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Assessment of the risks of transmission of myrtle rust (*Austropuccinia psidii*) spores by honey bees (*Apis mellifera*)

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Executive summary

Assessment of the risks of transmission of myrtle rust (*Austropuccinia psidii*) spores by honey bees (*Apis mellifera*)

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May 2018

Honey bees (*Apis mellifera*) have been observed foraging on and collecting myrtle rust (*Austropuccinia psidii*, "MR") spores, which would result in spores being brought back to their hives. If these spores remain viable within the hive, the long-distance movement of hives could be a means of spreading this plant pathogen. To help quantify this risk, we sought to determine the rates of spores being brought into hives by foraging bees, and to assess the survival of spores inside the hive environment.

We detected MR spores on returning forager bees and in pollen stores inside hives. We found that MR spores remain viable (able to germinate) within beehives and on worker bees for at least 9 days (limit of this test).

The greatest risk for New Zealand is that many bee hives are now being transported great distances into remote locations to produce honey, specifically mānuka honey, and this may lead to the transfer of spores to areas that would not have been exposed to the spores through wind movement. Mānuka plants are susceptible to myrtle rust and therefore this commercial activity could inadvertently spread myrtle rust into otherwise unaffected regions.

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1 Introduction

Austropuccinia psidii, (commonly known as myrtle rust; MR), is a fungal pathogen of plants in the family Myrtaceae with a broad host range. The pathogen was first recorded in North Island, New Zealand in May 2017, and has since been detected at locations through North Island and at the top of South Island.

Plant species in the Myrtaceae family in New Zealand have considerable cultural, ecological and economic importance, and the pathogen has the potential to have severe negative impacts to these values through the loss of populations of these plant species. As a primarily wind-dispersed pathogen, eradication or management of the pathogen is complex. An understanding of the potential transmission pathways and their relative risks is important for making decisions about how to avoid or mitigate the spread of the pathogen to new areas.

Western honey bees (*Apis mellifera*) are managed in New Zealand for honey production and crop pollination. The most economically valuable honey crop comes from the myrtaceous mānuka (*Leptospermum scoparium*), but beekeepers also seek honey crops from pōhutukawa (*Metrosideros excelsa*) and rātā species (*Metrosideros spp*), both in the Myrtaceae family. In addition, honey bees will visit most other native and exotic members of the family.

Honey bees have been observed actively collecting MR spores (Shaw 1999; Carnegie et al. 2010), and this behaviour could lead to the build-up of spores within a hive that could then be transferred to new susceptible hosts or new outbreak-free locations. Of principal concern is the long-distance movement of hives from MR outbreak areas into remote back-country sites for mānuka honey harvest; a movement that could potentially transmit the pathogen in a direction contrary to prevailing winds.

To determine the relative risk of honey bees as vectors of MR, it is necessary to first determine whether spores can survive within a hive environment and whether spores are actively brought into hives by bees. The environment within a hive near the brood is kept at a constant 36°C at relatively high humidity, and bees are highly effective at grooming themselves which could reduce the probability of spores remaining and surviving on bees and in the hive.

The aim of this project was to assess the relative risk of MR spore transmission via the movement of honey bees (*A. mellifera*) or beehives by assessing the movement of spores into hives by bees and assessing the survival of spores once in the hive.

Due to legal constraints around the handling and research of MR in New Zealand, the field work was conducted in Brisbane, Queensland (QLD), Australia with our partners at the Queensland University of Technology (QUT) and the Queensland Department of Agriculture and Fisheries (QDAF).

2 Methods and results

2.1 TRIAL DESIGN AND SAMPLING PROTOCOL

The overall design of the study was to place honey bee hives at locations in and around Brisbane, QLD, Australia, in proximity to active MR outbreaks for a period of 2 weeks to allow the bees opportunity to forage on the spores. At the end of this 2–week exposure, samples of returning foragers and pollen stores were taken from the hives. The hives were then moved to containment screen houses for 2 weeks for further research on spore persistence and viability without further exposure to natural sources of spores. Experiments were conducted to test and develop methods to assess:

- The presence of MR spores on foraging bees in the field
- The presence of MR spores in pollen stores in hives from the field
- The viability of spores over time in hives
- The persistence and viability of spores on bees in hives.

The first trial was conducted primarily to test feasibility of the methodology, particularly to see if hives would survive in screen houses. In December 2017, single hives were placed at three locations in close proximity to sporulating MR outbreaks; Moggill (Brisbane), The Channon (New South Wales; NSW) and the Tallebudgera Valley (SE Queensland). The foraging behaviour of bees was observed in the field. After 2 weeks, samples were taken of up to 25 pollen-foraging bees returning to each hive and 50 bees from within each hive, along with samples of pollen cell contents, before the hives were moved into containment.

