Hort Innovation

Final Report

Novel technologies to assist rapid and sensitive detection of Brown Marmorated Stink Bug

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Delivery partner:

Cesar Australia

Project code:

AS19000

Project:

Novel technologies to assist rapid and sensitive detection of Brown Marmorated Stink Bug (AS19000)

Disclaimer:

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Contents

Public summary

The Brown Marmorated Stink Bug (BMSB) is an insect pest native to China, that has spread and established in other continents, largely linked to ongoing trade links. New Zealand and the East and South West coastlines of Australia have suitable climates for this pest to thrive. This bug can survive on a wide range of fruits and vegetables, and has the ability to spread long distances through flight or as a hitchhiker. The damage it causes impacts both the aesthetics and yield of the crop. When present on grapes, it has been shown to lead to a 37% decline in yield, as well as the chemicals it produces significantly taint the wine and juice. It is therefore imperative that this pest is detected quickly and accurately when it makes it to, and past, the border to ensure a quick response and reduce the damage caused.

This project focussed on using environmental DNA (eDNA) technologies to improve the detection and surveillance of BMSB. All living organisms shed fragments of their DNA into their environment. This eDNA can be collected using a variety of means, and tested either for specific species of interest, or more broadly for all species present. This project has applied both of these approaches. A single species test for BMSB was developed and tested to ensure it was highly sensitive and specific - detecting only BMSB and not other closely related insects at acceptable thresholds. In order to assess how well eDNA methods worked in the field, a test was also developed for a surrogate species common in New Zealand and Australia, the Green vegetable bug (GVB). Simple methods for collecting GVB eDNA were developed and evaluated. The most practical and effective of these was sampling of leaves into a sealed bag, collection of eDNA by agitation in water, with eDNA then concentrated by filtration, extracted, and tested. The effects of GVB density and the window of detection were investigated, over a time course from 1 - 72 hours. We are able to robustly detect a single GVB when present on a leaf for at least 16 hours in dry conditions. Multiple species (including BMSB) can also be identified using these techniques, as well as other in-field collections, allowing for broader surveillance and monitoring.

Other outcomes of this work included a workshop with international eDNA experts, where the latest ideas and technologies were shared; development of a Pest Bites video, which details the ways to survey and identify BMSB; and a review of the literature for the wine and horticultural industries on the impact and management of BMSB. The materials for a guide and workshop to train industry staff in the use of eDNA diagnostics are currently being produced.

Keywords

Brown Marmorated Stink Bug; BMSB; eDNA; pest detection; pest surveillance; pest monitoring, wine, grapevine, viticulture, fruit and vegetable crops.

Introduction

The Brown Marmorated Stink Bug (BMSB) is a species native to China that has become an established pest in other continents when spread via trade routes. Globally the regularity and volume of goods being traded internationally is increasing. New Zealand and the East and West coastlines of Australia provide suitable climate conditions for the establishment of this pest. At the New Zealand border, the number of interceptions numbered over 1000 from 2005 to January 2017. Seasonal measures indicate that imports from 34 countries pose a high risk for BMSB transmission. Post-border detections have been steadily increasing over recent years.

The impact of BMSB establishment in Australia and New Zealand would vary depending on point of entry and spread. BMSB has the ability to survive on a wide range of fruits and vegetables, and can disperse long distances through flight, or as a hitchhiker. Damage to crops can be both aesthetic, as well as significantly reducing yield through direct and indirect damage. All stages of the life cycle are able to survive on grape, with as little as five BMSB per cluster leading to a 37% decline in yield (Smith et. al, 2014). Volatile compounds produced by BMSB also significantly taint wine and juice.

The sensitivity and speed of detection is key for the success of containment and eradication activities. This can have significant impacts on the supply chain, and the ability of growers to show proof of freedom. This project was undertaken in order to develop a testing strategy that allows for the rapid and sensitive diagnosis of BMSB presence, aiding in ongoing surveillance and biosecurity preparedness.

Genetic technologies based on environmental DNA (eDNA) can be sensitive and cost-effective ways to detect individual species, or monitor broad groups of species. DNA is continually shed by all living organisms in their environment, and detecting species from their trace eDNA requires practical methods for collection, and the development of assays for analysis in the lab or field. There are two main detection methods that use different assay platforms: single species assays, and multiple species assays (metabarcoding). Single species assays are highly sensitive, and allow for the amplification and identification of a small, selected region of DNA from a single target species. Here, the test result is quantitative amplification of target DNA, if present. Metabarcoding amplifies a single DNA region from multiple species, and then reads the sequence of amplified molecules by high-throughput DNA sequencing to resolve species by comparing with a compiled reference database. The type of species amplified varies based on assay design, e.g., targeting all of the insects in the sample, or more broadly, all of the eukaryotes. In this project, both types of detection were used - single species quantitative polymerase chain reaction (qPCR) assays to allow for sensitive detection of BMSB, and also metabarcoding for detecting BMSB.

By developing the sampling methods, assays and training resources for the industry, this project has increased awareness and understanding around BMSB identification, monitoring and detection. The outcomes facilitate more sensitive surveillance methods, and decrease the time required for detection or to establish proof of freedom. An increase in adoption of these technologies would ideally help to reduce the likelihood of establishment of this key pest species, decrease loss of yield, and minimise disruption of key trade pathways.

Methodology

Field testing of eDNA sampling approaches

Field testing was performed at Indulge Farms (Silvan, Victoria, Australia) across areas that varied in observed GVB nymph density and plant damage, using:

- spray aggregation: spraying fruit and leaves and collecting run-off for filtering
- leaf rolling: using small commercial paint-rollers to adsorb eDNA from leaf surfaces, followed by filtering of roller wash water
- bag aggregation: picking leaves into bags, agitating in water, then collecting eDNA by filtration.

Detailed sampling methods are described in Appendices 3 and 4.

Laboratory experiments using grapevine or apple leaves and the Green Vegetable Bug (*Nezara viridula*; GVB) as a surrogate for BMSB were performed to define the period of exposure and density of insects required before robust eDNA detection could be expected using qPCR and metabarcoding techniques. Thus, laboratory experimental set up, sample collection, DNA extraction and qPCR assays based on the GVB mitochondrial cytochrome oxidase I (COI) gene were first designed and validated.

qPCR primers/probes were designed using GVB sequence available on NCBI and validated using locally-sourced GVB DNA and Sanger sequencing. The specificity of the qPCR assay was tested using plant DNA and the DNA of closely related species such as BMSB. See Appendix 2 for extended details of assay conditions.

Sample collection and DNA extraction methods were tested in laboratory and field conditions to establish the most practical and sensitive approaches for eDNA detection.

Glasshouse testing of eDNA sampling approaches

In addition, effects of wash buffer, and efficacy of filtering versus collection of particulate matter by centrifugation, was tested in the laboratory using glasshouse experiments. After further refinement of sampling approaches, effects of insect density and the window of detection were investigated.

Time of exposure

In a glasshouse (20C), one GVB was added to sterile tissue culture pots containing 2 grapevine leaves (collected from the PFR site). Insects were left to walk and feed on leaves for 30 minutes, 2 hours, 16 hours, 24 hours, 48 hours and 72 hours. A total of four to six biological replicates at each time point, as well as a negative control (leaves without insect), was taken. After the insects were removed, leaves were transferred into a ziplock bag and washed using 50 ml of sterile water by shaking the bag at 350 rpm for 5 minutes. The washed water was put through a filter to collect cells and free DNA. qPCR on the extracted DNA was performed as described above (and in Appendix 2).

Density

Experiments in a glasshouse (20C) examined detection sensitivity. A total of 1, 3, 5 or 10 GVB were added to sterile tissue culture pots containing 2 grapevine leaves (collected from PFR). Insects were left to walk and feed on leaves for up to 72 hours. A total of two biological replicates for each density, and negative control (leaves without insect), was performed. Sample collection, DNA extraction, qPCR assay and statistical analysis were performed as above for *Time of exposure*.

Statistical analysis

To compare GVB detection over time generalised linear mixed effects (GLMM) models were performed in R (R Core Team 2022) using the package brms: Bayesian Regression Models using 'Stan' (Bürkner, 2017). The fixed effect was time (numeric predictor), with technical replicate fitted as a random effect. The detection response was modelled as either presence / absence, with a Bernoulli error distribution, or as a continuous variable for qPCR cycle number (Cq) with a hurdle gamma distribution (since the data contained a high proportion of zeros). Models were validated and assessed using prior and posterior predictive checks, effective sample sizes, and R-hat diagnostics.

To compare GVB detection as a function of density another GLMM model was fit, with a fixed effect of GVB count. A Bernoulli error distribution was selected for presence /absence data, and Gaussian error distribution for Cq data. Models were validated and assessed as above. For all Bernoulli models, a normal(0,2) weakly informative prior was specified on the

slope to aid model convergence.

Ongoing experiments

Time and density effects on apple leaves

Another experiment to quantify the time of exposure and density using apple leaves has been performed with a higher number of biological replicates to further validate methods and results on another economically important crop, and provide greater statistical power. Insects were left to walk and feed on apple leaves for 2 hours, 6 hours, 16 hours, 24 hours, 48 hours and 72 hours (one insect per sample), and 3 and 5 insects were left to walk and feed on apple leaves for 2 hours and 24 hours. A total of 8 biological replicates for each treatment and negative controls (leaves without insects) were performed. Sample collection, DNA extraction, qPCR assay and statistical analysis will be performed as above. In addition, samples will also be tested by metabarcoding to compare sensitivity against the targeted qPCR approach.

