Research in Support of the Guam Coconut Rhinoceros Beetle Eradication Project

Biological Control of Coconut Rhinoceros Beetle

Semiannual Report for USDA APHIS Grant 13-8515-1555-CA

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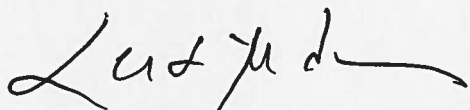and
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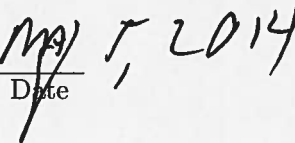AgResearch New Zealand

May 4, 2014*

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1 Summary

- Two insect pathogens have been introduced to Guam under USDA-APHIS import permits: for biological control of the coconut rhinoceros beetle (CRB): Oryctes nudivirus (OrNV) and green muscardine fungus (GMF), *Metarhyzium majus.* The host range of both pathogens is limited to Dynastinae, the subfamily of scarab beetles containing CRB, which is the only representative of this subfamily on Guam. Half of the current grant funds support a collaboration with Dr. Sean Marshall and Dr. Trevor Jackson of AgResearch New Zealand to work on the virus, and the remaining half is allocated for to measure the efficacy of GMF. Dr. Marshall made two trips to Guam, during January and February 2014 to perform bioassays in the quarantine laboratory at the Guam Plant Inspection Facility.

- **OrNV.** This virus has been very effective in reducing CRB damage keeping it at low levels elsewhere in the Pacific. However, laboratory bioassays indicate that the Guam CRB may be resistant to all strains of the virus tested to date. The primary goal of current research is to find a strain of OrNV which is highly pathogenic for the Guam CRB beetle population, and to autodisseminated this strain. Although results obtained during this project have demonstrated that the Guam CRB population is not resistant to OrNV infection, the possibility remains that the Guam CRB population has a degree of tolerance towards OrNV (or possibly to a subset of OrNV isolates). A genetic marker has recently been developed that distinguishes the Guam CRB population from other CRB populations (see the Supplementary Data section). The recently identified Hawaiian CRB population shares the same genetic marker that is present in the Guam CRB population. While the number of CRB populations tested for genotypes outside of Guam is still relatively small (i.e. Samoa, Fiji, PNG, Malaysia, Diego Garcia), OrNV is known to infect CRB in these other regions. The correlation of the genetic difference observed in Guam compared to other CRB specimens and the difficulties in establishing OrNV infection in the Guam population raises the possibility that the Guam (and Hawaiian) CRB populations are less susceptible to OrNV infection. We note that conclusive evidence for this hypothesis is not currently available for the Guam (or Hawaiian) population and therefore requires further research. However, Zelazny et al. (1989) have previously reported tolerance towards OrNV infection and transmission within some CRB populations.

- **GMF.** For the past 3 years, we have been importing GMF spores produced by Dr. Ambrosio Alfiler of the Philippine Coconut Authority. The spores are distributed in two ways: by direct application to CRB breeding sites and by dusting adult male CRB for autodissemination. The fungus has established on Guam indicated that we find it in breeding sites far from any release sites. Prior to the initial release of GMF, we found no CRB larvae or adults infected with GMF on Guam. The objective of the current grant is to measure the impact of this pathogen on the Guam CRB population. This work has been delayed because the technician tasked with this work quit unexpectedly. A replacement has just been hired.

- **Assistance to the Hawaii CRB Project.** We obtained the first 4 beetles detected in the current Hawaiian infestation for dissection and genotyping. All were females and two of theses were gravid. DNA from the 4 CRB adults collected in Hawai were compared to DNA samples from other CRB populations in the Pacidic using restriction fragment length polymorphism (RFLP) analysis. The Guam and Hawaii populations have DNA which breaks into 253 bp fragments. DNA fragments of this size are absent in DNA samples from Diego Garcia, Fiji, Samoa, and PNG. Thus the Hawaii population may have originated from Guam or a currently unknown common source.

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3 Results Guam Oryctes Nudivirus Bioassays

Project Aims

1. Obtain and Disseminate Virus Strains Which Kill Guam's Rhino Beetles.
Oryctes nudivirus (OrNV) has been effective in reducing coconut rhinoceros beetle (CRB) population levels and keeping them at low levels elsewhere in the Pacific. Our objective is to obtain samples of virus strains which are highly pathogenic for the Guam CRB population.

2. Determine Why Previously Tested Virus Strains Failed to Kill Guam's Rhino Beetles. Suppression of CRB populations on Pacific Islands using OrNV has been very successful over the past 50 years, and has become the major tactic in the region. Several successful CRB biocontol projects using OrNV have been run by the Secretariat of the Pacific Community (SPC). Failure of OrNV on Guam may mean that we are dealing with a virus-resistant population of beetles. It is of regional importance to determine reasons for this in order to prevent similar failures elsewhere.

Project Summary

The initial attempts to infect CRB with OrNV were based on the food incorporation method, which involved feeding CRB banana contaminated with OrNV or a control without OrNV. Treated banana was placed into a jar containing a single beetle and the standard rearing material (i.e. damp commercially produced garden centre sphagnum peat moss). Analysis of bioassay data showed no demonstrable effect of the virus on gut morphology or beetle mortality and suggested that OrNV infection did not establish. Failure to establish infection in these initial experiments was unexpected.

Further investigation included testing of freshly prepared OrNV-X2B (the same isolate used in the initial bioassay, but a different batch), the OrNV-B isolate, a mixture made up from 5 different OrNV isolates (PNG, I, V23b, S2A, and PV505), and a crude preparation of OrNV obtained from the infected gut of a field collected Fijian CRB. Analysis of bioassay results again provided no evidence for virus infection among the OrNV treated Guam CRB.

As previous use of OrNV in other Pacific and SE Asian regions have successfully used the food incorporation dosing method to infect CRB (e.g. Fiji, Samoa, Malaysia), the failure to establish OrNV infection in the Guam population suggested one of three main possibilities: 1) an unanticipated difference was present in the initial bioassay experiments, 2) the virus isolates provided to Guam had somehow become inactivated, or 3) the Guam population was potentially resistant to OrNV infection.

The experiments conducted for this research project were set up to identify potential reasons for the previous failure of OrNV to infect Guam CRB, and to determine if any of the available OrNV isolates could infect the Guam CRB population. Results from this current project have demonstrated that several OrNV isolates (including the commonly used OrNV-X2B isolate) are able to infect Guam CRB, which indicates this population is not resistance to OrNV. However, further experiments are required to conclusively determine if the Guam CRB population is more tolerant to OrNV infection, at least to some isolates, and whether infective strains can be transmitted to healthy beetles.

Based on insights gained during this project, it is likely that a combination of factors contributed to the failure of the initial attempts to infect Guam CRB with OrNV. Influences include possible unintended negative effects of using sphagnum peat moss (as opposed the more widely used coconut wood or sawdust) in the bioassay set up, and that the genetically distinct Guam CRB may be more tolerant to OrNV challenge than other populations. Results to date suggest that the Guam CRB is less

susceptible than other Pacific populations to OrNV infection, but further experimentation is necessary to confirm this and find ways to overcome the potential limitation of the virus.

Recommendations:
1) Establishment of OrNV infection in Guam CRB using the food incorporation method can be improved by placing the treated food item (e.g. banana slice) into an empty jar and allow feeding for 48-72 hours before placing rearing material into the incubation container. This prevents the sphagnum peat moss from contacting the virus prior to consumption, which likely prevents interaction with any antimicrobial activities associated with the peat moss, and reduces the possibility for desiccation due to absorption of moisture away from virus.
2) Use of droplet feeding and direct hemocoelic injection methods were two alternative methods that also proved to be useful in establishing OrNV infection in Guam CRB. These alternative methods appear to be somewhat more consistent in producing infection within individuals. In particular, the hemocoelic injection method provided more consistent infection of CRB over the feeding methods; however care must be taken to ensure sterility during the inoculation procedure.
3) Using the improved infection methods, a programme for release of lab infected adult CRB could be designed to introduce OrNV into the Guam outbreak population. Establishment of OrNV in other CRB populations within the Pacifc region has greatly assisted efforts to manage pest numbers and the palm damage associated with CRB outbreaks.
4) Conduct further validation experiments to determine if the Guam CRB population may be more tolerant to specific OrNV isolates, and to identify which isolates may be most appropriate for regular release into the population.
5) Maintain the use of other CRB control strategies, since use of OrNV alone is unlikely to control the Guam CRB outbreak. In places where CRB populations are being successfully managed, other CRB control strategies have continued to be carried out alongside OrNV release (e.g. removal or management of decaying organic matter, destruction of potential breeding sites).

Results and Discussion

Detection of Infection in CRB Treated With OrNV Isolates

CRB bioassays were setup to test OrNV infectivity using populations primarily from Guam (see Methods section), though a few individuals were obtained from Fiji for testing. It is important to note that although shipping of live CRB between Guam and Hawaii is established, shipping companies and airlines were reluctant to import live CRB into Guam from international sources, despite having valid import permits issued from USDA APHIS. As a result it was only possible to conduct limited direct comparative infectivity assays between Guam beetles and a few Fijian CRB adults (only 4 survived shipping, the remaining 40+ having perished during or shortly after transit). Additionally, a colleague in Malaysia has kindly agreed to assist us by offering to set up and monitor a parallel bioassay (in his Malaysia lab) to test infectivity of the Malaysian OrNV isolate (MalB) against Malaysian adult CRB. Results from the Malaysia bioassay were not yet available at time of writing.

To determine if a feature of the initial Guam food incorporation assay may have contributed to the unexpected failure in establishing OrNV infection, a modification of the Guam food incorporation treatment was tested (see the Methods section for full details). Briefly, the modification involved feeding individual CRB adults treated banana within an empty feeding container for the first 48-72 hours before adding the rearing material. In addition, droplet feeding and direct hemocoelic injection methods were investigated as alternative approaches for establishing infection (see the Methods section for full descriptions). Using the methods outlined above, bioassays were set up and monitored over 6-8 weeks. Where feasible, a standardized virus stock (standardized at a titre of 1×10^6 infectious units per millilitre (IU/ml) of virus was determined by cell culture based methods) was used as a source of inoculum. Mortality was used as an initial indicator to assess the effectiveness of these infection methods in establishing OrNV infection in Guam CRB. The total combined results obtained from all dosing methods (modified food incorporation, direct hemocoelic injection, and droplet feeding) are summarized in Table 1. The various dosing methods employed are presented individually in Table 2. In Table 1, comparison of the various OrNV treatments against control treatments revealed a significant difference in observed mortality for OrNV-I, X2B, TAS, TAP. The titre of the OrNV-TAP and MalB virus could not be properly titred since TAP was derived from the cell

pellet fraction collected from a cell culture produced TAS batch, while MalB consisted of virus freshly isolated from infected Malaysia CRB guts. In the case of MalB, molecular testing confirmed the correct isolate was present, however MalB was not directly injected as it was a freshly obtained from CRB guts collected from Malaysian CRB and it could not be confirmed as being 100% free from other microbes. When the results are broken down in to the individual treatments (Table 2), it is clear that direct hemocoelic injection of OrNV was likely to be the primary cause of mortality for the CRB. While the OrNV-TAP treatment was not significant, it is possible that the virus titre in the TAP sample was lower than anticipated. The cell pellet fraction was included as a pilot test to determine how useful application of the cell pellet fraction may be for infecting CRB.