The containment cages were 7 x 7 x 3 m insect-mesh screen houses located at QUT environmental research facility at Samford (SERF). Each hive was kept in a separate screen house and fed sugar syrup, water and a pollen substitute. While in containment, hive worker bees (50 per day) were collected daily or at 2–day intervals over a 2 week period. In the first trial we were able to demonstrate the viability of keeping colonies alive for up to 3 weeks with this methodology.

In the second trial, six hives were placed for 2 weeks at sites of known MR outbreak at four sites in Brisbane in January 2018 (Moggill, Kenmore, Samford and three subsites within the Brisbane Botanic Gardens at Toowong). After 2 weeks, forager bees and pollen cell contents were collected from the hives. Hives were then moved into containment at SERF for two rounds of further research.

The presence of spores on bees and pollen was initially examined by washing of bees, precipitation by centrifugation and light microscopy. Published quantitative PCR (qPCR) methods were then modified, optimised and used to detect and quantify the spores (from DNA copy number).

Little active foraging on rust by bees was observed in the field and bees in screen houses were found to carry no or very little distinguishable residual spores. Methods were therefore developed to assess viability of MR spores over time in hives using both pure spores and pure spores on live bees placed inside the hives during containment in the SERF screen houses.

In trial one (December 2017), MR spores suspended in 0.05% Tween®80 were dried onto small, concave wax blocks that were secured inside plastic queen bee cages to prevent direct contact with bees inside the hive, and then placed on brood frames within the hive. Four samples per hive were prepared in this manner. Wax blocks were removed on days 0 (control), 1, 4 and 7. Wax contamination of samples in the first trial led to a modified protocol in which pure dry spores were placed inside a lidless 0.5 ml Eppendorf tube with a paper plug, placed within plastic queen bee cages and sampled in the same manner as in trial 1. This was used and repeated in two rounds in trial two (January/February 2018), with the addition of a fifth sample taken on day 9 in round two.

In this second trial, an additional method was developed to test persistence of spores on live bees. MR spores provided by QDAF were used to coat bees collected from the hives, which were then placed in batches of five bees in plastic queen bee cages with a sugar cube. This method ensured that the bees would remain alive over the period of the trial (as bees can be fed by other bees through the cage), while minimising contact with other bees and ensuring that the inoculated bees could be recovered and sampled on subsequent days. Bees remained alive in the cages and were free to groom and consume the spores. Four cages of bees were prepared for each hive in the first round of trial two, and then were sampled on days 0 (control), 1, 4 and 7. This was repeated in in the second round with the addition of a fifth sample taken on day 9.

A second control involved two samples of the spores provided by QDAF that were immediately run through the germination protocol to assess for germination without being placed on wax, in tubes or on bees. These controls are hereafter called “controls”, while the day 0 controls which were placed on bees or in tubes are referred to as “day 0” samples.

2.2 SPORE VIABILITY

Methods were developed to assess the viability of MR spores over time in hives using both pure spores and pure spores on live bees placed inside the hives during containment in the SERF screen houses. The viability of spores using germination assessment was tested using pure spore samples and live bees coated in spores in trial two, from methods developed in trial one. Spore viability and visual presence of spores in pellets were assessed using a germination test on water agar adapted from published methods (Salustiano et al. 2008).

Viability of pure spores in hives

In trial one (December 2017), MR spores suspended in 0.05% Tween80 were placed onto 1 cm concave wax blocks and dried before placement in queen cages in hives as described in Section 2.1 or in a controlled environment 26°C in the dark. After collection from the hive, spores were removed from the wax by gentle agitation in 0.05% Tween80, then pelleted by centrifugation and re-suspended in 100 µL of either paraffin or 0.05%Tween80 in water. The suspension was pipetted onto an agar plate and incubated at 22°C in the dark. Samples suspended in water/Tween80 were covered in paraffin wax on the agar plate before incubation. In both rounds of trial two, dry spores that had been placed in hives in 0.5 ml Eppendorf tube were placed directly onto water agar, covered in paraffin wax, and incubated as with the samples in trial one.

After incubation for 24 to 48 h, germinated and un-germinated spores in each of ‘three fields of view’ on each plate were counted using an inverted microscope. All spores in one or several fields of view were counted and the numbers germinated and not germinated were recorded. Up to 50 spores were counted for each sample, leading to a maximum possible spore count of 300 for any given sample point (i.e. samples from bees and tubes from six hives).

