Final report

Biology of pasture mealybug and identification of natural enemies

Project code: B.PAS.0004

Prepared by: Caroline Hauxwell, Queensland University of Technology

Date published: June 2022

PUBLISHED BY

Meat and Livestock Australia Limited

PO Box 1961

NORTH SYDNEY NSW 2059

This is an MLA Donor Company funded project.

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government and contributions from the Australian Meat Processor Corporation to support the research and development detailed in this publication.

Abstract

Pasture dieback causes unhealthy growth and death in a range of introduced and native grasses across Queensland and into northern NSW, resulting in large losses in beef production areas. Pasture mealybug, *Heliococcus summervillei* Brookes, is the primary cause of pasture dieback. This project aims to describe the life history of pasture mealybug, provide an understanding of the link between mealybug and pasture dieback, and identify natural enemies.

A detailed understanding of mealybugs, including species identification, life history and identification by molecular markers, was completed. Laboratory assays determined the sexual reproductive strategy of the mealybug, and its relative survival and development on different grass varieties. Diversity within the mealybug population is very low is possibly a new incursion of a variant of *H. summervillei.*

Life history studies and seasonal monitoring in the field identified the highly seasonal nature of the mealybug and the windows of opportunity for targeting of management interventions. Seasonal scouting is critical to targeting and management of mealybugs . Seasonal management such as slashing should target vulnerable mating adults and foliar-feeding early instars during late spring and summer. Further research on integrating management strategies (slashing, grazing, insecticides) with the seasonal and reproductive biology of the mealybug to quantify impact on susceptible mealybug populations and benefits within farm practice is strongly recommended.

Transcriptome analysis demonstrated the mechanism of disruption of the jasmonic acid/salicylic acid pathway by which feeding by the mealybug renders the grass susceptible to pathogens such as *Fusarium*, resulting in 'pasture dieback'. Interactions between the mealybug, its microbial symbionts, and the plant should be studied further to understand these mechanisms more clearly and to identify pathways of resistance or tolerance in pasture varieties.

Analysis of small RNAs identified a number of viruses in plant material that might be amplified as a result of this suppression. No *Closteroviruses*, which are amplified in Mealybug-induced Pineapple Wilt disease, were found, but the interaction between disruption of the grass immune response and possible amplification of endogenous viruses requires further study.

Natural enemies of the mealybug were identified by collection of live mealybugs and cadavers, and systematic sampling techniques were evaluated. Two parasitoids were identified, one a new record for Australia and one, the most abundant, a new species of genus *Parectromoidella* (*Hymenoptera*, *Encyrtidae*). Parasitoids are widely distributed and appear to have alternative hosts resulting in greater abundance of the wasps in early spring, but they cannot be relied on to control the pasture mealybug at current levels of abundance. Augmentation of beneficial insects is an important area for further investigation.

The confirmation of *H. summervillei* as the causal agent of pasture dieback is of critical importance to industry. Graziers and agronomists can now move forward with confidence to identify management strategies that suit their farm operations.

The identification of key points in the seasonal and reproductive biology of the mealybug during which damage occurs and management can be targeted is critically important. These findings and the quantitative methods developed, will inform current management and development of proven strategies suitable for a range of farm operating models and systems.

Executive summary

Background

Pasture dieback causes unhealthy growth and death in a range of introduced and native grasses across Queensland and into northern NSW, resulting in large losses in beef production areas. Pasture mealybug, *Heliococcus summervillei* Brookes, is the primary cause of pasture dieback. This mealybug was previously reported to have caused severe pasture dieback in Queensland in 1926 (Summerville 1928) and the 1930s (Brooks 1978), in New Caledonia in 1998 (Brinon et al 2004), and more recently in Puerto Rico and Barbados.

Understanding the biology of the mealybug *Heliococcus summervillei*, its growth, reproduction, seasonal interaction with pasture grass, and natural enemies, is critical to understanding the progression of dieback, evaluating treatments, and targeting effective controls.

This project aims to describe the life history of pasture mealybug, provide an understanding of the link between mealybug and pasture dieback, and find natural enemies to the mealybug.

Objectives

The project objectives, as outlined in the research agreement, are as follows:

1. Provide a detailed understanding of mealybugs (including species identification, life history and identification by molecular markers) based on field, laboratory and glass/screen house studies
2. Identify natural enemies of mealybug species
3. Make preliminary recommendations on role of natural enemies in mealybug control
4. Develop standardised field sampling protocols for mealybugs and in conjunction with project partners
5. Outline of one draft manuscript for journal publication
6. Provide to MLA periodic information summaries suitable for general media on the role and management of mealybugs in relation to addressing pasture dieback
7. A two-page summary outlining key facts and findings of mealybugs and how to identify mealybugs associated with dieback

Methodology

A detailed understanding of mealybugs (including species identification, life history and identification by molecular markers) was completed using laboratory and glass/screen house studies. The laboratory assays were used to determine the reproductive strategy of the mealybug, its survival and development on different grass varieties.

Natural enemies of the mealybug were identified by collection of live mealybugs and cadavers, and systematic sampling techniques including sweep nets and transect sampling. Parasitoids were identified and quantified across seasons, and a small number of pathogens were isolated. These techniques allowed the development of recommendations for the potential of natural enemies in control of the mealybug and recommendations for augmentative approaches for both parasitoids and pathogens.

Standardised field sampling protocols for mealybugs were developed through modification of sampling techniques used in New Zealand for the pasture mealybug *Balanococcus p*oa, and grape mealybug.

Standardised observation recording sheets were developed and shared with project partners. Training materials were developed and disseminated on identification of symptoms ('what to look for' and how to quantify pasture mealybugs.

Workshops, media outputs, farm visits, presentations at Beef Week, a webinar, panel discussions with NABRAC and other activities were used to provide information to MLA, the MLA communications team, dieback program participants and with graziers and others in livestock industries, and to communicate findings and implications for mealybug and dieback management throughout the project

Results have been analysed and used to draft two manuscripts for journal publication. Provide to MLA periodic information summaries suitable for general media on the role and management of mealybugs in relation to addressing pasture dieback

Results/key findings

This work has confirmed the identity of the mealybug *Heliococcus summervillei* and demonstrated beyond doubt that it is the causal agent of pasture dieback. Diversity within the population is very low, both in morphology and in DNA marker sequences. The diversity of the insect and its symbionts suggest this might be an incursion of a new variant within the species and require some further investigation.

Feeding by mealybug disrupts the Jasmonic Acid/Salicylic Acid pathway and other plant immune responses, rendering the grass vulnerable to secondary infections such as *Fusarium*. Interactions between the mealybug, its microbial symbionts, and the plant should be studied further to understand these mechanisms more clearly and to identify pathways of resistance or tolerance in pasture varieties.

Life history studies and seasonal monitoring in the field identified the highly seasonal nature of the mealybug and the windows of opportunity for targeting of management interventions. Adult mealybugs do not feed but shelter and mate in the shaded spaces beneath dense clumping grasses. Management is especially important in the warmer seasons when the small and medium nymphs emerge to feed on flushing foliage: these are the instars that feed on grass and cause 'dieback'. Life history studies also led to development of rapid screening assays for fast throughput of variety screening and testing of possible controls, including microbial controls and endophytes.

A small number of natural enemies have been identified. These include two new records of parasitoid wasps (Hymenoptera: *Encyrtidae*), one of which is a new species. Augmentation of parasitoids seems likely to be needed. Further work should determine if commercial *Anagyrus* species will parasitise *H. summervillei*, a potential option to boost control in the field, or if *Parectromoidella*, a close relative, will parasitise other, more easily-reared mealybugs used in commercial mass production such as citrus mealybug. Further work on parasitoid host range, and biocontrol with *Anagyrus* species, is recommended.

The parasitoids found were predominantly 1 species, a new species of *Parectromoidella*. The parasitoid *Calipteroma* reported from the 1926 outbreak was not found by QUT but might have been found by DAF Qld. This new species of Parectromoidella was found concurrent with the geographic range of *H. summervillei*, and it appears to be abundant in the field well before the peak in *H. summervillei* numbers. It is worthy of further study. Dr Noyes (NHM, London UK) has suggested that a revision of the genus is needed to identify this species. With the rapid aging of qualified taxonomists and the great need for identification of biological control agents as incursions increase, there is an opportunity to develop a PhD project to study the identity, host range and potential augmentation of parasitoids of pasture mealybugs and train a young expert in the techniques.

QUT has been actively promoting understanding of the link between mealybug biology and dieback, and monitoring by graziers in order to target management. Articles, field days, a webinar, and training

in the use of monitoring and sampling, including use of sampling kits, have been provided and training materials have been developed, including filming of two short videos on ‘what to look for’ and ‘how to sample’ mealybugs.

Conclusions

The project has confirmed the identity of the mealybug *Heliococcus summervillei* and demonstrated beyond doubt that it is the causal agent of ‘pasture dieback’.

Diversity within the mealybug population is low. Only one physical variant was observed, even though historic and international samples show there are significant morphological variations between populations (Brookes 2007). The last record of the 1926 variant (Summerville 1928) was found by Dr. M. Schutze in a sample submitted to Biosecurity Qld in 2016 (M. Schutze, pers com).

Transcriptome analysis showed how feeding by the mealybug disrupts the JA/SA pathway and other plant immune responses, rendering the grass vulnerable to secondary infections such as *Fusarium*. We know from insecticide studies that removing the mealybug allows the grass to recover, i.e. the immune responses must be restored once the mealybug is removed. The mechanism by which this occurs requires further examination. Interactions between the mealybug, its microbial symbionts, and the plant should be studied further to understand these mechanisms more clearly and to identify pathways of resistance or tolerance in pasture varieties. Further work on mechanisms of mealybug-induced susceptibility across grass varieties, including transcriptome analysis, is strongly recommended.

Other possible causes of dieback including fungal pathogens such as *Fusarium* have been eliminated, though there is an association with Badnaviruses and possibly Dicistroviruses that should be investigated a little further. The suppression of the plant defence responses could induce susceptibility to the virus from which the grass recovers, a mechanism similar to the inducement of Pineapple Wilt Virus by mealybugs and recovery after treatment with insecticides. The ecology and biology of the virome of pasture grasses, possible vectors, and interactions with the mealybug impacts on the grass immune system would be a useful area of further study.

The sex ratio in the summer is roughly 1:1. *H. summervillei* mealybug is predominantly sexually reproductive, though it may reproduce parthenogenically in soil over winter. Mating occurs in the dense thatch layer of summer pastures. Mated females turn pink before producing young. Only early instars of both sexes feed on plants and cause ‘pasture dieback’. Adults of both sexes do not feed. Females overwinter in soil, in contrast to reports by Summerville (1928). Overwintering populations of dispersed females are difficult to manage and sample.

Seasonal scouting is critical to targeting and management of mealybugs. Seasonal management strategies such as slashing should target vulnerable mating adults and foliar-feeding early instars during late spring and summer.

Parasitoids are low in abundance but probably have alternate hosts in the landscape. We know little about the parasitoids of other mealybugs and scales across Queensland, including the *Solenopsis* mealybug that is often found on *Parthenium* and neighbouring cotton crops. Further study is required to identify the parasitoid and scale insect hosts in pastures and surrounding vegetation and to determine the host range of and parasitism rates in *H. summervillei* are increasing. However, unlike New Caledonia, the current outbreak in Queensland has already gone on for at least 8 years, and there is no sign of an increase in natural enemies (Brinon 2004).

Augmentation of parasitoids seems likely to be needed. Further work should determine if commercial *Anagyrus* species will parasitise *H. summervillei*, a potential option to boost control in the field, or if *Parectromoidella*, a close relative, will parasitise other, more easily-reared mealybugs used in commercial mass production such as citrus mealybug. Further work on parasitoid host range, and biocontrol with *Anagyrus* species, is recommended.

The parasitoids found were predominantly 1 species, a new species of *Parectromoidella*. The parasitoid *Calipteroma* reported from the 1926 outbreak was not found by QUT but might have been found by DAF Qld. This new species of Parectromoidella was found concurrent with the geographic range of *H. summervillei*, and it appears to be abundant in the field well before the peak in *H. summervillei* numbers. It is worthy of further study. Dr Noyes (NHM, London UK) has suggested that a revision of the genus is needed to identify this species. With the rapid aging of qualified taxonomists and the great need for identification of biological control agents as incursions increase, there is an opportunity to develop a PhD project to study the identity, host range and potential augmentation of parasitoids of pasture mealybugs and train a young expert in the techniques.

Benefits to industry (2-3 sentences)

The confirmation of *H. summervillei* as the causal agent of pasture dieback is of critical importance to industry. Graziers and agronomists can now move forward with confidence to identify management strategies that suit their farm operations.

The identification of key points in the seasonal and reproductive biology of the mealybug during which damage occurs and management can be targeted is critically important. These findings and the quantitative methods developed, will inform current management and future development of proven and quantifiable strategies suitable for a range of farm operating models and systems.

Life history studies have also led to development of rapid screening assays for fast throughput of variety screening and testing of possible controls, including microbial controls and endophytes.

A small number of beneficial insects have been identified. Augmentation of beneficial insects is an important area for further investigation.

Future research and recommendations

Feeding by mealybug disrupts the JA/SA pathway and other plant immune responses, rendering the grass vulnerable to secondary infections such as *Fusarium*. Interactions between the mealybug, its microbial symbionts, and the plant should be studied further to understand these mechanisms more clearly and to identify pathways of resistance or tolerance in pasture varieties.

Analysis of small RNAs identified a number of viruses in plant material that might be amplified as a result of this suppression. No *Closteroviruses*, which are amplified in Mealybug-induced Pineapple Wilt disease, were found, but the interaction between disruption of the grass immune response and possible amplification of endogenous Badnaviruses and (possibly) Dicistroviruses requires further study.

Further research on integrating management strategies (slashing, grazing, insecticides) with the seasonal and reproductive biology of the mealybug to quantify impact on susceptible mealybug populations and benefits within farm practice is strongly recommended.

A small number of beneficial insects have been identified. These include two new records of parasitoid wasps (Hymenoptera: *Encyrtidae*), the most abundant of which is a new species of *Parectromoidella*. Though these parasitoids are widely distributed concurrent with *H. summervillei*, and appear to have alternative hosts in spriing, they cannot be relied on to control the pasture mealybug at current levels of abundance.

Dr John Noyes (NHM, London UK) has suggested that a revision of the genus *Parectromoidella* is needed to identify this species. With the rapid aging of qualified taxonomists and the great need for identification of biological control agents as incursions increase, there is an opportunity to develop a PhD project to study the identity, host range and potential augmentation of parasitoids of pasture mealybugs and train a young expert in the techniques.

Augmentation of beneficial insects is an important area for further investigation. Further work should determine if commercial *Anagyrus* species will parasitise *H. summervillei*, a potential option to boost control in the field, or if *Parectromoidella*, a close relative, will parasitise other, more easily-reared mealybugs used in commercial mass production such as citrus mealybug. Further work on parasitoid host range, and biocontrol with *Anagyrus* species, is recommended.

Table of Contents

Background

Pasture dieback causes unhealthy growth and death in a range of introduced and native grasses across Queensland and into northern NSW, resulting in large losses in beef production areas. Pasture mealybug, *Heliococcus summervillei* Brookes, was previously reported to have caused severe pasture dieback in Queensland in 1926 (Summerville 1928) and the 1930s (Brooks 1978), in New Caledonia in 1998 (Brinon et al 2004), and more recently in Puerto Rico and Barbados.

This project conducts original research that provides a detailed understanding of the biology of *H. summervillei*, including species identification, life history and molecular markers. This is based on field, laboratory and glass/screen house studies. The project describes the life history of pasture mealybug, provides an understanding of the link between mealybug and pasture dieback, and identifies natural enemies.

At the start of the project, it was not clear that *H. summervillei* was the primary cause of pasture dieback. Previous work funded by MLA had used next generation sequencing to investigate possible fungi or bacteria as causal agents. There were no consistent patterns of occurrence of any pathogens across leaf, root or soil samples analysed. *Fusarium* species and variants tended to be abundant at all field sites. The bacterial symbiont of *H. summervillei*, *Candidatus Tremblaya phenacola* was found consistently in dieback-affected material and was the most abundant bacterium in affected material (determined from read abundance). However, this work was flawed because the sampling design was based on an assumption that infected and uninfected plants would be easy to differentiate between and within sites, something we now know is not the case. It is likely that affected and unaffected plants were not clearly differentiated in the sampling.

This project included the provision to test for interactions between the mealybug and plant pathogens. While this was not a major focus of this study, we made use of available materials and extensive field collections of plant and rhizospheric material to focus on two possible groups: *Fusarium* species and strains, and viruses associated with mealybugs.

Mealybugs are known to vector important plant viruses, particularly *Badnaviruses* and *Closteroviruses* (such as Mealybug-induced Pineapple Wilt, and infection that has many similarities with pasture dieback). High-throughput sequencing of small RNAs (sRNA) from plants has been used successfully to identify the viruses infecting them, including previously unknown viruses, even in extremely low titre, symptomless infections (Kreutz et al 2009). We used this approach to screen a small number of samples of uninfected grasses from the glasshouse and grass infested with mealybug, and field material for the presence of small RNA that might indicate infection by a virus.

One important factor in the biology of the mealybug is the process by which the insect can cause pasture dieback on large, dense tussocks of grass. Mealybugs are very small, less than 0.2mm across, and could not consume enough plant sap to kill these large grasses. *H. summervillei* also appears to cause dieback in very low numbers. How can such a small insect kill a large plant? One hypothesis is that feeding by the mealybug supresses plant defence responses.

In plants recognition of biotic attacks leads to the accumulation of signalling hormones such as jasmonic acid (JA) and salicylic acid (SA), which induce expression of downstream defence-related genes. Antagonistic cross-communication between these signalling pathways is a cost-efficient adaptive strategy that allows plants to tailor their defence responses to specific attackers: the JA signalling pathway is recognized as a key regulator of defence against necrotrophic pathogens and insects, whereas SA is a key regulator of defence against biotrophic pathogens. Several studies have found that certain insects, including mealybugs, can suppress JA mediated defences in plants to benefit their performance.

The Solenopsis mealybug, *Phenacoccus solenopsis*, has been shown to supress the plant defences by disrupting JA/SA cross-talk that modulates induced plant defences (Zhang et al 2015). In that system, mealybug feeding decreased JA production and JA-dependent defense gene expression, but

increased SA accumulation and SA-dependent gene expression. In this project we use transcriptome analysis to identify the effect of feeding by *H. summervillei* on gene expression in American buffel grass, *Cenchrus ciliaris*, to determine if the mealybug has a similar effect on the plant defence pathways.

Natural enemies (predators, parasitoids and pathogens) of pest species are an important part of agroecosystems that can prevent or reduce the severity of outbreaks. Unspecified (and unquantified) 'natural enemies' were posited as the reason for the relatively rapid 'disappearance' of *H. summervillei* and pasture dieback in the New Caledonia outbreak of 2018 (Brinon et al 2004). Two natural enemies of *H. summervillei* were reported in the 2916 outbreak in Cooroy, Qld: the predatory beetle *Cryptolaemus montrouzieri*, and the parasitoid wasp *Leptomastic guttatipennis* Gurault 1915 (*Encyrtidae : Tetracneminae*) (later redescribed as *Callipteroma sexguttata* Motschulsky 1863 (Trjapitzin and Triapitsyn 2018)) (Summerville 2018).

