FINAL REPORT

Title:

Development of a pheromonal attractant for detecting small, incipient populations of brown treesnakes on Hawaii

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For:

Cooperative Service Agreement No. 06-7483-0602(RA) between Hawaii Invasive Species Council and United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA/APHIS)

Citation:

Mathies, T. 2008. Development of a pheromonal attractant for detecting small, incipient populations of brown treesnakes on Hawaii. Unpublished report, QA 1438. National Wildlife Research Center. Fort Collins, CO.

Abstract

The overall goal of this research was to investigate the potential of the brown treesnake sex pheromone as a tool for detecting and controlling incipient populations on Hawaii. Specific objectives included demonstrating the existence of the female sex pheromone, determining whether its bioactivity is retained following chemical fractionation, and characterizing the chemical identity of the pheromone. The first objective was met. Responses of "free-ranging" adult male brown treesnakes to substrate-born skin secretions of adult females were investigated in an outdoor semi-natural enclosure at the U.S. Fish and Wildlife Service's Ritidian Unit of the Guam National Wildlife Refuge, Guam. Males were given a simultaneous choice of investigating poles to which the either scent of a vitellogenic female (enlarging ovarian follicles) or non-vitellogenic female (reproductively quiescent ovaries) had been applied just prior to onset of male nocturnal activity, or a no-scent control pole (3 replicates per treatment, per night, per female). Males spent more time, and exhibited a greater frequency of investigative "head-backups", on poles contacted by vitellogenic females than non-vitellogenic females or no-scent controls. These results show that females produce the sex pheromone when undergoing ovarian growth and that the pheromone is secreted on the female's skin. Pheromone is transferred onto substrate as the female moves and males show preferential interest in substrates contacted by reproductive females over non-reproductive females. In a very limited test of whether the pheromone might also be airborne (1 night, using all females: vitellogenic and non-vitellogenic), no males visited caged females placed within the enclosure (no substrate scent trails leading to cages). Objectives 2-5 have not yet been met. Work towards the second and third objectives is in progress. Ovarian growth has been experimentally induced in 11 captive female brown treesnakes, and the first detectable evidence of vitellogenesis was found in three females the week of 30 June 2008). Twelve additional females are currently being maintained in a non-reproductive state (controls). There are 49 captive males that will be used to bioassay female pheromone. Bioassays of female skin secretions are expected to begin in early July and will also be conducted on skin secretion samples that have been fractioned using column chromatography. Fractioning coupled with bioassays will allow us to identify the fraction containing the pheromone. If this work is successful, we will attempt to meet the fourth objective by characterizing the pheromone using Gas Chromatography-Mass Spectrometry. Completion of work involving bioassays is expected by August 30, 2008. Field-testing on Guam of an apparatus using a pheromone-containing sample to direct males into a trap (fifth objective) is dependent on success of the ongoing work above. A prototype pheromone-based trap has been developed, but is untested. Travel to Guam for this purpose is tentatively scheduled for September 2008. Results of ongoing work will be provided to HISC following completion.

Introduction

Hawaii's ability to prevent the establishment of the brown treesnake (*Boiga irregularis*) in Hawaii relies mainly on interdiction measures conducted on Guam. The second line of defense is at Hawaii's ports of entry and relies mainly on the use of detector dogs and visual sightings. It is inevitable that some snakes will eventually pass undetected beyond the port environs and breeding populations may become established. Hawaii presently has few practical tools for detecting brown treesnakes once they have dispersed beyond cargo areas. The main tool, (brown treesnake trap containing a live mouse bait), may not be effective in Hawaii because, unlike Guam, Hawaii is a relatively food-rich environment — snakes may have little incentive to enter a trap. Trapping and other less available methods (e.g., visual searches, field-trained detector dogs) are relatively costly to implement and maintain in a ready state.

The purpose of this work was to investigate the female brown treesnake sex pheromone as a tool for detecting and capturing brown treesnakes in low-density situations, such as an incipient population. In the few snake species studied, the female sex pheromone consists of a non-volatile homologous series of long-chain saturated and unsaturated methyl-keytones that are expressed along the dorso-lateral trunk region (Mason 1992). During the reproductive season, this pheromone is produced in the liver under the control of estrogen and is present in its active form in circulating plasma (Garska and Crews 1981). Females passively deposit the pheromone on the substrate as they move throughout the environment creating a chemical trail (Mason et al. 1989; Mason 1992). Males rely primarily on their olfactory senses to locate reproductive females (Noble 1937), specifically the vomeronasal organ, a chemo-receptive organ implicated in the detection of, and response to, sex pheromones (Halpern and Martinez-Marcos, 2003). Males use this mechanism to follow the pheromone trails of females (Halpern and Kubie, 1980, Ford, 1986).

The five objectives of this work, as outlined in the Research Proposal, were:

Objective 1. Experimentally demonstrate that female skin extracts have bioactivity (i.e., female extracts *vs*. controls are attractive to males).

Objective 2. —Experimentally demonstrate that bioactivity of extracts remain intact following the first fractionation of samples.

Objective 3. —Conduct a second, finer-scale, fractionation of the bioactive samples. Experimentally demonstrate that bioactivity of one or more of these fractions retain bioactivity.

Objective 4. — Conduct chemical analyses of the bioactive fractions obtained following bioassays conducted under Objective 3. In addition, conduct chemical analyses skin extracts of adult males, non-reproductive females and reproductive females to demonstrate and qualify differences.

Objective 5. — Conduct initial field evaluations of pheromonally-based apparatus for directing brown treesnakes into a trap.

Work under Objective 1 has been completed and results are presented below in the section Objective 1. Work under Objectives 2, and 3 is in progress. The NWRC research protocol and Amendments for this work are provided in Appendix 1 and 2, respectfully. Current status of the research is provided below in the section Objectives 2 and 3. Work under Objective 4 has not been initiated and will be dependent on whether Objectives 2 and possibly 3 are achieved. Main work under Objective 5 has not been initiated and is also dependent successes of Objectives 2 and 3. There has been limited work under this objective and it is presented under section Objective 5.

