This would give beekeepers the ability to identify at-risk colonies and intervene early through queen replacement or supplementary feeding.

5. Breeding for larval resistance to chalkbrood would be a valuable industry strategy that would complement other efforts that aim to ensure hygienic behaviour and genetic diversity. To do this effectively, beekeepers must be able to identify resistant colonies. With the help of researchers, colonies can undergo exposure assays to identify resistant stock, but a more practical field-based assay would facilitate uptake in selecting for this important trait.
Introduction

Chalkbrood is a brood disease of the honey bee, *Apis mellifera*, caused by the fungal pathogen *Ascosphaera apis*. It is considered a stress-related disease, with multiple interacting genetic and environmental factors influencing the severity of outbreaks. Several recent investigations into chalkbrood disease outlined the factors that affect disease establishment and spread, and these emphasised the need to examine the host-pathogen relationship in the context of a multi-factorial environment (Aronstein and Murray 2010, Jensen et al. 2013, Evison 2015). Hornitzky (2001) also provided a valuable literature review on chalkbrood for the Australian beekeeping industry that encouraged more focus on developing chalkbrood-resistant bees and called for chemical treatment options to assist beekeepers during outbreaks. In recent years, there is evidence that chalkbrood disease has become a more significant issue for many beekeepers in Australia (Roberts, Anderson et al. 2015) and overseas (Aronstein and Murray 2010). However, it is unclear what factors are driving current outbreaks in Australia and how these influence differences in colony susceptibility within apiaries, which is often observed by beekeepers.

Genetic factors that influence chalkbrood outbreaks relate to the immune response of individual larvae and social immunity behaviours of colonies, and their interaction with the virulence of different chalkbrood strains (Gilliam et al. 1983, Vojvodic et al. 2011, Evison et al. 2013, Evison et al. 2015). A common strategy for beekeepers to address these genetic factors is to replace the queen of infected colonies with presumably more resistant genetic stock. This relies on the new genetics having better innate larval resistance and/or greater hygienic behaviour, however this is typically unknown. There has been renewed interest in enhancing hygienic behaviour – the early detection and removal of infected larvae – in the Australian population. This follows on from earlier research in Australia and overseas that showed the value of hygienic behaviour in disease management (Gilliam et al. 1983, Oldroyd 1996, Wilkes and Oldroyd 2002, Manning 2010). This latest research has demonstrated that there are encouraging high baseline levels of hygienic behaviour already present in Australia, but this surprisingly did not correlate with lower infection levels of chalkbrood (Gerds et al. 2018, Gerds 2020). This is consistent with a study by Harpur et al. (2014), which found little correlation between hygienic behaviour and innate immunity (i.e. antimicrobial activity of worker haemolymph) and also supported recommendations that beekeepers consider the innate larval susceptibility of hygienic colonies when breeding for chalkbrood resistance (Jensen et al. 2013).

Innate larval immunity to infection has received relatively little attention in Australia, despite it being an essential factor in preventing chalkbrood outbreaks. However, one Australian study (Lee et al. 2013) showed that even genetically similar colonies can vary in larval susceptibility to geographic isolates of chalkbrood. Overseas research has provided more insight into the host-pathogen genetics of chalkbrood and has demonstrated important variation in larval susceptibility and complex strain-specific interactions affecting virulence (Jensen et al. 2009, Vojvodic et al. 2011, Evison et al. 2015). Only recently have multiple genetic strains been identified in Australia and a comparison of some strains did not find virulence differences (Gerds 2020, Gerds et al. 2021), therefore it is still unclear how this diversity influenced current chalkbrood infections.

It is also well-established that colonies with higher genetic diversity have reduced incidence of chalkbrood (Tarpy 2003, Tarpy and Seeley 2006), which suggests that insufficient mating of queens can increase colony susceptibility to infection. The underlying mechanisms behind this are not fully understood, as high genetic diversity does not always correlate with physiological measures of immunity (Wilson-Rich et al. 2012, Lee et al. 2013, Evison et al. 2016). However, it appears that higher intra-colony genetic diversity may decrease disease outbreaks by providing a more stable larval immune response to infection (Simone-Finstrom et al. 2016). Most recently, Chapman et al. (2019) identified evidence of under-mating in Australian queens, particularly in autumn, which could be contributing to disease susceptibility.
Environmental factors that influence chalkbrood outbreaks relate to the range of climatic, nutritional, chemical, pathogen and hive management stressors that colonies are exposed to and which interact to exacerbate infections. Maintaining optimal brood temperature is important for colony health and it is well-established that brood chilling, such as from unbalanced brood-to-worker ratios, can increase the likelihood of chalkbrood outbreaks (Koenig et al. 1987, Flores et al. 1996, Jensen et al. 2013). Hence, beekeepers often report problems with chalkbrood during spring colony growth and in small nucleus colonies. However, outbreaks can occur regardless of temperature, as optimal mycelial growth for chalkbrood is similar to average hive temperatures (Vojvodic et al. 2011). Agrochemicals and pathogens other than chalkbrood are also linked to compromised immune systems (Doublet et al. 2015, Goulson et al. 2015, Sánchez-Bayo et al. 2016, Pamminger et al. 2018) and disruption of colony dynamics (Becher et al. 2014, Perry et al. 2015), and this can result in poor brood care and increased larval susceptibility to chalkbrood. Interestingly, there is also evidence that chalkbrood outbreaks in summer were associated with *Nosema ceranae* infections in spring (Hedtke et al. 2011). Another important underlying factor is the level of spores in hives, as spores can contaminate food stores and comb (Koenig et al. 1987, Koenig et al. 1987, Gilliam et al. 1988, Flores et al. 2005). As such, comb replacement strategies and gamma irradiation are recommended management strategies for beekeepers (Simone-Finstrom et al. 2018).