This project conducts both longitudinal and widespread systematic sampling to identify parasitoid wasps (*Hymenoptera: Encyrtidae*) of *H. summervillei* from mealybugs collected in the field. It describes the wasps identified using taxonomic keys with expert advice from Dr John Noyes, Natural History Museum, London. We use DNA sequence markers to identify the wasps collected in sweep nets by comparison with known sequences from validate specimens in the NCBI database.

Encyrtid wasps (*Hymenoptera*: superfamily *Chalcidoidea*) are important parasitoids of scale insect, including mealybugs (*Homoptera*: *Coccidae: pseudococcidae*), and are important biological control agents. Amongst these, the tribe *Anagyrini* (*Hymenoptera: Encyrtidae*) are used widely in biological control of pest mealybugs, both for introductions in classical biological control and in mass rearing for release in crops, particularly orchard and protected cropping (Noyes and Hayat 1994). Identifying the parasitoids of *H. summervillei* in the field may indicate potential candidates for mass release and further study.

We compare the seasonal abundance of these wasps in emergence studies with longitudinal and geographic sampling using sweep nets and emergence traps in the field, and with mealybug abundance studies. We then make preliminary recommendations on role of natural enemies in mealybug control, and some key future work.

Finally, early recognition of symptoms and how to identify mealybugs is essential for timely interventions and management. The data on the seasonal field biology from previous outbreaks of *H. summervillei* is scant, with only basic observations from the 1926 outbreak (Summerville 1928). Sampling methods to determine biology are better described for other mealybugs such as the New Zealand pasture mealybug *Balanococcus poa* (Maskell, 1879), and for the grape mealybug, Pseudococcus maritimus (Geiger et al 2001).

In this project we develop standardised field sampling protocols for mealybugs to determine the seasonal abundance of mealybugs, their size and potential for population growth, and distribution on plant, thatch and soil, to improve targeting of appropriate controls. We produced a range of training materials, kits and information through MLA, including contributing to the MLA publication on what to look for in Pasture dieback, and several MLA articles on how to effectively target management of the mealybug through monitoring. We actively disseminated information and demonstrated sampling, mealybug identification, and management with graziers and agronomists, while collecting on properties, through grower information days on farm (with Ag Force and PGW Seeds), MLA webinar, Beef Week, and with NABRAC.

Objectives

1.Provide a detailed understanding of mealybugs (including species identification, life history and identification by molecular markers) based on field, laboratory and glass/screen house studies

2.Identify natural enemies of mealybug species

3.Make preliminary recommendations on role of natural enemies in mealybug control

4.Develop standardised field sampling protocols for mealybugs and in conjunction with project partners

5. Outline of one draft manuscript for journal publication

6.Provide to MLA periodic information summaries suitable for general media on the 7.role and management of mealybugs in relation to addressing pasture dieback

8.A two-page summary outlining key facts and findings of mealybugs and how to identify mealybugs associated with dieback

3. Methodology

3.1 Provide a detailed understanding of mealybugs (including species identification, life history and identification by molecular markers) based on field, laboratory and glass/screen house studies

3.1.1 The mealybug

Mealybugs were collected from pastures with symptoms of dieback at 39 locations from North Qld, Queensland Central Highlands, Central Queensland, Toowoomba region, the Sunshine Coast, Brisbane, Gold Coast, and Northern NSW. Some of these locations (such as Banana station and the Arcadia Valley) included multiple sites to determine within-population diversity.

Sixty-six (66) samples were submitted to Dr Mark Schutze, Biosecurity Qld, for identification by comparison with the specimens from the 1926 outbreak (Summerville 1928). Thirteen (13) of these were specimens of other mealybugs that were submitted at the request of the landholder or were found on pasture grasses (such as the Rhodes grass mealybug), weeds, or surrounding vegetation close to dieback-affected pastures.

DNA was extracted from 121 mealybugs from 21 locations, including samples from the same locations as samples sent for morphological identification, and 8 samples known to be other species than *H. summervillei*. Multiple mealybugs from some locations were included to provide comparison of variation within and between populations. Three regions of the mealybug nuclear genome (18s, 28s and Dynamin) were amplified. Full primer details are given in Table 1.

Table 1: Primers used in this project

Target Region	Primer Details		
28S (~650bp)	Forward	S3660	5'-GAGAGTTMAASAGTACGTGAAAC-3'
	Reverse	A335	5'-TCGGARGGAACCAGCTACTA-3'
18S (~600bp)	Forward	2880	5'-CTGGTTGATCCTGCCAGTAG-3'
	Reverse	B	5'-CCGCGGCTGCTGGCACCAGA-3'
Dynamin (~300bp)	Forward	3006F1.1	5'- CCGGAYATGGCGTTCGAAGCTA-3'
	Reverse	3006R2.1	5'-TCTTCGTGGTTGGTGTTCATGTACGC-3'

Sanger sequencing was performed by Macrogen (Seoul, South Korea). Raw data in the .abi format was imported into R 4.2.0 using sangeranalyseR 1.6.1 and base calling and quality trimming were performed using default parameters. Sequence length distribution was visualised for each genetic marker. A minimum length cut-off was chosen for each marker, and all sequences with lengths below the cut-off were discarded.

Reference sequences were added to the 28S dataset from NCBI GenBank, allowing the taxonomic position of *H. summervillei* to be compared to the other *Heliococcus* mealybugs for which sequences

were available. The fall armyworm *Spodoptera frugiperda* was chosen as an outgroup, being closely related to both the scale insects and parasitoid wasps sequenced in this study.

Sequence alignments were generated for each marker using DECIPHER 2.24.0, and alignments were visualised with ape 5.6-2. The 28S alignment was truncated between 300 and 1200 base pairs due to the excessive length of a single reference sequence. A UPGMA tree was built using phangorn 2.9.0. This tree was optimised using a Generalised Time Reversal model, with optimisation of variable size proportion, and stochastic rearrangement, again with phangorn. The resulting tree was rooted by an outgroup, if one was present, and 100 bootstrap replicates were created. The resulting phylogenetic trees were plotted using ggtree 3.4.0. Where expert taxonomic identification was performed by Dr Mark Schutze of the Department of Agriculture and Fisheries (Queensland, Australia) this taxonomy was indicated on the phylogenetic tree. When sequences from samples identified as *H. summervillei* did not form a clade with the other mealybug sequences we performed a BLAST search in NCBI GenBank and removed the taxonomic assignment for any sequences that most closely matched a parasitoid wasp.

3.1.2 Laboratory life history (see below for field / seasonal biology)

Detailed life history studies were undertaken in the laboratory on Buffel grass and Rhodes grass.

In the first study, one hundred and twenty (120) mealybugs were followed from birth to death in 4 replicates. Neonates were collected from adult females in batches of 20 and raised on Buffel grass leaf in petri dishes at 26°C and observed between 1 and 3 days until death.

A second detailed study was conducted on both Buffel and Rhodes grass. In this study mealybugs were first reared in cohorts for 20 days (the age at which males begin to pupate), then separated into individual glass vials on cut leaf to prevent mating. Those that survived more than 24 hours (68 on buffel grass and 59 on Rhodes grass) were observed daily until death. Recorded data included date of death, pupation (males), change to pink colour (an indicator of sexual maturity in males and of reproductive capacity in females), and production of young (if occurred). Analysis included sex ratio and times of different instars, and calculation of median survival time (time at which 50% of those that died were alive) on the two different grasses by GLM in R Studio.

A third study determined the sex ratio, distribution of male and female mealybugs on the plant, and the reproductive strategy (sexual or asexual reproduction). Second instar mealybugs were collected from grasses grown and infested in insect screenhouses at the QUT research facility at Samford (SERF) in summer 2021: 100 from the upper leaf and 100 from the crown. These were reared for 1 week in individual containers until male mealybugs were observed to pupate. Male and female mealybugs were then either reared separately or combined in mating trios: two male pupae to one female 3rd instar nymph and reared on cut leaf until death. Recorded data included date of death, change to pink colour (and indicator of sexual maturity in males and of reproductive capacity in females), and production of young (if occurs). A second collection to determine sex ratio was made in summer 2022.

3.1.3 Transcriptome analysis: demonstrating the pathway by which mealybug causes ‘pasture dieback’.

3.1.3.1 Mealybug collection and maintenance

Mealybugs were collected from colonies maintained on American buffel grass plants by gently brushing the insects from the leaves using a fine wet paintbrush.

3.1.3.2 Infestation of plants

To contain the mealybugs to one area of the plants for feeding and sampling, we created leaf cages suitable for narrow, linear leaved monocot plants (Figure x). The clip cages consisted of a 120 mL collection tube with the bottom removed to allow for airflow. One opening is sealed by mesh and

the other is sealed by a soft pliable stopper made of cotton wool enclosed in mesh. The shape and size of these cages allowed for the most natural position of grass plants with the least damage to the leaf tissue. All mealybugs remained contained and accounted for during the experiment.

Twelve (12) healthy 8-week-old buffel plants were divided evenly into two groups: treatment and control. Plants within groups were further divided evenly into two sampling time points: 24 hrs and 72 hrs. Plants in the treatment group were infected with 15 mealybugs per plant, a mixture of 2nd and 3rd instars. Five (5) leaves from each biological replicate were inserted it to a leaf cage containing the pre-collected mealybugs. Leaves from control plants were inserted into empty cages. Leaf samples were collected from infested and control plants at 24 and 72 hrs. Feeding by mealybugs was evidenced at each time point by accumulation of honeydew on leaves and cages.

3.1.3.3 RNA isolation and quantification

Infected and non-infected leaves were flash frozen in liquid nitrogen and stored at -80 for downstream analysis. 100 mg of leaf frozen leaf tissue was ground into fine powder in liquid nitrogen. Total RNA was isolated using TRIzol (Invitrogen) method followed by Qiagen minelute clean up (Qiagen, Valencia, CA). RNA was digested with DNase using the RNase-Free DNase kit (Qiagen, Valencia, CA), subsequently eluted in 14 µl RNase-free water, and stored in -80 °C. RNA concentration was quantified by Qubit® (Invitrogen) according to the manufacturer's instruction

3.1.3.4 Library preparation and RNA sequencing

Libraries were prepared using illumina stranded mRNA prep kit (Illumina, San Diego, CA, USA) following the manufacturer's instructions. mRNA was purified from total RNA using oligo(dT) magnetic beads. First strand cDNA was synthesized using reverse transcriptase and random hexamers followed by the second strand synthesis. The cDNA fragments were 3' adenylated followed by anchor ligation. PCR was used to selectively amplify the anchor ligated DNA fragments and add dual indexes. The cDNA concentration of the library was determined using a Qubit® (Invitrogen) according to the manufacturer's instructions.

3.1.3.5 Read Mapping and gene expression quantification

Bioinformatics analysis was performed using tools available in Galaxy https://usegalaxy.org.au/. The quality of the sequencing data was assessed using the FastQC. Sequences were trimmed for quality and adaptors removed using Trimmomatic software. The sequences used for further analysis had a Phred score with a confidence level above 20. Trimmed transcript sequences were mapped to the annotated reference genome *Seteria italica* (NCBI Assembly: GCF_000263155.2) and quantified using RNAstar. Differential gene expression in response to mealybug infestation was analysed using DEseq2. Gene expression counts were normalized using fragments per kilobase of exon per million mapped reads (FPKM). Differentially expressed genes (DEGs) were determined with a log2 fold change > 2.0 cut-off and an absolute P-value of ≤ 0.05.

3.1.3.6 Pathway analysis

Gene ontology was performed using Galaxy tool Goseq. Pathway analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation service and KOBAS 2.0. http://kobas.cbi.pku.edu.cn/

3.1.4 Other causes: viruses

The presence of viruses in pasture grasses was tested by examination for small RNA fragments (Krauts et al 2009). Samples of leaf were collected from uninfected grasses grown in the glasshouse (as above), from grasses infested with mealybug in the screenhouse, and from grasses with symptoms of dieback and low levels of mealybug presence in the field as follows:

Controls: American buffel, Mekong brizantha, Gatton Panic
Treated: (infested with mealybug in screenhouses): Bluegrass, Gatton Panic, Rhodes Grass
Symptomatic, collected from the field: Rhodes grass

3.1.4.1 RNA isolation and quantification

RNA isolation and quantification was conducted as above (transcriptome) Infected and non-infected leaves were flash frozen in liquid nitrogen and stored at -80. 100 mg of leaf frozen leaf tissue was ground into fine powder in liquid nitrogen. Total RNA was isolated using TRIzol (Invitrogen) method followed by Qiagen minelute clean up (Qiagen, Valencia, CA). RNA was digested with DNase using the RNase-Free DNase kit (Qiagen, Valencia, CA), subsequently eluted in 14 µl RNase-free water, and stored in -80 °C. RNA concentration was quantified by Qubit® (Invitrogen) according to the manufacturer's instructions.

Samples were sent to AGRF for small RNA Library Prep with bead selection and NEXTFLEX Small RNA v3 Library Preparation. Detections of viruses was conducted using the QUT VirReport pipeline and the VirusDetect pipeline available at Standford University.

3.1.5 Other causes: *Fusarium*

Soil samples were collected from pastures at every point along transects of 5 or 10 metres. Fungi were isolated from roots and associated soil using methods described by Islam (2018) https://eprints.qut.edu.au/117674/.

Colonies were classified into morphotypes based on appearance and isolated by sub-cultured onto fresh medium. Isolates were then characterised by extraction of DNA, PCR amplification of the ITS marker and Sanger sequencing (by Macrogen).

Sequences were trimmed and quality-controlled using the sangeranalyseR package (Chao et al., 2021) with default parameters in RStudio 4.2.0 (RStudio Team, 2020). Resulting sequences were compared to the NCBI ITS region fungal reference database using the blastn search tool (National Center for Biotechnology Information, 2022). Representative species identifications were applied to the isolates grouped by morphology.

All Fusarium sequences were aligned with the complete listing of Fusarium ITS sequences from the NCBI reference material database. Alignment was conducted using the MUSCLE method in MEGA11. A Maximum-Likelihood phylogeny was constructed with the Tamura-Nei substitution model in MEGA11 using default parameters and 500 bootstrap replications (Tamura, Stecher, and Kumar, 2021).

Representative sequences were determined by choosing the first isolate from each date and site that appeared in the lowest level phylogenetic clades. Representative sequences were included with associated sequences from the NCBI reference material database in a new maximum likelihood phylogeny (as above).

3.2 Identify natural enemies of mealybug species

Data on incidence of predators (specifically *Cryptolemus*) was recorded at all sites if found.

3.2.1 Parasitoids

3.2.1.1 Collection Sites

Initial sampling at Brendale, QLD, collected mealybugs from which a single parasitoid emerged.

Seventeen (17) pasture sites at which mealybugs and symptoms of pasture dieback occurred were sampled over two years (May 2020- April 2022) across central and Southern Queensland and Northern New South Wales.

3.2.1.2 Live mealybug collection and rearing

Eight (8) sites were sampled fortnightly or monthly throughout the sampling period: Brendale, Samford Ecological Research Facility (SERF) and Kin Kin were sampled monthly May to October 2020 (winter), then fortnightly from October 2020 with additional sites at Maudsland (Creek and

Paddock), Wolvi and Nobby's Creek (NSW). Sampling was restricted on some weeks due to COVID lockdowns, state border restrictions and weather. Sampling was conducted monthly from April 2021 to March 2022. Sampling at Wolvi and Nobby's Creek was discontinued due to site recovery from mealybug and a site at Biggenden was added.

Pasture mealybugs (*H. summervillei)* were collected at 5m intervals along a 50m transect. A sample of surface area approx. 25cm^2 was dug up using a post hole shovel and searched destructively for mealybugs, recording the instar and location each mealybug was found (Leaf/stem, thatch/surface, roots/soil). Live mealybugs were collected into 50ml falcon tubes with grass and kept cool during transport to the laboratory. Only live mealybugs were collected: no mummified mealybugs were collected from the field.

Mealybugs were removed from field samples and the number with black marks (indicating parasitism) was recorded. Mealybugs were then reared in glass scintillation vials with up to 20 mealybugs per vial. The top of the vial was covered with fine fabric voile to prevent moisture build-up. Fresh grass was added to vials every 3-4 days. Vials were examined every 2 to 3 days for mummies or wasp emergence, which were collected and stored in 80% ethanol at -20°C

3.2.1.3 Sweep nets and Emergence traps

Insect collections were made at 17 sites affected by pasture mealybug across Central and Southern Queensland and Northern New South Wales. Sweep nets (38cm diameter insect sweep with a 74 cm tapered net bag) were used sampled at 3 random locations within the sampled area for each site visit. The net was swept five times towards the ground cover for each collection, taking ~1m steps per sweep. The contents of nets were emptied nets into large ziplock bags and kept on ice during transportation then frozen at -18°C.

Emergence traps (Bugdorms™ soil emergence trap white 60x60x60cm with 108x32 mesh, MegaView Science Co., Ltd., Taichung, Taiwan) (Lingbeek et al. 2021) were installed at two (2) locations (Brendale and SERF) from May 2020 until March 2022. Three dorms were placed at random points across each of the mealybug-affected areas, and GPS coordinates were recorded. Bug dorms were initially paced at other sites but were removed due to destruction by cattle.

Approximately 100ml was 100% propylene glycol was used as the collection trap to preserve the samples (Nakamura et al. 2020). Collection reservoirs were replaced once a month with a clean tube with propylene glycol and stored at ~26°C. After each collection, the dorms were moved into a new area for the next collection period.

Invertebrates were from sweep nets and emergence bug dorms were initially sorted into seven orders or morphotypes based on body shapes (spiders, ants, beetles, flies and wasps, leafhoppers, mealybugs, and 'other'). Flies and wasps, which are often very small (under 3mm) were more carefully searched. Any known parasitoids of *H. summervillei* identified from emergence from mummies, or from literature, were extracted. Care was taken to try to identify any samples of *C. sexguttata* (Summerville 1928). All samples were preserved in propylene glycol or 80% ethanol at -20°C.

3.2.1.4 Identification of emerging wasps and confirmation of host identity

The identity of 4 mealybugs was confirmed by Dr Mark Schutze, Queensland Biosecurity, by examination of the mummies or whole mealybug and comparison with the specimens of *H. summervillei* identified by Brooks. These were: one mummy from Theodore (collected 27/11/20), two from Taroom (collected 8/12/2021) and one whole mealybug with black marking from Gaeta (April 2021).

Specimens collected in 2019 were sent to Dr Chris Burwell (Queensland Museum) for identification. All other specimens were identified to genus level using a dichotomous key for encyrtid wasps focused on the funicle/pedicle length (Noyes and Hayat 1984). Specimens were later confirmed from

photographs in consultation with Dr. John Noyes (Natural History Museum), and international expert in Encyrtid wasps.

