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Chemically mediated sexual behavior of the brown tree snake, *Boiga irregularis*[1]

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Abstract: The brown tree snake (*Boiga irregularis*) is an invasive pest species responsible for serious economic and ecological damage on the Pacific island of Guam, including the extirpation or extinction of nine native forest birds. Bioassays utilizing the courtship and mating behaviors of the brown tree snake were developed to begin isolating the female sex pheromone of this species. Filter paper treated with hexane extracts of female skin and solvent controls were presented to captive males in their home cages. Males displayed significantly more courtship behaviors to extracts than controls. These lipid extracts were subsequently fractionated by column chromatography. Fractions and controls were presented to males in a randomly selected sequence and male courtship behavior assayed. Significant differences in the number of courtship behaviors displayed to each of the fractions and the controls were observed. Fractions 1 through 4 each elicited courtship behavior from males, with fraction 4 eliciting courtship behaviors in 6 of 8 trials, while controls elicited no courtship behaviors from males.
Keywords: Boiga irregularis, brown tree snake, courtship behavior, Guam, introduced pest species, sex pheromones.

Résumé: Le serpent brun arboricole (*Boiga irregularis*) est une espèce nuisible introduite, causant d'importants dommages écologiques et économiques sur l'île de Guam (Océan Pacifique). Cette espèce est, en effet, responsable de l'élimination voire l'extinction de neuf espèces indigènes d'oiseau forestier. Des dosages biologiques utilisant les comportements d'accouplement et de cour de cette espèce ont été faits afin d'isoler la phéromone liée au sexe femelle. Du papier filtre traité avec des extraits d'hexane de la peau des femelles, ainsi que des solvants témoins ont été présentés à des mâles captifs. Ces derniers ont manifesté un comportement de cour significativement plus vigoureux en présence d'extraits. Ces extraits lipidiques ont été fractionnés par la suite dans une colonne chromatographique. Les produits de fractionnement (les fractions) et les témoins ont été présentés aux mâles de manière aléatoire et leur comportement de cour évalué. On a observé des différences significatives du nombre de comportement de cour en fonction des fractions et des témoins. Chacune des fractions 1 à 4 a provoqué un comportement de cour chez les mâles, la fraction 4 induisant 6 fois sur 8 un tel comportement alors qu'aucun comportement de cour n'a été stimulé par les témoins.
Mots-clés: Boiga irregularis, serpent brun arboricole, comportement de cour, Guam, espèce nuisible introduite, phéromones sexuelles.

Introduction

Since the first pheromone was identified in the silk moth, *Bombyx mori*, well over a thousand insect sex pheromones have been identified (Abelson, 1985). In contrast, very few vertebrate pheromones of any kind have been identified in the same time period (Duvall, Müller-Schwarze & Silverstein, 1986). Notable exceptions include pheromones identified in the domestic pig, *Sus scrofa* (Patterson, 1968), the goldfish, *Carassius auratus* (Stacey & Sorenson, 1986), the Asian elephant, *Elaphas maximus* (Rasmussen, *et al.*, 1996), the Japanese red-bellied newt, *Cynops pyrrhogaster* (Kikuyama *et al.*, 1995), and the red-sided garter snake *Thamnophis sirtalis parietalis* (Mason *et al.*, 1989; 1990).

The relative lack of success in identifying the chemical structures of vertebrate pheromones is generally attributed to difficulties in developing robust bioassays (Albone, 1984). It is often difficult to replicate the social or environmental context in which vertebrate pheromones operate under the controlled conditions necessary to conduct a successful bioassay. Also, many vertebrate semiochemicals consist of dozens of compounds which can lose their signal function when separated into fractions (Albone, 1984; Duvall, Müller-Schwarze & Silverstein, 1986). However, snakes, more than most vertebrates, are like insects in that they rely on the detection of chemical stimuli for information from their environment and chemical signals release stereotypical behaviors that can be observed in the laboratory (Carpenter, 1977; Mason, 1992). Thus, it is possible to create unequivocal bioassays designed to isolate pheromones (Mason *et al.*, 1989;1990; Mason, 1993).

The brown tree snake, *Boiga irregularis*, is a nocturnal, rear-fanged colubrid native to Australia, Papua New Guinea and the Solomon Islands (Fritts, 1988; Rodda, Fritts & Chiszar, 1997). Although this species is primarily arboreal, it spends a significant amount of time on the forest floor when searching for prey (Fritts, 1988; Rodda, Fritts & Chiszar, 1997). This snake has a generalized diet consisting of birds, reptiles, amphibians and mammals (Savidge, 1988). Brown tree snakes can reach lengths of up to 3 meters and masses of up to 2 kilograms (Fritts, 1988; Rodda, Fritts & Chiszar, 1997).