Objective 1.

Methods

From July 18 to September 8, 2006, NWRC personnel conducted initial field and laboratory work on Guam on the development of a pheromonal attractant for the brown treesnake. The working hypothesis of the study protocol (QA-1372; enclosed with 1st Quarterly Report) was that females produce the pheromone only when reproductive; that is, when ovarian follicles are undergoing vitellogenesis. Because the brown treesnake has no synchronous breeding season on Guam, and females are thus reproductive sporadically, the first goal was to determine whether sufficient numbers of vitellogenic females could be obtained. To this end, all snakes collected by USDA Wildlife Services, Guam between 11 July and 1 September were screened for adult females. These females were then examined for yolking (= vitellogenic) follicles (Figure 1). A total of 13 vitellogenic females were found (all other adult females were non-reproductive = non-vitellogenic) and this number was sufficient for conducting studies. The 13 vitellogenic females along with 13 nonvitellogenic females and adult males were housed singly outdoors at WS facilities on Andersen Air Force Base.

FIGURE 1. Abdomen of female brown treesnake (*Boiga irregularis*) is palped for enlarging ovarian follicles.

Two test systems for evaluating male response to female scent were investigated. The first system investigated involved releasing males onto grass lawn plots to which female scent was applied by directing a female along a predetermined and controlled heading. Males were then released onto plots and their propensity to follow the scent trail was evaluated. This test system proved unworkable and was abandoned due to the reluctance of males to exit release boxes and to behave normally upon exit. The second test system investigated proved satisfactory and is described below under Experiment 1.

Experiment 1.

Fifteen adult male brown treesnakes (snout-vent length > 1100 mm) were each implanted intra-abdominally with a unique identifying PIT tag and then released into a roughly 21 x 21 m snake enclosure located at the Guam National Wildlife Refuge (Figure 2A.) This enclosure was preexisting and contained well-developed native vegetation similar to the naturally snake-containing area outside the enclosure. Three test stations were set up within the enclosure parallel to, and just within, the vegetation edge: one each on the north, west, and east sides. Each station had a sling apparatus for horizontally suspending three wooden poles (Figures 2B, C). Poles were branchless saplings with lengths and diameters of approximately 3 m and 50 cm, respectfully. Poles were suspended in vegetation, one below the other, with the uppermost pole approximately 162 cm above the ground (Fig. 2C). At one end of a pole triage, the ends of all three poles passed through the circular antenna of an AVID PIT tag reader (Figure 2B). The reader was connected to a data logger that recorded time and PIT tag number when a PIT tag passed through the reader. Each pole triage was monitored by a video camera with infrared illumination connected to a VCR that recorded date and time (Figure 2D).

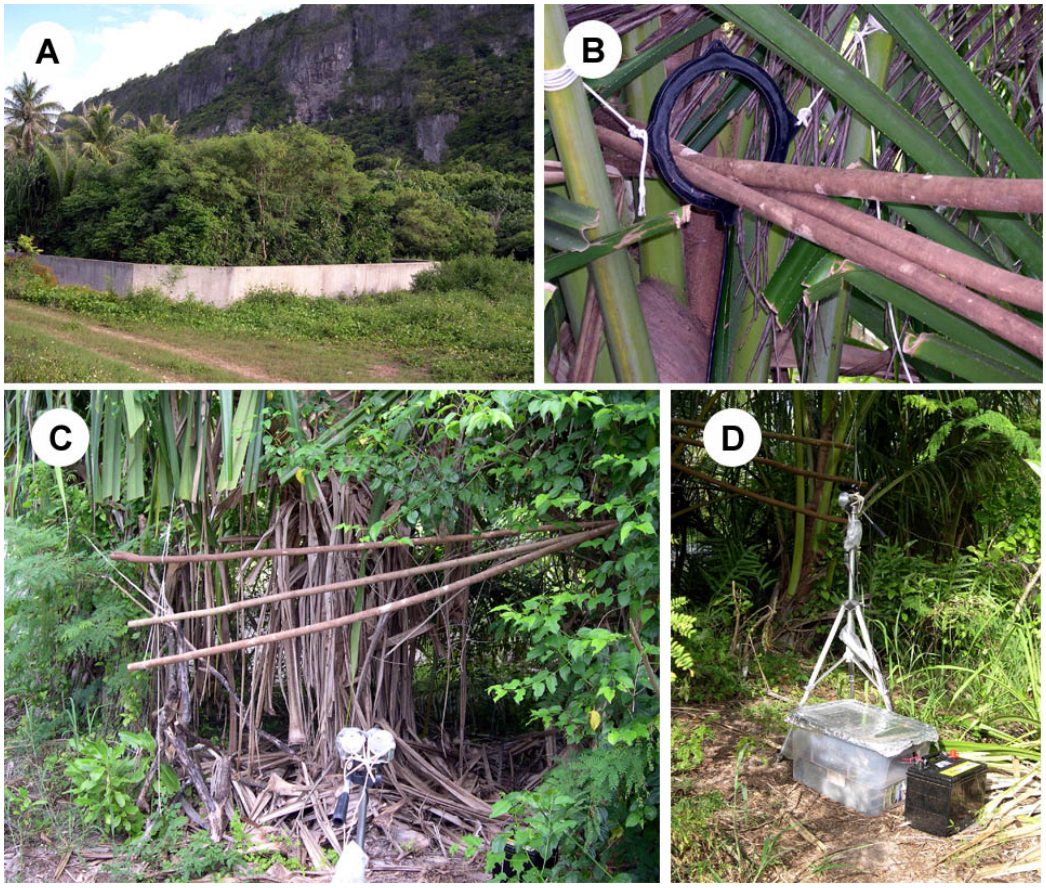

FIGURE 2. A. Snake enclosure at Guam National Wildlife Refuge. B. AVID PIT reader with ends of three scented test poles passing through reader hoop. C. Station on west side of snake enclosure with three scented test poles. D. Infrared camera system at station on west side of snake enclosure.

