

AN ABSTRACT OF THE THESIS OF

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In animals, reproductive behaviors serve to attract individuals together during the breeding season and to coordinate the behavioral and physiological states of individuals so that mating can successfully occur. In snakes, the various reproductive behaviors including courtship, mating, courtship inhibition, male combat and trailing are mediated primarily by pheromones. Pheromones are naturally produced chemical signals that influence the physiology or behavior of a conspecific when released by one individual into the environment. The research included in this study was designed to characterize the role pheromones play in the mediation of reproductive behaviors of the brown tree snake (*Boiga irregularis*) a rear-fanged, arboreal colubrid native to Australia, Papua-New Guinea and the Solomon Islands. Much attention has been paid to this species as it is an introduced pest species on the Pacific island of Guam where it has caused considerable economic and ecological damage.

In order to create behavioral bioassays designed to experimentally determine the role pheromones play in the mediation of brown tree snake reproductive behavior, the complete repertoire of courtship, mating and male combat behaviors were described.

Male brown tree snakes display combat and courtship behaviors similar to other colubrid species while female brown tree snakes take a very active role in courtship, displaying behaviors that both release and inhibit male courtship. These data represent the first description of any reproductive behaviors in this species and one of only a few studies examining the reproductive behaviors of a tropical species. A behavioral bioassay measuring the release of male courtship behavior was utilized to isolate and characterize the female sex pheromone of this species as a suite of non-polar, non-volatile skin lipid molecules. Using another bioassay, the pheromonal inhibition of male courtship behavior by a volatile pheromone originating from female cloacal secretions was characterized. Lastly, pheromone mediated mate attraction was examined by determining the abilities of male brown tree snakes to follow conspecific pheromone trails during the breeding season. Male brown tree snakes possess the ability to follow both male and female pheromone trails but did not, however, discriminate between male and female pheromones trails when given a choice.

**Pheromonal Mediation of Reproductive Behavior in the Brown Tree Snake**

by

**Michael J. Greene**

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Michael J. Greene, Author

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## **Dedication**

**This thesis is dedicated to my parents Cheryl and Michael Greene  
in acknowledgement of their love, support and encouragement.**

# **1. Pheromonal Mediation of Reproductive Behavior in the Brown Tree Snake, *Boiga irregularis***

## **General Background**

Reproductive behaviors consist of a series of motor patterns that ultimately result in the fertilization of gametes and the subsequent passing of parental genetic information to offspring. Because of the central importance of reproduction to animals, a variety of reproductive behaviors have evolved including courtship and mating behaviors as well as associated behaviors that serve to attract males and females together during the breeding season and to bring individuals into a physiological and behavioral state where courtship and mating can successfully occur (Blum, 1985). Examples of these sex-related behaviors include nest building (Evans, 1997), territoriality (Mathis *et al.*, 1995), mate choice (Gould and Gould, 1997), male combat (Shine, 1993; Pellis, 1997) and mate attraction (Cardé and Baker, 1984; Roelofs, 1995).

Reproductive behaviors are mediated by a complex interaction of internal factors including genetics and physiology as well as by the detection of external information such as environmental stimuli and social signals which are conveyed as visual, tactile, auditory and chemical information (Bermant and Davidson, 1974). These different factors all play a role in eliciting reproductive behaviors and coordinating reproductive events with other conspecifics. Typically, animals utilize a combination of stimuli to signal between conspecifics in order to mediate reproductive behaviors, however a particular form of communication often plays a dominant role over the others (Cardé and Baker, 1984; Stadler, 1984; Harris and Foster, 1995).

Chemical communication is a fundamental aspect of most species' biology and for most species it represents a dominant sensory modality (Eisner and Meinwald, 1995). The detection of chemicals from the environment is a central component of virtually every activity important to an animal's ability to reproduce and survive. These activities including food collection (Burghardt, 1990; Byers, 1995), defense (Berenbaum, 1995; Daly, 1995) and reproduction (Vandebergh, 1983; Cardé and Baker, 1984; MacDonald *et al.*, 1990; Eisner and Meinwald, 1995). Additionally, chemical signaling is thought to be the ancestral mode of communication because it is a prominent trait of all taxa and is the sensory modality used by more primitive, unicellular organisms (Eisner and Meinwald, 1995).

The mediation of reproductive behaviors by chemical signals occurs through the use of pheromones, naturally produced chemicals that when released into the environment by one individual cause a subsequent behavioral or physiological change in a conspecific upon detection (Karlson and Luscher, 1959). Pheromones act either by releasing specific behaviors or have longer term, primer effects by causing changes in another individual's physiological state (Albone, 1984). They are known to mediate many reproductive behaviors including courtship and mating behaviors along with behaviors involved with mate choice, mate attraction and location, sex and species recognition, male agonistic behaviors such as combat (Duvall, *et al.*, 1985; MacDonald *et al.*, 1990). As a result of their central importance in reproduction, an understanding of pheromonal communication is necessary to understand the proximate mechanisms that control reproductive behaviors in animals.

Pheromones can be classified into two groups according to their chemical characteristics and the medium by which they are transported (Albone, 1984; Stadler, 1984; Halpern 1992). The majority of pheromones are relatively simple, low-molecular weight compounds that are either volatile in air or diffuse easily through water and can be detected by a receiver over relatively large distances from their source. These pheromones have been generically termed olfactory pheromones (Mustaparta, 1984; Stadler, 1984). Other pheromone systems, in contrast, utilize what have been termed contact pheromones which are usually high-molecular weight compounds of low volatility that must be contacted directly with an animal's sensory organ in order to be detected (Stadler, 1984; Halpern, 1992).

The vast majority of research concerning pheromones has been conducted in the insects (Bell and Cardé, 1984; Roitberg and Isman, 1992; Cardé and Bell, 1995; Eisner and Meinwald, 1995) where over a thousand insect pheromones have been identified to date (Abelson, 1984). Sex attractant pheromones alone have been identified in greater than 1600 insect species from over 90 families in 9 orders (Mayer and McLaughlin, 1991; Roelofs, 1995). In addition, there are many other pheromone mediated insect behaviors that have been studied even though the pheromone that mediates the behavior has not been identified (Aldrich, 1995; Howard and Akre, 1995).

The success investigators have had in determining the roles pheromones play in mediating insect behaviors is due to the fact that chemical communication is the dominant sensory modality used by insects and that pheromones release relatively simple, stereotyped behaviors in the absence of other sensory inputs (Silverstein, 1984).

Importantly, many of these pheromone mediated behaviors can be elicited in the laboratory under artificial conditions so that they can be exploited in bioassays.

The 'response guided approach' is an experimental paradigm that takes advantage of these insect characteristics to examine pheromone communication (Albone, 1984). This approach employs the particular behavior being studied in a bioassay where the experimental animal displays its normal behaviors in response to chemical stimuli taken from the emitting source. Typically, the starting material containing the pheromone is taken as a solvent extract. Fractions of the crude extracts can then be created and presented to the experimental subject in the bioassay. If the animal displays the appropriate behavior to a fraction, components of the pheromone are known to be present in that fraction. In this manner, the experimental animal effectively "chooses" fractions containing the pheromone and only compounds that are biologically meaningful to the animal are isolated. This technique is not only useful in isolating pure fractions of pheromones for chemical analyses, but it also provides an experimental paradigm that allows causal links between a pheromone and a particular behavior to be made.

In contrast to the abundance of pheromones identified in insects, only a handful of vertebrate pheromones have been isolated and positively identified. Notable examples include a releaser of female courtship behavior in the domestic pig, *Sus scrofa* (Patterson, 1968), a primer pheromone that increases male milt volume and a releaser of spawning behavior in male goldfish, *Carassius auratus* (Sorenson and Scott, 1994), the female sex attractant pheromone of the Asian elephant, *Elaphas maximus* (Rasmussen, *et al.*, 1996) and the male sex attractant of the Japanese red-bellied newt, *Cynops pyrrhogaster* (Kikuyama *et al.*, 1995). Other important examples include primer pheromones that

mediate female reproductive physiology under different social conditions in the house mouse (Novotny *et al.*, 1990) and the female sex pheromone of the red-sided garter snake *Thamnophis sirtalis parietalis* (Mason *et al.*, 1989, 1990). Numerous other pheromone systems have been identified, however the chemical structures of these pheromones have not been causally linked to specific behavioral or physiological effects in other conspecifics through the use of bioassays (see MacDonald *et al.*, 1990 for examples).

The low number of known pheromone structures in vertebrates is mainly a result of the multisensory nature of vertebrates which possess highly developed brains and sophisticated sensory systems that allow vertebrates to rely on a combination of sensory inputs to mediate a particular behavior (Albone, 1984). As a result, it is often difficult to provide the correct combination of environmental and social signals necessary to release a specific behavior, making it very difficult to create an effective bioassay in artificial laboratory and field situations. In addition, many vertebrate pheromones consist of complex mixtures of chemicals that lose their signal function when separated into fractions, making the response guided strategy of pheromone difficult to employ. As such, there are few instances in vertebrates where pheromones will elicit a behavior sufficiently simple, robust and stereotyped enough to be used in a bioassay (Albone, 1984; Novotny *et al.*, 1990).

### **Snakes as Model Organisms**

More than most vertebrates, snakes rely upon the detection of chemical information from their environment as their dominant sensory modality (Mason, 1992). In addition, snakes are much like insects in that pheromones release relative simple, stereotyped



behaviors that are generally displayed only in specific contexts such as during courtship. These traits conveniently allow for the construction of behavioral bioassays that allow for the identification of snake pheromones and for the characterization pheromone signaling in snakes. As such, snakes are considered excellent vertebrate models for the study of pheromonal communication (Mason, 1992; Halpern, 1992).

Snakes have highly developed chemical senses which reflect the importance of chemical signaling to their biology (Halpern, 1987; Halpern 1992). Snakes possess an olfactory system that detects primarily volatile chemicals and well developed vomeronasal organs that are distinct from the olfactory system and function to detect mostly non-volatile chemical signals (Halpern and Kubie, 1983; Halpern, 1992). In snakes, the vomeronasal system seems to be utilized in the detection of pheromones more often than the olfactory system. The vomeronasal organ detects pheromones associated with courtship, aggregation, mate attraction via the trailing of conspecifics, male agonistic behaviors, prey trailing and prey attack (see Halpern, 1992 for a review). Detection occurs by way of tongue-flicks which carry molecules from the source of the pheromone into the mouth where they are delivered to the vomeronasal ducts located in the roof of the snake's mouth (Halpern and Kubie, 1983; Halpern, 1992).

Most commonly, pheromonal mediation of snake reproductive behavior is manifested through the release of male courtship behaviors by female sex pheromones located in female skin lipids (Noble, 1937; Mason *et al.*, 1989, 1990; Mason, 1993). Early studies by Noble (1937) demonstrated that female sex pheromones in garter snakes (*Thamnophis*) are located in the female's dorsal skin lipids and serve to release male courtship behavior and play a role in mate attraction. Since then, a number of anecdotal

reports of courtship behavior being released by pheromones have been reported (Mason, 1992). However, few studies have examined this phenomena in an experimental manner, allowing few conclusions to be made about the fundamental characteristics of snake pheromones and the behaviors they are thought to mediate. These characteristics include information such as the chemical identity of the pheromone, pheromone biosynthesis, pheromone release into the environment and the subsequent detection of the pheromone by other conspecifics (Mason, 1992).

The most extensively studied pheromone system in snakes is the female sex pheromone of the red sided garter snake (*Thamnophis sirtalis parietalis*). This pheromone has been identified as a series of long chain saturated and monounsaturated methyl ketones (Mason *et al.*, 1989, 1990; Mason, 1993). In bioassays, the sex pheromone was shown to be attractive to males as demonstrated by the release the of male courtship behaviors (Mason *et al.*, 1989).

When male and female skin lipids were mixed and presented to males in a bioassay, male courtship behavior was inhibited (Mason *et al.*, 1989). Subsequent chemical analyses revealed that this male sex recognition pheromone was composed primarily of squalene and presumably serves to allow males to differentiate other males during mating aggregations (Mason *et al.*, 1989). Behavioral evidence exists in other species to support the use of pheromones in sex recognition (Vagvolgyi and Halpern, 1983).

A subset of male *T. s. p.* elicit courtship from other conspecific males (Mason and Crews, 1985). These males, known as “she-males” seem to have a selective advantage during mating, as they mate up to twice as often as normal males (Mason and Crews,

1985). Chemical analysis of she-male skin lipids revealed that these males produced the female sex pheromone. In addition, she-males did not have squalene, the male sex recognition pheromone of this species, in their skin lipids (Mason, 1992). Few other studies have attempted to chemically isolate or identify snake pheromones and this research represents the most sophisticated study of reptilian pheromone communication (Mason *et al.*, 1989, 1990; Mason, 1993).

Trailing behavior to locate mates during the breeding season is mediated by skin lipids, possibly by sex pheromones, passively deposited on the ground as snakes move through their environment (Noble, 1937; Ford and Low, 1983; Ford, 1986). The ability of male snakes to trail female conspecific pheromone trails during the breeding season seems to be ubiquitous in snakes and has been documented in numerous species representing 5 snake families (see Ford, 1986 for a review). The detection of trailing pheromones allows males to determine the sex, species and direction of the trail producing individual (Noble, 1937; Ford, 1978, 1982; Ford and Low, 1983).

Male-male ritualized combat behavior is released by a pheromone located in the skin of male snakes (Schuett and Gillingham, 1989; Andr  n, 1986; Secor, 1990) and there is evidence that volatile pheromones, originating from male skin, may also play a role in mediating combat behavior in some species (Andr  n, 1982). In snakes, male combat serves as a contest between males for access to females during the breeding season. Winning or losing a combat bout can have significant influence on the ability of a male to successfully mate with a female with winners typically going on to court females and losers reverting to a submissive stature where they avoid both male and female conspecifics (Schuett and Duvall, 1996).

Female garter snakes become unattractive to males after mating because of a pheromone deposited near the female's cloaca (Devine, 1977; Ross and Crews, 1977; R.T. Mason, unpublished). This pheromone, which appears to be relatively nonvolatile, acts in an antagonistic manner to the female sex pheromone by preventing males from initiating courtship. It is unclear whether this pheromone is produced by the female in order to signal her mated status to males (Devine, 1977) or by the males in order to prevent the female from copulating multiple times with different males (Ross and Crews, 1977).