This species was accidentally introduced on Guam sometime during or shortly after World War II (Rodda, Fritts & Conry, 1992). Like most other Pacific islands, Guam had no endemic snake species, presenting the brown tree snake with an environment containing no specialized predators and

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prey naive to snake predation. As a result, this species has flourished, reaching densities of nearly 13 000 brown tree snakes per square mile in the tropical rain forests of the island (Rodda, Fritts & Conry, 1992).

With such high densities, *Boiga irregularis* has become a serious pest for a variety of reasons. This snake has caused the extirpation of nine of twelve native forest bird species on Guam, including the extinction of three endemic species (Savidge, 1987). It is also responsible for dramatically reducing populations of introduced reptiles and mammals, native lizards, the endangered Mariana fruit bat and domestic animals (Wiles, 1987; Savidge, 1988; Fritts & McCoid, 1991; Rodda & Fritts, 1992). A rear-fanged colubrid, this snake readily bites people, including dozens of sleeping babies (Fritts, McCoid & Haddock, 1990; 1994). In addition, thousands of power outages resulting in severe financial loss have been attributed to brown tree snakes short-circuiting power lines while searching for prey (Fritts, Scott & Savidge, 1987).

There is great concern that the brown tree snake will be inadvertently introduced to other areas in the Pacific, resulting in a repeat of the disaster observed on Guam. Brown tree snakes have already been discovered on Wake Island, Diego Garcia and Okinawa, Japan (Fritts, 1988). Brown tree snakes have been discovered on Hawaii on at least seven occasions near airports and military bases, arriving primarily by aircraft from Guam (Fritts, 1988; Rodda, Fritts & Chiszar, 1997). This is of major concern, as Hawaii has one of the highest numbers of endangered and threatened species in the world (Office of Technology Assessment, 1993).

As part of an ongoing study examining the basic reproductive physiology and behavior of the brown tree snake, we have made the first observations of courtship and mating behavior in this species using members of our captive colony (Greene & Mason, unpubl. data). The courtship behavior of this species closely parallels that of other colubrids and adheres to the triphasic schema developed to describe colubrid courtship in a standardized manner (Gillingham *et al.*, 1977; Gillingham, 1979). In this species, males will court females only after tongue-flicking the skin of an attractive female, indicating that a sex pheromone is present in the female integument.

We report here initial results from bioassays we have developed to isolate and characterize the brown tree snake female sex pheromone. Isolation and identification of this pheromone may prove useful in controlling the brown tree snake (Mason, in press). Currently, the most effective means of controlling brown tree snake populations is by trapping with live prey as bait, requiring an expensive breeding and maintenance program (Rodda *et al.*, 1992). This trapping method is only effective in the near vicinity of the trap where a snake could detect volatile prey odors or see the prey in the trap. Trails of synthetic pheromone could be applied to the forest floor as trails leading away from a trap in several different directions, much like the spokes of a wheel, to lead snakes to the trap from the surrounding area by eliciting trailing behavior. This would not only be an effective method for capturing snakes on Guam but also for monitoring for the presence of brown tree snakes on other islands, such as Oahu, Hawaii, that do not presently have populations of brown tree snakes.

Material and methods

Animals used in this study were collected on Guam and have been housed under laboratory conditions for the past 5 years (Greene *et al.*, 1997). The captive colony contains 7 female and 10 male snakes, housed in Plexiglas cages designed specifically for arboreal reptiles (Mason *et al.*, 1991). The cages contain branches for climbing and hide boxes attached to the roof of the cages. Room temperature cycles daily from 24°C to 30°C and relative humidity is maintained at approximately 80% using a room humidifier and daily spraying of the inside of the cages with water. Light (14L:10D) is provided by overhead fluorescent lights and by natural light entering the snake room through windows. Snakes are fed mice or chicks every three weeks and water is available *ad libitum* for drinking and soaking.

All experiments were conducted between 1900 and 0200, the time when brown tree snakes are most active under the test conditions. As this was during the scotophase, lighting was provided by a 7 watt incandescent red light. All experiments were videotaped and subsequently analyzed by an observer blind to the experimental conditions.

Male responses were recorded for a 1-hour period following the introduction of a female ($n = 7$) into his cage, in order to determine which females in the captive colony were sexually attractive during the time of the study. Subsequently, a single shed skin from a sexually attractive mature female was placed in the cage of a male ($n = 10$) for 20 minutes and the male courtship behaviors were recorded to determine if, as is the case for other snakes, the pheromone was present in the skin (Noble, 1937; Mason, *et al.*, 1989). In addition, a single shed skin from another male was presented to the males ($n = 5$) in their individual home cages for 20 minutes to act as a control.