There were three treatment scents, those of vitellogenic females, non-vitellogenic females, and a no-scent control. Each of the three treatment scents were applied to a different pole at each station. Within each pole triage, scent treatment type was randomized with respect to pole placement (i.e., “upper”, “middle”, “lower”). Female scent was applied to poles by placing a female at one end of a pole and letting her crawl to the opposite end (Figure 3). Scent application was performed within 3 h of dusk. One pair of females (1 vitellogenic and 1 non-vitellogenic) was tested each night with each female used once. Thus, for example, on one test day the same vitellogenic female was allowed to crawl on three poles, one at each station. Poles were used once and then discarded.

FIGURE 3. A scent trail is applied to test pole by placing a female brown treesnake (*Boiga irregularis* one end of a test pole and coaxing her to crawl to the opposite. Pole is then hung in vegetation at camera station inside enclosure containing males (see text).

Data on male behavior on poles was extracted from videotapes and identity of males was determined from data from AVID PIT tag readers. Data recorded were treatment type of pole investigated by male, length of time head on pole, and number of "head back-ups" conducted when investigating a pole. The close proximity of the three poles to one another allowed a male to be on one pole while its head investigated another pole. Therefore, all observations are based on the location of a male's head. A "head back-up" is a behavior we observed males performing when actively crawling along a pole. As a male moves forward, it regularly flicks the pole with its tongue. Tongue-flicking is a sensory-gathering behavior used by snakes to deliver odorants to the vomeronasal organ. During a head back-up, the male pauses, pulls its head back so that its nose is directed down onto the pole, and repeatedly flicks its tongue on one spot. We assume that males perform this action when they encounter a scent of interest, in this case the scent of a female, and that they gain additional information about that female from the repeated tongue flicks.

Experiment 2.

We also performed a limited test of whether the pheromone might be airborne. To do so, home cages of the 13 vitellogenic and 13 non-vitellogenic females were placed within the enclosure described above in Experiment #1 (Figure 4.). Cage tops and bottoms were covered with ¼ in wire hardware cloth and were thus highly ventilated. Cages were arranged on the ground under forest canopy in two parallel rows about 60 cm apart, with vitellogenic females in one row and nonvitellogenic females in the other. A video camera system as described in Experiment #1 was placed at the head of the aisle between the two rows such that snake activity at cages was monitored. This experiment was conducted on one night immediately following the last test night in experiment described above. It was hypothesized that if the female pheromone were airborne, males would appear at the cages of females and attempt to gain entrance to cages of interest.

FIGURE 4. Home cages of vitellogenic and non-vitellogenic female brown treesnakes (Boiga irregularis) placed for one night within outdoor enclosure containing free-ranging male brown treesnakes and filmed overnight.

Immediately following the conclusion of Experiment 2, all females were euthanized and their ovaries inspected to confirm follicular state.

Results & Discussion

Experiment 1.

Mean time spent investigating poles differed among treatment types, although statistical significance was marginal (Fig. 5; one-factor ANOVA: $F_{2, 108} = 3.14$, $P =$

0.047). Male contact of poles on which vitellogenic females had crawled was longer than those crawled on by non-vitellogenic females or no-scent controls. Mean number of head back-ups per second differed among treatment types (Fig. 6; one-factor ANOVA: $F_{2,103}$ = 8.32, P = 0.0004). The number of head back-ups per second was higher on poles on which vitellogenic females had crawled compared to those crawled on by non-vitellogenic or no-scent controls.

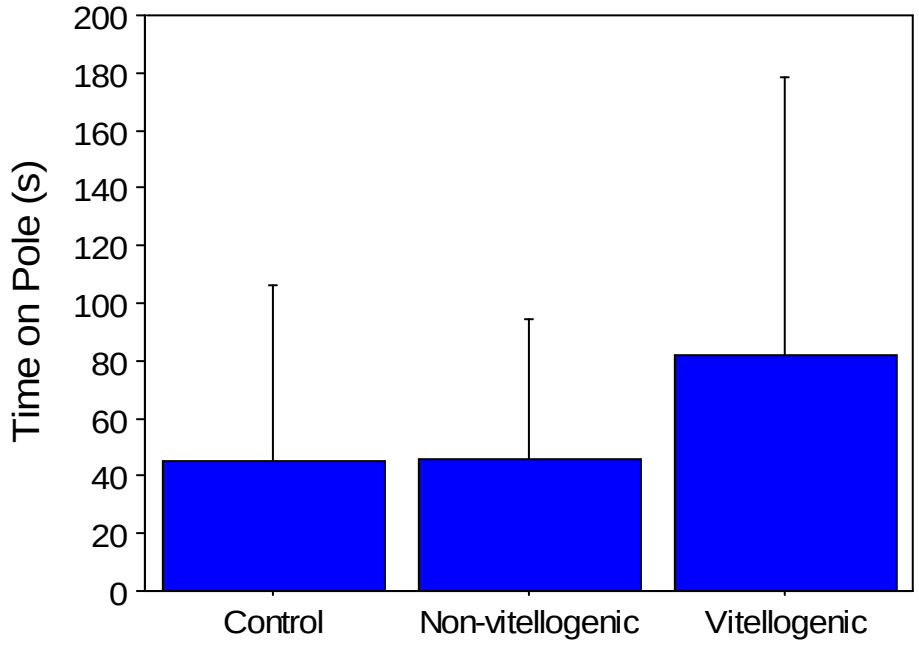

FIGURE 5. Mean length of time spent by male brown treesnakes (*Boiga irregularis*) on experimental poles to which had been applied the scent of a vitellogenic female, non-vitellogenic female, or a no-scent control. Means ± one standard error are shown.