Large-scale glasshouse experiment on brassica plants

To further validate and generalise these results, a final experiment is underway using insect cages and entire brassica plants. These experiments will again test the effects of insect density and exposure time on plants. The window of detection after insects have been removed from plants will also be examined. The sampling approaches established from field and laboratory testing to date will be used (leaf washing and filter collection of eDNA), and the sensitivity of species-specific qPCR assays will again be compared with metabarcoding.

Photos/images/other audio-visual material

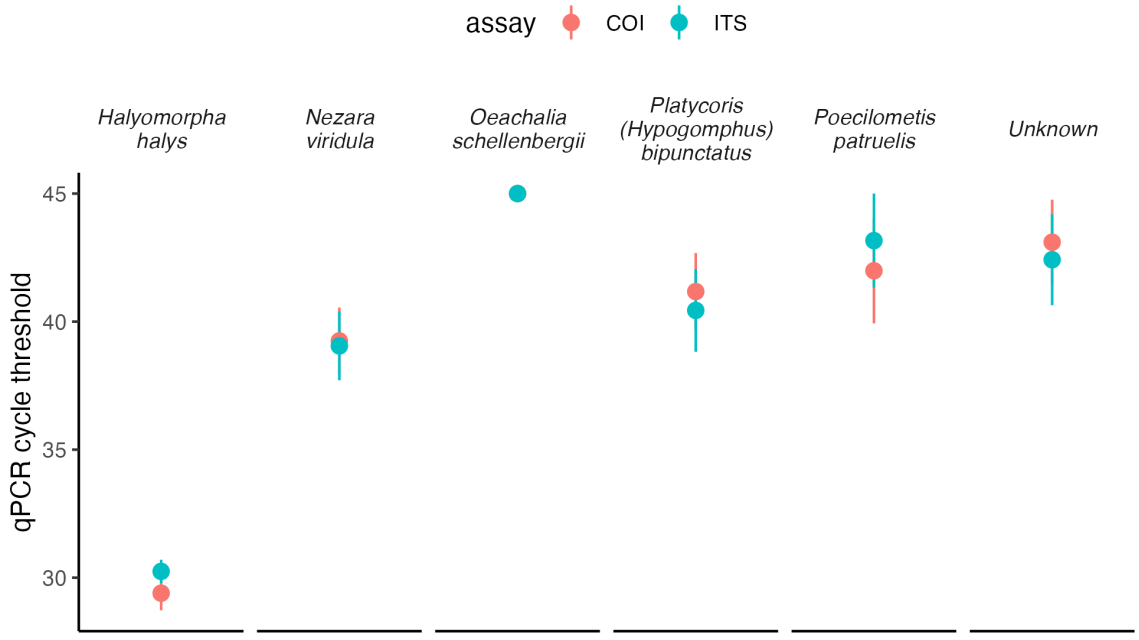
Figure 4: A) Spray aggregation on raspberry plant; B) Tree rolling; C) Filtering water collected from spray aggregation or tree rolling technique using a syringe and Sterivex. Image credits: EnviroDNA Pty Ltd.

The above figure is from the confidential report produced by EnviroDNA pty ltd., entitled:' Early indicators assessment using eDNA approaches for High Priority Plant pests (HPPPs)', on June 18th, 2021.

Results and discussion

Assay development

Specimens of BMSB, the surrogate GVB, and likely off-target species were sourced from Western Australia (WA), New South Wales (NSW) and the Unites States of America (USA) by EnviroDNA (Appendix 1). BMSB samples from WA and NSW were collected from pre- and post-border interceptions (2017 to 2019). Samples from the USA were collected from lab colonies and the field. DNA was extracted from all samples and Sanger sequenced at the mitochondrial COI region (1 unique sequence or haplotype was found in the BMSB specimens from USA and 5 were found in the specimens from WA). Two BMSB specific qPCR assays that focus on the ITS1 nuclear gene (Valentin et al., 2015) and the COI mitochondrial gene (Dhami et al., 2016) were optimised and tested on all BMSB and off-target samples.



Results are summarised in the above figure, showing the qPCR cycle number required to amplify a standardised input of test DNA (fewer cycles reflect greater assay specificity, with maximum cycle number capped at 45). Mean and standard error are shown for each assay and species tested. Both assays showed acceptable specificity for BMSB (approximately 500-fold greater amplification for BMSB than for any off-target species). The COI assay showed greater efficiency over the ITS assay, and was selected for use in the remaining project activities.

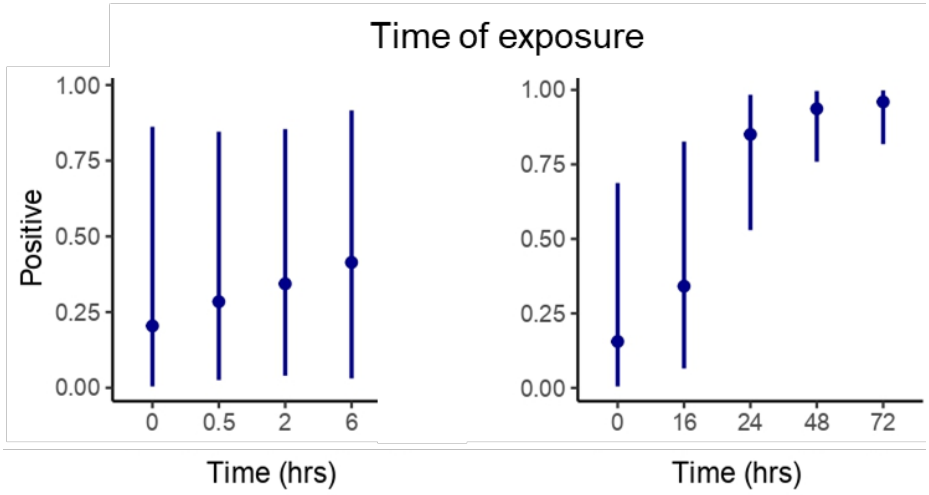
Sampling approaches

Results from field trials (Australia) were used to refine sampling methods and assays in the glasshouse (New Zealand). All field-tested methods tested were found to be suitable for collecting GVB DNA from areas where nymphs were sighted, however spray aggregation and leaf-rolling were less time-efficient than the simpler leaf collection/washing approach. We therefore carried this method forward for refinement and further testing in controlled conditions.

In laboratory testing, collecting eDNA by alternative buffers provided no obvious advantage over water. Centrifugation, theoretically allowing collection of smaller particles than water filtration, was not superior in sensitivity to filtration of water using a 0.45 micron pore polyethersulfone (PES) filter. These results showed that simple collection methods that do not require specialised equipment suffice for efficient, sensitive detection of GVB.

Effects of time on leaf for detection of GVB by qPCR

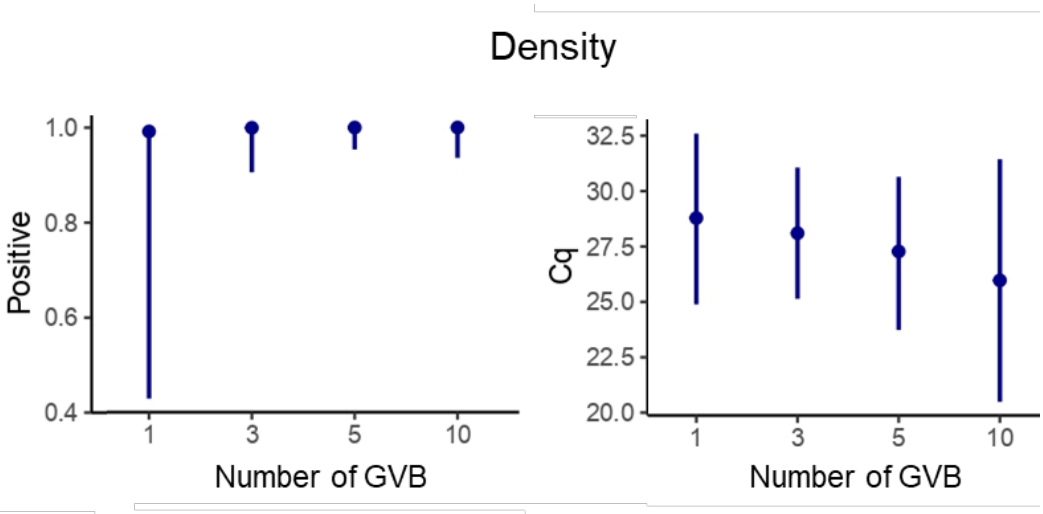
In Bayesian analysis, explanatory variables are considered important (“significant”) if the 95% credible interval (95% CI) of their estimated effects does not overlap zero. The figure below shows the estimated detection probability as a function of time since GVB were introduced to vine leaves. GVB was not reliably detected from 0 to 6 hours, although detection probability showed a steady increase with time over this period. Detection probability increased sharply after 16 hours. Robust detection was seen after 24 hours, with little added sensitivity between 48 and 72 hours.



Conditional effect plot of detection probability (using binary detection / non-detection as the response variable) for a single GVB on leaves for different time treatments. Mean values with 95 % credible intervals are shown.