Spore viability on bees

We initially assessed the feasibility of visual identification of spores and spore viability (germination) in material washed from bees collected in either individual forager bees, or groups of forager bees, or bees from within the hives. Bees were washed by mechanical agitation for 15 min in 1 mL (individual) or 40 mL (groups of 5 up to 25 bees) of either 0.05% Tween80 in water or 0.05% Tween80 in paraffin, and the material recovered (which included pollen collected by the bees) was pelleted by centrifugation.

Viability of spores on bees was assessed in trial two. Bees were collected from each hive into 50 ml falcon tubes and chilled briefly on ice, then tipped gently to coat with dry spores provided by QDAF. Coated bees were then placed in batches of five bees in plastic queen bee cages with a sugar cube. Bees remained alive in the cages and were free to groom and consume the spores and be fed by nest mates. Four cages of bees were prepared for each hive in round one of trial two, and sampled on days 0, 1, 4 and 7. This was repeated in the second round with the addition of a fifth sample taken on day 9. Bees were then washed to remove spores in 0.05% Tween80 and spore viability assessed as above.

Washing with Tween80 in water recovered a far greater quantity of material than washing in paraffin with Tween80, and significantly more material was recovered from forager bees from the field than from bees within the hive. Almost no material (including pollen) could be recovered from any bees collected during containment in screen houses in trial one.

We found that spores could be identified visually via microscope but that no spores were detected by visual inspection in our initial inspection of pellets (the majority of which was pollen) from foraging or hive workers.

Spores placed on wax substrates inside hives could be recovered 2 weeks later, confirming that this method was viable as a test of spore recovery in hives. However, a greater concentration of spores was recovered from the wax in the controlled environment than in the hive, and bees were observed harvesting the wax and possibly spores in the hive. In addition, wax contamination resulting from washing the spores from the tablet was found to interfere with microscopic examination of spore germination. The protocol was therefore modified to use spores placed in 0.5 mL Eppendorf tubes with paper plugs, and this method was used in two rounds in trial two.

Spore germination using the modified protocol (wash in 0.05% Tween80 in water to recover spores, then cover with paraffin) gave better results compared with germination in 0.05% Tween80 in water without paraffin.

Spore germination after 2 weeks of storage on wax in the controlled environment (incubator) and in hives was not significantly different: 22% and 28%, respectively.

Forty-eight samples recovered from Eppendorf tubes or from bees coated with spores (39% of 122 samples taken in trial two) were found to contain spores, of which only eight samples had fewer than 50 spores detected. Spore recovery rates remained relatively constant for samples of bees, with spores recovered from more than a third of samples on day 9 (Figure 1). In contrast, no spores were recovered from day 0 samples from tubes and remained low on day 1, but by day 9 spores were recovered in all six tube samples (Figure 1).

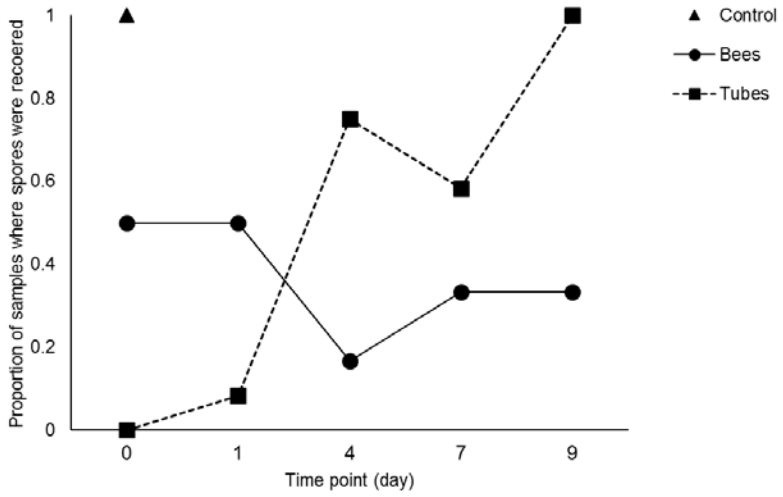

Figure 1. Proportion of samples where myrtle rust (*Austropuccinia psidii*) spores were recovered, from control samples in the laboratory ("control"), live bees coated in spores ("bees"), and spores placed in Eppendorf tubes ("tubes"), on days 0 (the day the hives were moved into containment), 1, 4 and 7 in both rounds of trial two, and also on day 9 in the second round.

Mean germination rates of positive control samples kept in the lab were 20%. Germination rates on samples taken from hives varied between 3% (tubes on day 1) and 16.2% (bees on day 1). Germination was recorded in all samples apart from discs/tubes on day 0, and germination rates on day 9 in round two of trial two were 8.2% for bee samples and 14% for samples in tubes.

