Olfaction in Coconut Rhinoceros Beetle

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Chemosensory Responses to Chemical Stimuli and Concentration Effect in the Coconut Rhinoceros Beetle

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Abstract

The ability for animals to detect chemicals in their surrounding environment whose source is located at some distance from the body is essential to animal survivorship; food and predators can be detected and discriminated, along with pheromones emitted from members within the same species. This study aimed to characterize olfactory AP responses to an array of chemical stimuli, including the aggregation pheromone oryctalure, which has not been previously studied. In addition, the effect of increasing concentration on frequency rate was evaluated. Results show no difference in AP frequency and amplitude in liked vs disliked chemicals, suggesting that discrimination takes place in signal transduction pathways and higher brain centers that are responsible for processing olfactory information. It was also observed that AP frequency and amplitude increase with increasing concentrations of acetic acid and oryctalure ($p = 5.23 \times 10^{-5}$; $p = 5.36 \times 10^{-4}$, respectively), providing evidence for the response of AP characteristics in accordance with stimulus strength. Implications from this study can be used in conservation efforts on Guam, where oryctalure in Standard Pheromone Traps can be replaced with a stimulus that elicits a more maximal response in CRB.

Introduction

The ability for animals to detect chemicals in their surrounding environment whose source is located at some distance from the body is essential to animal survivorship; food and predators can be detected and discriminated (Hansson, 1999). Insects accomplish chemoreception using olfactory organs that are concentrated at the anterior end. In arthropods, the primary olfactory organs are generally located on the antennae or antennules, which are covered with sensilla. Located at the tip of sensilla is a small pore that enables odorant molecules to cross the exoskeleton and bind to odorant receptors on the dendrites of neurons, causing a graded response and triggering an action potential (Moyes & Schulte, 2016).

In insects, the ability to communicate with other members within a species is based on olfactory communication via pheromones, chemicals released from an animal that elicit a specific response, including social interaction, mate choice, and mate identification (Birch *et al*., 1982; Wyatt, 2009). Products aimed at targeting pests take advantage of the specificity in response elicited by pheromones, most particularly sex pheromones: chemicals that are released to detect or attract potential mates (Gomez-Dias *et al*., 2013). Biocontrol agents utilize insect pheromones to control insect pests, such as oryctalure for the coconut rhinoceros beetle *Oryctes rhinoceros*, an invasive species to Guam that has wreaked havoc on the island's coconut palm trees.

Olfaction and its effect on behavior has been most intensively studied in insects and crustaceans, but none so far have looked into the electrophysiological responses of chemical stimuli specifically in *O. rhinoceros*, or the coconut rhinoceros beetle (CRB). This study proposes a preliminary study and methodology of testing chemosensory responses in *O. rhinoceros* that give insight to the AP responses

elicited when the CRB is chemically stimulated. Particularly, this study aims to characterize olfactory responses to an array of chemical stimuli, including the aggregation pheromone oryctalure. More specifically, we seek to support the following hypotheses: a) Characteristics of the AP response are distinct in "liked" versus "disliked" stimuli; b) AP frequency increases with increasing concentration of the stimulus. Not only is it important to the survivorship of the organism to be able to discriminate among sources, but knowing the strength of the source is also of importance.

Methods

Preparation of beetle and PowerLab setup

Four male *O. rhinoceros* individuals were anesthetized by being placed in a freezer. After 30 minutes, beetles were dissected using fine-tip scissors by physically separating the anterior end that constitutes the pronotum and discarding the rest of the body. Both front tibias were cut as close to the base as possible and discarded. Due to its hard exoskeleton, a Dremel was used to drill a small hole approximately 10 mm below the base of the tibia on the beetle's ventral side. A ground electrode was then placed into the resulting hole, which was drilled ipsilateral to the antenna being cut. Using fine-tip scissors, the very tip of the antenna was cut in order to allow the placement of a recording electrode into the antennae in a length-wise fashion. The ground electrode (green) and recording electrode (positive or negative) were connected to a BioAmp cable on the PowerLab system. LabChart from ADInstruments was used on a Mac desktop system to gather recordings and data.

Stimulation and extracellular recordings

Recordings made from this study were extracellular; thus, responses received via LabChart are a result of compound action potentials. Oryctalure and banana were used as the "liked" stimulus, while 45% acetic acid was used as the "disliked" stimulus. Three minutes of observation preceded stimulation, which included a control test with water. Stimulation occurred by soaking a Q-tip with the respective stimulus and being placed approximately 5 mm above the beetle's antennae for two seconds. Upon stimulation, a comment was prepared and added with the chemical name. Five minutes of LabChart data collection would follow, along with behavioral responses noted. The control (water) would then be introduced before another chemical stimulation. This "ON-OFF" stimulation attempted to return the beetle to pre-stimulus conditions. The aforementioned procedures were repeated for each chemical being tested.

