USDA APHIS Grant AP17PPQFO000C312
Progress Report 2
Performance Period: February 1, 2018 - August 31, 2018

Coconut Rhinoceros Beetle Biological Control

Aubrey Moore, University of Guam

September 24, 2018

Contents

1 Staffing

- Ian Iriarte, my graduate student and technician returned to the University of Guam to work on this project on April 11, 2018. Ian will be developing a coconut rhinoceros beetle damage assessment survey, which is one of the objectives of this project, as his Master's thesis topic.

- Funding from the Department of Interior, Office of Island Affairs was used to hire an insect pathologist for a 2 year term. Dr. James Grasela was recruited and started work at UOG on June 24, 2018.

2 Laboratory Bioassays of OrNV Isolate Dug42

- Permit applications to allow importation of *Oryctes rhinoceros* nudivirus (OrNV) isolates were submitted to USDA-APHIS and the Guam Department of Agriculture. These were approved. The first isolate to be imported under these permits was collected from CRB Guam biotype adult beetle caught in the Dumaguete area of Negros Island, Philippines during field work conducted there by Dr. Aubrey Moore (UOG), Ian Iriarte (UOG), and Dr. Sean Marshall (AgResearch, New Zealand) in January 2017. The isolate, Dug42, was cleaned up and propagated in insect cell culture at AgResearch New Zealand prior to being sent to Guam for pathogenicity testing (5).

- Field-collected CRB-G adults which were challenged with a high dose of OrNV-Dug42 by application of virus suspension to mouth-parts showed no significant response (Appendix B: Technical Report: Per Oral Bioassay of OrNV-Dug42).

- In a second laboratory bioassay, ORNV-Dug42 was injected directly into the haemocoel of adult beetles. There was no significant response.(7).

- Three environmental cabinets which will allow control of temperature, relative humidity, and lighting during bioassays were procured and have been installed.

3 CRB Damage Survey

- A 360 degree digital camera and accessories were purchased.

- A protocol for roadside CRB damage surveys using digital imagery was developed and trial runs were made.

- A workflow for scoring CRB damage from digital imagery is under development.

4 Regional Collaboration

4.1 Training Workshop for Commonwealth of the Northern Mariana Islands

- A workshop on CRB was organized for participants who work on the CRB problem in the Commonwealth of the Mariana Islands (CNMI). The workshop was held from July 30 through August 3 at the University of Guam. The agenda is attached (8).

4.2 Participation in the Society for Invertebrate Pathology Annual Meeting

Moore, Grasela, Iriarte and Quitugua attended the International Congress on Invertebrate Pathology and Microbial Control & the 51st Annual Meeting of the Society for Invertebrate Pathology to participate in a symposium at this conference entitled *The challenge of a virus resistant rhinoceros beetle to palm production in the Pacific and prospects for microbial control* organized by Trevor Jackson and Sean Marshall of AgResearch New Zealand and to participate in a meeting to discuss a regional response to *Oryctes rhinoceros* Biotype G which is has invaded Guam, Hawaii, Palau, Papua New Guinea, and the Solomon Islands. CRB-G is a serious threat to coconut production, oil palm production, ornamental palms of value to tourism, and sustainability of island ecosystems in general. Without a Pacific-wide regional control effort, CRB-G will spread throughout the Pacific and beyond.

At the symposium, Moore made an oral presentation entitled *Attempted microbial control of coconut rhinoceros beetle, Oryctes rhinoceros, Biotype G on Guam using Oryctes rhinoceros nudivirus and Metarhizium majus*. He was also coauthor of a second presentation entitled *Progress with control of a virus resistant coconut rhinoceros beetle* presented by Sean Marshall.

At the meeting, we discussed how to strengthen existing collaboration among partners within Asia and the Pacific who are working on developing an effective response to CRB-G. All present agreed on a free exchange of information and biological samples. Meeting notes are documented in a wiki I developed to facilitate sharing information within the CRB-G action group at http://guaminsects.net/CRBG.

Collaboration among partners working to develop an effective response to CRB-G will be essential to building an effective response. Experts at the meeting agreed that finding an isolate of Oryctes rhinoceros nudivirus which can be used as an effective classical biocontrol agent for CRB-G is the most feasible way to control this pest and a search for new virus isolates must take priority. Dr. Nur Ain Farhah from Malaysia offered to send OrNV isolates from her lab for testing against CRB-G at the University of Guam.

Presentations and discussions at the SIP meeting are documented at http://guaminsects.net/CRBG/index.php?title=SIP_Meeting,_Australia,_2018.

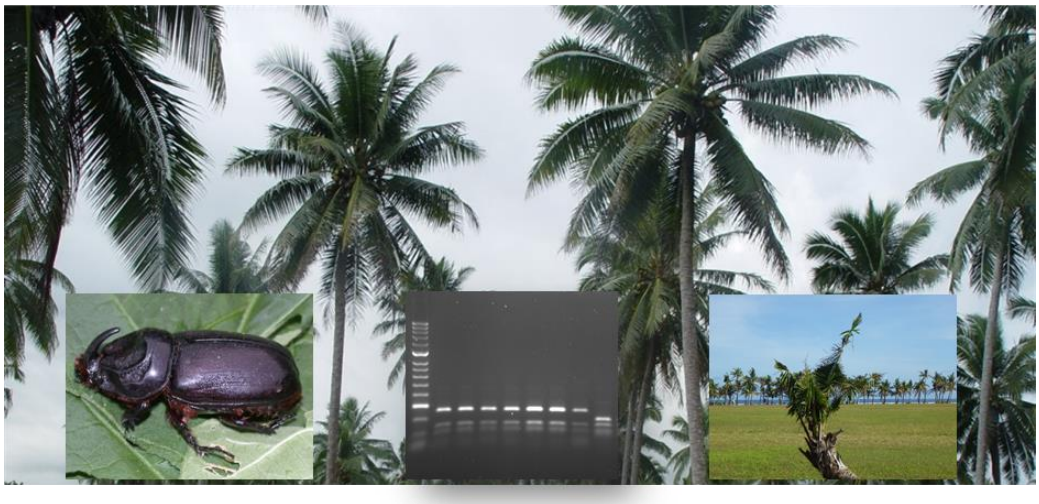
5 Appendix A: Technical Report: Diagnostics for OrNV Infection of CRB-G Collected in the Philippines

See following page.