Table 1: Combined total CRB mortality data from OrNV challenge assays.

Treatment Type	n=	% Mortality	SEM[a]	p-value[b]
Control	55	29.1	6.2	
OrNV-I	37	51.4	8.3	0.048*
OrNV-X2B	50	58.0	7.1	0.003*
OrNV-TAS	25	72.0	9.2	0.001*
OrNV-TAP	18	66.7	11.4	0.010*
OrNV-MalB	46	45.7	7.4	0.100

[a] Standard error of the mean for mortality observations.
[b] p-values based on comparison of the control treatment to each of the OrNV treatments. p-values were calculated using a Fisher's exact test from a 2-proportions test. Significance at the >95% confidence interval is indicated by an asterisk (*).

Table 2: Combined CRB mortality data for individual OrNV dosing methods.

Dosing Method	Treatment Type	n=	% Mortality	SEM[a]	p-value[b]
Food Incorporation	Control	22	31.8	10.2	
	OrNV-I	14	28.6	12.5	1.000
	OrNV-X2B	22	50.0	10.9	0.358
	OrNV-TAS	10	40.0	16.3	0.703
	OrNV-TAP	8	62.5	18.3	0.210
	OrNV-MalB	22	40.9	10.7	0.755
Direct Injection	Control	23	30.4	9.8	
	OrNV-I	23	65.2	10.2	0.012*
	OrNV-X2B	24	62.5	10.1	0.041*
	OrNV-TAS	15	93.3	6.7	<0.000*
	OrNV-TAP	10	70.0	15.3	0.057
	OrNV-MalB	NA			
Droplet Feeding	Control	10	20.0	13.3	
	OrNV-I	NA			
	OrNV-X2B[c]	4	75.0	25.0	0.095
	OrNV-TAS	NA			
	OrNV-TAP	NA			
	OrNV-MalB	24	50.0	9.6	0.141

[a] Standard error of the mean for mortality observations.

It is important to remember that bioassays to prove mortally for pathogens causing long term or chronic infections are inherently difficult (Jackson & Saville, 2000; Lacey, 1997). For the CRB-OrNV bioassays, the length of time required to run the mortality assays is a major issue as it is necessary to maintain individual insects for long enough to enable the assay to distinguish specific treatment mortality from age- or handling-related mortality. The issue encountered for demonstrating that the OrNV treatment causes disease and mortality was the relatively high CRB mortality rate in the control treatments. This is not a unique feature specific to the experiments conducted in this project, but is an ongoing issue for CRB-OrNV pathogen testing more generally. Therefore, to establish that OrNV infection was the likely cause of death, CRB were dissected and assessed for OrNV infection through histological and PCR analyses of tissue samples.

Figures 1 to 3 provide exemplar photos of gross visual, histological, and PCR observations representing OrNV infection versus non-infected CRB specimens. Visual gross examination (Figure 1) of healthy gut tissue generally exhibits a thin gut with a brown colouration, while OrNV infected gut tissue generally correlates (though not always) with a swollen and whitish appearance (early stage or light infections may present as only slightly swollen and/or a light brown colour). Gross visual evaluation is only possible for live, moribund, or freshly deceased CRB, so could not be relied upon for diagnosis. Histological analysis (Figure 2) of healthy gut epithelium tissue shows the columnar cells forming ordered undulating columns with rows of nuclei arranged at the basal end of the columnar cells, cells of the regenerative crypts appear as distinct large circular clusters, and the gut lumen appears free of cell-like vesicles with portions of peritrophic membrane generally being easily observed. Histopathology of *Oryctes* nudivirus infected gut tissue reveals a disarrayed appearance of the normally neat columns of columnar cells, the regenerative crypt clusters appear swollen, the lumen is partially or completely filled with individual round cell-like vesicles that have been sloughed from the gut epithelium, and the peritrophic membrane is often not observed. Histological analysis is a reliable diagnostic method for mid to late stages of OrNV infection, but early stage infection can be difficult to distinguish. PCR analysis (Figure 3) to specifically detect the presence of OrNV DNA within a sample is another method to identify infected OrNV CRB, and is able to assist in confirming diagnoses made from histology (and gross visual examination, when possible). Due to the high sensitivity of the PCR method, a protocol has been developed to distinguish between samples that are infected with OrNV (i.e. virus has replicated within the tissue) versus specimens that may only be contaminated with OrNV (e.g. account for OrNV dose applied in bioassay, or accidental cross contaminated during tissue dissection).

Although diagnosis was not possible for all individuals in the bioassay experiments (in part due to the advanced state of decay observed for several individuals, but also the large numbers of insects), gut tissue from 17 OrNV treated CRB specimens were analysed by both histopathology and PCR detection for presence of OrNV infection. Evidence for OrNV infection (based on histology and PCR methods) was detected in a total of 9 specimens (2 out of 9 from food incorporation, and 7 out of 8 from direct hemocoelic injection). Virus infection was observed in CRB treated with OrNV-I, TAS, TAP, and X2B strains (results for OrNV-MalB were not available at time of writing). It is worth noting that evidence for OrNV infection in has never been observed (gross visual, PCR, or histological) in any wild caught or control treated Guam CRB specimens.

Figure 1: Partially dissected abdomens of *O. rhinoceros* displaying gut tissue (highlighted by red arrow) commonly observed in healthy and OrNV diseased adults (left and right panels, respectively). The key aspects to note from the gut tissues are: thin with dark brown colouration (healthy) versus swollen with milky or light brown colouration (infected).
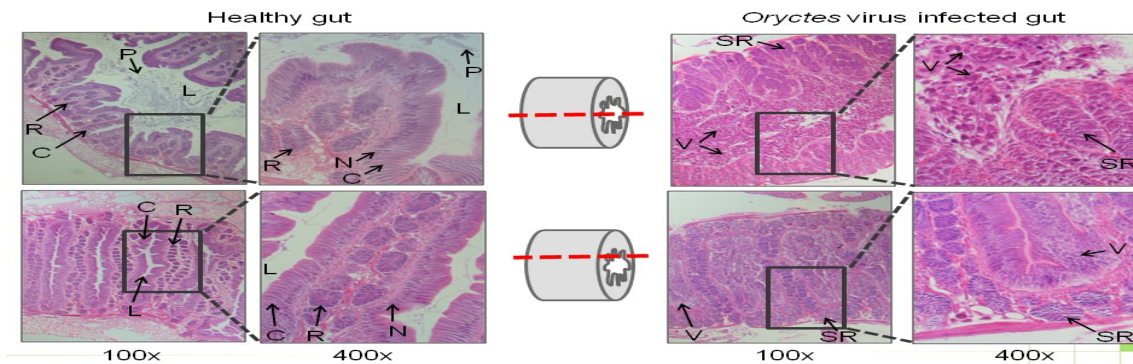

Figure 2: Histological differences observed between healthy and OrNV infected CRB gut tissue. The photo panels show examples of hematoxylin and eosin stained tissue (longitudinal) sections viewed under a light microscope. Compact dark blue/violet staining highlights DNA material (e.g. nucleus), while the cytoplasmic material stains pink with a diffuse blue tint. The central diagram indicates the approximate orientation (red line) of the exemplar tissue sections (grey cylinder).

The series of photos labelled as 'healthy gut' photos (left-hand side) shows sections viewed at magnifications of 100-fold (left) 400-fold (right). The columnar cells (C) of the healthy gut epithelium form ordered undulating columns, and as observed at 400x magnification, the rows of nuclei (N) are arranged at the basal end of the columnar cells (small dark blue staining). The cells of the regenerative crypts (R) appear as distinct large circular clusters (dark blue stain). The lumen (L) of healthy guts appears free of cell-like vesicles, with portions of peritrophic membrane (P; containing food particles) being generally easily observed.

The series of photos labelled as '*Oryctes* nudivirus infected gut' photos (right-hand side) show an infected gut viewed at magnifications of 100-fold (left) 400-fold (right). Although the 'waviness' of the gut epithelium can still be observed, the neat columns of columnar cells have disappeared and the regenerative crypt clusters appear swollen (SR; compare with healthy gut). Additionally, the gut lumen is completely filled with individual round cell-like vesicles (V) that have been sloughed from the gut epithelium; neither 'clear' space nor peritrophic membrane can be observed.

Figure 3: Example agarose gel from PCR analysis to detect OrNV infection in CRB gut tissue. Following PCR amplification using primers specific to OrNV, reaction products are separated by electrophoresis on an agarose gel. Lanes showing a 945 base pair PCR product indicate presence of OrNV infection (lanes 2, 4, and the positive control), whereas lanes missing the DNA band are

negative for presence of OrNV (lanes 1, 3, and the negative control). PCR conditions are set up to distinguish between present of OrNV DNA via infection (i.e. due to replication in the host) versus low levels of contaminating virus that may be present in specimens (e.g. original dosing or accidental cross contamination of specimens during dissection).

Based on observations obtained from the bioassay experiments, it appears that the Guam CRB are susceptible to OrNV infection via dosing by the ingestion and direct hemocoelic injection methods described. Furthermore, analysis suggests that at least three of the OrNV isolates (X2B, I, and TAS; diagnosis of bioassay results for MalB are pending) were able to establish infection in Guam CRB that eventually led to death (Table 1). As Guam CRB are not resistant to OrNV infection it should be possible to use OrNV as a biocontrol agent to assist in managing the Guam CRB population. However, prior to establishing a full OrNV biocontrol release programme, further experiments should be conducted to validate that lab infected insects are able to transmit OrNV to uninfected Guam CRB.

Since establishing OrNV infection in Guam CRB proved to be much more difficult than expected, it is recommended that further experiments be conducted to determine which OrNV isolates are would be most appropriate to use against the Guam CRB. Although it was not possible to test every known isolate within this project, Guam has been supplied with aliquots of the all the current cell culture captured OrNV isolates. Cell culture captured OrNV isolates supplied to Guam include: X2B, B, I, PNG, TAS, MalB, PV505, V23b, B36, MSA, SSA, Ap371, SEY, DVO, 22A, NSA, VFC3, XMS, NSB, S2A, and 46P. Please note that isolates X2B, B, I, PNG, TAS, MalB, PV505, V23b, NSA, and S2Aare known to be active (based on cell culture analysis), while aliquots of isolates B36, MSA, SSA, Ap371, SEY, DVO, 22A, VFC3, XMS, NSB, and 46P were taken from the few remaining original stock ampules that Dr Allan Crawford made during the 1980's (as these are original vials, some stocks may have lost activity over time).

OrNV isolates that are able to infect Guam CRB can subsequently be tested to determine which isolate(s) would be most effective as a biocontrol agent against the Guam CRB. These future experiments should include standardization of CRB selection for bioassay testing (e.g. adults of a known age and weight range), administration of a measured OrNV dose (e.g. based on cell culture titration), and incubation using a standard protocol (e.g. monitored over a known period of time under similar environmental conditions). Furthermore, consideration of characteristics such as the amount of feeding, mortality, fecundity, and rate or reliability of OrNV transmission should be taken into account. From the results presented here, there is evidence to suggest there may be some differences between isolates with respect to virulence and transmission. This is not wholly unexpected as previous studies have indicated that differences in virulence do exist between OrNV isolates (Crawford & Zelazny, 1990; Crawford et al, 1986; Moslim et al, 2010; Ramle et al, 2005; Wang et al, 2011; Zelazny, 1979; Zelazny et al, 1990).