3.2.1.5 Molecular identification of wasps

A reference library of wasp DNA 'barcode' sequences was prepared to DNA was extracted from wasps. Molecular identification of wasps was conducted to support the conventional taxonomy, and to provide reference markers to identify wasps in un-emerged mummies.

Wasp specimens collected from the site at Brendale, QLD, using sweep nets. Specimens were classified to family level using a morphological taxonomic key (Lucid Key for Australasian families of Hymenoptera). DNA extraction was conducted using QuickExtract (LGC, Biosearch TechnologiesLtd.) method and PCR amplification using the Hymenopteran-specific 28S-D2 primer. Sanger sequencing was conducted by Macrogen. Sequence data was corrected and compared to reference sequences from the NCBI database using Geneious Prime.

3.2.2 Pathogens

A small number of mealybug cadavers recovered from quadrat searches were apparently infected by fungal pathogens. Fungi were isolated using selective media and identified from morphology and PCR amplification and Sanger sequencing of the ITS4 DNA 'barcode', as for *Fusarium*, above.

3.3 Make preliminary recommendations on role of natural enemies in mealybug control

Preliminary recommendation on the role of natural enemies are discussed in the results and conclusions.

3.4 Develop standardised field sampling protocols for mealybugs and in conjunction with project partners

3.4.1 Development of sampling methods

Detailed sampling was conducted at 8 locations over 2 years as described above.

- QUT's facilities at SERF (Samford),
- A beef grazing property Brendale (Brisbane)
- A beef grazing property Kin Kin (northern Sunshine Coast),
- A slashed acreage property at Wolvi (northern Sunshine Coast),
- Two locations at a beef grazing property Maudsland (Gold Coast),
- A site at Murwillumbah (Northern NSW).
- A site at Biggenden, Queensland

Eight (8) sites were sampled fortnightly or monthly during the sampling period. Brendale, Samford Ecological Research Facility (SERF) and Kin Kin were sampled monthly May to October 2020 (winter), then fortnightly from October 2020 to April 2021 (summer) with additional sites at Maudsland (Creek and Paddock), Wolvi, and Nobby's Creek (NSW). Sampling at Wolvi and Nobby's Creek was discontinued in the 2021/22 season due to site recovery from mealybug and a site at Biggenden was added. Sampling was conducted monthly from April 2021 to March 2022.

A further 10 pasture sites at which mealybugs and symptoms of pasture dieback occurred were sampled systematically during the two years (May 2020- April 2022) across central and Southern Queensland and Northern New South Wales: Mundubbera, Moura, Banana, Rockhampton (2 sites), Theodore, Arcadia Valley, Taroom, Jambin, and Mulgildie. Sampling was restricted on some weeks due to COVID lockdowns, state border restrictions and bad weather.

Sites were sampled at 10 points at 5m intervals along a 45m transect using 2 methods: a 50cm x 50cm quadrat search of grass, thatch and soil surface, and a 25cm x 25cm dug sample of grass, thatch, soil and roots. The different instars of mealybug, location in the sample (soil, crown, leaf), soil moisture, and degree of dieback symptoms were recorded.

Initial dug samples were returned to the laboratory and searched destructively, a labour-intensive process requiring transport of large amounts of material. A process was developed by comparing number of mealybugs observed in complete destructive sampling to 10 minutes intensive sampling similar to an approach used to quantify mealybugs in grass variety and insecticides spray trials (reported B.PAS 0006).

It was found that 10 minutes intensive sampling typically completes the search of dug samples and records the majority of mealybugs, and this was incorporated into a field sampling method. This method was then tested in field using both quadrats and dug samples (placed on a plastic sheet) were initially searched intensively for 10 minutes and then until no more mealybugs were observed. This confirmed that both methods found all or most of the mealybugs in the first 10 minutes, creating a simple sampling method that could search 10 data points along a transect in 2 hours.

3.4.2 Training

The sampling method was tested with the agronomists and graziers using either 10 dug samples on a 45m transect, or 5 samples on a 25m transect, depending on the time availability for the grazier/agronomist and the number of mealybugs in each sample. Property managers or agronomists at the Biggenden, Moura, Kin Kin, Mundubbera and Mulgildie, Banana, Rockhampton, Arcadia Valley, and Mullumbimby properties were trained in the procedure after QUT had completed the initial survey and sampling. All graziers were followed up with visits by QUT staff in September (and October) 2020

Sampling methods and reporting sheets were modified for both ‘expert’ and ‘graziers’. The ‘expert’ site and dieback assessment sheet was finalised and shared with all MLA dieback program participants and NSW DPI in November 2020.

Storyboards for training material were developed. What to look for (symptoms, where to look for mealybugs, use of a hand lens), sampling procedures (dug sampling and searching along a transect) and collection of mealybugs in the field were photographed. The steps in these processes were described using PowerPoint with instructions at each step to create ‘story boards’ on ‘what to look for’ and ‘How to sample’.

Storyboards were used as a basis for filming in a pasture dieback site in April 2022 and are now being edited prior to uploading.

3.5 Outline of one draft manuscript for journal publication

See ‘results’ and other sections, below.

3.6 Provide to MLA periodic information summaries suitable for general media on the role and management of mealybugs in relation to addressing pasture dieback

Communications were provided frequently to MLA program leaders and comms team through reports, emails, a webinar, on-line participant meetings, material for specific media outputs, and a national science panel review in April 2022. A summary has been provided separately for all 3 QUT projects.

The ‘expert’ site and dieback assessment sheet was finalised and shared with all MLA dieback program participants and NSW DPI in November 2020.

3.7 A two-page summary outlining key facts and findings of mealybugs and how to identify mealybugs associated with dieback

A summary of key findings is provided in results. Two articles on key facts and management strategies for graziers have been published by the MLA comms team and a third is in preparation for spring 2022. A summary of key findings is provided in results.

The 'expert' site and dieback assessment sheet was finalised and shared with all MLA dieback program participants and NSW DPI in November 2020.

4 Results

4.1 Provide a detailed understanding of mealybugs (including species identification, life history and identification by molecular markers) based on field, laboratory and glass/screen house studies

4.1.1 Description of the mealybug.

H. summervillei were collected and identified at 39 locations across Queensland and Northern New South Wales (Fig. 1). Some of these locations included multiple sites (such as Banana station and the Arcadia Valley) to determine within-population diversity.

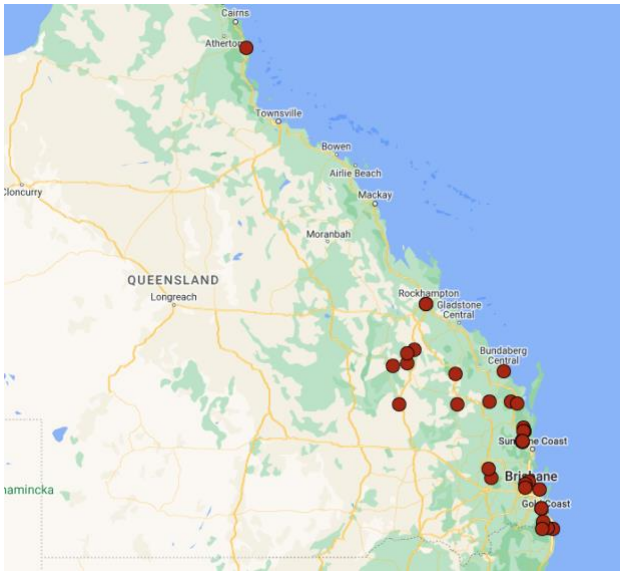

Figure 1: Locations of mealybugs collected and identified as *H. summervillei*

Sixty-six (66) samples of adult female mealybugs were sent to Dr Mark Schutze at Biosecurity Qld for identification by comparison of morphology with voucher specimens. These included 13 mealybugs associated with pastures (weeds, nearby vegetation, or submitted by graziers) that were thought not to be *H. summervillei*, but to confirm identity (Table. 2).

Table 2: Other mealybugs recovered from pasture, weeds, and nearby vegetation and crops. Identifications of mealybugs was confirmed by Biosecurity Qld.

Mealybug species	Host plant
Saccharicoccus sacchari	Sugar Cane
Vryburgia brevicruris	Bluegrass
Antonina graminis	Rhodes grass, Setaria, *Urochloa sp.*
Trionymus ascripticius	Balloon bush
Phenacoccus solenopsis	Parthenium
Monophlebulus sp	Brigalow
Coccus longulus	Leucaena
Hypogeococcus festerianus	Harrisia cactus
Icerya aegyptiaca	*Urochloa sp.*

The mealybug causing pasture dieback was confirmed by Biosecurity Qld as a variant of the species *Heliococcus summervillei* Brooks (*Pseudococcidae*, *Phenacoccinae*). This species has a history of causing 'pasture dieback' in Australia (1926 and 1938), and internationally in New Caledonia (1998), Barbados (2020) and Puerto Rico (2019).

Detailed comparisons with the original specimens collected by Summerville (1928) show a slight difference in anatomy (Schutze et al 2019): there are fewer translucent pores on the hind tibia than reported in the samples collected in 1926, with some similarities with the Pakistan samples (Summerville 1928, Brooks 1978). This initially led to suggestions that the recent samples were a different species, but a review of all findings by national experts (Schutze, Tree and Gullan) determined that this was simply variation within the species.

There were also minor differences in samples from the recent outbreak of *H. summervillei* in Barbados. Dr Zee Ahmed, Florida State Collection of Arthropods, reported that "*The consistent difference in the character between Barbados samples and the original illustration [Brooks 1978] seems like the presence of additional crateriform tubular ducts, one on the head and one in the medial region on each of abdominal segments III and II. The number of large crateriform ducts is more than 100 on the dorsum of specimens from Barbados (74-86 in the Australian sample and 78-106 in specimens from Pakistan), and cerarian setae have more than 4 somewhat lanceolate setae (2 to 3 in species description of H. summervillei). More than six small setae of similar shape (4 to 6 in species description), anal ring setae appear to be of the same size as of anal lobe setae in Barbados samples."*

It appears then that there is some variation in morphology in different outbreaks and populations of *H. summervillei* across time and location. This begs the question: how diverse is the population in the current Australian outbreak, and could it be an incursion of a new variant?

DNA was extracted from 121 mealybugs, which included 8 other mealybugs from surrounding vegetation. Minimum length cut-offs were chosen from the expected sequence lengths and empirical sequence length distributions (Figure 2), and all sequences below the cut-offs were excluded from downstream analysis.

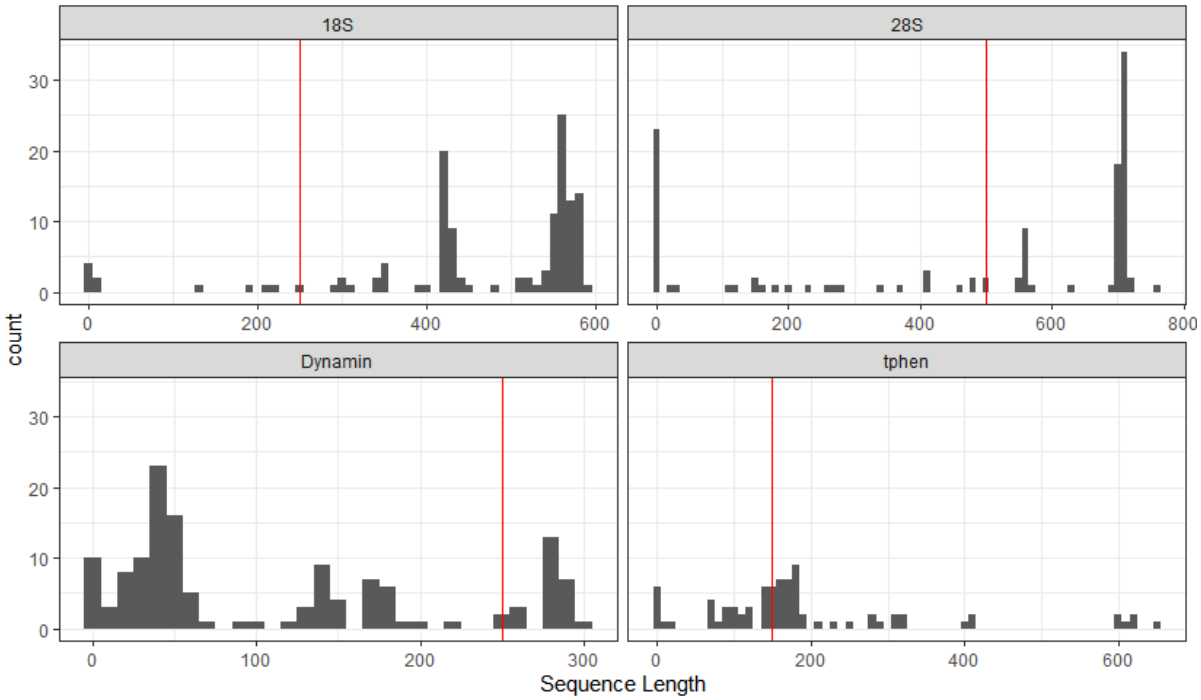

Figure 2: Histogram showing distribution of sequence lengths for each marker gene. Red lines indicate the chosen minimum length cut-off for each gene.

Molecular analysis (Sanger sequencing of 18s, 28s and Dynamin 'DNA barcodes') demonstrated that there is very little diversity within the *H. summervillei* population in the current outbreak (Figs.3 &4). Variation across the state was no greater than variation within sites. This confirms that the *H. summervillei* in the current outbreak is a single species in all sites sampled across central and southern Queensland and New South Wales. It also demonstrates that DNA sequence analysis, particularly using 28s, can be used to differentiate *H. summervillei* from other mealybug species in and around pastures.

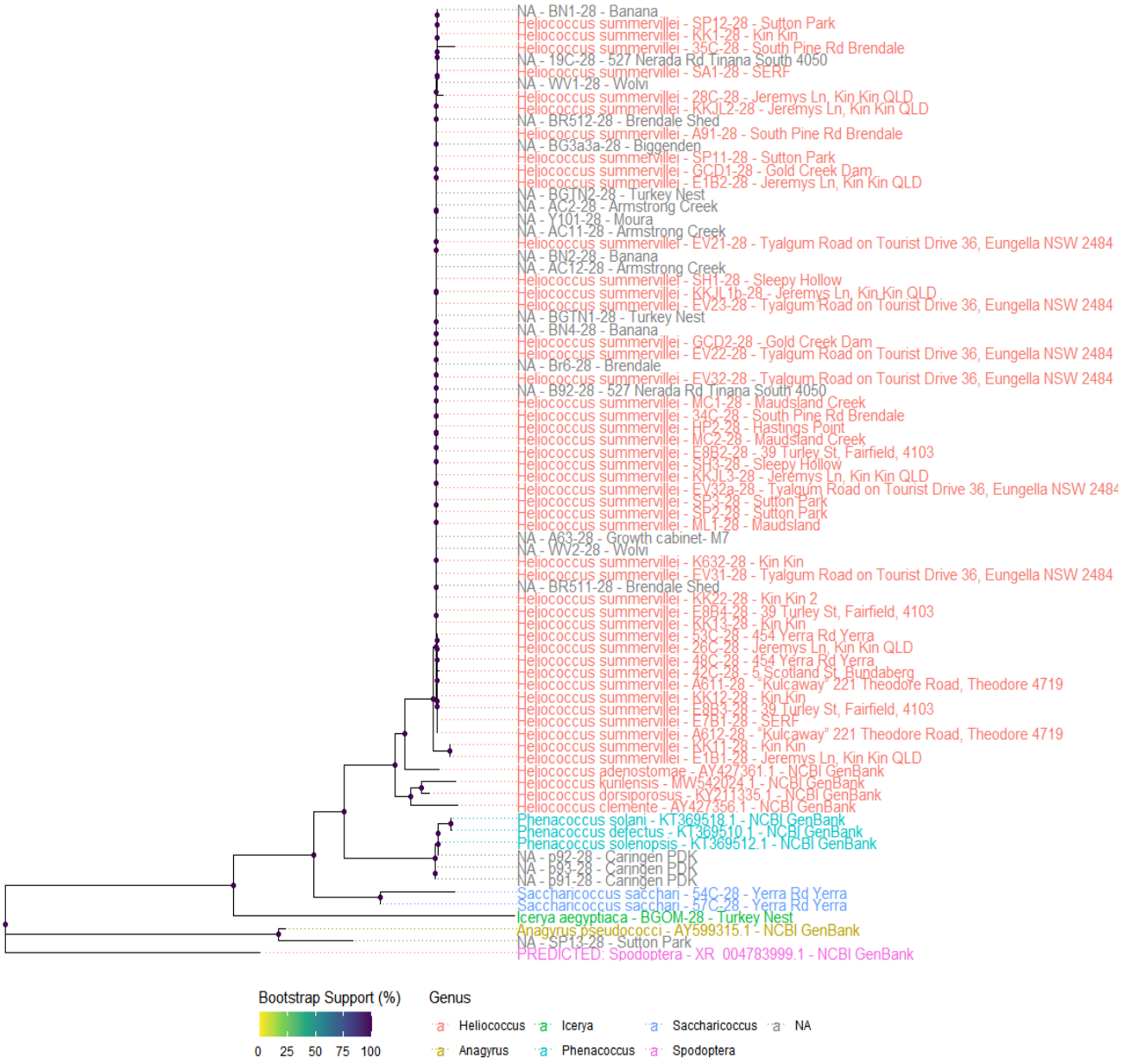

Figure 3: Phylogenetic tree produced using 28S gene. Tip labels include taxonomic ID from a database or expert examination, PCR or NCBI GenBank ID, and the location from which each sample was collected. Tip labels are coloured by the genus to which samples from the same collection were assigned by Biosecurity Qld (if confirmed). Node points are coloured by bootstrap support value.