### **Biology of the Brown Tree Snake**

The purpose of this research is to characterize the role pheromones play in mediating the reproductive behaviors of the brown tree snake, *Boiga irregularis*, a rear-fanged colubrid native to the eastern and northern coastal areas of Australia, Papua New-Guinea, and the Solomon Islands (Cogger, 1992). The primary habitat of this species is in the tropical rain forests of these areas where they are nocturnal, spending the night actively foraging for an generalized diet of lizards, small mammals, birds and bird eggs (Savidge, 1988). Their prey is killed by a combination of constriction and envenomation (Rodda *et al.*, 1997). The venom is injected into the prey through grooves in their enlarged rear fangs and is not considered toxic to humans (Weinstein *et al.*, 1991). Brown tree reaches snout-vent-lengths of up to 3 m and masses of up to approximately 2 kg and have a relatively slender body plan characteristic of arboreal snakes (Lillywhite and Henderson, 1993; Rodda *et al.*, 1997). This species possess a sexual dimorphism where males tend to be longer and heavier than females as fully mature adults (Shine, 1996).

The brown tree snake was accidentally introduced to the Pacific island of Guam during or after World War II (Rodda *et al.*, 1992). Like many other Pacific islands, no native snake species have ever existed on Guam. As a result, over the next 50 years this species flourished on Guam until a recent study found densities of brown tree snakes estimated to be nearly 13,000 snakes per square mile (Rodda *et al.*, 1997).

As Guam's brown tree snake population increased from the time of its introduction it became a serious menace to humans on the island. The snakes climb power lines in search for prey and, in many instances, will cause power outages. This has been a major problem for the island (there have been 1200 snake caused power outages between 1978 and 1994) and has caused severe losses in revenue and equipment (Rodda *et al.*, 1997). The snakes also enter houses where they can be very aggressive towards people if forced into a defensive position and will repeatedly strike at the threat (Johnson, 1975). This has resulted in numerous cases of snake bites, with approximately one in a thousand hospital visits to a Guam hospital being reported (Fritts *et al.*, 1990). They will also prey on pets and small domestic animals such as rabbits and chickens (Fritts and McCoid, 1991).

The most dramatic result of the colonization of brown tree snakes on Guam has been the decimation of the island's avifauna. The dense brown tree snake population has caused a dramatic decrease in the number of birds on the island (Savidge, 1987). As a result of snake predation, nine of twelve species of native forest birds have been completely eliminated from the wild on Guam. Three of these species were endemic to Guam and are now extinct (Savidge, 1987). The remaining species can be found on snake-free neighboring islands of Guam. With such a major loss in prey, the snakes now survive on

rodents, lizards, small domestic animals and the endangered Mariana fruit bat (Wiles, 1987; Rodda and Fritts, 1992; Savidge, 1987).

Unfortunately, the brown tree snake problem is not limited to Guam. Individual brown tree snakes have been discovered on many islands immediately surrounding Guam such as Wake island, Diego Garcia, Okinawa, as well as Oahu, Hawaii (Rodda *et al.*, 1997). Of particular concern is the island state of Hawaii, where there have been at least seven brown tree snake sightings since the mid-1980s (Rodda *et al.*, 1997). Current management and research efforts are focused on controlling the spread of the brown tree snake while also controlling the population levels on Guam itself to avoid another disaster like that on Guam (Rodda *et al.*, 1997).

### **Summary of Research and Experimental Goals**

The overlying goal of these studies was to gain a comprehensive understanding of the role pheromones play in mediating the reproductive behaviors of the brown tree snake. More specifically, this research had the following experimental goals: 1) to catalog the repertoire of brown tree snake courtship, mating and combat behaviors, 2) to isolate and characterize the female sex pheromone of this species with the ultimate goal of identifying its chemical components, 3) to examine the ability of brown tree snakes to follow conspecific pheromone trails under breeding conditions and 4) to characterize the pheromonal inhibition of male courtship by females, a novel pheromone system in reptiles.

The behaviors described in Chapter 2 of this thesis are the only published accounts of the reproductive behaviors of the brown tree snake, or any member of the genus *Boiga*,

and represent the most comprehensive examination of the reproductive behaviors of any tropical snake species. The behavioral descriptions presented are characterized using standardized terminology to allow for comparisons to be made with northern temperate species, on which the vast majority of behavioral research has been conducted in snakes.

As a result of the behavioral observations presented as part of this study, six potential pheromone systems were found in the brown tree snake: 1) a female sex pheromone that releases male courtship behavior, 2) a pheromone that is released by females in response to male courtship and acts to inhibit male courtship behavior, 3) a male sex pheromone that releases female courtship behavior, 4) a male combat pheromone that releases combat behavior, 5) a female trailing pheromone that acts in mate attraction and 6) a male trailing pheromone that males follow to locate males under breeding conditions. In subsequent experiments, these behaviors were exploited in behavioral bioassays designed to experimentally determine the function of specific pheromones and also to identify the source of the pheromones, the method of pheromone release into the environment, the behavioral effects of the pheromones and the chemical composition of the pheromones.

Despite the numerous anecdotal reports describing the release of snake courtship behavior by pheromones, few investigators have examined this phenomena experimentally, despite the obvious importance of sex pheromones to snakes. In Chapter 3, a bioassay measuring male courtship behavior in response to pheromone solvent extracts presented on filter paper was used to determine that the female sex pheromone alone was sufficient to release male courtship behavior. In addition, the female sex pheromone was characterized as a suite of non-polar, non-volatile skin lipid molecules using this technique.

In Chapter 4, the ability of brown tree snakes to follow pheromone trails produced by sexual attractive conspecifics was examined, as this behavior likely represents the mechanism for mate location in the wild. As has been shown in nearly all snakes tested to date (Ford, 1986), male brown tree snakes follow female pheromone trails under breeding conditions. However, this is the first experimental study where males have demonstrated the ability to follow male pheromone trails and the only known study to examine the trailing behavior of a tropical colubrid species. The trailing pheromones in the brown tree snake are non-volatile skin lipid components that may be identical in chemical composition to the male and female sex pheromones.

Lastly, in Chapter 5, an experiment designed to characterize the pheromonal inhibition of male courtship behavior by females was conducted. This pheromone, which is voluntarily released during courtship, originates from female cloacal secretions. This is a novel pheromone system in reptiles and may represent a mechanism for females to reject males that are deemed unsuitable as mates. This pheromone, in contrast to the female sex pheromone and trailing pheromones, is a volatile signal that is probably detected through the olfactory system because behavioral effects are observed even when males do not tongue-flick the pheromone directly.

By taking a comprehensive experimental approach designed to characterize the roles pheromones play in mediating brown tree snake's reproductive behavior, the results of these experiments provide sophisticated understanding of pheromonal communication in the brown tree snake. Thus, these results contribute to our understanding of the role pheromones play in mediating reproductive behaviors in snakes and vertebrates in general as few studies have examined a single vertebrate species to such an extent. In addition, these results



provide a basis for future research designed to positively identify the pheromones characterized in this study and for subsequent studies examining the potential uses of these pheromones as part of a brown tree snake management (Mason, in press).

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## 2. The Courtship, Mating and Combat Behaviors of the Brown Tree Snake, *Boiga irregularis*

### Abstract

The brown tree snake, *Boiga irregularis*, is an introduced pest species on the island of Guam where it has caused significant economic and ecological damage. Despite the need for basic information about this species in order to manage it effectively, little is known about the brown tree snake's reproductive behavior. We report here the repertoire of reproductive behaviors displayed by this species. The courtship behavior of the brown tree snake parallels that of other colubrids, following the triphasic schema developed to standardize descriptions of colubrid courtship, although there are differences in the specific behaviors displayed. Unlike most other colubrids, female brown tree snakes employ courtship behaviors normally displayed only by males. In addition, females seem to possess the ability to inhibit male courtship through the use of a pheromone located in a liquid secretion from the cloaca. Male brown tree snakes display ritualized combat behavior that is similar to what has been observed in other colubrid species.

### Introduction

Reproductive and ritualized combat behaviors have been described in detail for many species of snakes (Davis, 1936; Lowe, 1948; Shaw, 1951; Carpenter and Ferguson, 1977; Andr  n, 1986; Secor, 1987; Schuett and Gillingham, 1988; Secor, 1990). These

behaviors tend to be “ritualistic” in snakes and can be divided into clearly identifiable motor patterns that can be compared between species when standardized terminology is used (Carpenter and Ferguson, 1977; Gillingham, 1979; Carpenter 1980; Secor, 1987). The vast majority of these reports, however, have been conducted on northern temperate species, while far fewer descriptions of tropical snake behaviors have been published (Barker *et al.*, 1979; Gillingham and Chambers, 1982).

The purpose of this paper is to describe the repertoire of courtship, mating and ritualized combat behaviors displayed by the brown tree snake (*Boiga irregularis*), a rear-fanged colubrid native to the forests of Australia, Papau New Guinea and the Solomon Islands (Cogger, 1988). This species is nocturnal and forages for a generalized diet of mammals, reptiles, amphibians and birds which are killed by a combination of constriction and envenomation (Savidge, 1988; Greene, 1989; Vest *et al.*, 1991; Shine, 1991). The snake is arboreal, but spends much of its time on the forest floor (Cogger, 1992), and has a relatively slender body that is characteristic of arboreal snakes (Lillywhite and Henderson, 1993). Brown tree snakes can reach snout-to-vent lengths of up to 3 meters, masses up to 2 kilograms and are sexually dimorphic with males being longer and heavier than females (Fritts, 1988).

Sometime during or after World War II, the brown tree snake was accidentally introduced onto the island of Guam where it proliferated in some areas, reaching densities of up to 13,000 snakes per square mile (Rodda *et al.*, 1992). Subsequently, the snakes have caused the extinction or extirpation of 9 native forest birds and have affected nearly all vertebrate populations on the island through predation (Savidge, 1987; Wiles, 1987; Rodda and Fritts, 1992). The snakes have also become economic pests, causing power



outages while searching for prey on power lines and preying upon domestic animals and pets (Fritts *et al.*, 1987; Fritts and McCoid, 1991). In addition, snake bites are common on Guam, resulting in approximately 1 of 1250 hospital visitors being treated for snake bites (Fritts *et al.*; 1990, 1994). Current management efforts are focused on reducing the number of brown tree snakes on Guam and preventing their introduction to other areas of the Pacific (Fritts, 1988). There have been at least 7 sightings of brown tree snakes on Hawaii and the snakes may have become established on other islands in the Pacific (e.g. Saipan).

Despite organized efforts to manage this economically and ecologically important pest species, very little is known about its basic reproductive biology (McCoid, 1994; Whittier and Limpus, 1996). To date, no descriptions of brown tree snake reproductive behaviors have been reported. A comprehensive knowledge of a pest species' reproductive biology is crucial to management efforts. The brown tree snake management plan (Brown Tree Snake Control Committee, 1996) calls for research on brown tree snake reproduction to develop strategies aimed to eliminate reproductive adults and their progeny. We report here, for the first time, the sequences of male courtship behavior, female courtship behavior and ritualized male combat behavior in the brown tree snake.

## **Materials and Methods**

The animals used in this study were collected in the field on Guam and have been housed in our laboratory for the past 6 years under an established laboratory protocol (Greene *et al.*, 1997). The snakes (10 males and 7 females) were housed in Plexiglas

cages designed specifically for arboreal reptiles (Mason *et al.*, 1991) and were fed a diet consisting of thawed frozen mice or chicks every 3 weeks. Male snakes ranged in snout-to-vent length from 132.5 cm to 199.5 cm (mean  $\pm$  SD:  $162.1 \pm 22.1$  cm) and in mass from 350 g to 1175 g ( $647.5 \pm 265.7$  g). Females ranged in snout-to-vent length from 129 cm to 156 cm ( $137.9 \pm 9.9$  cm) and in mass from 300 g to 525 g ( $385.7 \pm 81.5$  g). Temperatures ranged from 23 to 30°C (mean = 25 °C) and relative humidity ranged between 75 and 80% in the room. During the study, lighting (14L:10D) was provided by overhead fluorescent lights and ambient sunlight entering the room through windows.

Reproductive behaviors were induced in our captive colony of brown tree snakes by reducing the ambient temperature in the snake colony room, a common technique used by snake breeders and researchers (Crews and Garrick, 1980). We have induced reproduction in our captive colony three times using this procedure, beginning in December of 1995, December of 1996 and December of 1997. A seven week cooling period of 5 °C below normal room temperature (25 °C) was sufficient to bring all members of the snake colony into breeding condition. During the cooling period, humidity remained unchanged and the amount of light entering the snake room changed slightly, decreasing until winter solstice and then slowly increasing. Courtship and combat behaviors were observed from all snakes in the colony for the next eleven months until the next cooling period was begun.

Experiments were conducted during scotophase, between 1900 and 0200, when the snakes were normally active. Observations of reproductive behaviors were made by introducing male-female or male-male pairs into an arena constructed of clear Plexiglas. The arena has walls measuring 1.25 m on each side, providing 1.6 m<sup>2</sup> of floor area. The

snake pairs were left together in the arena for 1 hour and their behaviors were recorded by an observer hidden behind a blind or by a video camera. The arena was cleaned using soap and water, rinsed and dried between trials. Lighting was provided by a red 7 watt incandescent light mounted above the arena. Twenty courtship trials and twenty male-male combat trials were conducted. The snakes had no courtship, mating or combat experience during the past 6 years in captivity. No females were present during the combat trials.

The courtship and combat behaviors displayed by males were observed along with the courtship behaviors displayed by females. Behaviors were described using standardized terminology established in the literature (Gillingham *et al.*, 1977; Gillingham, 1979; Gillingham, 1980). To allow comparison to other studies of colubrid male courtship behavior, the triphasic system developed by Gillingham (1979) that breaks male courtship into 3 phases (Phase I: tactile-chase, Phase II: tactile-alignment and Phase III: intromission) was incorporated into the description of male courtship behavior. The following descriptions catalog all behaviors observed during courtship and combat trials and place the behaviors in the temporal order in which they were generally observed.