Potential Explanation(s) For Failure Of Initial Attempts To Infect CRB With OrNV

Results from the current experiments were unable to conclusively identify a specific reason for the initial failure of establishing OrNV infection in Guam CRB; however, three possible explanations (or combinations thereof) were identified: 1) inactivation of virus by desiccation, 2) inactivation of virus by putative antimicrobial activity present in the sphagnum peat moss used for rearing, or 3) the Guam CRB population has a degree of tolerance to OrNV infection.

1) OrNV is susceptible to inactivation under desiccating conditions. In laboratory assays, Guam CRB were observed to bury the banana slices prior to consuming them. If the bait is not entirely consumed prior to (or shortly after) burial, it is possible that the peat moss environment could significantly decrease the moisture activity to a point where OrNV becomes inactivated. While the peat moss is moistened, moisture activity levels may not have appropriate to ensure infectivity. Unlike many biocontrol viruses (e.g. baculovirus), OrNV is not encased in a protective protein crystal matrix (Crawford & Sheehan, 1985; Huger, 2005). OrNV is enveloped in a lipid membrane containing proteins that is required for infection; however, this envelope is easily damaged by desiccation and other environmental factors (Marshall et al, in preparation; Payne, 1974; Payne et al, 1977; Zelazny, 1972).

2) The standard food incorporation method for infecting CRB with OrNV places the treated banana directly onto the rearing material for consumption. The typical CRB rearing material traditionally used for adults is lightly moistened coconut peat or sawdust compost (with or without steer manure). In Guam, the adult CRB are maintained in moistened sphagnum peat moss. Peat moss has similar properties to coconut peat or sawdust, but it is more readily available and less expensive. However, various antimicrobial properties have been associated with sphagnum peat moss (Klavina et al, 2012; Painter, 1991), and anti-viral activity for some viruses has been documented in the literature (Klöcking & Helbig, 1991; Witthauer et al, 1976). Therefore, it is possible that the anti-microbial factors putatively present in sphagnum peat moss are effective in inactivating OrNV.

3) Although results obtained during this project have demonstrated that the Guam CRB population is not resistant to OrNV infection, the possibility remains that the Guam CRB population has a degree of tolerance towards OrNV (or possibly to a subset of OrNV isolates). A genetic marker has recently been developed that distinguishes the Guam CRB population from other CRB populations (see the Supplementary Data section). The recently identified Hawaiian CRB population shares the same genetic marker that is present in the Guam CRB population. While the number of CRB populations tested for genotypes outside of Guam is still relatively small (i.e. Samoa, Fiji, PNG, Malaysia, Diego Garcia), OrNV is known to infect CRB in these other regions. The correlation of the genetic difference observed in Guam compared to other CRB specimens and the difficulties in establishing OrNV infection in the Guam population raises the possibility that the Guam (and Hawaiian) CRB populations are less susceptible to OrNV infection. We note that conclusive evidence for this hypothesis is not currently available for the Guam (or Hawaiian) population and therefore requires further research. However, Zelazny *et al*. (1989) have previously reported tolerance towards OrNV infection and transmission within some CRB populations.

Although results from these experiments were unable to provide a single explanation for the apparent difficulty of infecting Guam CRB with OrNV, it seems highly likely that a combination of the factors mentioned has contributed to the difficulty in establishing OrNV infection in Guam CRB.

Methods

Bioassay methods for challenging Guam Coconut Rhinoceros Beetle with *Oryctes* Nudivirus

Numerous methods for inoculating CRB have been used over the years (Bedford, 1980; Bedford, 1981; Bedford, 2013; Jackson, 2009; Zelazny, 1978; Zelazny et al, 1987). Protocols for the bioassay methods used within these experiments are described below. Furthermore, a few additional methods have also been described that may prove useful for inoculating Guam CRB with *Oryctes* nudivirus.

Food Incorporation - Adult banana feeding

The typical route to establish *Oryctes* nudvirus infection is via oral transmission, with food incorporation being the simplest dosing method for lab inoculation (Jackson, 2009). However, it is important that the entire OrNV dosed food piece is consumed within a short period of time to prevent inactivation of the virus inoculum (and to ensure a full dose has been consumed).

To set up the bioassay, weigh individual CRB and randomly assort the adults into either control (i.e. no OrNV is added to buffer) versus experimental (i.e. OrNV is included with buffer) treatment groups. If a variety of adult sizes are available ensure that the developmental range between the control and treatment groups is matched (e.g. if 10 large and 10 small adults are available, each treatment group should have similar numbers of small and large adults).

Slice a thin piece of banana (~3-4 mm thick, use 1 per CRB), cut the slice in half and let this air dry for ~5-10 min. To each half of the banana add 50 µl of the treatment dose (control or virus) to each half of the banana slice and air dry briefly until liquid has mostly absorbed into the banana. Make a 'sandwich' from banana halves and add to the empty container with CRB. Lay the container on its side (to provide room for the adult to move and eat banana) and leave for 1-2 days (record amount of banana eaten) before adding peat material. If necessary (e.g. original banana hardly eaten, or

wanting to apply a larger dose), re-feed with freshly treated banana (virus titre likely drops significantly after ~2 days). The advantage of routinely re-feeding is that it improves the chances of a larger dose being administered.

After all (or the majority) of the banana has been consumed, add peat material up to ~3-4 horizontal body heights of the container (~100 ml using the 400 ml mason jars). This waiting period will allow observation of feeding and prevent virus from being absorbed into the surrounding peat medium. Continue to observe larva ~2-3x per week and record the 'health' of individual beetles at each observation (e.g. normal activity, slow moving/lethargic, slight twitching, etc.).

Food Incorporation – Adult Droplet Feeding

Droplet feeding is another commonly used method for oral delivery of virus to insects (Jackson, 2009; Lacey, 1997) and has been used to inoculate CRB in other regions (e.g. Fiji, Maldives).

To set up the bioassay, weigh individual CRB and randomly assort the adults into either control (i.e. no OrNV added to buffer) versus experimental (i.e. OrNV included in buffer) treatment groups. If a variety of adult sizes are available ensure that the developmental range between the control and treatment groups is matched (e.g. if 10 large and 10 small adults are available, each treatment group should have similar numbers of small and large adults). One to two days prior to setting up the droplet feeding bioassay, remove CRB from the rearing material to allow them to dry out slightly. This will encourage the insects to drink the entire droplet that they are being treated with.

On the treatment day, set up a piece of plastic sheeting for each treatment (large enough to accommodate all beetles for that treatment) and secure each sheet to the lab bench with tape. Prepare enough solution to allow dosing of all beetles for the specific treatment type. The droplet feeding solution is prepared so that the solution that has a final sucrose concentration of 10%. This is most easily achieved by adding a freshly made (or sterile) 50% sucrose stock solution (e.g. dissolve 5 g white table sugar in water with a final volume of 10 ml) to the treatment solution to create a final sucrose concentration of 10% (e.g. addition of 40 μl of a 50% sucrose to 200 μl of virus will allow 10 beetles to be treated with 20-24 μl of this solution). Using strongly sticking tape (e.g. double sided or manual looping of tape), attach tape to plastic sheeting so that adult CRB will be spaced well apart from each other (i.e. unable to touch plus room to manipulate a pipette). Stick the backs of individual adults onto the tape so belly faces up, and check that the insects cannot easily free themselves from the tape. Apply a 20-30 μl droplet of the OrNV-10% sucrose solution to the mouth parts of the adult CRB. Surface tension generally maintains the droplet as a small ball over the mouth parts. This setup allows visual observation to determine when the entire droplet has been consumed. If the droplet falls away from the mouth, try placing it back over the mouth. If this still does not work, either dry the region and try again, or record that a full dose was not given to that individual. Once the droplet has been consumed, place the treated CRB into a container with a slice of banana and leave for 1-2 days (record amount of banana eaten). Note that after administration of the first dose it may be desirable to re-dose with a second droplet later to ensure sufficient virus has been applied. Re-dosing is generally carried out 1-2 days after the initial dose is given. Addition of the peat rearing material to the jar can be done once all dosing has been completed. Continue to observe larva ~2-3x per week and record the 'health' of individual beetles at each observation (e.g. normal activity, slow moving/lethargic, slight twitching, etc.).

Direct Hemocoelic Injection Methods - Adult hemocoel injection

Previous reports (Zelazny, 1978) have indicated that adult CRB can be infected via direct injection of OrNV into the hemocoel. Dosing in this manner may be advantageous if the adults are not feeding enough to ensure administration of a full dose. However a surface sterilized insect and a pure inoculum (i.e. free from all other organisms except the virus) are absolutely required to prevent death by septicaemia via accidental introduction of other microbes into the hemocoel (Lacey, 2012). A sterile virus inoculum can be obtained from cell culture produced virus or other sterile virus isolation methods (e.g. filtration through a 0.2 µm filter, though be aware that, while rare, some microbes are able to pass through this sized filter).

Set up the bioassay by randomly assorting larva into control (buffer only injection) versus experimental (virus isolate injection) treatment groups. If a variety of adult sizes are available ensure that the developmental range between the control and treatment groups is matched (e.g. if 10 large and 10 small adults are available, each treatment group should have similar numbers of small and large adults).

Prior to injection, surface sterilize the leg joint areas on the underside of the adult by squirting liberally with a 5% bleach solution and laying the adult on its back for 2-3 min, rinse with sterile water, apply 70% ethanol (let soak 2-3 min), rinse site with sterile water, and proceed with injection. Note that if the insect is reasonably clean the bleach treatment may not be necessary. If the adult is especially dirty, rinse off well with tap water prior to the sterilization treatment.

For the injection, prepare a sterile 1 ml syringe with a sterile 30 gauge needle. Slowly fill the syringe (avoid creating bubbles) with the desired amount of sterile virus (e.g. if injecting 5 beetles, aspirate enough solution to allow for at least 5 beetles). Remove air bubbles from syringe-needle unit by holding the unit so the needle end is up, draw back solution from end of the needle into the syringe, gently tapping syringe until the bubbles are (mostly) gone, and finally pushing the plunger to fill the end of the needle. To inject, lift the left hind leg to expose the soft tissue, insert the needle (be careful to avoid internally located gut tissue) and inject desired volume (~100-150 µl can be handled by adult CRB, 200 µl is too much), and hold for a few moments before withdrawing the needle. This is to allow the fluid to enter the body cavity to minimize the amount that can flow back out as a result of injection pressure. As CRB are quite strong, it is helpful to have one person hold the insect while a second carries out the injection. Larva can also be injected (again around the leg-body junction), but are not as robust as the adults and will often suffer a higher mortality rate as a result of the injection.