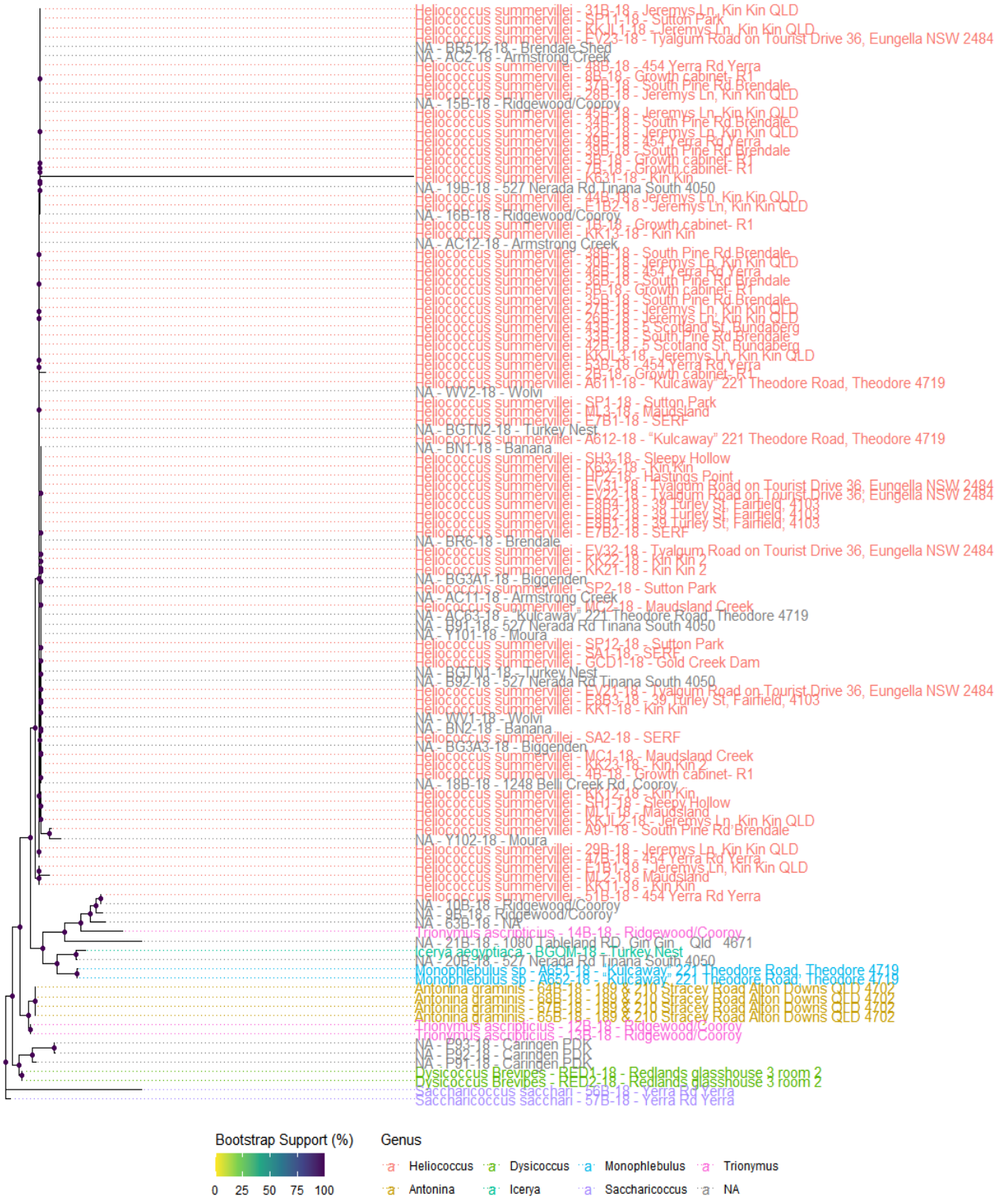

Figure 4: Phylogenetic tree produced using 18S gene. Tip labels include taxonomic ID from a database or expert examination, PCR or NCBI GenBank ID, and the location from which each sample was collected. Tip labels are coloured by the genus to which samples from the same collection were assigned by Biosecurity Qld (if confirmed). Node points are coloured by bootstrap support value.

The lack of diversity in the population using both physical characteristics and sequence data in the current outbreak suggests firstly that the original 1926 variant found by Summerville is no longer

widespread in the population, and secondly that the variant in the current outbreak may be an incursion of a new variant. Dr Schutze did identify a single sample of leaf in the Biosecurity Qld. collection, submitted from Atherton in 2016, in which both variants were present. No examples of the 1926 variant have been found since.

The lack of genetic diversity in the population suggests that the variant in the current outbreak may be an incursion of a new variant. Unfortunately, there is no data on genetic markers or variants in populations from overseas and it has not been possible for us to obtain overseas material for comparison (though contact with Dr Ahmed and collaborators on the Barbados outbreak has been good). The only sequences available from *H. summervillei* specimens are from the cytochrome c oxidase 1 gene, preventing direct genetic comparison of samples from this study with existing resources. Obtaining additional sequences from other closely related species would help clarify the taxonomic position of *H. summervillei*.

There are alternative options to investigate any diversity in the current Australian populations: endosymbiont DNA sequences in Russian wheat aphid have been found to be a high-resolution population genetic marker that is extremely useful in studies of invasion over a relatively short evolutionary history time frame (Zhang et al 2014). Further work on variation in the *Candidatus Tremblaya phenacola* symbiont cross the *H. summervillei* population will be conducted on samples already in the collection as part of post graduate studies after the end of this project.

4.1.2 Laboratory life history

Both sexes appear to have 4 instars. Males pupate in the 3rd instar and emerge as winged adults (4th instar). The ratio of males to females of second instars collected from grasses in the screenhouse was 50:50, but the ratio of insects reared from neonates in the laboratory was lower, at 25% male (1:3, M:F). Survival of males was much lower in Rhodes grass where only 1 male pupa emerged.

Second instar mealybugs collected from crown or leaf of grasses in summer 2020/21 showed an interesting distribution. In the crown the ratio M:F was 2:1, on the leaf it was M:F 0.3:1. In 2022 the pattern was closer to 50:50 male and female both on the leaf and in the crown (Fig. 5).

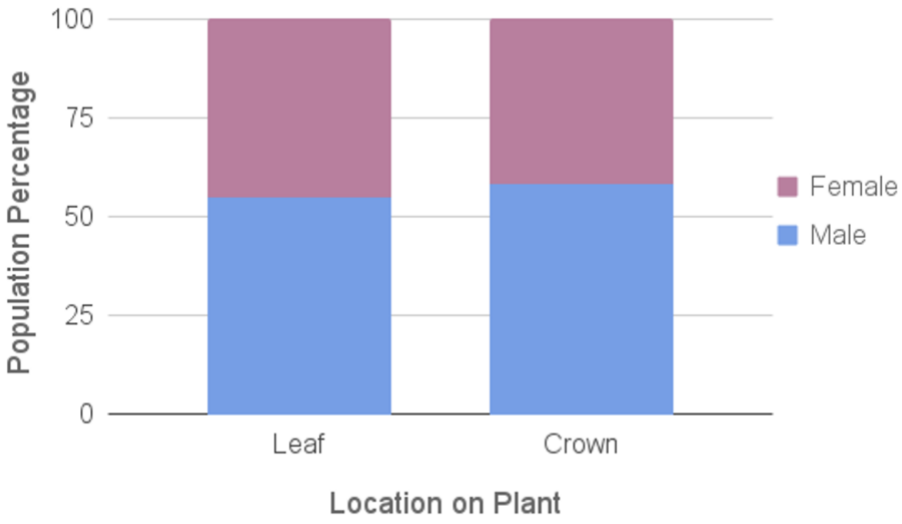

Figure 5: Distribution of male and female second instar nymphs collected from crown or leaf and reared to pupation (males) or 4th instar in the laboratory, summer 2022.

The method used in here (collecting second instars) probably biases the result towards distribution of early instars rather than mature adults. We know from field samples (below) and glasshouse

studies (B.PAS 0003) that mature females remain mostly in the crown of the plant (and reported by Summerville 1928). It appears that the males may also pupate in the crown, possibly to mate with maturing females, while early instars climb onto the leaf, possibly to disperse in wind, and return to the crown to mate and reproduce.

Male 3rd instar nymphs, pupae and adults are easy to recognise (elongated, pink). Males pupate at around 15 to 20 days in the 3rd instar and emerge as pink, winged adults at 20 to 23 days. Males were more short-lived than females, with a maximum observed lifespan of 29 to 33 days depending on temperature. Observation during screenhouse monitoring of mealybugs (B.PAS 003) suggests that males are produced mainly during the peak summer breeding season and are not observed at all in winter.

Mature females turn pink only when mated, with first production of young between 37 and 45 days. This is about half the time recorded by Summerville (1928). Most of the young (average 106 young per female, n=48) are produced in the first 24 hours after maturing (turning pink) in the laboratory and on average females produce young for 7 days. Most females die 10 days after beginning reproducing, some may live much longer (88 to 100 days in different assays).

Females do not appear to feed once they begin production of young, but instead disperse in containers and cages, with an apparently thigmotactic behaviour, hiding in corners and crevices in cage materials. This aligns with their observed behaviour in the field, where mature females disperse, hiding under logs, dry cowpats and in soil during cool and dry conditions. The majority of mealybugs recovered in the field from April to September are found in such close, hidden locations.

Reproduction in all assays was exclusively sexual. Unmated females raised as individuals either when collected as second instars from grass or from laboratory reared cohorts remain white and do not reproduce. White final instar females have never been observed to produce young in the laboratory. However, large, white females surrounded by neonates have been collected in the field in soil during winter, and it is possible that there is a parthenogenic life stage during the winter under field condition. Facultative parthenogenesis has been observed in other mealybug species particularly when males are not available. It may be that unmated (white) females produce young asexually over winter.

4.1.3 Evaluation of mealybug survival and development on different grass varieties

There was a significant difference in mealybug development and survival on the different grass varieties. Fewer mealybugs survived, and those that died did so earlier and smaller on Rhodes grass than on than on Buffel grass. A similar pattern was observed in Mekong brizantha and Gatton Panic compared to American buffel (reported in B.PAS 0006)

Early instar larvae die in significant numbers (approximately 30% die between days 10 and 20) on both varieties, but the proportion of death was higher on Rhodes grass. A preliminary analysis of the median survival time (ST_{50}) of mealybugs between 10 and 20 days of age showed that 50% of the mealybugs that died on American buffel grass had died by 15 to 16 days of age, while 50% of the nymphs on Rhodes grass had died by 13 days of age, i.e median time to death was more rapid on Rhodes grass (Figure 6).

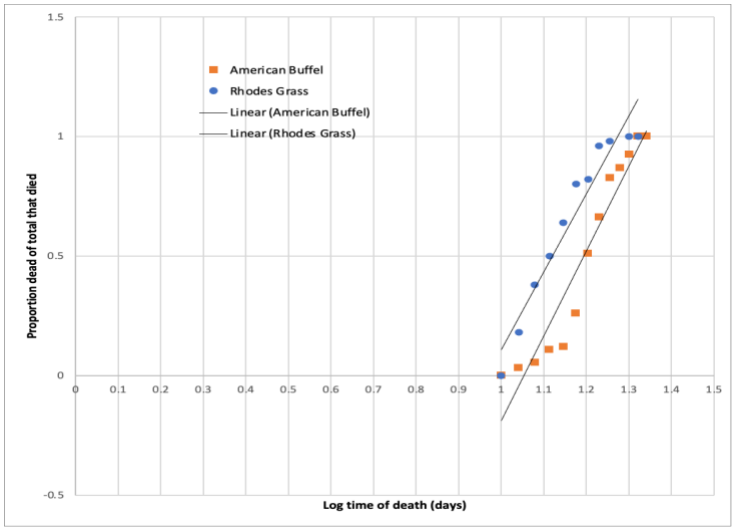

Figure 6: Plot of proportion mortality against log of time to death (in days) between 10 and 20 days post-partum of cohorts raised on American buffel grass (orange) and Rhodes grass (blue).

The results of this first assay suggested that conventional bioassay techniques such as survival time analysis could be used in laboratory populations as a rapid test (10 days to 2 weeks) to determine the relative impact of tolerant and susceptible grass varieties on mealybugs. This is a useful adjunct to more costly, seasonal and time-consuming screenhouse tests that determine the impacts of mealybugs on the grass.

This method was successfully used to compare tolerant or resistant grass varieties by a comparison of survival time and development, reported in testing pasture varieties in B.PAS 0006. Survival on Mekong brizantha was less and time to death shorter than on Gatton Panic, on which survival and median time to death were in turn less than on American buffel. These patterns of increased mortality, shorter median time to death and reduced development on less favourable hosts also mirror the pattern of resistant varieties in screenhouse infestation assays reported in B.PAS 0006. However, the laboratory ST50 assay were more statistically robust and sensitive in detecting differences between varieties, and can be conducted rapidly, and year-round, using laboratory populations

In summary, laboratory life history studies on American buffel and Callide Rhodes grass demonstrated that the mealybug, *Helioccocus summervillei* Brooks, is exclusively sexually reproductive under laboratory conditions and that the sex ratio is 1:1 during the summer season. There is limited evidence of asexual reproduction in the field over winter.

Laboratory experiments confirmed that mature, mated females turn pink and do not feed, but are highly mobile and disperse before producing young. Males pupate in the third instar. Adult males do not feed and disperse by flying.

4.1.5 Mealybugs as the primary cause of pasture dieback

Laboratory infestation of American buffel grass with the mealybug resulted in rapid development of symptoms of dieback (5 days) and at low numbers of mealybug (5 or less).

4.1.5.1 Transcriptome analysis

Transcriptome analysis of American buffel grass infested with *H. summervillei* results in rapid (24 hour and 72 hour) changes in gene expression (Fig. 7).

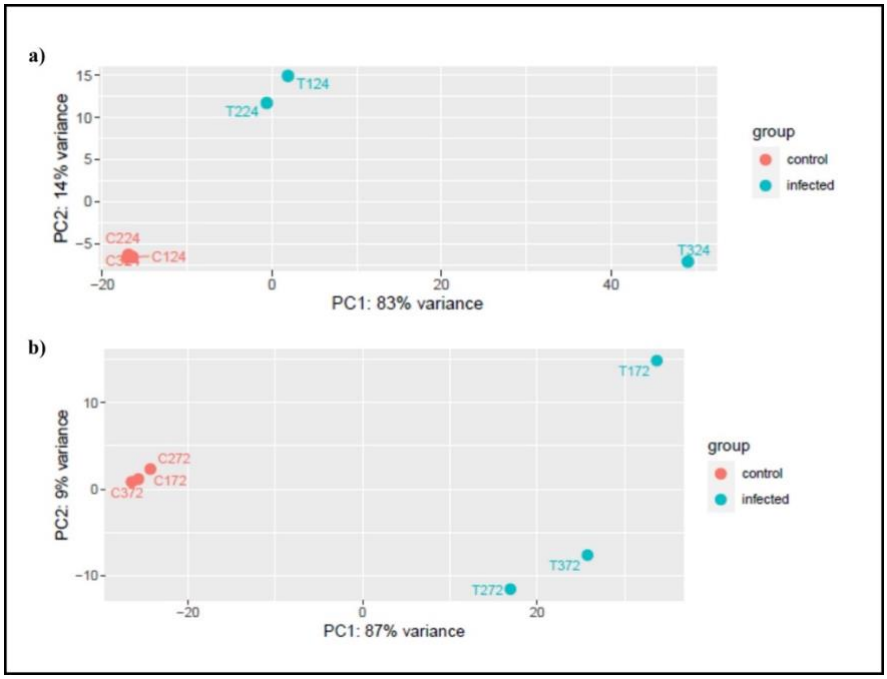

Figure 7. Evaluation of RNA-seq DEseq2 data. Principle components analysis (PCA) maps showing the largest component of variance. a) 24 h post infection PC1 (83% of the variance) and PC2 (14 % of the variance). b) 72 h post infection PC1 (87% of the variance) and PC2 (9% of the variance).

H. summervillei feeding over 24 and 72 hrs resulted in significant induction of several Salicylic acid (SA) related genes and downregulation of several Jasmonic acid (JA) related genes (Fig. 8).

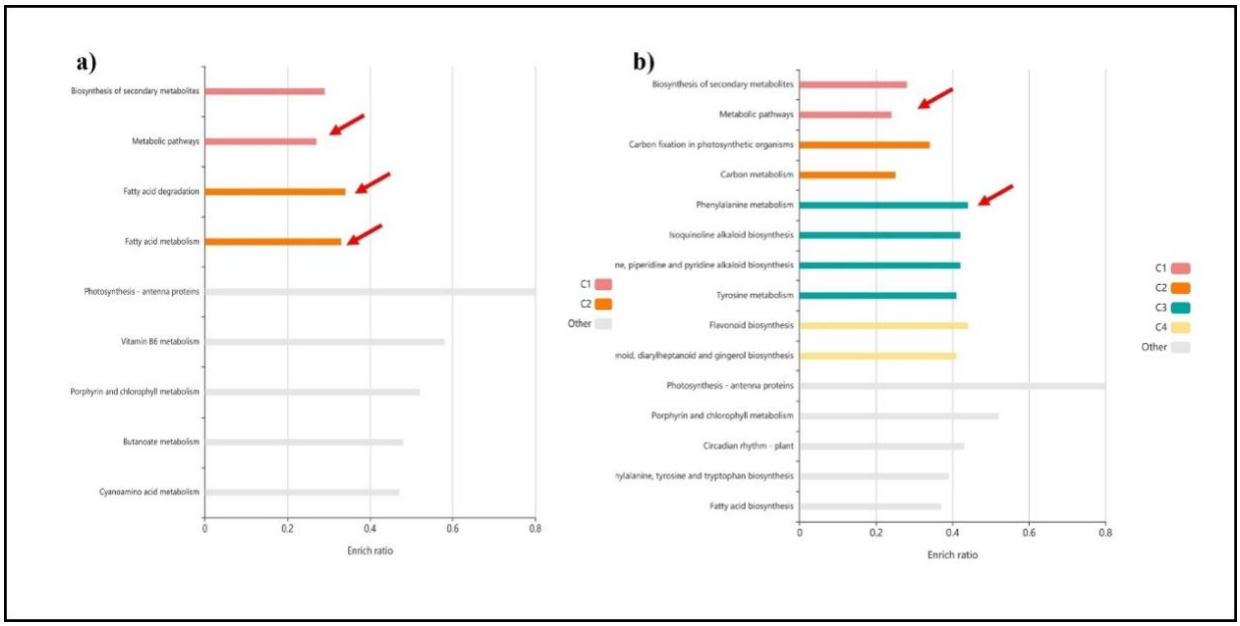

Figure 8. KEGG Enrichment analysis relative to untreated control a) 24 h post infection b) 72 h post infection. Y axis represents significantly (P< 0.05) enriched pathways categories, X axis represents enrichment ratio (number of genes differentially expressed/background genes higher enrichment ratio indicates greater enrichment). Red arrows indicate enriched pathways related to JA and SA synthesis. KEGG pathway categories: C1 (pink) = global metabolic pathways, C2 Orange = lipid metabolism, C3 (green)= aminoacid metabolism, C4 (yellow)= biosynthesis secondary metabolites

Specifically, differential expression analysis of RNA sequencing data revealed induction of SA-responsive regulatory proteins (TGAL6, NPR1), SA biosynthetic genes (PAL, CM2), a repressor of JA

dependent genes (TIFY11b), and SA dependant genes (PR1, CHIT1) (Fig 9). The function of these genes is listed in Table 3.

In addition to modulation of SA/JA signalling we found transcription factor WRKY2 to be successively downregulated at both time points (Fig 9, Table 3) compared to uninfected control?. WRKY proteins play pivotal roles in regulation of transcription associated with stress responses, and are their expression is typically upregulated in response to biotic stress such as fungal attack (Muthamilarasan et al 2015). KEGG pathway analysis showed this gene fell into the plant-pathogen interaction pathway and is responsible for regulating defence responses to fungus and fungal effectors. The downregulation of this gene after exposure to mealybug suggests an active downregulation of these key genes, making the plant more vulnerable to biotic attack, including fungi.

