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Establishing the disease status of the Asian honeybee in the Cairns region

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by Dr John Roberts and Dr Denis Anderson

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Foreword

This research provides important knowledge on the pathogens introduced with the Asian honeybee, *Apis cerana*, in the Cairns region that may impact the Australia honeybee industry. This knowledge was essential for addressing the concerns of the United States and other countries that exotic pathogens may have transferred from *A. cerana* to the local European honeybee, *Apis mellifera*, and be spread via imports of Australian bees. This research is a key step in re-establishing trade of Australian bees with the United States and for maintaining other export markets under threat.

The Australian honeybee industry and apiarists will benefit from this research through the identification of exotic pathogens that may affect the health of *A. mellifera*. There are also potential benefits to commercial beekeepers, particularly those based in Eastern Australia that are currently unable to export live bees to the United States.

The findings of this research showed that no new honeybee pathogens have been introduced to Australia with the establishment of *A. cerana* in the Cairns region. There is also no indication that novel pathogen strains have spread from *A. cerana* to *A. mellifera*, but at least one pathogen has spread from the local *A. mellifera* and is infecting *A. cerana*.

This project was funded from industry revenue that is partially matched by funds provided by the Australian Government.

This report is an addition to RIRDC's diverse range of over 2000 research publications and it forms part of our Honeybee R&D program, which aims to improve the productivity and profitability of the Australian bee keeping industry through the organisation, funding and management of a research, development and extension program that is both stakeholder and market-focussed.

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Craig Burns
Managing Director
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We thank Louise Morin for reviewing this report and thank Dave Alden of RIRDC for his support as project manager.

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Executive Summary

What the report is about

This report describes the identification of pests and diseases of the Asian honeybee (*Apis cerana*) in the Cairns region of Australia and outlines the likely spread of exotic pathogens to the local European honeybee (*Apis mellifera*) population.

This research is important for understanding potential risks to the health of Australia's managed and wild *A. mellifera* populations. It is also essential for addressing the concerns of the United States and other countries that exotic pathogens may be spread via imports of Australian bees. This research is a key step in re-establishing trade of Australian bees with the United States and for maintaining other export markets under threat.

Who is the report targeted at?

The report is targeted at the Australian beekeeping industry and apiarists for the better management of bee pathogens and also at decision-makers who develop policy in regards to exports of live bees in the State and Commonwealth Governments and in governments overseas.

Where are the relevant industries located in Australia?

The Australian honeybee industry is represented across all states and territories. Through management of *A. mellifera*, this industry supplies honey and other bee products, including queen and packaged bees, for domestic users and international export markets. The industry has an estimated gross value of production of $75 million annually with live bee exports estimated to be worth $6 million dollars when the US market was accessible. The honeybee industry also provides paid pollination services to the horticultural and agricultural sectors valued in excess of $4 billion annually.

While this research will benefit the honeybee industry as a whole, commercial beekeepers based in Queensland and New South Wales that want to export live bees are likely to see more direct benefits. Commercial and amateur beekeepers closer to the Cairns region will also benefit from this research, as they are more likely to be directly impacted by exotic pathogens carried by *A. cerana*.

Background

Apis cerana, has been present in Cairns since 2007 and it is estimated that this population now consists of several hundred colonies. This bee is the Java strain of *A. cerana*, which occurs naturally in Indonesia and has spread to Papua New Guinea and Solomon Islands. Little is known about the pathogens that are present in this bee population or if novel pathogens can be spread to the local *Apis mellifera*. Establishing the pest and disease status of this *A. cerana* population is needed to determine potential pathogens that threaten the productivity of the Australian honeybee industry and its ability to export live bees to countries where *A. cerana* is not present.

In 2010, the United States prohibited the trade of Australian live bees because of uncertainty over the potential transfer of pathogens from *A. cerana* to the local *A. mellifera*. Other countries, such as Canada are also reviewing whether to continue importing Australian bees. The disease status of Asian honeybees as a whole is not well known, except for their ability to host parasitic mites such as *Varroa*. It is thought they carry a similar range of pathogens to those found in *A. mellifera*. But some of these pathogens are unique strains that may differ in virulence and their transferability to other bee species is unknown. Therefore, knowledge of the pathogens carried by *A. cerana* in the Cairns region and their ability to spread to *A. mellifera* is essential for re-establishing and maintaining international markets for Australian live bees.

Aims/objectives

The project had five objectives:

1. Collect samples of live and dead brood and adult bees of *A. mellifera* and *A. cerana* (Java strain) from across the Cairns region

2. Collect and examine comb from both species for signs of abnormalities and diseases

3. Test collected samples for the presence of described and undescribed honeybee pathogens including viruses, fungi, protozoa and bacteria

4. Where possible, identify the (likely) transmissibility of identified pathogens from *A. cerana* to *A. mellifera* in the Cairns region

5. Provide a set of diagnostic techniques to enable diagnosticians to identify pathogens in *A. cerana* from the Cairns region

Methods used

Multiple colonies of *A. cerana* and *A. mellifera* from across the Cairns region were sampled during July 2012. Sampling involved examining available brood for signs of disease and collecting live and dead bees that would be laboratory tested for pathogens.

Several approaches were used to identify honeybee pathogens. Non-viral pathogens (e.g. bacterial and fungal diseases) were identified primarily by diagnostic polymerised chain reaction (PCR) testing. To detect bee viruses we used injection techniques to amplify viruses in bee pupae, tested bee samples by serology with virus antiserum, used ultracentrifugation to isolate virus particles from bee samples, performed diagnostic PCR testing for bee viruses and applied deep sequencing techniques to detect known and uncharacterised viruses.

Results/key findings

A total of eight colonies of *A. cerana* and fourteen colonies of *A. mellifera* were sampled from across the Cairns region. Four colonies of *A. cerana* had brood available for examination and all *A. mellifera* colonies were inspected for pests and brood disease. There was no evidence of parasitic mites in any colony and small hive beetle was found at low levels only in *A. mellifera* colonies.

PCR testing of non-viral pathogens positively identified chalkbrood and *Nosema ceranae* from both *A. cerana* and *A. mellifera.* Serological testing and diagnostic PCR for bee viruses identified black queen cell virus (BQCV) and Kashmir bee virus (KBV) from both *A. cerana* and *A. mellifera*. Deep sequencing of *A. cerana* and *A. mellifera* adults identified additional viruses including Lake Sinai virus 1 and 2 (LSV1 and LSV2) and several uncharacterised viruses that may or may not be honeybee pathogens.