Lipid extracts were obtained by soaking freshly shed skins from attractive females ($n = 25$ skins) in hexane for 4 hours. The lipid extract was collected in a 250 ml round-bottom flask, the solvent was evaporated and the lipid yield was measured on a digital scale. A total of 664 mg of crude skin lipids were obtained and dissolved in 4 ml of hexane. A 1 ml aliquot of the extract was applied to a 10 cm × 8 cm filter paper and was allowed to air dry. A blank control consisting of untreated filter paper, whole solvent and lipid controls were produced by adding hexane or vegetable oil and hexane to a filter paper. The skin lipid extract and controls were presented to males in their individual home cages, in a random order for 20 minutes and courtship behaviors displayed by males were recorded (Figure 1). Only the highest ranking behavior attained in the male courtship sequence was used in the subsequent statistical analysis (Table I).

The crude skin lipid extract was then separated into 20 fractions using an Alumina activity III liquid chromatography column with a solution of hexane and diethyl ether as the mobile phase (Mason *et al.*, 1989; 1990), resulting in subsequent fractions containing more polar compounds. For each fraction the solvent was evaporated, the yield measured using a digital scale and the lipids dissolved with 4 ml of hexane. Pieces of 8 cm × 10 cm filter paper were treated with each fraction by applying approximately 1 ml aliquots

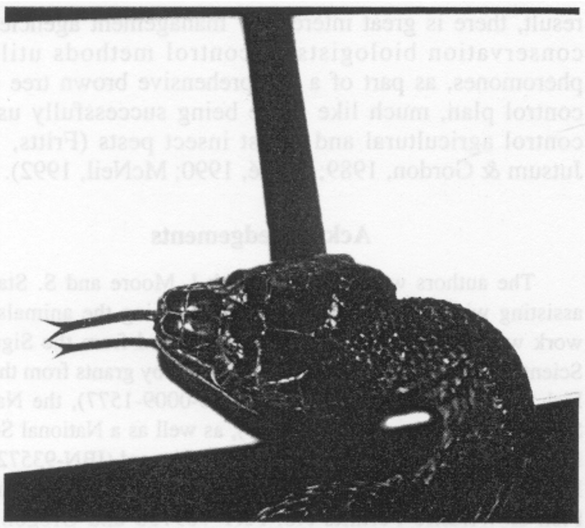

FIGURE 1. A male brown tree snake investigating a filter paper treated with female skin lipid extract with tongue-flicking behavior during a bioassay test.

TABLE I. Graduated scale of courtship behavior for the male brown tree snake, *Boiga irregularis*

Ranking	Behavioral description
0	Male fails to investigate the female or only briefly investigates the female with tongue flicks.
1	Male rapidly tongue-flicks the integument of the female, displays head-jerking behavior and probes the female's body with snout.
2	Male chin rubs the dorsum of the female with surging head movements.
3	Male aligns body with female's.
4	Male attempts cloacal apposition (tail-search copulatory attempt) by maneuvering his tail under the female's tail.
5	Copulation.

of concentrated fraction to the filter paper and allowing the hexane to evaporate.

A preliminary screening for courtship behavior was conducted by presenting males (n = 10) with the sequential samples in order, each one for 10 minutes It became clear that the active components occurred in fractions 1 through 4, so a second series of assays were carried out by presenting fractions 1 through 4 and a control (fraction 5 through 20 combined) to each male (n = 10) in a randomized sequential order. Each 20 minutes trial was conducted in a male's home cage with a 15-minute break between each test. As in the crude skin lipid extract bioassay, the presence of courtship behavior was assayed. In several cases, the data collected were not used in the analyses, as the males displayed defensive behaviors or did not tongue-flick the filter paper once during the entire trial. In snakes, tongue-flicking serves to bring chemical signals to the vomeronasal organ, located in the roof of the snake's mouth, where detection occurs (Halpern & Kubie, 1980).

Chi-squared tests based upon proportions were used to determine if there were significant differences in the number of trials where males displayed courtship behaviors to the various stimuli in the bioassays. Specifically, differences between fractions 1 through 4 and the control were examined along with differences between fractions 1 through 4 only. Statistics were analyzed using the SigmaPlot statistics program (©Jandel Scientific Corporation).

Results

All females in our captive colony elicited vigorous courtship displays from males, demonstrating that the females were sexually attractive during the study. Included in the male courtship display were head-jerking behavior, a rapid, rhythmic jerking head movement and snout-probing, a behavior where males firmly press their snout against the female's side or dorsum while tongue-flicking with only the tips of the tongue exposed. In addition, males displayed chin rubbing, a behavior where the males pressed their chin on the female's dorsum while tongue-flicking and moving along her body in forward surging movements. These behaviors are unique to courtship and are only observed in a reproductive context. Other behaviors included body alignment, where males aligned their bodies along the length of the female and tail-search copulatory attempts, where the males attempted to align their cloacae with the female's.

