POTENTIAL BIOLOGICAL CONTROL OF THE COCONUT RHINOCEROS
BEETLE ON OʻAHU, HAWAIʻI

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By:

Mason Harry Russo

Thesis Committee:
Dr. Zhiqiang Cheng, Committee Chair
Dr. Mark Wright
Dr. Michael Melzer

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ABSTRACT

Many islands throughout the Pacific have had their palm trees devastated by *Oryctes rhinoceros* (Coleoptera: Scarabaeidae: Dynastinae), the coconut rhinoceros beetle. *O. rhinoceros* primarily feeds on coconut palms, *Cocos nucifera*, but can feed on a variety of other palms and plants of economic value. Integrated pest management is often used to control *O. rhinoceros*, with a combination of mechanical, cultural, chemical, and biological control methods. *O. rhinoceros*'s spread across the Pacific was influenced by agriculture, shipping, war, and tropical storms. In 2013 it was detected on the Hawaiian Island of Oʻahu. Due to the regulations associated with bringing in biological control agents to the Hawaiian Islands, a survey was conducted to identify local entomopathogenic fungus strains to test on the *O. rhinoceros* larvae. Soil samples from around Oʻahu were collected and 73 strains of *Beauveria* spp. and *Metarhizium* spp. were tested on lab reared *O. rhinoceros* first instar larvae. *Heterorhabditis indica* and *Steinernema feltiae* entomopathogenic nematodes were collected on Oʻahu for trials as well. *O. rhinoceros* larvae were reared in the University of Hawaiʻi Arthropod Containment Laboratory, with field caught adults brought into the colony weekly. These larvae reflected the field population on Oʻahu, and were used in laboratory assays involving entomopathogenic nematodes and fungi. The entomopathogenic nematode testing did not yield substantial mortality, while the entomopathogenic fungal strains yielded greater than 60% mortality with five prominent strains. Promising entomopathogenic fungi results led to field testing on field caught larvae as well.

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CHAPTER 1. Introduction: A review of *Oryctes rhinoceros* life history, geographical

distribution, containment methods, and its introduction to the island of Oʻahu.

<u>Invasive Species and Hawaiʻi</u>

The introduction of invasive species has detrimental effects on island ecosystems, often

reducing the biodiversity of the endemic or native species; negatively impacting a variety of

economically important industries such as tourism, fisheries, agriculture, and infrastructure, and

causing human and animal health issues (Reaser et al. 2007). Invasive species can cause

extinctions and may alter regional biodiversity faster than habitat fragmentation or destruction

(Sandlund et al.2002). Sandlund et al. (2002) notes that after the introduction of a new species,

the population typically enters a lag phase in which the species undergoes natural population

growth and range expansion. The length of this phase varies by species, as some environments

are more favorable than others for expansion. This is also the phase where eradication is possible

if all stages of the insect can be targeted effectively, typically through chemical and mechanical

control.

The islands of Hawaiʻi are geographically isolated and climatically suitable for invasive

species to establish and thrive. The isolation of the Hawaiian archipelago has resulted in a

number of adaptive radiations and diversity of endemic species that are particularly susceptible

to invasive introductions, owing to their evolution in isolation of other species. An estimated

72% of recorded extinctions in the United States are species that were endemic to Hawaiʻi (Cox

1999). While Hawaiʻi is only 0.2% of the total land area of the United States, it has 25% of the

federally listed endangered species. These high extinction rates are due to the many

anthropogenic disturbances to the islands. The early Polynesian settlers brought more than 40

species with them that contributed to the first wave of extinctions. Cox (1999) notes that from European arrival to 1998, there have been over 900 recorded invasive or non-native species which have become established in Hawaiʻi. That number is likely to have risen since then and it is expected that introductions through anthropogenic activities will continue.

The classic 'tens rule' with invasive species introduction indicates the likelihood of an organism establishing or becoming a pest species. If 1000 species were to be introduced and 100 escaped into the wild, it is likely ten will be established and one will become a pest species (Williamson and Fitter 1996). That individual pest species is then likely to alter the biodiversity of the area negatively and possibly permanently. The International Union of the Conservation of Nature and Natural Resources (IUCN) has identified 100 of the World's worst alien species, ranging from aquatic and land plants, aquatic and land invertebrates, fungi, amphibians, fish, reptiles, birds, mammals, and micro-organisms (Lowe et al. 2000). In my opinion, the tens rule is unlikely to be entirely applicable to Hawaiʻi in regard to some species on this list and its favorable climate. Many of the species on the IUCN's list have already been established in Hawaiʻi, affecting endemic and naturalized species. Many naturalized species have cultural relevance, as they were brought over on canoes by early Polynesian voyagers such as banana, taro, and coconut palms.

Oryctes rhinoceros Introduction

The coconut rhinoceros beetle, *Oryctes rhinoceros* (Coleoptera: Scarabaeidae: Dynastinae) is one of the many invasive species introductions that have arrived on the island of Oʻahu and it has the potential to devastate the naturalized and native palms on the island. Unlike other new introductions, this species has a very high chance of becoming widely established, considering its history of invading many other islands in the Pacific (Bedford 1980). An

eradication program run by the Hawai'i Department of Agriculture (HDOA) with an annual budget of approximately $1.5 million is in place (Rysin et al. 2018). Since the 2013 introduction, *O. rhinoceros* has been confined to a small area of O'ahu using trapping, sanitation and green waste management.

There are lots of incentives for eradication within this contained area, due to the environmental and economic damage that could occur to O'ahu's urban landscape. Urban palm trees increase aesthetic value in residential and urban areas (Vargas et al. 2007). Within O'ahu's landscape are an estimated 50,419 coconut palms in beach parks, non-beach parks, streets, resorts, residential, community/business areas, and industrial areas (Rysin et al. 2018). Costs of palm tree removal, replacement, and maintenance are about $2,525 per tree and a United States Department of Agriculture report indicated that control costs of an expanded *O. rhinoceros* population would be around $3 million annually. Pathway analysis showed that if eradication occurred on O'ahu, there would be a 50% reduction in the chance of a mainland introduction (Kumar and Bigsby 2018). If *O. rhinoceros* were to be widely established on the Hawaiian Islands, the chance of a mainland introduction could make this estimate increase. An assessment of mainland agricultural impacts estimated damage losses could reach $900 million annually for the conterminous United States if *O. rhinoceros* were to become widely established (Rysin et al. 2018).

Feeding and Hosts

Understanding the background of this invasion and the life history of this pest are essential in order to control the infestation. *O. rhinoceros* primarily feeds on the coconut palm, *Cocos nucifera* and oil palm, *Elaeis guineensis.* It also has been known to be a minor pest of other economically important plants such as sugarcane, banana, and pineapple (Baker 2002 and

Hinckley 1973). During nocturnal feeding, the adults feed into the apical meristem of the palm, typically resulting in fronds emerging with a characteristic "V" cut (Figure 1). Feeding on the crown of the palm leaves this distinctive sawed off appearance and can easily be used to visually detect adult infestations. The detriments of crown feeding vary from immature coconut drop off, to poor frond health, and eventually tree death with heavy infestations. Other feeding methods leave distinctive holes, when the beetles directly chew through the outside of a frond (Figure 2). Extensive crown feeding can result in tree death, in which these dead standing palms become ideal growth sites for larvae.

Figure 1: Characteristic V-cut on palm leaves after crown feeding

Figure 2: Adult CRB damage sign on palm frond

Population Expansion Across the Pacific

Locating oviposition and breeding sites is the best way to mitigate an infestation, but the variety of larval developmental sites makes it difficult to eradicate an infestation. Coconut stumps, logs, and decaying organic matter are ideal for larval growth (Bedford 1980, Hallett et al. 1995, Moore et al. 2016). Egg to adult development of *O. rhinoceros* on Guam is estimated to take between 3.5-8 months according to life history estimates (Moore 2019). *O. rhinoceros* have

been successful in colonizing many Pacific islands. The first two World Wars contributed to an initial population expansion due to increased transportation between Pacific Islands, and war damage on palm trees (Bedford 1980.) Both war and natural disasters typically result in numerous dead standing palms, rapidly increasing population growth. This can also occur when larvae are inadvertently transported in organic matter such as mulch. Oviposition in mulch combined with agricultural transport, can expand a population to new parts of an island easily. The lack of specificity with adult breeding and oviposition sites contributes to why *O. rhinoceros* is difficult to control once a population has established. With the Oʻahu infestation, experiments indicated that smaller particle mulch was the oviposition preference of wild caught females (Manly et al. 2018). Manly (2018) suggested that milling mulch piles with larger pieces of mulch could deter oviposition or decrease beetle reproductive success. Furthermore, this research indicates that searching the bottom of mulch piles will narrow the search for larvae.

Before its recent expansions, *O. rhinoceros* had an endemic range that included much of Southeast Asia. This encompassed West Pakistan, India, the Maldive Islands, Ceylon, Hainan, Taiwan, Hong Kong, Vietnam, the Malayan Peninsula, Bali, Islands of Java, Sumatra, Lombok, Kalimantan, Celebes, Ceram, and Indonesia (Bedford 1980). Since then, its range has expanded extensively, facilitated by war transport and damage, tropical storms, and especially globalization. From World War Two to modern day, populations of *O. rhinoceros* have been found in Western Samoa, American Samoa, Fiji, Tonga, Tokelau Islands, Wallis Island, Palau, Papua New Guinea, and Oʻahu, Hawaiʻi (Bedford 1986, Marshall et al. 2016).