The importance of nutritional resilience as a general strategy in disease management has also become increasingly understood in recent years (Somerville 2005, Brodschneider and Crailsheim 2010, Huang 2012). Higher pollen diversity and quality has been shown to improve the immunocompetence and survival of adult bees and colonies (Alaux et al. 2010, Alaux et al. 2017) and can reduce the effects of Nosema infection (Mattila and Otis 2006, Di Pasquale et al. 2013, DeGrandi-Hoffman et al. 2016). There appears to be nothing known about how colony nutrition affects the development of chalkbrood outbreaks, although it is likely an important factor in larval susceptibility, as there is evidence that diet quality improved larval resistance to the opportunistic fungal pathogen *Aspergillus fumigatus* (Foley et al. 2012). There is also growing evidence of the importance of the honey bee’s gut microbial community (i.e. microbiome) on colony health (Engel et al. 2016, Raymann and Moran 2018). The relatively simple bee gut microbiome is affected by diet and can influence disease resistance (Hamdi et al. 2011, Schwarz et al. 2016). It has even been shown to have a protective role against the bacterial brood diseases American foulbrood and European foulbrood (Forsgren et al. 2010, Vásquez et al. 2012). Investigating the link between honey bee nutrition, gut microbiome and chalkbrood resistance in Australia is a valuable area of research with broader implications for bee health.
Objectives

In this study, colonies from several apiaries in southern NSW that experienced chalkbrood infections in spring/summer 2018 were examined, and genetic and environmental factors that could have influenced the health of colonies investigated. For healthy and infected colonies, key genetic factors (colony genetic diversity, baseline immunity and chalkbrood strain diversity) and environmental factors (food stores, pollen diversity, gut microbiome, background spore contamination and Nosema spore levels) were compared. In addition, the virulence of three chalkbrood strains currently circulating in Australian apiaries was assessed. From this data, several key factors that influence the health of these colonies were identified. The identification of these factors will enable further research into, and the development of, strategies to reduce chalkbrood outbreaks.

The specific project objectives were:

1. Examine factors influencing the development of chalkbrood infections in Australian colonies
2. Examine factors affecting within- apiary variability of chalkbrood infection
Methodology

Study design and sampling

Chalkbrood-infected colonies were identified for the study between November 2018 and February 2019, and were from five NSW commercial beekeeper apiaries. A total of 32 infected and 32 healthy colonies were sampled across these apiaries.

Sampling involved visual inspection of frames in the brood box for chalkbrood-infected larvae (i.e. mummies), with each frame photographed for later examination. Collected from each hive were: a random sample of about 50 worker bees off a brood frame, 10 uninfected late-stage larvae, six chalkbrood mummies (three white and three black where possible), and stored pollen from about 20 cells. All samples were stored at -20 °C before use, with the uninfected larvae stored at -80 °C before use.

Sampled colonies were re-inspected in February 2019 to determine whether infected hives were still infected (i.e. chronic infection) or had recovered (i.e. acute infection), and if healthy colonies remained uninfected. Based on this, 15 chronically infected colonies and 15 healthy colonies were selected for the analysis of genetic and environmental factors.

Figure 1. Samples were collected from chalkbrood-infected and healthy colonies across five NSW commercial beekeeper apiaries.
Figure 2. Colony experiencing a chalkbrood outbreak with infected larvae (mummies) expelled.
**Genetic factors**

**Colony genetic diversity using microsatellite markers**

To measure colony genetic diversity, a set of seven microsatellite markers were used to genotype about 24 workers from each of our core 30 samples, as well as from three additional Gundaroo colonies with low acute infections. DNA was extracted from bee legs using Chelex resin and microsatellite markers amplified in two multiplex assays using Phusion U Multiplex PCR master mix (ThermoFisher Scientific). Microsatellite marker primers (Solignac et al. 2003, Shaibi et al. 2008) and fluorescent probes are given in Table 1. PCR products were sent to Biomolecular Research Facility (ANU) for capillary fragment analysis. Marker alleles were determined manually using Peak Scanner™ (ThermoFisher Scientific). Worker genotypes were then analysed with Colony (Jones and Wang 2010) to determine the number of males ($m$) the queen has mated with and their frequency in each colony. Effective mating frequency ($me$) was then estimated using the equation given in Nielson et al. (2003) and used recently for Australian colonies by Chapman et al (2019). This measure accounts for unequal proportions of offspring produced from each drone father (i.e. paternity skew) and is robust to small sample sizes.