Effects of GVB density on detection by qPCR

No significant effect of GVB density on detection probability across the tested range (1-10 bugs, 72 hours exposure) was found, although detection was more variable when only 1 bug was present. These results show that the developed sampling approaches and GVB assay can be highly sensitive, but more data are required to test the potential for estimating differential pest pressure at large spatial scale.



Conditional effect plot of GVB detection probability (Left: detection / non-detection as the response variable. Right: qPCR cycle as the response variable) for different GVB densities (exposed for 72 hours). Mean values with 95 % credible intervals are shown.

Given extensive pandemic-related setbacks the final goal for this work of large-scale field-testing of these methods in a formal modelling approach (site occupancy-detection models; SODM) to determine appropriate sampling intensity has not yet been completed. In place of this, the generation of data in a more realistic glasshouse setting is ongoing, and joint statistical analysis of effects across three plant species from qPCR and metabarcoding will provide more precise estimates of sensitivity, and the window of detection (in the absence of rain).

Outputs

Table 1. Output summary

Output	Description	Detail
BMSB qPCR assays	Method development and validation of qPCR assay for BMSB and GVB.	See details in above methodology section (Appendix 2), finalised version will be submitted once the glasshouse trial has concluded.
BMSB metabarcoding assays	Method development for detecting BMSB in metabarcoding assays from tissue and eDNA samples.	Not completed. Results and protocol will be submitted once the glasshouse trial has concluded.
eDNA sampling approaches	Local scale eDNA sampling methods have now been developed and these methods have been written up as sampling approaches.	Methods have been developed, and finalised versions will be reported after the conclusion of the glasshouse trials (including publication in a peer-reviewed journal). Descriptions of trial protocols are in Appendix 4.
PestBites episode	Video on how to identify BMSB found on the Cesar Australia YouTube channel.	Episode can be found here. For website address see Appendix 6.
Literature review	Literature review on BMSB and implications for the wine industry.	See Appendix 7.
Real time in-field detection protocols	A method that allows for extraction of eDNA from a sample in the field, and a test which results in a detection/no detection readout	Using the Franklin Biomeme in-field detection was achieved in a pilot study. However due to restrictions and delays surrounding COVID this is not ready for industry adoption.
Training materials for eDNA diagnostics	Guides and materials for the training of industry staff for the use of eDNA technologies	This is ongoing, and will be finalised after the glasshouse trials have concluded. The guides will be informed by the outcomes and findings of this trial, and delivered closely after.

Outcomes

Table 2. Outcome summary

Outcome	Alignment to fund outcome, strategy and KPI	Description	Evidence
Development of validated BMSB qPCR assay	Hort Frontiers Strategy (HFS) People and Enterprise, and Plant and Resources	A test for BMSB allows for sensitive and rapid detection, improving the biosecurity toolkit and ensuring productive, profitable growers through prevention of loss of yield.	Validated to stage 3-4 according to the Thalinger et al. 2021 scale.
Development of metabarcoding assay for detecting BMSB	Hort Frontiers Strategy (HFS) People and Enterprise, and Plant and Resources	A surveillance method for BMSB allows for ongoing monitoring, improving the biosecurity toolkit and ensuring productive, profitable growers through prevention of loss of yield.	Tested on both eDNA samples and also insect soups. COI assay more effective for soups, 16S more effective for eDNA samples
Development of local eDNA sampling strategy	Hort Frontiers Strategy (HFS) People and Enterprise, and Plant and Resources	Developing sampling strategies that can be adapted based on the crop and carried out easily will benefit industry staff carrying out monitoring, and help protect growers crops	Sampling methods have been trialled in the field, as well as the lab. A glasshouse trial will be the final step in sampling strategy development. Methods will be described and reported at the conclusion of this process.
Development of real-time in-field eDNA methods	Hort Frontiers Strategy (HFS) People and Enterprise, and Plant and Resources	The ability to detect an incursion rapidly in the field is key to containment and eradication. This reduces the likelihood of establishment, protecting growers' crops.	The Franklin qPCR machine has been trialled for detecting GVB with success from tissue and eDNA samples. Further work is still required to optimise DNA extraction from eDNA samples.
eDNA workshop	Hort Frontiers Strategy (HFS) People and Enterprise	This allows for the exchange of ideas and knowledge, but improves innovation.	This was carried out in Lincoln, New Zealand on the 23rd of May 2023.
Optimising eDNA surveillance protocols	Hort Frontiers Strategy (HFS) People and Enterprise, and Plant and Resources	Optimised eDNA surveillance protocols allow for improved chances of BMSB detection, protecting growers' crops.	Optimised eDNA protocols will be finalised after the conclusion of the glasshouse trial.
Training video	Hort Frontiers Strategy (HFS) People and Enterprise, and Plant and Resources	The Pest Bites training video allows for the information related to BMSB identification and detection to be disseminated to a larger audience in an easily	The training video detailing ways to identify and survey for BMSB can be found on Cesar Australia's YouTube channel. Website link is in the appendices.

		digestible fashion.	
Literature review and associated industry articles.	Hort Frontiers Strategy (HFS) People and Enterprise, and Less Wate	The review provides critical information for the wine industry, which may help to prevent waste of product through BMSB volatile taint, and helps to ensure productive, profitable growers.	Literature review is complete, pending final review. Associated industry articles still to be completed after the glasshouse trial concludes.
eDNA in-field training guide	Hort Frontiers Strategy (HFS) People and Enterprise, and Plant and Resources	The in-field training guide will allow for the adoption of eDNA detection methods for BMSB within the industry, improving biosecurity and protecting crops.	Awaiting finalisation of in-field eDNA methods based on the glasshouse trials before the training guide is developed.

Monitoring and evaluation

Table 3. Key Evaluation Questions

Key Evaluation Question	Project performance	Continuous improvement opportunities
Has the project developed new technology that is now available for industry uptake?	The project has developed sampling approaches for eDNA sample collection, a qPCR assay, and a metabarcoding assay for industry use.	Further work could be done to trial the sample collection methods in various crops.
To what extent has the project met the needs of industry levy payers?	The project has met the needs of industry levy payers by generating eDNA assays which allow for a more sensitive and rapid detection of BMSB, negating the need to capture whole individuals.	Having rapid in-field tests developed to the point where they are ready for deployment would help meet needs for the most rapid detection available and associated reduction in response time.
To what extent were the target engagement levels of industry levy payers achieved?	Industry levy payers were engaged in this project through the consultation with growers in the planning stage of initial field trials for eDNA sample collection methods. The eDNA experts meeting included both Australian and New Zealand border security experts who are a key demographic for downstream usage. Unfortunately, COVID did impact the timing of the meeting, as an in-person meeting was not able to occur until much later in the project than originally planned. The ability of field trials to be carried out with growers during the appropriate seasons for high GVB density was also impacted.	Engagement with the target groups will be ongoing with the extension activities highlighted above.
Did the project engage with industry levy payers through their preferred learning style?	A variety of engagement styles were utilised in this project. These included the production of videos and more in-depth review documents.	Ongoing work will include more extension activities- which shall include the generation of eDNA guides (video and written), as well as articles through grower newsletter or magazines, as well as social media engagement highlighting these resources.
What efforts did the project make to improve efficiency?	This project improved efficiency by using GVB as a common surrogate species for BMSB, allowing for field and laboratory experiments to progress without waiting on rare BMSB incursions or samples. Efficiency was also improved by conducting controlled trials in the laboratory whereby the factors of time and density could be studied. This produces cleaner data than in-field trials, and allows for more accurate interrogation of these conditions. It has informed the work to be carried out in the ongoing glasshouse trial, which will mimic field conditions more closely.	The large numbers of filtered eDNA samples that will be generated in the glasshouse and ongoing time course and density experiments that are to be generated at Plant and Food Research will be extracted by EnviroDNA - where there are specialised laboratories and existing workflows. It also provides a model for sending terrestrial samples collected in the field by end-users for screening. EnviroDNA laboratories are the site where metabarcoding will be carried out. This will improve efficiency of the experimental process moving forward.

Recommendations

Practical applications of the findings of this project include the Pest Bites video, which can be used by grower or industry officers to aid in the identification and detection of BMSB. An eDNA sampling and detection guide will also be finalised and provided to industry. These allow for the adoption of this technology on a wider scale.

Growers or pest inspection officers will be able to use the eDNA guide in order to appropriately collect leaf or frass samples in the field, which can be sent for BMSB detection using the qPCR assay in cases of suspected incursion. This assay can also be deployed into routine surveillance activities.

Activities that would ensure full value from this work would include the eDNA guide to be developed, both as a video and written format available online for all industry technical staff. Further, articles covering the outcomes of this project in various grower and/or industry journals or newsletters would increase awareness, allowing for the work to reach a wide audience and ensure full value from the project.

The use of the metabarcoding assay to be developed for surveillance can be used to monitor not only BMSB, but any other insect pest present in the reference databases. It also has the added benefit of informing growers of the presence of any beneficials in their crops, which may reduce the need for insecticide spraying.

Future R&D work could include developing sampling approaches to grape collection containers- to ensure that tainted fruit can be excluded from inclusion in processing. This could reduce time and resources spent processing grapes which will need to be discarded, reducing costs.

Airborne eDNA sampling approaches could be developed, using passive or active air samplers in crops for ongoing surveillance. Potentially, the same metabarcoding and qPCR assays developed here could be applied to this new sample type.

