

SEX RECOGNITION IN THE LEOPARD GECKO,
Eublepharis macularius (SAURIA: GEKKONIDAE)
Possible Mediation By Skin-Derived Semiochemicals

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Abstract—Male leopard geckoes, *Eublepharis macularius*, rely on skin-derived semiochemicals to determine the sex of conspecifics. Males respond to other males with agonistic behavior while females elicit courtship behavior from males. While females were shedding, males responded to them with agonistic behavior. The same females were courted both before and after shedding. An initial survey of hexane-extracted skin lipids from male and female geckoes revealed fatty acids common to both sexes. Several steroid analogs of cholesterol were unique to males while long-chain methyl ketones were unique to females. Results are discussed in the context of skin lipids serving as pheromones in reptiles.

Key Words—Sex recognition, Gekkonidae, gecko, semiochemicals, sex behavior, agonistic behavior, skin lipids, fatty acids, steroids, methyl ketones.

INTRODUCTION

Sex recognition in lizards is thought to rely primarily on two sensory modalities: vision and chemoreception. Only one of these is of primary importance within a taxon, however (Camp, 1923). For example, gekkonids have long been recognized as utilizing chemical communication in many aspects of their social behaviors (Evans, 1961; Carpenter and Ferguson, 1977), but no experimental studies have been conducted on these lizards.

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Greenberg (1943) observed and quantified social behavior of a gekkonid under laboratory conditions. He proposed two primary mechanisms of sex recognition in the banded gecko, *Coleonyx brevis*: visual displays of stereotyped behaviors and chemoreception of skin-derived chemicals. Greenberg observed that sex recognition in the gecko was not acquired at a distance and close contact was essential before sex recognition occurred. He noted that male *Coleonyx* readily distinguished other males from females and responded accordingly with agonistic or fighting behavior to the former and courtship to the latter. Greenberg hypothesized that females might be identified as mating partners through chemical stimuli. To test this hypothesis, he devised an ingenious experiment. In the majority of courtship displays observed, the male approached from the rear and took a grip on the female's tail. Females were courted further while males were ignored or attacked. Greenberg anesthetized four male and four female geckoes and surgically exchanged their tails. Courting males responded by courting the males with the female's tails. Conversely, when a courting male gripped the male tail of the female, these females were not courted but attacked. He concluded that male *Coleonyx* respond to a chemical stimulus emanating from the skin, reinforcing behavioral cues such as fighting and receptivity to courtship.

The present study continues these investigations of chemical communication in the leopard gecko, *Eublepharis macularius*, a gecko with courtship and agonistic behaviors similar to those of *Coleonyx* (see Gutzke and Crews, 1988). Unlike Greenberg's study, the present study investigates the role of chemical communication in geckoes by observing untreated, intact animals. Based on studies involving other reptiles (Andren, 1982; Radcliffe and Murphy, 1983), we hypothesized that during shedding, those chemical cues contained in or on the female's skin would be temporarily unavailable or in such low concentrations as to be undetectable by males. If skin-derived chemical cues are the primary means by which males distinguish gender, one might expect males to behave differently toward shedding males and females as opposed to the same individuals both before and after shedding. In order to test this hypothesis we observed pairs of geckoes (male-male and male-female) and recorded their behavioral interactions at three distinct periods: preshedding, shedding, and postshedding. Essential in this regard, unlike Greenberg's study, is the fact that in the present study, the male and female stimulus animals were not anesthetized and thus were free to display normal behaviors to the male with which they were paired.

Because Greenberg and others had hypothesized that sex recognition in geckoes is mediated by semiochemicals sequestered on the skin of both males and females, an initial investigation was undertaken to isolate and chemically characterize the skin surface lipids that may serve as semiochemicals in this

lizard. The skin lipids were analyzed by gas chromatography-mass spectrometry (GC-MS).

METHODS AND MATERIALS

Description of Behaviors Measured. Mating behavior in the leopard gecko begins with the male rapidly vibrating his tail and approaching the female slowly. The male then touches the female and begins to lick her tail. He then grips and shakes the female's tail. The biting involved is gentle and does not result in any discernible wounds. The male then shifts his grip to the female's back, neck or head, simultaneously moving his body parallel to hers. If the female is receptive she will raise her tail and allow the male to appose his cloaca to hers and intromission soon follows. A nonreceptive female will terminate courtship by fleeing from the male or biting him (Gutzke and Crews, 1988).