Oryctes rhinoceros nudivirus Control and New Biotype

However, up to a point, all of those invasions to new islands were controlled with the use of *Oryctes rhinoceros* nudivirus (OrNV), which was found to effectively target the beetles after a new introduction. Due to the nudivirus, *O. rhinoceros* was considered a pest that could be controlled in the 1960's and 1970's. On islands with large populations, there were beneficial results from mass releasing nudivirus-infected adults to spread the virus to uninfected areas of the island (Zelazny and Alfiler 1991). Once *O. rhinoceros* adults are infected, they are able to spread the virus to other breeding sites, which results in a significant reduction in lifespan and oviposition. In the Maldives, multiple strains of a nudivirus were released with evidence of slight resistance to certain strains in their *O. rhinoceros* population, while other strains were extremely effective in population reduction (Zelazny et al. 1990). Classical biological control was tested with *Scolia ruficornis* wasps, but mass rearing and release was not explored for *O. rhinoceros* population control (Catley 1969).

The dynamic of *O. rhinoceros* infestations changed with its introduction on Guam, where the release of OrNV beetles to combat the recent infestation, made no impact (Marshall et al. 2017). This was then hypothesized and proven to be a new haplotype of *O. rhinoceros*. This would be characterized as CRB-G, which is resistant to OrNV exposure. Marshall et al (2017) explains that CRB-G has been identified in Port Moresby, Papau New Guinea; Oʻahu, Hawaiʻi, and the Solomon Islands. Reil et al. (2018) hypothesized that a reverse invasion could occur, where the virus-resistant haplotype could reenter areas that were previously managed by OrNV. Biotyping varied geographic populations revealed that Taiwan, Guam, and Hawaiʻi had the CRB-G haplotype, while American Samoa, Hainan, and Thailand all had the susceptible (CRB-S) biotype (Figure 3). Palau had both haplotypes present. The origin of CRB-G is still undetermined, but the presence of it in its native range of Taiwan could indicate an incipient

second invasion. Either way, without the OrNV management tool for CRB-G, new methods need to be tested to manage *O. rhinoceros* in its new invasive range.

Figure 3: CRB haplotype map. (Green markers indicate native range, brown indicate first detected in 20th century, red markers indicate first detected in 21st century. Open circle indicates CRB-G biotype detection, Filled circle indicates population is entirely CRB-G biotype. Moore 2019 http://aubreymoore.github.io/crbdist/mymap.html)

Integrated Pest Management Approaches

Mechanical control and sanitation are integral tools to slow the spread of *O. rhinoceros* and provide long term control after its introduction. This involves the removal of dead palm trunks, burning palm debris, surveying potentially infected sites and trapping surveys (Bedford 1980, Hallett et al. 1995). Pulverization of logs and potentially infested material are also effective sanitation methods. In Malaysia the planting of ground cover crops over breeding grounds showed some efficacy as well (Manjeri et al. 2014). *Centrosema pubescens* and *Pueraria javanica* are both low growing perennial herbs that decreased larval growth by covering the organic matter that could potentially have been used for oviposition.

Adult trapping remains one of the most accurate ways to monitor and assess infestations, and there has been constant effort to improve their efficiency. The vane trap is frequently utilized

for catching *O. rhinoceros* adults and can be easily hung up with pheromone lures (Bedford 1980). Lure development has shifted over the past few decades, with chrislure (ethyl dihydrochysanthemumate) as the conventional attractant for the vane traps. This shifted to ethyl chrysanthemumate, which was later proven to be more effective (Maddison et al. 1973). In an attempt to further increase the efficacy of this trap, a different compound was isolated from *O. rhinoceros* specimens leading to the use of ethyl 4-methyloctanoate in vane traps (Hallett et al 1995). This is the conventional trapping pheromone that is used today in both vane and barrel traps.

Guam testing

The infestation of *O. rhinoceros* on Guam started in 2007 and was initially contained to a small region of the island. The eradication program used mass trapping, sanitation, and detector dogs; but the infestation spread to all parts of the island by 2010 (Moore 2019). Population suppression was attempted with OrNV and failed due to resistance, which led to the discovery of the CRB-G biotype mentioned earlier. Typhoon Dolphin, in 2015, increased the population density of *O. rhinoceros*, with the decaying plant material in rural areas providing numerous breeding sites that could not be accessed for beetle control. With such a widespread population, extensive research has been conducted with minimal restrictions in recent years.

One study in Guam looked at using radio-tagged beetles to locate new breeding grounds. This involved releasing multiple adults that could be tracked and lead to success in finding new sites. However, there were beetles that they were not able to recover (Moore et al. 2016). This was due to external factors including inaccessible locations and beetles flying out of range of the receiver. This research provided valuable information such as the flight distance for most of the specimen (52.8m to 564.6m).

Another method that was tested was the use of acoustics for *O. rhinoceros* detection. Acoustic monitoring techniques have been used for a variety of insects, typically implemented by detecting vibrations and then recording the frequency. These frequencies are species specific and have been recorded for both *O. rhinoceros* larvae and adults (Mankin et al. 2009, Mankin et al. 2011). The adults produce distinctive chirping sounds that have been recorded during various activities. This includes feeding on dead palms, live palms, or movement within the soil. These frequencies were recorded and isolated, with potential for field detection in the future. The inclusion of acoustic detection methods in a control program is feasible to implement and the success on Guam could pave the way for Oʻahu testing (Mankin and Moore 2010). This would help with detecting insects in healthy trees before feeding symptoms emerge and could improve surveying methods for the urban infestation on Oʻahu.

The infestation of *O. rhinoceros* in Guam spread throughout the entire island within a few years despite people implementing a similar eradication program to that which is currently being conducted on Oʻahu (Moore 2019). Oʻahu has avoided a major outbreak but faces the same challenges in dealing with CRB-G.

Oʻahu Population and Chapter Conclusion

CRB-G was first detected on Oʻahu, Hawaiʻi, near the naval Joint Base Pearl Harbor-Hickam in 2013. Since then it has slowly expanded its range despite an intensive eradication program. Pearl City and Iroquois Point have harbored consistent populations. Some minor expansions to Mānoa and Waimanalo have been detected by trapping, but no known establishment has occurred in those locations. There has been difficulty in eliminating or even finding breeding sites in infested areas and detector dogs are being trained to locate them. Research funding has been provided to help find new ways to control the Oʻahu population.

Epsom salt trials ($MgSO_4$) were successful at killing larvae in breeding material, but preemptive application to all potential breeding sites is infeasible. Sandlund et al. (2002) noted that containment can end suddenly for invasive species, resulting in a population that cannot be controlled.

The only successful eradication so far of *O. rhinoceros* was implemented on Niuatoputapu or Keppel Island, which is a very small island and involved most of the islanders taking an active role in locating and eliminating breeding sites (Bedford 1980). This eradication approach was successful. Even with all of the control methods researched, it is very difficult to accomplish complete eradication without an intensive process and involved community. The role of the citizen scientist to recognize and report invasive species to the scientific community has become more popular in recent years (Gallo and Waitt 2011). Public outreach on Oʻahu is an integral part of the eradication program and can help identify new populations and breeding sites. The following chapter will focus on the use of augmentative biological control, which could be added to the current integrated pest management (IPM) program for controlling *O. rhinoceros* in infested areas of Oʻahu.

CHAPTER 2. Entomopathogenic fungi and nematodes as potential augmentative biological control agents against the coconut rhinoceros beetle.

Entomopathogens occur naturally in many soils and can be utilized to target pest species if isolated, propagated, and applied properly. Entomopathogenic fungi (EPFs) are very diverse and occur in a range of fungal taxa, including the Phylums Zygomycota, Ascomycota, Deuteromycota, Chytridiomycota, and Oomyctoa (Shah and Pell 2003). The classes Entomophthorales and Hyphomycetes, in Zygomycota and Deuteromycota respectively, include most of the EPFs species researched today. Their life cycle has the ability to synchronize with insect host stages and the ideal environmental conditions for infection. Shah and Pell (2003) detail the impact of EPFs on a wide diversity of insect orders including many Hemipteran pests such as aphids, scales and white flies. They can target many Dipteran species such as mosquitoes, as well as some Lepidopteran, Orthopteran, and Coleopteran hosts.

Hyphomycetes includes the genus *Metarhizium* which is very effective for insect control. Insect mortality occurs after the fungus has penetrated the insect body, and releases toxins that overwhelm the host defense response. After contact with the insect host, fungal hyphae penetrate the insect cuticle and then consume the nutrients within the body cavity (Bidochka 2008). The conidia grow on the outside of the cadaver, which can infect other nearby insects. After sporulation, the spores can disseminate to new hosts or persist until conditions are favorable. A study assessing EPF dissemination potential showed that *Metarhizium* spread up to 10 meters to uninfected *O. rhinoceros* larvae contained in above ground breeding boxes (Fernando et al. 2010). Besides the potential for spores to spread, EPFs have the potential to persist after application at high infectivity levels. *Metarhizium* spp. was applied to the top layer of soil in *O. rhinoceros* larval feeding sites, resulting in high infection rates and persistence in the soil for

more than a year (Latch and Falloon 1976). For preemptive soil application insect hosts do not have be present for initial application. Resting spores can form that persist in the soil, and activate once the host is available.

There are a few successful examples utilizing EPF biological control as a long term practice. The gypsy moth, *Lymantria dispar*, was a widely spread pest species in the northeastern United States after an accidental introduction. *Entomophaga maimaiga* was found to be effective in control and has been utilized to reduce populations (Shah and Pell 2003). This was accomplished by using infective cadavers, which spread the fungus to healthy larvae, and thus protect the oak trees. Another effective EPF application method is through the dispersal of a mycoinsecticide. Shah and Pell (2003) highlights another EPF success in Africa, where the widespread devastation due to the Desert locust, *Schistocerca gregria*, and several other grasshopper species, combined with the environmental concerns of chemical applications led to the search for different control methods. Using *Metarhizium* fungus located in Niger, West Africa, they were able to formulate, develop, and patent Green Muscle®, a mycoinsecticide that caused greater than 70% mortality after two weeks.