Refereed scientific publications

Journal article

NA

Whole book

NA.

Chapter in a book or paper in conference proceedings

NA

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Intellectual property

No project IP or commercialisation to report

Acknowledgements

Not applicable

Appendix 1: sourcing of reference material

Primary contacts of BMSB and off-target samples.

Australian State or Country	Primary Contact	Position and Organisation
Western Australia	Jacquie Otley	Entomologist; Department of Agriculture, Water and Environment (DAWE)
New South Wales	Martin Horwood	Senior Biosecurity Officer; Local Land Services (LLS)
Victoria	Linda Semeraro	Entomologist; Agriculture Victoria
United States of America	Joshua Milnes	Pest Biologist; Washing State Department of Agriculture (WSDA)

Appendix 2: GVB qPCR detailed methods

GVB qPCR was performed using a CFX96 Touch Real-Time PCR detection system (Bio-Rad). A ten-fold dilution series of the GVB DNA starting at 10 ng/µL was used to generate a standard curve and determine the efficiency of the qPCR assay as well as the limit of detection. Each sample was performed in triplicate. Each 20 µL reaction mixture contained 4 µL of DNA template, 10 µL of SensiFast Probe Hi-ROX Kit mix (2x), 0.8 µL of each primer at a concentration of 10 µM (400 nM final concentration), 0.2 µL of 10uM probe (100 nM final concentration). The PCR conditions were 5 min at 95°C (initial denaturation), and then 50 cycles of 10 s at 95°C (for denaturation), followed by annealing of 30 s at 60°C.

Primers and probe used were:

GVB_Forward ATTATTACTACTTTCATTACCT

GVB_Reverse TGAAATTAATCCGAATCCTGGT

GVB_Probe ACAGATCGGAACTTTAATACATCATTCT

Appendix 3: eDNA extraction protocol (Using the Qiagen DNeasy Blood & Tissue Kit)

1) Transfer leaves using clean forceps [cleaned between each sample by rinsing in 10% bleach, sterile water, and 100% ethanol before being flamed] into a small Ziploc bag, and add 50 ml of sterile water to the bag containing the sample.
2) Place bags into a shaker at 350 rpm for 5 minutes, before cutting the corner of the bag with scissors, and squeezing all the water from the bag into a sterile 50 ml falcon tube.
3) Transfer the water into a 50 ml sterile syringe with a 0.45 micron pore filter attached. Pass water through the filter and discard the water, and cap the filter to create a closed system.
4) Filters are processed according to the Qiagen protocol.
5) Store eDNA sample at -20 degrees celsius.

Appendix 4: eDNA sampling approaches

- Paint rolling/swabbing
    - A small paint roller (e.g., Bunnings I/N: 1662564) was dampened with sterile water and used to swab or roll the surfaces of raspberry plants with positive visual GVB detection.
    - Rollers were removed and placed into a ziploc bag filled with water, agitated and squeezed to remove excess water, then water was filtered by syringe fitted with a 0.45 micron PES filter.
- Water run-off
    - Plants and surrounding areas with positive GVB sightings were sprayed with water and run-off (100-200 mL) collected into pre-bleached buckets
    - Water contents were syringe filtered
- Aggregation
    - Leaves of plants with positive GVB sightings were taken and placed into a ziploc bag containing ~200 mL water
    - Contents were vigorously shaken then syringe filtered

All filters were extracted according to protocol using Qiagen PowerSoil kits beforeGVB qPCR assay.

Appendix 5: eDNA for terrestrial biosecurity workshop report

A workshop was run on Tuesday 22nd May at Plant & Food Research, Lincoln, New Zealand. The workshop was a hybrid of in-person and online. Attendance was largely based on invites to Australasian researchers known to be working in the eDNA space as well as border security personnel from Australia's Department of Agriculture Fisheries and Forestry (DAFF) and New Zealand's Ministry for Primary Industries (MPI).

Attendee	Place of employment	Country	Attendance
Rebijith Balan	MPI	NZ	in person
Simon Bulman	Plant & Food Research	NZ	in person
Andrew Cridge	Scion	NZ	online
Manpreet Dhami	Manaaki Whenua Landcare Research	NZ	online
Uday Divi	DAFF	Aus	online
Andrew Dopheide	MWLCR	NZ	
Dianne Gleeson	University of Canberra	Aus	in person
Geoff Grossel	DAFF	Aus	online
Jessi A Henneken	Dept of Energy, Environment and Climate Action (DEECA)	Aus	online
Justine Larrouy	Plant & Food Research	NZ	in person
Dongmei Li	MPI	NZ	in person
Mackenzie Lovegrove	EnviroDNA	Aus	in person
Craig Marston	DAFF	Aus	online
Francesco Martoni	Dept of Energy, Environment and Climate Action (DEECA)	NZ	online
Rebecca McDougal	Scion	NZ	online
Luke Noble	EnviroDNA	Aus	online
Steve Pawson	University of Canterbury	NZ	online
Luciano Rigano	MPI	NZ	in person

The workshop was arranged around a series of presentations from Australasian researchers involved in terrestrial eDNA research.

Dianne Gleeson. National eDNA Reference Centre for Australia and biosecurity projects at University of Canberra

Dianne Gleeson presented two talks, with the first covering the eDNA reference centre (ERC). The second addressed specific biosecurity related research from UC (especially projects by Alejandro Trujillo). Uday Divi (DAFF) also joined the workshop at this point. Uday is responsible for the DAFF "eDNA testing programme", and it was noted that Uday could well have given the first talk i.e., the reference centre is integrated with the activities of DAFF in the testing programme.

The focus of the ERC is both ring testing and certification of lab procedures through NATA (equivalent of IANZ). The reference centre is also doing/coordinating ring testing of eDNA protocols (from themselves and other research groups). Di mentioned many collaborations with overseas labs to benchmark protocols and to gain access to material from unwanted organisms. In depth testing is important as lab results may not be replicated in the field. As an example from ERC, detection of invasive turtles from sites known to contain animals was negative and only became possible after "putting sandpaper on rocks".

Since the completion of the iMapPests programme, the ERC has been doing some work with the sentinels. Smaller air filtering units have been developed by SARDI for deployment at ports by DAFF (mentioned by Rohan Kimber at the last iMapPests meeting) but may still be valued at $30-40K each according to Francesco Martoni.

Varroa was the subject of a multi-agency research programme (including ERC and AgVic) which featured the transport of varroa free hives from the Chathams to Wellington, where eDNA techniques were benchmarked.

Later in the talks, Di presented the results of ongoing eDNA screening for khapra across the importation chain. This work involved large numbers of samples and the collection of data from many different collection points. An important observation was that the sites for produce packing were an important source of contamination. Large scale data management and interrogation was an important trend. Ultimately, Di indicated that her team is looking to design a detection device that sits in a cargo container during transport and can be eDNA screened before clearance.

Craig Marston. eDNA implementation at DAFF

Craig's talk was particularly focussed on the implementation of a major initiative to test khapra beetle detection at the border using dust/vacuuming approaches. Craig described development of the DAFF eDNA programme as to some degree opportunistic. Given the focus on dust/vacuuming, sieving of collected material was a key axis point for khapra detection. DAFF initially investigated detection of exuviae (shed skins) of khapra in 100g vacuumed material. The exuviae apparently provided insufficient DNA for detection. Single intact larvae in the same system were still not detectable until PCR inhibition was dealt with. At that point it became detectable with regularity – with, I think, "the bullet" for extraction.

An important storyline in Craig's talk was the follow-up to a positive eDNA detection for khapra. A shipping container containing khapra was released into the community, DAFF and the ERC have spent a large amount of effort testing eDNA methodology for tracing khapra in the post border environment (for example, in warehouses, shopping locations).

The best approach for detecting ants (a major DAFF focus alongside khapra and BMSB) is not yet clear. An important observation was that BMSB does not seem to be easily detectable from dust.

Described the structure of DAFF response teams. NAQs is super busy with incursions from the north!

Mackenzie Lovegrove/Luke Noble. Terrestrial eDNA sampling approaches

Mackenzie described the testing of a wide range of methodologies for eDNA capture in field situations i.e., away from the border. Ladybug trapping as they are predatory and contain DNA which may be from pest species. Described a large project in Melbourne to localise three species of mosquitoes in 35 sites.

In a sugarcane project, wipes are being used to capture eDNA from the surface of plants; DNA is extracted directly from the wipes.

EnviroDNA are currently testing a number of different approaches for capturing eDNA from air. Much of this work is being done in a natural area close to Melbourne where the fauna is quite well understood. Drones with active filter devices, handheld equipment and MWAC passive samplers are all being tested.

Steve Pawson. Ultraviolet lights and molecular diagnostics for wide area surveillance of recent invasive species establishment

Described a multi-research agency project based around the Port of Tauranga (New Zealand), funded by the Biological Heritage programme. UV light traps in combination with DNA metabarcoding are being tested for identifying insects present in the port. This project comes after Scion has spent many years collecting and identifying insects at the port using traditional methods.
Non-destructive DNA extraction as used in iMapPests, testing of different primer sets, development of New Zealand

specific DNA reference databases. In the next season, a new generation of semi-automated traps will be tested at the port.
An important part of this talk was dedicated to ongoing consultation with Māori groups around the development of the New Zealand specific DNA reference databases.