None of the honeybee pathogens detected were new to Australia. There was also no evidence that novel strains had spread from *A. cerana* to the local *A. mellifera* population. For all non-viral pathogens detected, *A. cerana* carried the same strains known from *A. mellifera* and for the viruses detected, *A. cerana* carried variant strains to those of *A. mellifera*. However, it appears that chalkbrood and possibly the local strains of BQCV and LSV may have spread from *A. mellifera* to *A. cerana*.

The diagnostic techniques described in this report will enable other diagnosticians to screen for honeybee pathogens in *A. cerana* and *A. mellifera*.

The findings of this research will benefit the Australian honeybee industry by providing information that is needed for managing the health of *A. mellifera* and for accessing export markets for Australian live bees.

Implications for relevant stakeholders

The implications of this research for the Australian beekeeping industry are that they are informed of the level of risk from exotic pathogens that could affect the management of *A. mellifera*. These findings also have important implications for the trade of Australian bees with overseas markets, particularly with the United States. Our results did not support their concerns that exotic pathogens could be spread from *A. cerana* to *A. mellifera* and then be introduced to the United States via Australian bee imports. This information is needed by overseas policy makers in order to review current protocols for importing Australian live bees.

The spread of pathogens from *A. mellifera* may also have an impact on the health of this *A. cerana* population. The accumulation of pathogens could negatively affect the successful spread and build-up of *A. cerana* beyond the Cairns region. The likelihood of this will become clearer following the transition to management stage, but could have important beneficial implications for both industry and the wider community.

Recommendations

The following recommendations are targeted at decision makers:

- That a strategy be developed for future monitoring of pathogens in this *A. cerana* population. The sampling limitations of this study may have left some pathogens undetected, especially those at low prevalence during the sampling period. Ideally, additional future testing will include two or three sampling periods throughout the year.

- That some *A. cerana* colonies be allowed to establish and be used for better monitoring of pests and diseases. Access to colonies with brood for examination was found to be difficult in this study. Established colonies are more likely to have detectable brood disease and a greater build-up of pathogens. Following the transition to management there may be an opportunity for suitable colonies to be monitored and periodically sampled.

- That novel viruses detected in this study be further characterised. The detection of a possible calicivirus in both *A. cerana* and *A. mellifera* is particularly interesting, as it may be BVX or BVY. We potentially have a unique opportunity to better understand a long known bee virus for which there is little known.

Introduction

The Asian honeybee *Apis cerana* was detected in Australia near the port area of Cairns, Queensland in 2007. Despite initial efforts to eradicate this exotic pest, it has become established in the region and was declared to be non-eradicable by 2010. With the establishment of *A. cerana* in Australia there is an inherent risk it may have also introduced exotic pathogens. Although it was soon established that the bee did not introduce *Varroa* mite (D. Anderson pers. obs.), there is very little known about other pests and diseases in this population or their potential to be transmitted to local European honeybees (*Apis mellifera*). Establishing the disease status of this *A. cerana* population is needed to determine potential pathogens that pose a risk to Australia's beekeeping industry.

The health status of *A. cerana* as a whole has been poorly studied except with regard to their host interaction with parasitic mites (Anderson & Trueman 2000). It is generally thought they carry a similar suite of pathogens and viruses to those affecting *A. mellifera*. However, some of these pathogens are in fact unique strains that may have differences in virulence and their ability to be transmitted to other bee species is mostly unknown. For instance, *A. cerana* carries a serological strain of sacbrood virus (called Thai sacbrood or Chinese sacbrood) that is shown to have high virulence in this bee (Bailey et al. 1982; Choe et al. 2012). This strain is yet to be found infecting *A. mellifera*, but has been found in other bee species including *Apis dorsata* and *Apis florea* (Allen & Ball 1996). A unique strain of the bacterial disease, European foulbrood, has also been reported from *A. cerana* in India (Bailey 1974). There is little known about this strain and more recent infections of *A. cerana* in China and Korea are reported to be the *A. mellifera* strain (Kang et al. 2012; Zhou et al. 2000). The potential for novel pathogens or strains carried by Asian honeybees to impact *A. mellifera* still remains largely unexplored.

Initial investigations of the Cairns *A. cerana* population showed it belongs to the Java haplotype of this bee, which occurs in Indonesia, Papua New Guinea and Solomon Islands. The disease status of this haplotype has not been well studied, but is known to carry the Asian strain of sacbrood virus which can have high virulence locally (D. Anderson pers. obs.). While examinations of the Cairns population have determined that no parasitic mites were introduced, the presence of other pathogens and particularly viruses remains unknown. Exotic pathogens from this bee put at risk the health of *A. mellifera* in Australia and can greatly impact on the beekeeping industry. The uncertainty of novel pathogens has already had a significant impact on Australia's live bee exports. In 2010, the United States prohibited imports of Australian bees because of the risk of exotic viruses being spread from *A. cerana* to the local *A. mellifera* and then into the United States via exports from Australia. Other countries such as Canada are also reviewing their current protocol for importing Australian bees (Clarke 2012). Of specific concern for the United States was the introduction of slow paralysis virus. This is a relatively rare virus that has not been reported from Australia or *A. cerana*, but has been associated with Varroa-induced colony mortality (de Miranda et al. 2010). Determining what pathogens are carried by the *A. cerana* in Cairns and whether they have infected the local *A. mellifera* is essential for managing the health of Australia's *A. mellifera* and to maintain and re-establish international markets for Australian live bees.

Objectives

1. Collect samples of live and dead brood and adult bees of *A. mellifera* and *A. cerana* (Java strain) from across the Cairns region

2. Collect and examine comb from both species for signs of abnormalities and diseases

3. Test collected samples for the presence of described and undescribed honeybee pathogens including viruses, fungi, protozoa and bacteria

4. Where possible, identify the (likely) transmissibility of identified pathogens from *A. cerana* to *A. mellifera* in the Cairns region

5. Provide a set of diagnostic techniques to enable diagnosticians to identify pathogens in *A. cerana* from the Cairns region

Methodology

Sample collection and brood inspections

Samples of *A. mellifera* and *A. cerana* were collected from across the Cairns region over 2 weeks in July 2012. These samples included adult bees and brood to be tested for pathogens by diagnostic PCR and also live brood to be used for amplifying viruses.

All *A. mellifera* colonies were managed hives provided by a local beekeeper, Mr Maurie Damon. These colonies are generally kept at single locations, although some are occasionally moved around the Cairns region. The Department of Agriculture, Fisheries and Forestry (DAFF) provided all the *A. cerana* colonies for this study through calls from the public during the sampling period. Some colonies were recent swarms with no comb yet produced, although several colonies were more established with multiple brood combs.