The lack of recovery of MR spores in the majority of samples would artificially reduce mean germination rates, so the zero recovery data points were removed for further analysis. To assess whether spore germination rates on bees and tubes declined over the seven or nine days of the rounds in trial two, a Hierarchical Generalised Linear Model (HGLM) was used, where the response variate was the number of germinated spores out of the binomial totals. The fixed effect was Sample*Time*Site and the random effect was Round, modelled by beta distribution. The analysis was performed by GenStat 17.

There is no evidence to suggest difference between samples or sites (p-value > 0.1). There is limited evidence to suggest a difference over time (p-value = 0.07; Figure 2), but there is moderate evidence of a difference between rounds (p-value = 0.013).

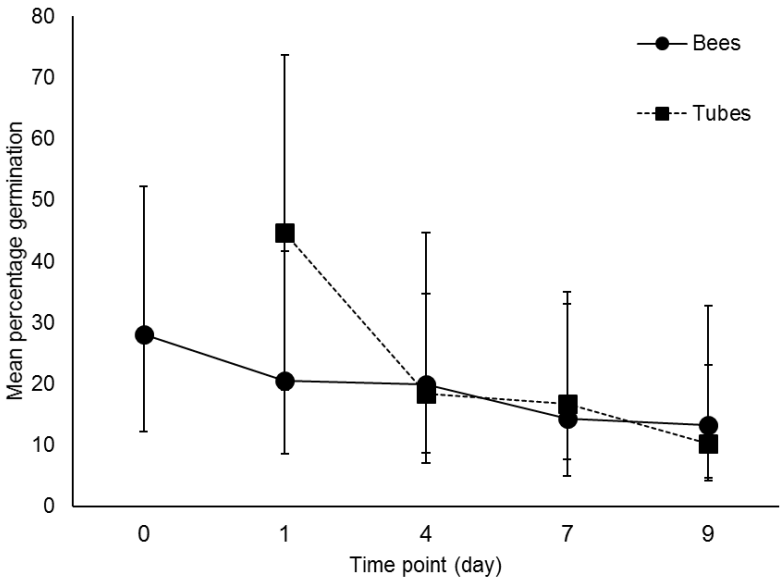

Figure 2. Predicted mean germination rates (from Hierarchical Generalised Linear Model (HGLM) of myrtle rust (*Austropuccinia psidii*) spores from live bees coated in spores ("bees"), and spores placed in Eppendorf tubes ("tubes"), on days 0 (the day the hives were moved into containment), 1, 4 and 7 in both rounds of trial two, and also on

2.3 QPCR ASSESSMENT

The method of Baskarathevan et al. (2016) was adapted using the qPCR primers PpsiITS1F/R/P to detect MR uredospores from samples taken from hives in the field and in screen houses.

Bees and pollen cell contents were collected from nine hives at the end of a 2-week placement at sites of known MR outbreaks in south east Queensland and northern NSW: at three sites in December 2017 (Moggill, The Channon (NSW) and the Tallebudgera Valley) and at four sites in Brisbane in January 2018 (Moggill, Chapel Hill and Samford, and three places within the Brisbane Botanic Gardens at Toowong). Bees were washed individually or in batches of 5 or 25 to remove spores by agitation for 20 min in 0.05% Tween80. The bees were removed from the wash, which was then centrifuged, the supernatant removed and the pellet dried. The pellet was frozen in liquid nitrogen and ground with a micropestle, re-suspended in extraction buffer (SDS, Tris, EDTA, and RNase) and incubated at 50ºC for 30 min. Protein contaminants were removed by precipitation with potassium acetate and DNA was precipitated with isopropanol, washed twice with 70% ethanol, dried and re-suspended in 200 µL of Tris/EDTA buffer and stored at -20ºC. All samples, including dilutions of purified spores, were extracted in the same way.

For qPCR, 3µl of extract was used in a reaction which included PpsiITS1F/R primers and PpsiITS1P Taqman probe, SensiFAST Probe (no Rox) reaction buffer and water. Samples were amplified in a Rotor gene 6000 Real Time Thermocycler (72 well carousel) using a 2 step PCR profile: one initial cycle at 95ºC for 5 min followed by 40 cycles at 95ºC for 15 sec and 60ºC for 45 sec. Fluorescence was detected using the Green channel and Auto-Gain optimisation was used before first acquisition. During analysis, dynamic tube and slope correct options were used.