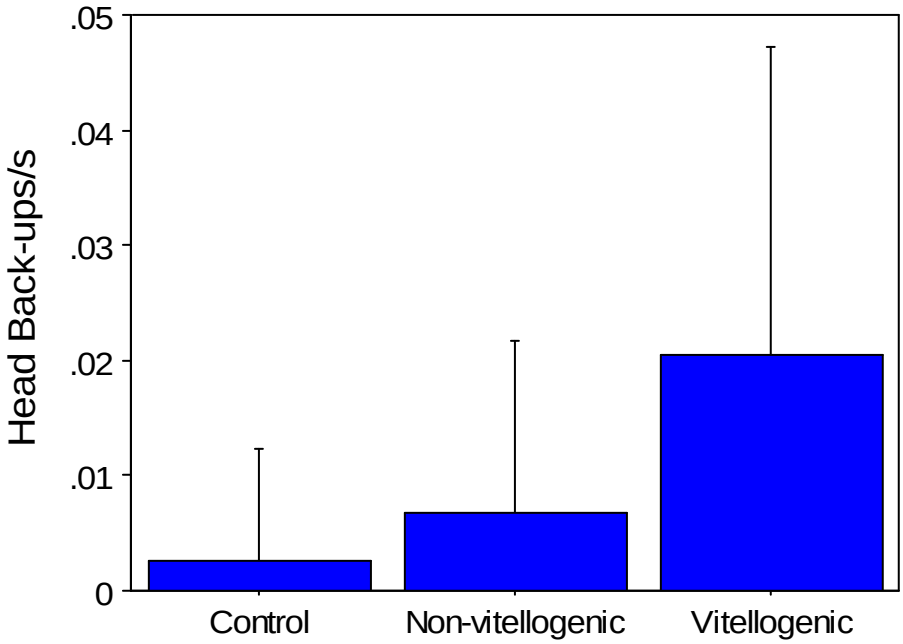

FIGURE 6. Mean number of investigative head back-ups per second (see text) by male brown treesnakes (*Boiga irregularis*) on experimental poles to which had been applied the scent of a vitellogenic female, non-vitellogenic female, or a no-scent control. Means ± one standard error are shown.

Males investigated all the three scent treatment types although somewhat more time was devoted to poles on which scent of vitellogenic females had been applied. This considerable variability has a number of possible non-competing causes. First, it is possible that once males determined that a vitellogenic female was "present" on a given night, they may have began widely searching, becoming less discriminatory about the subsequent experimental poles they contacted. Second, there was a no *a priori* way for us to know whether a particular vitellogenic female was actually attractive to males (i.e., secreting the sex pheromone). It is likely that pheromone secretion increases to a maximum during a certain period in vitellogenesis or that the pheromone is intermittently released during this period. Both these factors, if real, would tend to obscure any real trends. The number of head back-ups males initiated while investigating poles presents the best evidence for the hypothesis that females produce the sex pheromone only during vitellogenesis: males stopped and tongue-flicked poles of vitellogenic females at a higher rate than poles of non-vitellogenic females or controls. The observation that males conducted no more head back-ups per second on poles of non-vitellogenic females than the no-scent controls suggests these females are not releasing the pheromone and therefore do not breed while in this condition.

Experiment 2.

During the one night of testing to see if the pheromone was airborne, no males appeared at cages of any females, even though skin secretions of at least some of the

vitellogenic females had been recently been proven attractive to males. This experiment was very limited. Regardless, if the pheromone does have airborne bioactivity, it stands to reason that some males should have been attracted because the un-naturally large number of vitellogenic females assembled in the enclosure had the potential to produce an ultra-strong pheromone signal. Regardless, the possibility of an airborne mechanism should receive some additional inquiry.

Upon completion of Experiment 2, all vitellogenic females were euthanized and the ovaries dissected out to confirm states of follicular development. All 13 females had fully enlarged ovarian follicles (Figure 7). However, follicles in three females appeared to be undergoing atresia.