Francesco Martoni. eDNA at iMapPests and beyond

Francesco discussed a wide range of projects that he is involved with at DEECA. The central theme of the presentation was the use of DNA metabarcoding. Francesco listed a number of projects where students incorporated metabarcoding as one chapter in their thesis. One student with a thrips project that had a chapter on metabarcoding. Other projects were on identification of baited ants (Biosecurity Innovation Programme) and a marine biosecurity detection programme for the Port of Melbourne.

A lack of voucher specimens was noted as a continuing problem for metabarcoding as a biosecurity tool. This was later repeated by MPI. A great photograph of an ant after non-destructive DNA extraction showed the retention of diagnostic features on the insect surface.

Francesco emphasised the need to be ahead of the curve with development of reference sequence databases. Predicting which taxa may be part of a future biosecurity issue is impossible. While Francesco is generating a large number of sequence datasets, it was unclear if these contribute to a larger database.

Francesco doesn't feel like metabarcoding is yet an accepted diagnostic tool for biosecurity. To help this, he would like to see a set of agreed protocols for metabarcoding researchers.

Andrew Cridge. Detection of invasive plant species using pollen eDNA

Andrew Cridge from Scion discussed an MBIE Smart Idea project (3 years) which aimed to test the use of pollen metabarcoding for the detection of unwanted plant pests. A network of traps was established in the Dunedin region. New information on the distribution of plants in the vicinity was gained, such as the unexpected presence of threatened species. Tools from the project are being deployed in the Bay of Plenty where the regional council is aiming to demarcate the spread of an invasive plant species.

Jessi Henneken. Monitoring beehives using eDNA
Jessi presented an exploratory study of honeybee hives. Metabarcoding DNA monitoring was paired with GPS monitoring of hives. The study captured data from four hives x four months. Most of the insect detection was, unsurprisingly, of honeybee (85%). However, aphids and planthoppers were detected. The researchers also picked up "*D. krausii*" a recently released biocontrol agent. These insects may be sheltering in the hives, or (this was thought less likely) remnant DNA may have been captured on honeybees.

Justine Larrouy. eDNA detection of the Green Vegetable Bug, a model pentatomid

Justine presented the results of recent experiments on GVB detection. This work utilised laboratory insect colonies and was aimed at understanding exposure times and number of insects required for GVB detection. Species-specific qPCR was combined with a grapevine leaf washing and filtering approach for collecting eDNA. Under this experimental set-up, GVB could be detected after 24hrs but detection after 48 and 72 hours was much more robust. At 72 hours, the number of GVB present (1-10) made little difference to detection levels.

Mackenzie Lovegrove. Which pest species are suitable for eDNA detection? (Boosting Diagnostics)

Mackenzie presented a comprehensive profile of 34 high priority pest species that had been categorised based on criteria such as distribution, hosts, and life history. This information was used to establish the opportunities for eDNA detecting these pests – with an emphasis placed on possible DNA aggregation sites. A top 10 list of pests that might be detected by eDNA included leaf miners, the Spotted Lanternfly, and psyllids.

Mackenzie described extensive research showing successful detection and identification of leaf miner insects from eDNA left in leaf mines. These methods have been used to detect invasive leaf miners in Australian agriculture settings.

Ministry for Primary Industries – final discussion

At the end of the presentations, there was a general discussion among the participants present in Lincoln. MPI described the operations projects they have recently completed or are just initiating.

Rebijith mentioned a recent MPI operations project aimed at detecting Glassy Winged Sharpshooter DNA at feeding sites in leaves. Detection was very poor until there was penetration or scratching of the leaves to release saliva etc. An eDNA project to detect mosquitoes was run by Chandan Pal before his departure from MPI.

Currently see a benefit for eDNA looking for pests of bees especially bee mites. This currently takes a huge amount of effort to screen. Following the talk from EnviroDNA, leaf miners were mentioned as a potential research target. The reference sequences for leaf miners from New Zealand are small in number.

Uncertainty around unknowns in eDNA datasets is still a problem for MPI (Luciano Rigano). Does an eDNA detection represent a real and live incursion? Better markers for bacteria and – for some taxa – fungi, was also desired before eDNA could be helpful.

Conclusions

- Ports or border facilities are a major focus for eDNA detections in Australia.
- Detection of khapra beetle in dust from ports is a major area of research i.e., vacuuming is one major technique for eDNA aggregation. Detection of khapra is based upon qPCR, rather than a more general detection method (such as those based on DNA sequencing).
- Successful biosecurity detections of khapra using eDNA mean that this approach has moved from purely research to implementation as a genuine border detection tool.
- BMSB and ants are other important terrestrial eDNA targets in Australia, but the best methodologies for their detection are unclear. BMSB does not appear to be easily detected from dust.
- No eDNA aggregation methods other than vacuuming are yet proven. Suitable methods for eDNA aggregation in field situations remain under investigation.
- Many research groups are exploring options for capturing airborne eDNA.
- Large scale DNA datasets are being generated alongside information on imports. Curation and interrogation of such big data will only become more important. A related issue is that accurate reference DNA datasets are crucial for eDNA applications based on sequencing.
- MPI New Zealand has seen a number of exploratory experiments with eDNA but there is little implementation as yet.

Appendix 6: Pest Bites video

Pest Bites video on BMSB identification can be found on the Cesar Australia Youtube channel at

https://www.youtube.com/watch?v=-nQiJNdbhwI

Appendix 7: 'Brown marmorated stink bug (*Halyomorpha halys* (Stål)) impact on wine grape quality and considerations for vineyard management in Australia: a review'

Brown marmorated stink bug (*Halyomorpha halys* (Stål)) impact on wine grape quality and considerations for vineyard management in Australia: a review.

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Abstract
The invasive brown marmorated stink bug (BMSB, *Halyomorpha halys* Stål) (Hemiptera: Pentatomidae) is a hemimetabolous insect pest that attacks over 300 plant hosts including commercially grown crops of economic importance, such as wine grape. In 1996, the pest was detected outside is native range in Asia, in the USA soon followed by detections in parts of Europe. In the USA, BMSB is of concern to wine grape growers and vintners. If BMSB were to reach Australia, there is concern about the impact on both yield and wine grape quality. BMSB can cause direct loss through feeding on the wine grapes. If BMSB are harvested with the wine grapes, they may contaminate the juice and final wine. Here, our review focuses on the effect of pre-harvest damage on yield loss and postharvest contamination of BMSB on wine grape quality, and management considerations for Australian viticulture and vinification.

Keywords
Halyomorpha halys, BMSB, viticulture, wine taint, yield loss, post-harvest, grape quality, invasive species

Introduction

The brown marmorated stink bug (BMSB, *Halyomorpha halys* Stål) (Hemiptera: Pentatomidae) is a hemimetabolous insect pest that is native to North and South Korea, Japan, and China (Lee et al., 2013a). Brown marmorated stink bug is polyphagous, attacking over 300 plant hosts, many of agricultural importance, including, vegetables, ornamentals and wine grapes (Holtz and Kamminga 2010, Mohekar et al. 2017). Its feeding habit involves piercing the fruit or reproductive structures of hosts (McPherson and McPherson 2000).

Brown marmorated stink bug is capable of long-distance flight (Nielson et al 2013; Lee & Leskey 2015; Wiman et al 2014) and walking dispersal (Lee et al 2014) and frequently moves among crops and wild host plants as they mature during the growing season. Like many other pentatomids (Saulich & Musolin 2014), prior to overwintering, BMSB seek out and cluster in secluded dark areas, where they remain dormant until spring, at which time they disperse to feed on hosts and reproduce (Toyama et al. 2006, 2011). In its native range, BMSB is known to be an arboreal species (Bernon et al. 2005) that overwinters in dead standing trees such as oaks, locusts, and paulownias (Lee et al. 2014). In addition to natural overwintering sites, BMSB will move into structures to overwinter (Kobayashi & Kimura 1969; Wantanbe et al. 1994; Hamilton 2009; Inkley 2012; Leskey et al. 2012). BMSB is also considered a nuisance pest due to its habit of entering households and other dwellings and buildings, where it seeks winter retreats and releases unpleasant smells from stink glands when disturbed (Zhu et al 2012).

The invasive insect was first detected outside of its eastern Asian native range in 1996, in the United States (Hoebeke 2002, Hoebeke and Carter 2003). It has since spread, to Canada (Fogain and Graff 2011), parts of Europe (Wermelinger et al. 2008, Arnold 2009, Heckmann, 2012, Callot and Brua, 2013), Georgia, Abkhazia, and Russia and Chile, South America (Gapon 2016).

This pest is not found in Australia, although it does pose a high biosecurity risk due to its tendency to hitchhike on imported goods.

The effect of pre-harvest damage on yield loss and postharvest contamination of BMSB on wine grape quality, and the management considerations for Australian viticulture is the focus of this review. In Australia, the grading of grapes is currently subjective, and done through taste, smell, vineyard observations and a knowledge of history, often combined with some basic chemical analyses. However, there are attempts to make the process more objective by utilising the chemical composition of winegrapes to differentiate between grape grades (Wine Australia, 2020).