After completing the injection, place the adult into a clean (preferably sterile), empty, individually labelled container with a slice of banana and leave for 1-3 days to observe for death. Death within this time frame is likely due to injection damage and/or handling injury. Record the date/time of dosing, amount of dose administered, and any special notes (e.g. significant amount of fluid leaks back out, the needle potentially inserted too far into the body, etc.). Once it is clear that the insect has survived the injection process itself, peat material can be added to the containers (~2-3 body heights, which is ~100 ml using the 400 ml mason jars). Continue to observe all insects ~2-3x per week and record 'health' status at each observation (i.e. active, slow moving/lethargic, slight twitching, etc.).

DNA and Tissue Preservation for diagnosis of OrNV infection

Moribund (indicated by a darkening in larval colouration) and dead larva should be preserved for future analysis. It is preferable to preserve adults and larva either prior to death or within a few hours after death (to avoid excessive decay). For preservation, dissect out the gut tissue as per instructions in the manual "Collection of *Oryctes rhinoceros* tissue for analysis". Briefly:

- For DNA analysis, place a 0.5-1 cm piece of the midgut into propylene glycol (PPG) for DNA preservation (95-100% ethanol will also work), and store at -20°C until ready to ship (or extract DNA).
- For histological analysis, place ~1-2 cm of the midgut into FAA fixative (final concentrations of 5% formaldehyde/2.5% acetic acid/50% ethanol made up as an aqueous solution) for histological analysis; note that 10% formalin, also known as 4% paraformaldehyde solution, will also work though we've found it doesn't preserve insect tissue quite as well as FAA). Store FAA fixed tissue at room temperature (~20-25°C) until a package is ready for shipping (or preparation for histological analysis).
- It is important to use enough PPG or FAA to completely cover/submerge the specimen (a 10x volume is generally recommended for initial fixation), to allow quick preservation of the tissue.
- Place the remaining gut and/or cadaver into an appropriate container and store at -80°C (-20°C will be ok for a few months) until virus extraction is required. The cadaver may be discarded if the DNA and histology analyses confirm the specimen is not infected with OrNV infection; however, if virus is present, this collection process ensures viable virus can be recovered from infected cadavers.

Bioassay methods requiring validation prior to use in a Guam CRB control programme

Direct Hemocoelic Injection Methods - Larva hemocoel injection

Previous reports have indicated that larva are susceptible to OrNV and that replication can occur outside of the gut (Huger, 2005; Zelazny, 1972). Although direct hemocoelic injection of Guam CRB larva was tested, the results were inconclusive due to the high mortality rates (likely as a result of damage due to the injection process) observed within the control treatments. As successful use of OrNV within a control programme will rely on transmission between adults, the larval hemocoelic injection method was not investigated further. However, the larval stage may be advantageous to use for bulk production of cell culture produced virus and also for testing susceptibility of CRB populations against various OrNV isolates. Note that a surface sterilized insect and a pure inoculum (i.e. free from all other organisms except the virus) are absolutely required to prevent death by septicaemia via accidental introduction of other microbes into the hemocoel (Lacey, 2012). A sterile virus inoculum can be obtained from cell culture produced virus or other sterile virus isolation methods (e.g. filtration through a 0.2 µm filter, though be aware that, while rare, some microbes are able to pass through this sized filter).

Set up the bioassay by randomly assorting larva into control (i.e. buffer only) and experimental (i.e. virus isolate with buffer) treatment groups. If a variety of developmental stages are available ensure that the developmental range between the control and treatment groups is matched (e.g. if 10 first and 10 third instars are available, each treatment group should have similar numbers of first and third instars).

Prior to injection, surface sterilize the grub by immersing a 5% bleach solution for 2-3 min, rinse with sterile water, apply 70% ethanol (let soak 1-2 min), rinse site with sterile water, and proceed with injection. Note that if the insect is reasonably clean the bleach treatment may not be necessary. If the larva is especially dirty, rinse off well with tap water prior to the sterilization treatment.

For the injection, prepare a sterile 1 ml syringe with a sterile 30 gauge needle. Slowly fill the syringe (avoid creating bubbles) with the desired amount of sterile virus (e.g. if injecting 5 lava, aspirate enough solution to allow for at least 5 larva). Remove air bubbles from syringe-needle unit by holding the unit so the needle end is up, draw back solution from end of the needle into the syringe, gently tapping syringe until the bubbles are (mostly) gone, and finally pushing the plunger to fill the end of the needle. To inject, hold larva just firmly enough so it wiggle too much, insert the needle into leg-body junction point with needle travelling parallel to the gut (be careful to avoid internally located gut tissue!), inject the desired volume and hold for a few moments before withdrawing the needle. This is to allow the fluid to enter the body cavity to minimize the amount that can flow back out as a result of injection pressure. Volumes injected need to be appropriate for the larval size; we found that approximately 20-40 µl can be injected into 1st instar, 100 µl for 2nd instar, and up to 200 µl for 3rd instar. Larvae are not as robust as the adults and will often suffer a higher mortality rate as a result of physical handling and the injection process.

After injection, wait 2-3 days before adding some peat/manure material to allow wound site to heal over and to enable identification of injection damaged larva. Continue to observe larva ~2-3x per week and record 'health' of beetle at each observation (i.e. active, slow moving/lethargic, slight twitching, etc.).

Food Incorporation - Larva feeding options

To improve oral dosing of larva via feeding, it is necessary to test if larva will eat food other than fresh or rehydrated manure within at least a 24 h period. If it is possible to identify a food source that the CRB grubs will readily feed on, then feeding may be a more convenient dosing method. Such oral dosing allows control over the dose provided (i.e. can visualize amount of bait eaten), and introduces the virus to the targeted gut tissue. This method will require testing to determine if it will be a useful method for the Guam CRB control programme.

Set up a feeding test using a variety of easily obtainable food sources (e.g. carrot, parsnip, apple slice, sterilized manure). Select 1st, 2nd and 3rd instar larvae to determine which stage is the 'best' feeder and amounts they are able to readily consume. Observe rate of consumption. It may be

necessary to remove larva from 'peat' medium a few days prior to setting up the assay. This will help to dry them out and perhaps make them willing to consume the offered food. A pre-feeding step, whereby a small piece of food is offered, will identify larva that will readily feed.

If a food source that is consistently eaten quickly cannot be found, a useful alternative method may be to directly apply the dose via oral injection using a blunted 30 gauge needle (Lacey, 2012). Another alternative oral dosing method is the submersion method (Bedford, 1980; Zelazny, 1978), and while not used in this project it has been utilized in previous CRB control programmes.

OrNV transmission from infected (lab or imported) Guam CRB to uninfected Guam CRB

This is an alternative dosing method that is based on previous studies (Zelazny, 1976) and anecdotal observations in regions where OrNV is present in the CRB population (e.g. Fiji, Samoa, Malaysia; if traps are not cleared within a day of catching most of the individuals caught in the trap develop OrNV infection). Therefore, by mixing Guam CRB with CRB populations collected from regions known to have high OrNV (or with lab infected CRB), an improvement on OrNV transmission into the virus free Guam CRB population may be possible. This method will require testing to determine if it will be a useful method for the Guam CRB control programme.

After a shipment of foreign adult CRB arrives into Guam, collect any dead CRB, and (if possible) remove a small piece of gut (~0.5 cm) for DNA analysis, and another piece for histological analysis. Store the rest of the cadavers at -80°C (or -20°C) as a source of inoculum to inoculate a new Guam cohort. With the remaining live 'foreign' CRB, let the insects re-adjust from their travel for a few days prior to setting up the bioassay. The live imported CRB adults can be pooled into 1 or more containers with the addition of a minimal amount of their peat medium (i.e. it should be able to easy to see the insect). Be sure to keep these imported beetles in well labelled containers to distinguish them from the Guam beetles. It is worth noting that the frass is likely to contain infectious amounts of virus (Zelazny, 1976), therefore, the frass and bedding material from OrNV infected beetles may provide a viable source of virus inoculum (save and place into a 'population mingling box'). However, maintaining 'foreign' CRB as individuals is preferable if other types of experiments are to be carried out (e.g. comparative bioassay) to prevent contamination should OrNV or other infectious diseases be present.

To set up the transmission bioassay, label the Guam CRB elytra (e.g. diamond pen, laser etcher). Place the labelled Guam adults into a box with a minimal amount of slightly moistened coconut wood chips or sawdust (it should be easy see the container bottom). To this same box add the foreign beetles (i.e. likely to be virus infected); no need to label foreign CRB since the Guam beetles are labelled and it avoids causing any extra handling stress to the imported CRB. Any fresh frass or bedding material collected from these putatively infected 'foreign' beetles could be added to the box as well. Record observations every 2-3 days to determine which beetles die first.

It will be important to diagnose the cause of death to determine if mortality was due to OrNV infection. It would also be advisable to keep the cadavers as a potential source of inoculum. This will require dissection to observe gut appearance, and use of an appropriate fixative for preserving DNA (propylene glycol) for PCR and tissue (FAA or 10% formalin) for histology.

PCR detection for presence of OrNV

DNA was extracted from CRB tissue using a Genomic DNA Isolate kit (Bioline). The primers used to amplify a 945 base pair (bp) fragment of the OrNV genome were OrNV15a (5'-ATTACGTCGTAGAGGCAATC-3') and OrNV15b (5'-ATGATCGATTCGTCTATGG-3') (Richards et al, 1999). Each 25 µl PCR reaction constituted 0.25 µl i-StarTaq DNA Polymerase (iNtRON Biotechnology), 2.5 µl 10x PCR buffer (iNtRON Biotechnology), 0.5 µl dNTP mixture (10 mM), 0.5 µl OrNV15a (10 µM), 0.5 µl OrNV15b (10 µM), 2 µl diluted (1 in 100) DNA template, and 18.75 µl water. PCR amplifications were performed in a C2100 (BioRad) thermocycler with a cycling profile of 35 cycles of 94°C denaturation (30 s), 50°C annealing (45 s), 72°C extension (1 min) with an initial

denaturation of 3 min at 94°C and a final extension of 5 min at 72°C. An 8 μl aliquot of each PCR reaction was separated by agarose gel electrophoresis (1%, 0.5xTBE), stained with RedSafe (iNtRON Biotechnology) and fluorescence visualized over UV light. Photographs were recorded using an UVIdoc HD2 gel doc (UVItech).

Histological detection of OrNV infection
Preparation of sectioned tissue slides for histological analysis within this project used standard protocols for embedding, sectioning and staining CRB specimens were used. These were carried out by Gribbles Veterinary Christchurch, New Zealand (christchurch.vetlab@gribbles.co.nz).

A great deal of specialist equipment and expert training are required to successfully embed, section, and stain tissue specimens for histological analysis. Therefore, it will likely be most efficient for the CRB control programme to contact a local medical or veterinarian service lab in Guam to see if they are willing to carry out these services on CRB specimens.

CRB rearing
CRB rearing was carried out using a standard protocol as described in the manual "CRB Rearing" (see http://guaminsects.net/anr/content/coconut-rhinoceros-beetle-eradication-project-technical-reports).

Supplementary Data

Distinguishing Guam CRB Populations From Other CRB Populations

The research described below was not specifically part of the research activities funded for this project; however, these results are included here because of their valuable contribution to the Guam CRB eradication efforts.