Testing for concentration

Dilutions for acetic acid were made using the following equation: $M_1V_1 = M_2V_2$, creating dilutions that consisted of 6%, 12%, 25%, and 45% acetic acid. Oryctalure was purchased online via ChemTica with no known concentration listed, thus dilutions were made by adding 5 mL and 3 mL distilled H_2O. The aforementioned procedures on stimulation and observations were followed for concentration effect.

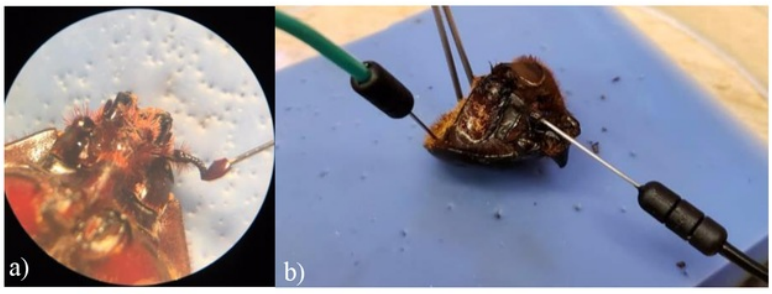

Figure 1 a) Recording electrode placed into the distal end of the CRB antenna as seen under a dissecting microscope. **b)** Electrode connections on the CRB, which consist of a ground electrode placed below the tibia and a recording electrode inserted into the antenna.

Statistical Analysis

For each stimulus, frequency and amplitude were collected and organized via Microsoft Excel and analyzed for differences in means via R Commander ver. 3.5.0 using an Analysis of Variance (ANOVA) if normally distributed or a Kruskal-Wallis if not normally distributed where transformation of data was not possible. Frequency was calculated by first solving for the rate, or spikes per minute:

$$SPM = [(n - 1) / \Delta T\ (sec)]\ x\ 60$$

then applying SPM to the following equation:

$$f = SPM/60.$$

Change in amplitude was noted by determining baseline using the Marker and Waveform Cursor on LabChart and measuring the difference of the response spike peak as referenced by the baseline. Thirty peaks were manually measured for each stimulus.

Results

O. rhinoceros beetles were stimulated with banana, 45% acetic acid, oryctalure, and water (as control). Kruskal-Wallis and ANOVA results indicate a statistically significant difference in mean amplitude ($p = 2.3 \times 10^{-16}$; Fig. 1) and mean frequency ($p = 1.08 \times 10^{-8}$; Fig. 2) across the four stimuli, respectively. Mean amplitude was calculated at 76.16 µV for water, 92.6 µV for banana, 145.4 µV for oryctalure, and 169.6 µV for acetic acid. Mean frequency was calculated at 403.3 Hz for water, 511.9 Hz for banana, 619.6 Hz for oryctalure, and 635.5 Hz for acetic acid.

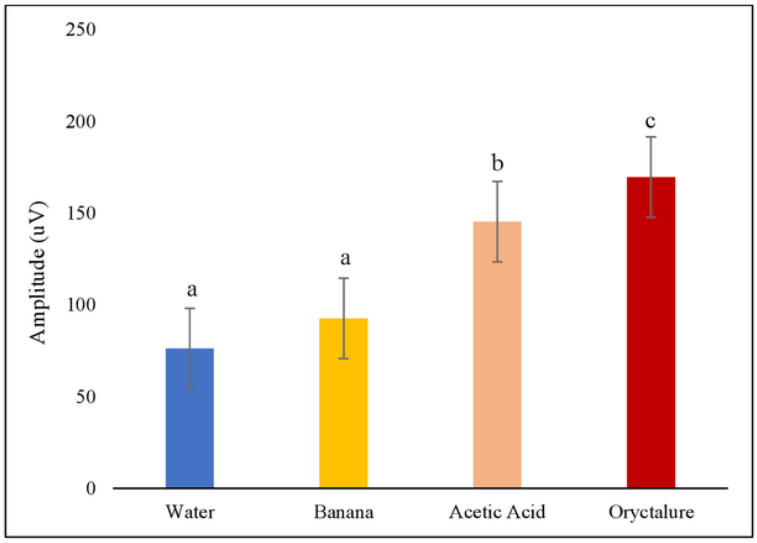

Figure 1. Bar graph showing the average in mean amplitude across four stimuli, including the control. Kruskal-Wallis test showed that there was a statistically significant difference in median amplitude between the different stimuli, $p = 2.3 \times 10^{-16}$. Letters above bars denote differences based on post-hoc Nemenyi test.