Incursion and establishment risk

Exotic plant pest transmission pathways are often closely associated with the movement of people or products (Paini et al. 2016; Weeks et al. 2020; Inspector General of Biosecurity, 2020). The number of exotic pest interceptions at Australian ports between 2012 and 2018 was reported as more than 104,000 linked to air cargo, and 35,800 linked to sea freight (Inspector General of Biosecurity, 2019). Exotic bees, Brown marmorated stink bug, and Giant African land snail comprised the majority of National Priority Plant Pest interceptions (Inspector General of Biosecurity, 2019).

High profile interceptions of BMSB have been featured in the media. In November 2018, a cargo ship originating in China was ordered to turn around without making port at Freemantle after an offshore inspection by federal Department of Agriculture and Environment biosecurity officers detected an infestation of BMSB as well as other exotic species on board (Fresh Fruit Portal, 2018). In that same year the stink bug was found within Melbourne city limits, brought into the city on imported ceramics and machinery from Italy, which prompted an exotic pest eradication response (Agriculture Victoria, 2021; Stock and Land, 2018).

Each year seasonal measures over the Australian Spring to early Autumn are prescribed by the federal Department of Agriculture and Environment to limit the risk of BMSB importation on cargo. The situation is an evolving one. As of 2021, 37 countries had been identified as target risk and subject to sanitary and inspection measures by the Australian government, with a further seven classed as an emerging risk (DAWE, 2021).

The climactic suitability of many parts of the country for supporting BMSB, particularly eastern Australia, overlaps with several major agricultural and horticultural production zones. In addition to being a key biosecurity risk to many horticulture (e.g. apple and pear, rubus, stonefruit, cherries, vegetables), cotton, wine and nursery industries, it also represents a threat as a significant public nuisance due to its tendency to aggregate in man-made structures during its overwintering diapause (Lee et al., 2013a). The economic impacts of BMSB should it establish in New Zealand were recently quantified (NZIER 2017). They showed that BMSB would significantly reduce horticulture yields, for example a yield loss of 47% and 45% was predicted for apples and pears respectively, if BMSB were left uncontrolled. Further, even with chemical-based control, losses of 25% were forecast (NZIER 2017).

Damage in vineyards

Period of risk

BMSB is a landscape-scale threat that can invade vineyards from wooded habitats, other nearby crops, and in the spring, from human-made structures (Nielsen et al. 2016). Adult BMSB emerge from their overwintering sites in late winter/early spring and subsequently disperse to early season hosts. In its

invasive range in the US, after leaving overwintering sites it is unclear where they go, however in Asia, they are reported to utilize arboreal hosts (Rice et al. 2014). Oviposition begins once critical diapause-terminating cues are met (Leskey & Nielsen 2018). BMSB may move frequently among different wild and cultivated host plant species, feeding alternately among them. As with most crops including wine grapes, there is a strong edge effect, with higher populations found in rows near the edge of blocks (Basnet et al 2015). The level of BMSB management required during each growing season can vary according to the size of the adult population that survives the winter (Nielsen et al. 2016).

In its native range, BMSB is reported as an occasional pest of grape (Ohira 2003), however, in parts of its invasive range in the US, BMSB has arisen as a significant pest of grapes (Hamilton 2009, Leskey et al. 2012, Pfeiffer et al. 2012). In the US, BMSB has been found infesting wine grapes in New Jersey, Oregon, and Virginia (Nielsen et al. 2016). To date, significant populations have not been recorded in juice grape production areas such as Erie, Pennsylvania or in New York State. In the mid-Atlantic and Williamette Valley regions, vineyards neighbouring alternative host crops and non-crop plants such as peach, apple, wheat, broadleaf maple, English holly, catalpa, tree of heaven, and empress tree may experience dispersal into grape when the alternative host is harvested or when the daylength decreases into August–October. It has also been noted that when soybean or corn fields reach suitable maturation stages, BMSB will leave the vineyard and move into those fields (Nielsen et al. 2016). A study in vineyards in Virginia showed that overwintering adult bugs were first detected in vineyards in May (this is equivalent to November in Australia); however, the timing of first detection differed among vineyards. Egg masses were found primarily in June and July, and were usually found on the lower surface of grape leaves, although they were occasionally on the upper leaf surface, on the fruit, or on the rachis. All developmental stages of BMSB were found in vineyards, suggesting that grape can serve as a reproductive host. Substantial variation in BMSB densities was found among vineyards and throughout the growing season. The first instars were found on egg masses and after moulting, dispersed throughout the grape vines. The date on which the first egg mass was collected was considered as a biofix. Based on a degree day model, there were sufficient degree days for completion of one generation in Virginia vineyards.

In an Australian context, if BMSB were present, and based on the Virginia US scenario we might expect them to emerge from their overwintering sites around late August, early September. They would subsequently disperse to host crops, before moving into vineyards before late spring, i.e. November.

Feeding damage

BMSB feed directly on the fruiting or reproductive structures of host plants (McPherson and McPherson 2000). In vineyards, BMSB can directly affect yield and quality by feeding on the fruit. This damage may result in secondary pest attacks, or infection by other pathogens (Basnet et al., 2015; Leskey et al., 2012). This often results in fruit collapse because of progressive necrosis (Lee, 2015; Leskey et al., 2012). The movement of high numbers of BMSB into buildings, including wineries, can provide another opportunity for BMSB to affect wine. If the pest is harvested with the wine grapes, it has the potential to may contaminate the juice and final wine (Tomasino et al. 2013), although this aspect has received little attention by the global research community.

As wine grapes do not have to be marketed directly to consumers, some visible cosmetic feeding damage is acceptable as long as there are no other negative repercussions such as reduced yield. Grapevines are susceptible to BMSB damage from fruit set. All life stages, except for the newly hatched first nymph stage,

can cause damage to grape bunches (Basnet et al. 2015, Tomasino 2013). BMSB can cause direct injury to grape berries (Hamilton 2009) and can feed on the grape rachis and cause subsequent abscission of the clusters (Pfeiffer et al. 2012).

BMSB feeding begins with the insertion into the grape berry of the stylet and the injection of watery saliva containing digestive enzymes, causing the cellular breakdown of host-plant tissue that enables the pre-digested contents to be consumed (Leskey et al. 2018). BMSB feeding leads to soft sunken areas at the grape feeding site that become slowly discoloured. Damage incurred post-veraison is most severe and results in black necrotic spots that grow over time, resulting in berry deformation (Basnet 2014).

There is the potential for an increase in disease incidence associated with feeding by BMSB; these may be either pathogens vectored directly by BMSB or indirectly where pathogens use the feeding site as an entry point into the berry (Jones and Lambdin 2009). However, this has not been a significant issue to date. A study in the US on Chardonnay and Concord grapes showed that there was relatively little incidence of disease as a result of BMSB feeding (Smith et al 2014). In a vineyard with modest levels of grape powdery mildew (*Erysiphe necator* Schw.) and bunch rot (*Botrytis cinerea* Pers.:Fr.) and BMSB, harvest results revealed minimal incidence of disease (Smith et al. 2014).

Economic loss to vineyards

BMSB has caused substantial damage to the vineyards of Virginia, US where the pest has reached economic, i.e. high levels (Pfeiffer et al. 2012). In New York (NY) State, the third leading grape producer in the US, BMSB is potentially an economic threat to the grape industry in cool-climate regions, but again only at high densities (Smith et al 2014). In a study by Smith et al (2014) in NY State, BMSB were caged on Chardonnay and Concord grape clusters for two weeks during and after the period of fruit set. The authors showed a strong positive correlation between density, and both number and percentage of berries damaged for nymphs and adults. At harvest during late summer, cluster weight for both Chardonnay and Concord cultivars was shown to decrease with increased density of adults. Further, adult females had a greater effect than adult males, while nymphs had little impact on cluster weight (Smith et al 2014). The authors concluded that further studies were required to confirm these results and that they were unable to make recommendations on BMSB thresholds for intervention at this stage.

Wine taint caused by BMSB

In vineyards, BMSB may be collected along with clusters at harvest, and can be transported in lugs or bins to the winery. If crushed with the berries, they can impart a noticeable odour or flavour, referred to as taint (Kelly 2010).

BMSB primarily secretes tridecane and trans-2-decenal when stressed (Baldwin et al. 2014). Trans-2-decenal (T2D) is considered to be the main component of BMSB taint due to its strong “green”, “cilantro”-like aroma (Mohekar et al. 2017b). Tridecane is an odourless compound, and its effect on wine quality remains unknown (Mohekar et al. 2017a).

White wine

Pinot gris has been found to be free of T2D even at BMSB density of 1 per cluster (Mohekar et al. 2016). In pinot gris, concentrations of T2D and tridecane were greatest after a hard press, which was expected as BMSB would still be alive during pressing and may continuously produce stress-related compounds.

However, at the finished wine stage, only low levels of tridecane (9.69 µg/L) were measured. Trans-2-decenal appeared to be completely removed during alcoholic fermentation. This was thought to be due to compounds being driven off by carbon dioxide or T2D reacting with SO_2, or being converted to its corresponding alcohol n-decanol during fermentation (Mohekar et al. 2017a). A sensory analysis that compared Pinot gris with and without 9.7 µg/L tridecane did not result in a discernible difference (Mohekar et al. 2016), showing that tridecane does not appear to affect white wine quality. The authors concluded that the risk of BMSB contamination affecting white wine is low, as pressing, which is the critical processing step and was responsible for the largest amounts of stress compounds released, occurs prior to fermentation. Thus, the limited studies to date show that BMSB taint for white wines is not problematic with respect to wine quality, at least within the bounds of the study parameters.