For all *A. mellifera* and *A. cerana* colonies we made observations on colony health by looking for dead or dying adult bees and by examining multiple brood combs (when available) for signs of disease. We searched for symptoms of the brood diseases chalkbrood, American foulbrood (AFB), European foulbrood (EFB) and sacbrood disease. We also examined colonies for the presence of *Varroa* mites, *Tropilaelaps* mites and small hive beetle (*Aethina tumida*).

Samples of dead/dying adult bees and pupae suspected of being infected with brood diseases were collected in 1.7 ml collection tubes. Live adult bees were collected in screw-top containers with approximately 200 bees collected from each *A. mellifera* colony and up to several hundred bees collected from each *A. cerana* colony. All samples were transported on ice to the laboratory and stored at -20 °C.

Microscopic detection of non-viral honeybee pathogens

Adult bees were examined microscopically for the presence of the internal pathogens *Nosema ceranae, N. apis*, tracheal mites (*Acarapis woodi*) and *Malpighamoeba mellificae*.

For detection of *Nosema* spores, groups of 10 bees were homogenised with a mortar and pestle in 4 ml of distilled water. The homogenate was examined for *Nosema* spores on a microscope slide at 400x magnification under a light microscope (Leica Microsystems). A total of 100 bees per *A. cerana*

colony and 50 bees per *A. mellifera* colony were examined. If *Nosema* spores were detected, 30 bees were examined individually to determine the level of infection. We also dissected out and examined the Malpighian tubules from 100 *A. cerana* and 30 *A. mellifera* per colony at 100x magnification under a light microscope (Leica Microsystems) to detect tracheal mites and Malpighamoeba.

Virus amplification in honeybee pupae

Live brood from several *A. cerana* and *A. mellifera* colonies was collected and brought to the Northern Australia Quarantine Strategy (NAQS) laboratory based at the Cairns Airport. White-eyed stage pupae were extracted from the brood comb and following the methods of Anderson (1984) were used to amplify viruses that were present in the colony.

Separate adult and larval extracts were created for each colony by grinding 30 bees in 10 ml of 0.5 M potassium phosphate buffer pH 8. The homogenate was evenly distributed among ten 1.7 ml centrifuge tubes followed by 100 µl of diethyl ether added to each tube and briefly vortexed. Then 200 µl of chloroform was added to each tube, shaken vigorously for 30 seconds and centrifuged for 2 minutes at 12 000 rpm. The supernatant, which contains possible virus particles, was collected from each tube and pooled for each sample.

To amplify viruses, approximately 1 ml of diluted bee extract was passed through a 0.22 µm bacterial filter and injected into the abdomen of white-eyed pupae from the same colony between the inter-segmental spaces. For each colony, approximately 50 pupae were injected with adult and larval extracts. Injected pupae were placed in 90 mm petri dishes lined with filter paper and kept in an incubator at 30 °C for up to seven days. Pupae were monitored daily for signs of infection, which can include stopped development, fluid build-up and discolouration of the body and eyes. Individual extracts of several pupae displaying signs of infection were made by the same methods stated above and re-injected into white-eyed pupae of the same bee species to further amplify potential viruses.

At the end of the 2 week sampling period, all injected pupae were collected in individual 1.7 ml centrifuge tubes and transported on ice to the CSIRO Black Mountain laboratory in Canberra, ACT and stored at -20 °C.

Serological analysis for honeybee viruses

All injected pupae were tested serologically for honeybee viruses at the CSIRO Black Mountain laboratory. This approach is less sensitive than molecular methods but is valuable by only detecting viruses present at replicative levels. There are also several honeybee viruses for which we have no genetic information and can only be detected serologically.

Extracts were made from each pupa as described above using 900 µl of 0.5 M potassium phosphate buffer, 100 µl of diethyl ether and 200 µl of chloroform. Conventional Ouchterlony gel-diffusion tests (Ouchterlony 1964) were used as described by Bailey (1982) with 2% NaCl added to the gels. This modification is needed to detect the Asian strain of sacbrood virus (Thai sacbrood). Twelve different virus antisera were used which included; acute bee paralysis virus (ABPV), Kashmir bee virus (KBV), sacbrood virus (SBV), black queen cell virus (BQCV), slow paralysis virus (SPV), cloudy wing virus (CWV), chronic bee paralysis virus (CBPV), bee virus X (BVX), bee virus Y (BVY), Arkansas bee virus (ArBV), Apis cerana virus-1 (ACV1) and Apis cerana virus-2 (ACV2). Antisera for ACV1 and ACV2 were made against 2 uncharacterised viruses that were isolated from *A. cerana* in PNG during previous studies. Only adult samples were tested against antisera for CPBV, BVX and BVY because they are only found in adult bees. Positive reactions to antisera were identified from a precipitation line forming between the antisera and sample wells.

Isolation and purification of honeybee viruses

Using ultracentrifugation methods we aimed to isolate and purify viruses from adults and larvae. For each colony, 50 adult bees were homogenised with a mortar and pestle in 30 ml of 0.5 M potassium phosphate buffer and 3 ml of diethyl ether. The homogenate was filtered through gauze into screw-top centrifuge tubes and 3 ml of chloroform was added. After vigorous shaking for 30 seconds, tubes were centrifuged at 6 000 rpm for 30 minutes in an Avanti J-E centrifuge. Supernatants were transferred to Ultraclear SW28 tubes (Beckman Coultier) and centrifuged at 25 000 rpm for 3.5 hours at 4 °C in a Beckman L-80 ultracentrifuge. Pelleted samples were redissolved in 1 ml of 0.01 M potassium phosphate buffer. The concentrated samples were then passed through a CsCl gradient in Ultraclear SW41 tubes (Beckman Coulter) by ultracentrifugation at 38 000 rpm for 17 hours at 4 °C. Potential virus bands were removed from the gradient using a glass pipette and transferred to a fresh SW41 tube. Buffer was added to the tubes and potential virus particles were pelleted by centrifugation at 38 000 rpm for 2 hours at 4 °C then resuspended in 1 ml of 0.01 M potassium phosphate buffer.

PCR detection of honeybee pathogens

We used diagnostic PCR to compliment the serology results in detecting viruses and to confirm the presence of non-viral pathogens. PCR protocols are available for most known honeybee pathogens and provide greater sensitivity than serology for detecting viruses in low abundance.