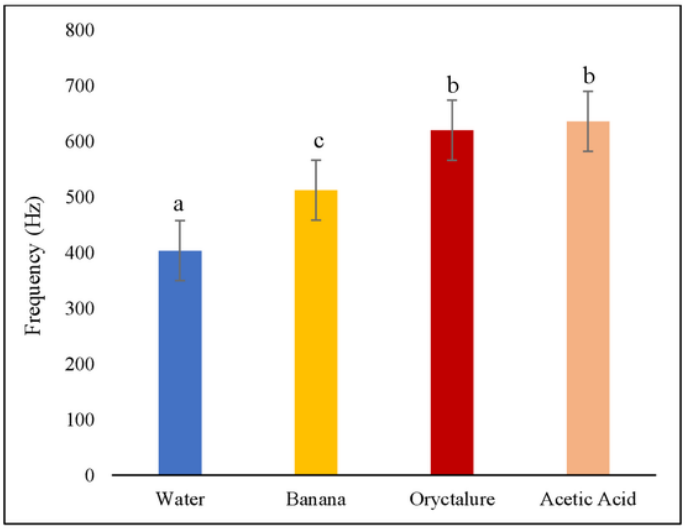

Figure 2. Bar graph showing the average in mean frequency across four stimuli, including the control. ANOVA results indicate a statistically significant differences in at least two means, $p = 1.08 \times 10^{-8}$. Letters above bars denote which means are similar or different from one another, based on Tukey post-hoc results.

O. rhinoceros beetles were also tested for a concentration effect, where it was hypothesized that an increase in chemical concentration would also result in an increase in frequency and amplitude. Acetic acid dilutions consisted of 6% acetic acid, 12% acetic acid, 25% acetic acid, and 45% acetic acid. Oryctalure was diluted first using 5mL H_2O and again with 3mL H_2O. Kruskal-Wallis results for differences in average amplitude in both acetic acid (p = 3.43 x 10-13) and oryctalure (p = 2.40 x 10^{-12}) were statistically significant. Mean amplitude was calculated at 185.8 µV for 6% acetic acid, 192.6 µV for 12% acetic acid, 195.3 µV for 25% acetic acid, and 265.1 µV for oryctalure (Fig. 3). For oryctalure dilutions, mean amplitude was calculated at 143.6 µV for oryctalure + 5mL H_2O, 201.4 µV for oryctalure + 3mL H_2O, and 206.0 µV for pure oryctalure (Fig. 4).

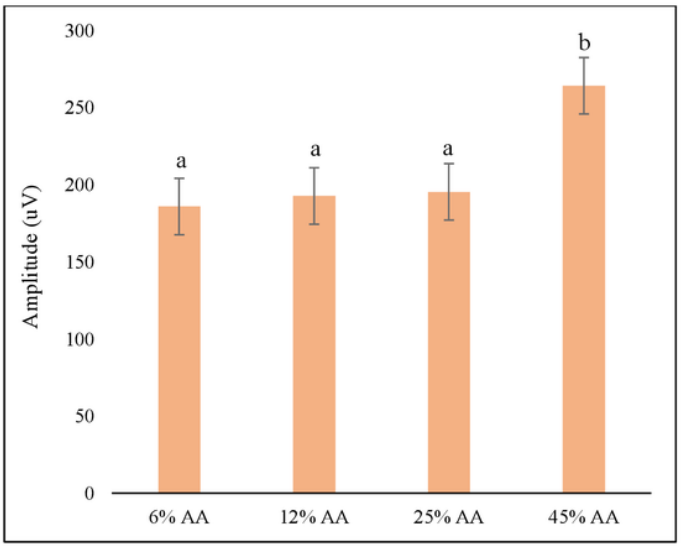

Figure 3. 45% acetic acid was diluted to 6% AA, 12% AA, and 25%% to test for concentration effect. Bar graph showing the average in mean amplitude across four acetic acid dilutions. Kruskal-Wallis test showed that there was a statistically significant difference in median amplitude across the acetic acid dilutions, p = 3.43 x 10^{-13}. Letters above bars denote differences based on post-hoc Nemenyi test.