Red wine

Trans-2-decenal has been found in red wine made with BMSB contaminated grapes (Tomasino et al. 2013a). It has been shown that T2D has a negative effect on red wine quality, significantly decreasing consumer preference at a concentration as low as 4.8 µg/L, the determined consumer rejection threshold (CRT) (Mohekar et al. 2014). For a discussion on the use of CRTs (preference) as opposed to detection thresholds (DTs) see Mohekar et al. (2017b). In pinot noir, a wine that does not normally contain T2D, concentrations above this level can add green, musty, herbal characteristics to wine, which are not desirable (Mohekar et al. 2017b). Further, reductions in favourable pinot attributes including dark fruit, red fruit and floral characteristics have been observed (Mohekar et al. 2017b).

A study on merlot and pinot noir (also white grapes, Pinot gris – see above) red wines, showed that the concentrations of both (E)-2-decenal and tridecane increased with an increase in BMSB density (Mohekar et al. 2017a). The study showed that destemming/crushing produced the highest concentrations of (E)-2-decenal, and pressing produced the highest concentrations of tridecane. Both compounds were greatly reduced during fermentation (as in white wine), but pressing after fermentation resulted in an increase in the concentrations of both stress compounds. It is thought that the concentrations were reduced because of compounds being driven off by carbon dioxide, as the winemakers could distinctly smell BMSB stress compounds when mixing the cap with the fermenting juice. All final treatment wines contained tridecane, and a 2014 Pinot noir wine also contained (E)-2- decenal in the finished wine. The presence of BMSB stress compounds in the red wines after pressing is of concern given the minimal decrease in the concentrations of the taint compounds in the wine post-pressing and the low reactivity of BMSB stress compounds in the wine matrix. BMSB taint in must decreases during fermentation, with the critical processing point, pressing after red wine fermentation (Mohekar et al 2017a)..

Thresholds for taint

In red wine, it has been shown that as low as three BMSB per cluster in the vineyard can result in finished wine with 2.02 µg/L of T2D (Mohekar et al. 2017b). The same BMSB density may also result in T2D concentration at or above the CRT when winemaking causes stress to BMSB, resulting in higher secretion of taint compounds (Mohekar et al. 2017b). Additionally, previous work suggests that the presence of dead BMSB can also result in wine containing tridecane but not T2D (Mohekar et al. 2017a). Based on the known consumption rejection threshold for T2D, Mohekar et al (2017a) recommended a threshold limit of 3 BMSB/cluster. These threshold levels can be applied to guide a pesticide application program in the vineyard. Additionally, it provides a valuable reference point for future research on taint due to BMSB.

Taint removal

Due to the negative impact of BMSB taint on wine quality and consumer preference, efforts are needed to minimize the concentration of these taint compounds in finished wine.

However, as yet there is little known of methods to remove taint caused by BMSB from wine (Mohekar et al. 2018). Modifications in wine making protocols may reduce BMSB taint concentrations in final wine, as winemaking processes are known to alter aroma composition (Mohaker et al. 2017a).
Alterations in harvesting, pressing and fermentation have shown potential in reducing BMSB taint in finished wine (Mohekar et al. 2017a). The effect of press type was trialled in a study on pinot noir. At a density of 3 BMSB/cluster, the use of a bladder press versus a basket press produced higher concentrations of tridecane. A similar result was observed at a BMSB density of 0.3 bug/cluster (authors did not show this data). A bladder press presses the grapes from the centre of the press outward, while a basket press presses the grapes from the top and bottom. Both of these presses have a different application of pressure that can alter final composition (Mohekar et al. 2017a). Thus, it is possible that taint levels in the finished wine could be modified by using an appropriate press that minimises the release of taint compounds.

The modification of wine processing may not be always be appropriate however, as it can restrict the style of wine made from BMSB contaminated grapes. Additionally, process modification may not be sufficient against high BMSB densities and finished wine may still contain T2D or tridecane (Mohekar et al. 2017). Therefore, post-fermentative measures are required to be able to produce a desired wine style while minimizing taint levels.

Wine industries rely on fining agents to correct wine faults and to improve wine quality (Fudge et al. 2012, Pickering et al. 2006). Wine sensory characteristics such as flavour, colour and mouthfeel can be adjusted by fining agents (Cosme et al. 2012; Threlfall et al. 1999). Fining agents are chosen for their affinity to unwanted compounds in wine through mechanisms such as hydrophobic interaction, hydrogen bonds, Vander Waals interaction and electrostatic interactions (Fudge et al. 2012; Braga et al. 2007). The fining agent, along with unwanted or taint compounds are then removed by racking, centrifugation or filtering. Commonly used fining agents such as bentonite, gelatin, casein and activated charcoal have previously been used on taint compounds (Tomasino 2016). Oak has been used effectively to mask lady beetle green aroma characteristic taint in red and white wine (Pfeiffer et al., 2012). In addition to fining agents, reverse osmosis filtration has also been explored as a viable option for taint removal (Fudge et al. 2011). In this process, selective taint removal can be achieved by carrying out filtration under pressure. Reverse osmosis is often combined with an adsorption or ion-exchange column to remove taint compounds more efficiently (Fudge et al. 2011). Another possible technique to reduce BMSB taint in wine is through aging. As wine ages, complex reactions occur that are known to change wine composition and sensory characteristics (Ebeler et al. 2001; Styger et al. 2011).

Recently, a study treated red wine grapes containing trans-2-decenal with fining agents and put them through reverse osmosis filtration (Mohekar et al. 2018). The authors assessed tridecane and T2D concentrations in red and white wine at bottle aging durations of 0, 6, 12 and 24 months. Reverse osmosis was found to be partially successful in removing T2D concentration from finished wine. And while tridecane and T2D concentrations decreased during bottle aging, post-fermentative fining treatments (gelatin, egg albumin, potassium caseinate, bentonite, yeast lees and French oak bean, medium plus toast) were not capable of removing these compounds in tainted wine. Although French oak did not alter the concentration of tridecane and T2D in red wine, it did mask the expression of BMSB-related sensory characters. The authors concluded that due to the ineffectiveness of removing BMSB taint post-

fermentation, focus should be placed on managing BMSB pre-harvest to reduce numbers in grape clusters so that the taint risk is reduced in the wine (Mohekar et al. 2018).

Management Considerations for Australian Vineyards

Several studies have measured adult dispersal between and among hosts throughout the growing season. In vineyards, as with many hosts, the highest densities of BMSB occur along the edges (Basnet et al. 2015), which can be exploited for management purposes. Further, given that high densities of BMSB reportedly cause the most damage, limiting population growth by targeting the overwintering and early season populations would be advantageous. Below are a number of tactics that are utilised or being considered for BMSB pest management.

Cultivar

Included in the wide range of hosts BMSB has been shown to feed on are two species of cultivated grapes, *Vitis labrusca L.* and *V. vinifera* L. (Bernon 2004, Wermelinger et al. 2008, Hamilton 2009, Pfeiffer et al. 2012). There are no reports of resistant, or tolerant cultivars.

Host use and preferences

In the US, an electivity analysis identified that BMSB is more commonly found on non-Asian host plants than on Asian host plants (Martinson et al. 2016). BMSB females oviposit more frequently on crops with long fruiting periods, including several fruiting vegetables (Zobel et al 2016). There is evidence that BMSB displays a preference for some host plants over others (Bergmann et al. 2016, Nielsen et al. 2016, Zobel et al. 2016). The stink bug also requires plants with fruiting structures to complete development and both host plant phenology (Acebes-Doria et al. 2016) and host utilisation is strongly related to host quality (Nielsen et al. 2009, 2011, Zobel et al. 2016).

Biological Control

Biological control is a viable management option that has received increasing attention for eradicating and suppressing a range of pest species, including BMSB. Several species of *Trissolcus* (Hymenoptera: Scelionidae) egg parasitoid wasps have been identified as the most effective biological control agents of BMSB. *Trissolcus japonicus* [syn. *Trissolcus halyomorphae*] and *Trissolcus mitsukurii* are especially efficient at controlling BSMB populations in both its native and introduced range, with *T. japonicus* parasitism rates at up to 70% and an average annual rate of 50% reported in China (Qiu 2007).

In 1962, *T. mitsukurii* was imported from Japan into Australia as a potential green vegetable bug (GVB; *Nezara viridula*) egg parasitoid and released in all states apart from the Northern Territory. Field recoveries were made in South Australia, New South Wales, and the Australian Capital Territory within several years of release. However, the current distribution of *T. mitsukurii* and its non-target hosts are unknown. The presence of this parasitoid could offer significant potential for control of BMSB, with augmentative release of biological control agents to manage BMSB increasingly proposed (e.g. Roversi Pio *et al.*, 2016; Stahl *et al.,* 2019). Parasitization rates can reach 70%, with annual mean parasitization of 50% (Yang et al. 2009). Thus, the ecological relationships BMSB has with other stink bugs and the associated parasitoid complex within its native and introduced range will need to be considered in the context of using these natural enemies as part of biological control-based strategies.

Biological control agents that are not known to be present in Australia, and could be considered for classical biological control include *T. japonicus.* While high parasitism rates of this pest have been reported, it does not appear to have constrained the native distribution of BMSB, as it parasitizes other stink bugs (Qiu 2007) and competes with other parasites (Qiu and Yang 2010). Other species in the genus

Trissolcus hold potential. In an 8-week survey in China, Koppel (2010) found BMSB eggs to be parasitized by *Trissolcus halyomorphae* Yang in four different host plants in Nanjing, Kunming, and Xi'an.