For detection of non-viral pathogens, DNA was extracted by different methods suited to each pathogen. For *Nosema* and EFB, 500 µl of spore-positive bee homogenate was placed in a 1.7 ml centrifuge tube with 0.1 g of glass beads (1 mm diameter) and vortexed at 3000 rpm for 1 minute. DNA was then extracted using the High Pure PCR template preparation kit (Roche Diagnostic). For chalkbrood, DNA was extracted from 1 mg of an infected larva (chalkbrood 'mummy') using the PowerPlant Pro DNA isolation kit (MOBIO laboratories). For EFB and AFB, suspected diseased brood was first resuspended in 400 µl of distilled water then 200 µl transferred to a 1.7 ml centrifuge tube. The sample portion allocated for AFB detection was boiled for 10 minutes to release the DNA. The portion allocated for EFB detection was used with the High Pure PCR template preparation kit. PCR reactions for all non-viral pathogens were carried out in a total volume of 10 µl containing 1 x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM forward primer, 0.4 µM reverse primer, 1 U *Taq* DNA polymerase (New England Biolabs) and 1 µl DNA template. PCR cycling conditions and primers sequences for each pathogen are shown in Table 1.

To detect honeybee viruses, RNA was extracted using the Purelink viral RNA extraction kit (Invitrogen) following the manufacturer's instructions. RNA was isolated from extracts prepared as above from injected pupae, group adult and larval samples and from diseased *A. cerana* brood from Gordonvale. Extracted RNA was converted to cDNA using the Phusion RT-PCR kit (Finnzymes) following the manufacturer's protocol and using 100 ng oligo(dT) primers. PCR reactions for all viruses were carried out in 10 µl reactions consisting of 1 x Phusion HF Buffer, 0.2 mM dNTPs, 0.5 µM forward primer, 0.5 µM reverse primer, 0.2 U Phusion Hot Start II DNA polymerase (Finnzymes) and 1 µl cDNA template. PCR cycling conditions and primers sequences for each virus are shown in Table 1.

PCR reactions were analysed on 1.5% agarose gels stained with GelRed (Jomar Biosciences) and viewed under a UV illuminator. Positive PCR products were sequenced then analysed with MEGA5 (Tamura et al. 2011).

Deep sequencing analysis for honeybee pathogens

A pooled adult bee sample for both *A. cerana* and *A. mellifera* were chosen for deep sequencing analysis to detect viruses. Using the group adult extracts made by ultracentrifugation, we pooled 200 µl from each colony and passed this through a 0.22 µm bacterial filter. We then mixed 170 µl of the filtered samples with 5 µl of RNase, 5 µl DNase and 20 µl of DNase I buffer before incubating at 37°C for 2 hours. RNA was extracted from the treated samples using the Purelink viral RNA extraction kit. Illumina libraries were prepared from the extracted RNA and 250 bp paired-end sequences were generated on an Illumina MiSeq at Micromon (Monash University, Clayton, Victoria). Raw reads were quality trimmed and assembled using Trinity (Grabherr et al. 2011) with a minimum contig length of 1000 bp. Assembled transcripts were then identified through BLASTn and promising candidates were individually examined.

Table 1. Diagnostic primers used to detect honeybee pathogens.

Pathogen	Primers	PCR cycling conditions	Reference
Nosema ceranae	F: CGGCGACGATGTGATATGAAAATATTAA R: CCCGGTCATTCTCAAACAAAAAACCG	94 °C (15s), 60 °C (30s), 72 °C (45s)	Martin-Hernandez *etal (*2007)
Nosema apis	F: GGGGGCATGTCTTTGACGTACTATGTA R: GGGGGGCGTTTAAAATGTGAAACAACTATG	94 °C (15s), 60 °C (30s), 72 °C (45s)	Martin-Hernandez *etal* (2007)
Chalkbrood	F: GTTTCCGTAGGTGAACCTGC R: ATATGCTTAAGTTCAGCGGGT	94 °C (30s), 52 °C (30s), 72 °C (45s)	Anderson *etal* (1998)
American foulbrood[a]	F: TCGAGCGGACCTTGTGTT R: CTATCTCAAAACCGGTCAGAG	94 °C (30s), 59 °C (30s), 72 °C (45s)	Lauro *etal* (2003)
American foulbrood[b]	F: CTTCGCATGAAGTCATG R: TCAGTTATAGGCCAGAAAGC	94 °C (30s), 59 °C (30s), 72 °C (45s)	Lauro *etal* (2003)
European foulbrood	F: CTTTGAACGCCTTAGAGA R: ATCATCTGTCCCACCTTA	94 °C (30s), 60 °C (30s), 72 °C (45s)	McKee *etal* (2003)
Sacbrood virus	F: GCTGAGGTAGGATCTTTGCGT R: TCATCATCTTCACCATCCGA	98 °C (10s), 58 °C (10s), 72 °C (45s)	Chen *etal* (2004)
Black queen cell virus	F: TGGTCAGCTCCCACTACCTTAAAC R: GCAACAAGAAGAAACGTAAACCAC	98 °C (10s), 56 °C (10s), 72 °C (45s)	Benjeddou *etal* (2001)
Kashmir bee virus[c]	F: AATGTGGGTTGATACATTGAAAGA R: CGTTGCGGGATTTCCAGA	98 °C (10s), 60 °C (10s), 72 °C (30s)	Meuss *etal* (2010)
Chronic bee paralysis virus	F: AGTTGTCATGGTTAACAGGATACGAG R: TCTAATCTTAGCACGAAAGCCGAG	98 °C (10s), 56 °C (10s), 72 °C (30s)	Ribiere *etal* (2000)
Acute bee paralysis virus	F: TGAGAACACCTGTAATGTGG R: ACCAGAGGGTTGACTGTGTG	98 °C (10s), 56 °C (10s), 72 °C (30s)	Tentcheva *etal* (2004)
Slow paralysis virus	F: GATTTGCGGAATCGTAATATTGTTTG R: ACCAGTTAGTACACTCCTGGTAACTTCG	98 °C (10s), 56 °C (10s), 72 °C (45s)	deMiranda *etal* (2010)
Deformed wing virus	F: CTCGTCATTTTGTCCCGACT R: TGCAAAGATGCTGTCAAACC	98 °C (10s), 56 °C (10s), 72 °C (30s)	Singh *etal* (2010)
Lake sinai virus 1	F: CTTGAAACTCAGGGCTTCGTTAC R: AGGGATGACGGAGCACAATT	98 °C (10s), 50 °C (10s), 72 °C (30s)	Runckel *etal* (2011)[d]
Lake sinai virus 2	F: TTCCGGTCTGTCCTTTACGC R: AAGCGCAAGGATGCAAAGTG	98 °C (10s), 50 °C (10s), 72 °C (30s)	Runckel *etal* (2011)[d]

[a] AFB external primers are used on the DNA samples
[b] AFB internal primers are used on the PCR product from the external primers
[c] Israeli acute paralysis virus (IAPV) is a strain of KBV and is detectable with these primers
[d] Primers were modified from the original reference for greater strain specificity

Results

Honeybee collections and brood inspections

A total of eight colonies of *A. cerana* and fourteen colonies of *A. mellifera* were sampled from across the Cairns region (Figure 1, Table 2). Several *Apis cerana* colonies were from recent swarms, but four colonies were up to six months old and had produced brood comb. These four colonies were inspected for brood pathogens and were also used for virus amplification. All fourteen *A. mellifera* colonies contained brood for inspection and five of these colonies were used for virus amplification.