## **Results**

### *Male Courtship Behavior.*

#### **Phase I:**

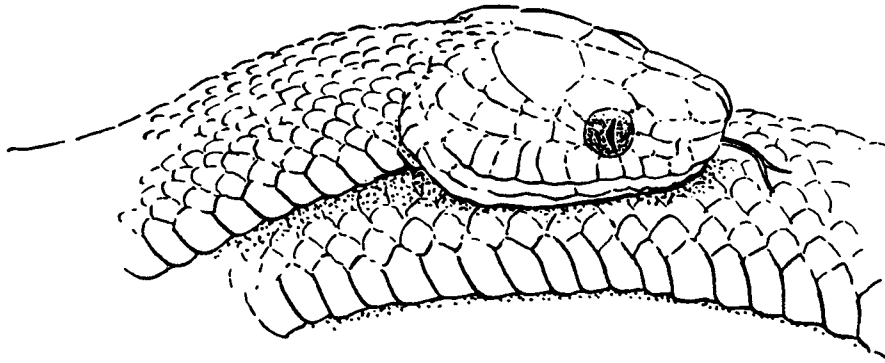
Courtship begins when a male tongue-flicks the dorsal integument of the female. In snakes, the detection of sex pheromones occurs in the vomeronasal organ of snakes via

tongue-flicking (Halpern, 1992). After tongue-flicking the female's dorsum, the male displays head-jerking behavior where the male rapidly tongue-flicks the female while rhythmically jerking his head in a lateral direction. The male will mount the female soon after displaying head-jerking by placing his chin on the female's dorsum while subsequently displaying chin-rubbing where the male advances along the female's body while pressing his chin to the female's dorsum (Figure 2.1). Chin-rubbing is accompanied by forward-body jerking, rhythmic forward surging movements of the male's head and body, and periodic snout-probing, where the male presses his snout to the lateral side of the female's body while displaying short tongue-flicks only with the tips of its tongue. After several minutes of chin-rubbing, the male will periodically display head-lifting by coiling his head and neck region into an 'S' formation and bobbing it up and down perpendicularly to the ground. At this point in the courtship sequence, males will display chase behavior by following a retreating female or chase-mount behavior by pursuing a female while remaining mounted on the female's body and displaying chin-rubbing. A period of chase-mount behavior eventually leads to body-alignment where the bodies of the courting pair are aligned side by side or the male's body is mounted on the female's body (Figure 2.2).

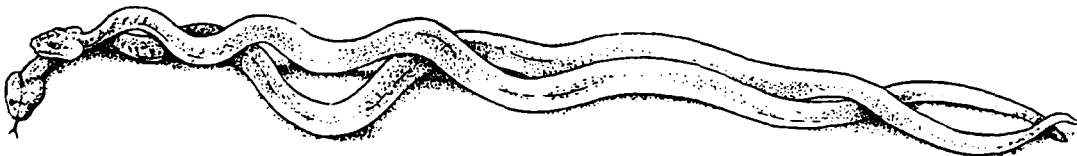
#### Phase II:

Tail-search copulatory attempts follow, in which the male repeatedly attempts to align his cloaca with the female's by wrapping his tail under the female's (Figure 2.3). Mating was observed only twice during this study, as most mating pairs were separated before reaching this phase to avoid confounding other studies being conducted

**Figure 2.1** A male chin-rubbing the dorsum of a sexually attractive female during courtship. Chin-rubbing behavior is accompanied by forward body jerks.



**Figure 2.2.** A male and female during body-alignment. The male is mounted on the dorsum of the female and is displaying chin-rubbing behavior.

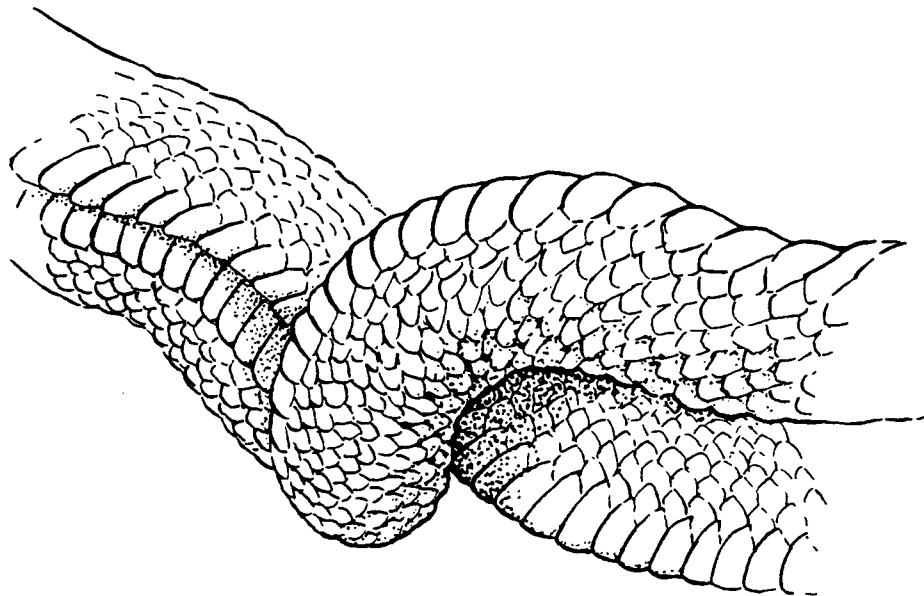


simultaneously with the same snakes (Chapter, 3). In the two complete mating trials, the males displayed one and six tail-search copulatory attempts, respectively. No caudocephalic waves or body writhing were displayed by the males.

Phase III:

If the female is receptive to the male's courtship, the male will intromit one of his hemipenes into the female's gaped cloaca. During copulation, the male remains motionless except for periodic tongue-flicking. No copulatory plugs were deposited into the female's cloaca at the termination of copulation.

**Figure 2.3 A male displaying tail-search copulatory attempts.** The male's body is mounted on the female's dorsum (the tails of the snakes extend to the left of the page). In an attempt to oppose his cloaca to the female's, the male has wrapped his tail under the female's so that the ventral surfaces of both snakes are adpressed near their cloacae.



Times spent in the three phases of courtship were recorded for the two complete mating sequences observed. The first mating pair spent 7.3 minutes in Phase I, 0.1 minutes in Phase II and 12.3 minutes in Phase III of courtship for a total time of 19.7 minutes. The second mating pair spent 16.0 minutes in Phase I, 0.7 minutes in Phase II and 15.8 minutes in Phase III, a total time of 32.5 minutes. Combining the times for the two pairs, a mean time of 11.7 minutes was spent in Phase I of courtship, a mean time of 0.4 minutes in was spent in Phase II and the pair spent a mean time of 14.1 minutes in Phase III. The two mating pairs spent a mean time of 26.1 minutes in the entire courtship sequence.

#### *Female Courtship Behavior.*

Tongue-flicking a male generally releases a short (less than 20 seconds) display of head-jerking behavior from females. Typically a female that displays head-jerking will mount the male by moving her head along the male's dorsum or it will move along side the male's body towards the male's head. Upon reaching the male's head, the female maneuvers her body under or directly in front of the male's snout in an apparent attempt to elicit tongue-flicks from the male. This typically elicits courtship behavior from the male. The female then retreats from the male while displaying head-lifting behavior identical to that displayed by courting males. Courting males, in response, follow the female (chase and chase-mount behavior) which eventually leads to body-alignment.

Male snakes cannot force copulation, therefore mating occurs only if a female is receptive to male courtship and gapes her cloaca to allow the male to insert one of his hemipenes. Like the male, the female remains nearly motionless during copulation.

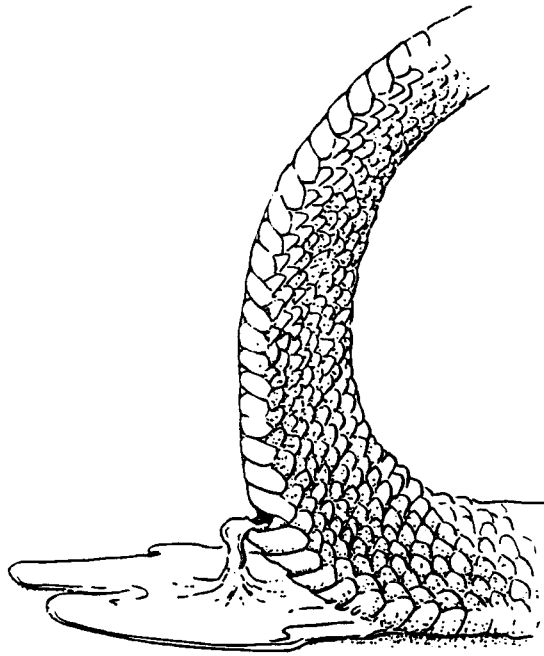
Female brown tree snakes do not display any noticeable tail movements immediately preceding cloacal gaping as has been reported in other species (Shaw, 1951; Holman, 1960; Murphy *et al.*, 1978).

Male courtship may elicit female behaviors that apparently serve to discourage male courtship. Often, females will hold their heads in a defensive display, as if about to strike (although strikes at males during courtship have never been observed) while slowly moving away from the male snake and displaying tail-lashing behavior. Females also responded to male chin-rubbing with body-bridging and body-bumping at the point where the male's head makes contact with the female's body.

In 6 of 20 courtship trials, continued attempts at courtship by males resulted in the female lifting her tail perpendicularly to the ground and releasing a bolus of liquid from the cloaca (Figure 2.4). The liquid was clear with a yellow or white precipitate that was observed to originate from the urogenital opening and the paired cloacal glands located in the tail. This liquid appeared and smelled identical to what is released when brown tree snakes of both sexes are disturbed. When disturbed, a fine spray of cloacal gland secretions is released and the snake opens its urogenital opening and releases a bolus of this liquid. Tail-lifting behavior, however, is only displayed by females during courtship and never when snakes are disturbed. In all courtship trials where this liquid was released, the females did not previously display any behaviors associated with defensive displays (Johnson, 1975).



**Figure 2.4 A female brown tree snake displaying tail-raising behavior and releasing a bolus of cloacal secretions in response to male courtship.**



*Male Responses to Female Courtship Behavior.*

After head-lifting displays by females, males would often respond with head-lifting behavior themselves. In addition, males would orient towards females or would approach and tongue-flick females. Upon approaching the female, the male would often move directly to the female's head and would posture its head above the female's as in hovering behavior displayed during combat. Males were also observed pinning the female's head which usually resulted in the female quickly pulling her head free. In other cases, males approached and mounted a female displaying head-lifting and resumed courtship.

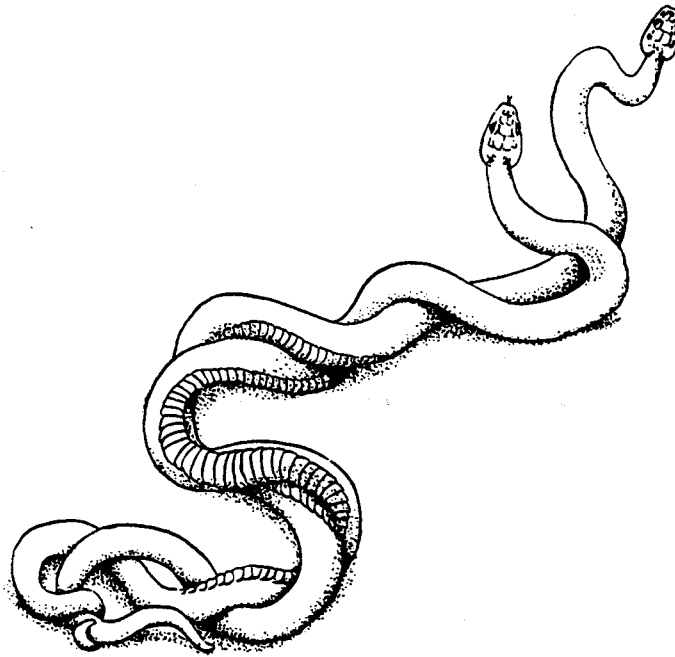
Male behavior changed noticeably after females lifted their tails and released cloacal secretions. Prior to this event, males displayed vigorous courtship behavior. After female cloacal secretions were released, however, males stopped displaying courtship

behavior, except for periodic, weak head-nodding, and generally stayed near the female, following her around the arena. Identical behaviors were observed in all 6 cases, although in only 1 of these cases did a male tongue-flick the female cloacal secretions directly.

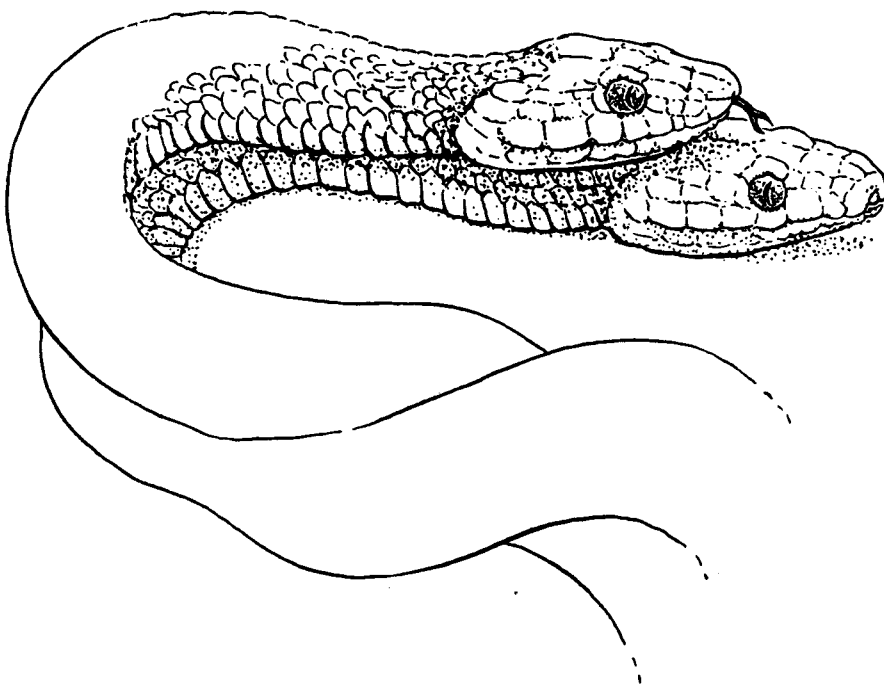
#### *Male Combat Behavior.*

In every case, ritualized combat behavior began only after males tongue-flicked their opponent's integument. In response to tongue-flicking each other, opposing males display head-jerking behavior. Simultaneously, the males mount their opponent's body and display chin-rubbing accompanied by forward-body jerking behavior. Typically, the chin-rubbing initially occurs in the direction of the opposing male's tail and then towards the opposing male's head. During this phase of combat, males will often display body-bridging, where they bridge the section of their bodies upon which their opponent is chin-rubbing, or body-bumping behavior, repeated body-bridges at the point of chin-rubbing. Males will also periodically display snout-probing to each other and will display vigorous bouts of head-lifting when chin-rubbing temporarily ceases (Figure 2.5). Head-lifting by one male was generally followed by a head-lifting display by its opponent or, if separated, the opponent would approach the head-lifting male and continue combat. Eventually, body-alignment is displayed when the bodies of both males are aligned side by side or one male is mounted on the other male's body.

**Figure 2.5** Two males in a typical combat posture with head-lifting and body-coiling displayed.



**Figure 2.6** Two males displaying head-pinning behavior during combat.



Upon reaching body-alignment, the males begin to display head-pinning behavior where males attempt to pin their opponent's head to the ground using their chin or a U-shaped loop of their body formed just posterior to the head (Figure 2.6). This is often accompanied by hovering in which a male will hold its head and body above its opponent's head instead of using a physical pinning action. During head-pinning, males remain in a position parallel to the ground.