Based on the hypothesis that DNA barcoding genes may be useful in determining the source of the Guam CRB population and the source of other CRB incursions, a small pilot experiment was set up to see if putative genetic differences in CRB *cytochrome oxidase I* (*COI*) gene could distinguish between populations of CRB from various regions within the Pacific, including Guam. Sequence results from this pilot investigation confirmed that CRB collected from Guam is the same species as (i.e. *Oryctes rhinoceros*) as is present in Fiji, PNG, and Samoa. Further analysis of *COI* sequences revealed a specific genetic difference that correlated only with the Guam insects. Using these *COI* differences, a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay was developed to allow the Guam genotype to be distinguished from other CRB genotypes.

Due to the unexpected difficulties in establishing OrNV infection in the Guam CRB population, it was further hypothesized that the correlated genetic difference may be a useful marker for apparent virus resistance in the Guam population. Analysis of CRB specimens from several additional locations is now being conducted, and to date also includes Malaysia, Diego Garcia, and the newly discovered Hawaiian invasion. Table S1 summarizes results of the current efforts to detect the Guam-like genotype from various regions. Figure S1 provides a representative example of PCR-RFLP results observed. The PCR-RFLP results from the current data set has revealed that only Hawaii shares the Guam PCR-RFLP pattern. This suggests that the Hawaiian invasion has either come from the same source as the CRB that originally invaded, or that CRB was accidently transferred from Guam into Hawaii.

Table S1: PCR-RFLP differentiation of the Guam-like *Oryctes rhinoceros* genotype from genotypes identified in specimens collected from other nations.

Specimen Location	n =	Matches to the PCR-RFLP pattern for Guam
Guam	11	11

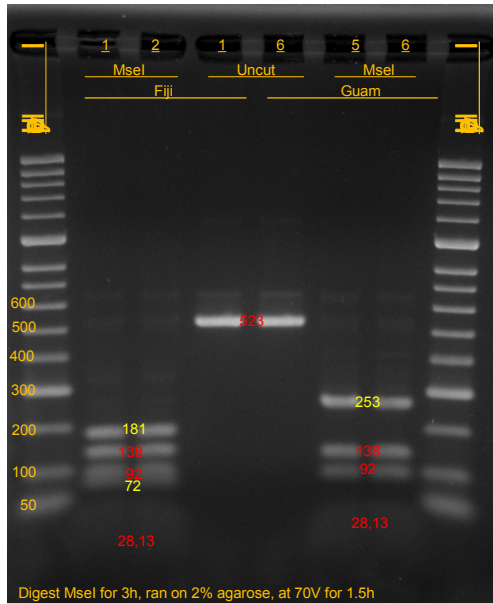| Hawaii	4	4
Fiji	14	0
Samoa	7	0
Papua New Guinea	92	0
Malaysia	47	0
Diego Garcia	2	0

Figure S1: Representative results from a PCR-RFLP analysis demonstrating the difference between the Guam-like CRB genotype from the Fijian CRB genotype, which is same as the commonly observed CRB genotype. Following PCR amplification of a 523 base pair (bp) fragment of the *COI* gene from *O. rhinoceros*, the *COI* amplicon was digested with the restriction enzyme MseI for 3 hours with remaining fragments separated on a 2% agarose. Numbers on figure next to the DNA bands indicate fragment size in base pairs. The DNA banding pattern is as follows: the two end lanes are a DNA size ladder, lanes 1, 2, 5, and 6 are MseI-digested DNA from PCR amplicons, and lanes 3 and 4 represent undigested DNA from the PCR amplicon.

Method

PCR-RFLP method for detecting the Guam-like CRB genotype

DNA was extracted from CRB tissue using a Genomic DNA Isolate kit (Bioline). The primers used to amplify a 523 base pair (bp) fragment of the CRB *COI* gene were C1-J-1718Oryctes (5'-GGAGGTTTCGGAAATTGACTTGTTCC-3') and C1-N-2191Oryctes (5'-CCAGGTAGAATTAAAATRTATACCTC-3') (Marshall, publication in progress). Each 25 µl PCR reaction constituted 0.125 µl i-StarTaq DNA Polymerase (iNtRON Biotechnology), 2.5 µl 10x PCR buffer (iNtRON Biotechnology), 0.5 µl dNTP mixture (10 mM), 0.5 µl C1-J-1718Oryctes (10 µM), 0.5 µl C1-N-2191Oryctes (10 µM), 2 µl diluted (1 in 50) DNA template, and 18.75 µl water. PCR amplifications were performed in a C2100 (BioRad) thermocycler with a cycling profile of 35 cycles of 94°C denaturation (30 s), 50°C annealing (45 s), 72°C extension (1 min) with an initial denaturation of 3 min at 94°C and a final extension of 5 min at 72°C. A 5 µl aliquot of each PCR reaction was checked by agarose gel electrophoresis (1%, 0.5xTBE), stained with RedSafe (iNtRON Biotechnology) and fluorescence visualized over UV light. Photographs were recorded using an UVIdoc HD2 gel doc (UVItech). Successfully amplified *COI* PCR products (8 µl) were each combined with 0.2 µl Mse1 (10U/µl; New England BioLabs, NEB), 1 µl 10x NEB Buffer#4, 0.1 µl 100x NEB BSA and 10.7 µl

water, and incubated at 37°C for 3 h. Digested samples (10 μl) were mixed with DNA loading dye, loaded onto on a 2% agarose gel in 0.5xTBE buffer. The gel was electrophoresed using 60 V for 1.5 h, stained with RedSafe and DNA fluorescence detected over UV light. Photographs were taken using an UVIdoc HD2 gel doc.

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4 Protocol for Collection of Oryctes rhinoceros tissue for diagnostic analysis

Protocol for Collection of *Oryctes rhinoceros* tissue for diagnostic analysis
Version 22/04/2014
Prepared by: Sean Marshall (AgResearch, NZ)

This is an update to methods described in "Biological Control Of Rhinoceros Beetle In The Pacific Using *Oryctes* Virus Operational Protocols (Version B, April 2010)", which will be revised accordingly.

<u>**Collection of *Oryctes rhinoceros* tissue samples for diagnostic analyses**</u>

1)   Following collection of live beetles from pheromone traps or breeding sites, they should be placed into individual containers that are marked with the collection number. Storing insects individually reduces the risk of virus contamination. The containers should be transported in a cool bin (so that they do not overheat) to the laboratory where they must be stored in cool conditions until needed (e.g. for gut extractions). Beetles can be stored at 15°C for 2-3 days (or frozen and kept at -20°C for long term storage, though take care to avoid minimize thawing prior to dissection and this is only relevant for DNA extractions).

    a. Beetles collected from pheromone traps – transfer of virus between beetles readily occurs within the confines of a pheromone trap, particularly if insects are left longer than overnight. If more than a 1 beetle has been caught in a trap, please record the trap number the beetles were from and the timing between trap inspections.

2)   Record the location that individual *Oryctes rhinoceros* where collected from and include as much relevant detail as possible. For example:

    a. A unique insect identification number, the location and trap number (e.g. GPS coordinates, nearest distinguishing landmark), the date of collection, gender of the beetle, dissection date, and the gut characteristics should be recorded.

    b. If possible take photographs of the area for documentation of damage within the area
        i.   If time permits conduct a damage assessment for the area.

    Below is an example datasheet for recording information

Insect ID	Site	Collection date	gender	Dissection date	Insect and gut condition	PCR result	Histology

3)   Label each individual specimen tube using short codes that correspond with each individual beetle specimen. Use a permanent (non-smudge) marker pen for labelling.

4)   Dissect out the gut as described in Chapter 3 of the document "Biological Control Of Rhinoceros Beetle In The Pacific Using *Oryctes* Virus Operational Protocols (Version B, April 2010)" (see below for a copy of this chapter).

    a. During dissection, photograph the gut to document visual appearance. The photo can be used to cross reference with the written insect gut condition.

b. **Note**: If larvae have been collected, simply dissect out a portion of the upper thoracic region ('soft' part just behind the head, though the head itself can potentially be used as well) ...please try to avoid the hind gut region as this contains a lot of microbes)

c. **Note**: (only relevant if genotyping insect species or populations!) if gut dissection is not feasible then remove whole legs from the body (a minimum of 2 legs, with leg muscle intact) as this will suffice for genotype analysis of the insect (however it will not be possible to reliably test for present of *Oryctes* nudivirus infection)

Instructions for Gut Dissection

From: Chapter 3 of "*Biological Control Of Rhinoceros Beetle In The Pacific Using Oryctes Virus Operational Protocols (Version B, April 2010)*"

~~3~~ Dissection (Trevor Jackson)

Adult beetles must be dissected before disease diagnosis can be carried out. The method of storage of the dissected sample will depend on the diagnostic test being carried out.

Equipment for extraction of beetle gut

- Heavy and fine dissecting scissors
- Fine forceps
- Paper tissues (i.e. 'Kleenex', paper towels, or similar)
- Gloves (lab examination style)
- 0.1 M HCl* (wash bottle or spray bottle)
- 70-95% ethanol
- 2-ml tubes with O-ring
- Rubbish bag or bin for used tissues etc.

*inactivates DNA (e.g. on your equipment, bench surface), ethanol doesn't inactivate DNA.

Extraction of mid gut

Before starting, the bench surface and equipment should be wiped with 0.1 M HCl to inactivate any DNA and with 70% ethanol to remove any residues. Alternatively, if HCl and/or ethanol are not available, use a disinfectant wipe (e.g. bathroom cleaning wipe), but be sure to use a clean portion of the cloth with each wipe to minimize spread of putative contaminants.

To open the body cavity and expose the gut, the head and pronotum are removed by cutting across Line A, slightly to the posterior of the junction between the pronotum and the second thoracic segment with heavy scissors. The posterior section is then opened by cutting with fine scissors along lines B and C separating the dorsal and ventral cuticle.
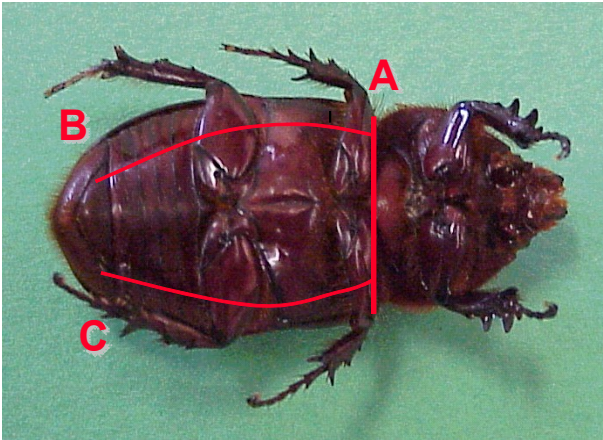
Left is ventral view; Right is dorsal view (with head, elytra, and wings removed).

Heavy dissecting scissors are used to cut off the head and pronotum along line A.
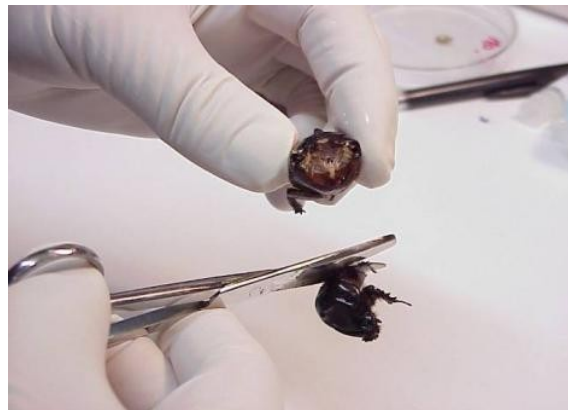
To open up the abdomen it will be necessary to carefully insert the fine dissecting scissors along the line of the alimentary tract in order to cut through the wing muscles. The abdomen can then be opened along lines B and C. As an alternative to cutting along the ventral portion of the abdomen, one can carefully cut along the dorsal portion of the abdomen (as per the B and C lines). To avoid piercing the gut, angle the scissors up and away from the body.