Brood inspections revealed very little signs of pests and diseases in both the *A. cerana* and *A. mellifera* colonies. Small hive beetle was the only pest species identified in the study and was only found at low levels in all *A. mellifera* colonies inspected. There was no evidence of parasitic mites in any colony, which is consistent with the initial examinations of this population carried out in 2007. Chalkbrood was the most common brood disease identified, with low level infections found in *A. mellifera* colonies from Mareeba, Green Hill, Oak Beach and Edge Hill and a significant infection in the *A. cerana* colony from Gordonvale. We collected 125 infected drone brood (chalkbrood 'mummies') from this one colony, but interestingly there was no evidence of worker brood being infected. In all cases, the capped cells of infected drones were further sealed around the edge and the characteristic hole in *A. cerana* drone cell cappings was plugged (Figure 2).

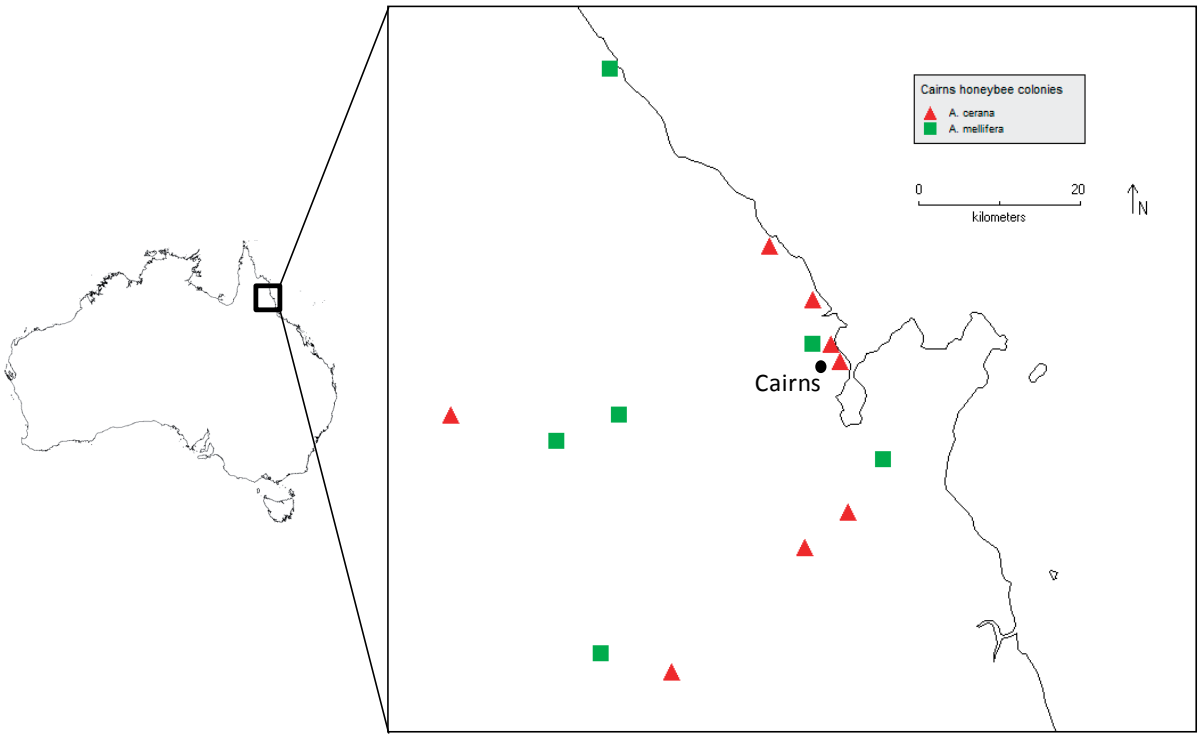

Figure 1. Map of *Apis cerana* and *Apis mellifera* colonies sampled from the Cairns region of Queensland, Australia.

Table 2. Honeybee colonies examined for pests and diseases.

Sample/Location	Species	# colonies	Brood inspected	Non-viral pathogens detected[2]	Viruses detected[2]
Atherton	*A. mellifera*	3	yes[1]	---	BQCV, KBV
Mareeba 1		4	yes[1]	chalkbrood, SHB, *N. ceranae*	KBV, LSV2
Mareeba 2		1	yes	---	LSV1, LSV2
Green Hill		3	yes[1]	chalkbrood, SHB, *N. ceranae*	KBV, LSV2
Oak Beach		2	yes[1]	chalkbrood, SHB	KBV
Edge Hill		1	yes[1]	chalkbrood, SHB	KBV, LSV1, LSV2
Little Mulgrave	*A. cerana*	1	yes[1]	*N. ceranae*	KBV
Cairns 1		1	yes[1]	---	KBV
Mareeba 3		1	no	---	KBV
Trinity Beach		1	no	*N. ceranae*	---
Machan's Beach		1	no	*N. ceranae*	---
Gordonvale		1	yes[1]	chalkbrood, *N. ceranae*	BQCV, KBV
Cairns 2		1	yes[1]	---	KBV
Yungaburra		1	no	---	LSV1

[1] Brood was injected for virus amplification
[2] Detected by serology and/or PCR

Figure 2. Brood diseases observed in the *Apis cerana* colony at Gordonvale. (a) Potential bacterial infection found in a drone cell. (b) Sealed drone cells with a chalkbrood 'mummy'. (c) Open drone cells from (b) showing chalkbrood 'mummies'.

The Gordonvale colony was the only *A. cerana* colony to show brood disease and was also the most established (Figure 3). This colony was estimated to be up to six months old and appeared to have recently swarmed based on the presence of several used queen cells and the composition of the hive. In addition to chalkbrood, possible bacterial disease was found in three drone cells. These infections had a strong odour and were light brown in colour, but were not particularly ropey in texture (Figure 2). These were possible symptoms of EFB or AFB infections and were collected for further analysis. Several other diseased pupae found in the Gordonvale colony were suspected of succumbing to viral infections. Nine drone pupae and one queen pupa were found inside capped cells and collected for further analysis. In contrast, there were no signs of bacterial diseases or viral infections in any *A. mellifera* colonies inspected.

Figure 3. *Apis cerana* colony at Gordonvale, Queensland with brood diseases.