CRB diagnostics for *Oryctes* nudivirus infection from *Oryctes rhinoceros* tissue (Guam)

Sean Marshall and Nicky Richards

July 2018



Report for University of Guam (Guam)
CLIENT REPORT NUMBER: TBA

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CONTENTS

1. EXECUTIVE SUMMARY

The coconut rhinoceros beetle (CRB; Oryctes rhinoceros) is a major pest of coconut and oil palm, but the discovery and release of Oryctes rhinoceros nudivirus (OrNV) in the 1960s and 70s suppressed the pest such that no new invasions of uninfested islands by CRB were reported for over 30 years after implementation of the biocontrol programme. Surprisingly, a highly damaging outbreak was reported from Guam (2007), which could not be controlled by OrNV. Subsequently, new invasions have been reported from Port Moresby, Papua New Guinea (2009); O'ahu, Hawai'i (2013); and Honiara, Solomon Islands (2015). Genetic evidence has shown that all of these outbreaks have been caused by a previously unrecognized haplotype, CRB-G, which appears to be tolerant to OrNV isolates commonly released in the Pacific region for managing CRB populations (Marshall et al., 2017). Entomologists working on this problem agree that the most feasible solution is establishment of biological control using an isolate of OrNV that is highly pathogenic to CRB-G.

The University of Guam and AgResearch are collaborating in an effort to identify an OrNV isolate that is able to infect CRB-G. The aim of the current work was to begin testing new candidate *Oryctes* nudivirus isolates that may be effective in managing outbreak populations of CRB-G. In one line of experimentation, a series of host-OrNV dosing passages was carried out in an attempt to select for a putative low abundance mutation that may have been present to in a mixed virus solution. Unfortunately, evidence for OrNV infection was not able to be demonstrated. However, previous work identified a candidate adult CRB-G specimen (Dug42) from Philippines as being infected with OrNV (based on visual observation, DNA detection of virus, and pathohistological analysis). We were able to purify OrNV from this gut tissue to obtained the OrNV isolate Dug42. OrNV-Dug42 has been propagated in the permissive cell line DSIR-Ha-1179 and aliquots sent to University of Guam so that a pathogen challenge assay could be set up against Guam collected CRB-G.

Recommendations:

- Based on discussion with the Guam-based team, further refinement of passaging methodology is recommended if further 'witch's brew' selection experiments are to be attempted.
- OrNV-Dug42 has been propagated in the permissive cell line DSIR-Ha-1179 and aliquots sent to University of Guam so that a pathogen challenge assay could be set up against Guam collected CRB-G. Further investigation is required to determine if OrNV-Dug42 is able to cause infection and/or mortality in CRB-G.
- It is still important to continue searching for alternative OrNV isolates (and other potential biocontrol agents) in regions known to harbor CRB-G populations, particularly where CRB caused palm damage is not being reported as an issue.

2. BACKGROUND

The coconut rhinoceros beetle (CRB; *Oryctes rhinoceros*) is a major pest of coconut and oil palm, but the discovery and release of *Oryctes rhinoceros* nudivirus (OrNV) in the 1960s and 70s suppressed the pest such that no new invasions of uninfested islands by CRB were reported for over 30 years after implementation of the biocontrol programme. Surprisingly, a highly damaging outbreak was reported from Guam (2007), which could not be controlled by OrNV. Subsequently, new invasions have been reported from Port Moresby, Papua New Guinea (2009); O'ahu, Hawai'i (2013); and Honiara, Solomon Islands (2015). Genetic evidence has shown that all of these outbreaks have been caused by a previously unrecognized haplotype, CRB-G, which appears to be tolerant to OrNV isolates commonly released in the Pacific region for managing CRB populations (Marshall et al., 2017).

Entomologists working on this problem agree that the most feasible solution is establishment of biological control using an isolate of OrNV that is highly pathogenic to CRB-G. The objective of this collaborative project between the University of Guam and AgResearch (New Zealand) was to begin testing new candidate *Oryctes* nudivirus isolates that may be effective in managing outbreak populations of CRB-G. Two approaches are currently being pursued: 1) a series of CRB-G-OrNV dosing passages was carried out in an attempt to select for a putative low abundance mutation that may be present within a mixed virus solution (a 'witch's brew' experiment); and 2) a traditional pathogen challenge bioassay using new OrNV isolates against CRB-G adults to identify effective candidate OrNV against CRB-G.

3. METHODS

The molecular DNA methods used for this work were based on those previously described in Marshall et al. 2017.

3.1 Genomic DNA extraction from *O. rhinoceros* gut tissue

DNA was extracted from the gut tissue supplied using the ZR Genomic DNA Tissue MiniPrep (Zymo Research) column system following manufacturer instructions. DNA elution was carried out using 100 µl of elution buffer from the appropriate kit, and aliquots of eluted DNA samples were subsequently used for further analyses.

3.2 PCR detection of *O. rhinoceros* nudivirus infected *O. rhinoceros* beetles

O. rhinoceros gut tissue dissected from moribund or dead bioassay specimens had DNA extracted as described above. The PCR protocol for detection of OrNV was based on that described in Richards et al. (1999), and has been subsequently modified by using undiluted and diluted DNA template (from undiluted down to 1 in 5 0000) to better distinguish infection from mere presence due to dosing with OrNV for the pathogen challenge assay. The primer pairs 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 contained 0.2 µl Taq DNA Polymerase (Thermo Fischer Scientific), 2.5 µl 10x PCR buffer, 2 µl $MgCl_2$ (25 mM),2 µl dNTP mixture (10 mM), 0.5 µl OrNV15a (10 µM), 0.5 µl OrNV15b (10 µM), 2 µl diluted DNA (reactions of 1 in 100, and 5 000), and 15.3 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) alongside a 100 bp ladder, stained with RedSafe (iNtRON Biotechnology) and fluorescence visualized over UV light. Photographs were recorded using an UVIdoc HD2 gel doc (UVItech). Detection of OrNV PCR product in the 1 in 5 000 dilution was considered here as indicative of OrNV infection, while no detection within undiluted DNA template is consider completely free of OrNV presence.