Fingers are used to pull apart the dorsal and ventral cuticle to expose the gut. Fine forceps can then be used to lift out the midgut. To avoid tearing the gut, carefully tease the gut from the surrounding tissue by gently pulling on the gut, releasing the pressure (before it breaks), grabbing another section with the forceps, and gently pulling on this new portion and again release the pressure; repeat these steps as required until the gut is free of the body. It may be necessary to cut the posterior attachment point where the gut meets the rectal opening.

Place gut sample into a 1.5-2mL tube containing propylene glycol (for DNA preservation), or FAA (for histology preservation)

Clean instruments and hands between each sample. Do not reuse paper tissues.
If using disinfectant wipes, use a 'clean' area for cleaning action (e.g. wipe first scissor blade with one corner, wipe second blade with another corner). Also remember to wipe off gloves as these will be contaminated with beetle tissue from the dissection.

Gender determination
        Female - Possess hair at the posterior end of the body. Horn on the head tends to be smaller than males, though this is not a reliable character. Release of eggs may be seen upon squeezing of abdomen, or presence of eggs may be observed within body cavity upon dissection.

Male - Posterior end of body is smooth (and may be shiny). Upon dissection, the male sex organs will be visible (distinctive hook shaped chitinous appendage)

Visual identification of infected midgut for isolation of OrNV from beetles

Dissection Equipment for extraction of beetle gut:
- Heavy and fine dissecting scissors
- Fine forceps
- Tissues (i.e. 'Kleenex', paper towels, etc)
- Gloves
  - M HCl* (wash bottle or spray bottle)
- 70-95% ethanol
- 1.7-2-ml tubes with O-ring
- Rubbish bag or bin for used tissues etc.

*inactivates DNA (e.g. on your equipment, bench surface), ethanol doesn't inactivate DNA

Extraction of mid gut
- Before you start wipe the bench surface and your equipment with 0.1M HCl to inactivate any DNA and with 70 % ethanol to remove any residues. Alternatively, if HCl and/or ethanol are not available, use a disinfectant wipe (e.g. bathroom cleaning wipe), but be sure to use a clean portion of the cloth with each wipe to minimize spread of putative contaminants.
- To open the abdomen and expose the gut, the head and pronotum are removed by cutting across Line A, slightly to the posterior of the junction between the pronotum and the second thoracic segment with heavy scissors. The posterior section is then opened by cutting with fine scissors along Lines B and C separating the dorsal (soft) and ventral (hard) cuticle.
  - To open up the abdomen it may be necessary to carefully insert the fine scissors along the line of the alimentary tract in order to cut through the wing muscles.
- Use fingers to pull apart the dorsal and ventral cuticle. The gut is now exposed. Use fine forceps pull out the midgut.
- Place gut sample in the tube containing propylene glycol (for DNA preservation)
- Clean instruments between each sample. Do not reuse tissues. However, if using disinfectant wipes, use a 'clean' area for cleaning action (e.g. wipe first scissor blade with one corner, wipe second blade with another corner). Also remember to wipe off gloves as these will be contaminated with beetle tissue from the dissection.
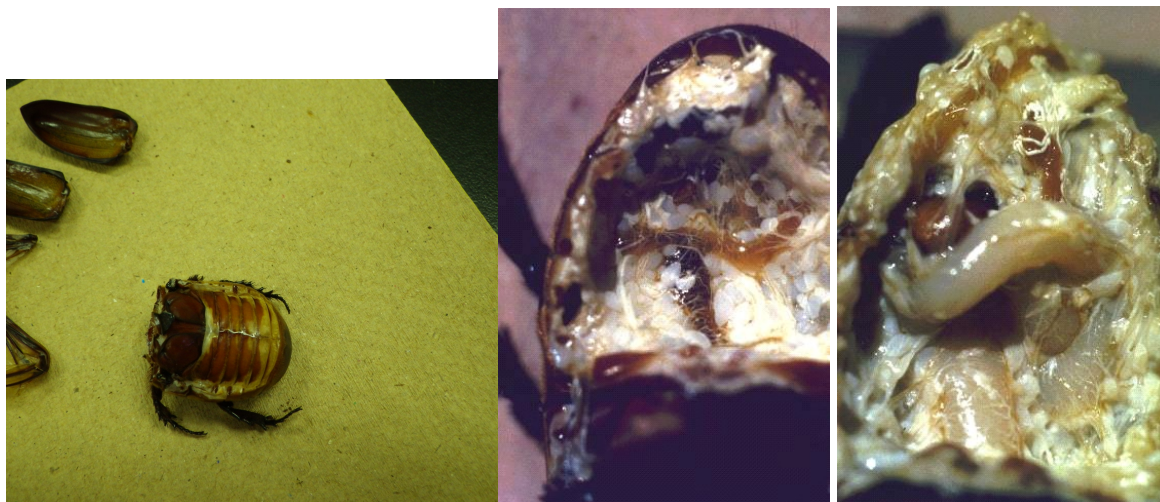

Figure 3: Dorsal view of *Oryctes rhinoceros* with elytra and wings removed and lines showing points to cut to expose gut material (left panel). Partially dissected abdomen of *O. rhinoceros*, displaying commonly observed healthy versus disease phenotypes (middle and right panels, respectively). The key aspects are: dark vs milky, thin vs swollen, empty vs full of organic matter.

Storage of gut tissue for diagnostics

The method of storage of the gut sample will depend on the diagnostic method being used (i.e. DNA vs histology). The midgut can be divided into two pieces (or more if required) to provide samples for both types of analysis.

1) For **DNA/PCR** analysis, the tissue sample should be immediately stored in 0.5-1ml of propylene glycol (enough to full immerse the tissue), gently mix to ensure tissue is fully submerged (if required; gentle mixing helps remove to remove air bubbles). Samples for DNA analysis are best stored at -20°C (preserved for several months to years). However, if a freezer is not available, storage at 4°C for 4 weeks or room temperature (up to 24°C) for 1 week is possible (note that some DNA degradation will occur). The propylene glycol solution has been pre-aliquoted into tubes within the collection kit

    a.   The advantage of using propylene glycol as a DNA preservative is that samples are considered to be preserved and are also non-toxic and so may be transported differently than for ethanol (see Transport of Samples, below).

        i. If propylene glycol is not available, ethanol can be used to preserve tissue for DNA analysis. However, 95-100% ethanol should be used (70% is fine for a few days…not weeks), and airlines generally have strict policies on carrying ethanol (i.e. limited quantities and requires special packaging)

2) For **histology**, also see *Preservation of Tissue for Histological analysis* under the Appendices Section. Briefly, the gut sample should be immediately stored in FAA (5% formaldehyde, 2.5% glacial acetic acid, 50% absolute (99-100%) ethanol, 42.5% water), gently mix to ensure tissue is fully submerged (if required; gentle mixing helps remove air bubbles). If FAA is not available, 10% neutral buffered formalin (10% NBF = 4% formaldehyde solution) can be used. Leave samples in fixative for a minimum of 24 hours. Samples may be left in fixative for several days to weeks prior to downstream processing.

    a. The fixation solution can be pipetted (e.g. 1 ml aliquots into 1.7 ml specimen tubes) before dissections begin if a large number of samples are to be processed.

    b. Both FAA and 10% NBF contain hazardous chemicals (see below). Please use appropriate protection when handling.

    c. The advantages of FAA fixative are:

        i. Tissues are fixed with minimal cell disruption and samples can be further processed for light or electron microscopy if required.

       ii. Tissues can be stored in the fixative at room temperature without the samples becoming brittle or the need to change fixative (as is required for 10% NBF).

Shipment of Tissue Samples for Analysis

Sending the packages containing **only** propylene glycol as a preservative (for DNA/PCR analysis):

    1) Place tissue samples (immersed in propylene glycol in a 1.7-ml screw- top tube with an O-ring seal) into a sealable plastic container and line with absorbent material (e.g. cellulose packing, thick paper towel or cotton wool) to soak up any spillage that may occur during transportation;

        a. If a sturdy plastic container is not available, a sealable sturdy plastic bag may be used.

2) Place the sturdy container inside a plastic bag and seal.
   a. If sturdy plastic container is not available, use a second a sealable sturdy plastic bag may be used.

3) Place the double sealed contents into a cardboard box (or other sturdy/crush proof material).
   a. If a box is not possible use a padded courier bag or envelope, or bubble wrap the samples.

4) A description of contents must be prominently attached to the outside of the final shipping packaging (see appendices for example template). For example, if the specimens are packed into a box that is then placed inside a courier bag, the description of contents must be prominently attached to the outside of the courier bag so that it can be easily seen by the biosecurity inspectors at the NZ border. Mark the outside of the package as:

   > For scientific research.
   > Dead, preserved tissue specimens from *Oryctes rhinoceros* (coconut rhinoceros beetle).
   > Preservative used is mono-propylene glycol (PPG; non-restricted chemical).
   > Package contains *[insert # of vials]* vials containing preserved/non-viable specimens, and therefore are therefore considered to be non-regulated under the provisions of the Dangerous Goods Regulations.

5) Send package to the following address (see appendices for example template):

   > Sean Marshall
   > AgResearch Limited (Lincoln Research Centre)
   > cnr Springs Road and Gerald Street, Lincoln
   > Private Bag 4749
   > Christchurch 8140
   > New Zealand
   > T +64 3 321 8800   T (DD) +64 3 325 9935

6) An 'export invoice' or 'pro forma' invoice is often required to be filled in for international courier packages (see appendices for example template). This generally involves a separate piece of paper attached to the outside of the package that contain the receivers address and contact details, a description of goods (as per step 4 above), cost of goods, and the senders contact information. For research specimens the cost of goods please fill out a value <NZ$10. The specimens themselves have no commercial/market value, so the 'cost of goods' is just the packaging.
   a. Please contact your courier service for advice on whether an export invoice is required.
   b. PPG is **not** classified as a hazardous substance under the Material Safety Data Sheet, and preserved/non-viable biological substances are considered to be non-regulated under the provisions of the Dangerous Goods Regulations.

7) Please note that while an import permit is not required for shipping **preserved** specimens into NZ (*all other specimens require an import permit*), the description of contents must be

visible on the outside of the final packaging and must match the actual contents of the package. If the inspector counts 30 vials and the description says there are 20 vials or 35 vials, the package may be destroyed and/or legal action taken.

> ***NB*** *all non-preserved specimens require an import permit. For NZ biosecurity staff, preserved means all organisms associated with the specimens being sent are non-viable (e.g. dissected gut tissue will be non-viable, however it will contain associated microbes, and these microbes must also be dead!).*

> **a.** Further information can be found in the document "Import Health Standard for the Importation into New Zealand of Nonviable Animal Specimens from All Countries" (see http://www.biosecurity.govt.nz/files/ihs/inespeic.all.pdf).