### Table 1. Microsatellite markers used for genotyping colony workers

<table>
<thead>
<tr>
<th>Multiplex 1</th>
<th>Fluorescent probe</th>
<th>Forward primer$^1$</th>
<th>Reverse primer$^2$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A7</td>
<td>FAM-GCCTTCCTCCGCGGCA</td>
<td>CCCTTCCTCTCTTTATCTTCC</td>
<td>GTTAGTGCCCTCTCTTGTC</td>
<td>Solignac et al. 2003</td>
</tr>
<tr>
<td>Ap43</td>
<td>VIC-GCCTGCAACCGCACGC</td>
<td>GCCGGTGCCAGCCCGC</td>
<td>CGAAGGTTGTTCAACGGCC</td>
<td>Solignac et al. 2003</td>
</tr>
<tr>
<td>A113</td>
<td>VIC-GCCTGCAACCGCACGC</td>
<td>CTCGAATCGTGGTGCTCC</td>
<td>CCTGATTCTGCAACCTTGAC</td>
<td>Shaibi et al. 2008</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Multiplex 2</th>
<th>Fluorescent probe</th>
<th>Forward primer$^1$</th>
<th>Reverse primer$^2$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A8</td>
<td>FAM-GCCTTCCTCCGCGGCA</td>
<td>CCAAGGTTAAGTTAAAGAC</td>
<td>GGCCTTGAAAGTTCTTGAC</td>
<td>Solignac et al. 2003</td>
</tr>
<tr>
<td>A24</td>
<td>FAM-GCCTTCCTCCGCGGCA</td>
<td>CACAAGTTCAAAAGCTAC</td>
<td>CACATGGAGAGTACAGC</td>
<td>Solignac et al. 2003</td>
</tr>
<tr>
<td>A79</td>
<td>VIC-GCCTGCAACCGCACGC</td>
<td>CGGAGTGCCGAGGATCTTC</td>
<td>GTCGTCGGACAGGATGCG</td>
<td>Solignac et al. 2003</td>
</tr>
<tr>
<td>B124</td>
<td>VIC-GCCTGCAACCGCACGC</td>
<td>GCAACAGGTCGGGTTAGAG</td>
<td>CAGGATAGGTTGATTAAGCAG</td>
<td>Solignac et al. 2003</td>
</tr>
</tbody>
</table>

$^1$ Probe sequence added to 5’ end of forward primer

$^2$ Pigtail sequence GTTTCTT added to 3’ end of reverse primer

**RNA high-throughput sequencing for immune gene analysis**

To investigate baseline immunocompetence, RNA from three pooled uninfected larvae from each of the 30 core colonies was extracted. Individual larvae were disrupted with a plastic pestle in 600 uL lysis buffer (PureLink RNA mini kit, ThermoFisher Scientific) and centrifuged at high speed for two minutes before equal volumes of supernatant from each larva were placed into a single tube per colony. Then 350 uL of mixed supernatant was used for RNA extraction following the recommended protocol. RNA samples were then preserved using RNATable (Sigma-Aldrich) and sent to Novogene (Hong Kong) for sequencing using Illumina PE150 RNA-Seq. Raw sequence data was processed on CSIRO’s high-performance computing clusters. Raw data was quality trimmed using Trimmomatic, and trimmed reads were then mapped to the honey bee genome (GCF_003254395.2_Amel_HAv3.1) with HISAT2 and transcriptome assembly done with StringTie (Pertea et al. 2016). Gene and transcript count matrices were then extracted for differential gene expression analysis using the R packages edgeR (Robinson et al. 2010) and limma (Ritchie, Phipson et al. 2015).

**Chalkbrood strain analysis**

From infected colonies, three white and three black chalkbrood mummies per hive were collected in individual tubes and stored at -20 °C before use. Isolates of *A. apis* strains were made from each of the three white mummies collected using methods described in Jensen et al. (2013). In a biological cabinet, mummies were sterilised in a 10% sodium hypochlorite solution for 10 minutes, then rinsed twice with sterile distilled water. One-third of the mummy was cut into three small pieces and placed on MY-20 (Malt Yeast) agar plates and sealed with parafilm. Mycelial growth was visible after plates
were incubated for 36 hours in the dark at 34 °C (Figure 3). A single hyphal tip was aseptically dissected and transferred to a new MY-20 agar plate and incubated for a further three days. A loopful of aerial hyphae was collected in 100 μl of Prepman Ultra Sample Preparation reagent (Applied Biosystems) and DNA extracted by heating at 100 °C for 10 minutes, followed by high-speed centrifugation and collection of the DNA-containing supernatant. Each isolate was PCR amplified and sequenced with the primer sets EF1α and Scaffold 300, as described by Jensen et al. (2012). Based on variable sites in these two sequence markers, each isolate was assigned to strain A, B, F or G.

Environmental factors

Colony resources

From each colony all frames in the brood box were photographed and the frame area (cm²) covered by brood, honey and pollen was manually calculated using the Fiji image analysis software (Schindelin et al. 2012) (Figure 4). The core 30 samples (15 chronic infection and 15 healthy colonies) were analysed, and the pollen area for the remaining acutely infected samples was then calculated.

Figure 3. Mycelial growth of Ascosphaera apis cultured from infected larvae.

Figure 4. Frames from the brood box were photographed and area of brood, honey and pollen were estimated using Fiji image analysis software.
Pollen analyses for species diversity and chalkbrood spore contamination

Stored pollen samples were separately mixed in 4 ml of 0.05 M potassium phosphate buffer to create a homogenous pollen solution.

For pollen diversity analysis, 100 uL of pollen solution from the 30 core colonies was centrifuged at high speed for five minutes and the supernatant removed, before 550 ul of lysis buffer and 20 ul proteinase K (NucleoSpin DNA Food extraction kit, Macherey-Nagel) were added and the recommended protocol to extract DNA followed. The sequence marker ITS2 was amplified via a two-step protocol. The first PCR step amplified the target region using the gene-specific primers S2F and 4rev (Chen et al. 2010), and the target region was then extended with a generic binding sequence (from Illumina 16S protocol) to bind the second step primers. The second PCR step added the Illumina 8-base dual barcoding indexes and flowcell attachment sequences. DNA libraries were sequenced on an Illumina MiSeq 2 x 250 bp run at the Ramaciotti Centre for Genomics (UNSW). Sequence data was analysed using the USEARCH pipeline (Edgar 2010) and unique operational taxonomic units (OTUs) identified with the ITS2DB (Ankenbrand et al. 2015) and PlantAligDB trnL_GH databases (Santos et al. 2019). Differences in alpha and beta diversity between infected and healthy colonies were then investigated using the phyloseq and vegan R packages (Oksanen, Kindt et al. 2007).