3.3 Isolation and propagation of live OrNV from infected tissue

Isolation of OrNV from infected CRB gut tissue proceeded as follows. Dissected gut tissue (putatively containing live OrNV) was initially macerated using a microhomogenizer a 2.0 ml tube containing 50 mM Tris pH 8.0 buffer. The macerate was clarified by pelleting the cell debris and passing the crude supernatant material sequentially through a series of sterile disc filtration units (0.8, 0.45, and 0.2 µm pore sizes).

Propagation of virus was carried out using the DSIR-Ha-1179 cell line, which were maintained using standard insect cell culture techniques as previously described (see Crawford, 1982; Crawford and Sheehan, 1985; Lynn, 2002), but with a few minor changes. Briefly, the Ha1179 cells were routinely grown at 27 °C as an adherent culture in surface treated tissue culture flasks containing PS100 medium made up as follows: Graces' Insect medium (Invitrogen or Sigma), 2.95 g/L tryptose phosphate broth (Sigma), TC100 vitamins (Sigma), adjusted to pH 6.2 with KOH, and supplemented with 10% fetal bovine serum (Invitrogen). For cell line maintenance, once cells reached 80-90% confluence (~10-14 days), cells were gently aspirated off the bottom of the flask using a transfer pipette and sub-cultured at a 1:5 ratio of cells to fresh medium. Production of OrNV essentially followed the method previously described (Crawford and Sheehan, 1985) for infection. Cells were grown to approximately 25% confluence in 25 cm^2 tissue culture flasks, infection was initiated by directly adding an aliquot of OrNV directly to flasks containing cells with medium. The infected flasks were gently rocked to mix contents, followed by stationary incubation at 27 °C for 14 days. Virus was harvested from infected cells by dislodging cells from the bottom of the flask (via transfer pipette), centrifugation (10 min at 100 *g*) to pellet the cell material, and the virus containing supernatant collected and subsequently stored at 4 °C. The virus titre for a given sample was measured by using of a tissue culture infectious dose 50 (TCID50) assay, which uses a 50% endpoint dilution technique (in 96-well plates) and statistical analysis (based on the Reed-Muench or Spearman-Kärber formulae) (Finney, 1952; Reed and Muench, 1938) to calculate the infectious per millilitre (IU/ml).

4. RESULTS AND DISCUSSION

4.1 OrNV detection

Following genomic DNA extraction of 27 tissue samples from the 'witch's brew' experiment, a dilution PCR analysis of DNA extracted from *O. rhinoceros* tissue was used to detect the incidence of OrNV infection from the *O. rhinoceros* gut tissue specimens from the 'witch's brew' specimens provided for analysis. The results are summarized in Table 1, with Figure 2 showing the original agarose gel images. OrNV amplicons were not detected any of the 1 in 5000 DNA dilutions; only specimens GU2-1 and 2-2 at a 1 in 100 dilution displayed an OrNV.

Generally, true OrNV infection displays distinct and easily distinguished amplification at the 1 in 5000 dilution and very robust at the 1 in 100 dilution. As no positive PCR reactions were detected at the 1 in 5000 dilution it is unlikely that active virus infection was established from the original 'witches brew' experiment. Further discussion with the Guam-based team highlighted the observation that the material obtained from successive passaging became

highly viscous and also contained *Metarhizium majus* fungal spores. Based on this, further refinement of the passaging methodology is recommended if further 'witch's brew' selection experiments are to be attempted.

Table 1: Summary of OrNV detection results from subset of *Oryctes rhinoceros* gut tissue received by AgResearch from the University of Guam.

Tube Label	OrNV Detected	Comment
GU1-1	No	No OrNV infection
GU1-2	No	No OrNV infection
GU-3	No	No OrNV infection
GU-6	No	No OrNV infection
GU-9	No	No OrNV infection
GU-12	No	No OrNV infection
GU-15	No	No OrNV infection
GU-18	No	No OrNV infection
GU-21	No	No OrNV infection
GU-24	No	No OrNV infection
GU-27	No	No OrNV infection
GU-30	No	No OrNV infection
GU-33	No	No OrNV infection
GU-36	No	No OrNV infection
GU-39	No	No OrNV infection
GU-42	No	No OrNV infection
GU-45	No	No OrNV infection
GU-48	No	No OrNV infection
GU2-1	Yes (only 1:100)	Potential OrNV infection
GU2-2	Yes (only 1:100)	Potential OrNV infection
GU2-3	No	No OrNV infection
GU2-3	No	No OrNV infection
GU2-4	No	No OrNV infection
GU2-5	No	No OrNV infection
GU2-6	No	No OrNV infection
GU4-1	No	No OrNV infection
GU4-2	No	No OrNV infection