A serial dilution of spores was prepared from a stock solution (~440,000 spores/mL estimated by counting; D0). Dilutions of 10^{-1} (D1), 10^{-2}(D2), 10^{-3} (D3), 10^{-4} (D4), 10^{-5} (D5) were prepared and 0.8 mL of each extracted and amplified in qPCR as described above to generate a standard curve for quantification. The D1–D3 dilutions generated the linear phase of the standard curve, while very high (D0) and low (D4 and D5) concentrations were detected but could not be quantified consistently (Table 1). DNA extracted from dilutions D1–D3 was used to prepare standard curves for all qPCR runs incorporating field and screen house samples. D4 and another sample were also included in all runs to check for consistencies between runs. The standard curve was generated using the auto-threshold function based on D–D3. Reactions were run in triplicate. No template controls (NTC) were included in each qPCR run.

Table 1. The number of spores extracted as estimated from standard curves generated from D1–D3 in seven separate quantitative PCR runs. Red numbers indicate values used for standard curves.

	Estimated number of spores extracted [average of three samples]						
D1 - 44,000 spores/mL [35,000 spores extracted]	34,880	36,988	35,747	35,352	34,784	34,952	34,903
D2 - 4400 spores/mL [3500 spores extracted]	3,524	3,134	3,355	3,431	3,544	3,510	3,519
D3 - 440 spores/mL [350 spores extracted]	349	370	357	354	347	350	349
D4 - 44 spores/mL [35 spores extracted]	121	n/a	150	139	158	152	147
positive sample	n/a	30	21	26	26	35	36

The total DNA obtained from each sample extraction varied. The detection of MR did not correlate with the total DNA extracted; very dilute total DNA samples gave positive results while some high concentrations of total DNA were negative. The qPCR output for all runs included cycle threshold (Ct) for each sample replicate, the corresponding calculated concentration (spores extracted) for each replicate estimated from the standard curve, Rep. Ct which was the average Ct for the three replicates of each sample and the corresponding concentration (Rep. Calc. Conc.) determined from the standard curve.

Tables 2, 3 and 4 give the Rep. Ct and Rep. Calc. Conc. (i.e. the estimated total number of spores in the original extract) for each sample. Very low levels of myrtle rust spores were only detected in later cycles making quantitation unreliable. No amplification was detected in the NTC.

MR was positively identified using this qPCR in samples from all sites in all trials, apart from samples from the hive located at Samford in trial two (Table 4; Figure 4). The highest estimated spore count per extract was 71, in a sample taken from pollen in a cell in a hive in trial one

Table 2. Estimated myrtle rust (*Austropuccinia psidii*) spore counts from samples taken from hives immediately after a 2-week period at sites of known myrtle rust outbreak in south east Queensland (QLD) and northern New South Wales (NSW) in December 2017. Blanks indicate no detection (below detection threshold), while * indicates cycle threshold (Ct) value (>35) was observed in only one replicate. Rep. Ct. is the average Ct for three replicates, and Rep. Calc. Conc. is the corresponding concentration determined from the standard curve.

	qPCR Sample ID	Rep. Ct (std dev)	Rep. Calc. Conc.	Source of samples
Trial 1: Field 6 December 2017	22/1-1	35.31 (2.33)	11	pollen from single cell in hive, Moggill
	22/1 -2	34.8 (0.15)	71	pollen from single cell in hive, Moggill
	22/1 -3	*		pollen from single cell in hive, Moggill
	22/1 -4			pollen from single cell in hive, Moggill
	22/1 -5	37.97 (0.77)	10	single forager bee, Moggill
	22/1 -6			single forager bee, Moggill
	22/1 -11	*		single leg from forager bee, Moggill
	22/1 -7	35.47 (0.88)	47	wash from 25 bees from within hive at The Channon
	22/1 -8	34.88 (0.4)	67	wash from 25 bees from within hive at The Channon
	22/1 -9	35.14 (3.82)	57	wash from 25 bees from within hive at Tallebudgera
	22/1 -10	36.21 (0.22)	29	wash from 25 bees from within hive at Tallebudgera

Table 3. Estimated myrtle rust (*Austropuccinia psidii*) spore counts (Rep. Calc. Conc.) from samples of stored pollen taken from hives immediately after a 2-week period at sites of known myrtle rust outbreak in SE Queensland in January 2017. Blanks indicate no detection (below detection threshold), while * indicates Ct value (>35) was observed in only one replicate. Rep. Ct. is the average Ct for three replicates, and Rep. Calc. Conc. is the corresponding concentration determined from the standard curve.