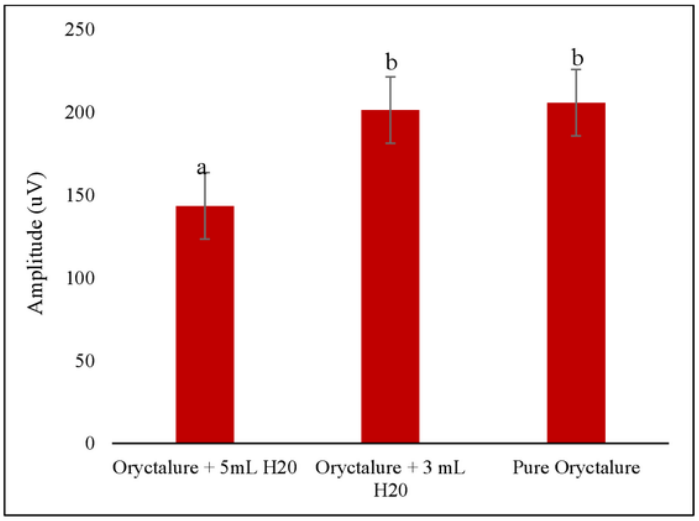

Figure 4. Concentration effect on amplitude was tested by diluting oryctalure with 5mL water and 3mL water. Bar graph showing the average in mean frequency across oryctalure dilutions. Kruskal-Wallis results indicate a statistically significant difference in median amplitude across the oryctalure dilutions, $p = 2.40 \times 10^{-12}$. Letters above bars denote differences based on post-hoc Nemenyi test.

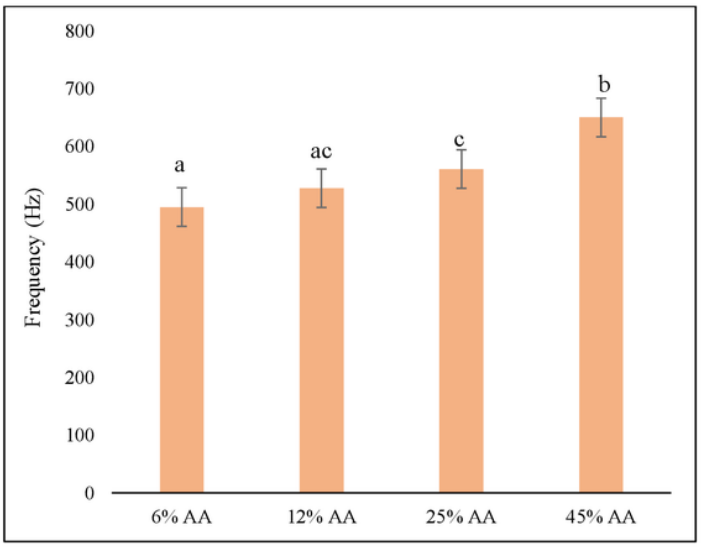

Figure 5. Bar graph showing the average in mean frequency across acetic acid dilutions. ANOVA test showed that there was a statistically significant difference in mean amplitude across the acetic acid dilutions, $p = 5.23 \times 10^{-5}$. Letters above bars denote differences across means, according to a post-hoc Tukey test.

In addition, ANOVA results indicated mean frequency statistically differed across dilutions for both acetic acid and oryctalure (p = 5.23 x 10^{-5}; p = 5.36 x 10^{-4}, respectively.) Mean frequency was calculated at 494.9 Hz for 6% acetic acid, 527.4 Hz for 12% acetic acid, 560.7 Hz for 25% acetic acid, and 650.0 Hz for 45% acetic acid (Fig. 5). For oryctalure dilutions, mean frequency was calculated at 617.7 Hz for oryctalure + 5mL H_2O, 709.4 Hz for oryctalure + 3mL H_2O, and 771.7 Hz for pure oryctalure (Fig. 6).