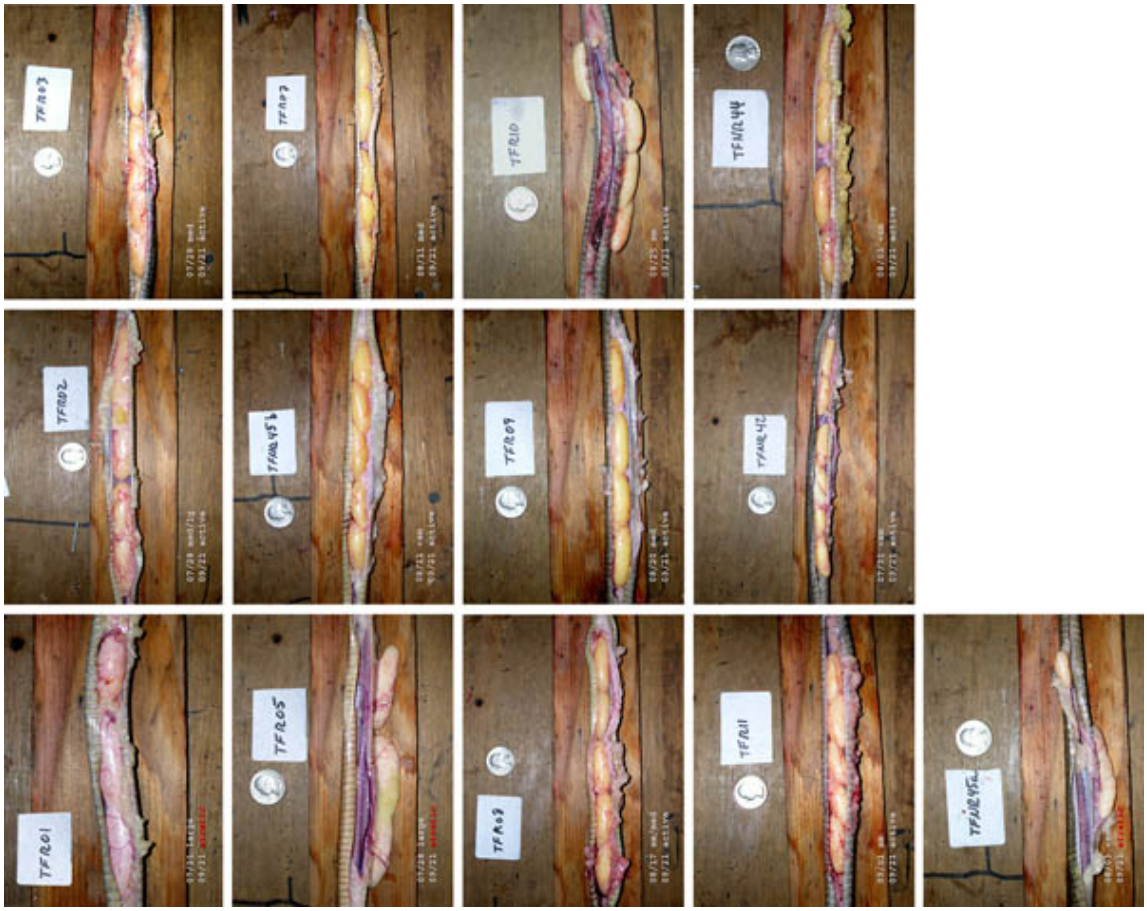

FIGURE 7. Photos of follicle-containing ovaries of the 13 reproductive females used in this study. Ovaries of all females contained fully enlarged follicles. Follicles in three females appeared atretic (1st, 2nd, and 5th photos, left to right, bottom row). It is not known when during the study follicles in these three females began atresia.

Objectives 2&3

Methods

Work toward these objectives is in progress. Methods are provided in the NWRC study protocol, QA-1438 and QA-1438 Amendment 1 in Appendices 1 and 2, respectfully. Work accomplished to date is as follows. According to methods in Mathies and Miller (2003, 2004), we have attempted to induce ovarian growth in one group of females (n = 11). Ovarian growth was not induced in a second group of female that will serve as controls (n = 12). At the outset of this work, all females were held in a common room at a constant 28.5°C and 75% relative humidity. To induce ovarian growth one group of females was moved into a second room on 17 March 2008 where they were held at a constant 25°C and 75% relative humidity. These female were kept at these reduced temperatures, and then on 13 May 2008 they were returned to the common room holding the other group of females (a 58-day cool period). At this time, we began abdominally palping females every 1-2 weeks for developing ovarian follicles. On 30 June 2008, the first vitellogenic follicles were detected in three of the females that had been kept under cool temperatures. Successful induction of ovarian growth is the critical go/no-go step in this work. We expect that ovarian growth will become detectable in additional females over the next two weeks. We have 49 adult male snakes that will be used to bioassay female pheromone. Fractioning of samples and bioassays of those samples will begin in July 2008.

Objective 4

Methods

No work has yet been conducted under this objective. Discussions are in progress with NWRC Chemistry section personnel on the steps necessary to characterize the pheromone using Gas Chromatography-Mass Spectrometry capability at the NWRC.

Objective 5

Methods

Under Objectives 2 and 3, we will be collecting pheromone samples from females and reserving some of these under stable storage conditions. Under Objective 4, we will transport these samples to Guam for testing in the field. Testing on Guam is tentatively scheduled for September 2008. This work has four main goals: 1) develop and field-test a pheromone based snake trap, 2) investigate distances males are willing to follow a substrate-based pheromone trail, 3) investigate longevity of pheromone trails in the field, and 4) investigate efficacy of a pheromone-based trap relative to the standard live mouse-baited trap.

We will likely test a number of trap designs, the differences being primarily in the door design and nature of the guide pole or rope leading into the trap. A prototype trap has been constructed (Figure 8), but has not been field-tested. The NWRC protocol (QA) for this work has not yet been written.

FIGURE 8. Prototype pheromone-based trap for the brown treesnake (*Boiga irregularis*). The female sex pheromone is applied to the wooden pole leading into the trap. In the field, the pole will be connected to a much longer pheromonally-inoculated guideline (not shown). The second panel shows a detail of the wooden pole leading into the trap door. Plastic bars hanging in the trap doorway swing in, allowing a snake to enter trap, but do not swing out.

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Appendix 1.

QA-1438

National Wildlife Research Center
Wildlife Services
Animal and Plant Health Inspection Service
United States Department of Agriculture

Study Protocol

1. Title: Chemical fractionation and bioassay of the female brown treesnake sex pheromone

2. Study Director: Tom Mathies

3. Sponsor: USDA/APHIS/NWRC

4. Testing Facility: National Wildlife Research Center, 4101 LaPorte Avenue, Fort Collins, Colorado 80521-2154

5. Background and Justification:

The brown treesnake is an invasive predator accidentally introduced to the island of Guam shortly after WW II. The devastating effect of the snake on Guam's native wildlife is well documented and the economic costs continue to be appreciable. Since the snake's arrival on Guam, it has become pervasive throughout Guam and on all of Guam's military installations. Constant inspection and control operations by the military and USDA/Wildlife Services are now necessary to deter the snake from stowing away on transport and cargo and establishing new populations in recipient locations such as Hawaii. Development of attractants, including pheromones, has been identified as a line of research for reducing population levels of the snake on Guam

Work conducted under QA-1373 showed that male brown treesnakes preferentially investigated residual substrate scent of females whose ovaries contained vitellogenic follicles over females that were non-reproductive. The present study will build on those findings by investigating whether adequate bioactivity (male attraction) is maintained following chemical fractionation of female skin secretions. Fractionation is the first step towards chemical characterization of the female sex pheromone and synthesis for use as a brown treesnake attractant/control tool.

6. Objectives:

1. Identify the period of maximal pheromone secretion with respect to follicular development
2. Determine if level of male investigative response to female scent is conserved following chemical fractionation of scent

Hypotheses:

1. Male investigative response varies with extent of vitellogenic follicle development in female.
2. Male investigative response is greater toward scent of vitellogenic females than that of non-vitellogenic females or other males.
3. Male investigative response does not differ between scent of proven vitellogenic females and at least one of fractions from their scents.

7. NWRC Approved Project Title:

This study is being conducted as part of the approved Brown Treesnake Project of the Product Development Program under Hawai'i Invasive Species Council Funding.

8. Regulatory Compliance/Guidelines:

Regulatory Standard:

X	None, non-regulated study
	CFR Title 40, Part 160: Good Laboratory Practice Standards (FIFRA);
	CFR Title 21, Part 58: GLP Standards for Nonclinical laboratory Studies, (FFDCA)

9. Study Classification Information

Does this study include any or all of the following?