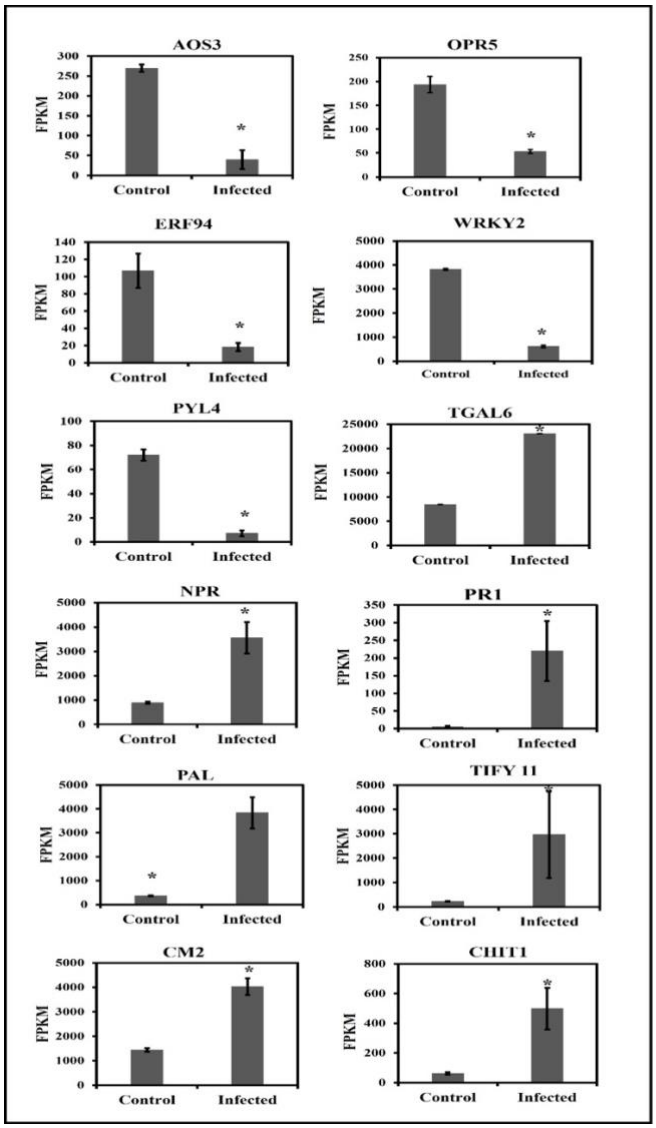

Figure 9. Significant gene changes in American buffel grass 72 hours post infection with mealybugs. Data is shown as FPKM (fragments per kilobase of million mapped reads) ± SE n=3 *=P<0.01.
JA related genes: AOS3 (Allene oxide synthase 3), OPR5 (12-oxophytodienoic acid reductase), ERF94 (Ethylene-responsive transcription factor), PYL4, (Pyrabatin resistance-like 4).
SA related genes: TGAL6 (TGA like transcription factor 6), NPR1 (Non-expresser of PR genes 1), PR1 (Pathogenesis related protein 1), PAL (Phenylalanine ammonia lyase), TIFY11 (JAZ domain-containing protein), CM2 (Chorismate mutase 2), acidic endochitinase (CHIT1).
Transcription factor WRKY2 is significantly downregulated.

Table 3. Functional annotation of genes of interest showing log changes (up or down regulation) relative to uninfected control at times after start of mealybug feeding. N/A = no difference from control detected.

GENE NAME	LOG2(FC) 24 HRS	LOG2(FC) 72 HRS	ANNOTATED FUNCTION/ PROPOSED FUNCTION
OPR5	-1.3	-1.8	Enzyme in the octadecanoid pathway required for the synthesis of JA precursor *cis*-12-oxo-phytodienoic acid (OPDA)
ERF94	-0.7	-2.4	Positive regulator in the ethylene responsive branch of the JA pathway
AOS3	N/A	-2.5	Catalyses the first step in the biosynthesis of jasmonic acid
PYL4	N/A	-3.1	ABA receptor involved in JA signal transduction through the regulation of regulatory transcripts.
WRKY2	-1.0	-2.6	Positively regulates JA responsive defence genes. Role in defence against fungal pathogens.
TGAL6	1.1	1.4	Co-regulator of SA dependent gene expression and involved in transcriptional repression of JA responsive genes.
NPR1	N/A	2	Master regulator of SA dependent gene expression and essential for SA/JA cross talk
PR1	N/A	4.7	Marker gene for SA accumulation in plants
PAL	2.3	3.3	Shikimate pathway enzyme required for the biosynthesis of SA and other secondary metabolites
TIFY11	N/A	3	Transcriptional repressor protein of JA responsive genes
CM2	0.95	1.5	Shikimate pathway branch point required for cytosolic phenylalanine production
CHIT1	N/A	2.9	SA dependent gene, part of the pathogenesis-related protein family

GO analysis of transcriptome sequences for 24 hours post infection show that cinnamic acid biosynthesis and ammonia-lyase activity are over-represented (Fig 10). This is important because plants synthesise SA via cinnamate and PAL.

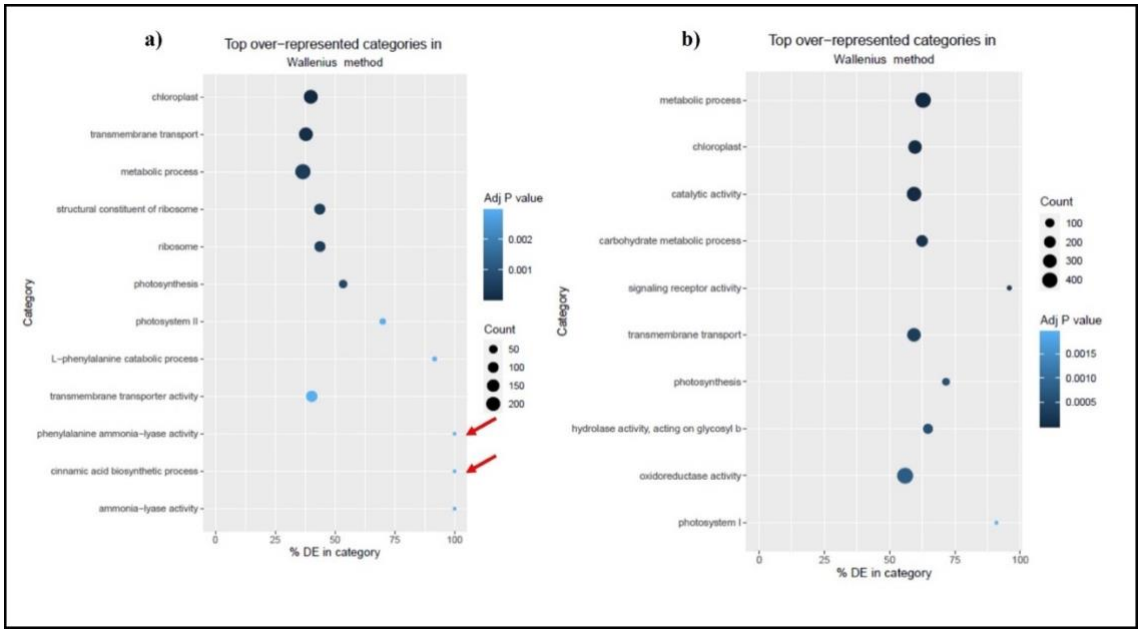

Figure 10. GO Enrichment analysis of transcriptome sequences. Top 10 significantly enriched Go terms, a) 24 h post infection b) 72 h post infection. Y axis represents GO categories, X axis represents percentage of genes in category that are significantly (P< 0.05) differentially expressed. Red arrows indicate enriched categories related to SA synthesis: cinnamic acid biosynthesis and ammonia-lyase activity are over-represented.

These changes effectively shut down Jasmonic acid/Salicylic acid pathway and down-regulate critical genes involved in defence against fungal pathogens. Disruption of levels of Jasmonic acid/Salicylic acid (the main mechanisms of plant defence against fungal pathogens) has been previously shown to be an effect of feeding by the Solenopsis mealybug on its host plant (cotton).

These findings support the hypothesis that feeding by *H. summervillei* modulates the antagonistic SA/JA crosstalk to induce SA related genes and suppress the more effective JA defences in buffel grass. These JA signalling suppression mechanisms and/or SA induction mechanisms could be synthesised directly by *H. summervillei* or its endosymbionts, and subsequently released in oral secretions.

This work demonstrates the pathway by which the mealybug affects the plant immune responses, making pasture grasses susceptible to infection by a range of fungal pathogens, and opens up new avenues of research in insect/symbiont pest/plant biology and screening for resistant pasture varieties. Analysis of the data continues for publication. Further research is planned under a PhD project proposed to begin in 2023.

4.1.5.2 Other causes: viruses

The aim of the virus screen was to see if the approach using deep sequencing of small RNA could detect any pattern of viruses associated with infestation by *H. summervillei* and 'pasture dieback' across a variety of grass species when compared to glasshouse controls. The analysis appears at first glance to suggest that some viruses, specifically some Badnaviruses and the insect-pathogenic Dicistroviruses, are associated with screenhouse-infested or field collected grass samples when compared to glasshouse controls (Table 4).

Table 4: Small RNA fragments of plant viruses identified in grass samples grown in glasshouses without exposure to mealybugs (controls) or infested with mealybugs in a screenhouse (Treated) or collected from mealybug-infested plants with symptoms of dieback from the field (Field).

Sample	Targeted species generic name	Mean coverage	Read count
Control buffel	Banana streak virus	13.11	374
Control buffel	Sugarcane bacilliform virus	5.56	5626
Control mekong	Enset leaf streak virus	25.58	29566
Control mekong	Pineapple bacilliform CO virus	7.87	1365
Control mekong	Sugarcane bacilliform Guadeloupe D virus	29.36	29994
Control mekong	Sugarcane bacilliform virus	9.81	12614
Control Panic	Banana streak virus	14.75	977
Treated bluegrass	Sugarcane bacilliform Guadeloupe A virus	0.12	41
Treated bluegrass	Sugarcane bacilliform virus	0.12	41
Treated Mekong	Banana streak OL virus	1.46	85
Treated mekong	Melicytus chathamicus endogenous virus Badnavirus	8.87	219
Treated mekong	Sugarcane bacilliform Guadeloupe A virus	2.36	883
Treated mekong	Sugarcane bacilliform Guadeloupe D virus	4.53	1558
Treated mekong	Sugarcane bacilliform virus	2.34	873
Treated Panic	Banana streak CA virus	0.52	183
Treated Panic	Banana streak OL virus	1.1	65
Treated Panic	Dioscorea bacilliform virus	1.11	29
Treated Panic	Melicytus chathamicus endogenous virus Badnavirus	0.9	23
Treated Panic	Planococcus ficus-associated dicistrovirus 1	1.47	692
Treated Panic	Sugarcane bacilliform Guadeloupe A virus	0.64	227
Treated Panic	Sugarcane bacilliform Guadeloupe D virus	1.41	497
Treated Panic	Sugarcane bacilliform virus	1.48	42
Treated Rhodes	Dioscorea bacilliform virus	0.39	10
Treated Rhodes	Leibnitzia anandria dicistrovirus	16.82	8276
Treated Rhodes	Planococcus ficus-associated dicistrovirus 1	51.77	26993
Treated Rhodes	Sugarcane bacilliform Guadeloupe D virus	0.62	213
Field Rhodes	Dioscorea bacilliform virus	0.28	7
Field Rhodes	Planococcus ficus-associated dicistrovirus 1	57.87	39153
Field Rhodes	Sugarcane bacilliform Guadeloupe A virus	0.23	83
Field Rhodes	Sugarcane bacilliform virus	0.27	96

Closer examination suggests that any association with a potential virus causal agent is not strongly supported by this data. Dicistroviruses are primarily insect pathogens and might only be found in infested and field-infested plants as a result of transfer of viruses infecting the mealybug to the plant in saliva or by contamination (honeydew).

Similarly, two Badnaviruses (*Melicytus chathamicus* endogenous virus Badnavirus and *Dioscorea* bacilliform virus (DBV)) are only found in infested and field infested samples. Badnaviruses can be vectored by mealybugs, however, the pattern does not hold: DBV is found in panic and both Rhodes Grass samples, but not in treated bluegrass or Mekong brizantha, while *Melicytus chathamicus* endogenous virus Badnavirus is only found in treated panic and treated Mekong Brizantha. These

may be endogenous viruses of the grasses. No evidence of *Closteroviruses* (vectored by mealybugs and amplified in Mealybug-induced Pineapple Wilt disease) was found.

Overall, the method confirms the potential for rapid and cost-effective screening for plant viruses in grass samples using small RNA analysis. The presence of at least one Badnaviruses and one Dicistroviruses in all treated samples does suggest that a small amount of further analysis, including additional samples and careful sequence analysis, is justified.

4.1.5.3 Other causes: Fusarium

Thirty nine (39) sites with differing severity of 'pasture dieback' were systematically sampled for *Fusarium* across Qld and NSW. Three hundred and seventy-seven (377) isolates were and amplicons containing partial ITS 1 and 2 regions and whole 5.8S regions were sequenced, quality controlled, and initially identified from BLAST searches.

Comparison of sample sequences with references from the NCBI database show that species and variants vary widely across sites. There is no consistent pattern of any one or any group of species or variants associated with 'pasture dieback' (Fig.11).

The diversity of *Fusarium* species and variants at any site was also not associated with severity of pasture dieback symptoms as determined by proportion of transect points affected by mealybug and with symptoms of pasture dieback at 17 sites across Queensland and New South Wales (P = 0.4106). This was reported in the final report for B.PAS 0006.

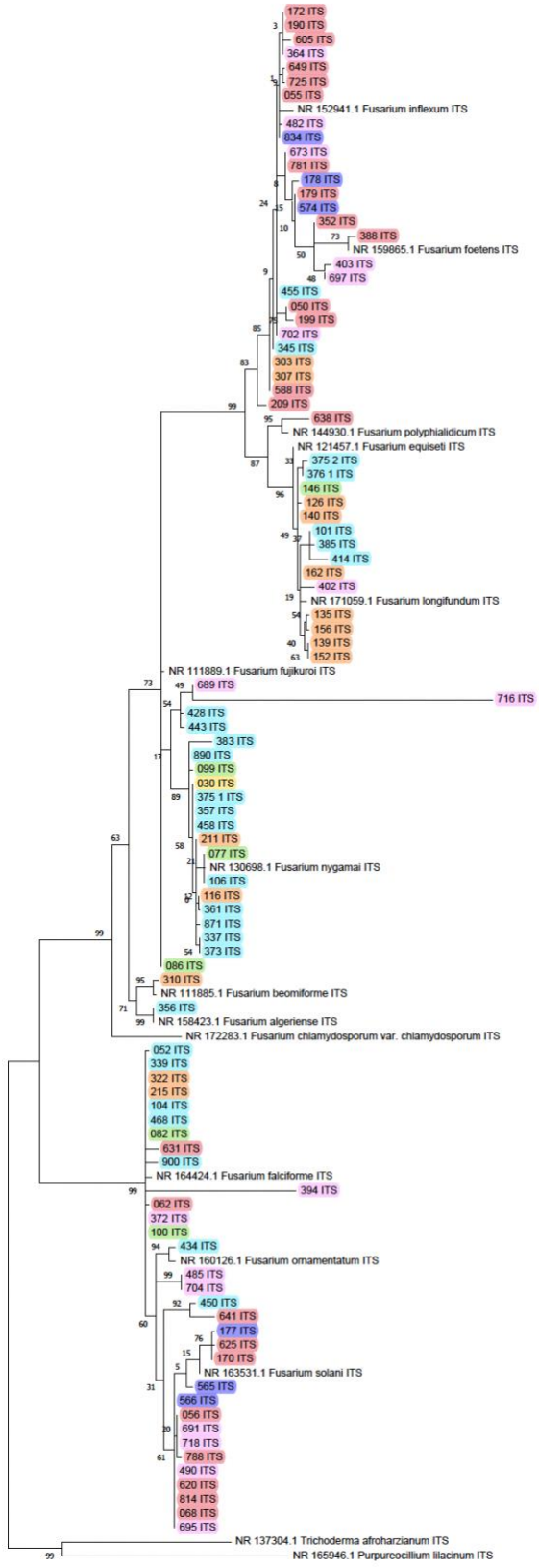

Figure 11: Maximum-likelihood tree of a sub-set of ITS gene sequences of *Fusarium* isolates and validated specimens in the NCBI database. Tip labels show taxonomic ID (NCBI GenBank ID) or the isolate ID code. Tip labels coloured by location isolated: Cyan = Banana, orange = North Burnett, pink = Gympie, red = Gold Coast and NNSW, green = Rockhampton, blue = South-East Queensland, yellow = Roma).

4.2 Identify natural enemies of mealybug species

4.2.1 Parasitoids

4.2.1.1 Mealybug collections to identify parasitoids of H. summervillei

Mealybugs were collected across an area from Rockhampton (Central Queensland), West to Theodore and Taroom, and south to Everest (NSW) 2,645 *H. summervillei* collected from field sites.

Parasitoids emerged from medium (3rd instar) mealybugs, with only 1 emerging from a larger 4th instar mealybug. This is similar to many other parasitoids of mealybugs, and 3rd instar mealybugs have been found to be the best for rearing mealybug parasitoids, they have the highest parasitisation rate. (Fiaz et al., 2013)(Tena et al., 2017) (Amarasekare, 2007).

Every mealybug from which a wasp emerged had a characteristic black spot, most likely resulting from melanisation where the female wasp's ovipositor penetrated the mealybug, and melanisation around the head (Fig. 12). Mealybugs with these black markings were therefor counted as parasitised but either the egg had been encapsulated (Blumberg 1997) or the mealybug or wasp died before the wasp could emerge.

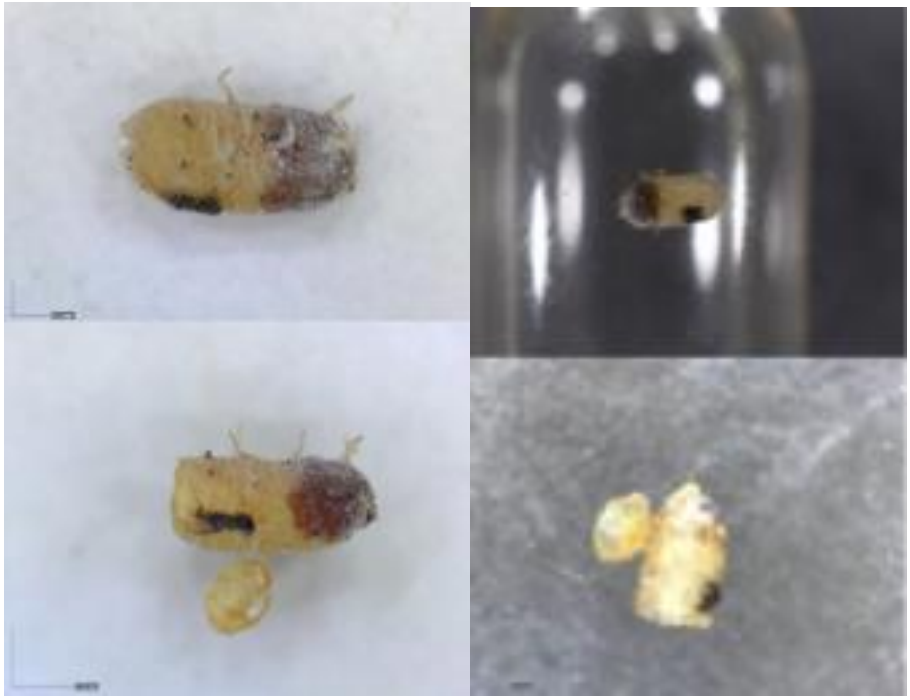

Figure 12: Upper: Mealybug hosts before wasp emergence with characteristic 'black spot' and melanisation around the head.
Lower: Mummified hosts after emergence. Photos: Edward Bryans, QUT.