For spore contamination, 1 ml of pollen solution was collected for each sample in a 2 ml screw-cap tube containing a 3.2 mm stainless steel ball bearing and 0.5 mm glass beads. Samples were centrifuged at high speed for five minutes and the supernatant removed before adding 250 ul of lysis buffer (PureLink Genomic Plant DNA Purification kit, ThermoFisher Scientific). Samples were disrupted using a FastPrep bead beating system then continued with the DNA extraction kit following the recommended protocol. Pollen DNA was tested for *A. apis* DNA by quantitative PCR (SensiFAST SYBR No-ROX kit, Bioline) using primers developed by Klinger et al (2015). All samples were run in duplicate and amplified DNA was quantified by comparison to a serial dilution prepared from a sample with known spore levels estimated microscopically (Human et al. 2013).

Gut microbiome analysis

The adult gut microbiome for the 30 core colonies was investigated by extracting DNA from the abdomens of five adult bees per colony. Each abdomen was rinsed in 70% ethanol and then sterile water before being placed in a 2 ml screw-cap tube with a 3.2 mm stainless steel ball bearing and 0.5 mm glass beads, 200 uL lysis buffer (High Pure PCR Template Preparation kit, Roche) and 40 uL proteinase K. Samples were disrupted using a FastPrep bead beating system then continued with the DNA extraction kit following the recommended protocol. DNA was then sent to Novogene (Hong Kong) for amplicon sequencing of the V4 region of the 16S rRNA gene to characterise and compare the gut microbiome (i.e. bacterial community) in chalkbrood-infected and healthy colonies. Sequence analysis was done using the QIIME 2 analysis pipeline (Bolyen et al. 2019) to identify unique operational taxonomic units (OTUs), assign taxonomy to these OTUs and investigate differences in alpha and beta diversity between infected and healthy colonies.

Nosema spore levels

To explore the relationship between chalkbrood infection and Nosema infection, 30 adult bees were macerated in 5 ml 0.05 M potassium phosphate buffer in an extraction bag (Bioreba). After that, 10ul was pipetted onto a slide with a counting chamber (Improved Neubauer) and Nosema spore levels were estimated under 400x magnification (Leica DM2500), as described by Hornitzky (2005).
Comparison of chalkbrood strain virulence

An in-hive larval inoculation experiment was conducted to compare the virulence of three chalkbrood strains (A, B and F) and investigate how past infection history influenced chalkbrood susceptibility.

Following the methods of Jensen et al. (2013), mating plates with MY-20 agar were set up for each strain by combining isolates from a different colony/mummy. Isolates were first tested for mating type using primers developed by Aronstein et al. (2015) to ensure mating and spore production. Mating plates incubated at 34 °C for six weeks until black fruiting bodies (spore cysts) were visible where the hyphae of the two mating types met. For each strain, fruiting bodies were collected aseptically in 20 μL sterile distilled water and put into sample tubes. Spore cysts were homogenised using a plastic pestle to release the spores, with increasing volumes added to a final volume of 1 ml. Samples were left for 30 minutes to allow larger particles to settle before 500 μL supernatant was transferred to a fresh tube. Spore concentration was then calculated from a 10^2 dilution using a haemocytometer (Improved Neubauer), as described in Human et al. (2013). Spore solutions were adjusted with sterile water to normalise spore concentration to 2 x 10^5 spores/ml for each strain. Spore solutions were mixed 1:1 with sterilized honey solution (1:1, honey:water) to be used for larval inoculations.

Six hives were used for the experiment, three of which had experienced significant chalkbrood infections during the previous spring. Inoculations involved removing a brood frame with many three-day-old larvae from each colony and using a pipette to carefully give 5 μL of spore inoculum to 40 larvae per treatment (i.e. a dose of 1 x 10^3 spores per larva), and 40 larvae received 5 μL honey solution as the control group. Acetate paper was overlayed to mark the location of inoculated larvae and frames then returned to the colony to allow cell capping. Four days later, frames with inoculated larvae were removed and incubated in the lab at 34 °C for five days. Counts were then done on the number of larvae that were capped or removed (i.e. hygienic behaviour) after inoculation and the number of larvae that had pupated or became infected (i.e. mummies) after five days of lab incubation (Figure 5). Data analysis was carried using GraphPad Prism version 8.

Figure 5. Inoculated larvae were uncapped and assessed for infection after five days in the lab
Results

Genetic factors

Colony genetic diversity using microsatellite markers

Colony genetic diversity has been shown to have a positive effective on colony productivity and survival (Mattila and Seeley 2007, Tarpy et al. 2013) and supports disease resilience (Tarpy 2003, Tarpy and Seeley 2006, Seeley and Tarpy 2007, Delaplane et al. 2015, Simone-Finstrom et al. 2016). Using microsatellite markers, 759 worker bees from 33 colonies were genotyped to compare the genetic diversity of infected and healthy colonies. From the worker genotypes, the queen genotypes were deduced to then estimate the number of males \((m)\) the queen had mated with and the effective mating frequency \((m_e)\) for each colony.

Infected colonies generally had fewer patrilines and a lower effective mating frequency than healthy colonies. Infected colonies had an average of 12.24 patrilines and healthy colonies an average of 14.19 patrilines \((t_{31} = 1.86, p = 0.072)\). This difference was even more pronounced for \(m_e\) with an average of 13.08 and 20.72 for infected and healthy colonies, respectively \((t_{31} = 2.397, p = 0.023)\) (Figure 6). There was also a significant overall effect of infection status for \(m_e\) \((F_{1,23} = 7.81, p = 0.010)\), with no significant effect of location \((F_{4,23} = 2.04, p = 0.133)\) or an interaction \((F_{4,23} = 0.84, p = 0.842)\).