X	Animals -- please complete and attach **Animal Use Appendix**
	Plants -- no additional appendix required
	Microbiological/Biohazardous Materials -- please complete and attach **Microbiological/Biohazardous Materials Use Appendix**
	Chemical Analysis -- please complete and attach **Analytical Chemistry Appendix**
	Literature review only -- no additional appendix required
	Statistical or economic analysis only -- no additional appendix required
	Use of a test, control, references substance, bait or device -- complete and attach **Test, Control and Reference Materials / Device Formulation and Use Appendix**

10. Methods/Procedures:

Induction of vitellogenesis in females: vitellogenesis will be induced following Mathies et al. 2004. In brief, females in which vitellogenesis is to be induced will be housed separate from other females in a room where air temperature inside cages is held at approximately 22°C for six weeks. At the end of this period, females will be moved back to higher temperatures within the room containing the other females and males. Beginning at this time, females in all groups may be offered as many thawed mice as they will consume one to two days each week. All females will be palped abdominally once a week to check for presence of enlarging (vitellogenic) follicles.

Bioassay: The bioassay will consist of a set of stereotypic behavioral responses elicited from males by female scent or chemical fractions thereof. The rate of tongue-flicking will be one measured response. Other response types will be identified based on post testing observations (i.e., from videotape examinations).

Bioassay Apparatus/Sample Acquisition: The apparatus will consist of 12 modular holding chambers, each divided into two areas. One area of each chamber will hold a male. The other area will hold a scent sample from a female. A sliding panel will separate the two areas. This panel will be raised to allow male access to scent samples. Video cameras placed above the assay apparatus will record male snake activity in all 12 chambers. Sides of chambers will be opaque so that males cannot see each other. Scent samples will be obtained by swiping a cotton gauze pad moistened with a mixture of chloroform, hexane, and methanol over integument of the female. These solvents will then be evaporated off pads. Each bioassay will use six males, thus two separate assays may be conducted per night.

Skin Scent Fractions: Three solvent-containing cotton-gauze pads will be used to wipe the dorsum of each female. One pad will be extracted in chloroform, another in hexane, and the other in methanol. Each pad will be placed in a 50 mL glass screw-top tube for 24 h with enough solvent to submerge pad. The pad will then be removed and the solvent evaporated off by placing the tube in a water bath at ~40ºC and directing a stream of nitrogen gas onto the solvent surface until the final desired solvent volume is achieved. Final test samples to be presented to males will be obtained by applying a fraction to cotton gauze pads, evaporating off the solvent prior to testing.

Presentation of whole scent samples and fractions thereof: Samples will be placed in a linear arrangement across the back of a holding chamber, opposite the end containing a male. Order of samples will be randomized within each chamber.

11. Experimental Design and Statistical Analyses:

Assignment of snakes to groups: assignment of females to the female groups will be randomized. Assignment of males to the male groups will be randomized.

Test groups: There will be two test groups of 15 adult females each, vitellogenic females and non-vitellogenic females. There will be one test group of 15 males. The two female test groups will be referred to hereafter as the VIT and NON-VIT groups, respectively. Males will be referred to as the TEST MALE group.

Males in bioassays: There will be 36 males used in bioassays. These males will be referred to hereafter as BIOASSAY MALES. Males in this group do not include any of those in the TEST MALE group (above). Each assay will use six randomly selected BIOASSAY MALES.

Experiment 1: When vitellogenesis is first detected in a VIT female, that female will be bioassayed once a week until those follicles become atretic. A previous study found the interval from first detection of vitellogenesis to onset of atresia to be approximately two months (Mathies et al. 2004). In each bioassay, a NON-VIT female and a TEST MALE will be randomly selected and tested on the same dates as the VIT female. Because dates of detectability of vitellogenesis are likely to vary among females, assays for each VIT female may have their own unique start and end dates. Each BIOASSAY MALE will be presented with one sample each of the following four test samples: VIT female, NON-VIT female, TEST MALE, and a blank sample.

Experiment 2: This experiment builds on, and is conducted in parallel, with Experiment 1 using the same VIT females. It is anticipated that as vitellogenesis progresses, male preference for VIT females *vs*. NON-VIT females will become apparent. The day after this preference becomes manifest for a female, a skin scent sample will be fractioned that day. That evening, a bioassay will be conducted by presenting each BIOASSAY MALE (same males used previous night) with a side-by-side choice of each fraction along with a blank sample (control) and a whole scent sample from that VIT female. This procedure will be followed once a week in parallel with testing in Experiment 1.

Statistical analyses: The statistical experimental unit will be the bioassay conducted for a female on a single date. Thus, observations from the six males within an assay will be averaged for each male behavioral response metric. Male response will be analyzed using a two-way repeated–measures analysis of variance where treatment [test group (Experiment 1); fraction type (Experiment 2)] is the between-measures factor, bioassay date is the within-measures (repeated) effect, and mean male response (based on n = 6 per assay) is the response metric.

12. Description of Environmental Conditions and Monitoring Requirements:

Three animal rooms will be used to house snakes and conduct assays (one common room, one to induce vitellogenesis in VIT females, one to conduct testing). Lights in all rooms will ramp on at 1330 h and off at 0130 h MST (L:12 h; D:12 h). These times were chosen to avoid acclimating snakes away from their accustomed light cycle [Guam time (GMT + 10 h)]. Air temperature will be 22°C in the room used to induce vitellogenesis (see also Methods/Procedures: *Induction of vitellogenesis in females*:). Relative humidity

(RH) will be the maximum achievable at 22°C. Air temperature in the rooms used to house all other snakes and conduct bioassays will be 28-29ºC. RH will be 80-90%. Temperature and RH will be measured and stored within temperature and RH data loggers placed within cages and the bioassay apparatus. Data from the data logger will stand as the data used to set and tune room environmental controls. Temperature and RH monitoring sensors and equipment intrinsic to the building environmental system will not be used to determine temperature and RH in the room. Light intensity will be determined before study start. Living Water Misters may be used in all housing/testing locations. Off/on times and frequency will be determined before study start.