In developing an IPM plan including EPFs, optimizing the release method and frequency for EPF treatment, is essential for successful treatment. In some approaches, monthly releases are necessary to reduce a population below economically damaging levels. The fungus can also persist throughout the season if the pest is consistently susceptible, allowing EPFs to maintain their presence in the area as an inoculative treatment.

This is a characteristic that EPFs have in common with entomopathogenic nematodes, where host availability determines control throughout the season. Entomopathogenic nematodes (EPNs) are free living soil nematodes that can be utilized to target specific insect hosts.

However, they are very susceptible to high temperature, dehydration, and sunlight (Leite et al. 2018). These disadvantages can be countered with proper application timing and release methods. Liete et al. (2018) showed that nematode storage and transportation can be improved with vermiculite and polyacrylamide gel as protectants to prevent loss of specimens before field application. The most commonly utilized nematode Families in insect biological control are Steinernematidae and Heterorhabditidae. Steinernematidae utilizes the bacterial symbionts *Xenorhabdus*, while Heterorhabditidae uses *Photorhabdus,* enterobacteriaceae which are pathogenic to the insect hosts (Stuart et al. 2006). The nematode infective juvenile (IJ) stage enters the insect hosts and releases the enterobacteriaceae which weakens the insect immune system. From there, host mortality can occur within 24-48 hours after contact, and the nematodes and bacteria feed within the host for multiple generations. Then the IJs exit the insect, with thousands emerging seeking a new host.

Rearing can be relatively easy with favorable insect hosts such as mealworms, *Tenebrio molitor* and wax moths, *Galleria mellonella* (Shapiro et al. 2012). *In vivo* production is utilized, in which favorable hosts are inoculated with EPNs and the cadaver is then placed on a white piece of paper surrounded by water. This method is known as the White trap, which results in emerging IJs migrating to the water, and also allows for species quantification and identification (White 1927). The amount of IJ emergence varies by the size of the insect host, which is why *G. mellonella* is a preferred host for large scale production with only 25-200 IJs needed for infection. *In vitro* application uses a culture of the appropriate bacterial symbiont to grow entomopathogenic nematodes. There are tradeoffs to each approach, where the *in vivo* growth has low costs, but high labor requirements, while *in vitro* growth has high initial costs but minimal labor requirements.

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The simplicity of rearing EPNs has facilitated their development into biological control agents that are filling a growing market of bio-pesticides, with some potential field success. In the case of the Colorado potato beetle, *Leptinotarsa decemlineata*, it was determined that *Steinernema feltiae* and *S. carpocapsae* yielded higher adult mortality than other EPN strains, along with sufficient control of earlier instar larvae at 20°C and 25°C (Tridan et al. 2009). The strains tested in this study caused promising mortality in *L. decemlineata* and have been hypothesized to perform well in the field if developed into an IPM program. EPNs have already been developed commercially for agricultural application in some crops. Control of the alfalfa snout beetle, *Otiorhynchus ligustici* was verified with field application on 87 fields (Shields and Testa 2017). *In vivo* rearing was developed for large scale production with *S. carpocapsae, S. feltiae,* and *Heterorhabditis bacteriophora* for this field testing. Making applications at sunset with a fertilizer stream nozzle, larval control was achieved for the first year after application, with EPN detection in the soil continuing for six years after application. The EPNs persisted in the soil with a corn/alfalfa crop rotation and helped reduce alfalfa damage during the entire six-year period. The success with EPNs in agriculture has allowed them to be accepted by some growers as a feasible method for insect control in some crop systems.

Both EPNs and EPFs are potentially valuable additions to pesticides in an IPM program because they are effective under appropriate application conditions, environmentally friendly, and have no negative impacts on human health. In a study looking at control of the invasive southern masked chafer, *Cyclocephala lurida*, entomopathogen application yielded similar or better control in comparison to systemic insecticides (Wu et al. 2014). Two marketed EPF products containing *Beauveria bassiana* or *M. anisopliae* were tested in conjunction with *H. bacteriophora* on third instar larvae of *C. lurida*. The EPF-EPN interaction produced additive

effects in target pest mortality under laboratory conditions. However, this study also showed that the commercial EPF strains were not as suitable for this pest and more virulent strains should be developed. It can be useful to isolate the strains from the target species under laboratory conditions first, and then to verify that the pest will be targeted in the field by the entomopathogen. It is possible that the EPF/EPN choice may not reduce pest populations in the field below the economic injury level, even if laboratory trials have high mortality. If field mortality is confirmed, this will ensure that the given isolate is the most effective method for control of that pest. This process can be applied to the *O. rhinoceros* infestation on Oʻahu, ultimately determining if entomopathogens can reduce larval populations in the field.

The efficacy of locally extant entomopathogens on Oʻahu has been assessed on larvae of *O. rhinoceros* with varied results (See Chapter two results). Although *Metarhizium* was tested in 1913 on the coconut rhinoceros beetle (Bedford 1980), there has been no EPF or EPN research conducted on Oʻahu's CRB-G infestation. In field tests on Guam, *Metarhizium magus* yielded larval field mortality reported by Dr. Roland Quitugua. The *O. rhinoceros* population on Guam remains widespread, but this could change if future strains are found with higher mortality rates. On Oʻahu, the *O. rhinoceros* infestation has a small population that is difficult to target because infestations are not easily located. This makes it very challenging to target potential larval development sites with EPFs. If EPF's could be mass released, this could facilitate *O. rhinoceros* population suppression. For example, there is another invasive species the oriental flower beetle, *Protaetia orientalis,* which could potentially be exploited to achieve improved *O. rhinoceros* management by EPFs. *O. rhinoceros* and *P. orientalis* are morphologically similar at earlier life stages and develop in the same breeding sites (Watanabe and Melzer 2017). If entomopathogens could be found that cause significant mortality in both species, this would be an effective way to

preemptively treat the infested areas of Oʻahu for *O. rhinoceros*. Hypothetically, fungal spores could constantly be recycled in breeding sites by *P. orientalis* and when *O. rhinoceros* adults oviposit in these areas, larval mortality should occur owing to increased spore presence.

Previously, 73 strains of *Metarhizium* spp. and *Beauveria* spp. were collected on Oʻahu by Dr. Jing Li in the University of Hawaiʻi at Mānoa Turf and Landscape Management Lab, with preliminary studies tested on lab reared *O. rhinoceros* to assess mortality rates. The following experiments narrowed this selection to the top five most virulent strains from ten promising strains. I also was encouraged to test these strains in conjunction with Epsom Salt ($MgSO_4$), which should decrease larval growth rates, while increasing larval vulnerability to entomopathogens. Promising strains for field testing were selected and grown on rice for preliminary trials. Two EPN species were reared and tested as well on *O. rhinoceros*. EPF testing was conducted on *P. orientalis.*

MATERIALS AND METHODS:

Laboratory trials with O. rhinoceros

O. rhinoceros specimens were collected in the field by CRB response personnel and then bred in captivity at the University of Hawaiʻi Arthropod Containment Laboratory (UH-ACL). Soil was previously collected by Dr. Jing Li from the Makua Keaau, Kealia Trail, Waimea Botanical Garden, Wahiawa Botanical Garden, Hoʻomaluhia Botanical Garden, Ewa Beach Park, Liliʻuokalani Botanical Garden, Makiki District Park, Ala Moana Beach Park, Ala Wai Golf Course, Lyon Arboretum, Koko Crater Botanical Garden, and the University of Hawaiʻi at Mānoa campus. Mealworms, *Tenebrio molitor*, were placed into these soil samples and

Metarhizium fungus was isolated from specimens onto PDA/protein plates. Earlier testing by Dr. Jing Li indicated that Lyon Arboretum (LA), Koko Head Botanical Garden (KO), and the University of Hawaiʻi at Mānoa campus (CP) strains were effective on *O. rhinoceros* first instar larvae. However, these tests were conducted using a small sample size and further experiments were needed to assess which strains would be the most virulent. *Metarhizium* samples were plated on a PDA/protein mixture (Becton, Dickinson and Company, Difco^{TM} Potato Dextrose Agar), which consisted of 9.75 g Potato Dextrose Agar powder, 2.5g Peptone Powder (Fisher Science Education), and 250 ml deionized water. This was autoclaved and poured into dishes (Fisherbrand Petri Dish, Slippable lid, 100mm x 15mm). Once the medium had set, fungus was plated using a wire loop.

Prior to pathogenicity trials, a 0.1% Tween 80 was prepared and poured into the fungal plates to create a spore suspended solution. A rubber scraper was used to scrape the fungus off of the agar without breaking the surface of the gel. This yielded a spore suspension for each treatment, which was then vortexed for one to two minutes until thoroughly mixed. This was then diluted by a factor of 100 with deionized water and vortexed again. This diluted solution was then quantified using a hemocytometer to assess the spore concentration. If the solution yielded $1x10^8$ or higher spore concentration, then the solution was acceptable for the trial. Another solution was then made with the same inoculum that was one fifth the concentration of the initial solution. If the spore counts were below the threshold, spores were scraped off of more plates and vortexed into the mixture until the desired spore concentration was achieved.