![Figure 6. Total number of patrilines (m) and effective mating frequency (m_e) of infected (n=17) and healthy (n=16) colonies.](image)

Tarpy (2003) showed that genetically diverse colonies are less likely to contract a severe chalkbrood infection. Our data supports this, with these infected colonies experiencing chronic moderate-high chalkbrood levels. However, infected colonies still had estimates that were generally higher than \(m_e > 7\), which is considered sufficiently mated (Tarpy, vanEngelsdorp et al. 2013). This threshold is to ensure colony survival and productivity and may underestimate the required genetic diversity to improve disease resilience. Using the average \(m_e\) of 13 for the infected hives as a threshold, 71% of infected colonies had \(m_e < 13\) but only 25% of healthy colonies had \(m_e < 13\). Tarpy (2003) also had queens inseminated by 24 drone fathers to observe benefits against chalkbrood, although reduced disease severity has also been shown in experiments that used 10 drone fathers compared with single-mated queens (Tarpy and Seeley 2006, Seeley and Tarpy 2007).

Chapman et al. (2019) recently assessed the mating frequency of Australian commercial queens and found that while queens were generally sufficiently mated \((m_e > 7)\), about one-third of autumn-
produced queens were potentially under-mated. However, with an average $m$, of 10.6 and 9 in spring and autumn, respectively, perhaps the mating frequency of Australian queens needs to be higher to improve chalkbrood resilience.

**RNA high-throughput sequencing for immune gene analysis**

Larval immunity has an important role in chalkbrood infections and here it was investigated whether constitutive expression of immune genes (i.e. baseline immunocompetence) was a key factor influencing colony chalkbrood infections. Using an RNA high-throughput sequencing approach of uninfected larvae, total gene expression of infected and healthy colonies was compared. Many genes were differentially expressed but none were found significant between the two groups, due to large within-group variation (Figure 7).

Examination of specific immune gene encoding for antimicrobial peptides showed constitutive expression was generally low, although two chalkbrood colonies with very high infection levels had approximately two-to-eight-fold increases in expression for several immune genes (Figure 8). The results do not indicate that baseline immunocompetence had a significant role in the infection status of these colonies. However, it is possible that the induced immune response may be an important factor influencing infection that needs further investigation.

Other studies have considered immune gene expression following pathogen exposure, with abaecin, defensin-1 and hymenoptaecin showing up-regulation after chalkbrood infection and links to resistance (Aronstein et al. 2010, Evison et al. 2016, Nie et al. 2020). Similarly, abaecin and hymenoptaecin levels have been found to correlate with resistance to AFB and high genetic diversity respectively (Evans and Pettis 2005, Simone-Finstrom et al. 2016). Interestingly, in this investigation, these genes were highly expressed in two highly infected colonies and this could be an immune response by resistant larval genotypes in these colonies.

**Figure 7.** Differential gene expression of total larval genes (18,914 honey bee genes) between infected and healthy colonies. Log fold-change expression was not significant for any genes after multiple test correction. Blue lines mark 1 log fold-change.
Figure 8. Gene expression (log counts per million) for key immune genes encoding antimicrobial peptides in larvae from infected and healthy colonies.

Chalkbrood strain analysis

Three chalkbrood-infected larvae (mummies) were genotyped from 31 colonies using two gene sequence markers (EF-alpha and SCA300) to identify the diversity of chalkbrood strains infecting these colonies. Based on these markers, there are seven recognised genetic strains of chalkbrood found around the world (Jensen et al. 2012). Here, it was found that strain A was the most dominant strain infecting colonies across apiary sites studied, with strains B, F and G at less than 20% prevalence (Figure 9). It was also found that strain diversity was significantly higher in colonies with higher chalkbrood levels ($t_{28} = 2.12$, $p = 0.043$). A recent wider screening of chalkbrood strains in Australia has also found strain A to be most common, but did not find any one strain was associated with high infections (Gerdts et al. 2021).

Figure 9. Diversity of chalkbrood strains infecting colonies across five NSW apiary sites and the observed number of infected larvae (mummies) in colonies with a single strain or multiple strains present.
Environmental factors

Colony resources

Image analysis of brood box frames found that chalkbrood-infected colonies had on average significantly less pollen stores than healthy colonies (Figure 10), but there was no significant difference in the amount of brood or honey. Chalkbrood infection had a significant effect on pollen stores \((F_{(1,47)}= 11.22, p = 0.002)\) accounting for 12.0% of the variance, with apiary site \((F_{(4,47)}= 4.31, p = 0.005)\) accounting for 18.5% of the variance and no significant interaction.

Healthy colonies were then compared with chronic, acute or low infection status colonies (Figure 11). This showed that chalkbrood infection had a significant effect on pollen stores \((F_{(3,53)}= 5.76, p = 0.002)\) and there was a significant linear trend for higher pollen levels in healthier colonies \((F_{(1,53)}= 16.54, p < 0.0002)\). Higher pollen levels provide more nutrition for the developing brood and help with resilience against disease (Foley et al. 2012). Therefore, colonies with insufficient pollen stores may be at greater risk of a chalkbrood outbreak.

Interestingly, there was no difference between chronic and acute infection colonies, suggesting that other factors may determine a colony’s recovery from chalkbrood infection. The pollen levels were not recorded at the second inspection, but perhaps recovered colonies had a greater ability to collect pollen and improve brood nutrition.