13. List number and title of Standard Operating Procedures (SOPs):

AC/CO 014.00 Brown Tree Snake Maintenance
AC/CO 016.00 Animal Quarantine Procedures at Fort Collins
HS 004.00 Personal Protective Equipment
HS 008.00 Hazard Communication

14. List of Records to be Maintained:

A study log book and data sheets will be used to record assignment of snakes to groups, individual body masses and lengths, follicular status, and spatial arrangement of test samples within bioassay apparatus. Activity of bioassay males will be recorded on videotape. Data will be extracted from videos and recorded on datasheets. Data for room temperatures and RH will be stored as computer files.

15. Permits/Certifications:

This study is part of the objectives as identified in the Brown Treesnake Control Plan, as approved June 28, 1996 by the Aquatic Nuisance Species Task Force, and 2) under terms of the Memorandum Agreement among the U.S. Department of Defense, U.S. Department of Agriculture, the Government of Guam, and the state of Hawaii. A Federal Fish and Wildlife Permit (number MA097832-0) authorizes NWRC to import and maintain alive up to 300 brown treesnakes a year from Guam. This permit is effective 02/16/2007 to 02/15/2012.

16. Endangered Species Act Compliance:

Is there a possibility that the study, as proposed, will or may affect threatened or endangered (T&E) species?
Yes:_____ No:___X__, this study will have no effect on any T&E species.

17. Historical Resources:

Does the study involve any major ground disturbance or otherwise have the potential to adversely affect historic resources?

Yes:_____ No:__X___

18. National Environmental Policy Act Compliance:

Does this study qualify for categorical exclusion[1] from further NEPA analysis?

Yes:__X___ No: _____ Unsure: _____

19. Employee and Public Safety:

USDA/APHIS/WS safety regulations will be followed in all activities. Protective clothing will be worn as appropriate (HS 004.00).

20. Schedule:

Proposed Experiment Start Date: __June 1, 2007_____

Proposed Experiment Termination Date: __December 31, 2007_____

Proposed Study Completion/Archive Date: __December 30, 2008_____

[1] Categorical exclusion is based on consideration of all environmental issues relevant to this study, including consideration of cumulative impacts on wild animals and other environmental parameters, such as removal caused by the study combined with other reasonably foreseeable removals by other causes (e.g., sport harvest, wildlife damage management actions, and any other known causes of mortality) pursuant to APHIS NEPA Implementing Procedures at 7 CFR Part 372.5(c)(2)(i) which categorically exclude:

> "Research and development activities . . . that are carried out in laboratories, facilities, or other areas designed to eliminate the potential for harmful environmental effects--internal or external--and to provide for lawful waste disposal.

or at 7 CFR Part 372.5(c)(1)(i) which categorically exclude:

> ARoutine measures, such as . . . surveys, sampling that does not cause physical alteration of the environment, testing . . . removals . . . (This) may include the (lawful) use . . . of chemicals, pesticides, or other potentially hazardous or harmful substances, materials, and target-specific devices or remedies, provided that such use . . . : (A) . . . is localized or contained in areas where humans are not likely to be exposed, and is limited in terms of quantity . . . B) . . . will not cause contaminants to enter water bodies . . . (C) . . . does not adversely affect any federally protected species or critical habitat; and (D) . . . does not cause bioaccumulation.@

21. Staffing:

Title	Activity	SY
Study Director	Design and Analyses	0.08
	Data Collection	0.20
	Reports and Publications	0.04
	Archiving Review	0.02

22. Principal Investigators, Cooperators and Consultants:

USDA/Wildlife Services/Guam (collection of test subjects)
1060 Army Drive, Suite 103C
Barrigada Heights, GU 96913.

23. Related protocols:

QA-1372 (Overall research focus)
QA-1437 (Specifically Animal Transport)

24. Cost Estimate for Each Fiscal Year:

	FY-07
A. Salaries and Benefits	$13,200
B. Facilities (in addition to existing facility or space costs)	$3,250
C. Equipment	$250
D. Supplies	$600
E. Operating Costs (e.g., travel, misc. services, etc.)	$0
TOTAL	$17,300

25. Staff qualifications:

All study participants (T. Mathies and R. Mauldin) have curriculum vitae on file that verify their training and qualifications for the work preformed in this study.

26. Archiving:

All raw data, documentation, records, protocols, specimens, correspondence and other documents relating to interpretation and evaluation of data, and final reports generated as a result of this study will be retained in the archives of the National Wildlife Research Center at Fort Collins, Colorado.

27. Protocol Amendments:

Any changes in this protocol will be documented on the Study Protocol Amendment Form, reviewed by appropriate personnel (e.g., IACUC, IBC, ACP, QA, etc.), and

signed and dated by the Study Director, Research Program Manager and Sponsor. Amendments will be distributed to all study participants as appropriate.

28. References:

Mathies T., E. A. Franklin, and L.A. Miller. 2004. Proximate cues for ovarian recrudescence and ovulation in the brown treesnake *(Boiga irregularis*) under laboratory conditions, Herpetological Review, 35:45-46.

29 Appendices:

Animal Use Appendix

Signature Page:

_____ _____
Study Director Date

Concur:

_____ _____
Research Program Manager, NWRC Date

Approved:

_____ _____
Director, NWRC Date

Processing:

QAU Received: _____ QAU Processed: _____

IACUC (see IACUC approval date in Animal Use Appendix)
IBC (see IBC approval date in Microbiological/Biohazardous Materials Use Appendix

Animal Use Appendix

A. Animal description:

1) Species: brown treesnake (*Boiga irregularis*)
2) Strain and substrain (if applicable): "Guam form"
3) Number and sex: Males: 56; Females: 33. Of these snakes, 5 males and 3 females are collected and imported as spares to offset possible losses during shipping.
4) Snout-vent length range: 960 to 1400 mm
5) Age: unknown, but sexually mature

B. Rationale for involving animals, for appropriateness of species, and for numbers:

1) Rationale for involving animals: The complexity of the processes being studied cannot be duplicated or modeled using in vitro models. There is not enough information known about the processes being studied to design non-living models. Efficacy of the test substances cannot be determined any other way than by direct testing.
2) Rationale for appropriateness: brown treesnake is the target species
3) Rationale (include calculations as appropriate): No sample size calculations were attempted. Sample sizes (15 snakes per each of the three tests groups) are low, but in line with those used in other published studies on bioassays of snake sex pheromones. The repeated measures design (i.e., repeated testing of individuals) should increase statistical power.