Sending the packages containing ethanol, FAA, and/or 10%NBF as preservatives:
The International Air Transport Association (IATA) Dangerous Goods Regulations permits the transport of small volumes of flammable and corrosive liquids in approved containers (LabPak-1). See web site: www.iata.org. The LabPak-1 shipping containers protect against potential leakage of the preserving chemicals from the sample tubes. They can be obtained from Thermofisher Scientific NZ Ltd (http://www.thermofisher.co.nz).

1) Wrap the sample tubes containing the tissue samples in absorbent material (e.g. cellulose packing, thick paper towel or cotton wool) and place inside a sealable plastic bag before placing inside shipping container. Bubble wrap or absorbent material can be used to stop movement of sample package inside the container.

2) A warning label 'Dangerous Goods in Excepted Quantities' must be displayed on the outside of the package (example of label shown below).). For example, if the specimens are packed into a box that is then placed inside a courier bag, the description of contents must be prominently attached to the outside of the courier bag so that it can be easily seen by the biosecurity inspectors at the NZ border. Complete the label with appropriate details as follows and glue on one side of the container that shows "infectious substance" biohazard label. When sending:
    a. For ethanol samples you only need to tick class 3 and write UN1170 and the proper shipping name is ethanol.
    b. For FAA samples you need to tick class 3 write UN1170 and UN1198 with the proper shipping names being ethanol and <10%formaldehyde solution (respectively).
    c. For 10% NBF samples (i.e. if FAA is not used as the histology fixative) you need only tick class 3 and write UN1198 and the proper shipping name is <10%formaldehyde solution.

3) A description of contents must be attached to the outside of the shipment. For LabPak-1 boxes this can be glued on the "documentation" panel. However, if the labpak-1 box is then placed inside a courier bag, the description of contents (and the dangerous goods label) must be prominently attached to the outside of the courier bag so that it can be easily seen by the biosecurity inspectors at the NZ border. Mark the outside of the package as:

For scientific research.

Dead, preserved tissue specimens from *Oryctes rhinoceros* (coconut rhinoceros beetle).

Preservative used is *[insert all that apply: ethanol, 4% formaldehyde, or FAA (50% ethanol / 5% formaldehyde / 2.5% acetic acid)]* with not more than 1ml of solution per vial.

Package contains *[insert # of vials]* vials containing specimens.

This volume qualifies as very small amounts of dangerous goods qualify and are therefore considered to be non-regulated under the "de minimis" provisions of the Dangerous Goods Regulations.

4) Send package to the following address:

Sean Marshall
AgResearch Limited (Lincoln Research Centre)
cnr Springs Road and Gerald Street, Lincoln
Private Bag 4749
Christchurch 8140
New Zealand
T +64 3 321 8800   T (DD) +64 3 325 9935

5) An 'export invoice' or 'pro forma' invoice is often required to be included for international courier packages. This generally involves a separate piece of paper attached to the outside of the package that contain the receivers address and contact details, a description of goods (as per step 4 above), and cost of goods. For research specimens the cost of goods please fill out a value <NZ$10. The specimens themselves have not market value, so the 'cost of goods' is just the packaging.

   b. Please contact your courier service for advice on whether an export invoice is required.

6) Please note that while an import permit is not required for shipping **preserved** specimens into NZ (*all other specimens require an import permit*), the description of contents must be visible on the outside of the final packaging and must match the actual contents of the package. If the inspector counts 30 vials and the description says there are 20 vials or 35 vials, the package may be destroyed and/or legal action taken.

   NB all non-preserved specimens require an import permit. For NZ biosecurity staff, preserved means all organisms associated with the specimens being sent are non-viable (e.g. dissected gut tissue will be non-viable, however it will contain associated microbes, and these microbes must also be dead!).

   a. Further information can be found in the document "Import Health Standard for the Importation into New Zealand of Nonviable Animal Specimens from All Countries" (see http://www.biosecurity.govt.nz/files/ihs/inespeic.all.pdf).

7) As long as each ethanol or FAA preserved specimen is packaged: 1) within individual vials, 2) with each vial contains not more than 1ml of solution per vial, and 3) the single shipped package contains no more than or 100 ml of fixative solution, then this volume qualifies

under the very small amounts of dangerous goods quantity and is therefore considered to be non-regulated under the "de minimis" provisions of the Dangerous Goods Regulations.

Appendices:

Preservation of Tissue for Histological analysis

Safety Information for Fixation Chemicals
By nature FAA and 10% NBF solutions contain harmful chemicals and should be handled with special precautions.

Formaldehyde is a dangerous chemical that is found in both the FAA and 10% NBF fixatives. Formaldehyde can be purchased as a liquid or in solid form. More information can be found at: http://swehsc.pharmacy.arizona.edu/exppath/resources/formaldehyde.php.

Liquid forms of formaldehyde should be stored tightly sealed; since exposure to air encourages the oxidation of formaldehyde to formic acid (37% formaldehyde is usually shipped with 10-15% methanol to inhibit this change). Formaldehyde should be stored at room temperature (cold temperatures encourage the formation of trioxymethylene with a resulting white precipitate). The shelf life of an unopened 37% formaldehyde stock solution is ca 1 year if stored in the dark at 20-25°C. Once opened, the solution should be used within 3-4 months. Therefore if possible purchase in small volumes. Solutions containing ca 4% formaldehyde should be used within 1-2 months, if not sooner. Suggested sources of formaldehyde include Sigma-Aldrich: #533998-500ML Formaldehyde histological grade, 37 wt. % in H_2O or Fluka: #47629 Formaldehyde solution.

An alternative to formaldehyde solutions is crystalline paraformaldehyde, which can be used to make up the required solutions, but it is hazardous to workers if recommended safety procedures are not followed. If you are not familiar with making solutions from the crystalline form, it is recommended that a premade formaldehyde solution is purchased.

Acetic acid is a component of the FAA fixative. Stock solutions of acetic acid (e.g. glacial or solutions >10%) are strong acids and should be handled with care. It is often purchased as glacial (meaning concentrated; e.g. Scharlau: #AC0352). Diluted acetic acid solutions can also be used as long as the final concentration in FAA is 2.5%.

Preparation of FAA Fixative

Prepare FAA in small volumes as needed (see Table below). Store at room temperature (20-25°C) and use within ca 2 months. The formaldehyde should be gently shaken before use to avoid a concentration gradient in the bottle.

Table: Directions for making 200 ml of FAA fixative (scale volume as required).

Stock Solution	Volume	Final Concentration
37% formaldehyde solution	25 ml	5%
Glacial acetic acid	5 ml	2.5%
95% ethanol	100 ml	50%
distilled water	70 ml	42.535%

Preparation of 10% NBF Fixative

An alternative fixative to FAA is 10% neutral buffered formalin (10% NBF; 10% formalin is an old name for a 4% formaldehyde solution). It may be easier and safer to buy premade 10% neutral buffered formalin. Use within printed expiry date if purchased premade or 3 months if self-made. Prepare self-made fixative in small volumes as needed (see Table below). Store 10% NBF at room temperature (20-25°C).

Table: Directions for making 100 ml of 10% NBF (scale volume as required).

Solution	Volume/weight
37-40% formaldehyde	10 ml
Sodium phosphate (monobasic) NaH_2PO_4 x $1H_2O$	0.4 g
Sodium phosphate dibasic (anhydrous) Na_2HPO_4	0.65 g
Distilled water up to 1000 ml	ca 90 ml

Example Address and Description of Content label templates
(NB Revise wording as necessary, print, cut out, and attach to package.)

Template for shipping address and for Description of Contents:

Example of an Export Invoice

DESCRIPTION OF CONTENTS

| Sender:
Name
Company
Address

Phone:
Fax:
E-mail:

Consignees:
Name:
Company:
Address:

Phone:
Fax:
E-mail: | **Date:**

Comments:
Package contains:

For example:
For scientific research.
Products are intended for research purposes in Consignees' laboratory only.

Dead, preserved/non-viable tissue specimens from *Oryctes rhinoceros* (coconut rhinoceros beetle).
Preservative used is mono-propylene glycol (PPG; non-restricted chemical). |

TYPE OF PACKAGING	DETAILED DESCRIPTION OF CONTENTS	QUANTITY	COMMERCIAL VALUE*
Cardboard outer and Styrofoam inner	*Example…* *Dead, preserved tissue specimens from Oryctes rhinoceros (coconut rhinoceros beetle)* *Preservative used is* mono-propylene glycol (PPG; non-restricted chemical). *Specimens contained in sealable vials labelled as Fiji#1- 1 to 20.* Preserved specimens are non-viable biological substances and are therefore considered to be non-regulated under the provisions of the Dangerous Goods Regulations.	. *30 vials containing specimens*	*NZ$10*

I hereby confirm that the information contained in this statement is true and that the contents of this shipment are as stated above.

Signature: _____

Title: _____   **Date:** _____

5 Hawaii Beetle Dissections

Research in Support of the Guam Coconut Rhinoceros Beetle Eradication Project

Hawaii Beetle Dissections

Prepared by
Aubrey Moore
University of Guam Cooperative Extension Service
and
Sean Marshall
AgResearch, New Zealand

January 17, 2014*

Four beetles from Hawaii were sexed, measured and dissected to look for eggs. Sample 1 was an interception in the arrivals baggage area of the Honolulu International Airport. The remainging three specimens were from pheromone trap CRB4 deployed on a golf course at the Hickam Air Force Base. All were female; two were gravid but we did not find embryos.

1 Notes

20140114.001 (interception # APWJI????; International airport on 2013/??/?? baggage carousel)

Female
  Body length: 49 mm (L)
  Elytra dimensions: 27 x 21 mm (LxW)

Dissection: many eggs easily observed, no obvious embryo within the 1 egg that was dissected (1, 2, 3).

20140114.002 (interception # APWHI133641003001; trap#CRB4 on 2013/12/30 near airforce base)

Female
   Body length: 44mm (L)
   Elytra dimensions: 25 x 19 mm (LxW)
   Dissection: initial tissue appeared to be undergoing initial putrification, no obvious eggs seen (4).

20140114.003 (interception # APWHI140021003001; trap#CRB4 on 2014/01/02 near airforce base)

Female
   Body length: 44mm (L)
   Elytra dimensions: 27 x 21 mm (LxW)
   Dissection: many eggs easily observed, no obvious embryo within the 1 egg that was dissected (5).