Figure 10. Pollen area (cm²) in infected (n=33) and healthy (n=24) colonies. Infected colonies had significantly less pollen \((p < 0.05)\) at Majura and Bimbimbie apiary sites.
Figure 11. Pollen area (cm²) in colonies with chronic (n=14), acute (n=11) or low (n=11) chalkbrood infections and healthy (n=21) colonies.

Pollen diversity analysis

Pollen underpins colony nutrition and research shows that colonies containing a diversity of pollens are found to have better overall health (Alaux et al. 2010, Huang 2012, Di Pasquale et al. 2013, Alaux et al. 2017). To examine the influence of pollen diversity in chalkbrood-infected and healthy colonies, deep amplicon sequencing of the ITS2 gene was used to identify the diversity of plant species contributing to the stored pollen of our study colonies.

A total of 32 plant species were detected across the study colonies, with between 16 and 31 taxa found in each colony (Figure 12). The main floral resources used by colonies at these sites were from the Asteraceae (various small flowering plants/weeds), Brassicaceae (wild mustard, canola), Myrtaceae (Eucalyptus, Leptospermum) and St John’s wort (*Hypericum perforatum*).

No significant differences in the diversity or relative abundance of species present in the stored pollen of healthy and chalkbrood-infected colonies were found (Figures 13). Not surprisingly, there were significant differences in the mix of pollens collected between apiary sites (PERMANOVA; Gower distance, $F = 7.23$, $R^2 = 0.56$, $p = 0.001$). Therefore, despite healthy colonies having greater pollen stores, this did not appear to translate into greater pollen diversity, with both healthy and chalkbrood-infected colonies collecting equally diverse pollens.
Figure 12. Proportion of ITS2 reads for 32 distinct plant taxa present in the pollen of chalkbrood (n=15) and healthy (n=15) colonies.

Figure 13. Species richness of pollen collected by chalkbrood-infected and healthy colonies.
**Chalkbrood spore contamination**

Contamination of pollen by chalkbrood spores is a known transmission pathway for infection of larvae and was investigated here using an optimised quantitative PCR detection assay targeting the *A. apis* 5.8S rRNA gene (Klinger et al. 2015). Using this assay, chalkbrood spore levels in stored pollen were compared. It was found that there was significantly higher contamination of pollen in chronic infection colonies than healthy colonies ($t_{28} = 2.45$, $p = 0.021$) (Figure 14). It is unknown whether spore levels were higher in these colonies before they experienced an outbreak or if infection caused spore levels to increase. Given that spore contamination of acute and low-infection colonies (and many chronic infection colonies) was similar to healthy colonies, the research suggests spore contamination of pollen is unlikely to be a strong factor in chalkbrood outbreaks. However, higher spore contamination could influence whether a colony suffers a chronic infection or an acute infection and recovers.

To the best of our knowledge this is the first study to use molecular quantification of spore contamination of pollen in honey bee colonies (see Klinger et al. 2015 and de Sousa Pereira et al. 2019 for related uses). This could be further developed as a valuable diagnostic tool for chalkbrood and useful to investigate spore contamination of hives during infection.

![Figure 14. Quantification of chalkbrood spores in the stored pollen of chalkbrood-infected (n=28) and healthy colonies (n=15)](image)

**Gut microbiome**

Deep amplicon sequencing of the 16S ribosomal RNA gene was used to compare the gut bacterial community (microbiome) of adult bees in chalkbrood-infected and healthy colonies. Consistent with other studies of the honey bee microbiome (Engel et al. 2016, Jones et al. 2018, Raymann and Moran 2018), the core set of bacterial species dominating each sample was identified: Lactobacillus, Gilliamella, Snodgrassella, Bifidobacterium, Bartonella and Frischella (Figure 15). The sequencing found eight Lactobacillus species with *L. melliventris* most abundant, two Gilliamella species, two Snodgrassella species, three Bifidobacterium species, two Bartonella species and two Frischella species. A total of 111 bacterial operational taxonomic units (OTUs) with at least 10 reads were found, with the core microbiome taxa representing 90.4% of the sequencing reads and present in all samples.

However, no evidence of gut microbiome dysbiosis (i.e. disturbed gut health) in chalkbrood-infected colonies was found, with no significant differences in species relative abundance, diversity or
composition (Figure 15 and 16). A recent Australian study found healthy colonies have more culturable aerobic environmental bacteria compared with chalkbrood-infected colonies (Khan et al. 2020). While not directly comparable, when we looked at the relative abundance of the ‘non-core’ microbiome taxa (9.6% of the sequencing reads) there was no significant difference. It would be interesting to quantify specific taxa (rather than relative abundance) identified by Khan et al. (2020) for further comparison.

Figure 15. Relative abundance of core bacterial species dominating the gut microbiome of chalkbrood-infected and healthy colonies.

Figure 16. Observed OTUs (alpha diversity) in the gut microbiomes and relative abundance of non-core (environmental) gut bacteria in chalkbrood-infected and healthy colonies.
**Nosema spore levels**

Nosema was investigated as a potential factor that could exacerbate chalkbrood infection as there is some correlative evidence for a positive relationship between the two pathogens (Hedtke et al. 2011). Nosema spore levels were estimated by microscopic examination of 30 macerated adult bees under 400x magnification. There was no significant difference in Nosema spore levels between chalkbrood-infected and healthy colonies (Mann-Whitney U = 95.5, p = 0.49), although the highest spore levels were detected in chalkbrood-infected colonies (Figure 17).