When first instar larvae were ready for the trial, they were transported to the Hawaiʻi Department of Agriculture Plant Quarantine (HDOA-PQ) facility for testing to reduce the chance for contamination of the captive laboratory reared population. In five different trials, multiple

strains of the fungi were tested and narrowed down to find the most efficient isolates. LA-003, LA-016, LA-022, LA-025, LA-026, LA-028 CP-003, CP-004, KO-001, and KO-002 were tested. This included promising strains that were tested by Dr. Jing Li, along with new strains that I hypothesized would be effective. In a sixth trial, 1X $MgSO_4$ concentration was mixed into the soil prior to fungal application, ideally slowing larval development. 1X $MgSO_4$ is reached by mixing 3.175 g of $MgSO_4$ with 100 ml of deionized water, and the uniform solution is added to a Magenta Box (6 cm x 6 cm x 7 cm) full of sterile substrate. At the test site, one ml of each treatment was pipetted into a cup with a first instar larva and then approximately 25 ml of sterile substrate was added to fill the cup. The substrate was composed of mulch milled through a 1.66 mm steel screen, yielding small particulate material, which was then autoclaved at 121°C. Individual cups were necessary to avoid cannibalism and decrease insect stress. The cups were stored in an incubator at 25°C. Each strain was tested on 16 larvae at the two concentrations, along with a 0.1%Tween 80 control, and an untreated control. Mortality recordings were made on days 3, 7, 10, and 14 by searching each cup for larval survival.

Field trial with O. rhinoceros

The *O. rhinoceros* for this experiment were collected in the field by the state CRB response team, from a breeding site near Leeward Community college on the south side of Oʻahu and transported in plastic screw top bottles to Waiwa Road in Pearl City. There is a Hanakehau, or learning farm located there where a controlled breeding site was set up.

Using this location would ensure that the beetles are not transported to uninfested locations, thus minimizing transportation and decreasing larval stress. To ensure that the experimental insects could easily be recovered after treatment, the experiment was housed in a

large plastic tub. The plastic tub was approximately 1.9 cm thick polyethylene with dimensions of approximately 91 cm tall and 135 cm by 50.8 cm width. Four plastic containers measuring 34 cm x 29 cm x 8.9 cm containing approximately nine liters of mulch were buried within the plastic breeding tub. Surviving larvae and dead larvae at the end of the experiment were recorded and transported in screw top plastic bottles to the building and placed in a walk in freezer.

There were few opportunities in which enough larvae were found in the field for testing. The first attempt at a field trial took place in November 2018. This trial was to be conducted with fungus suspension treated soil, but excessive rain flooded the trial and drowned all of the treatments. The second trial started in March 2019 and Strain LA-016 was inoculated on rice as a substrate to maintain the spores in the field. A similar method is used by Novozymes® to produce their Met52 Granular bioinsecticide. With this product, the recommended rate of application is to uniformly apply 500 g to 1.5 kg/m^3 of moist soil medium (Crop production Met52). Three boxes containing 12 or 13 larvae were treated with 0.550 kg of rice. These 14-liter boxes were filled with nine liters of the same mulch found at the original breeding site. A fourth box was set up as a negative control. There were varied larval stages collected in the field, and similar instars were distributed between the treatments. All of the boxes received 10 second instar larvae, with some first or third instar larvae mixed into the treatments as well. A 10g sample of the rice was quantified at 7.5×10^6 spores per ml. Observations were made on Day 7, Day 13, and Day 19.

Laboratory trial P. orientalis

First and second instar specimens were collected in mulch at Mililani Agriculture Park by the CRB response team. Specimens were divided based on health and size within each replicate

for each instar. Based on results from previous trials, isolate KO-002 was selected as the fungal strain for application. Fifteen milliliters of water and Tween 80 (0.1%) was prepared and poured into fungal plates. A rubber scraper was used to scrape the fungus off of the agar without breaking the surface of the gel. This was done twice leaving 30 milliliters of suspended spores for each treatment. Spore concentration was quantified using a hemocytometer and serial dilutions were then made as described. If the spore counts were below this threshold, spores were scraped off of more plates and vortexed into the mixture until the requirement was met. The spore count for this trial was 5.35×10^8 and 1.07×10^8 for each treatment. One milliliter was directly applied to both first and second instar larvae and they were placed in individual cups to avoid cannibalism and decrease insect stress. The substrate used was composed of mulch milled through a 1.66 mm steel small screen, yielding small particulate material. This was from the same material used for *O. rhinoceros* rearing. Each treatment had 4 replicates of 4 specimens each. The cups were stacked and stored in an incubator at 20°C.

OFB-1 trial

Another fungal suspension trial was conducted using *Metarhizium* isolated from a *P. orientalis* specimen (Figure 4). This followed the same protocol as laboratory trials 1-6 and was conducted at HDOA-PQ on *O. rhinoceros* 1st instar larvae.

Figure 4: *Metarhizium* growth on OFB

Entomopathogenic Nematode Laboratory Trial

Coconut rhinoceros beetle specimens were collected in the field by CRB response personnel and then bred in captivity at the UH-ACL. *Heterorhabditis indica* OM160 originated from the East side of Oʻahu and were provided by Dr. Roxana Myers (USDA-ARS) within mealworm *Tenebrio molitor* cadavers, and extracted on White traps. Dr. Koon-Hui Wang (UH Mānoa) provided locally isolated *Steinernema feltiae* nematodes. A wax moth colony, *Galleria mellonella*, was started with specimens collected from local beekeepers. The wax moth larvae were used to rear the nematodes in larger quantities. Aliquots of 1000 and 500 infective juveniles (IJs) per milliliter were diluted for different application rates using a rectangular counting tray (Luc et al 2005). This tray has four rectangular boxes within a large rectangular box. Counting the four inner boxes is representative of half of the sample in the five milliliter tray.

The experiment took place in an incubator (30°C) in the UH-ACL and tested mortality on first instar larvae. Individual cups with substrate were necessary to avoid cannibalism and decrease insect stress. The substrate was composed of mulch milled through a 1.66 mm steel screen, yielding small particulate material. Each treatment had four replicates of four larvae each. The treatments included an untreated soil control, water soil control, 996 IJ's of *S. feltiae*, 498 IJ's of *S. feltiae*, 993 IJ's of *H. indica*, and 497 IJ's of *H. indica*. For all of the treatments excluding the soil control, one milliliter of treatment was directly applied to the specimen prior to the cup being filled with mulch. Observations were made daily from day three after treatment to day seven, and every other day up to day 12.

Statistical analyses were conducted for these trials running a one way Analysis of Variance using R Studio. This was run to assess the difference between treatments in relation to mortality, including blocks as factors. Blocking was included to see if the location of larval cups in the incubator made a difference between treatments. If a significant effect was evident, a Least Significant Difference mean separation test was run. Interval plot graphs were generated using Minitab 14.

RESULTS:

These trials helped identify the most virulent strains that were collected. Early trials indicated CP-003, LA-016, KO-001, and KO-002 were the most effective strains, while LA-003 and CP-004 were not as lethal to *O. rhinoceros* larvae (Figure 5). Repeating this trial narrowed this down further to only LA-016, KO-001, and KO-002 (Figure 6). Due to the high success rate of LA-016, a trial was set up to assess more strains from Lyon Arboretum. Figure 7 shows the addition of LA-022, LA-025, LA-026, and LA-028. Of these, LA-025 and LA-026 showed promising results and were included in future trials. All of these strains were then tested, with promising mortality results from KO-001, KO-002, LA-016, LA-025, and LA-026 (Figure 8). Unfortunately, it was difficult to indicate whether EPF mortality was the direct cause of death in some cases, because the cadavers would typically desiccate in the cup, but spore presence was noticed on some specimens.

Figure 5: EPF trial 1. Results of entomopathogenic fungus isolate screening on coconut rhinoceros beetle larvae. Mean mortality per trial; columns with the same letters were not statistically different, LSD, P > 0.050. ($F_{3,13}$=6.2228, P < 0.0001)

Figure 6: EPF trial 2. Results of entomopathogenic fungus isolate screening on coconut rhinoceros beetle larvae. Mean mortality per trial; columns with the same letters were not statistically different, LSD, $P > 0.050$. ($F_{3,13}=6.217$, $P < 0.0001$)