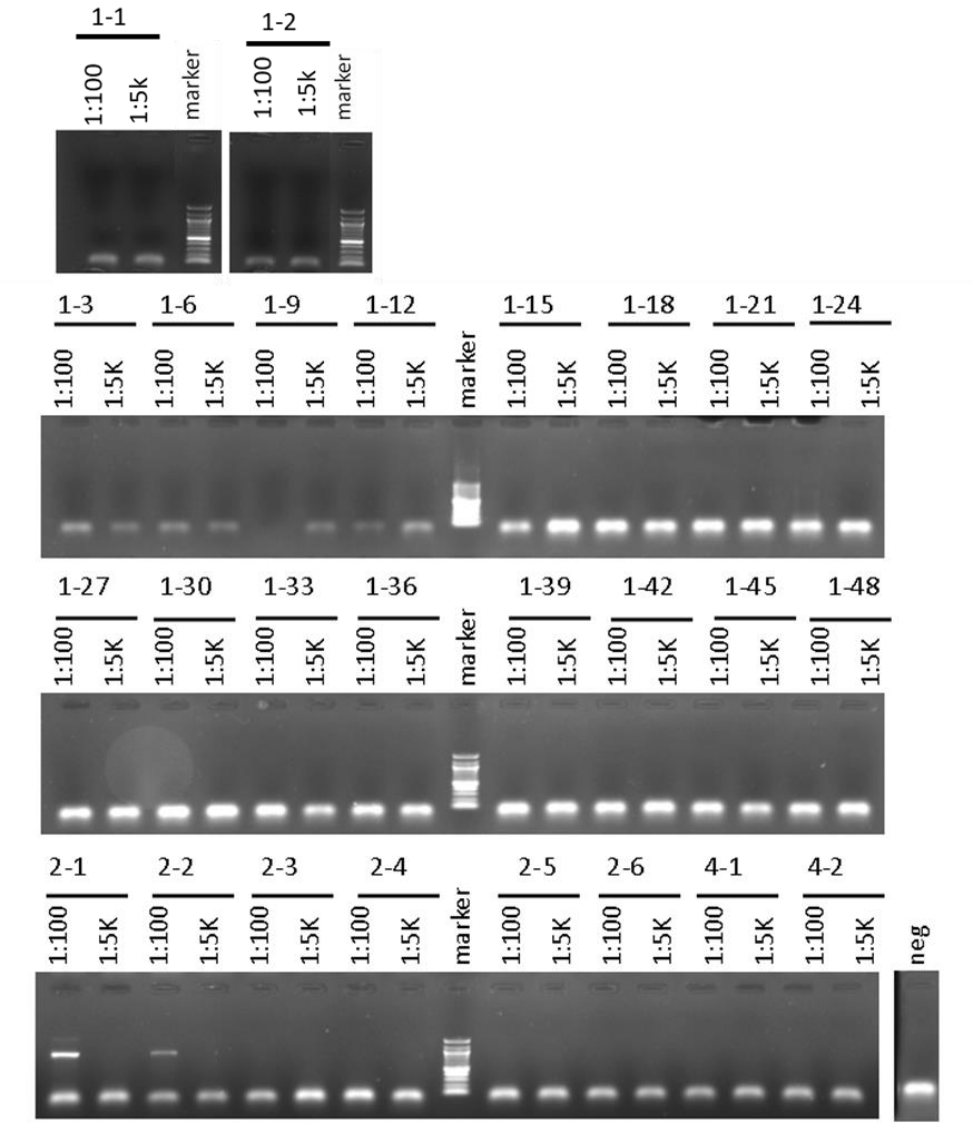


Figure 2: Agarose gel PCR assay results for OrNV detection.
Presence of a 945 base pair PCR product indicates OrNV DNA is present. Obvious detection of OrNV PCR product in the 1 in 5000 (1:5K) dilution is considered as indicative of OrNV infection. The marker (M) used has DNA 'ladder rungs' every 100 base pairs.

4.2 Isolation and propagation of OrNV-Dug42

From a previously identified OrNV infected adult CRB-G specimen (Dug42), *Oryctes* nudivirus was purified from the gut tissue and designated as OrNV isolate Dug42 (OrNV-Dug42). Further propagation was carried out using the permissive cell line DSIR-Ha-1179, whereby the characteristic cytopathic effects were observed and virus was readily detected from this material. Ten 1 ml aliquots of OrNV-Dug42 titred at ~1.0 x 10^6 infectious units/mL were sent to University of Guam for further testing within a pathogen challenge assay against Guam collected CRB-G adults.

5. CONCLUSIONS AND RECOMMENDATIONS

- The PCR haplotyping results indicated that all specimens analyzed were CRB-G.
- Data from the 'witch's brew' experiment for establishing of OrNV infection suggested no virus was present. Although two OrNV PCR positive result (GU-2-1 and 2-2) were observed at the 1 in 100 dilution point. They were not observed at the 1 in 5000 threshold, which has been previously validated to distinguish true infection from spurious contamination.
- Discussion with the Guam-based team highlighted the observation from the 'witch's brew experiment' that the material collected from successive passaging became highly viscous and also contained *Metarhizium majus* fungal spores. Based on this, further refinement of passaging methodology is recommended if further 'witch's brew' selection experiments are to be attempted.
- OrNV-Dug42 has been propagated in the permissive cell line DSIR-Ha-1179 and aliquots sent to University of Guam so that a pathogen challenge assay could be set up against Guam collected CRB-G. Further investigation is required to determine if OrNV-Dug42 is able to cause infection and/or mortality in CRB-G. For future investigations, additional collection and preservation of tissue for histological analysis may assist with interpretation of results from pathogen challenge experiments.
- It is still important to continue searching for alternative OrNV isolates (and other potential biocontrol agents) in regions known to harbor CRB-G populations, particularly where CRB caused palm damage is not being reported as an issue.

6. ACKNOWLEDGEMENTS

We thank Dr Aubrey Moore and the University of Guam for their collaboration and continued support of this work.

7. REFERENCES

Crawford, A. M., 1982. A coleopteran cell line derived from *Heteronychus arator* (Coleoptera: Scarabaeidae). In Vitro. 18**,** 813-816.

Crawford, A. M., et al., 1985. A physical map of the *Oryctes* baculovirus genome. Journal of General Virology. 66**,** 2649-2658.

Finney, D. J., 1952. Statistical method in biological assay. Hafner.

Huger, A. M., 1966. A virus disease of the Indian rhinoceros beetle, *Oryctes rhinoceros* (Linnaeus), caused by a new type of insect virus, *Rhabdionvirus oryctes* gen. n., sp. n. Journal of Invertebrate Pathology. 8**,** 38-51.

Lynn, D. E., 2002. Methods for maintaining insect cell cultures. Journal of insect science (Online). 2**,** 9.

Marshall, S.D.G., Moore, A., Vaqalo, M., Noble, A., Jackson, T.A. 2017. Journal of Invertebrate Pathology 149, 127-134

Reed, L. J., Muench, H., 1938. A simple method of estimating fifty per cent endpoints. American Journal of Epidemiology. 27**,** 493-497.

Richards, N. K., Glare, T. R., Aloali'i, I., Jackson, T. A., 1999. Primers for the detection of *Oryctes* virus from Scarabaeidae (Coleoptera). Molecular Ecology. 8**,** 1552-1553.

6 Appendix B: Technical Report: Per Oral Bioassay of OrNV-Dug42

See following page.