An extended bout of head-pinning results in body-coiling in which the bodies of the opposing males become entwined from just posterior to the neck region to the cloaca and the males constrict their opponent's body (Figure 2.5). Combat bouts last for hours in this species and observations have been made of bouts lasting greater than 2 hours before a winner was decided. The winner of the combat bout is determined when the loser attempts to flee from its opponent. Fleeing is typically accompanied by lashing of the body and tail.

## **Discussion**

The repertoire of courtship behaviors displayed by the brown tree snake closely parallels those of northern temperate colubrid species (Secor, 1987). As such, male courtship behavior follows the triphasic schema developed to describe male courtship behavior in colubrid snakes (Gillingham *et al.*, 1977; Gillingham, 1979). In this species, Phase I is characterized by head-jerking, mounting behavior, chin-rubbing, snout-probing, head-lifting, chase-mount behavior and body-alignment. Phase II is initiated with the first

tail-search copulatory attempt and terminates with intromission and Phase III is comprised of intromission and coitus (Gillingham, 1980).

The duration of time that males spent in Phases I and III of courtship was comparable to what has been observed in other colubrids, although the amount of time spent in Phase II of courtship was shorter than has been reported for most colubrids (Secor, 1987). Only the gray-banded kingsnake, *Lampropeltis mexicana alterna*, has been reported with a comparably short Phase II (Murphy *et al.*, 1978).

Although brown tree snakes follow the same general pattern of courtship as other colubrids, they differ in the types of behaviors prominently displayed by males during courtship. Secor (1987) identified body-jerking, writhe/writhe-bump behavior, the presence of caudocephalic waves and coital biting as major motor patterns in the repertoire of colubrid courtship behavior. With the exception of head-jerking behavior, male brown tree snakes do not display any of these behaviors.

Brown tree snakes display snout probing which has rarely been reported as a courtship behavior in other snakes (Gillingham, 1974). This behavior, which involves close range, rapid tongue flicking to the integument of the female, seems to be a mechanism to aid in the detection of the female sex pheromone. Behaviors similar to snout-probing are displayed by these snakes in other situations where the detection of chemical signals releases behaviors, such as feeding on dead prey items and following pheromone trails (Greene and Mason, unpublished). This may be a mechanism for the snakes to detect semi-volatile components of the sex pheromone using their olfactory system while simultaneously sampling non-volatile pheromone components via the vomeronasal organ.

In the brown tree snake, females display clearly identifiable behaviors during all stages of courtship that seem to effect the outcome of the courtship sequence. These behaviors seem to have two opposing functions depending on the context in which they are displayed: 1) to instigate courtship from males or to excite courting males or 2) to discourage male courtship. Both behavioral displays and pheromones seem to be involved with signaling the intent of the female.

Females display courtship behaviors identical to several of those displayed by males when females are seemingly attempting to elicit male courtship. These behaviors are displayed only after a female tongue-flicks the integument of a male, suggesting that a male sex pheromone that releases female courtship behavior is present in this species. Several studies have observed female head-jerking or body-jerking behavior (Gillingham, 1974; Murphy *et al.*, 1978; Gillingham, 1979; Secor, 1987). This is the first report, however, in which displays of mounting, chin-rubbing and head-lifting behaviors were observed in colubrid females. This demonstrates that female brown tree snakes take an active role in courtship, displaying these courtship behaviors to elicit male courtship and possibly to evaluate the quality of the courting male.

Head-lifting behavior could represent a mechanism for female mate choice in this species by allowing females to determine the success of courting males in prior combat bouts. There is precedence for this in viperid snakes (Andr  n, 1986; Schuett and Duvall, 1996). Female copperheads, *Agkistrodon contortrix*, display head-lifting behavior during courtship to evaluate the success of courting males in combat bouts prior to courtship (Schuett and Gillingham, 1988). Males that had recently won combat bouts responded to female head-lifting with a ‘challenge display’, a vertical posture displayed during the initial

stages of combat. In contrast, losers of combat bouts were repelled by female head-lifting. Male copperheads without prior combat experience responded to female head-lifting with challenge displays (Schuett and Duvall, 1996).

The use of head-lifting behavior by male and female brown tree snakes could be analogous to the mate selection system in copperheads (Schuett and Duvall, 1996). Male brown tree snakes respond to female head-lifting with head-lifting bouts of their own, by approaching and tongue-flicking the female, mounting the female, hovering over the female's head or pinning the female's head. Each of these are prominent behaviors in male combat of this species. No observations were made of males being repelled by female head-lifting, however, the males used in this study had no combat experience during the previous 6 years of captivity.

The release of cloacal secretions along with the visual signal of the raised tail could be a mechanism for female brown tree snakes to reject males deemed unacceptable or to signal to males that a female is not sexually active at that time. Male brown tree snakes in this study ceased courting females that released cloacal gland secretions even though they had been vigorously courting the females previously. After exposure to female cloacal gland secretions, these males still showed interest in the female by following the female around the cage, tongue-flicking her body, but not by displaying courtship behaviors except for periodic, weak head-jerking. In contrast, in other trials, males that voluntarily stopped courting females showed little interest in the female, being occupied with tongue-flicking the floor and walls of the cage instead of the female. These changes in male behavior are suggestive, at least, for the presence of a male courtship inhibitory pheromone in female cloacal secretions. Gillingham (1979) observed identical tail-raising

behavior in an analysis of the reproductive behavior of eastern North American rat snakes (genus *Elaphe*) following coital separation and defecation, although it was observed at a low frequency.

Selection might favor males that ceased courting females upon detection of signals from females that signal non-receptivity. Male snakes cannot force copulation, therefore, male mating success depends upon the female gaping her cloaca to allow intromission of the male's hemipene (Gillingham, 1979; Secor, 1987). Therefore, it would prove futile for a male to court a female after the female has signaled that the male is an unacceptable mate or that she is not reproductively competent at that time.

The brown tree snake ritualized combat display is initiated only after males tongue-flick each other. This suggests that a pheromone is present in the male integument that releases combat behaviors. Like other colubrids that display ritualized combat, the males of this species are larger than the females (Shine, 1978, 1996). The snakes remain in a horizontal posture during combat and display behaviors similar to those displayed by other colubrids including head-jerking, hovering, body-coiling, and head-lifting (Carpenter, 1980). Many colubrids display body-pinning during combat where a loop of a snake's body is used to pin the anterior portion of its opponent's body (Gillingham, 1980). Brown tree snakes, in contrast, use head-pinning where the same motions are utilized but to pin an opponent's head instead of its body.

Head-lifting during combat in the brown tree snake seems to be more vigorous than the analogous behavior displayed by other species. In other colubrids where head-lifting has been reported, head-lifting involves an elevation of a snake's head several centimeters above the ground (Brecke *et al.*, 1976; Gillingham, 1980). In contrast, brown



tree snakes bob their heads up and down nearly perpendicular to the ground. In this species, head-lifting may act as a solicitation display, serving as a visual signal that attracts other males, much like the challenge display seen in viperid combat bouts (Carpenter, 1977, 1984) Male brown tree snakes will display head-lifting and will approach other males and begin combat in response to head-lifting by other males.

Although studies have addressed the reproductive physiology of the brown tree snake (Shine, 1991; McCoid, 1994; Whittier and Limpus, 1996), this is the first description of this species' courtship, mating and combat behavior and represents one of the few descriptions of these behaviors in a tropical species. The results of this study provide an important basis for future studies on this species, including studies investigating the role of pheromones in mediating the reproductive behaviors of this species.

The courtship and combat behavioral sequences described as part of this study were initiated only after tongue-flicking was observed (never by visual or tactile signals alone) suggesting that these behaviors are released by pheromones. These putative pheromone systems include the female sex attractant pheromone, a male sex pheromone that releases female courtship behavior, a pheromone that releases male combat behavior and a potential courtship inhibition pheromone found in cloacal secretions from females. Further experiments are necessary, however, to characterize the role of these pheromones in mediating brown tree snake reproductive behaviors. It is possible that, if identified and synthesized, these pheromones could be used as part of a larger brown tree snake management plan as chemical attractants or reproductive inhibitors (Mason, in press).

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### Chapter 3

#### Chemically Mediated Sexual Behavior of the Brown Tree Snake, *Boiga irregularis*

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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 9 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.

## 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 *et al.*, 1986). Notable exceptions include pheromones identified in the domestic pig, *Sus scrofa* (Patterson, 1968), the goldfish, *Carassius auratus* (Stacey and 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 *et al.*, 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 *et al.*, 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 *et al.*, 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 *et al.*, 1997).

This species was accidentally introduced on Guam sometime during or shortly after World War II (Rodda *et al.*, 1992a). Like most other Pacific islands, Guam had no endemic snake species, presenting the brown tree snake with an environment containing



no specialized predators and 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 *et al.*, 1992a).

With such high densities, *Boiga irregularis* has become a serious pest for a variety of reasons. This snake has caused the extirpation of 9 of 12 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 and McCoid, 1991; Rodda and Fritts, 1992). A rear-fanged colubrid, this snake readily bites people, including dozens of sleeping babies (Fritts *et al.*, 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 *et al.*, 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 *et al.*, 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 and Mason, *in review*). 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 female 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.*, 1992b). 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. This would 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.

## Materials and Methods

Animals used in this study were collected on Guam and have been housed under laboratory conditions for the past 6 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 thawed frozen 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.

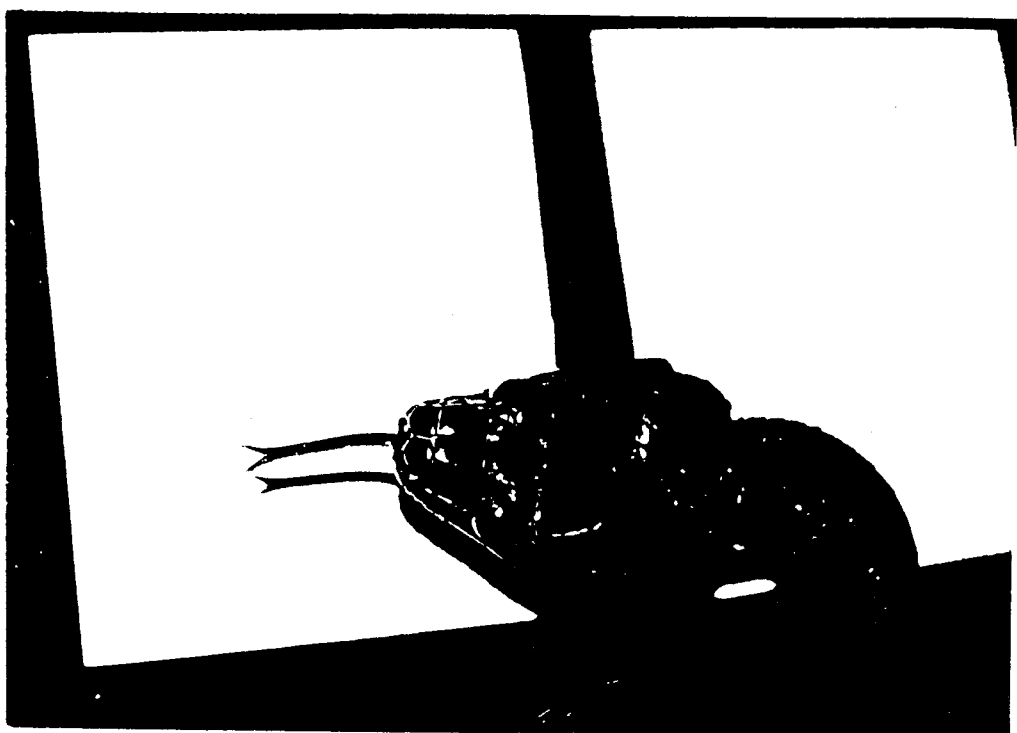
Male responses were recorded for a 1 hour period following the introduction of a female ( $n = 7$  courting pairs) into his cage, in order to determine which females in the captive colony were sexually attractive during the time of the study. Subsequently, a randomly chosen shed skin from a sexually attractive mature female was placed in the cage of a male ( $n = 10$  males) for 20 min 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). The shed skins used in this experiment were collected from all

seven females in the captive colony. Alternatively, a randomly chosen shed skin from another male was presented to 5 randomly chosen males in their individual home cages for 20 min to act as a control.

Lipid extracts were obtained by soaking freshly shed skins from attractive females (25 skins pooled from 7 females) 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 by 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 min and courtship behaviors displayed by males were recorded (Figure 3.1). Because male courtship behavior occurs in a predictable fashion (figure 3.1), only the highest ranking behavior attained in the male courtship sequence was used in the subsequent statistical analysis (Figure 3.2).

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 according to the methods of 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 by 10 cm filter paper were treated with each fraction by applying approximately 1 ml aliquots of concentrated fraction to the filter paper and allowing the hexane to evaporate.

**Figure 3.1.** A male brown tree snake investigating a filter paper treated with female skin lipid extract with tongue-flicking behavior during a bioassay test.



**Figure 3.2.** Graduated scale of courtship behavior for the male brown tree snake, *Boiga irregularis*.

<u>Ranking</u>	<u>Behavioral Description</u>
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.

A preliminary screening for courtship behavior was conducted by presenting males ( $n = 10$  males) with the sequential samples in order, each one for 10 min. 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 min trial was conducted in a male's home cage with a 15 min 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 and 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 (Figure 3.2). 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. 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. This sequence of behavior is unique to courtship and is only observed in a reproductive context (Chapter 2).

In the first experiment, 8 of 10 males responded to shed skins from attractive females with head-jerking and/or chin-rubbing behavior during the 20 min observation period (in 2 of 10 cases only tongue-flicking was observed). Males displayed head-jerking and chin rubbing after tongue-flicking the female shed skins and, in several instances, displayed vigorous chin rubbing with males pushing the shed skin along the floor of the cage. No other courtship behaviors were displayed by the males to the female shed skins. In contrast, males never responded (0 of 5 trials) to male shed skins with head-jerking or chin-rubbing behavior.

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 ( $\chi^2 = 21.600$ ,  $df = 3$ ;  $P < 0.001$ ; Table 3.1). Once again, males responded with head-jerking and chin rubbing to the crude skin lipid extract.

**Table 3.1. The response of male *Boiga irregularis* to female skin lipid extract and controls.** Only the highest ranking behavior attained in the male courtship sequence is presented and used in the statistical analysis.