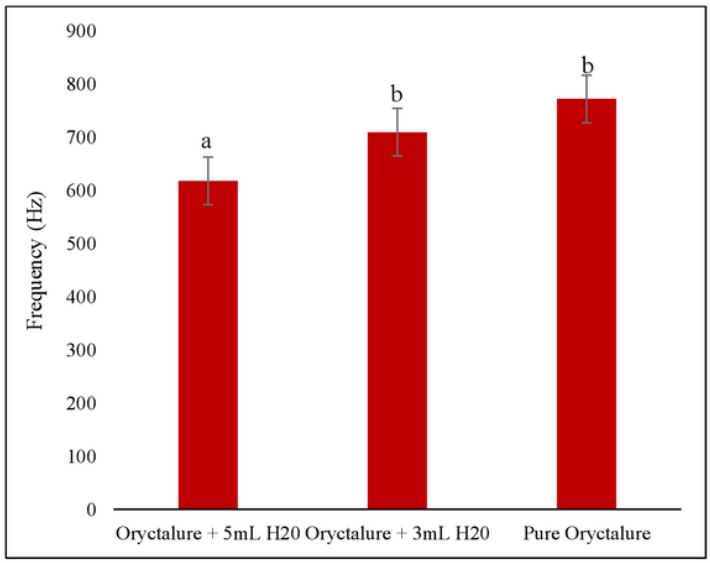

Figure 6. Bar graph showing the average in mean frequency across oryctalure dilutions. ANOVA test results indicate a statistically significant difference in mean amplitude across the oryctalure dilutions, p = 5.36 x 10^{-4}. Letters above bars denote differences across means, according to a post-hoc Tukey test.

Discussion

An organism's survival depends on its ability to discriminate between what is safe and unsafe (i.e., what is food and what is prey). When attempting to characterize the responses of "liked" versus "disliked" stimuli in *O. rhinoceros*, no clear

distinction in frequency, amplitude, or AP patterns were obvious as to draw a conclusion on how the AP characteristics might tell the beetle the nature of the stimulus. According to literature, how the beetle interprets environmental stimuli either as "safe" or "unsafe" may be occurring at higher brain centers that process olfactory information. In the insects whose olfactory systems have been studied, it has been shown that there are two areas of the insect brain where projection neurons of the antennal lobe send their axons: the lateral horn (LH) and mushroom body (MB) (Mobbs, 1982). Olfactory circuits with spatially distinct pathways allow the LH and MB to interpret the nature of the stimulus (Nowotny *et al*., 2014). Future studies examining olfaction in CRB may map out olfactory circuits and pathways that lead to higher brain centers, which ultimately discriminate the nature of the stimulus.

Published studies conducted on insect olfaction have investigated the effect of concentration on AP responses, where it has been shown that frequency shows a linear relationship when plotted against increasing concentration of stimuli (Maher *et al*., 2005; Fields *et al*., 2008; Silva *et al*., 2019). According to our results on frequency in both acetic acid and oryctalure of varying concentrations, *O. rhinoceros* has the ability to detect changes in the stimuli's strength, in which beetles exhibited an increase in frequency with increasing concentrations of both acetic acid and oryctalure pheromone. Bursts of action potentials delivered to the pre-synaptic cell of neurons is crucial in Ca^{2+} influx to initiate neurotransmitter release to the post-synaptic cell. Strong stimuli result in high bursts of APs down to the pre-synaptic cell, rapidly delivering neurotransmitters onto the respective post-synaptic cell, which ultimately elicits a strong response by the organism. This study has demonstrated the crucial encoding of stimulus strength by showing that primary neurons involved in olfaction in *O. rhinoceros* increase AP frequency and amplitude as a result of

stimulus strength. Although some differences are insignificant, an increase in sample size will most likely result in stronger evidence of our hypothesis.

In addition, the AP frequency resulting from stimulus strength is related to AP amplitude. Weak signals result in the opening of only a few ion channels, resulting in less ion influx and ultimately a small concentration of neurotransmitter release. In contrast, strong signals generate a high concentration of neurotransmitter release which allows many ion channels to open, thus allowing more ions to cross the cell membrane, resulting in a large change in membrane potential (Moyes & Schulte, 2016). As concentrations for acetic acid and oryctalure increased, the amplitude of response elicited by the CRB also increased as a reflection of the strength of the incoming stimulus

Results from this study can be strongly implemented in conservation efforts on Guam, where it has been shown that oryctalure used in Standard Pheromone Traps (SPTs) is not reducing *O. rhinoceros* populations significantly enough to rid the island of its invasiveness. As this study is the first on Guam to stimulate and record CRB olfaction via a range of chemicals at varying concentrations, it is possible to test the effectiveness of other pheromones that have the potential to replace oryctalure in SPTs. By observing the AP responses in *O. rhinoceros* across varying pheromones at varying concentrations, we can pinpoint a stimulus that elicits a stronger response when compared to oryctalure. When replacing oryctalure in SPTs with this superior stimulus, the possibility to eliminate the invasive coconut rhinoceros beetle on Guam can be made possible.

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Timeline of Research

March: Establish methodology with a cockroach as a model, including dissection of antenna, PowerLab BioAmp connections, chemical stimulation via Q-tip or pipette. Protocol was then moved onto CRB model, tested efficiency and modified accordingly. Late in the month, began testing liked versus disliked response in the CRB

April: Tested concentration effect in CRB

May: Gathered and analyzed data from LabChart Reader; prepared presentation & scientific paper.