Dumaguete OrNV bioassay 1

January 11, 2018

1 Dumaguete OrNV Isolate - Bioassay 1

1.1 Introduction

This notebook documents an initial bioassay of the Dumaguete isolate of *Oryctes* nudivirus.

1.2 Materials and Methods

1.2.1 Virus Sample

This virus was isolated from a single infected *Oryctes rhinoceros* Guam biotype specimen collected near Dumaguete, Negros Island, Philippines in January 2017.

1.2.2 Test Insects

Test insects were collected weekly from 31 coconut rhinoceros beetle pheromone traps (barrel traps) at the University of Guam Agricultural Experiment Station at Yigo. Beetles were held individually in numbered Mason jars partially filled with moist peat moss which were stored in an environmental cabinet at a temperature of 80 degrees F. Each was fed a slice of banana weekly. Beetles were reared an average of 46 days (range: 23 d to 75 d) prior to treatment.

1.2.3 Treatment

- 20 beetles were selected at random for treatment and another 20 were selected for experimental control
- 100 mg of sucrose was disolved in a 1 ml sample of the virus. Each beetle was given a 40 microlitre dose of this solution by pipetting a drop onto its mouthparts. A placebo was not given to beetles in the experimental control group.
- All beetles were provided a slice of banana on the day following treatment.

1.2.4 Observation procedure

- Beetles were observed weekly.
- Each beetle was weighed and its mass was entered immediately into a spreadsheet.
- A slice of banana was added immediately after each beetle was returned to its jar.

1.2.5 Analysis

- The data model includes 2 tables saved as Excel spreadsheets (see below).
- An empty **mass** field in the **observations** table indicates that the beetle died prior to observation.

1.3 Results and Discussion

- The beetles did not readily drink the 40 microlitre dose applied to their mouthparts. A food incorporation bioassay may be more efficient.
- During the 28 day bioassay period, 1 of 20 virus treated beetles died and 4 of 20 beetles in the experimental control group died. The difference in mortality is not significant (p = 0.34; Fisher's exact test). Post mortems indicated that all of the dead beetles were infected with *Metarhizium majus*. Guts looked normal.
- There was a significant difference in weight loss during the first week of the bioassay (p = 0.02; Welch's t-test). However, there was no significant difference in weight loss during succeding weeks. Difference in weight loss during the first week may be due to the fact that the virus treated beetles were handled much more than the contol group.
- In conclusion, results from this initial bioassay does not indicate pathogenicity to CRB-G for the OrNV Dumaguete isolate.

1.4 Calculations

```
In [22]: import pandas as pd
         import matplotlib.pyplot as plt
         import sqlite3
         import numpy as np
         from scipy.stats import ttest_ind, fisher_exact

         %matplotlib inline
         pd.options.display.max_rows = 4
```

1.4.1 import data from spreadsheet

```
In [23]: DATAFILE = 'aubrey.xlsx'

         df_jars = pd.read_excel(DATAFILE, 'jars')
         df_jars
```

```
Out[23]:     jar_number date_collected     sex treatment
         0           32     2017-10-02  Female     virus
         1           85     2017-10-02  Female     virus
         ..         ...            ...     ...       ...
         38        1263     2017-10-18    Male   control
         39        1633     2017-10-23    Male   control

         [40 rows x 4 columns]
```

```
In [24]: df_observations = pd.read_excel(DATAFILE, 'observations')
         df_observations
```

Out[24]:

	jar_number	observation_date	mass	note
0	32	2017-11-15	4487.0	NaN
1	50	2017-11-15	4136.0	mites
..	...	...	...	...
198	1263	2017-12-13	3036.0	NaN
199	1633	2017-12-13	3399.0	NaN

[200 rows x 4 columns]

In [25]:
```
# Create a dict containing pairs of dates.
# The index is an observation date and the value is the previous observation date

obs_dates = df_observations.observation_date.unique()
prev_obs_date = dict(zip(obs_dates[1:], obs_dates))

# Example usage:
# y = df_observations.observation_date[100]
# print y
# prev_obs_date[y.to_datetime64()]
```

In [26]:
```
# Add a new column "days_post_treatment" to "df_observations" and populate it

treatment_date = df_observations.observation_date.min().to_datetime64() # Assumes all t
df_observations['days_post_treatment'] = np.nan

for index, row in df_observations.iterrows():
    try:
        date = row.observation_date.to_datetime64()
        if date == treatment_date:
            days_post_treatment = 0
        else:
            days_post_treatment = date - treatment_date
            # Convert from timedelta in nanoseconds to integer days
            days_post_treatment = (days_post_treatment / np.timedelta64(1, 'D')).astype
    except:
        continue
    df_observations.loc[index, 'days_post_treatment'] = days_post_treatment

# Add a new column "mass_change" to "df_observations" and populate it

df_observations['mass_change'] = np.nan

for index, row in df_observations.iterrows():
    try:
        date = row.observation_date.to_datetime64()
        prev_date = prev_obs_date[date]
        prev_mass = int(df_observations.loc[(df_observations.observation_date==prev_dat
            (df_observations.jar_number==row.jar_number), 'mass'])
```

```
            mass_change = int(row.mass) - prev_mass
        except:
            continue
        df_observations.loc[index, 'mass_change'] = mass_change

df_observations
```

Out[26]:

	jar_number	observation_date	mass	note	days_post_treatment	mass_change
0	32	2017-11-15	4487.0	NaN	0.0	NaN
1	50	2017-11-15	4136.0	mites	0.0	NaN
..	...	...	...	...	...	...
198	1263	2017-12-13	3036.0	NaN	28.0	258.0
199	1633	2017-12-13	3399.0	NaN	28.0	-194.0

[200 rows x 6 columns]

In [27]:
```
df = df_observations.merge(df_jars, on='jar_number')
df
```

Out[27]:

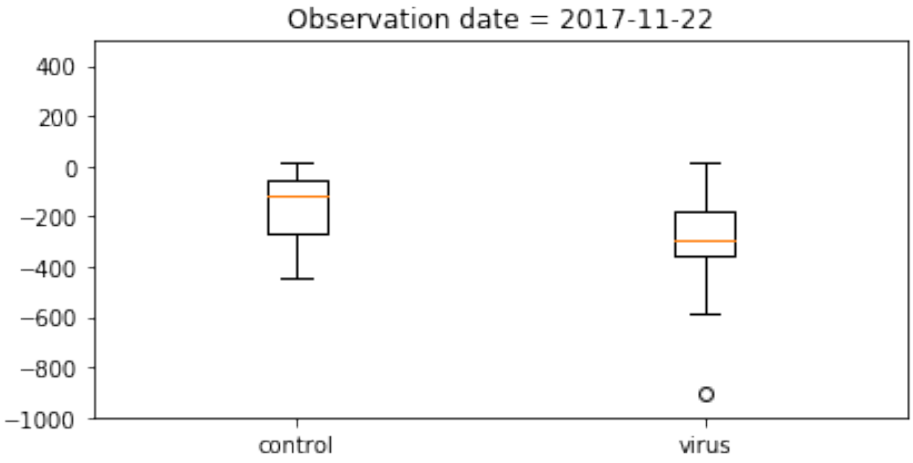
	jar_number	observation_date	mass	note	days_post_treatment	mass_change	date_collected	sex	treatment
0	32	2017-11-15	4487.0	NaN	0.0	NaN	2017-10-02	Female	virus
1	32	2017-11-22	3900.0	NaN	7.0	-587.0	2017-10-02	Female	virus
..	...	...	...	...	...	...	...	...	...
198	1633	2017-12-06	3593.0	NaN	21.0	447.0	2017-10-23	Male	control
199	1633	2017-12-13	3399.0	NaN	28.0	-194.0	2017-10-23	Male	control

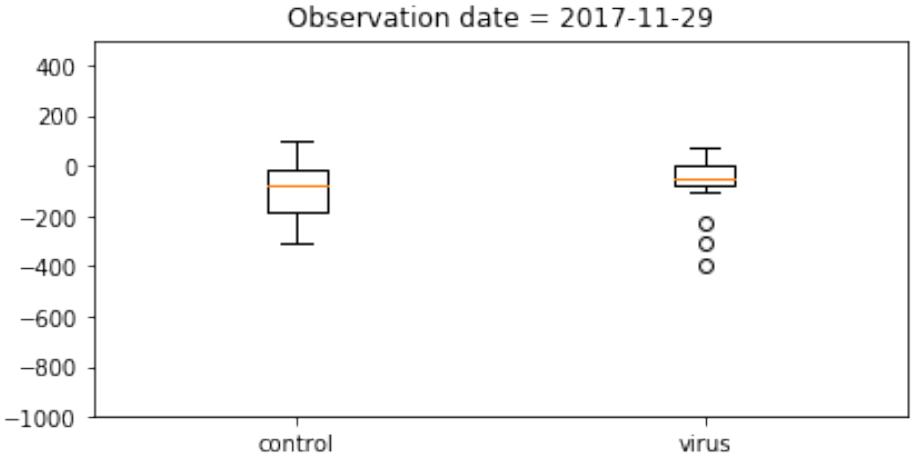
[200 rows x 9 columns]

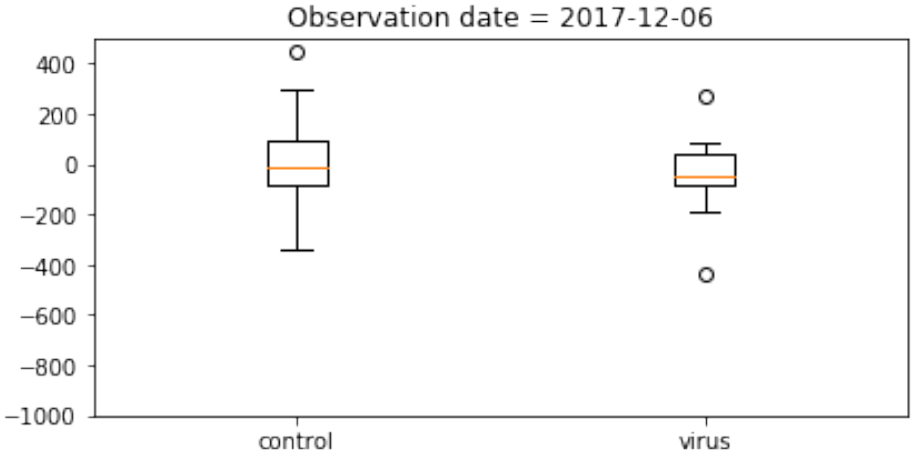
In [28]:
```
fig = plt.figure(figsize=(6, 12))
i = 0
print 'observation \tcontrol \t\tvirus'
print 'date \t\tmean mass change (mg) \tmean mass change (mg) \tp-value'
for date in prev_obs_date.keys():
    i += 1
    v = df.loc[(df.observation_date==date) &
```

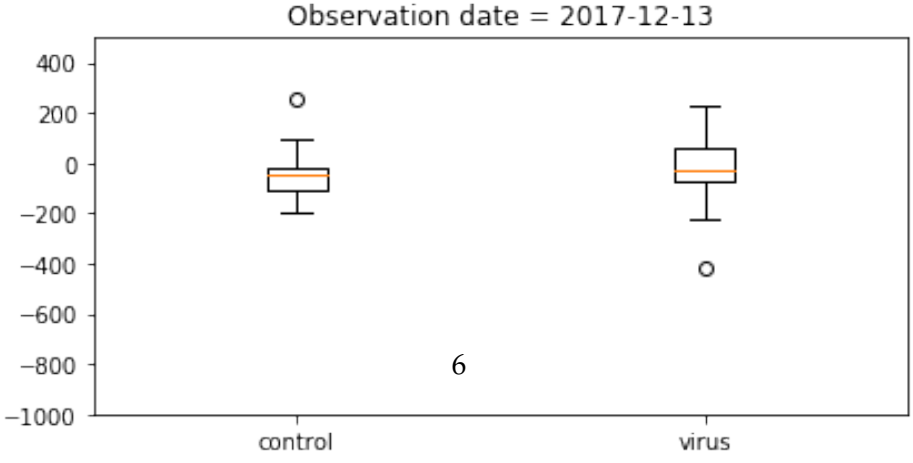
```
                                (df.treatment=='virus'), 'mass_change']
            v = v[-np.isnan(v)]
            c = df.loc[(df.observation_date==date) &
                                (df.treatment=='control'), 'mass_change']
            c = c[-np.isnan(c)]

            statistic, pvalue = ttest_ind(c, v, nan_policy='omit', equal_var=False)
            print '{}\t{:.0f} (n={})\t\t{:.0f} (n={})\t\t{:.4f}'.format(
                str(date)[:10], np.mean(c), len(c), np.mean(v), len(v), pvalue)
            ax = fig.add_subplot(4,1,i)
            title = 'Observation date = ' + str(date)[:10]
            ax.set_title(title)
            ax.boxplot([c.values, v.values])
            ax.set_xticklabels(['control', 'virus'])
            ax.set_ylim([-1000, 500])
        plt.tight_layout()
```

```
observation         control                 virus
date                 mean mass change (mg)         mean mass change (mg)         p-value
2017-11-22        -163 (n=20)                -298 (n=20)                0.0195
2017-11-29        -107 (n=19)                -70 (n=20)                0.3216
2017-12-06        3 (n=18)                -40 (n=20)                0.4429
2017-12-13        -41 (n=16)                -37 (n=19)                0.9350
```