	qPCR Sample ID	Rep. Ct (std dev)	Rep. Calc. Conc.	Source of samples
Moggill Jan 2018	aM 30 1	37.87 (0.24)	10	pollen from single cell in hive
	M 30 2	38.77 (0.36)	6	pollen from single cell in hive
	M 30 3	*		pollen from single cell in hive
	M 30 4	36.47 (0.07)	25	pollen from single cell in hive
	M 30 5	*		pollen from single cell in hive
Kenmore Jan 2018	K 30 1			pollen from single cell in hive
	K 30 2	35.64 (0.58)	42	pollen from single cell in hive
	K 30 3	*		pollen from single cell in hive
	K 30 4			pollen from single cell in hive
	K 30 5	38.52 (0.43)	13	pollen from single cell in hive
Hive 1 Botanical Gardens Jan 2018	B1 30 1			pollen from single cell in hive
	B1 30 2			pollen from single cell in hive
	B1 30 3	*		pollen from single cell in hive
	B1 30 4	39.4 (0.32)	8	pollen from single cell in hive
	B1 30 5	*		pollen from single cell in hive
Hive 2 Botanical Gardens Jan 2018	B2 30 1	*		pollen from single cell in hive
	B2 30 2	38.29 (0.74)	15	pollen from single cell in hive
	B2 30 3	37.91 (1.03)	19	pollen from single cell in hive
	B2 30 4	39.34 (0.76)	8	pollen from single cell in hive
	B2 30 5			pollen from single cell in hive
Hive 3 Botanical Gardens Jan 2018	B3 30 1	*		pollen from single cell in hive
	B3 30 2	36.66 (0.23)	42	pollen from single cell in hive
	B3 30 3	39.62 (0.09)	7	pollen from single cell in hive
	B3 30 4	*		pollen from single cell in hive
	B3 30 5			pollen from single cell in hive

Table 4. Estimated myrtle rust (*Austropuccinia psidii*) spore counts (Rep. Calc. Conc.) from samples of foraging bees taken from hives immediately after a 2-week period at sites of known myrtle rust outbreak in SE Queensland in January 2017. Blanks indicate no detection (below detection threshold), while * indicates Ct value (>35) was observed in only one replicate. Rep. Ct. is the average Ct for three replicates, and Rep. Calc. Conc. is the corresponding concentration determined from the standard curve.

	qPCR Sample ID	Rep. Ct (std dev)	Rep. Calc. Conc.	Source of samples
Samford Jan 2018	S1 30/1 1.1	*		1 forager bee
	S1 30/1 1.2			1 forager bee
	S1 30/1 1.3	*		1 forager bee
	S1 30/1 1.4			1 forager bee
	S1 30/1 1.5			1 forager bee
	S1 30/1 5.1			5 forager bees
	S1 30/1 5.3			5 forager bees
	S1 30/1 5.4			5 forager bees
	S1 30/1 5.5	*		5 forager bees
Moggill Jan 2018	MF1 29.1 1.1			1 Forager bee
	MF1 29.1 1.2			1 Forager bee
	MF1 29.1 1.3			1 Forager bee
	MF1 29.1 1.4	*		1 Forager bee
	MF1 29.1 1.5			1 Forager bee
	MF1 29.1 5.1			5 Forager bees
	MF1 29.1 5.2			5 Forager bees
	MF1 29.1 5.3	37.78 (1.48)	9	5 Forager bees
	MF1 29.1 5.4			5 Forager bees
	MF1 29.1 5.5			5 Forager bees
Kenmore Jan 2018	K1 29/1 1.1	36.88 (0.63)	12	1 Forager bee
	K1 29/1 1.2			1 Forager bee
	K1 29/1 1.3	37.5 (1.04)	8	1 Forager bee
	K1 29/1 1.4	*		1 Forager bee
	K1 29/1 1.5			1 Forager bee
	K1 29/1 5.1	36.14 (0.64)	20	5 Forager bees
	K1 29/1 5.2	34.73 (0.5)	48	5 Forager bees
	K1 29/1 5.3	36.29 (0.72)	18	5 Forager bees
	K1 29/1 5.4	36.33 (0.03)	17	5 Forager bees
	K1 29/1 5.5	*		5 Forager bees
Hive 1 Botanical Gardens Jan 2018	Bg1.1 29/9 1.1	*		1 Forager bee
	BG1.1 29/1 1.2			1 Forager bee
	BG1.1 29/1 1.3	35.19 (0.22)	5.2	1 Forager bee
	BG1.1 29/1 1.4	*		1 Forager bee
	BG1.1 29/1 1.5			1 Forager bee
	BG1.1 29/1 5.1	36.23 (0.79)	6	5 Forager bees
	BG1.1 29/1 5.2	*		5 Forager bees
	BG1.1 29/1 5.3	*		5 Forager bees
	BG1.1 29/1 5.4	33.17 (0.19)	42	5 Forager bees
	BG1.1 29/1 5.5	36.8 (0.51)	5	5 Forager bees
Hive 2 Botanical Gardens Jan 2018	BG2-1 29/1 1.1			1 Forager bee
	BG2-1 29/1 1.2			1 Forager bee
	BG2-1 29/1 1.3			1 Forager bee
	BG2-1 29/1 1.4	36.43 (0.91)	21	1 Forager bee
	BG2-1 29/1 1.5			1 Forager bee
	5.1?			
	BG2-1 29/1 5.2			5 Forager bees
	BG2-1 29/1 5.3	*		5 Forager bees
	BG2.1 29/1 5.4	34.47 (0.91)	19	5 Forager bees
	BG2.1 29/1 5.5	36.78 (1.14)	5	5 Forager bees
Hive 3 Botanical Gardens Jan 2018	B3 29 1.1	*		1 Forager bee
	B3 29 1.2	38.04 (0.66)	2	1 Forager bee
	B3 29 1.3	37.55 (0.4)	3	1 Forager bee
	B3 29 1.4			1 Forager bee
	B3 29 1.5			1 Forager bee
	B3 29 5.1	34.36 (0.77)	20	5 Forager bees
	B3 29 5.2	33.1 (0.65)	44	5 Forager bees
	B3 29 5.3	35.43 (0.82)	10	5 Forager bees
	B3 29 5.4			5 Forager bees
	B3 29 5.5	33.51 (0.3)	34	5 Forager bees