Behavior	Proportion of males that displayed courtship behaviors to:			
	Skin Lipid Extract (n= 9)	Lipid Control (n=9)	Solvent Control (n=9)	Blank Control (n=9)
Head-jerking:	0.11 (n=1)	0.00 (n=0)	0.00 (n=0)	0.00 (n=0)
Chin-rubbing:	0.56 (n=5)	0.00 (n=0)	0.00 (n=0)	0.0 (n=0)
Total:	0.67 (n=6)	0.00 (n=0)	0.00 (n=0)	0.00 (n=0)

Results of the second bioassay indicated that fractions 1 through 4 elicited significantly more male courtship behaviors than the control ( $\chi^2 = 12.631$ ,  $df = 4$ ;  $P = 0.013$ ; Table 3.2).

Males displayed head-jerking and chin rubbing to fractions 1 through 4 while never

**Table 3.2. The response of male *Boiga irregularis* to female skin lipid fractions 1-4 and control (fractions 5-20) in the bioassay.** Only the highest ranking behavior attained in the male courtship sequence is presented and used in the statistical analysis.

Behavior	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 (n=1)	0.10 (n=1)	0.14 (n=1)	0.13 (n=1)	0.00 (n=0)
Chin-rubbing:	0.14 (n=1)	0.20 (n=2)	0.43 (n=3)	0.63 (n=5)	0.00 (n=0)
Total:	0.28 (n=2)	0.30 (n=3)	0.57 (n=4)	0.76 (n=6)	0.00 (n=0)



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 ( $\chi^2 = 4.923$ ,  $df = 3$ ;  $P = 0.178$ ). There were no significant differences in the proportion of males displaying head-jerking between fractions 1 through 4 ( $\chi^2 = 0.0752$ ,  $df = 3$ ;  $P = 0.995$ ) or chin-rubbing between fraction 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% ethyl 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 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 and Gordon, 1989; Cardé, 1990; McNeil, 1992).

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#### 4. Trailing Behavior of the Brown Tree Snake, *Boiga irregularis*

##### Abstract

The ability of snakes to follow conspecific pheromone trails has significant consequences on their survival and reproduction. In particular, many studies have documented the ability of male snakes to follow female pheromone trails in order to locate potential mates during the breeding season. In this study, the ability of brown tree snakes to follow reproductively active conspecifics was tested in the laboratory using a Y-maze. A differential response was found in the ability of male and female brown tree snakes to complete trials. While males completed the majority of trials attempted, females completed significantly fewer trials, apparently due to lower activity levels under breeding conditions. Males displayed trailing behavior to both male and female pheromone trails, but did not discriminate between male and female trails when given a choice on the Y-maze.

##### Introduction

The ability to accurately follow pheromone trails is a prominent snake behavior that has been documented in 5 families of snakes (Ford, 1986; Mason, 1992). Field and laboratory studies have examined trailing behavior primarily in the context of mate location during the breeding season (Davis, 1936; Noble, 1937; Ford, 1979; Ford, 1982; Ford and Schofield, 1984; Lillywhite, 1985; Ford, 1986; Andr  n, 1986; Plummer and Mills, 1996), migration to and from winter hibernacula (Hirth, 1966; Heller and Halpern,

1981; Costanzo, 1989) and aggregation (Noble and Clausen, 1936; Finneran, 1949; Gelbach *et al.*, 1971; Brown and MacLean, 1983).

Laboratory-based studies conducted using Y-mazes have allowed for the characterization of snake trailing behavior. These studies have demonstrated that pheromone trails can communicate information concerning the sex, sexual attractivity and species of the trail producing individual (Noble, 1937; Ford, 1978, 1982; Ford and Scholfield, 1984; Ford and Low, 1983). Garter snakes (*Thamnophis*) can distinguish the direction in which pheromone trails are deposited on the substrate by examining the unequal deposition of skin odors on opposing sides of vertical objects in the environment (Ford and Low, 1983).

The brown tree snake, *Boiga irregularis*, is a nocturnal, rear-fanged colubrid native to Australia, Papua-New Guinea and the Solomon Islands (Rodda *et al.*, 1997). This species can reach snout-to-vent lengths of up to 3 m, masses of up to 2 kg and possess a relatively thin body typical of arboreal snakes (Lillywhite and Henderson, 1993; Rodda *et al.*, 1998). Brown tree snakes are generalist predators that actively forage for a diet consisting of mammals, reptiles, amphibians and birds (Savidge, 1988; Greene, 1989). This species is primarily arboreal, however, it spends a significant proportion of time on the ground and typically forages within the lower 3 m of vegetation (Cogger, 1992). The brown tree snake trails rat odors in the laboratory, suggesting that, like other snakes, trailing behavior is important in prey location for this species (Chiszar *et al.*, 1988).

The brown tree snake was inadvertently introduced to the island of Guam sometime during or after World War II (Rodda *et al.*, 1992). Upon its introduction this species flourished, reaching densities of up to 13,000 snakes per square mile in the most

suitable habitat (Rodda *et al.*, 1992). The snakes have effected virtually every vertebrate population on the island through predation and have caused the extirpation or extinction of 9 out of 12 native forest bird species (Savidge, 1987; Rodda *et al.*, 1997). In addition, the snakes are serious economic pests, having caused hundreds of power outages by crossing power wires while searching for prey on power lines and preying heavily on domestic animals (Fritts *et al.*, 1987; Fritts and McCoid, 1991).

Brown tree snakes display reproductive behaviors similar to other colubrid snakes species. Males of this species display stereotyped courtship behaviors which are released by a sex pheromone located in the skin lipids of the females as well as ritualized combat behavior which appears to be mediated by a pheromone located in the skin lipids of males (see Chapter 3; Greene and Mason, in press). In snakes, ritualized combat serves as a competition between males to gain hierarchical access to females during the breeding season (Andr  n, 1986; Schuett and Gillingham, 1989).

This species does not form large breeding aggregations, although small non-breeding, winter aggregations have been reported in its native and extralimited range (Pendleton, 1947; Covacevich and Limpus, 1973). Therefore, it is likely that brown tree snakes, like other snakes, utilize pheromone trails to locate conspecifics during the breeding season. In this study, the ability of the brown tree snake to trail reproductively active conspecifics was tested using a Y-maze in the laboratory.



## Materials and Methods

### *Husbandry*

Animals used in this study were collected on Guam and have been housed under laboratory conditions for the past 6 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). Males in the colony have a mean ( $\pm$  SD) snout-to-vent length (SVL) of  $162.2 \pm 22.1$  cm and a mean mass of  $647.5 \pm 265.7$  g. Females have a mean SVL of  $137.9 \pm 9.9$  cm and a mean mass of  $385.7 \pm 81.5$  g. The snake 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 during the study) is provided by overhead fluorescent lights and by natural light entering the snake room through windows. Snakes are fed thawed frozen mice or chicks every three weeks and water is available *ad libitum* for drinking and soaking. All snakes in the captive colony were mature adults in breeding condition during the study.

### *Y-maze Design.*

To test the ability of brown tree snakes to trail conspecifics, a Y-maze was constructed of clear poly-vinyl chloride tubing (PVC) with an internal diameter of 5.2 centimeters. The maze consisted of an initial stretch of tubing 96 centimeters long that connected to a Y-junction that separated into two arms, also 96 centimeters long, by a 45°

angle. The maze was elevated at an angle of 25° from the floor to entice the snakes into the maze as they are excellent climbers and prefer to move to the highest point possible when given a choice. All hide boxes used in the snake cages had entrance holes standardized in diameter so that they could be attached to the initial stretch of the maze using PVC adapters. Empty, clean hide boxes were attached to the ends of the arms, also using PVC adapters, for collection of the snakes at the conclusion of the trial.

The initial stretch, Y-junction and the arms could be taken apart for cleaning between trials. The maze components were cleaned by scrubbing the inside of the tubes and Y-junction with soap and water using a 2 inch diameter bottle brush modified with an extended handle so that the full length of the tube could be cleaned. Afterwards, the maze components were thoroughly rinsed with water and completely dried. Care was taken so that the inside of the maze components were not touched by human hands, so as not to contaminate the components with human skin lipids.

This maze design allows for minimal handling of the snakes, reducing the display of defensive behaviors in this relatively aggressive species. In addition, the design allows for easy cleaning and reassembly as well as for containment of these arboreal snakes in the maze. Importantly, by having a relatively small internal tube diameter, conspecific pheromone trails were laid down in a semi-natural manner, as trail producing snakes deposited odors on a horizontal surface (bottom of tube) and vertical surfaces (sides of the maze) (Ford and Low, 1983). Brown tree snakes, like other snakes species, are highly thigmotactic, preferring to travel along edges as opposed to open areas. This maze design, by having rounded edges and a small internal diameter, reduced concerns of thigmotactic behavior conflicting with trailing behavior (Costanzo, 1989).

*General Experimental Conditions.*

To characterize the ability of males to trail conspecifics, four experiments were completed: 1) males trailing female pheromone trail versus blank arm (N = 20 trials; males tested twice), 2) males trailing male pheromone trail versus blank arm (N = 18 trials; males tested twice but never against own trail), 3) males trailing male pheromone trail versus female pheromone trail (N = 10 trials) and 4) blank control (N = 10 trials, no pheromone trails applied to either arm). In addition, similar experiments were attempted, but not completed, to test the ability of females to trail male and female conspecifics. These experiments were terminated as the females were very inactive in relation to the males and typically would not leave their hide boxes at the start of the maze. In all cases, the order of the individuals tested was completely randomized.

As only 10 males were available in the captive colony, it was necessary to use males twice in experiments 1 and 2 to gain statistical power. In experiment 2, the trail producing males were never tested against their own pheromone trail, leaving the possibility of 18 total trials. However, males were never used more than once a night and there was a minimum of 2 days between trials where an individual was tested. As such, the trial was the statistical unit analyzed, not the individual male, and the trials were assumed to be independent of each other.

All trials were conducted at night between the hours of 2100 and 0200 when the snakes were normally active and the snake room was dark. Lighting was provided at a minimal level by a red 7 watt incandescent bulb that was placed 2 meters behind the maze so that the arms of the maze were lit evenly. During trials, the investigator observed the maze while hidden behind a blind. The environmental conditions were held constant

during the entire experimental period. There were no differences in either temperature or relative humidity between the nights when the experiments were conducted.

Trails were produced by allowing a randomly chosen snake of the desired sex to pass completely through the initial stretch, Y-junction and one arm of the maze a single time. The side of the Y-junction leading to the control arm was blocked with a piece of clean cloth so that no lipids were inadvertently added to the control side of the maze. The arm of the maze treated with a conspecific pheromone trail was randomly chosen in each case. In experiments 1 and 2, where males were tested twice, a different individual was used to produce the pheromone trail in the second trial. In experiment 3, where males were given a choice between male and female pheromone trails, the female trail was always applied immediately before the male trail in manner similar to the other experiments. As such, both male and female pheromone trails were present in the initial stretch of the maze while the Y-junction and arms of the maze contained only male or female trails alone. In experiment 3, a random male and female was chosen to produce the trail for each trial.

To begin a trial, a hide box containing a snake was removed from its home cage and attached to the initial stretch of the maze. The snake was then allowed to enter the maze at its own accord. Upon observing the emergence of the snake's head from its hide box, the investigator began timing the snake's progress up the maze. The trial was terminated when the snake's head entered the collection box attached to the arm of the maze that the snake had chosen.

The data from trials where snakes exhibited defensive behaviors such as striking or tail-lashing or where snakes did not enter the maze from their hide box after 30 minutes

were not used in the statistical analyses. In addition, trials where snakes did not display behaviors associated with trailing such as deliberate tongue-flicking behavior directed at the trail, periodic pauses and side-to side head movements (Brown and MacLean, 1983) were also excluded from statistical analyses. The arm chosen by the snake was observed along with the time it took the snake to complete the maze and the percentage of time spent in the Y-junction. The percentage of time spent in the Y-junction was measured as an indicator of the amount of time it took the snakes to decide which arm of the maze to enter.

### *Statistical Analyses.*

The trailing response of males was tested against a binomial distribution under the null hypothesis of equal proportions ( $H_0: p = q = 0.5$ ). All P-values reported are based upon two-tailed binomial tests. Time data were analyzed using a Kruskal-Wallis one way analysis of variance on ranks (as the data failed the Kolmogorov-Smirnov normality test) with a Dunn's post-hoc test (SigmaPlot, © Jandel Scientific). Statistical differences between the ability of male and female snakes to complete trials was tested using a Fisher's exact test based on proportions (SigmaPlot, © Jandel Scientific).

## **Results**

There was a dramatic difference in the capability of males and females to complete trials in this study (Table 4.1). Males completed 36 of 38 trials (95%; combining the data from experiments 1 and 2) while females completed only 5 of 16 trials (31%; combining all data collected from similar experiments) attempted (Fisher's exact test;  $P = <0.001$ ).

**Table 4.1 Trailing behavior of *Boiga irregularis*: Ability of males and females to complete Y-maze trials under breeding conditions.**

Condition	Trials Attempted	Trials Completed	Proportion Completed	Combined Proportion	P-value (Fisher Exact Test)
Males Trailing Females	20	20	1.00	36 of 38	< 0.001
Males Trailing Males	18	16	0.89	(0.95)	
Females Trailing Females	9	3	0.33	5 of 16	
Females Trailing Males	7	2	0.29	(0.31)	

Of the females that did complete trials, however, 4 entered the treated arm of the maze while only 1 entered the control arm (2-tailed binomial test,  $P = 0.376$ ). In the cases where females did not complete trials it was because the females did not enter the maze from their hide-boxes within the allowed 30 min time period.

**Table 4.2 Trailing behavior of *Boiga irregularis*: Results from three experiments are presented: 1) males trailing females versus a blank arm (experiment 1), 2) males trailing males versus a blank arm and (experiment 2) and 3) males given a choice of two blank arms (experiment 4).**

Condition	N	Trailed	Did not Trail	P (2-tailed binomial)
Males trailing females	20	16	4	0.012
Males trailing males	16	13	3	0.022
Males trailing blank maze	9	5	4	NS

(NS = not significantly different from control at  $P = 0.05$  (2-tailed binomial))

Male brown tree snakes displayed trailing behavior to both female and male pheromone trails (Table 4.2). In experiment 1, males entered the female treated arm of the Y-maze significantly more than blank control arm (2-tailed binomial test,  $P = 0.012$ ). In experiment 2, males entered the male treated arm of the maze significantly more than the blank control arm (2-tailed binomial test,  $P = 0.022$ ). No individual male failed to follow the pheromone trail more than once in experiment 1 or 2, lending support to the assumption that each trial was independent. When tested on a Y-maze where both arms were left blank (experiment 4), males chose the arms of the maze equally (2-tailed binomial test,  $P = 0.500$ ; Table 4.2).