![Figure 17. Nosema spore levels in adult bees from chalkbrood-infected and healthy colonies.](image)

**Multiple logistic regression for chalkbrood factors**

A multiple logistic regression was also conducted to determine whether any factors were significant predictors of a colony having a chalkbrood infection. After the significance of each predictor variable on infection status (dependent variable) was individually assessed, the mating number (m) and pollen area (per 1000 cm²) were included in the final model (Table 2). The model was significant ($\chi^2 = 12.25, p = 0.002$) with an $R^2$ (Nagelkerke) value of 0.41 indicating the model explained 41% of the variation.

The odds ratio for mating number was 1.49, indicating that a colony was 49% more likely to be healthy with each additional drone father. This suggests that a colony with $m = 14$ is 16 times more likely to be healthy than a colony with the minimal $m = 7$. The odds ratio for pollen area was higher, 3.31, indicating that a colony is 3.31 times more likely to be healthy with each 1,000 cm² of pollen, which is roughly one full side of a frame.

There was also a high overall predictive power of 75.76%, with the model correctly predicting 68.75% and 82.35% of infected and healthy samples, respectively. Based on this model, a colony with $m = 14$ and 2,000 cm² of pollen (i.e. two full frames) has a predicted probability of 18% of becoming infected. Whereas, if this colony has only $m = 7$ the probability of being infected increases to 79% and if the colony has $m = 14$ but very little pollen the probability of being infected is 58%.
Table 2. Variables included in the multiple logistic regression model for infection status

<table>
<thead>
<tr>
<th>Variable</th>
<th>B ± SE</th>
<th>Wald</th>
<th>p-value</th>
<th>Odds Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mating number ( (m) )</td>
<td>0.62 ± 0.28</td>
<td>2.172</td>
<td>0.046</td>
<td>1.49</td>
</tr>
<tr>
<td>Pollen area ( (1000 , \text{cm}^2) )</td>
<td>2.79 ± 1.21</td>
<td>2.305</td>
<td>0.035</td>
<td>3.31</td>
</tr>
<tr>
<td>Intercept</td>
<td>-6.50 ± 4.90</td>
<td>2.758</td>
<td>0.019</td>
<td>0.002</td>
</tr>
</tbody>
</table>

**Comparison of chalkbrood strain virulence**

In a separate experiment, in-hive larval inoculations were conducted with three isolated chalkbrood strains (A, B and F) to compare strain virulence and investigate whether past infection history influenced chalkbrood susceptibility.

The analysis identified significant differences in larval susceptibility to inoculation with strains A, B and F (Figure 18). Significantly more larvae were removed when inoculated with strain B \( (p = 0.010) \) and strain F \( (p = 0.003) \) compared with the control, and there was a significant overall trend of increasing larval removal between strains A to B to F \( (F_{1,28} = 9.770, \, p = 0.004) \). There was a similar trend of increasing larval infection between strains, however this was marginally non-significant \( (F_{1,28} = 3.519, \, p = 0.07) \) and no strain was significantly different to the control larvae.

No significant relationship between larval susceptibility (removal or infection) and past infection history of colonies for any strain was found. Although, there was notably higher infection with strain F in colonies that did not experience chalkbrood in the preceding spring.

![Figure 18. Average proportion of larvae that were hygienically removed, pupated or became infected after being inoculated with strain A, B, F or control. Significant difference \( (p < 0.05) \) with the control group is marked with an asterisk.](image)

In addition, multiple logistic regression was used to explore the effect of strain (A, B or F) and past infection history (healthy or infected) on the probability of an inoculated larva becoming infected (removed and infected data combined). A significant regression model was fitted to the data \( (\chi^2 = \)
211.3, df = 6, \( p < 0.001 \)), with all variables having a significant effect on infection (Table 3). Odds ratios for each strain indicated that larvae were 2.1, 3.0 and 3.6 times more likely to become infected following inoculation with strain A, B and F, respectively. Interestingly, past infection history had a significant influence on the model, with the odds ratio of 0.73 suggesting that larvae from colonies with a past infection history were less likely to become infected in the inoculation experiment.

Table 3. Variables included in the multiple logistic regression model for strain virulence

<table>
<thead>
<tr>
<th>Variable</th>
<th>B ± SE</th>
<th>Wald</th>
<th>p-value</th>
<th>Odds Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain A</td>
<td>0.76 ± 0.17</td>
<td>4.51</td>
<td>&lt; 0.0001</td>
<td>2.1</td>
</tr>
<tr>
<td>Strain B</td>
<td>1.08 ± 0.17</td>
<td>6.33</td>
<td>&lt; 0.0001</td>
<td>3.0</td>
</tr>
<tr>
<td>Strain F</td>
<td>1.29 ± 0.17</td>
<td>7.54</td>
<td>&lt; 0.0001</td>
<td>3.6</td>
</tr>
<tr>
<td>Infection history</td>
<td>-0.32 ± 0.12</td>
<td>2.62</td>
<td>0.009</td>
<td>0.73</td>
</tr>
<tr>
<td>Intercept</td>
<td>-0.58 ± 0.13</td>
<td>4.38</td>
<td>0.351</td>
<td>0.56</td>
</tr>
</tbody>
</table>

Overall, the results suggest important differences in the comparative virulence of three chalkbrood strains circulating in Australian honey bee colonies, with evidence that strains B and F are more virulent than strain A, and strain F may also be more virulent than strain B. This pattern was still evident despite the variation in susceptibility between colonies, suggesting this result could be more generalised. The result is consistent with an earlier lab-based study by Vojvodic et al. (2011) that showed strain F and G to be more virulent than strain A and, to the best of our knowledge, presents the first evidence of higher virulence for strain B. In contrast, Gerdts (2020) did not see a difference in virulence between strain A and strain G in lab-based assays. Direct comparison with this Australian study is difficult as we used different methods (in vivo versus in vitro) and made different strain comparisons.
Implications

The prevalence and severity of chalkbrood infections are influenced by multiple interacting factors. However, several factors with greater influence on chalkbrood infections were identified in this study and could be further investigated to reduce the impact of this disease in Australian colonies. Colony genetic diversity, the amount of stored pollen and differences in chalkbrood strains were significant factors between chalkbrood-infected and healthy colonies in this study and contributed to a significant logistic regression model with good predictive power for a colony’s infection status.