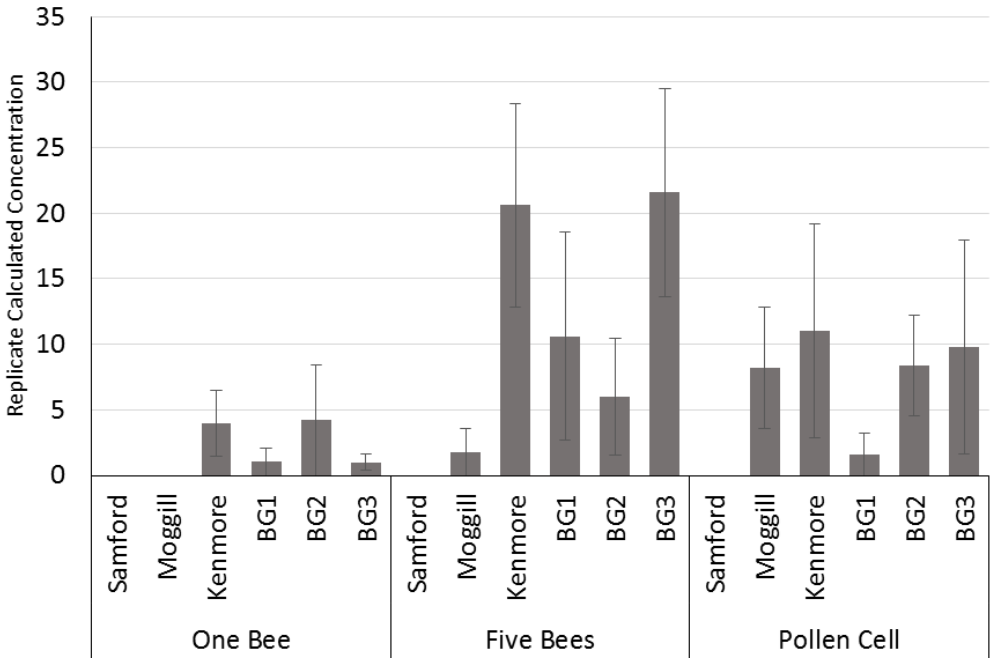

Figure 4. Mean replicated calculated concentrations of myrtle rust (*Austropuccinia psidii*) spores from one bee, five bee and pollen cell content samples taken from hives immediately following a two week period of foraging at myrtle rust outbreak locations around SE Queensland.

3 Discussion

Myrtle rust spores were recovered from hives after 9 days in isolation from any new source of spores, and showed 15% germination rates at this time point. This was true both for spores placed inside an Eppendorf tube with a paper plug and for spores that were used to coat live worker bees.

This result is important as it demonstrates that neither the internal environment of the hive nor the grooming behaviour of bees are sufficient to remove or kill all MR spores over a 9-day period. There is limited evidence for a decline in spore germination rates over the 9-day period, suggesting that spores may remain viable inside a hive considerably longer than 9 days.