Figure 7: EPF trial 3. Results of entomopathogenic fungus isolate screening on coconut rhinoceros beetle larvae. Mean mortality per trial; columns with the same letters were not statistically different, LSD, $P > 0.050$ ($F_{3,15}= 4.526$, $P < 0.0001$)
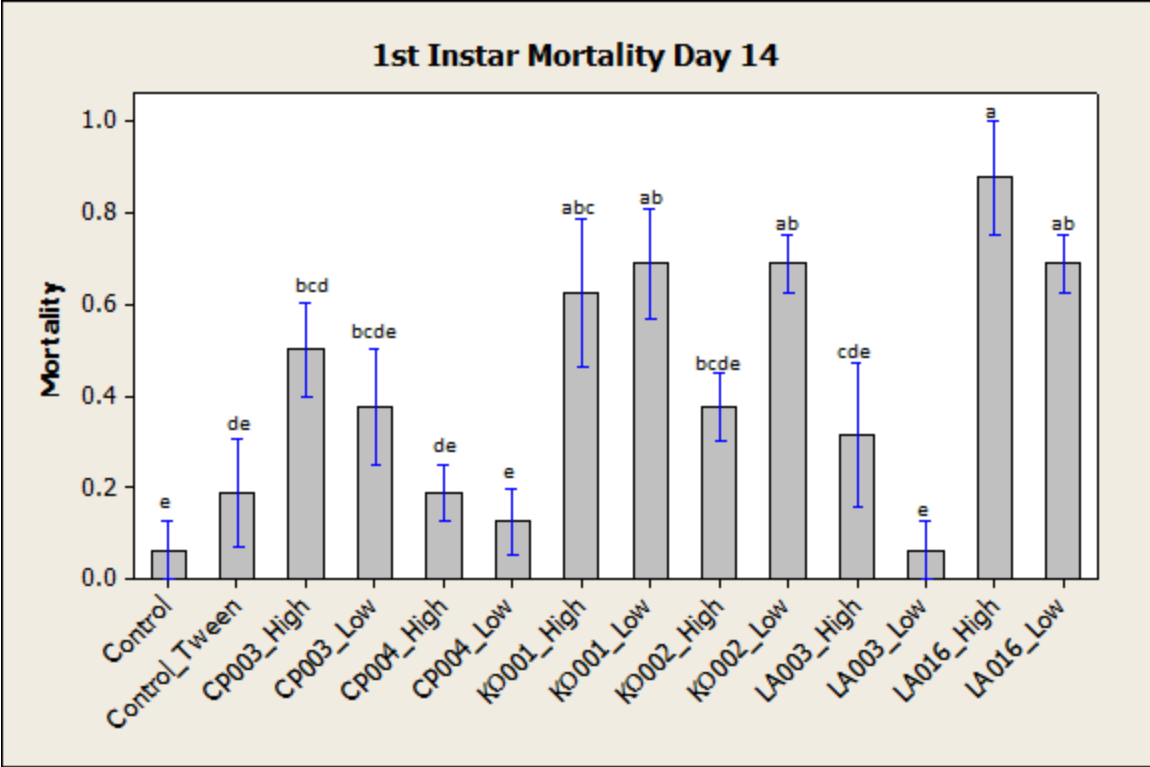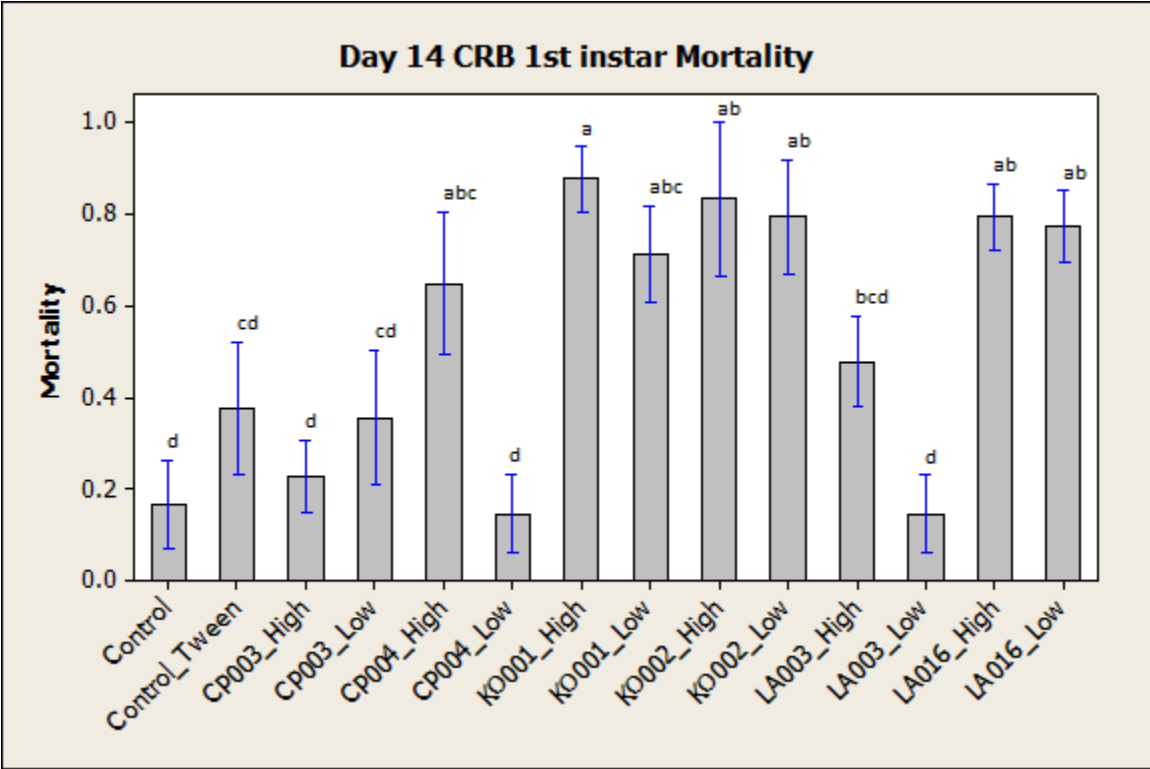
Figure 8: EPF trial 4. Results of entomopathogenic fungus isolate screening on coconut rhinoceros beetle larvae. Mean mortality per trial; columns with the same letters were not statistically different, LSD, $P > 0.050$. ($F_{3,11}=6.489$, $P < 0.0001$)

The mulch used for all these trials was very similar, but there were some issues with compost that started after the fourth trial. This was first noticed in the lab colony and evident in Figures 9 and 10. EPF Trial 5 had high control Tween and control mortality and no significance as a result of this. This was also what happened with the $MgSO_4$ trial (Figure 10). $MgSO_4$ did prevent second instar molting and increased mortality.
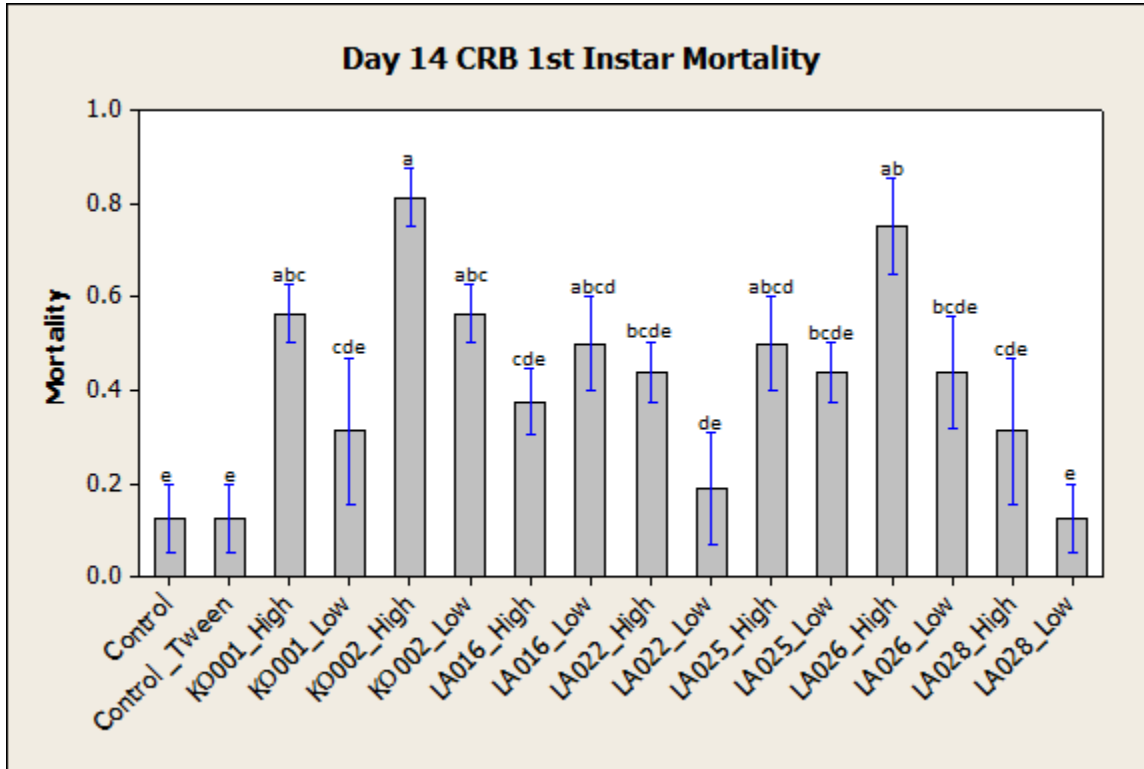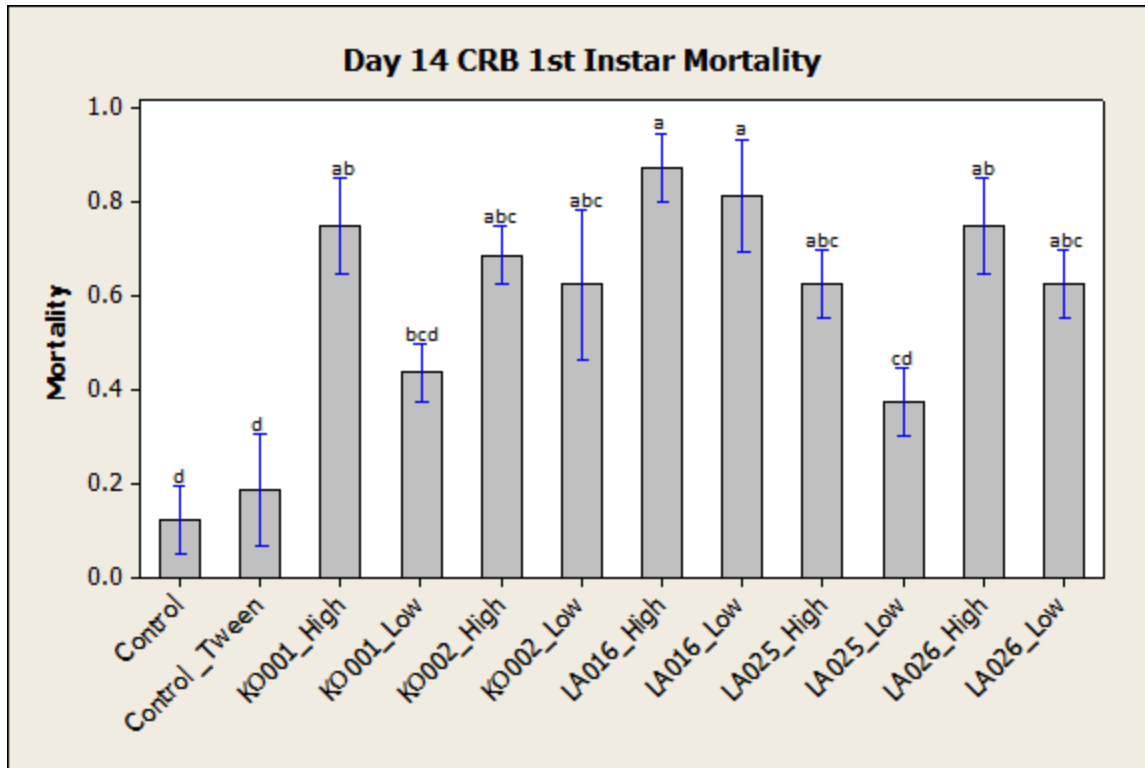
Figure 9: EPF trial 5. Results of entomopathogenic fungus isolate screening on coconut rhinoceros beetle larvae. Mean mortality per trial; columns with the same letters were not statistically different, LSD, P > 0.050. ($F_{3,11}$=1.7427, p = 0.1068)