Non-viral pathogens detected

The presence of non-viral pathogens was examined further in the laboratory by microscopy and molecular testing. Chalkbrood samples from *A. cerana* and *A. mellifera* brood were tested by PCR and were both confirmed as *Ascophaera apis* based on a 588 bp fragment of the ITS rDNA region. We also confirmed the species status by successfully mating it with the local Canberra isolate. Chalkbrood has been identified in *A. cerana* from China and India (Fries 2011), but has not been recorded from populations in Indonesia, PNG or Solomon Islands (where the Java strain occurs). We suspect that the *A. cerana* population in Cairns has acquired chalkbrood since arriving in Australia from the local *A. mellifera* population. The extent to which this pathogen is affecting the Cairns *A. cerana* population is unknown, but it does not appear to be serious as it was only detected in drone brood.

The potential bacterial samples collected from Gordonvale were also tested by PCR for identification. Surprisingly, these samples were not positively identified as either EFB or AFB. Based on the observed symptoms of these infections, we believe further investigation is still needed. There is little evidence of bacterial infections in *A. cerana*, however EFB has been reported from colonies in northern Asia (Kang et al. 2012; Zhou et al. 2000). While some of these reports suggest that EFB has spread to *A. cerana* from *A. mellifera*, Bailey (1974) also identified a distinct strain of EFB that may be endemic to Asian honeybees. It is still possible that the infections observed in the Gordonvale colony were a strain of EFB that was not detected by the diagnostic primers. EFB can be disguised by secondary bacterial infections such as from *Paenibacillus alvei*, which may have interfered with positive detection in this study (Forsgren 2010). However, it is important to note that secondary bacteria can also mask other causes of brood death including other pathogens, but also stressors such as chilling or poor nutrition.

Adult bees of *A. cerana* and *A. mellifera* were also microscopically analysed for the presence of *Nosema,* tracheal mites and Malpighameoba. Only *Nosema* was detected in these colonies but at relatively low levels. *Nosema* was found in adult *A. cerana* from Little Mulgrave, Gordonvale, Trinity Beach and Machan's Beach and in adult *A. mellifera* from Mareeba and Green Hills. We used PCR testing to identify the species present in each colony and found only *N. ceranae*. A comparison of a 218 bp sequence of the 16S rDNA region found no differences between the Cairns isolates and reference strains from Australia and Southeast Asia. It is difficult to determine if *N. ceranae* has spread between the two bee populations. But it is interesting that *N. ceranae* was first detected in Australia from *A. mellifera* collected in 2007 and 2008 and was most common in Queensland, while only *N. apis* was found in Western Australia and Tasmania (Giersch et al. 2009). There is growing evidence that *N. ceranae* is an endemic pathogen of *A. cerana* with a recent study in the Solomon Islands showing the spread of this pathogen to the local *A. mellifera* being linked with the invasion of *A. cerana* (Botias et al. 2012). Although examinations of Australian *A. mellifera* from the 1990s found only *N. apis* (Rice 1999; 2001), it will be difficult to determine if *N. ceranae* was introduced by the *A. cerana* in Cairns.

Honeybee viruses detected

Several methods were used to detect honeybee viruses: injection techniques to try and amplify viruses in bee pupae, ultracentrifugation of group bee extracts to isolate virus particles, serological analyses and molecular testing of bee samples.

Injected pupae showed signs of being infected with virus, although the symptoms were not consistent with our previous experiences with this technique. A total of 327 *A. cerana* and 596 *A. mellifera* pupae were tested serologically for known honeybee virus. For *A. cerana*, all pupae gave a positive reaction against the KBV antiserum. Precipitation lines were not always strong and were positioned closer than expected to the antiserum well. This observation and the prevalence of the reaction suggested it might be a non-specific reaction to bee protein. No other viruses were detected by serology in *A. cerana*. For *A. mellifera*, 106 pupae reacted to the KBV antiserum, although for many the reaction was similar to that found with *A. cerana*. However, several pupae from Edge Hill, Oak Beach, Atherton and Mareeba

gave clear positive reactions to the KBV antiserum. The only other virus detected was BQCV, which was detected in five pupae from Atherton.

Using PCR, we tested selected virus-positive pupae, group extracts of adult bees and larvae as well as diseased pupae from Gordonvale. Very few viruses were found in these samples with only KBV and BQCV reliably detected. However, detection of KBV was inconsistent with the serology results, as only 9 out of 70 pupae tested were positive. Furthermore, no adult or larval extracts of *A. mellifera* or *A. cerana* were positive for KBV. A 428 bp fragment of the RNA-dependent RNA polymerase (RdRp) gene was sequenced from the positive *A. cerana* and *A. mellifera* pupae and confirmed to be the same as a local KBV strain. Comparison with reference strains showed the Cairns isolates to be 96 – 98 % similar with KBV isolates from Spain, Korea, Canada and Australia. The PCR results suggest that non-specific proteins may have caused many of the serological reactions. This is further supported by our observations of the injected pupae, as there was not the rapid death and deterioration that usually occurs with KBV infections (ref). Alternatively, another strain of KBV may be present that responds poorly to the antiserum used and is not detected with our PCR primers.

PCR supported the serology results for BQCV, with a 632 bp fragment of the RdRp gene amplified from all five Atherton pupae. Surprisingly, none of the *A. mellifera* adult or larval extracts were positive, although BQCV was amplified from the *A. cerana* adult extract from Gordonvale and also from the dead queen pupa from Gordonvale. The lack of BQCV detected in pupae from Gordonvale is likely because the extract used for injection was a diseased drone pupa rather than a group adult or larval extract. This choice was made because of the limited pupae available at the time of injection. BQCV isolates from both *A. cerana* and *A. mellifera* were sequenced and compared with reference strains (Figure 4). There was 99 % sequence identity between the two strains, which also formed a distinct group from other BQCV isolates. Two serologically distinct strains (identified as B1 and B4) have been isolated from Australia previously (Anderson, unpublished). Here, the Cairns isolates were most similar to the B1 strain (97%), while the B4 strain groups among isolates from the United States and Asia. Therefore it is likely that the Cairns isolates are also serologically distinct from B4 and similar strains. Interestingly, there is no apparent relationship between BQCV isolates from the same host, with the Cairns *A. cerana* isolate more similar to Australian isolates rather than those of *A. cerana* from China and South Korea. This could indicate that the local BQCV strain has spread to *A. cerana*, but this cannot be confirmed without knowing the BQCV strain that naturally infects the Java haplotype in Asia.