Field abundance of the parasitoids in collections of mealybugs was low (1.8%). Of the 2,645 mealybugs collected, forty eight (48) had black markings indicating parasitism, 37 collected between November 2020 and March 2021, and 11 in December 2021. Nine (9) wasps emerged out *H. summervillei* collected from the field sites.

Table 5: Total mealybugs collected at 10 sites across south and central Queensland and northern New South Wales, of which the number with black dot indicating parasitism, and the number from which wasps emerged.

Site	Total mealybugs collected by site	Parasitised (black dot)	Emerged wasps
Banana	569	2	0
Biggenden	438	2	0
Brendale	90	1	1
Kin Kin	746	15	2
Maudsland	211	12	3
SERF	78	3	0
Nobby's Creek	23	2	0
Taroom	349	9	2
Theodore	63	1	1*
Wolvi	78	1	0
Total	2645	48	9

The two species of wasp emerging from *H. summervillei* were both Chalcid wasps of tribe *Anagyrini* (*Encyrtidae*: *Hymenoptera*). One was a new species of genus *Parectromoidella* Girault 1915 (Encyrtidae: Encyrtinae) and the second a new Australian record of a parasitoid *Yasumatsuiola orientalis* Trjapitzin, 1977 (Encyrtidae: Encyrtinae). The most abundant parasitoid was the new species of *Parectromoidella* (Fig. 12).

Figure 12: A new species of genus *Parectromoidella*, a Chalcid wasp. This was the most abundant parasitoid wasp isolated from *H. summervillei*.

Diagnosis: tarsi five-segmented; long funicle with six segments, with the first segment as long as pedicel; infuscate forewing reaching past the apex of gaster, with dark and light bands of satae; forewing linea calva interrupted on the dorsal surface by setae near posterior margin; post marginal vein nearly shorter than stigma vein on forewing; scutellum orange in colour without distinct tuft or bundle of setae; notaular lines absent; scape no more than three times as long as broad; malar space is longer than one-quarter of the eye; antennal toruli separated from mouth margin by 1.5 times the distance between Hypopygium reaching apex of gaster with the ovipositor not extended. (Noyes and Hayat 1984)

The Universal Chalcidoidea Database currently recognises nine (9) species of *Parectromoidella*: four from Queensland (*P. holbeini, P. lotae, P. lowelli* and *P. regalis*) (Girault 1922), four (4) from NSW (*P. abnormis*, *P. acacia*, *P. pacorus*, *P. thackerayi*) (Walker 1839, 1915; Girault 1917), and one (1) from the ACT (*P. laticincta*) (Girault 1932).

Distribution: All described species of genus *Parectromoidella* to date have been collected in Australia. Other undescribed species of the genus have been found in New Caledonia, Australia and New Zealand (Noyes and Hayat 1984). A full revision of the genus is required (Noyes, Pers. Comm 2021).

Y. orientalis only emerged from 1 mealybug collected in November 2021 in Theodore. The identity of the host mealybug was confirmed from the mummy as *Heliococcus* by Dr Mark Schutze, Biosecurity Queensland. Two specimens were also found in sweep nets from Theodore on the same date.

Females of this species have a distinct black and white colouration of the antenna (Noyes and Hayat 1994) (Fig 13). All 3 samples collected in this study were female and their identity was confirmed.

Figure 13: The parasitoid wasp *Yasumatsuiola orientalis* emerged from a parasitise H. summervillei collected in Theodore, Qld.

Only one species of genus *Yasumatsuiola* is currently recognized: *Yasumatsuiola orientalis* Trjapitzin 1977. *Y. orientalis* has been recorded previously in India and Southeast Asia, including China,

Thailand, Philippines and Indonesia) (Trjapitzin 1977, Noyes and Hayat 1994). Not previously recorded in Australia. *Y. orientalis* is a known parasitoid of *Ferrisia virgata* (striped mealybug), a polyphagous mealybug with hosts plants including legumes and cotton (Kaydan and Gullan 2012) that is itself only recently recorded in Australia.

Diagnosis: Taris five segmented; at least six segments, with the flagellum cylindrical in shape; scape longer than it is broad; forewing reaching apex of gaster; setae on forewing in distinct black and whites stripe pattern, giving the appearance of being infuscated; long marginal vein; no visible costal cell; scutellum black without any tuft or bundle setae; notaular lines absent; legs are partly infuscate; hypopygium reaching the apex of the gaster; mesopleuron and propleuron yellow/orange in colour; ovipositor not extended. (Noyes and Hayat 1984, 1994).

The Chalcid wasp reported as a parasitoid of *H. summervillei* by Summerville from the 1926 outbreak, *Callipteroma sexguttata* Motschulsky, 1863 (formerly *Leptomastix guttatipennis* Girault, 1915) (Summerville, 1928), was not found in any samples. *C. sexguttata* has been found throughout the Africa, Asia, Australia and Europe. It is a known parasitoid of three species of Pseudococcidae (*Birendracoccus saccharifolii, H. summervillei,* and one unidentified mealybug species found on *Cocculus trilobus*) (*Trjapitzin & Triapitsyn, 2018*). However, a picture likely of *C. sexuttata* was presented by DAF Qld at Beef Week 2021, so may still be present in some populations.

DNA sequencing confirmed that both Chalcid wasps are of the same tribe of *Anagyrini*, which includes the commercially-produced parasitoids of genus *Anagyrus* (Fig. 14) which are known to have a relatively wide host range across mealybug hosts.

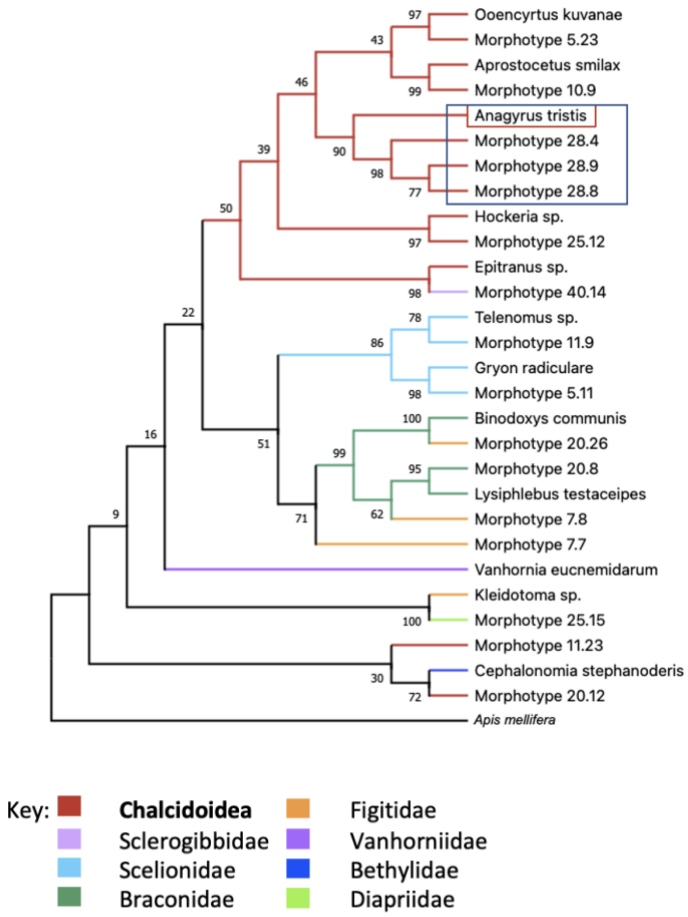

Figure 14: 28s sequence and comparison with reference sequence data for voucher specimens (NCBI) for identification of wasps extracted from Brendale sweep nets. Wasp morphotype 28, *Parectromoidella*, is closely-related to *Anagyrus tristis.*

4.2.1.2 Sweep nets

A total of almost five thousand insects (4824) were extracted from bug dorm and sweep net samples. 1,368 wasps were extracted from sweep nets. Of these, 166 were identified as parasitoids of *H. summervillei*. The majority were the same new and undescribed *Parectromoidella* species that emerged from *H. summervillei* collected in the field. Two further specimens of *Y. orientalis* were collected at Theodore in sweep nets, and on the same date as the specimen that emerged from an *H. summervillei* mealybug (Table 6).

Table 6: Number of *Parectromoidella* sp. And *Y. orientalis* parasitoid wasps collected in sweep nets at 17 sites across south and central Qld. and northern NSW.

Site	Number Parectromoidella	Number Y. orientalis
Banana	0	
Biggenden	5	
Brendale	35	
Emu Creek	63	
Everest	6	
Gaeta	0	
Jambin	1	
Kin Kin	0	
Maudsland	10	
Moura	0	
Mundubbera	0	
Nobby's Creek	8	
Rockhampton	2	
SERF	1	
Taroom	1	
Theodore	33	2
Wolvi	1	
Total	166	2

4.2.1.3 Distribution and seasonal abundance of parasitoids

The *Parectomoidella* sp. wasp was widely distributed across the range sampled, from Rockhampton, west to Taroom and south to northern NSW (Fig. 15).

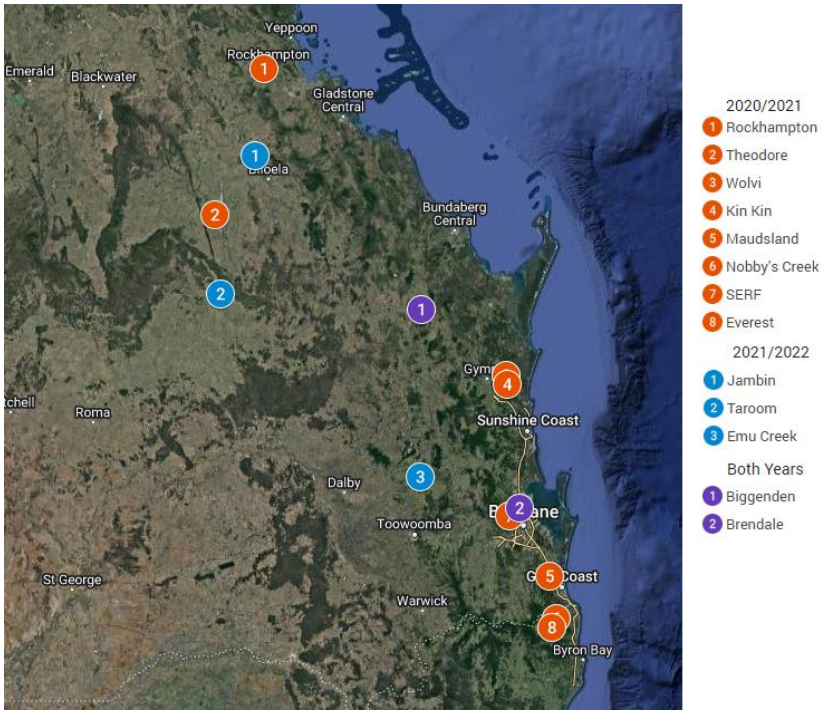

Figure 15: locations from which the parasitoid *Parectromoidella* was recovered by either collection of mealybugs or sweep nets.

Seasonal changes in proportion parasitism in collections of *H. summervillei* mealybugs at repeat sites (collections made fortnightly) (Fig. 16) and at all sites across the state (collected sporadically) (Fig. 17) show a similar pattern: the proportion of mealybugs parasitised peaked in March 2021, then dropped rapidly in April. Parasitism in collected mealybugs was not observed again until December 2021.

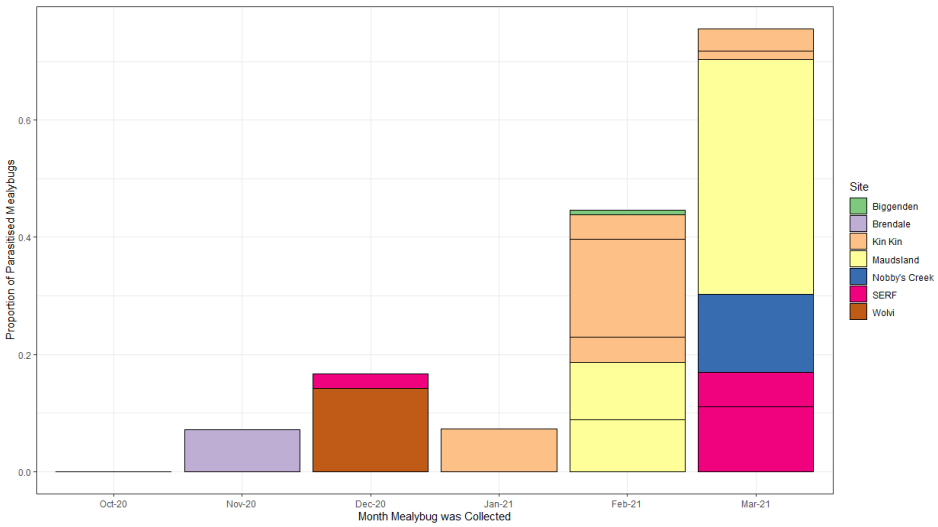

Figure 16: Proportion mealybugs parasitised by month at sites sampled every fortnight through the 2020/21 season

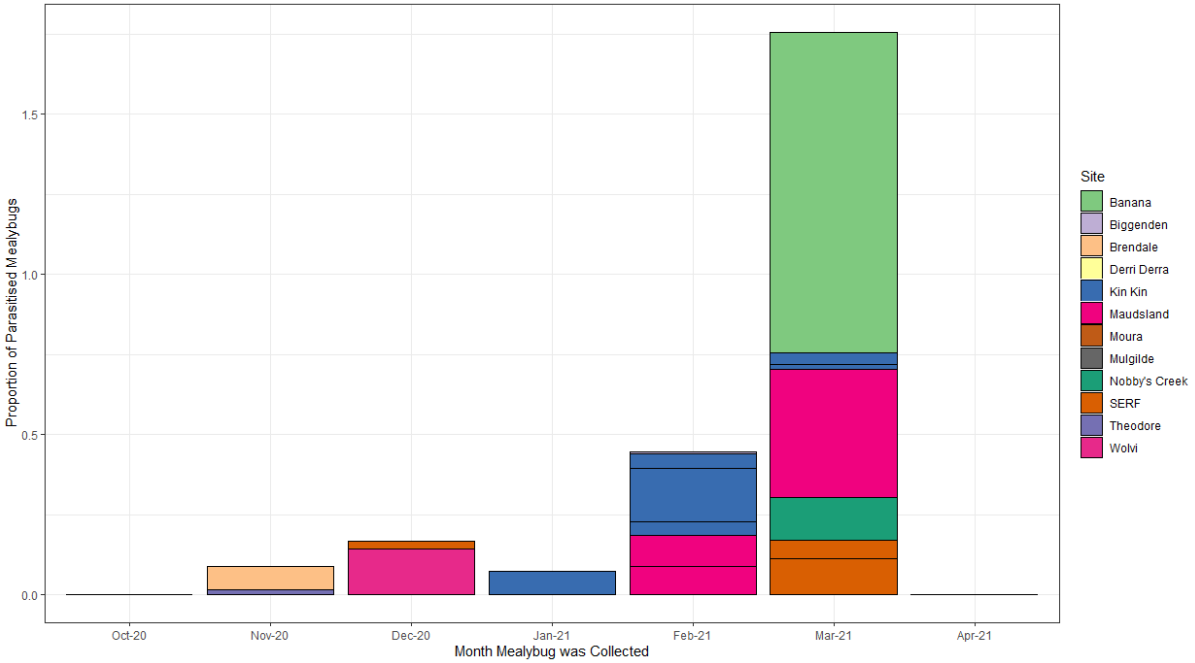

Figure 17: Proportion of mealybugs collected with parasitism (black dots) at all sites across Qld and Northern NSW Oct 2020 to April 2021.

This peak in parasitism in collected *H. summervillei* in March 2021 coincides with a peak in mealybug numbers observed the seasonal monitoring, and a subsequent sharp drop in mealybug numbers in April (section 4.4, below).

This is in contrast to abundance of collections from sweep nets.

Sweep nets and bug dorms.
Weekly or fortnightly sampling with sweep nets at 7 monitored field sites indicated that *Parectromoidella* wasp is most abundant in spring (October /November 2020), 2 to 3 months before the peak in abundance of mealybugs (February/March 2021) observed in the monitoring of mealybug abundance at those same sites (section 4.4, below).

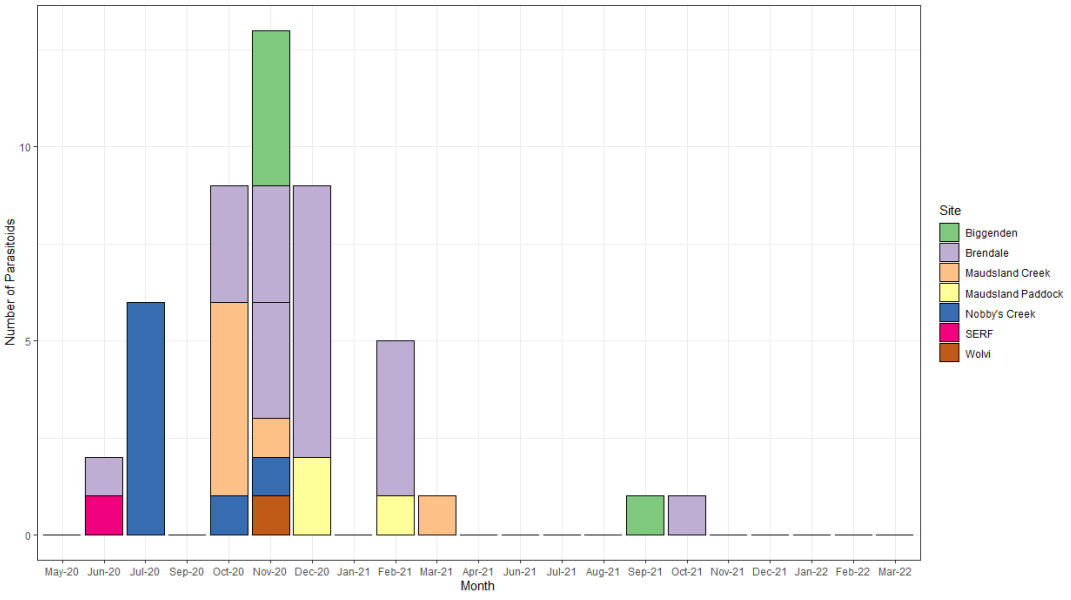

Figure 18: Number of the parasitoid wasp *Parectromoidella sp.* collected in sweep nets at sites sampled every month, May 2020 to March 2022.

The same pattern of abundance of parasitoid wasps of *H. summervillei* in sweep nets was observed at all sites sampled periodically across Queensland and Northern New South Wales, with peak abundance in November 2020 (2020/21 season) and subsequent sharp decline.