C. Source:

Snakes will be provided by USDA/APHIS/Wildlife Services, Guam.

D. Method of identification of animals:

Snakes will be maintained in individual uniquely numbered and labeled cages.

E. Trapping/Collecting:

N/A

F. Transport:

see Animal Transport Appendix, QA-1437

G. Handling/restraint:

In instances where it is necessary to handle snakes, restraint will be by hand

H. Quarantine:

AC/CO 016.00 Animal Quarantine Procedures at Fort Collins

To reduce chances of snakes carrying mites to NWRC, snakes will be sprayed on 1-2 occasions with an over-the-counter pet product for tick and flea control such as Frontline® spray prior to shipping from Guam. Any parisitide used will be approved in advance by the Attending Veterinarian

I. Housing/maintenance:

Snakes will be housed individually in clear plastic food storage boxes (60 X 39 X 13 cm). These are the same cages previously used to house African house snakes A plastic water bowl with an entrance hole cut in its outer wall of the double-sided wall will allow a snake to use the area beneath the bowl as a hide. Commercially purchased dead frozen fuzzy mice will be used as food and thawed just prior to use.

J. Disposition of animals:

At study end, animals will be euthanized as described below under **M. Euthanasia** or maintained at the NWRC for use in future studies.

K. Duplication of prior studies:

Automated literature search:

Date search performed: January 23, 2007
Keywords used: Pheromone, pheromonal, snake, brown treesnake
Period covered by search: 1969-2006, 1978-2006
Names of databases searched: Biosis, Zoological Record
Did the search reveal applicable alternatives (Y/N)? N

L. Pain or distress:

Consultation with Attending Veterinarian:

Name of Attending Veterinarian:____ Gordon Gathright _____
Date of Consultation: _____January 24, 2007_____

Is this study expected to cause more than momentary or slight pain or distress?

Yes:_____ No: __X____

No pain is anticipated.

If the Attending Veterinarian determines procedures may cause more than momentary or slight pain or distress, continue with the following items. If not, indicate as N/A.

1) Alternative procedures: N/A
2) Sedatives, analgesics, or anesthetics: N/A
3) Surgery: N/A

M. Euthanasia:

Animals that become Ill or injured during the course of the study will be euthanized using any of the above methods recommended below.

The 2000 Report of the AVMA Panel on Euthanasia (pg 687) lists only the following two general methods as being appropriate for reptiles:

1. Injectable agents—Sodium pentobarbital (60 to 100 mg/kg of body weight) administered intravenously or intraabdominally. Barbiturates other than pentobarbital can cause pain on injection.

2. Physical methods—all generally recognized methods are permitted. If decapitation is used, it must be followed by pithing or captive bolt.

Inhalant agents are not recommended because reptiles can voluntarily restrict breathing, enduring anoxia, and time to loss of consciousness may be greatly prolonged.

Society for the Study of Amphibians and Reptiles (SSAR) recommends methods for euthanasia be consistent those in the 2000 AVMA Report.

N. IACUC approval:
Date of IACUC Approval Letter:_____

Appendix 2.

National Wildlife Research Center **QA-1438**
AMENDMENT TO STUDY PROTOCOL

Study Director___Tom Mathies_____ **Amendment No.** _1_____ **Page** _1__ **of** ___

Changes in dates:

	No date changes	
X	Experiment Start Date:	(current): 1 June 2007 (revised): 17 February 2008
X	Experiment Termination/Completion Date:	(current): December 31, 2007 (revised): December 31, 2008
X	Study Completion/Archive Date:	(current): December 30, 2008 (revised): December 30, 2009

Additional protocol section/subsection/appendix to be changed:

10. Methods/Procedures:

An additional experiment is being added under this section (see revisions below)

Description of revisions: *(Please provide the level of detail normally required in the protocol)*

Experiment 3: The purpose of this experiment is to assess abilities of males in the BIOASSAY MALE group to follow lengthy substrate scent trails of females in the VIT, NON-VIT, and TEST MALE groups. The test apparatus will be constructed such that a BIOASSAY MALE placed within will be able to move through a branching system of interconnected troughs containing a scent trail. This system of troughs will be oriented horizontal to the room floor and placed up off the floor on supports. Troughs will consist of large diameter PVC pipe (8-12 in diameter) cut lengthwise. The system of troughs will span at least the length of the room where testing will occur, and may double back once. The scent of a VIT, NON-VIT, or TEST MALE will be applied to a linear substrate and this substrate will be placed within the system of troughs such that there is a single scent trail running from the start point to an endpoint within the trough system. The substrate will be poles (specific type to be determined, e.g., wood). Scent will be applied to poles by coaxing a snake to crawl the length of the pole. Snake-scented poles will then be placed end-to-end within the trough system to form an essentially continuous scent trail. Nylon mesh screen will be affixed over tops of troughs to prevent the BIOASSAY MALE from escaping the trough system. A BIOASSAY MALE placed within a trough

will be filmed overnight as described in the previous experiments. Each BIOASSAY MALE will be tested once a week in parallel with testing in Experiment 1.

Justification/reason(s) for changes and impact on study: *(If dates are changed, please provide a description of current status of study and remaining study plan/schedule.)*

Dates: This study commenced on 17 February with the beginning of the cooling period for females. The cooling period ended on 21 May. It is anticipated that testing will begin as early as the first week in July 2008. Testing would be complete by revised (above) end date of December 31, 2008.

The additional experiment is added because the utility of the pheromone attractant will partly depend on how well males follow the pheromone trail.

(Review by IACUC is required if changes involve or affect animals)

_____ _____

Study Director **Date**

_____ _____

Research Program Manager **Date**

QAU Received: _____ **QAU Processed: _____**