Observation date = 2017-11-22
400
200
0
−200
−400
−600
−800
−1000
control
virus


Observation date = 2017-11-29
400
200
0
−200
−400
−600
−800
−1000
control
virus


Observation date = 2017-12-06
400
200
0
−200
−400
−600
−800
−1000
control
virus

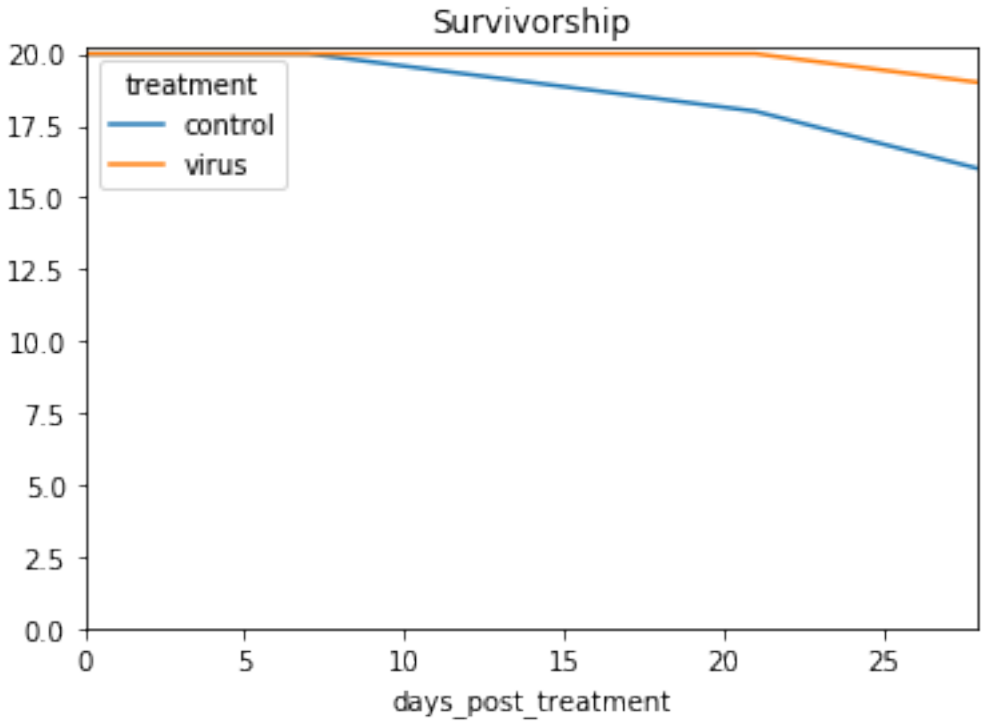

Observation date = 2017-12-13
400
200
0
−200
−400
−600
−800
−1000
control
virus

1.4.2 Mortality

In [29]:
```
df_groupby = df.groupby(['days_post_treatment','treatment'])['mass'].count().unstack()
df_groupby
```

Out[29]:
```
treatment             control  virus
days_post_treatment
0.0                        20     20
7.0                        20     20
...                       ...    ...
21.0                       18     20
28.0                       16     19

[5 rows x 2 columns]
```

In [34]:
```
myplot = df_groupby.plot()
ylim = myplot.axes.get_ylim()
myplot.axes.set_ylim(0, ylim[1])
myplot.set_title('Survivorship');
```



In [31]:
```
c_total = df_groupby.control.max()
c_alive = df_groupby.control.min()
```

```
        c_dead = c_total - c_alive
        v_total = df_groupby.virus.max()
        v_alive = df_groupby.virus.min()
        v_dead = v_total - v_alive
        print '{}\t{}\t{}'.format('', 'alive', 'dead')
        print '{}\t{}\t{}'.format('control', c_alive, c_dead)
        print '{}\t{}\t{}'.format('virus', v_alive, v_dead)
        print
        oddsratio, pvalue = fisher_exact([[c_alive, c_dead], [v_alive, v_dead]])
        print 'Fisher''s exact test p-value = {}'.format(pvalue)
```

```
        alive   dead
control 16      4
virus   19      1

Fishers exact test p-value = 0.341649341649
```

1.4.3 Holding time prior to treatment

```
In [32]: # Calculate days held prior to treatment

        pd.options.display.max_rows=100
        df_temp = df[df.observation_date==treatment_date]
        (df_temp.observation_date - df_temp.date_collected).describe()
```

```
Out[32]: count                         40
         mean            46 days 15:00:00
         std      18 days 08:07:05.397423
         min             23 days 00:00:00
         25%             33 days 00:00:00
         50%             44 days 00:00:00
         75%             57 days 00:00:00
         max             75 days 00:00:00
         dtype: object
```

7 Appendix C: Technical Report: Per Haemocoel Injection Bioassay of OrNV-Dug42

See the following page.