These findings add to extensive evidence that shows the benefits of higher effective mating to colonies. The mechanism for how genetic diversity supports disease resilience is not fully understood but can still be acted upon by industry to manage chalkbrood. Colonies with an effective mating level $>7$ have been considered adequate but this threshold is to ensure colony survival. Chapman et al. (2019) found that average $m_e$ was around 10 for Australian commercial queens, with one-third of autumn-produced queens with an $m_e < 7$. The results suggest that higher effective mating levels may be needed to develop chalkbrood resilience in hives.

Infected colonies were also found to have lower pollen stores, and this suggests that colony nutrition has a significant influence on infection. There is an interesting link between pollen stores and genetic diversity that this study highlights. Higher genetic diversity enhances the task allocation system in a colony, including pollen foraging (Eckholm et al. 2011), and has been linked to greater pollen use and brood care by nurse bees (Eckholm et al. 2015). Therefore, while higher $m_e$ can improve disease resilience through a more stable and effective immune response (Simone-Finstrom, et al. 2016), higher $m_e$ can also improve colony nutrition through increased pollen foraging, which has a positive nutritional effect on larvae that supports disease resilience (Dolezal and Toth 2018).

This knowledge could be used by beekeepers in several ways. The most practical strategy to implement is prophylactic pollen supplementation in spring, however the logistic regression model indicated that a well-fed colony still had a high chance of chalkbrood infection if the mating level was not high. Therefore, a likely more effective strategy is having a greater focus on increasing the average mating levels of commercial queens.

The comparison of chalkbrood strains circulating in Australian colonies highlighted the differences in virulence that are likely influencing disease prevalence and severity. However, further work is needed to better understand the impact of strains B and F on current outbreaks in Australia, as despite the evidence of higher virulence in this study, Gerdts et al. (2021) did not find chalkbrood severity in field colonies was associated with any one strain. The high prevalence of strain A and influence of host genetics and environment likely make it difficult to identify the effect of strain virulence. Whole colony inoculations with these strains would be valuable to directly test differences in infection severity.

Greater baseline immunity was not seen in healthy hives in this study but there may be differences in immune response after exposure to chalkbrood, something that has been shown in other studies (Aronstein and Murray 2010, Evison et al. 2016). Knowing that there is variation in larval resistance to chalkbrood, there is still opportunity for industry to undertake selective breeding for this trait. However, it will be important to consider the differences between chalkbrood strains as resistance mechanisms may not be equally effective against each strain. There is also likely an important interaction with colony genetic diversity, as increased genetic diversity can reduce the variation in larval immune response (Simone-Finstrom et al. 2016). Colonies with higher mating levels will have a greater diversity of larval genotypes with potential resistance to different chalkbrood strains. Breeding for chalkbrood resistance could even be achieved indirectly by selecting bees with high pollen foraging rates for colonies. This is a heritable trait (Page Jr et al. 1995) and this strategy aims to improve the nutritional resilience of colonies to pathogens more generally (Dolezal and Toth 2018).
Recommendations

This study identified queen mating levels and colony pollen levels to be key factors associated with chalkbrood infection. The research showed virulence differences between chalkbrood strains likely influence disease levels. The following recommendations have been made to improve understanding and the management of chalkbrood outbreaks in Australia.

1. Strategies to assist beekeepers to increase queen mating levels should be further developed and promoted. Oldroyd and Chapman (2018) suggested focusing on management practices to increase drone production, particularly in autumn. This could include feeding supplementary protein to encourage drone production and retention. Beekeepers should also adopt artificial insemination to increase queen mating levels and colony genetic diversity. The health benefits of higher genetic diversity are clear and warrant greater industry attention.

2. Beekeepers who purchase queens have no control of mating levels. However, managing the nutrition of colonies to maintain high pollen levels could be an effective strategy to prevent chalkbrood. Supplementary feeding to promote pollen foraging or providing supplementary protein are practical strategies that could build larval resilience to disease.

3. Further research is needed to explicitly test the impact of queen mating levels and colony pollen levels on chalkbrood. Determining thresholds for these factors to lower the risk of infection would be valuable for beekeepers. The current average $m_v = 10$ for Australian commercial queens (Oldroyd and Chapman 2018) is potentially too low. It is also unknown how effective various protein supplements are to achieve a desired nutrition threshold.

4. Innovative genetic testing technologies and hive monitoring equipment should be further explored to develop rapid screening tools that can detect queen mating levels and pollen foraging activity. This would give beekeepers the ability to identify at-risk colonies and intervene early through queen replacement or supplementary feeding.

5. Breeding for larval resistance to chalkbrood would be a valuable industry strategy that would complement other efforts that aim to ensure hygienic behaviour and genetic diversity. To do this effectively, beekeepers must be able to identify resistant colonies. With the help of researchers, colonies can undergo exposure assays to identify resistant stock, but a more practical field-based assay would facilitate uptake in selecting for this important trait.
References


Investigating factors that influence chalkbrood outbreaks in Australia

by John Roberts and Joel Armstrong
April 2021

AgriFutures Australia Publication No. 21-034
AgriFutures Australia Project No. PRJ-010815