Figure 10: EPF trial 6. Results of Epsom salt treated substrate and entomopathogenic fungus isolate screening on coconut rhinoceros beetle larvae. Mean mortality per trial; columns with the same letters were not statistically different, LSD, P > 0.050. ($F_{3,11}$=0.8351, p =0.607)

The *P. orientalis* trial yielded no statistically significant results (Figure 11); however KO002 yielded almost 70% mortality on second instar larvae, indicating that there should be further EPF trials. The mortality in the control Tween and control mortality were higher than expected, but this could be due to a variety of factors related to field caught specimens. The incubator settings were slightly different than previous *O. rhinoceros* trials as well. A fungal strain isolated from a control specimen in this trial was tested in September 2019. It yielded greater than 65% mortality ($P < 0.0001$) on *O. rhinoceros* first instar larvae, with minimal mortality associated with control Tween specimens (Figure 12).

Figure 11: EPF trial 8. Results of entomopathogenic fungus isolate screening on oriental flower beetle larvae. 1 after treatment indicates first instar larvae and 2 indicates second instar larvae. Mean mortality per trial; columns with the same letters were not statistically different, LSD, P > 0.050. ($F_{3,11} = 2.87$, p = 0.09284)
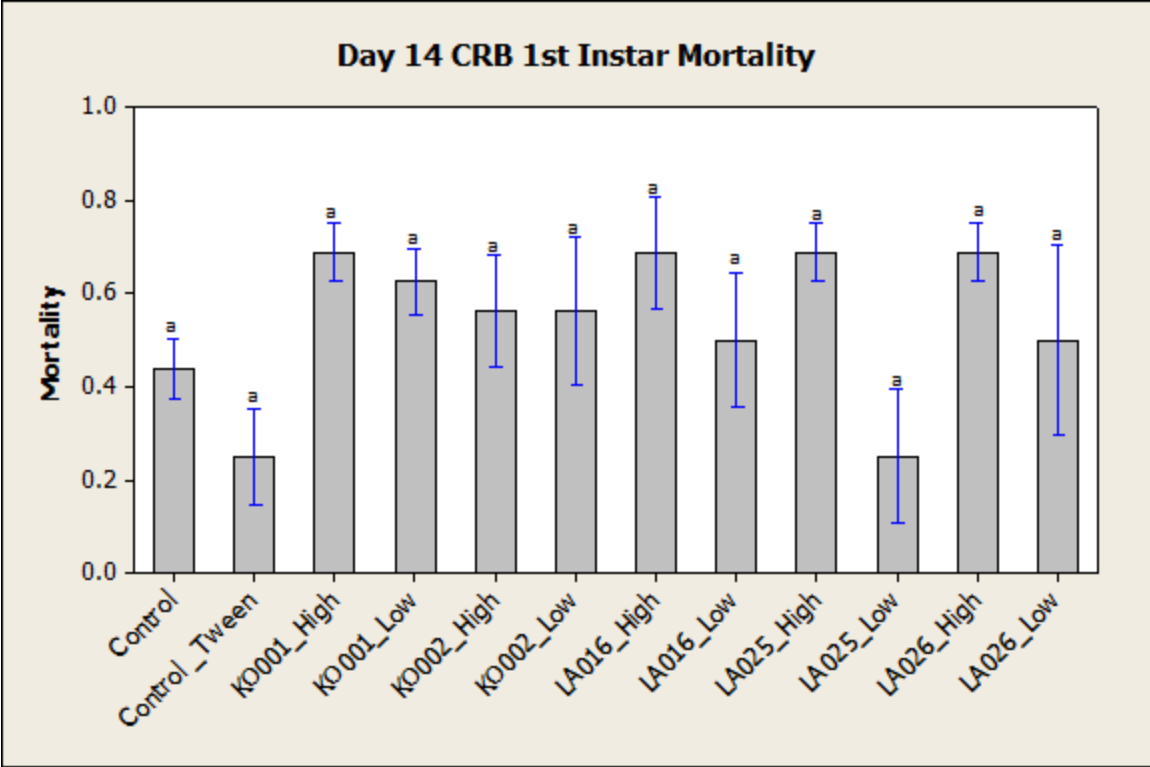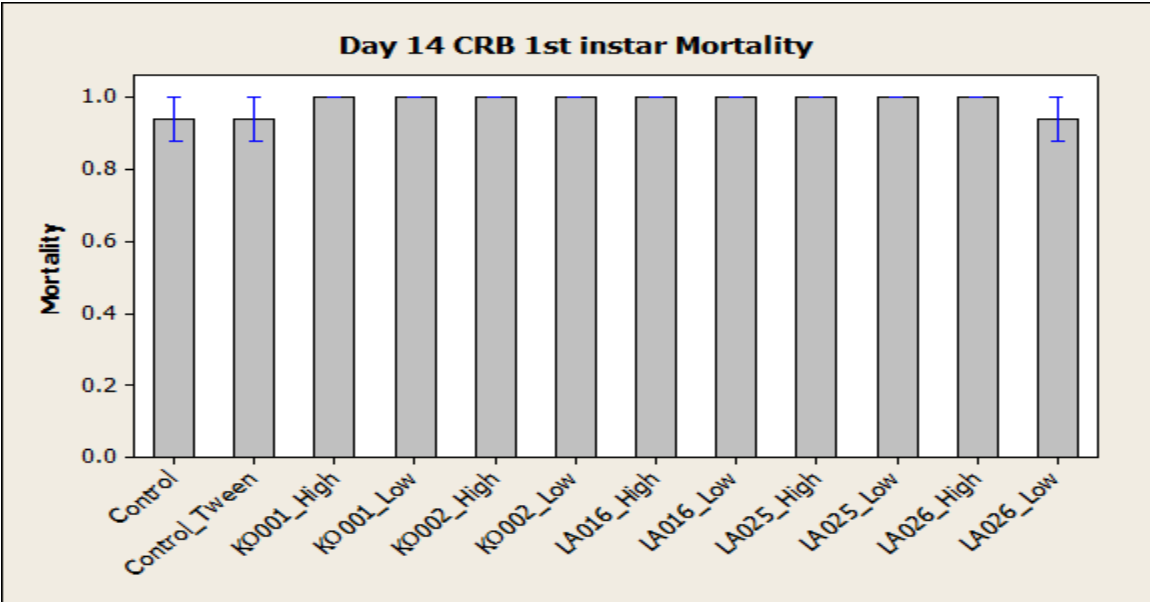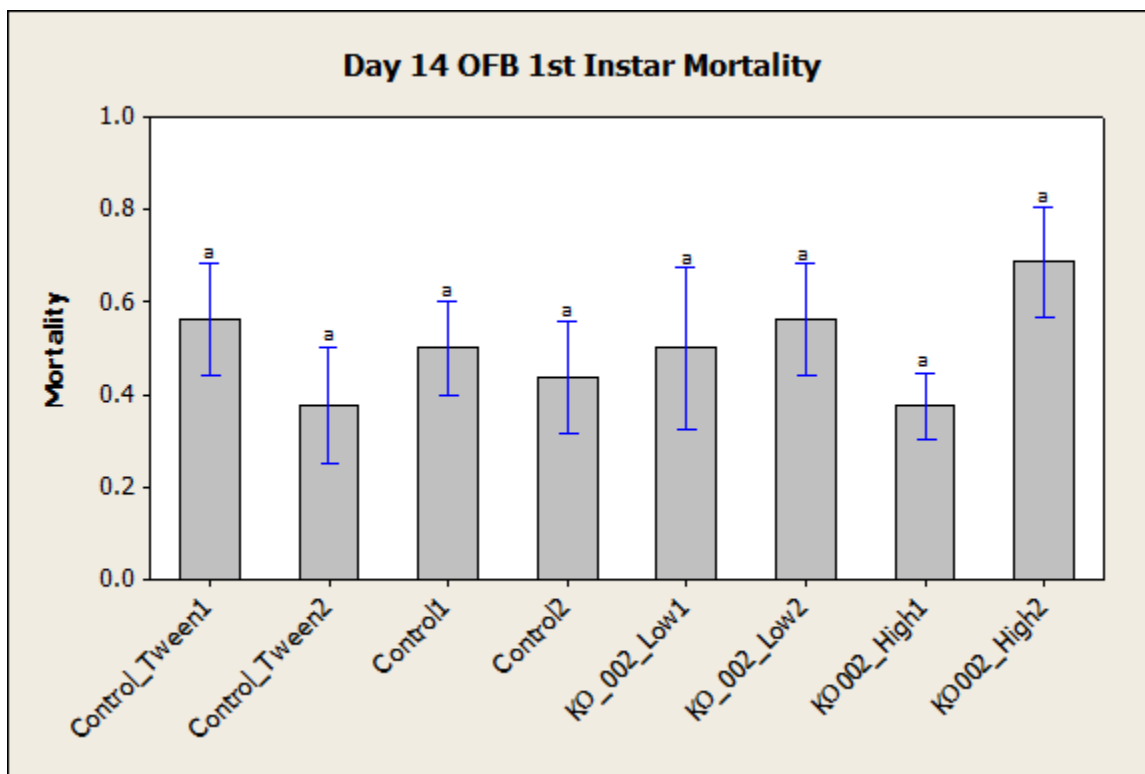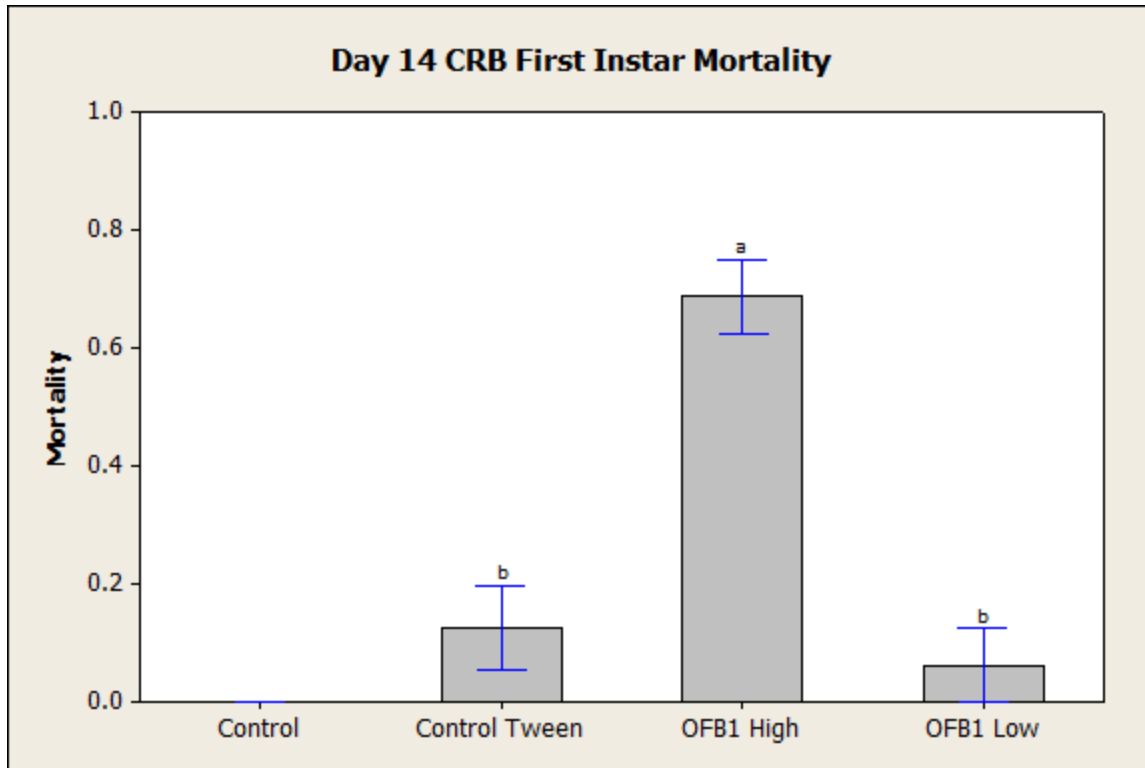
Figure 12: EPF Trial 9. Results of entomopathogenic fungus isolate screening on coconut rhinoceros beetle larvae. Mean mortality per trial; columns with the same letters were not statistically different, LSD, P > 0.050. ($F_{3,3}$= 46.2, P < 0.0001)