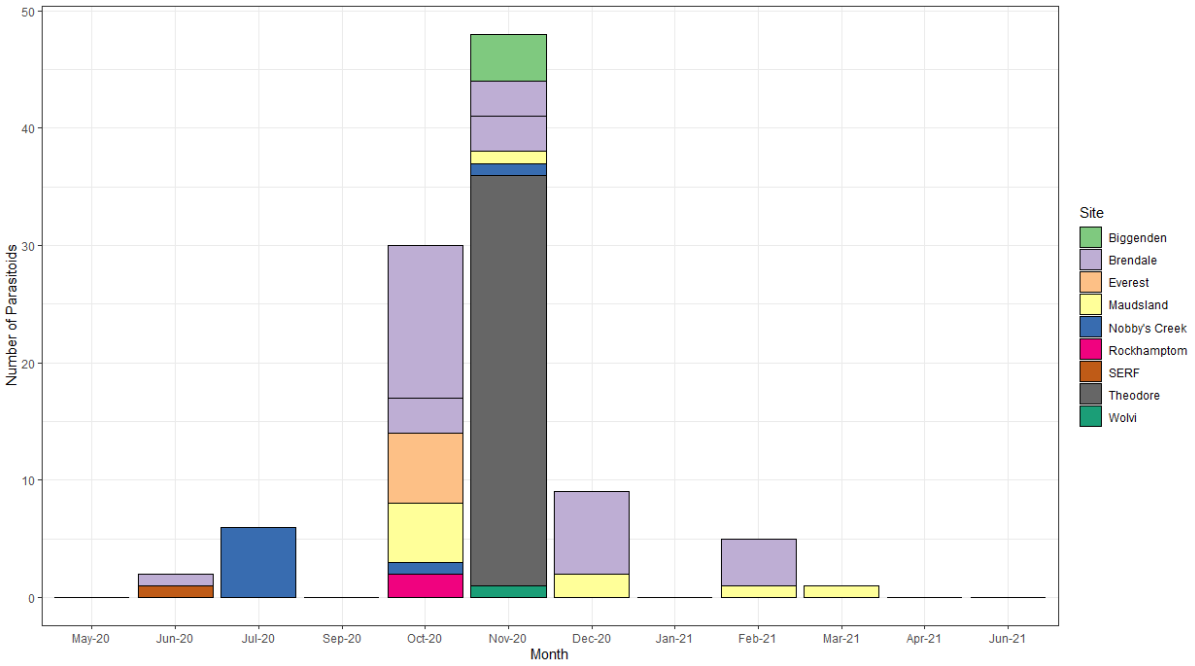

Figure 19: Number of the parasitoid wasp *Parectromoidella sp.* collected in sweep nets at sites sampled across in Queensland and Northern New South Wales, May 2020 to Jun 2021.

The short seasonal occurrence of *Parectromiodella* in mealybugs in February and March 2021, at both sites repeatedly sampled and at sites across the state (figs 18,19), and the far greater abundance at the same sites and across the state in October and November 2020 in sweep nets, suggests that this species of *Parectromoidella* primarily parasitises other hosts than the *H. summervillei* sampled, i.e those in the paddocks. Similarly, *Y. orientalis* has been previously only recorded on one species of mealybug, *F. virgata*, itself only recently introduced into Australia.

To date all of the parasitoids reported in *H. summervillei* are Chalcid wasps of the tribe *Anagyrini*. Wasps of this tribe are known to parasitise multiple hosts (Noyes and Hyatt 1994). It is possible that the primary host of these wasps is not as abundant during the later summer as during spring, and that the wasps then adapt to parasitise *H. summervillei*.

Further study is required to identify the parasitoid and scale insect hosts in pastures and surrounding vegetation and to determine if parasitism rates in *H. summervillei* are increasing. Further work should determine if commercial *Anagyrus* species will parasitise *H. summervillei*, a potential option to boost control in the field, or if *Parectromoidella* will parasitise other, more easily-reared mealybugs used in commercial mass production such as citrus mealybug.

4.2.2 Pathogens

Thirty six (36) isolates of fungi were isolated from mealybug cadavers. These included two species of genus *Trichoderma* (*T. ovalisporum and T. gamsii*) and the invertebrate pathogen *Purpureocillium lilacinum*. These fungi are also soil saprophytes and facultative endophytes in the rhizosphere of plants, and are known to be involved in boosting plant defences against pathogens and invertebrate pests. Epizootics not observed. Mealybugs collected live in the field (for parasitoid studies, above) died of non-specific causes including desiccation. The fungi from cadavers are more likely to be opportunistic infections of cadavers from fungi in the soil.

This result is best considered in the context of endophyte work conducted in B.PAS 0006.

4.3. Make preliminary recommendations on role of natural enemies in mealybug control

4.3.1 Parasites and predators.

Predators such as *Cryptolaemus*, ladybeetles, and lacewings were occasionally observed in the field, but their presence was patchy and inconsistent. Similarly, parasitism was low, and this after almost a decade of the current mealybug outbreak in Queensland. Biocontrols will require augmentation to be effective.

Mealybug collections are labour intensive but are the only way to confirm emergence of particular species of parasitoids from the target host. The number of wasps collected using sweep nets was far greater than those collected in bug dorms. Bug dorms were frequently trampled by cattle and are more expensive and prone to theft or vandalism. However, bug dorms will catch wasps emerging from the pasture, whereas sweep nets may capture wasps from outside the paddock that just happen to be flying through.

The number of parasitoids emerging from mealybugs with a 'black dot' was low. Knowledge of the abundance and diversity of parasitoids in un-emerged mummies might be obtained by use of DNA extraction and sequencing to identify the wasp by comparison with reference sequences. Overall, parasitism was low (1.8%), and emergence was lower. It needs to be higher to provide any meaningful level of control.

All of the parasitoids reported in *H. summervillei* are Chalcid wasps of the tribe *Anagyrini*. Wasps of this tribe are known to parasitise multiple hosts (Noyes and Hyatt 1994). It is possible that the primary host of these wasps is not as abundant during the later summer as during spring, and that the wasps then adapt to parasitise *H. summervillei*.

We know little about the parasitoids of other mealybugs and scales across Queensland, including the *Solenopsis* mealybug that is often found on *Parthenium* and neighbouring cotton crops. Further study is required to identify the parasitoid and scale insect hosts in pastures and surrounding vegetation and to determine the host range andif parasitism rates in *H. summervillei* are increasing. However, unlike New Caledonia, the current outbreak in Queensland has already gone on for at least 8 years, and there is no sign of an increase in natural enemies (Brinon 2004).

Augmentation of parasitoids seems likely to be needed. Further work should determine if commercial *Anagyrus* species will parasitise *H. summervillei*, a potential option to boost control in the field, or if *Parectromoidella*, a close relative, will parasitise other, more easily-reared mealybugs used in commercial mass production such as citrus mealybug.

The parasitoids found were predominantly 1 species, a new species of *Parectromoidella*. The parasitoid *Calipteroma* reported from the 1926 outbreak was not found by QUT but might have been found by DAF Qld. This new species of Parectromoidella was found concurrent with the geographic range of *H. summervillei*, and it appears to be abundant in the field well before the peak in H. summervillei numbers. It is worthy of further study. Dr Noyes (NHM, London UK) has suggested that a revision of the genus is needed to identify this species. With the rapid aging of qualified taxonomists and the greater need for identification of biological control agents as incursions increase, there is an opportunity to develop a PhD project to study the identity, host range and potential augmentation of parasitoids of pasture mealybugs.

4.3.2 Pathogens

Of the thirty six (36) isolates of fungi isolated from mealybug cadavers, the most promising biocontrols (*T. ovalisporum, T. gamsii* and the invertebrate pathogen *Purpureocillium lilacinum*) are also soil saprophytes and facultative endophytes in the rhizosphere of plants, known to be involved

in boosting plant defences against pathogens and invertebrate pests. Epizootics were not observed and the fungi from cadavers are more likely to be opportunistic infections of cadavers from fungi in the soil. This work is best considered in the context of endophyte work conducted in B.PAS 0006.

Spray trials with 'microbial' products (B.PAS 003) show limited impact on mealybugs. It's possible that application methods are important, since incorporation into potting mix has shown statistically significant impacts on mealybug abundance (B.PAS 0006). The benefits of fungal pathogens are more likely to accrue through soil/rhizospheric interactions, including pasture diversity, again explored in B.PAS 0006.

There are a small number of high quality fungal biopesticides available, and some limited work to test products such as Velifer (BASF Ltd) in screenhouses and laboratory screens should be considered.

4.4. Develop standardised field sampling protocols for mealybugs and in conjunction with project partners

4.4.1 Standardised field sampling

Systematic sampling and assay methods to quantify mealybug abundance, instar, and distribution in pastures and in artificial infestation of grasses in screenhouses were developed. These were used in fortnightly or monthly sampling at 8 field sites over 2 years (March 2020 to May 2022) across Central and Southern Queensland and northern New South Wales. The results are supported by weekly monitoring of mealybug populations in infested grasses over 12 months (May 2021 to May 2022) in screenhouses (B.PAS 0003).

It was found that 10 minutes intensive sampling typically completes the search of dug samples and records the majority of mealybugs. In field searches using both quadrats and dug samples (placed on a plastic sheet) it was confirmed both here and in B.PAS 0006 that most of the mealybugs were found in the first 10 minutes at the low (winter) densities, and that a representative proportion at higher, summer numbers. This is similar to results of methods established for monitoring of grape mealybug (Geiger et al 2001), creating a simple sampling method that can search 10 data points along a transect in 2 hours.

The sampling method was tested with the agronomists and graziers using either 10 dug samples on a 45m transect, or 5 samples on a 25m transect, depending on the time availability for the grazier/agronomist and the number of mealybugs in each sample. Property managers or agronomists at the Biggenden, Moura, Kin Kin, Mundubbera and Mulgildie, Banana, Rockhampton, Arcadia Valley, and Mullumbimby properties were trained in the procedure after QUT had completed the initial survey and sampling. All graziers were followed up with visits by QUT staff.

Sampling methods and reporting sheets modified for both 'expert' and 'graziers' were widely shared. The 'expert' site and dieback assessment sheet was finalised and shared with all MLA dieback program participants and NSW DPI in November 2020.

Storyboards for training material on what to look for (symptoms, where to look for mealybugs, use of a hand lens), sampling procedures (dug sampling and searching along a transect) and collection of mealybugs in the field were used as a basis for filming of 2 training videos in a pasture dieback site in April 2022 and are now being edited prior to uploading.

4.4.2 Seasonal biology of the mealybug, pasture dieback, and management

Mealybug numbers were found to correlate to symptoms of dieback across multiple sites, grass types and observers (Fig. 20). This supports other evidence that the mealybug *H. summervillei* as the causal agent of dieback.

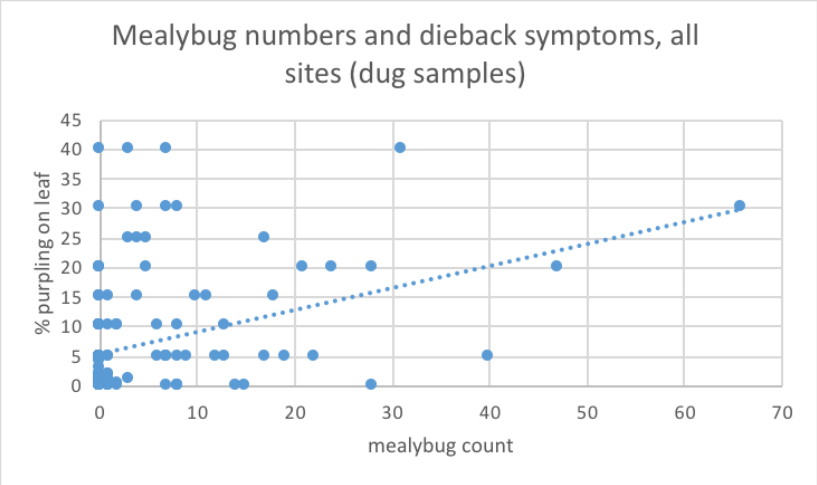

Figure 20: Estimates of dieback symptoms (purple colour) in live leaf against count of mealybugs in dug samples across multiple sites, staff and locations

Differences were observed in grasses and locations, with some sites having more severe symptoms with higher mealybug infestations (*Paspalum* at Murwillumbah NSW, Fig.21) and others with fewer mealybugs but significant symptoms (bluegrass at Biggenden, Fig. 22). Response of grasses to mealybug abundance may differ with tolerance of different grass varieties.

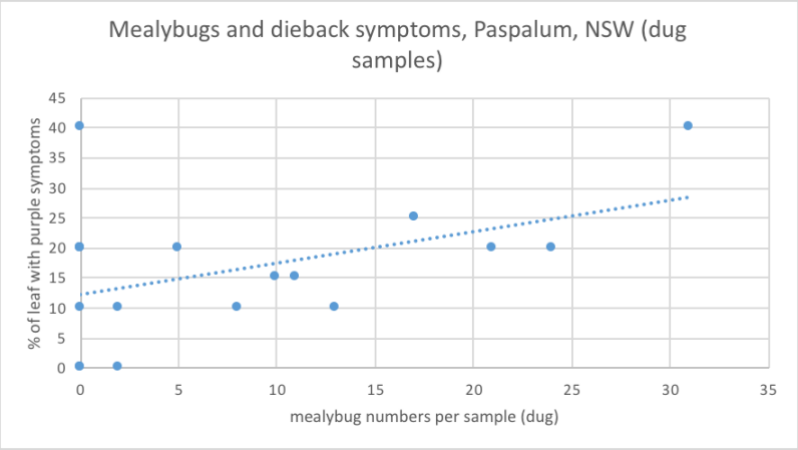

Figure 21: Estimates of dieback symptoms (purple colour) in live leaf against count of mealybugs in dug samples in Paspalum, NSW.

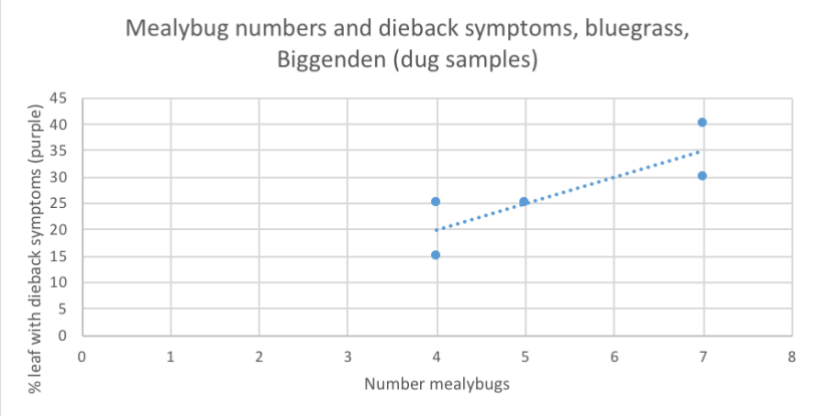

Figure 22: Estimates of dieback symptoms (purple colour) in live leaf against count of mealybugs in

dug samples in creeping bluegrass, Biggenden (Qld). Data was generated by graziers without supervision at one month after training.

Fortnightly or monthly sampling of field sites over 2 years and weekly monitoring of infested plants in screenhouses over 12 months confirmed that mature, mated (pink) females rapidly disperse into cracks in the soil and along roots (to a depth of up to 900mm along deeper soil cracks), under cowpats and other debris in the field, or under plant pots (in screenhouses) in late summer. Females with young were found at depths of up to 900mm underground in the depth of winter and through the cold and dry conditions (winter). The observation of the mealybug overwintering in soil contrasts with the description from the 1926 outbreak, where it was reported that pasture mealybugs were not observed in the soil (Summerville 1928).

It is also interesting to note that some white, apparently un-mated females were observed with associated live young in soil through winter, i.e. appear to be reproducing parthenogenetically (Fig. 23). Unmated females in the laboratory died without reproducing, and mated females turn pink. It is unlikely that flighted males could find females in the soil and females may reproduce parthenogenically when males are not available. The reproductive strategy of overwintering females requires further investigation.

Figure 23: Mature, white, apparently un-mated females with young are found through the winter in the soil at depths of up to 1m.

Females and young were found to re-appear in the upper soil and thatch layers in the spring and to become more apparent with warmer, wetter weather. Early instars move rapidly onto the leaves and feed actively during warmer, moist conditions and spring / summer flushes of grass growth (Fig 24).

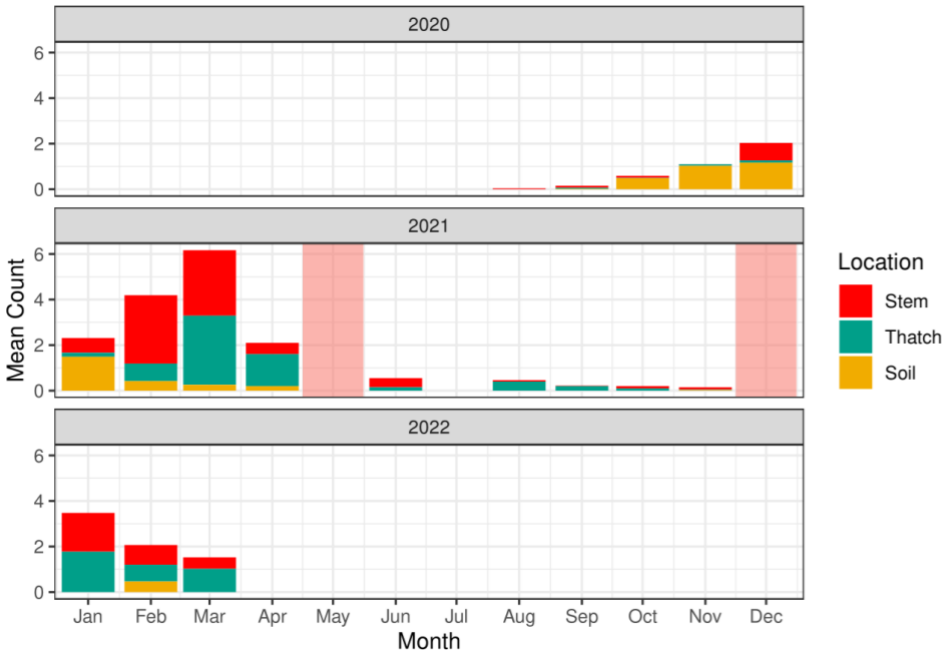

Figure 24. Distribution and abundance of mealybugs in the leaf/stem, thatch (upper soil and crown of grass) and soil over 2 years. Mean is of the number of mealybugs per transect point at all sites sampled fortnightly or monthly between March 2020 and April 2022. Shaded pink areas are months when sites could not be sampled due to COVID lockdowns or adverse weather.

Mature female mealybugs and pupae of males were mainly found in the crown and dense thatch layers of grasses during the warmer, wetter conditions of late summer January to March) in both years. Mealybugs in the field appear to mate and females produce young in the protection of the dense crown and thatch layer of grasses in mid to late summer. This overall pattern was also seen at individual sites (Fig. 25).