Non-hymernopteran biocontrols also present options. A tachinid fly in the genus *Bogosia* was reported parasitizing BMSB in Japan. The female laid an egg on the pronotum of the bug. After entering the host, the larva consumed the reproductive system, and sterilised the host. The tachinid larva then left the host to pupate. Parasitism of nymphs has also been observed (Kawada and Kitamura 1992). In the case of *Bogosia,* parasitism rates were reported between 6% and 7% (Kawada and Kitamura 1983) and >10% for overwintering adults (Kawada and Kitamura 1992). Further, a picorna-like virus, named *P. stali* intestine virus (PSIV), was found infecting the brown-winged greenbug and BMSB. Infected insects have an adult life span of about 13 days, compared to about a month in non-viruliferous adults (Nakashima et al. 1998). While classical biological control is an option, conservation or augmentative biological control strategies should be fully explored before the import of exotic parasitoids are considered due to the tedious and lengthy regulatory process, where success is not guaranteed.

Semiochemical

Trap crops

The highly polyphagous and mobile behaviour of BMSB has been exploited by investigating the use of trap crops as a management tactic. A trap crop is a plant that attracts arthropod pests away from nearby crops. The effects of using early maturing soybeans as a trap crop to protect mid- and late maturing varieties showed that more BMSB were observed on the early maturing soybeans than the later varieties (Osakabe and Honda 2002). Further, the study showed that BMSB populations did not increase in later varieties upon harvest of the early crop (Osakabe and Honda 2002). In another study, grain sorghum and sunflower were identified as the most attractive among five plant species (Nielsen et al. 2016). In pepper fields, sunflower alone used as a trap crop did not reduce injury (Soergel et al 2015); the use of sorghum plus sunflower as a trap crop species attracted BMSB, although injury to organic peppers was only minimally reduced (Mathews et al 2017). Another study used harmonic radar and protein markers to quantify retention within the sunflower and sorghum trap crop (Blaauw et al. 2017). The authors showed that the trap crop was more attractive than the pepper cash crop, however it did not act as a population sink. Identifying trap crops that are more attractive than Australian vineyards could offer a plausible control option for BMSB and warrants investigation.

Attract & Kill

A promising option under development is 'attract-and-kill' (Lee, 2015). Attract and kill, or lure and kill involves the combination of an attractant, (odour and/or visual cue) and a killing agent (pathogen or insecticide). This technology can be highly effective in controlling isolated and low-density populations of pests, and has the potential to add value to long-term pest management (Guerrero *et al.* 2014). This strategy shows promise for managing BMSB, with positive results of a proof-of-concept in US apple orchards (Morrison et al. 2019). The technique involves utilising the species' aggregation pheromone along with a pheromone synergist, methyl decatrienoate which attracts BMSB to the target area. These insects can then be sprayed in the target area with an insecticide to remove the individuals from the feeding population (Morrison et al. 2018). The benefit is that this reduces the area and amount of insecticide that needs to be applied. The authors concluded that attract and kill is an effective option for managing low to moderate BMSB populations in apple orchards, but requires optimisation to increase economic feasibility for grower adoption. Such a technique could also be useful in vineyards, and warrants further research.

Mass trapping

Mass trapping involves the use of olfactory stimuli to attract targeted organisms to a trapping device where they can be captured and killed. This strategy has been trialled against BMSB in the US, and has encountered several challenges. Traps, particularly those baited with aggregation pheromones, often result in increased plant injury near the trap (Prokopy et al. 2004), as pests are attracted to and arrive near the baited trap but remain outside the trap itself, termed 'spillover' (Switzer et al. 2009). As has been found with other stink bug species (Côkl et al. 2003), at close range, BMSB are thought to utilise substrate-borne vibrational signals that are involved in courtship (Polajnar et al. 2016). To improve trap capture by bringing adults together at close range and reducing spillover into crops, these signals could be trialled in combination with baited traps. There have not been any studies in vineyards testing the effectiveness against BMSB. The number of traps required in such strategies, and the cost of deploying and servicing the traps often mean such strategies are not economically feasible.

Mechanical Control

Given the chance of BMSB entering the wine processing building, or being harvested with grapes, even with pre-harvest management, it is important that post-harvest processes are managed to reduce the extent of any possible release of BMSB stress compounds into wine (Mohekar et al 2017).

BMSB infestations in residences or buildings can be reduced by sealing and screening openings (Pfeiffer et al. 2012). In the US, caulk has been utilised to seal light fixtures, exhaust fans and baseboards to keep those that have entered attics and basements from entering interior rooms (Day et al. 2011). Insecticides may be applied to the sides of buildings to prevent BMSB entering (Pfeiffer et al. 2012). Winemakers can utilise this knowledge to devise quality control measures.

Insecticides

Persistent insecticides are one of the most effective options to manage this pest, yet suppression has proved challenging and eradication has not yet been attempted (Lee et al., 2013b). Further, many of the insecticides that are used are not target-specific, and have led to secondary pest flare-ups. The most effective classes have been the pyrethroids and the neonicotinoids (Pfeiffer et al. 2012). While it has been possible to reduce populations immediately after a treatment, it has been more difficult to prevent reinfestation (Pfeiffer et al. 2012). In a glass-vial bioassay, bifenthrin was found to be highly toxic (Nielsen et al. 2008b). Other pyrethroids tested, with similar toxicity, were beta-cyfluthrin, cyfluthrin, fenpropathrin, and lambda-cyhalothrin. Recovery was recorded with all the pyrethroids. Neonicotinoids (dinotefuran, acetamiprid, and thiamethoxam) were also very toxic. The organophosphate phosmet had LD 50 values almost fourfold higher than other insecticide classes tested. This study showed that nymphs were more sensitive to insecticides than adults, and the larger females were more sensitive than males (Pfeiffer et al. 2012).

A list of insecticides ranked with a 'lethality index' (ranking materials from 0 to 100) was presented by Leskey (2011), including those that are registered in the US for grape. This index reflects both immediate mortality as well as the effect of recovery from initial paralysis. There was considerable variation in the lethality index within pesticide classes, e.g., dimethoate (93.3) and malathion (92.5) at the upper end of the range, and phosmet (20.0) near the lower end. Given the late season infestation seen in vineyards, and the potential problem of harvesting BMSB along with the fruit, the preharvest interval (the wait time between a pesticide application and when a crop can be harvested) is of great importance when considering pesticide choice.

PyGanic ®and clothianidin have both been successfully utilised in vineyards to control BMSB in grape clusters by applying the insecticides with an airblast sprayer late in the day preceding harvest (Pfeiffer et

al. 2010). A disadvantage of the pyrethroid class is the extremely damaging effect on populations of beneficial arthropods. It is common to see induction of secondary pest outbreaks, including spider mites and mealybugs. In the US, the latter is of concern in vineyards, since mealybugs are the vectors for grapevine leafroll virus (Pfeiffer et al. 2012). Sometimes there are also negative impacts of neonicotinoids on beneficial species. Transmission of grapevine leafroll virus is a concern in Australia, and therefore the risk of mealybug outbreaks, and other such secondary impacts, should be considered during management planning. As populations of BMSB are often higher at the edges of vineyards, if insecticides are needed, this should be where the focus of the control effort is centred (Pfeiffer et al. 2012).

Sterile Insect Technique

The sterile insect technique (SIT) is a plausible option to manage BMSB and has been successfully used to suppress, contain, prevent or eradicate insect pest populations from several insect orders including Diptera and Lepidoptera (Ref). SIT uses mass-reared insects that are irradiated before release to render them infertile. The success of SIT relies on sterile releases 'overflooding' the wild population, minimizing the possibility of wild males and wild females mating to produce viable eggs. This biologically-based approach has enjoyed significant success around the world including in Australia (e.g. Alphey, 2007), and works best when applied on an area-wide basis. SIT for BMSB management is currently being investigated by several countries including Italy, Korea, and the USA collaboratively with Plant & Food NZ (Suckling et al. 2019; Welsh et al. 2017). Empirical studies and population modelling strongly suggest that a synergistic interaction between released parasitoids and SIT may provide more rapid and cheaper eradication, or pest suppression than either technique alone (Sivinski 1996; Gurr & Kvedaras 2010).

Conclusion

BMSB has the potential to cause direct yield loss through its feeding habit on wine grapes, however loss through secondary infection appears to be of low risk. BMSB taint for white or rose wines has not been found to be problematic with respect to wine quality, and can be attributed to the fact that pressing occurs before fermentation in these varieties (Mohekar et al. 2017a). In red wine, BMSB appear to be problematic to wine quality if the insects are present during processing, as pressing is the critical step for release of stress compounds into wine at concentrations that would impact aroma perception. Trans-2-decenal is a volatile released by BMSB that has been shown to have a negative effect on red wine quality, significantly decreasing consumer preference at a concentration as low as 4.8 µg/L. The effect of tridecane on red wine quality has not been explored and warrants further research. There are a range of management options that are either available or entrain, that may be utilised by the Australian wine industry in the event of BMSB incursion and establishment. A landscape level approach is likely to prove most effective due to the pests vast host range, mobility and overwintering capability.

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17

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