The methods used to obtain these spore viability results were designed to maximise the chance of spore recovery, with bees coated in copious spores before being placed in queen cages and considerable quantities of spores placed on wax discs and in plastic tubes. The degree to which these results are comparable to the natural survival rate of spores within hives will depend on the loading of spores on foragers returning to the hive. However, observations of honey bees actively collecting spores showed that bees foraging on MR spores become coated with spores in a similar manner to the method used in this trial (G. Pegg, personal communication 2018).

The apparent lack of recovery of any spores at the day 0 time point for the tube samples may be due to over dispersion on the plates, and the increase in recovery rates for these samples may indicate an improvement in the method. However, the existence of this pattern in both rounds of trial two suggests that there may be more to understand about this phenomenon.

To determine whether bees were bringing MR spores back to their hives in these trials, we used qPCR analysis (following initial calibration trials) to estimate the number of spores in samples of pollen foraging bees returning to hives, bees sampled from within hives and the contents of pollen storage cells. Positive results were obtained from all hives on all sample dates, apart from the Samford hive in the first round of trial two. Without positive controls of bees directly caught while foraging on MR it is unclear how these figures relate to background amounts due to environmental contamination or amounts to be expected from bees foraging on spores. We aimed to collect these data, but were unable to find sufficient numbers of bees foraging on MR spores to allow for a sample collection. Determining the relative amounts of spores to be expected from spore-foraging bees should be a priority for future research, and will allow greater interpretation of these qPCR results.

Surveys of infected plants with active eruptions of spores at the field sites while hives were present showed little evidence of bees actively collecting spores. However, observations have been made by the authors and their collaborators of honey bees and native stingless bees collecting rust spores on occasion. It is likely that this behaviour depends on the relative availability of pollen, and so the phenomenon of bees actively collecting rust spores is relatively rare. Understanding the cues and triggers that drive this behaviour should be a priority for future research.

Bees have been implicated in the spread of other plant pathogens, including the fire blight pathogen *Erwinia amylovora* (Alexandrova et al. 2002), and the kiwifruit pathogen *Pseudomonas syringae* pv. *actinidae* (Pattemore et al. 2014). This study provides further evidence that honey bees may play a role in the transmission of plant pathogens, in this case MR, *A. psidii*.

Honey bees, and other insects, can spread plant pathogens such as MR locally as they forage and move from an infected plant to a susceptible plant. At this scale, the risk of transmission by honey bees is likely to be low compared with many other potential vectors including wind and human activity. However, there are several aspects of honey bee behaviour that may result in the spread of MR spores in a manner that would not have happened otherwise. Individual honey bees can forage 5 km from their hive, and thus spread the spores over this distance. This is especially significant if the direction of long-distance foraging bouts is across relatively un-modified habitat or in the opposing direction of prevailing winds. Furthermore, spores are likely to be transferred between individual foragers within a hive, similar to that seen with pollen and other plant pathogens (e.g. Pattemore et al. 2014), which could result in a 10 km diameter spread of spores from a hive.

In the case of MR, where spores are predominantly wind-dispersed, local spread of spores by honey bees is likely to be of low concern and limited by the flight distance of the bees. Other vectors or environmental movement (wind) could spread the spores over similar or greater distances.

However, managed honey bee colonies can be transported by vehicle over much greater distances, and this long distance movement could be counter to prevailing winds or could result in hives being moved into remote locations where other human activity is unlikely to have facilitated the spread of the spores. This is especially of concern in New Zealand where the most valuable honey crop is from mānuka (*L. scoparium*), a member of the susceptible Myrtaceae family. Hives being moved from locations with active MR outbreaks into remote mānuka sites risk moving spores into locations that might otherwise have been at relatively low risk due to prevailing wind directions. This would only be a risk if a) bees bring MR spores back into the hive, and b) if the spores can survive in the hive long enough to remain viable after these long distance moves.

This study has shown that a) MR spores are being brought into hives even when there is no observed evidence of active foraging on the spores by bees, and b) that these spores can remain on bees and remain viable in the hive for at least 9 days. These results suggest that the long-distance movement of hives needs to be considered as a potential risk for the transmission of MR spores, especially to remote locations.

As this study did not provide clear evidence of decline in either spore recovery or spore viability, future research should aim to extend the length of the trial to determine the decay curve for recovery and viability. In addition, the phenomenon of honey bees foraging on rust spores is not well understood, and future research should seek to understand the cues and triggers for this behaviour so that the role of honey bees in the spread of pathogens like MR can be better quantified.

4 References

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