20140114.004 (interception # APWHI140021003001; trap#CRB4 on 2014/01/02 near airforce base)

Female
   Body length: 42mm (L)
   Elytra dimensions: 25 x 19 mm (LxW)
   Dissection: initial tissue appeared to be undergoing initial putrification, no obvious eggs seen (6).
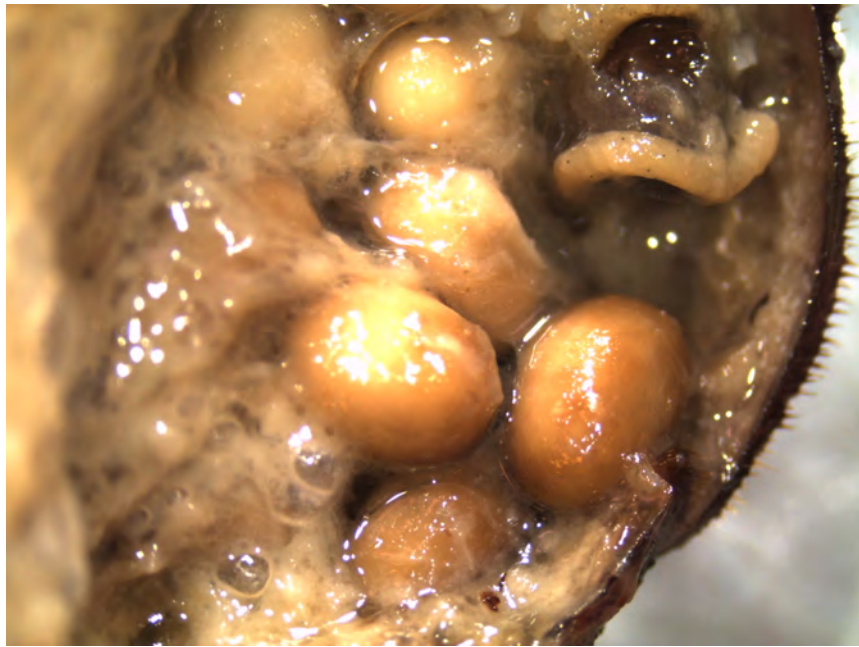
2 Ackowledgments

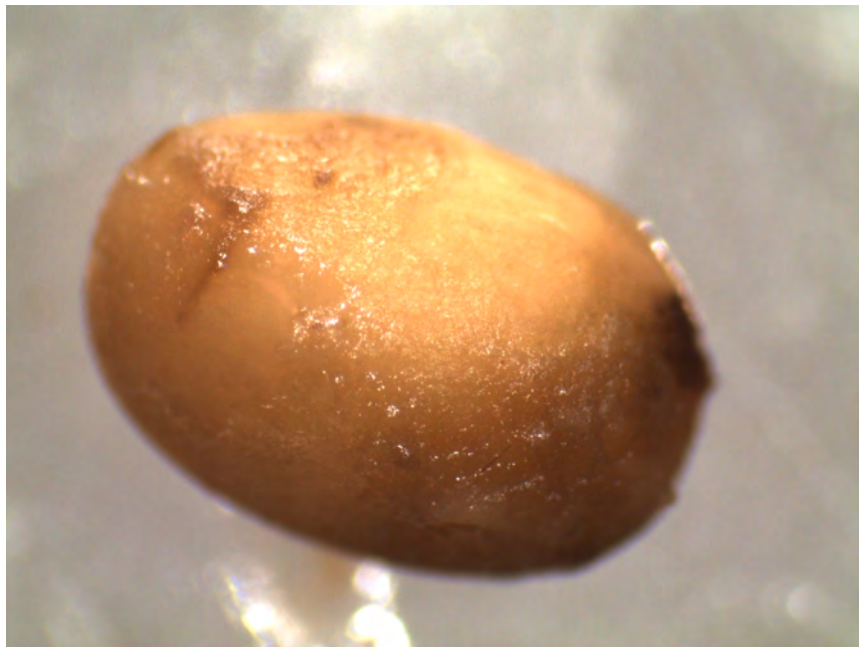Figure 1: Eggs in beetle 20140114.01

Figure 2: Egg from beetle 20140114.01

Figure 3: No obvious embryo in egg from beetle 20140114.01

Figure 4: Beetle 200140114.02.
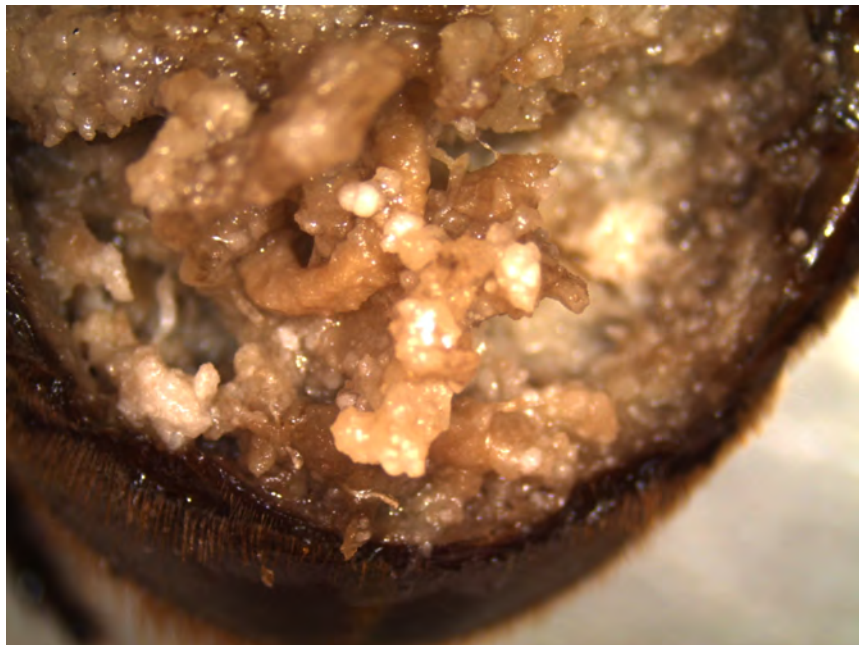
Figure 5: Eggs in beetle 200140114.03.

Figure 6: Beetle 200140114.04.

6 DNA Analysis of Hawaii CRB

Research in Support of the Guam Coconut Rhinoceros Beetle Eradication Project

DNA Analysis of Hawaii CRB

Prepared by
Aubrey Moore
University of Guam Cooperative Extension Service
and
Sean Marshall
AgResearch New Zealand

February 12, 2014[*]

DNA from four CRB adults collected in Hawai were compared to DNA samples from other CRB populations in the Pacidic using RFLP analysis. The Guam and Hawaii populations have DNA which breaks into 253 bp fragments. DNA fragments of this size are absent in DNA samples from Diego Garcia, Fiji, Samoa, and PNG. Thus the Hawaii population may have originated from Guam or a currently unknown common source.

1 Notes

DNA was harvested from hind femurs of CRB adults from Hawaii, Diego Garcia, and Guam and processed to find restriction fragment length polymorphism (RFLP). Collection data for the four Hawaii specimens are provided in a previous technical report. The RFLP results from the Hawaii samples match those from Guam. The results from Diego Garcia matched those from Fiji, Samoa, and Papua New Guinea.

See attachment3 for an image of the RFLP gels.

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[*]Revised February 12, 2014

C:/Documents and Settings/Administrator/My Documents/CRB Tech Reports/2014-01-17A Hawaii Beetle Dissections/CRB2014-01-17A.lyx

2 Ackowledgments

Thanks to Dan Vice USDA-APHIS Wildlife Services for collecting CRB adults on Diego Garcia on a recent trip. Thanks to USDA-APHIS-PPQ for releasing the Hawaii CRB specimens to us.

3 Attachment

RFLP of Oryctes rhinoceros COI (1718-2191 amplicon)
-2014/02/112 (gel 634.tif
-cut with MseI to distinguish Guam CRB populations
from other CRB populations (253bp band)

PCR used 1718Or-2191Or primers; Digest MseI for 3.5h, ran on 1.5% agarose, at 60V for 1.5h
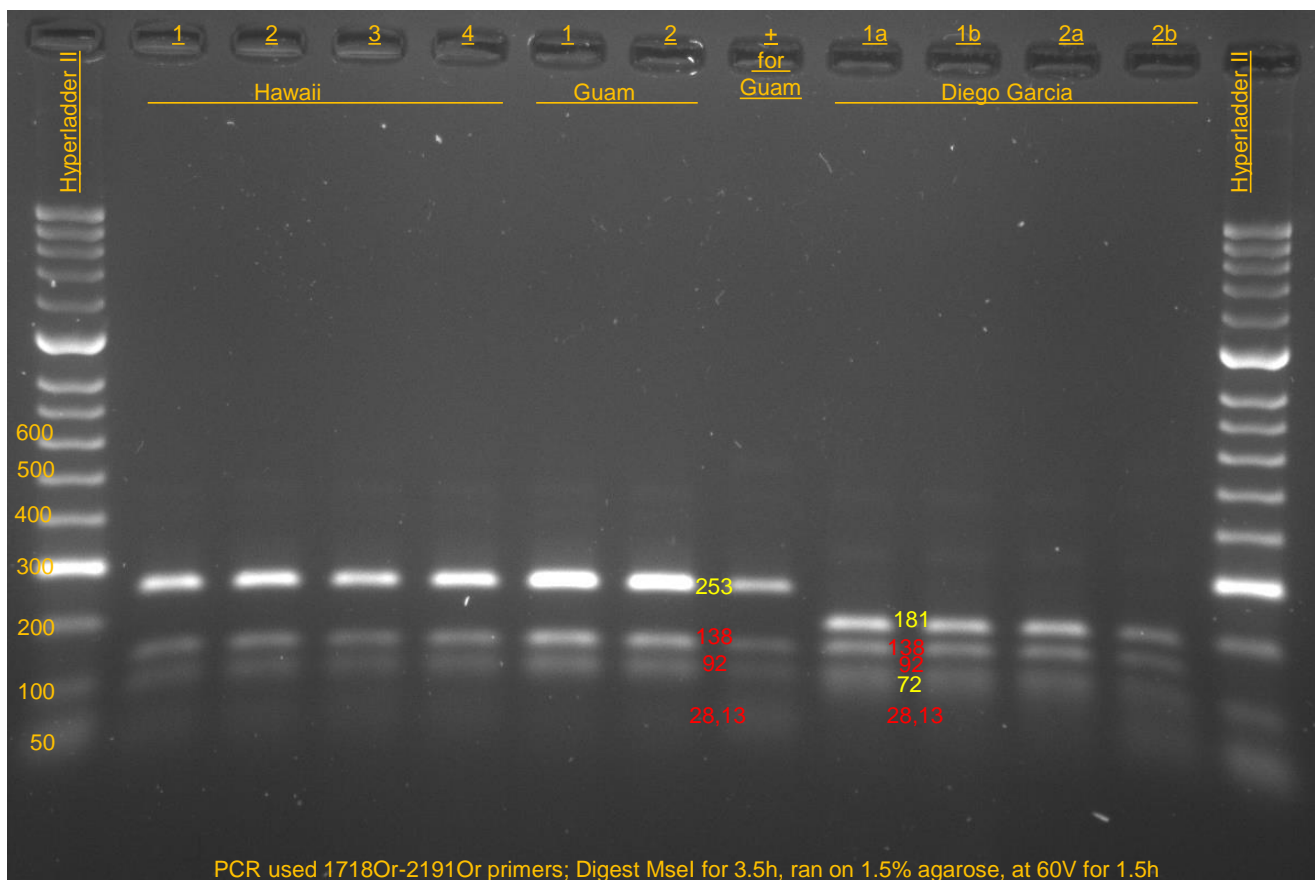
RFLP of Oryctes COI (1718-2191 amplicon)
-2012/09/18 gel 154.tif
-cut with MseI to distinguish
Guam from 'Fiji/common' populations