We also used ultracentrifugation to try and isolate viruses from group extracts of *A. cerana* and *A. mellifera* adults. Attempts to isolate viruses in CsCl gradients resulted in no clear virus bands, although a faint cluster of bands was observed in the Little Mulgrave *A. cerana* sample. The position of these bands was much higher in the gradient than expected for honeybee viruses, which suggests they are simply from bee protein. Nevertheless these bands were collected for further analysis. Following this result, group extracts were made from the injected pupae of *A. cerana* and subjected to ultracentrifugation. This resulted in single clear bands for the Little Mulgrave and Cairns 1 *A. cerana* pupae, which was also positioned high in the gradient. Although it is likely that these bands are bee protein, they too were collected for further analysis. However, as expected, PCR testing of these samples did not detect any viruses.

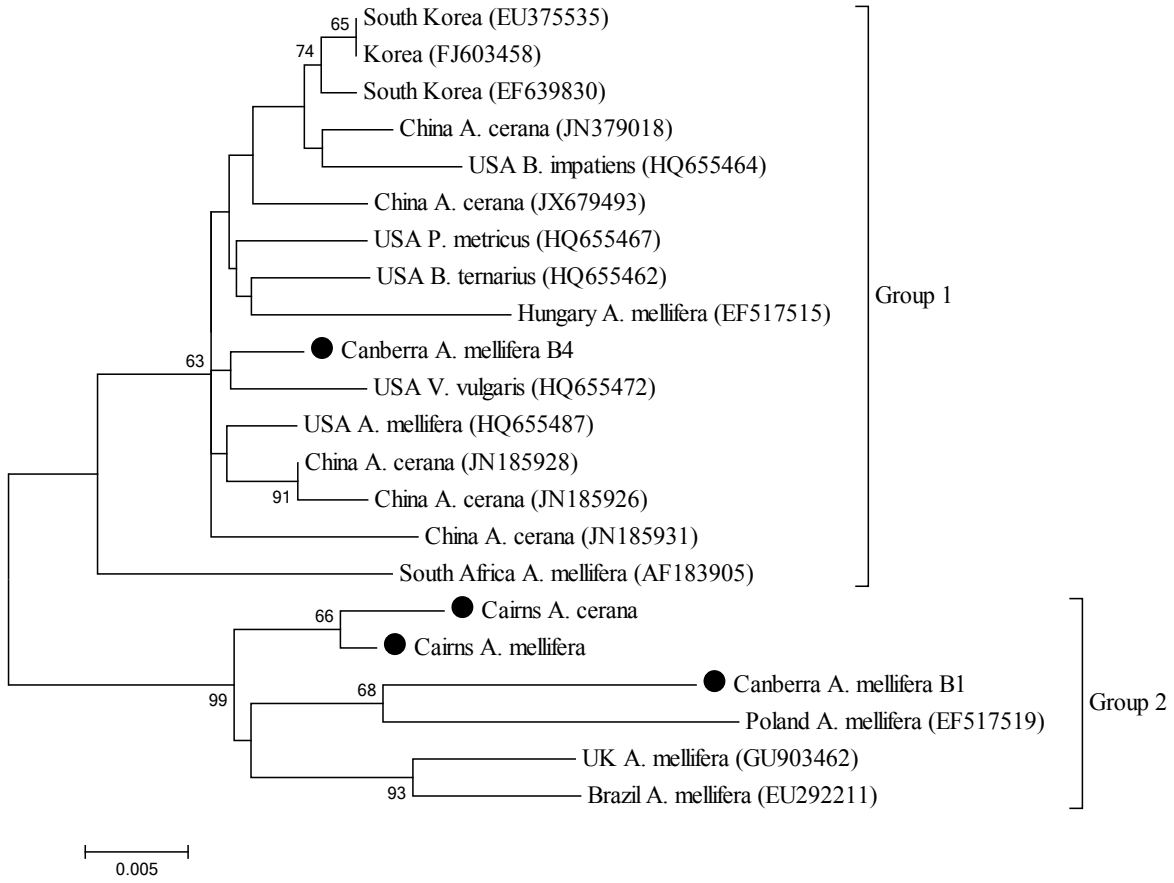

Figure 4. Neighbour-joining tree using a Tamura-3 parameter distance model of a 591 bp region of the RdRp gene of BQCV. Isolates from this study are indicated by a closed circle.

Virus detection by deep sequencing

Deep sequencing of pooled adult extracts for *A. cerana* and *A. mellifera* resulted in ~10.7 million reads of RNA sequence data for the two samples. After raw reads were quality trimmed, contigs greater than 1000 bp in length were assembled. From this dataset 40 contigs had significant similarity to viral sequence. Of these, BQCV was the only characterised honeybee virus detected. Single contigs covering approximately 90% of the BQCV genome were produced from both the *A. cerana* and *A. mellifera* samples. Consistent with the PCR results, virus strains were different for each bee species but were 99 % similar over the genome. It's surprising that KBV and SBV were not detected by this method, as these viruses are common in Australia and KBV was detected in some injected pupae. Various factors could have influenced this result including the seasonality of the viruses or the sample type used for sequencing, however we would expect detection of these viruses in subsequent surveys.

Two other viruses were sequenced from both *A. cerana* and *A. mellifera* that were most closely related to Lake Sinai Virus 1 and 2 (LSV1 and LSV2) (Figure 5). These viruses have recently been identified from *A. mellifera* in the United States and Spain but are not yet fully characterised (Granberg et al. 2013; Runckel et al. 2011). Contigs related to LSV1 were 90 % similar over common regions and each was approximately 77 % similar to the United States strain of LSV1. This level of difference may indicate that the Cairns isolates represent a closely related but distinct virus from LSV1. In contrast, contigs related to LSV2 showed high sequence similarity to the United States strain and likely represent geographical isolates of this virus. The Cairns isolates were 99 % similar over common regions and were 92 % similar to the United States strain of LSV2. We then used PCR primers

modified from those developed for LSV1 and LSV2 by Runckel et al. (2011) to screen adult and larval extracts from each colony. This detected LSV1 in the *A. mellifera* colonies Edge Hill and Mareeba 2 and the *A. cerana* colony Yungaburra. LSV2 was detected in the *A. mellifera* colonies Edge Hill, Green Hill, Mareeba 1 and Mareeba 2, but not in any *A. cerana* colony. Differences in the priming sites and low target abundance may explain the lack of detection in *A. cerana* colonies. The presence of these viruses from several colonies in this study and in similar studies overseas suggests they may be relatively common in honeybees. Runckel et al. (2011) have further shown that these viruses can replicate in *A. mellifera*. However, more research is needed to determine if they are truly bee viruses or perhaps bee viruses for which no sequence is known such as cloudy wing virus. But importantly, determine if they are pathogenic in honeybees.