Males demonstrated no preference for female pheromone trails over male pheromone trails when given a choice on the Y-maze (Table 4.3). Males chose the female treated arm 5 times and the male treated arm 5 times (2-tailed binomial,  $P = 0.623$ ).

**Table 4.3. Trailing behavior of *Boiga irregularis*: Ability of males to discriminate between male and female pheromone trails.**

N	Number of Males That Trailed Males	Number of Males That Trailed Females	P-value (2-tailed binomial)
10	5	5	0.623

There were no significant differences in the times to complete the maze between the four different trailing experiments (Kruskal-Wallis one way ANOVA on ranks,  $H = 3.722$ ,  $df = 3$ ,  $P = 0.293$ ). There were, however, significant differences in the percentage

of time males spent investigating in the Y-junction between the four trailing experiments (Table 4.4; Kruskal-Wallis one way ANOVA on ranks,  $H = 11.047$ ,  $df = 3$ ,  $P = 0.011$ ). Post-hoc analysis using the Dunn's method showed that males spent a greater percentage of time investigating the Y-junction in experiments 1 and 2 than in experiment 4, the blank control experiment, while no significant difference was found between experiment 3, the choice test, and experiment 4.

**Table 4.4 Trailing behavior of *Boiga irregularis*: Percentage of time spent investigating in the Y-junction of the maze.** Percentage of time calculated by dividing the time to complete the trial by the time spent in the Y-junction and multiplying by 100)

Condition	Percentage of Time in Y-Junction (seconds $\pm$ SD)	Significance (Dunn's Method)
Males Trailing Females	$13.7 \pm 10.2$	YES
Males Trailing Males	$12.7 \pm 6.0$	YES
Males Trailing Both Sexes	$7.3 \pm 4.8$	NO
Males Trailing Blank Maze	$5.6 \pm 3.8$	---

Head-jerking behavior (a behavior only displayed in the context of courtship and male ritualized combat in this species (Chapter 2) was displayed in 3 trials (by 2 different males) when males were following female trails and was observed in 2 trials (by 2 different males) when males were following male pheromone trails. In addition, all individuals that trailed displayed behaviors associated with trailing in other species, including tongue-flicks directed to the trail and periodic pauses with side to side movements of the head (Brown and MacLean, 1983; Lillywhite, 1984).



## **Discussion.**

The sexually dimorphic response in trailing behavior displayed by males and females in this study appears to be a result of decreased activity of females under breeding conditions. Under breeding conditions in the laboratory, males are much more active than females, spending their active hours exploring their cages, while females tend to remain confined to their hide boxes (M. Greene, personal observation). This difference in activity is absent when the snakes are not in breeding condition (M. Greene, personal observation). Given that of 5 females to complete trials, 4 followed conspecific trails, this response by females does not necessarily suggest that female brown tree snakes lack the ability to trail conspecifics.

Field data, collected in the brown tree snake's native range, corroborate these laboratory observations (Whittier and Limpus, 1996; Bull and Whittier, 1996). Activity patterns of brown tree snakes in Australia were estimated by examining the number of individuals collected per month over many years in museum collections (Bull and Whittier, 1996). Seasonal differences in activity levels between males and females were found. Males were more active than females, particularly during months where reports of mating existed and in months where females were in the late stages of ovarian development or gravid (Bull and Whittier, 1996; Whittier and Limpus, 1996). In other months, the numbers of males and females collected were approximately equal (Bull and Whittier, 1996).

The ability of male brown tree snakes to follow female pheromone trails most likely represents a mechanism for males to locate potential mates during the breeding

season. Similar responses have been reported in several other species, mostly in colubrids of the genus *Thamnophis* (Ford, 1986). However, few reports exist of trailing behavior in other taxa of snakes including those that display combat behavior such as the brown tree snake (Ford, 1986; Mason, 1992).

Field reports of the courtship and combat behavior of the Swedish adder (*V. berus*) have described the ability of males of this species to trail both conspecific males and females (Andrén, 1986). Upon locating a pheromone trail, a male adder will pause, investigate both directions and turn in the direction in which the trail was laid. Upon contacting and tongue-flicking the integument of a female, courtship will ensue (Andrén, 1986). Conversely, upon contacting another male, the snakes will initiate combat if a female is present (Andrén, 1982). The winner of the combat bout gains access to the local female by establishing a temporary territory that it will defend from other males (Andrén, 1986). Without the presence of a female, males typically do not interact aggressively (Andrén, 1986). Although other snake species have not been studied as extensively in the field, it appears that, under natural conditions, combat behavior occurs primarily in the presence of a female (Woodbury, 1941; Lowe, 1948; Moehn, 1967; Bennion and Parker, 1976; Shine *et al.*, 1981).

Male brown tree snakes may employ a strategy similar to that of *V. berus* by following male pheromone trails in order to locate males that are near or are courting females. By initiating combat, the trailing male may be able to displace the other male and gain access to the local female, as has been speculated for other species (Shine *et al.*, 1981). There would be little cost to this strategy, as combat would be unlikely to ensue without the presence of a female.

It would be expected that, given a choice between a conspecific male and female trails, males would prefer the female trail. This choice would, at least in a natural situation, lead directly to a female and would allow the male to avoid aggressive interactions with other males. However, in this study, males did not display a significant trailing response to either of the sexes, choosing the male trail equally as often as the female trail, indicating that males did not discriminate sex from the pheromone trails.

The lack of sex discrimination by males is further supported by data collected on the mean percentage of time males spent investigating the Y-junction of the maze during the four experiments. This variable was measured to quantify the amount of time it took males to decide which arm of the maze to enter. Males spent a significantly greater percentage of time investigating the Y-junction when presented with a female trail versus a blank arm (experiment 1) or a male trail versus a blank arm (experiment 2) than when presented with two blank arms (experiment 4). These data seem to reflect the additional amount of time needed by males to discern which arm of the maze the trail led into. In contrast, there was no significant difference in the percentage of time spent investigating the Y-junction when males were given a choice between an arm treated with a female trail and an arm treated with a male trail (experiment 4) and when males were given a choice between two blank arms (experiment 4).

There are two possible mechanisms to explain the lack of discrimination between male and female trails: 1) that the pheromone located in the skin lipids of males and females that releases trailing behavior is chemically identical in both sexes or 2) that the pheromones have different chemical structures, but that there is no behavioral discrimination by males. However, it is clear from behavioral observations and bioassays

that male brown tree snakes can discriminate between males and females via pheromones, as courtship with females or combat with males begins only after a male tongue-flicks another individual, making the first explanation unlikely (Chapter 2; Chapter 3).

The lack of behavioral discrimination by males may be an artifact of how the male and female trails were laid down in the maze. In the initial stretch of the maze, both male and female pheromone trails were present together. These trails then diverged in the Y-junction. Under natural conditions, a similar situation might signal to a trailing male that a male and female pair are in the near vicinity. If so, following either a male or female pheromone trail should lead to the courting pair, where the trailing male would have the opportunity to initiate combat. Also, in this situation, the trailing male might attempt to revert to vision in order locate the movement of the courting pair at short distances, as other species are known to do in the field (Hawley and Aleksuk, 1975; Andr n, 1982, 1986).

A comprehensive understanding of the basic biology of this species is needed in order to understand what traits make the brown tree snake an effective invasive species and to develop effective management strategies to control this snake on Guam and prevent its spread to other islands in the Pacific (Brown Tree Snake Control Committee, 1996; Mason, in press). The use of brown tree snake pheromones as chemical attractants has been proposed for use as part of a comprehensive control plan (Mason, in press). The results of this study provide evidence that the female sex pheromones and male combat pheromones of this species, when identified and synthesized, would be excellent candidates for chemical attractants on Guam.

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## 5. Pheromonal Inhibition of Male Courtship Behavior in the Brown Tree Snake, *Boiga irregularis*

### Abstract

In snakes, pheromones play a central role in mediating reproductive behaviors. We report here a novel pheromone system in the brown tree snake (*Boiga irregularis*) in which females release a pheromone that inhibits male courtship. We have made observations of female brown tree snakes releasing cloacal secretions (CS) during courtship which seem to cause courting males to cease courtship. Experiments were conducted to determine the role cloacal secretions play in the reproductive behavior of male and female brown tree snakes. Female CS caused a decrease in the amount of time males spent courting females and a decrease in the intensity of courtship when compared to a control treatment. Male CS did not, however, affect the amount of time females spent displaying courtship behaviors or the intensity of courtship. Neither male or female CS had any significant effects on male ritualized combat behavior including time males spent in combat or intensity of combat behaviors displayed. Therefore, the inhibition of brown tree snake reproductive behaviors is specific to female CS inhibiting male courtship behavior.

### Introduction

Snakes have highly developed chemical senses and rely upon the detection of pheromones to mediate reproductive behaviors (see Halpern, 1992 and Mason, 1992 for

reviews). Most commonly, pheromonal mediation is manifested through the release of male courtship behaviors by female sex pheromones located in female skin lipids (Noble, 1937; Mason *et al.*, 1989; Mason, 1993). The female sex pheromone of the red-sided garter snake (*Thamnophis sirtalis parietalis*), the only reptilian pheromone identified to date, has been identified as a series of long chain saturated and monounsaturated methyl ketones (Mason *et al.*, 1989, 1990; Mason, 1993). In addition, males of some species appear to have sex pheromones that act as releasers of female courtship behaviors (see Chapter 2) or act to distinguish males from females during courtship in breeding aggregations (Vagvolgyi and Halpern, 1983; Mason *et al.*, 1989; Mason, 1993).

Pheromones also mediate other behaviors associated with reproduction in snakes. Trailing behavior to locate mates during the breeding season is mediated by skin lipids, possibly by sex pheromones, deposited on the ground as snakes move through their environment (Noble, 1937; Ford and Low, 1983; Ford, 1986; Chapter 4). Male-male ritualized combat behavior is released by a pheromone located in the skin of male snakes (Schuett and Gillingham, 1989; Andrén, 1986; Secor, 1990) and there is evidence that volatile pheromones, originating from male skin, may also play a role in mediating combat behavior in some species (Andrén, 1982).

Female garter snakes become unattractive to males after mating because of a pheromone deposited near the female's cloaca (Devine, 1977; Ross and Crews, 1977; R.T. Mason, unpublished). This pheromone acts in an antagonistic manner to the female sex pheromone, preventing males from initiating courtship. It is unclear whether this pheromone is produced by the female in order to signal her mated status to males (Devine,

1977) or by the males in order to prevent the female from copulating multiple times with different males (Ross and Crews, 1977).

We report here a novel snake pheromone system used by female brown tree snakes (*Boiga irregularis*) to inhibit male courtship behavior. The brown tree snake is an arboreal, rear fanged colubrid native to the forests of Australia, Papua-New Guinea and the Solomon Islands (Cogger, 1992; Rodda *et al.*, 1997). This species was accidentally introduced to the island of Guam where it has caused significant economic and ecological damage including the extinction or extirpation of 9 bird species (Rodda *et al.*, 1990; Rodda *et al.*, 1997). The snakes reach snout-to-vent lengths of up to 3m and masses of up to 2 kg, with males being generally larger than females (Cogger, 1992; Rodda *et al.*, 1997)

Male courtship behavior in this species is released by a female sex pheromone consisting of a suite of non-polar, non-volatile skin lipid molecules (Greene and Mason, in press). Male brown tree snakes also display ritualized combat behavior, which presumably serves as a contest between males for access to potential mates during the breeding season (Chapter 2). In addition, male brown tree snakes follow pheromone trails of both male and female conspecifics under breeding conditions (Chapter 4)

Observations of brown tree snake courtship revealed that females play an active role in courtship (Chapter 2). Of particular interest were behaviors displayed by females that seemingly inhibited male courtship behaviors. In such cases, females were observed to lift their tails nearly perpendicular to the ground, gape their cloacae and release a bolus of liquid in response to male courtship. The cloacal secretions (CS) originated from both

the paired cloacal glands located in the snakes' tails and from the urogenital opening in the cloaca and consisted of a clear, viscous liquid containing a yellow or white precipitate.

Upon the release of the female CS, the behavior of courting males changed dramatically. Males that were vigorously courting females stopped displaying courtship in all cases where females were observed to release CS except for periodic, weak bouts of head-jerking, the first behavior displayed by males during their courtship sequence. In these cases, males typically remained near the female, following her around the cage and investigating her with tongue-flicks. These male and female behaviors have only been observed during courtship.

The experiments conducted as part of this study were designed to test the hypothesis that female brown tree snakes release CS to inhibit male courtship. In addition, the experiments ascertain whether this novel pheromone system is a specific mode of communication between females and males during courtship, whether this pheromone has general effects and can also be found in male CS or will have effects on male combat behavior.

## **Materials and Methods**

### *Husbandry.*

The animals used in this study were collected in the field on Guam and have been housed in our laboratory for the past 6 years under an established laboratory protocol (Greene *et al.*, 1997). The snakes (7 females and 10 males) are housed in Plexiglas cages designed specifically for arboreal reptiles (Mason *et al.*, 1991) and are fed a diet consisting

of thawed frozen mice or chicks every 3 weeks (Greene *et al.*, 1997). Males in the colony have a mean ( $\pm$  SD) snout-to-vent length (SVL) of  $162.2 \pm 22.1$  cm and a mean mass of  $647.5 \pm 265.7$  g. Females have a mean SVL of  $137.9 \pm 9.9$  cm and a mean mass of  $385.7 \pm 81.5$  g. Temperatures cycle from 23 to 30°C and relative humidity ranged between 75% and 80% in the room. Lighting (approximately 14L:10D) was provided by overhead fluorescent lights and ambient sunlight entering the room through windows.

#### *General Experimental Conditions.*

Four experiments were conducted to characterize the effects (sex specificity and context specificity) of cloacal secretions on male behaviors: 1) female CS versus a control on male courtship behavior (N = 7 male-female pairs; CS from female of pair), 2) male CS versus a control on male courtship behavior (N = 7 male-female pairs; CS from male of pair), 3) female CS versus a control on male combat behavior (N = 5 male-male pairs; CS from randomly chosen female) and 4) male CS versus a control on male combat behavior (N = 5 male-male pairs; CS from both males). The same control, a 5% aqueous solution of cologne, was used in each trial.

Different male-female or male-male pairs were randomly selected for each experiment. Within an experiment, the pairs were tested in each experiment using a switchback, or counterbalance, approach where on the first night of an experiment pairs were randomly treated with CS or a control solution. On the second night the experiment, the other treatment was applied to the pair.