Guam Coconut Rhinoceros Beetle Project Technical Report 7-19-2018

Protocol for injection of the Guam Coconut Rhinoceros beetle genotype (*Oryctes rhinoceros*) *w*ith nudivirus (OrNV)

James J. Grasela and Aubrey Moore

This was a first attempt at injection of adult beetles with a virus isolate (Dug42) obtained AgResearch, New Zealand. Adults have an extremely hard exoskeleton and according to the description of a previous laboratory injection protocol, which relied on finding a point on the ventral surface at the junction of the hind leg and the thoracic to inject, proved to be problematic. While holding the adult dorsal side down by hand, the beetle tends to flip-over while simultaneously attempting to move forward. There is no available source of CO_2 in our lab to anesthetize them, so the next best thing would have been to put them on ice or in a refrigerator for a short time (i.e. 5 min) before an attempt at inject. However, we tried the more difficult, expedient approach by not immobilizing the beetle and injected 20 µl of virus sample into each beetle with a sterile 30-gauge BD syringe. Another problem was that the small needle used for injection was too flexible, easily bent, and this probably caused some of the individual beetles not to receive the complete virus dose.

For the injection experiments, adult beetles were divided into three groups of 10 individuals with each group receiving a different treatment (sterile-activated virus, heat-inactivated virus, or filtered water as a control). Adult weights were measured just prior to injection. After treatment each beetle was transferred to a glass mason jar filled with moist, commercially blended steer manure and soil. Daily measurements of adult weight were taken until either the beetle showed possible viral symptoms or death from some other cause (e.g. bacteria).

Results

After two weeks post-injection none of the beetles showed signs of viral infection. However, there were some deaths during the experiment, but it was attributed to some other unknown cause. Table 1 shows the adult weights.

Day / I.D.	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Sterile virus														
1597	3.085	2.876	2.792	2.773	2.750	2.738	2.846		2.748		2.696			2.616
0865	2.510	2.649	2.664	2.305	2.338	2.263	2.401		2.272		2.315			2.293
0946	3.794	3.234	3.121	3.281	3.484	3.191	3.253		3.132		2.984			2.975
0819	5.387	4.945	4.815	4.805	5.075	4.655	4.957		4.648		4.677			4.607
3226	2.590	2.846	2.711	2.526	2.505	2.451	2.416		2.361		2.334			2.345
1369	3.578	3.354	3.273	3.276	dead									
0636	5.055	4.867	4.487	4.382	4.507	4.267	4.426		4.513		4.485			4.398
1423	3.353	3.352	3.254	2.932	3.084	2.761	3.000		2.832		2.754			2.726
1328	3.848	dead												
Heat-inactivated virus														
0267	4.770	5.129	4.721	4.384	4.292	4.258	4.245		4.197		4.167			4.213
0750	4.106	4.392	4.421	3.911	4.224	3.897	3.862		3.905		3.953			3.810
0259	3.954	3.903	3.505	3.485	3.755	3.430	3.506		3.535		3.382			3.314
1111	3.690	4.059	3.639	3.504	3.864	3.564	3.599		3.497		3.456			3.432
0087	3.643	3.750	3.782	3.468	3.671	3.510	3.502		3.403		3.518			3.410
1221	4.240	4.646	4.367	4.265	4.124	3.954	3.833		3.730		3.685			3.734
1841	5.800	5.139	5.666	5.126	5.059	5.009	5.119		5.077		4.931			5.019
1364	3.161	3.142	3.110	dead										
1618	3.949	3.673	3.666	3.615	3.573	3.567	nd		3.513		3.737			3.788
Control														
0941	3.473	dead												
0473	3.980	3.920	3.918	dead										
1202	3.550	4.079	3.917	3.877	3.850	3.677	3.606		3.600		3.577			3.507
0397	4.551	4.981	4.879	4.690	4.996	4.646	4.844		4.755		4.611			4.599
1288	4.250	4.063	3.988	3.848	3.809	3.717	3.691		3.612		3.526			3.447
0421	4.690	5.804	5.402	5.277	4.802	4.783	4.745		4.631		4.569			4.436
0898	5.177	5.146	5.136	5.128	5.130	5.143	5.098		5.060		5.066			5.025
3232	4.362	4.228	4.781	4.455	4.362	4.246	4.123		4.035		3.972			3.900
3235	1.750	1.725	1.728	1.727	1.712	1.707	1.682		1.641		1.607			1.580
0628	2.916	2.836	2.837	2.785	2.768	2.767	2.861		2.760		2.836			2.760

Table 1. Adult beetle weight (g)

8 Appendix D: Coconut Rhinoceros Beetle Workshop for CNMI

See following page.

University of Guam
2018 Coconut Rhinoceros Beetle Training

Venue: UOG Mangilao campus ALS 104

Monday July 30, 2018
9:00am - Biological Invasion of Guam, Dr. Aubrey Moore
10:30am – Guam CRB Introduction, Dr. Aubrey Moore
12:00pm – 1:00 pm - Lunch
1:00 pm – 4:00pm – UOG campus scouting, Roland Quitugua

Tuesday July 31, 2018
9:00am – Biosecurity gaps, Roland Quitugua
10:00am – Bird Loss project, Dr. Ross Miller
11:00am - Little Fire Ant, Dr. Ross Miller
11:45am – Greater Banded Hornet, Dr. Ross Miller
12:00pm - Lunch
1:00pm – 4:00pm - CRB scouting/sanitation, Roland Quitugua

Wednesday August 1, 2018
8:00am – CRB conference call
9:00 am – Rota CRB program, Troy Brown
10:45 – CRB Trapping, Roland Quitugua
12:00pm – Lunch
1:00pm – 4:00pm CRB scouting/sanitation, Roland Quitugua

Thursday August 2, 2018
9:00am – EpiCollect, Dr. Aubrey Moore
10:30am – Inaturalist, Dr. Aubrey Moore
12:00pm - Lunch
1:00pm - **(Tumon)** CRB scouting, Roland Quitugua
3:00pm - BBQ

Friday August 3, 2018
9:00am – CRB/Biosecurity recommendations, Roland Quitugua
10:30am – Species of concern
11:30am – Workshop survey
12:00pm – Lunch
1:00pm – Open topic