Several other viruses were identified from the deep sequencing analysis. Partial genomes of three known hemipteran viruses, Himetobi P virus (HiPV), Rhopalosiphum padi virus (RhPV) and Aphid lethal paralysis virus (ALPV) were found from *A. cerana*. However, these strains are quite variable from the reference genomes with only 88 %, 84 % and 91 % similarity found, respectively. None of these insect viruses were detected in *A. mellifera*, although we did find two plant viruses in *A. mellifera* that were not found in *A. cerana*. Partial genomes of Squash mosaic virus and Kennedya yellow mosaic virus were detected with 88 % and 73% similarity to the reference genomes, respectively. Further research will be needed before we will understand if any of these viruses are pathogenic to honeybees or in the case of the plant viruses, are vectored by honeybees.

In addition, several partial genomes were found in both *A. cerana* and *A. mellifera* that are of uncharacterised RNA viruses. One of these viruses had near complete genomes produced from both *A. cerana* and *A. mellifera* and based on conserved coding domain homology is likely an uncharacterised calicivirus. Remarkably, Bailey (1980) commented that BVX and BVY have similar properties to the mammalian caliciviruses. Therefore it is possible that one of these currently unsequenced viruses has been detected here. Adult extracts were negative when tested against antisera to BVX and BVY, but this may be due to low concentration. Several other partial genomes of uncharacterised viruses were recovered from *A. cerana* and *A. mellifera*, but further research will be needed to fully characterise these viruses and understand their importance for honeybees.

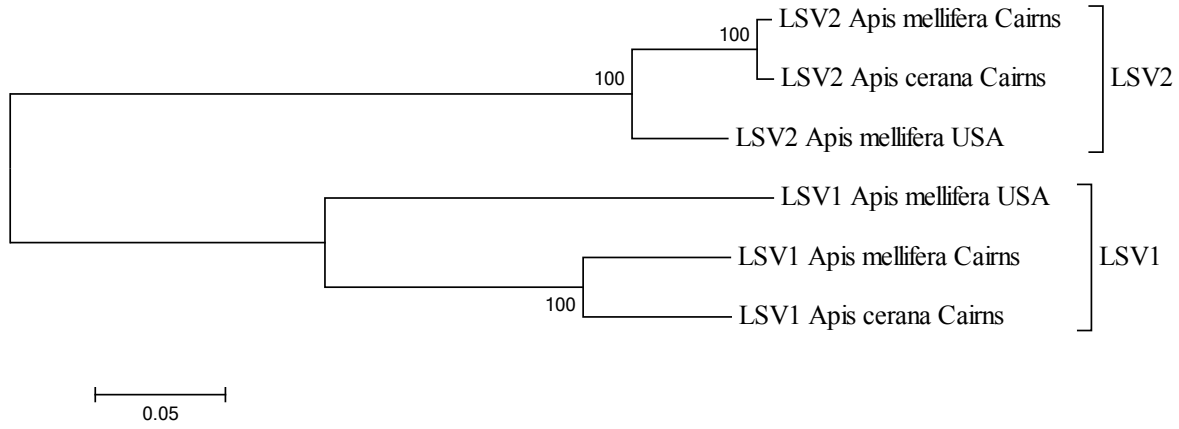

Figure 5. Neighbour-joining tree using a Tamura-3 parameter distance model of a 1170 bp overlapping region of LSV1 and LSV2.

Implications

The findings of this research have important implications for the Australian beekeeping industry through a better understanding of the level of risk from exotic pathogens affecting the management of *A. mellifera*. However, a limitation of this study was that there was only a single sampling period. Seasonal variation in abundance may have resulted in some pathogens going undetected. Clearly not all the pests and diseases of *A. mellifera* known from Australia were found here, so the possibility of some pathogens going undetected in *A. cerana* cannot be ruled out. Though, the likelihood of a serious pathogen being introduced and not being detected in this study or by the local beekeeping community would be low.

There are also important implications for the trade of Australian bees with the United States and other overseas markets. Our results did not support their concerns that exotic pathogens (including SPV) could be spread from *A. cerana* to *A. mellifera* and be introduced to the United States via Australian bee imports. *Nosema ceranae* is the only pathogen detected that may have been transferred to Australia's *A. mellifera*, but this is not an issue for the United States export market. Although not detected here, findings from another project looking at the Asian SBV (or TSBV) that infects the Java haplotype of *A. cerana* also indicate that this virus is not transferred to *A. mellifera*. The nature of the deep sequencing approach also results in the detection of uncharacterised viruses. However, further investigation of these viruses is needed to determine their importance for *A. mellifera* and should not impact on trade, especially in the absence of known pathogenicity or disease. This information is important for overseas policy makers in order to review current protocols for importing Australian live bees.

The spread of pathogens from *A. mellifera* may also have an impact on the health of this *A. cerana* population. We found evidence to suggest that chalkbrood and possibly local strains of BQCV and LSV were introduced to *A. cerana* after arriving in Australia. Because of the robbing behaviour of this bee it is likely to come in contact with many *A. mellifera* pathogens. Studies have already shown chalkbrood and EFB can been highly damaging to *A. cerana* in some areas of Asia. Therefore, the accumulation of pathogens may ultimately have a negative impact on the spread and densities of *A. cerana* beyond the Cairns region. The likelihood of this will become more apparent as the elimination of *A. cerana* colonies stops within the infested area following the transition to management stage.

Recommendations

- That there be a strategy developed for future monitoring of pathogens in this *A. cerana* population. The sampling limitations of this study may have left some pathogens undetected, especially those at low prevalence during the sampling period. Ideally, additional future testing will include two or three sampling periods throughout the year.

- That some *A. cerana* colonies be allowed to establish and be used for better monitoring of pests and diseases. Access to colonies with brood for examination was found to be difficult in this study. The Gordonvale colony is evidence that established colonies are more likely to have detectable brood disease and a greater build-up of pathogens. Following the transition to management there may be an opportunity for suitable colonies to be monitored and periodically sampled.

- That novel viruses detected in this study be further characterised. The detection of a possible calicivirus in both *A. cerana* and *A. mellifera* is particularly interesting, as it may be BVX or BVY. We potentially have a unique opportunity to better understand a long known bee virus for which there is little known.

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Establishing the disease status of the Asian honeybee in the Cairns region

By Dr John Roberts and Dr Denis Anderson

Pub. No. 13/082

This report describes the identification of pests and diseases of the Asian honeybee (*Apis cerana*) in the Cairns region of Australia and outlines the likely spread of exotic pathogens to the local European honeybee (*Apis mellifera*) population.

This research is important for understanding potential risks to the health of Australia's managed and wild *A. mellifera* populations.

The report is targeted at the Australian beekeeping industry and apiarists for the better management of bee pathogens and also at decision-makers who develop policy in regards to exports of live bees in the State and Commonwealth Governments and in governments overseas.

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