Experiments were conducted between 1900 and 0200, during scotophase, when the snakes were most active. The experiments were conducted in a clear Plexiglas arena

designed specifically for the observation of brown tree snake courtship behavior (Chapter 2). The arena was constructed with sides measuring 1.5 meters, providing 2.25 m<sup>2</sup> of floor area. Clear Plexiglas doors on opposite sides of the arena allowed access to the inside of the cage for cleaning and for transfer of snakes. Two red 7 watt incandescent lights, mounted above the roof of the arena, provided sufficient light for videotaping the trials through the walls. Data were later collected from videotape by an observer blind to the treatments. Between trials the floor and walls of the arena were cleaned using soap and water, dried using paper towels and allowed to air dry with the doors open for 30 minutes before the next trial was conducted. Environmental conditions (room temperature and relative humidity) remained constant over the entire testing period.

CS was collected immediately prior to a trial by gaping a snake's cloaca and applying light pressure anterior to the vent. Upon applying pressure, secretions sprayed from the papillae of the paired cloacal glands and an approximately 1 ml bolus of clear liquid containing a yellow precipitate was discharged from the snake's urogenital opening. The CS collected in this manner appeared and smelled identical to CS voluntarily released by females during courtship. The CS was collected in a clean 15 ml screw top vial and stored at room temperature until used in the experiment. All samples were used in a trial within 15 min after collection.

A trial began when a male-female or a male-male pair were placed in the arena and allowed to begin courtship or combat. After 90 seconds of courtship or combat behavior was displayed by males (in combat experiment, data were taken when either male of the pair was displaying combat behavior) at the level of chin-rubbing (courtship score 2, Figure 5.1 and 5.2), approximately 1 ml of cloacal gland secretion or control was added to

the arena during trials using a Pasteur pipette. Treatments were added to the arena through a hole in one of the cage walls that was covered with paper to prevent the snakes from observing the investigator adding the treatment. The treatment generally landed in the center of the arena floor while the snakes typically courted each other near one of the arena walls or in a corner.

A 10 min observation period followed the introduction of the treatment. During the observation period the amount of time that males spent courting or combating was recorded to quantify the inhibitory effects of CS on male behavior. To measure the intensity of courtship or combat, the amount of time that members of the pairs spent at each ethogram level was compared between the CS and control treatments. Lastly, to determine if CS has any effect on female courtship, the mean amount of time females spent actively courting males during experiments 1-2 was measured when treated with CS or the control.

All statistical comparisons were made within an experiment by comparing the time data collected during the CS treatment and the control treatment. The Wilcoxon signed rank test (SigmaStat, Jandel Scientific) was used to analyze the time data, as this test accounted for the dependency between the data collected for the two treatments and for the lack of normality of the time data.

## Results

### *Effect of CS on Time Males Spent Courting or Combating.*

In experiment 1, female CS reduced the amount of time males spent courting females when compared to the control treatment (Figure 5.3a; Wilcoxon signed rank test;

**Figure 5.1 An ethogram of courtship behavior for the male brown tree snake, *Boiga irregularis*.**

<u>Courtship Score</u>	<u>Behavioral Description</u>
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 mounts female and chin-rubs the dorsum of the female with surging head movements.
3	Male aligns body with female's.
4	Male attempts tail-search copulatory attempt by maneuvering his tail under the female's tail.
5	Copulation.

**Figure 5.2 An ethogram of ritualized combat behavior for the brown tree snake, *Boiga irregularis*.**

<u>Combat Score</u>	<u>Behavioral Description</u>
0	Male fails to investigate the opponent or only briefly investigates the opponent with tongue flicks.
1	Male displays head-jerking behavior.
2	Male mounts opponent and chin-rubs the dorsum of the opponent with surging head movements.
3	Male aligns body with opponent's.
4	Male attempts to pin opponent's head to the ground using head and body.
5	Male's body becomes entwined with opponent's while head-pinning continues.
6	Losing male flees from victorious male.



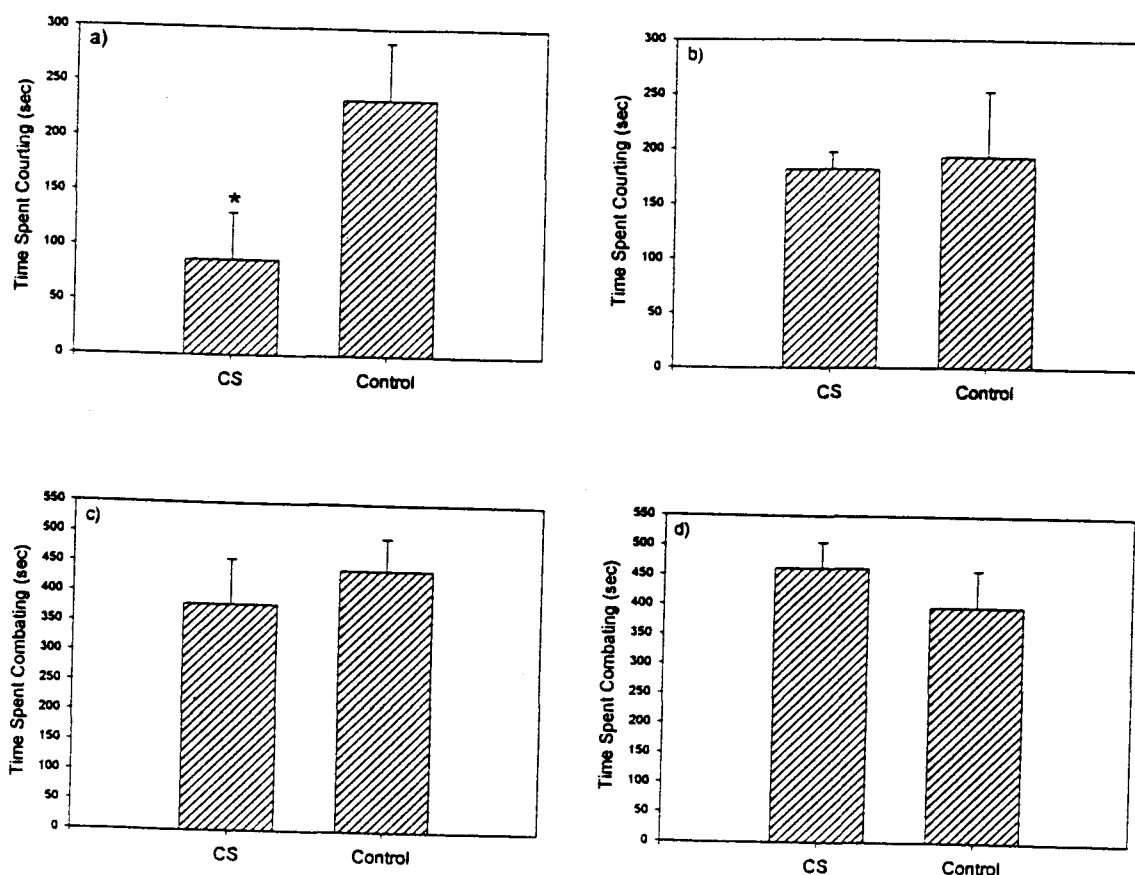
$p = 0.031$ ). In contrast, in experiment 2, there was not a significant differences in time spent courting by males when treated with male CS versus the control (Figure 5.3b; Wilcoxin signed rank test,  $p = 0.834$ ). Neither female CS (Wilcoxon signed rank test,  $P = 0.250$ ; experiment 3) nor male CS (Wilcoxon signed rank test,  $p = 0.188$ ; experiment 4) caused significant reduction in the mean times males spent combating when compared to the control treatment (Figures 5.3c-d).

#### *Effect of CS on the Intensity of Male Courtship.*

In experiment 1, female CS reduced the overall intensity of male courtship when compared to control treated males (Table 5.1). Although there was not a significant difference in the time males spent courting females at courtship score 1 (head-jerking) when treated with female CS or control, the female CS treatment had a lower mean (Table 5.1). However, there was a significant decrease in the mean amount of time males treated with female CS spent courting at courtship score 2 (chin-rubbing behavior) when compared to the control treatment (Wilcoxon signed rank test;  $p = 0.031$ ). Only one pair in each treatment reached courtship score 3, although when treated with CS the male spent less time at score 3 (body-alignment) than the control treated males (Wilcoxon signed rank test;  $p = 0.031$ ;  $P = 1.000$ ; Table 5.1).

In experiment 2, no significant reductions were found between the times males spent courting females at each courtship score (Figure 5.1) when treated with male CS or control (Table 5.1). In addition, no significant differences were found in the times males

**Figure 5.3** The mean amount of time ( $\pm$  s.e.m.) that male brown tree snakes spent either courting or combating in each experiment when treated with cloacal secretions (CS) or a control. (a) The mean time males spent courting females when treated with female CS or the control (experiment 1). (b) The mean time males spent courting females when treated with male CS or the control (experiment 2). (c) The mean time males spent combating when treated with female CS or the control (experiment 3). (d) The mean time males spent combating when treated with male CS or the control (experiment 4).



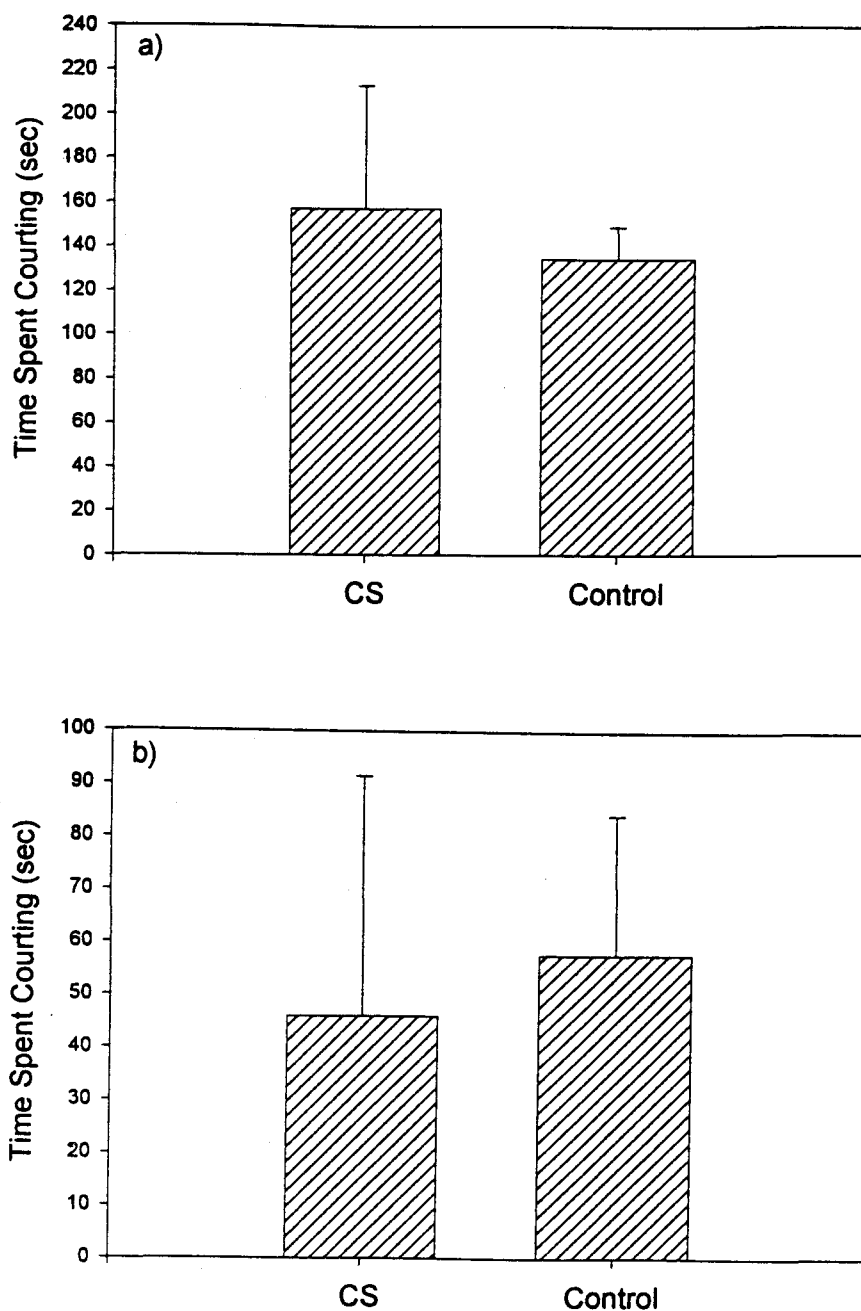
**Table 5.1 The effect of cloacal secretions (CS) and control treatments on the intensity of male courtship.** All values are listed as the mean  $\pm$  s.e.m. Statistical comparisons were made between the mean times males spent at each courtship score when treated with CS or the control. S.e.m. and p-values were not reported for data at courtship score 3 because the values each represent data taken from a single male.

Courtship Score	Experiment 1 (female CS versus control)			Experiment 2 (male CS versus control)		
	Female CS	Control	Wilcoxon p-value	Male CS	Control	Wilcoxon p-value
1:	67.8 $\pm$ 34.6	115.4 $\pm$ 34.8	0.297	58.8 $\pm$ 17.9	82.0 $\pm$ 16.5	0.156
2:	9.9 $\pm$ 5.9	101 $\pm$ 65.4	<b>0.030</b>	114.5 $\pm$ 27.8	94.3 $\pm$ 41.4	0.438
3:	9.4 $\pm$ 9.5	18.1 $\pm$ 18.1	1.000	7.7 $\pm$ 7.7	0.0 $\pm$ 0.0	1.000

**Table 5.1** The effect of cloacal secretions (CS) and control treatments on the intensity of male courtship. All values are listed as the mean  $\pm$  s.e.m. Statistical comparisons were made between the mean times males spent at each courtship score when treated with CS or the control. S.e.m. and p-values were not reported for data at courtship score 3 because the values each represent data taken from a single male.

Courtship Score	Experiment 1 (female CS versus control)			Experiment 2 (male CS versus control)		
	Female CS	Control	Wilcoxon p-value	Male CS	Control	Wilcoxon p-value
1:	67.8 $\pm$ 34.6	115.4 $\pm$ 34.8	0.297	58.8 $\pm$ 17.9	82.0 $\pm$ 16.5	0.156
2:	9.9 $\pm$ 5.9	101 $\pm$ 65.4	<b>0.030</b>	114.5 $\pm$ 27.8	94.3 $\pm$ 41.4	0.438
3:	9.4	18.1	-	7.7	0.0	-

**Figure 5.4** The mean amount of time ( $\pm$  s.e.m.) that females spent actively courting males when treated with cloacal secretions (CS) or the control. (a) The mean time females spent courting males when treated with female CS or the control (experiment 1). (b) The mean time females spent courting males when treated with male CS or the control (experiment 2).



spent combating at each combat score (Figure 5.2) when treated with female CS treatment or control (experiment 3) or when treated with male CS treatment or control (experiment 4; Table 5.2).