The *O. rhinoceros* field trial yielded 38-41% mortality in two of the replicates, but all of the specimens survived in the last replicate (Figure 13). With equal fungal treatments in all three boxes, it is interesting that one set of larvae were entirely unaffected. Only two second instar cadavers were recovered from fungus treated boxes, and no spores were visible to isolate for a future trial. The mortality for the remaining specimens could be attributed to the fungus in these cases, with decomposers in the box consuming the dead specimens. This was observed on a third instar cadaver that was almost entirely decomposed, with no visible conidia to isolate. Due to the lack of specimens found in the field, there has been no opportunity to repeat this trial or try it with more replicates. Lastly, the EPN trial yielded no significant results but did cause some

mortality in all of the treatment groups with zero control mortality by day six (Figure 14). Day

seven was the first sign of control specimen mortality in the trial.

CRB Field Mortality Day 19
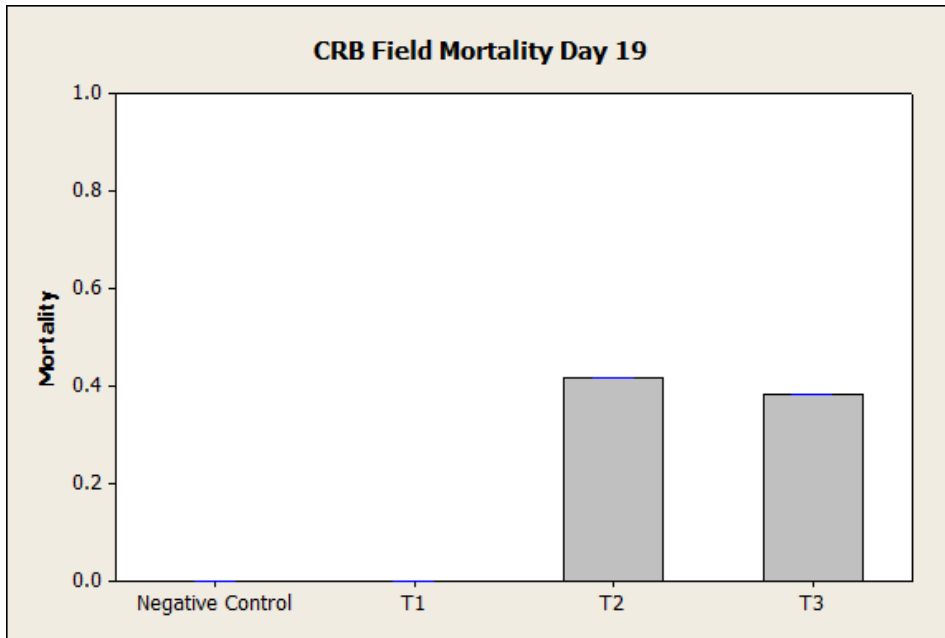
Figure 13: Fungal Field Trial 1. Results of entomopathogenic fungus isolate screening on coconut rhinoceros beetle larvae. T1, T2, and T3 represent the same EPF rice treatment. Mean mortality per trial; columns with the same letters were not statistically different, LSD, P > 0.050.

Day 6 CRB 1st Instar Mortality

Figure 14: EPN trial Day 6. Results of entomopathogenic nematode isolate screening on coconut rhinoceros beetle larvae. Mean mortality per trial; columns with the same letters were not statistically different, LSD, P > 0.050. ($F_{3,5}$=1.55, p = 0.23321).
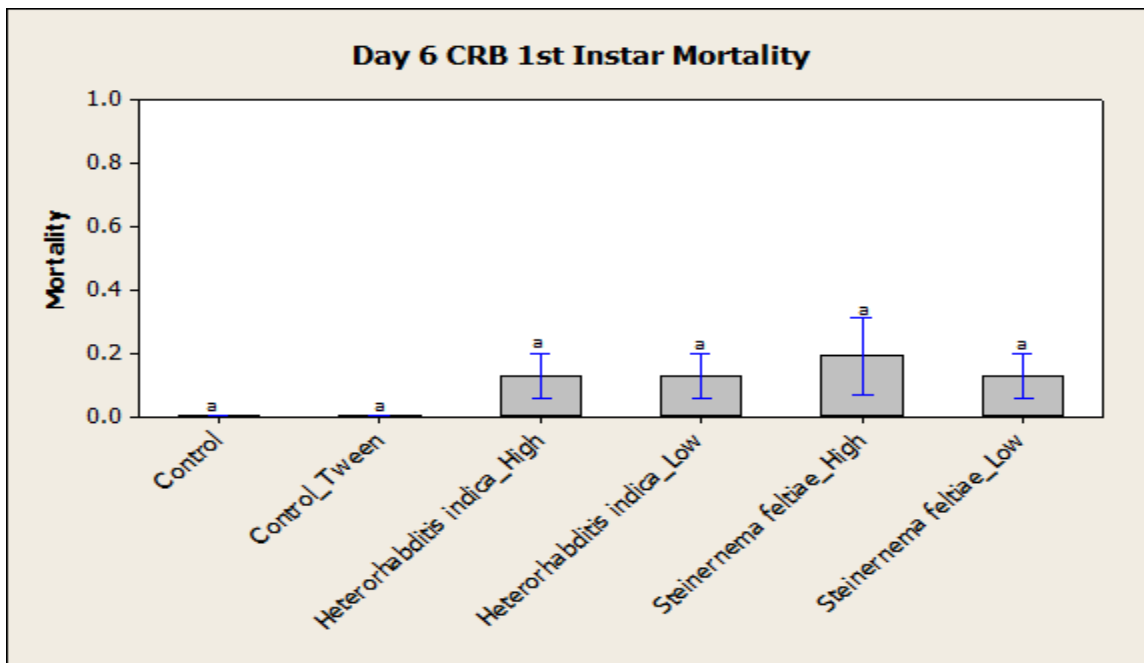
DISCUSSION AND SUMMARY:

The utilization of entomopathogenic fungal isolates for insect pest management has been accepted for many years, with over 171 mycoinsecticide products developed for various pests (Maina et al. 2018). Within this market are 12 dominant fungal species that have been altered for different application methods, using wettable powders, oil dispersions, granules, baits, suspensions, spray, and contact based powders. A recent estimate indicates that global bioinsecticide usage has grown by 10% annually, with mycoinsecticides making up 27% of this market. The demand for entomopathogenic organisms is unlikely to decrease as food security and biodiversity are continually threatened by pest species. As public approval and knowledge about mycoinsecticides increase, it is likely that this trend will continue and products will increasingly enter the market for IPM programs.