Two sites (Kin Kin and Maudsland Creek) were observed to have very few mealybugs in the summer of 2022 (Fig 25). While the reason for this absence can't be confirmed, both these sites were low lying and bordering on flowing creeks, prone to flooding. It is possible that the very wet weather in early 2022 had a significant adverse impact on the mealybugs in these locations.

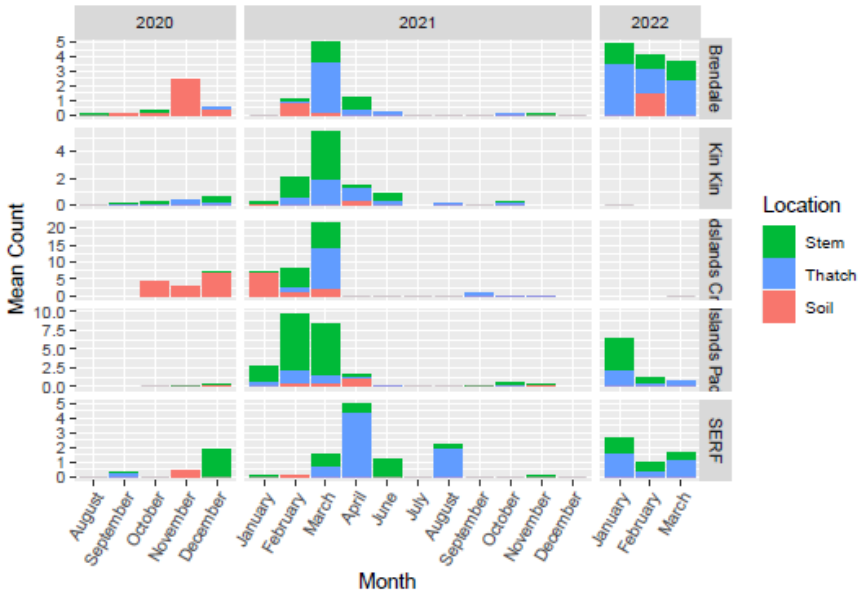

Figure 25: Seasonal distribution of mealybugs at individual sites monitored fortnightly or monthly, August 2020 to April 2022. Two sites (Kin Kin and Maudsland Creek) were observed to have very few mealybugs in the summer of 2022.

Mealybug behaviour is strongly seasonal and targeting the vulnerable life stages is critical for effective management. Dispersed, cryptic, and non-feeding females (winter / early spring) are a difficult target for monitoring and management. There is a narrow window in summer in which to manage vulnerable leaf-feeding instars, mating adults and reproducing females (Fig. 26).

Figure 26. Mean number of mealybugs per transect point at all sites sampled fortnightly or monthly between March 2020 and April 2022. Life stage (adult, nymph etc) is strongly seasonal, with peak abundance in late summer in both 2 years. Shaded pink areas are months when sites could not be sampled due to COVID lockdowns or adverse weather.

Since only the immature instars feed on leaf, the summer burst of flushing grass with late summer moisture is important to their survival and development. They are also the instars which cause the damage to the plant, when feeding. In very dry conditions, small instars move into the sheltered, humid, cooler space in the crown under the canopy of large clumping grasses like American buffel. Populations develop rapidly after summer rain and must be managed early. It is vital to monitor pasture during the spring for emerging populations and in summer for mealybugs and symptoms of dieback (yellow, red or purple discolouration of leaves). Insecticide applications (specifically Movento and Imidacloprid under permits from MLA) can be used to manage the emerging populations in small patches through spring and to reduce spread, so long as the grass is not hayed off or dry from drought: Movento (spirotetramat) requires healthy leaf for translaminar uptake.

Summer populations of mature females and male pupae in the dense crown and thatch, and abundance of feeding instars on the leaf, may be disrupted by grazing or slashing. Reducing thatch by slashing and grazing reduces spring and summer refugia for mealybugs.

Over winter the mealybugs will be hidden deep in soil in cracks, or in thatch, cracks in soil or under cowpats and logs, where spraying, slashing or burning will have little impact. Adult female mealybugs do not feed and systemic insecticides applied to leaf will have little impact. Increasing use of non-susceptible winter forages (legumes, brassicas and cereals) can provide fodder as an alternative to long, dense standing grass that provides good shelter for mealybugs.

Early recognition of symptoms and how to identify mealybugs is essential for timely interventions and management. Sampling by digging along transects across affected areas can be used to determine the area requiring treatment. Sampling will determine the abundance of mealybugs, their size and potential for population growth, and distribution on plant, thatch and soil to select appropriate controls. QUT training materials have been developed to address these needs and delivered at field days, presentations and site visits with individual property owners, managers and agronomists.

4.5 Outline of one draft manuscript for journal publication

A paper on the identification of the mealybug *Heliococcus summervillei* was presented at the 2019 Australian Entomological Society in partnership with Biosecurity Qld (Schutze et al 2019).

Peer reviewed publications: Analysis and modelling of the data is continuing towards publication of final results, and the final report will be updated by December 2022.

Two manuscripts (on the *H. summervillei* collection, description and systematics, and one on parasitoids) are in advanced draft. Two others (seasonal biology and transcriptome analysis) are drafted. Working titles are as follows:

i. Description, abundance and distribution of parasitoids of *Heliococcus summervillei* (Homoptera: *Pseudococcidae*) new to Australia.
ii. The mealybug, *Heliococcus summervillei* and ‘pasture dieback’.
iii. Seasonal phenology, spatial distribution and sampling for the mealybug *Heliococcus summervillei*, the causal agent of ‘Pasture Dieback’.
iv. Transcriptome analysis of the effects of the mealybug, *Heliococcus summervillei* Brooks on the JA/SA pathway in American buffel grass, *Chenchrus ciliaris*.

4.6 Provide to MLA periodic information summaries suitable for general media on the role and management of mealybugs in relation to addressing pasture dieback

Communications were provided frequently to MLA program leaders and comms team through reports, emails, a webinar, a presentation at an MLA panel event at Beef Week 2021, on-line participant meetings, material for specific media outputs, and a national science panel review in April 2022.

A summary of media outputs and extension activities has been provided separately for all 3 QUT projects.

See also 'Media outputs and engagements' summary provided for all projects.

Two Instructional video clips on the use of mealybug sampling kit and pasture dieback assessment tool have been filmed and are in editing. We expect to complete and upload shortly.

Assemble and distribute 25 expert and 150 producer Mealybug Sampling kits for shipping on request. These were assembled and distributed at a number of field days, along with instructions on how to sample and how to identify mealybugs. The most appreciated item in the kit was the hand lens, which made it possible for many graziers to see the mealybugs for the first time, and to appreciate just how small they are.

Remaining kits will simplified to include hand lenses, instructions on sampling, and mealybug collection tubes only. Soil and grass sampling materials will be removed after the project ends as we will not have the staff to process samples.

4.7 A two-page summary outlining key facts and findings of mealybugs and how to identify mealybugs associated with dieback

Information on how to identify mealybugs and pasture dieback was provided and included in the MLA joint publication.

Recommendations on seasonal management have been provided to the MLA Comms team and published September 2021 https://www.mla.com.au/news-and-events/industry-news/monitoring-mealybugs-in-spring-and-summer/) and December 2021.
A third article linking research to management is in preparation for publication in spring 2022.

Two instructional video clips on the use of mealybug sampling kit and pasture dieback assessment tool have been developed and filmed and are in editing prior to upload and sharing with MLA.

25 expert and 150 producer Mealybug Sampling kits were assembled and distributed.

Sampling and observation sheets were shared with all program participants in November 2020.

A summary of 'Media, outputs, and engagement' has been provided for all projects.

5. Conclusion

5.1 Conclusions

The project has confirmed the identity of the mealybug *Heliococcus summervillei* and demonstrated beyond doubt that it is the causal agent of 'pasture dieback'.

Diversity within the mealybug population is low. Only one physical variant was observed, even though historic and international samples show there are significant between populations (Brookes 2007).

Transcriptome analysis showed how feeding by the mealybug disrupts the JA/SA pathway and other plant immune responses, rendering the grass vulnerable to secondary infections such as *Fusarium*. Interactions between the mealybug, its microbial symbionts, and the plant should be studied further to understand these mechanisms more clearly and to identify pathways of resistance or tolerance in pasture varieties. Further work on mechanisms of mealybug-induced susceptibility across grass varieties, including transcriptome analysis, is strongly recommended.

Other possible causes of dieback including fungal pathogens such as *Fusarium* have been eliminated, though there is an association with Badnaviruses and possibly Dicistroviruses that should be investigated a little further. No Closteroviruses, which are amplified in Mealybug-induced Pineapple Wilt disease, were found. However, the interaction between disruption of the grass immune response and possible amplification of endogenous viruses would be an interesting area for further study.

The sex ratio in the summer is roughly 1:1. *H. summervillei* mealybug is predominantly sexually reproductive, though it may reproduce parthenogenically in soil over winter. Mating occurs in the dense thatch layer of summer pastures. Mated females turn pink before producing young. Only early instars of both sexes feed on plants and cause 'pasture dieback'. Adults of both sexes do not feed. Females overwinter in soil (in contrast to reports by Summerville 1928). Overwintering populations of dispersed females are difficult to manage and sample.

Seasonal scouting is critical to targeting and management of mealybugs. Seasonal management strategies such as slashing should target vulnerable mating adults and foliar-feeding early instars during late spring and summer.

Parasitoids are low in abundance but probably have alternate hosts in the landscape. We know little about the parasitoids of other mealybugs and scales across Queensland, including the *Solenopsis* mealybug that is often found on *Parthenium* and neighbouring cotton crops. Further study is required to identify the parasitoid and scale insect hosts in pastures and surrounding vegetation and to determine the host range of if parasitism rates in *H. summervillei* are increasing. However, unlike New Caledonia, the current outbreak in Queensland has already gone on for at least 8 years, and there is no sign of an increase in natural enemies (Brinon 2004).

Augmentation of parasitoids seems likely to be needed. Further work should determine if commercial *Anagyrus* species will parasitise *H. summervillei*, a potential option to boost control in the field, or if *Parectromoidella*, a close relative, will parasitise other, more easily-reared mealybugs used in commercial mass production such as citrus mealybug. Further work on parasitoid host range, and biocontrol with *Anagyrus* species, is recommended.

The parasitoids found were predominantly 1 species, a new species of *Parectromoidella*. The parasitoid *Calipteroma* reported from the 1926 outbreak was not found by QUT, but might have been found by DAF Qld. This new species of Parectromoidella was found concurrent with the geographic range of *H. summervillei*, and it appears to be abundant in the field well before the peak in *H. summervillei* numbers. It is worthy of further study. Dr Noyes (NHM, London UK) has suggested that a revision of the genus is needed to identify this species. With the rapid aging of qualified taxonomists and the great need for identification of biological control agents as incursions increase, there is an opportunity to develop a PhD project to study the identity, host range and potential augmentation of parasitoids of pasture mealybugs and train a young expert in the techniques.

5.2 Key findings

This work has confirmed that the mealybug *Heliococcus summervillei* is the causal agent of pasture dieback.

Life history studies and seasonal monitoring in the field highlights highly seasonal nature of the mealybug problem and identified the critical seasons and life stages for mealybug management.

Management is especially important in the warmer seasons when the small and medium nymphs emerge. These are the instars that feed on grass and cause 'dieback'. Adult populations shelter and mate in the shaded spaces beneath dense clumping grasses: monitoring and management are essential. Life history studies have also led to development of rapid screening assays for fast throughput of variety screening and testing of possible controls, including microbial controls and endophytes.

A small number of natural enemies have been identified. These include two new records of parasitoid wasps (Hymenoptera: Encyrtidae), one of which is a new species. Augmentation of parasitoids seems likely to be needed. Further work should determine if commercial *Anagyrus* species will parasitise *H. summervillei*, a potential option to boost control in the field, or if *Parectromoidella*, a close relative, will parasitise other, more easily-reared mealybugs used in commercial mass production such as citrus mealybug. Further work on parasitoid host range, and biocontrol with *Anagyrus* species, is recommended.

The parasitoids found were predominantly 1 species, a new species of *Parectromoidella*. The parasitoid *Calipteroma* reported from the 1926 outbreak was not found by QUT but might have been found by DAF Qld. This new species of Parectromoidella was found concurrent with the geographic range of *H. summervillei*, and it appears to be abundant in the field well before the peak in *H. summervillei* numbers. It is worthy of further study. Dr Noyes (NHM, London UK) has suggested that a revision of the genus is needed to identify this species. With the rapid aging of qualified taxonomists and the great need for identification of biological control agents as incursions increase, there is an opportunity to develop a PhD project to study the identity, host range and potential augmentation of parasitoids of pasture mealybugs and train a young expert in the techniques.

QUT has been actively promoting understanding of the link between mealybug biology and dieback, and monitoring by graziers in order to target management. Articles, field days, a webinar, and training in the use of monitoring and sampling, including use of sampling kits, have been provided and training materials have been developed, including filming of two short videos on 'what to look for' and 'how to sample' mealybugs.

5.2 Benefits to industry

The confirmation of *H. summervillei* as the causal agent of pasture dieback is of critical importance to industry. Graziers and agronomists can now move forward with confidence to identify management strategies that suit their farm operations.

The identification of key points in the seasonal and reproductive biology of the mealybug during which damage occurs and management can be targeted is critically important. These findings and the quantitative methods developed, will inform current management and future development of proven and quantified a strategies suitable for a range of farm operating models and systems.

Life history studies have also led to development of rapid screening assays for fast throughput of variety screening and testing of possible controls, including microbial controls and endophytes.

A small number of beneficial insects have been identified. Though these parasitoids are widely distributed and appear to have alternative hosts, they cannot be relied on to control the pasture mealybug at current levels of abundance. Augmentation of beneficial insects is an important area for further investigation.

6. Future research and recommendations

6.1 Further research

Feeding by mealybug disrupts the JA/SA pathway and other plant immune responses, rendering the grass vulnerable to secondary infections such as *Fusarium*. Interactions between the mealybug, its microbial symbionts, and the plant should be studied further to understand these mechanisms more clearly and to identify pathways of resistance or tolerance in pasture varieties..

No *Closteroviruses*, which are amplified in Mealybug-induced Pineapple Wilt disease, were found. However, the interaction between disruption of the grass immune response and possible amplification of endogenous Badnaviruses and (possibly) Dicistroviruses would be an interesting area for further study.

Further research on integrating management strategies (slashing, grazing, insecticides) with the seasonal and reproductive biology of the mealybug to quantify impact on susceptible mealybug populations and benefits within farm practice is strongly recommended.

A small number of natural enemies have been identified. These include two new records of parasitoid wasps (Hymenoptera: Encyrtidae), one of which is a new species. Augmentation of parasitoids seems likely to be needed. Further work should determine if commercial *Anagyrus* species will parasitise *H. summervillei*, a potential option to boost control in the field, or if *Parectromoidella*, a close relative, will parasitise other, more easily-reared mealybugs used in commercial mass production such as citrus mealybug. Further work on parasitoid host range, and biocontrol with *Anagyrus* species, is recommended.

The parasitoids found were predominantly 1 species, a new species of *Parectromoidella*. The parasitoid *Calipteroma* reported from the 1926 outbreak was not found by QUT but might have been found by DAF Qld. This new species of Parectromoidella was found concurrent with the geographic range of *H. summervillei*, and it appears to be abundant in the field well before the peak in *H. summervillei* numbers. It is worthy of further study. Dr Noyes (NHM, London UK) has suggested that a revision of the genus is needed to identify this species. With the rapid aging of qualified taxonomists and the great need for identification of biological control agents as incursions increase, there is an opportunity to develop a PhD project to study the identity, host range and potential augmentation of parasitoids of pasture mealybugs and train a young expert in the techniques.

6.2 Recommendations to industry

The confirmation of *H. summervillei* as the causal agent of pasture dieback is of critical importance to industry. Graziers and agronomists can now move forward with confidence to identify management strategies that suit their farm operations.

The identification of key points in the seasonal and reproductive biology of the mealybug during which damage occurs and management can be targeted is critically important. These findings and the quantitative methods developed, will inform current management and future development of proven and quantifiable strategies suitable for a range of farm operating models and systems.

A small number of beneficial insects have been identified. Though these parasitoids are widely distributed and appear to have alternative hosts, they cannot be relied on to control the pasture mealybug at current levels of abundance. Augmentation of beneficial insects is an important area for further investigation.

7. References

Brinon, L., Matile-Ferrero, D., & Chazeau, J. 2004 [Outbreak and regression of a grass infesting mealybug, introduced in New Caledonia, *Heliococcus summervillei* Brookes (Hemiptera, Pseudococcidae).]. Bulletin de la Societe Entomologique de France 109(4): 425-428.

Brooks, H.M. 1978. A new species of *Heliococcus* Sulc from Australia and Pakistan and a redescription of *Heliococcus glaczalis* (Newstead) (Homoptera: pseudococcidae) J. Aust. Ent. Soc. 1978, 17: 241-245.

Geiger, C., Daane, K., Bentley, W., Yokota, G. and Martin, L., 2001. Sampling program for grape mealybugs improves pest management. *California Agriculture*, *55*(3), pp.19-27. https://escholarship.org/uc/item/0x2013rq

Kreuze, J.F., Perez, A., Untiveros, M., Quispe, D., Fuentes, S., Barker, I. and Simon, R., 2009. Complete viral genome sequence and discovery of novel viruses by deep sequencing of small RNAs: a generic method for diagnosis, discovery and sequencing of viruses. *Virology*, *388*(1), pp.1-7. https://doi.org/10.1016/j.virol.2009.03.024

Muthamilarasan, M., Bonthala, V.S., Khandelwal, R., Jaishankar, J., Shweta, S., Nawaz, K. and Prasad, M., 2015. Global analysis of WRKY transcription factor superfamily in Setaria identifies potential candidates involved in abiotic stress signaling. *Frontiers in plant science*, *6*, p.910. https://doi.org/10.3389/fpls.2015.00910

Schutze, M., C. Hauxwell, D. Tree, G. Dickson, P. Gullan. 2019. The Mealybugs Strike Back: the return of Heliococcus summervillei and Queensland pasture dieback. Australian Entomological Society AGM and Scientific Conference, in association with the Society of Australian Systematic Biologists and Australasian Arachnological Society. https://eprints.qut.edu.au/232000/

Summerville, W.A.T. (1928). Mealybug attacking *Paspalum* grass in the Cooroy district. Qld. Agric. J, 30, 201-209.

Zhang, P.J., Huang, F., Zhang, J.M., Wei, J.N. and Lu, Y.B., 2015. The mealybug Phenacoccus solenopsis suppresses plant defense responses by manipulating JA-SA crosstalk. *Scientific reports*, *5*(1), pp.1-7. https://www.nature.com/articles/srep09354

Zhang, B., Edwards, O., Kang, L. and Fuller, S., 2014. A multi-genome analysis approach enables tracking of the invasion of a single R ussian wheat aphid (D iuraphis noxia) clone throughout the New World. *Molecular Ecology*, *23*(8), pp.1940-1951. **https://doi.org/10.1111/mec.12714**