#### *Effect of CS on Time Females Spent Courting Males.*

There were no reductions in the mean amounts of time females spent actively courting males during experiment 1, where females were treated with their own CS and the control (Wilcoxon signed rank test,  $P = 0.813$ ; Figure 5.4a), or in experiment 2, where male CS and the control were added to the arena (Wilcoxon signed rank test,  $P = 0.818$ ; Figures 5.4b).

### **Discussion**

The results of these experiments provide evidence that female CS inhibits male courtship behavior and confirms previous observations of male courtship inhibition by females in the brown tree snake (Chapter 2). Further, this pheromonal signal was present only in female CS and was effective in the context of courtship, not male ritualized combat. In addition, pheromonal effects were observed only on males in the experiments while never on females. These data represent a novel mechanism of courtship inhibition in snakes.

Observations made during the experiments provide insight into the chemical nature of this courtship inhibition pheromone. Brown tree snakes, like other snake species, are highly thigmotactic in nature, preferring to stay near edges such as the arena wall even

during courtship or combat. During the experiments, CS was added to the arena so that it landed in the center of the arena floor, away from the snakes that were typically located along the arena walls. As such, it was rare for a male to tongue-flick the CS added to the arena directly. In snakes, tongue-flicking serves to deliver non-volatile chemicals to the vomeronasal organs where they are detected (Kubie *et al.*, 1978, Halpern, 1987). As such, the behavioral effect of female CS on male courtship appears to be mediated via a volatile pheromone that is primarily detected by the olfactory system rather than the vomeronasal organ.

Most pheromones studied in snakes are non-volatile integumental odors (Noble, 1937; Kubie *et al.*, 1978; Gillingham and Dickinson, 1980; Andr  n, 1982; Mason *et al.* 1989, 1990; Mason, 1992), while little experimental attention has been paid to volatile snake pheromones (Watkins, *et al.*, 1969; Graves and Duvall, 1988). As snakes possess few glands that release volatile signals, particular attention has been paid to the paired cloacal glands located in the tail of all species of snakes. Studies have most often studied paired cloacal gland secretions in the contexts of alarm signals, although very little experimental evidence exists to support this idea (Graves and Duvall, 1988). The CS released by female brown tree snakes is composed, in part, of paired cloacal gland secretions along with liquid from the intestine that is released through the urogenital opening. It is impossible to determine from this study which source the courtship inhibition pheromone originates from, however, this study does provide some of the first experimental evidence implicating paired cloacal gland secretions in the mediation of snake behavior (Watkins *et al.*, 1969; Graves and Duvall, 1988).

In this study female CS did not seem to inhibit male courtship behavior through a generalized alarm response, as has been hypothesized as a function of CS (Graves and Duvall, 1988). If this were the case, you would not expect to observe sex and context specific responses like the ones observed. In this study, female CS effected males only during courtship and not during combat behavior while male CS had no effects on females or other males during combat. This does not, however, preclude the use of CS as an alarm pheromone or predator deterrent in another context outside of reproduction as brown tree snakes release CS when disturbed.

During courtship, observations of females releasing CS during courtship were always accompanied by tail-raising behavior. This suggested that a chemical and a visual signal were involved in inhibiting male courtship behavior. The results of this study indicate that the release of CS alone is sufficient to inhibit male courtship behavior. Although visual signals play a important role in snake courtship and combat (Carpenter, 1977; Gillingham, 1978, 1979; Secor 1987), pheromones are necessary to release these behaviors (Noble, 1937; Kubie *et al.*, 1978; Mason, 1989; Mason, 1992). It is possible that a more pronounced response would have occurred if the females in this experiment displayed tail-raising behavior or other behaviors associated with a lack of proceptivity in female brown tree snake including body-bumping and tail-lashing were present (Greene and Mason, in review).

The inhibition of male courtship behavior via the release of female CS may represent a mechanism for females to reject unsuitable mates or to signal that females are not sexually active at the time of courtship (Greene and Mason, submitted). Male snakes cannot force copulation, so females ultimately determine if copulation will be the outcome



of a courtship event. Therefore, there would be clear motivation for male snakes to stop courting a female after she has signaled that she is not receptive to his courtship. Further displays of courtship with the same female would prove to be a waste of energy, could result in the male missing other mating opportunities or could prove costly by attracting predators.

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## 6. Conclusion

The objective of the research presented in this thesis was to gain a comprehensive understanding of the role pheromones play in mediating the reproductive behaviors of the brown tree snake, *Boiga irregularis*. More specifically, this research had the following goals: 1) to catalog the repertoire of courtship, mating and combat behaviors (for the first time) in this species, 2) to isolate and characterize the female sex pheromone of this species, 3) to examine the ability of brown tree snakes to follow conspecific pheromone trails and 4) to examine and characterize the pheromonal inhibition of male courtship behavior by female brown tree snakes.

The data presented in this thesis are significant from a basic research level in that they further our understanding of how pheromones mediate vertebrate reproductive behaviors. Unlike most vertebrates, snakes such as the brown tree snake are excellent model systems to examine pheromonal communication because snakes respond to pheromones with relatively simple, stereotyped behaviors that can be elicited under artificial laboratory conditions without the benefit of other sensory inputs (Mason, 1992). Additionally, many snake pheromones are contained in integumental lipids that are relatively easy to separate into fractions that retain their biological activity (Mason *et al.*, 1990). The brown tree snake, a tropical species, offers an excellent comparison to similar research conducted on Northern temperate species such as garter snakes, genus *Thamnophis*, rattlesnakes, genus *Crotalus* and Swedish Adders, *Vipera berus*, among others (Halpern, 1992; Mason, 1992).

Additionally, as the brown tree snake is an invasive pest species in the Pacific, particularly on the island of Guam, this research provides valuable information that could aid in the control of this species. In particular, managers of the brown tree snake on Guam have called for research which develops potential chemical attractants to supplement current control measures (Brown Tree Snake Control Committee, 1996; Mason, in press). Currently, the most effective means of controlling brown tree snakes on Guam 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 when a snake is in the near vicinity of a trap and can detect the prey item by vision or by volatile prey odors. Synthetic pheromones could be applied to the forest floor in trails leading towards traps from several directions. This would be a potentially effective control method for capturing snakes because it would attract snakes from over large areas. This pheromone control method could aid in brown tree snake monitoring programs in areas where the snakes do not have established populations such as Oahu, Hawaii (Mason, in press). In addition, the Brown Tree Snake Management Plan (Brown Tree Snake Control Committee, 1996) calls for the development of chemical repellents and reproductive inhibitors to be used in controlling this species.

The descriptions of reproductive behaviors presented in Chapter 2 represent the first published account of the brown tree snake's courtship, mating and combat behavior. Additionally, this research is the first published account of the reproductive behavior of any member of the genus *Boiga* and represents the most comprehensive description of the reproductive behavior of any tropical snake species. Importantly, these observations

formed the basis of bioassays which were designed to experimentally examine the mediation of these reproductive behaviors by pheromones.

Specific behavioral observations (reported in Chapter 2) suggested that the brown tree snake possesses several pheromone systems that mediate reproductive behaviors. The hypothesized pheromone systems identified were 1) a female sex pheromone that released male courtship behavior, 2) a pheromone that inhibited male courtship behavior when released by females in response to male courtship, 3) a male sex pheromone that released female courtship behavior, 4) a male combat pheromone that released combat behavior from other males, 5) a female trailing pheromone that appeared to act in mate attraction and 6) a male trailing pheromone that appeared to allow males to locate other males during the breeding season. However, it is important to note that it is possible for some of the above pheromones to share the same chemical structure.

Subsequent experiments (Chapters 3 to 5) examined these potential brown tree snake pheromone systems in an experimental fashion by adhering to a paradigm known as the 'response guided approach' (Albone, 1985). In this approach, the release of specific behaviors by stimuli containing the pheromone are tested in a behavioral bioassay. Typically, the behaviors measured in the bioassay are displayed by the experimental subjects in an "all-or-none" fashion and are normally observed only in a specific behavioral context, such as during courtship or trailing (Albone, 1985).

To isolate a pheromone and characterize its chemical structure, solvent extracts can be taken from the pheromone's emitting source and tested in a bioassay. If the solvent extract contains the pheromone, it will release behaviors normally elicited by another conspecific. Further, solvent extracts can be separated into fractions with the use

of chromatography techniques. The experimental subjects then effectively “choose” the fractions containing components of the pheromone by displaying the appropriate behavior during the bioassay in response to the fractions. In this manner, only chemicals that are biologically meaningful to the animal are isolated for further analysis. Importantly, the response guided approach allows for causal links to be made between a pheromone and the display of a specific behavior.

In Chapter 3, the response guided approach was utilized in order to isolate the female sex pheromone of the brown tree snake and to investigate the chemical nature of the pheromone. By using an “all-or-none” bioassay that measured the presence or absence of courtship behaviors in response to a solvent extract of female skin lipids as well as to fractions created from the skin lipid extract it was possible to verify that the sex pheromone was located in the skin lipids of the female’s dorsum and that this pheromone was responsible for releasing male courtship behavior, even in the absence of the visual and tactile signals provided by females during courtship. Additionally, the bioassay allowed for the isolation of the female sex pheromone and for initial characterization of the pheromone’s chemical structure. It was determined that the pheromone was composed of relatively non-polar, non-volatile skin lipid molecules. Further chemical analysis is necessary to completely identify the chemical structure of this pheromone.

Although there are many anecdotal reports of female sex pheromones releasing male courtship behavior in snakes (see Mason, 1992 for a review), this study is one of only a few studies that have examined this phenomena in an experimental manner (Noble, 1937; Mason *et al.*, 1989, 1990; Mason, 1992). To date, only a single reptilian pheromone has been identified and chemically characterized, the sex pheromone of the

red-sided garter snake, *Thamnophis sirtalis parietalis* (Mason *et al.*, 1989, 1990). The data presented in Chapter 3 represent the only other system where a snake pheromone has been chemically characterized to such an extent. Once the female sex pheromone is identified and synthesized, it may also prove to be useful as a chemical attractant used in the control of the brown tree snake in the Pacific.

In Chapter 4, the ability of brown tree snakes to follow pheromone trails produced by reproductively attractive conspecifics was tested using a Y-maze constructed of clear polyvinylchloride tubing. In the first experiment, a differential response was found in the ability of males and females to complete experimental trials. Males completed the majority of trials attempted, entering and subsequently exiting the Y-maze by the completion of the trial, while females completed significantly fewer trials. This sexually dimorphic response appears to be attributable to lower activity levels in general by females under breeding conditions. In another experiment, males displayed a significant trailing response to female pheromone trails versus a blank treated arm on the Y-maze. This response likely represents a mechanism for males to locate potential mates during the breeding season, as this species is not known to form breeding aggregations. In a third experiment, males displayed a significant trailing response to pheromone trails produced by other males versus a blank arm treatment on the Y-maze. Males likely follow the pheromone trails of other males in order to locate males that are near or are courting females. By initiating combat with a male near or courting a female, the trailing male could displace his opponent and gain access to the female. In a final experiment, males did not demonstrate a preference for female pheromone trails over male pheromone trails when given a choice on the Y-maze. These data indicate that male



brown tree snakes lack the ability to discriminate sex from pheromone trails, although this result may be an artifact of how the male and female trails were applied to the Y-maze.

Although numerous studies have examined the ability of males to trail females during the breeding season (Ford, 1986; Halpern, 1992; Mason, 1992) this is the first study of its kind conducted on a tropical species of snake and is the first conducted on a primarily arboreal species. In addition, these are the first published experimental results showing male snakes trailing other males, although anecdotal reports of this phenomena exist in other species that display male combat behavior (Andrén, 1986). This study is particularly relevant to efforts aimed at using brown tree snake pheromones as control agents as it demonstrates that male brown tree snakes, and possibly females, will follow conspecific pheromone trails. This makes it theoretically possible, at least, to use synthetic pheromones as chemical attractants in trapping schemes.

In Chapter 5, the pheromonal inhibition of male reproductive behavior by females was examined. These experiments were based upon the observation reported in Chapter 2 in which the release of cloacal secretions (CS) by females seemed to cause males to cease courtship. In the study, female CS caused a decrease in the mean amount of time that males spent courting females as well as a decrease in the intensity of male courtship when compared to a control treatment. Conversely, male CS did not affect the amount of time that males spent courting females or the intensity of male courtship in comparison to control treatments. In addition, neither male nor female CS had any significant effects on the mean amount of time males spent displaying combat behavior in the presence of other males or on the intensity of combat displayed when compared to a control treatment. It

was concluded that the pheromonal inhibition of male brown tree snake courtship behavior is caused by the release of female CS during the act of courtship.

These data represent a novel pheromone system in reptiles and may represent a mechanism for females to reject courting males that were deemed unsuitable mates or alternatively as a signal to males that a female is not reproductively active. In addition, this pheromone system is interesting in that it involves a pheromone that is volatile in nature, which has rarely been reported in snakes, and one that is voluntarily released by the female, unlike sex pheromones which are located in skin lipids that are passively released into the environment. When identified and synthesized, this pheromone may prove to be a useful control technology if applied as a reproductive inhibitor of brown tree snakes on Guam. Being volatile in nature, a synthetic blend of this pheromone could be efficiently distributed throughout the environment by existing technologies developed for the pheromonal control of insects (Cardé, 1990; McNeil, 1992). If effective, the pheromone could reduce the number of matings during the breeding season, potentially reducing population levels over time.

Collectively, the results of these experiments provide a sophisticated understanding of the mediation of brown tree snake reproductive behaviors by pheromones and provide a sound basis for further research into this topic. It is clear that pheromones play a dominant role in brown tree snake reproduction, mediating both male and female courtship behaviors, mate location/attraction by use of pheromone trails and in releasing male combat behavior. Figure 6.1 provides a summary of these pheromone systems.

**Figure 6.1** A summary of known and hypothesized (in *italics*) pheromone systems in the brown tree snake, *Boiga irregularis*.

Reproductive Behaviors	Pheromone	Pheromone Action
Courtship:	Female Sex Pheromone	Releases Male Courtship Behavior
	Courtship Inhibitory Pheromone	Inhibits Male Courtship Behavior
	<i>Male Sex Pheromone</i>	<i>Releases Female Courtship Behavior</i>
Combat:	<i>Combat Pheromone</i>	<i>Releases Male Combat Behavior</i>
Trailing:	Male Trailing Pheromone	Releases Male Trailing Behavior
	Female Trailing Pheromone	Releases Male Trailing Behavior

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