Eight of 10 males responded to shed skins from attractive females with courtship behaviors, demonstrating that the sex pheromone is located in the skin and that this stimulus alone is sufficient to induce courtship behaviors. Males displayed head-jerking and chin rubbing after tongue-flicking the shed skin. In several instances, vigorous chin rubbing was displayed with males pushing the shed skin along the floor of the cage. No other courtship behaviors were displayed by the males. No behaviors were displayed to the male shed skins.

The results from the first bioassay demonstrated that the sex pheromone was extracted from shed skins by hexane, as the crude female skin lipid extract elicited courtship behaviors from 67% of males, but no behaviors were seen to the three controls (χ^2 = 21.600, df = 3; P < 0.001; Table II). Once again, males responded with head-jerking and chin rubbing to the crude skin lipid extract.

Results of the second bioassay indicated that fractions 1 through 4 elicited significantly more male courtship behaviors than the control (χ^2= 12.631, df = 4; P = 0.013; Table III). Males displayed head-jerking and chin rubbing to fractions 1 through 4 while never displaying any courtship behaviors to the control. There was a trend for an increase in courtship behaviors with extracts 1 to 4, although no significant differences were found (χ^2 = 4.923, df = 3; P = 0.178). There were no significant differences in the proportion of males displaying head-jerking (χ^2 = 0.0752,

TABLE II. The response of male *Boiga irregularis* to female skin lipid extract and controls

Behavior	Proportion of males that displayed courtship behaviors to: Skin lipid Extract	Lipid control	Solvent control	Blank control
Head-jerking	0.11	0.00	0.00	0.00
Chin-rubbing	0.56	0.00	0.00	0.00
Total	0.67	0.00	0.00	0.00

n = 9 in all assays.

TABLE III. The response of male *Boiga irregularis* to female skin lipid fractions 1-4 and control (fractions 5-20) in the bioassay

Behaviour	Proportion of males that displayed courtship behaviors to				
	Fraction 1 ($n = 7$)	Fraction 2 ($n = 10$)	Fraction 3 ($n = 7$)	Fraction 4 ($n = 8$)	Control ($n = 10$)
Head-jerking	0.14	0.10	0.14	0.13	0.00
Chin-rubbing	0.14	0.20	0.43	0.63	0.00
Total	0.28	0.30	0.57	0.76	0.00

df = 3; $P = 0.995$) or chin-rubbing between fractions 1 through 4 ($\chi^2 = 2.304$, df = 3; $P = 0.512$).

Discussion

Through bioassays which assayed for male courtship behavior we have isolated four female skin lipid fractions containing components of the female brown tree snake sex pheromone. The sex pheromone is composed of a suite of nonvolatile skin lipid molecules that appear to be nonpolar in nature as the four fractions that contain the pheromone eluted off of the chromatography column in 100% hexane or 98% hexane/2% diethyl ether.

The female sex pheromone of the red-sided garter snake, *T. s. parietalis*, a colubrid related to the brown tree snake, has been identified as a series of long chain saturated and monounsaturated methyl ketones (Mason *et al.*, 1989; 1990; Mason, 1993). A mixture of long chain methyl ketones, including a series of six ketodienes, have also been identified from the skin lipids of the brown tree snake (Murata *et al.*, 1991). However, no methyl ketones were found in female skin lipid fractions 1 through 4 when analyzed using gas chromatography/mass spectrometry, indicating that the female sex pheromone of brown tree snakes is composed of other compounds.

The importance of pheromones in the mediation of brown tree snake courtship behavior is reflected by the response of males in the bioassays. Shed skins and the female skin lipid extract elicited head-jerking behavior and chin rubbing, the initial behaviors of the male courtship sequence, from males without the additional sensory inputs normally provided by a female during courtship. However, the other male courtship behaviors, including body-alignment, tail-search copulatory attempts and copulation seem to require visual and tactile signals from the female to be displayed.

The objective of future experiments is to identify the brown tree snake female sex pheromone, concentrating our efforts on separating fractions 1 through 4 in order to isolate subfractions that retain activity in our bioassay that can be chemically analyzed for subsequent identification and synthesis, as well as for testing in the laboratory and in the field on Guam. If successful, this research will result in the identification of only the second reptilian pheromone and one of only a few vertebrate pheromones.

The introduced population of the brown tree snake on Guam represents one of the first opportunities to use a vertebrate pheromone to help manage an introduced pest species. No especially effective methods presently exist to control the brown tree snake in areas where it is established or to prevent its spread to other areas in the Pacific. As a result, there is great interest by management agencies and conservation biologists in control methods utilizing pheromones, as part of a comprehensive brown tree snake control plan, much like those being successfully used to control agricultural and forest insect pests (Fritts, 1988; Jutsum & Gordon, 1989; Cardé, 1990; McNeil, 1992).

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