As mentioned earlier, finding an isolate that can target both *P. orientalis* and *O. rhinoceros* would be advantageous for targeting multiple invasive species on Oʻahu concurrently. There was surprising behavior in the *P. orientalis* trial, in which some of the specimens exhibited an encapsulating behavior that protected some of the larvae from the spores (Figure 15). This behavior could help them avoid spores in the field. However, it is unlikely to entirely prevent infection because it inhibits their growth to new instars. There was high mortality in this trial, but no statistical significance. This could be attributed to the use of field-caught specimens. With *O. rhinoceros* trials, use of specimens from a lab colony ensured all of the individuals were from the same generation and of similar quality. Collection and transportation could have stressed *P. orientalis*, affecting the survivability of the larvae.

Metarhizium sp. was recovered from a control *P. orientalis* specimen, which could have acquired the fungus in the field or through possible lab contamination. A new strain (OFB-1) was

isolated and yielded significant results against *O. rhinoceros* in a subsequent trial. Only the high

concentration yielded statistically significant mortality, suggesting that higher spore density

increases larval mortality rates with this isolate (Figure 12). From that trial, one *O. rhinoceros*

specimen was recovered that yielded conidial growth (Figure 16). This was plated on PDA and

will be utilized for future trials as strains CRB-1.1 and CRB-1.2. Strain OFB-1 will be tested in

conjunction with these new strains in a future trial.

Figure 15: 1st instar casing.

Figure 16: *O. rhinoceros larvae* infected from OFB-1

The EPN trials did not indicate that they would be effective for *O. rhinoceros* larval

control. In the trials that were conducted, all of the dead *O. rhinoceros* larvae had EPNs emerge

onto White traps and these were reared on wax moths, yielding high enough concentrations for a

follow up trial. In the subsequent trial there was less mortality than the first EPN trial, indicating

that these varieties were not efficient in yielding *O. rhinoceros* mortality. Further research could

be conducted to identify and test different EPN species on larvae.

There have been successful experiments testing formulations of *Metarhizium* spp. on

various life stages of *O. rhinoceros* (Moslim et al. 2007, Moslim et al. 2009, Moslim and

Kamarudin 2014). Trials in India indicated that *M. anisopliae* could be applied to

vermicomposting piles with limited effect on decomposers, while eliminating *O. rhinoceros*

larval populations (Gopal et al. 2006). In Malaysia, rotting heaps of oil pam residues containing

all life stages of *O. rhinoceros* were sprayed and cover crops were planted overhead. This strategy created favorable conditions for fungal growth and there were reductions in all life stages 8 months after treatment (Moslim et al. 2007). In this trial they observed that the dead larvae were shrunken or covered with whitish fungus that matured to green. In EPF laboratory assays 1-5, similar larval remains were noticeable and associated with EPF mortality. Besides sprayable formulations, other granular methods have been tested against *O. rhinoceros* populations. Multiple studies have tested granular products such as Kaolin, rice bran, and lastly maize supplemented with palm kernel cake (Moslim et al. 2009, Moslim and Kamarudin 2014). Both of these experiments yielded greater than 90% larval mortality and explored different ways to increase sporulation and conidial density with these granular methods. Their methods could be implemented into the next field trial on Oahu by making improvements to the current rice procedure and altering the design of the trials.

Field conditions can inhibit the success of augmentative biological control agents, however there have been improvements in industry products to overcome these challenges (Maina et al 2018). It can be difficult to treat hard to reach field sites and novel methods have been tested to improve EPF dispersal. Another trial in Malaysia showed the potential for adults to cause larval mortality by dissemination of *M. anisopliae*. Specially designed vane traps would trap adults that were then exposed to entomopathogenic fungus, and then they were able to escape the trap from a separate chamber after exposure. This caused greater than 60% adult mortality and complete larval mortality in nearby field sites (Moslim et al. 2007). This strategy is effective for managing populations, but would only be practical with Oahu's population if the released specimen were sterilized. In another field trial testing *M. anisopliae* against *O. rhinoceros* larvae, results indicated that EPF's can persist for up to 24 months and were found 20

cm. deeper than their initial application depth (Latch and Falloon 1976). This indicated that surface applications of EPF's could effectively reduce larval infestations over a long period of time.

I recently reached out to a company that manufactures EPF products for insect control, explaining the *O. rhinoceros* infestation on Oʻahu and the recent research conducted with locally found isolates. They were responsive to my request for potential collaboration and have filed a permit for an isolate to be shipped out to them. Under an ideal scenario, they would be able to use the given isolate to develop a wettable powder product similar to their *B. bassiana* products. A new trial testing OFB-1, CRB-1.1, and CRB-1.2 is being conducted and the results could narrow down the choice of which isolate to send out. If one of these strains were sent to the company and developed into a formulation, long term field testing would be conducted on both *P. orientalis* and *O. rhinoceros* larvae of various stages. Success in the various field trials could indicate that augmentative biological control might be an option. On a broader scale, this product could be specifically labelled for *O. rhinoceros* and applied in the Pacific regions dealing with CRB-G infestations. With the threat of CRB-G re-invading areas currently under control by OrNV (Reil et al 2018), this research is relevant and practical for improving IPM programs across many Pacific Islands.

CHAPTER 3: CONCLUSIONS AND FUTURE WORK

The inadvertent spread of ecologically and economically damaging invasive insect species is unlikely to decrease in the years to come. Globalization provides pathways for many invasive species to reach new areas of ecological suitability, and it is infeasible for agricultural inspection agents to check everything that is entering. Suckling et al. (2019) notes that invasive arthropod introduction rates may be exceeding the capability for eradication. There has already been a three-fold increase in eradication programs in the last 50 years, with no indication that this trend is going to decrease.

The Hawaiian archipelago is susceptible to invasive species introductions as interisland travel, tourism, agricultural imports, and ports of entry can allow invasive species to arrive. Detection systems are essential in these areas to identify a potential pest and eradicate it, with early detection increasing the likelihood of a successful eradication (Tobin et al. 2013). Successful detection can occur with the implementation of both targeted and generalist insect collecting traps. In the case of *O. rhinoceros*, the vane trap is an effective way to monitor an infestation and to detect population expansions.

The *O. rhinoceros* infestation on Oʻahu highlights the difficulties with eradicating the CRB-G haplotype and the need for turning research into practical control strategies. It could be possible to find a new nudivirus strain that the beetles will be susceptible to, once again yielding long term biological control potential. Searching for *S. ruficornis* parasitic wasps on Oʻahu could be attempted as well, as they were able to parasitize up to 30% of the larvae in Western Samoa (Catley 1969). However, it is important to be cautious before declaring a biological program a

success, as the pest can adapt and become resistant to natural enemies, particularly when relying upon an insect pathogen to achieve classical biological control.

With the current situation, it will be important to focus on feasible ways to manage an *O. rhinoceros* infestation with a cryptic larval population. Improving trap efficacy for adults is not necessary because the vane traps are sufficient for monitoring the current infestation. Monitoring the transportation of green waste and palm debris away from infested areas is difficult to enforce, and further public outreach may help reduce accidental transportation of larvae or adults. Previous insecticide research has suggested that systemic insecticides such as acephate and imidacloprid are highly effective when consumed by *O. rhinoceros* adults (Kellar 2018). Recent laboratory trials have indicated that both dinotefuran and abamectin are lethal to adults after consumption. Multiple systemic insecticides will be tested on palm trees in future trials, targeting trees with visible infestations. This approach could greatly reduce adult populations, and lead to tree recovery.

Entomopathogens should be utilized in addition to the current eradication strategies at sites that are potentially infested with larvae in the infested areas of Oʻahu. Field testing of virulent strains that can cause significant mortality in *P. orientalis* and *O. rhinoceros* is the first step for turning this research into a usable treatment. The strains CRB-1.1 and CRB-1.2 potentially have the characteristics desirable for a viable field treatment option. However, the Lyon Arboretum and Koko Head Botanical Garden isolates are likely to be the desirable isolates for field testing. Development of a local strain into a sprayable formulation increases the opportunity for a variety of field trial designs on various instars of *O. rhinoceros*. This might result in the eventual implementation of a local commercialized augmentative biological control

agent being incorporated into the current integrated pest management program for *O. rhinoceros*

on Oʻahu.

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