Agonistic or fighting behavior is readily discernible from courtship behavior in the leopard gecko. In the fight pattern, the male raises high off the ground, fully extending his limbs and arching his back. Intense aggression is characterized by the male swelling his throat, followed by short dashes at his opponent and quick, vigorous bites. These bites frequently lacerate the skin and sometimes seriously damage it. Thus, the body posture of the male and the intensity of his bites unequivocally characterize the difference between courtship behavior and agonistic behavior.

Behavior Experiments. Subjects used in this research were sexually mature. Their care and maintenance have been described elsewhere (Bull, 1987). Ten male geckoes were randomly assigned as pairs with 10 female geckoes. The females were then presented to the males in the latter's home cage at three distinct times as determined by the stage in the female's shedding cycle. The preshedding state was defined as at least five days prior to clouding of the skin. Shedding was indicated as the time when the skin was opaque but still intact over the new skin underneath. Finally, postshedding was defined as one day after the complete loss of the old skin. Females were presented to the males once during each of these periods. The order, in terms of shedding condition, in which the females were presented to the male was systematically varied.

The test periods consisted of the female being introduced into the male's cage for 10 min or until the male courted and mounted the female or attacked and drove off the female. As mating may alter subsequent breeding behavior, each trial was concluded before mating or damage was inflicted on the female. Five male geckoes were paired with another five males in a similar fashion.

Isolation and Identification of Skin Lipids. Analytical grade hexane was obtained from Fisher Scientific. Hexane was purified by stirring over H₂SO₄

overnight, decanting and stirring over K_2CO_3 for 4 hr. The solution was filtered, dried over $MgSO_4$ for 1 hr, and redistilled before use.

The five adult male and nine female geckoes were killed with an overdose of Brevital sodium and placed dorsal side down in a separate 500-ml glass beaker with 5–10 ml of hexane. Care was taken that the head and cloaca did not touch the solvent in order to prevent contamination of the wash with body fluids. Each beaker was covered with aluminum foil and sealed with parafilm and left overnight at room temperature. The bodies were removed and the solution filtered through a sintered glass funnel to remove particulate matter and inorganics present on the skin. The extracted lipids were pooled and the solvents removed on a rotavaporator at $50^\circ C$. The resulting viscous semisolid mixture was redissolved in fresh hexane, transferred into 7.0-ml amber glass vials with Teflon caps, and stored at $-20^\circ C$.

Capillary gas chromatographic analyses were performed on a Finnigan-MAT 4920 with a combined quadropole mass spectrometer. This system is supported by the INCOS data system and includes a library of over 42,000 spectra. The capillary column was a fused silica 4-m BP1 ($0.5 \mu m$ film) (Scientific Glass Engineering). Samples were analyzed under electron impact (EI) mode. Helium was used as a carrier gas in this instrument at 12 psi. The conditions of the GC-MS were as follows: injector temperature at $290^\circ C$, source at $150^\circ C$, detector at $250^\circ C$, EM voltage at -2200 volts. The temperature program was set at $60^\circ C$ for 1 min. ramping to $290^\circ C$ at $10^\circ C/min$. Samples ($1 \mu l/10 mg/ml$) were injected by means of a 5-sec splitless injection.

RESULTS

The results of the behavior tests were unambiguous. The test males all courted their respective stimulus females during the preshedding and postshedding phases of the test (Figure 1). However, during the time when the females were shedding, all the males responded to them with aggressive behavior. Indeed, one female was attacked and had to be removed from the experiment. Thus, each male responded to the same female with both courtship behavior and aggressive behavior depending on the shedding stage of the female. No differences in the behavior of the females were detected during the course of this study. Males always exhibited aggressive behavior towards other males irrespective of the shedding stage of the stimulus male.

Identities of components by GC-MS were made by comparison to published spectra and comparison of GC retention time and similarity to synthetic standards. The mass spectral data revealed at least 33 components of the skin lipids of male and female geckoes (Figure 2, Table 1). The majority of the components were shared in common by both males and females. For instance, fatty acids with carbon chain lengths of C_{16} and C_{18} were the major peaks in

Females

Pre-shedding (5 days prior to clouding)		Shedding		Post-shedding (1 day post-shedding)	
N	Results	N	Results	N	Results
9	Males display sexual behavior	10*	Males display aggressive behavior	9	Males display sexual behavior

* One animal was severely injured and was removed from the experiment

FIG. 1. Behavioral responses of male leopard geckoes, paired with conspecific females at three distinct phases of the female's shedding cycle.

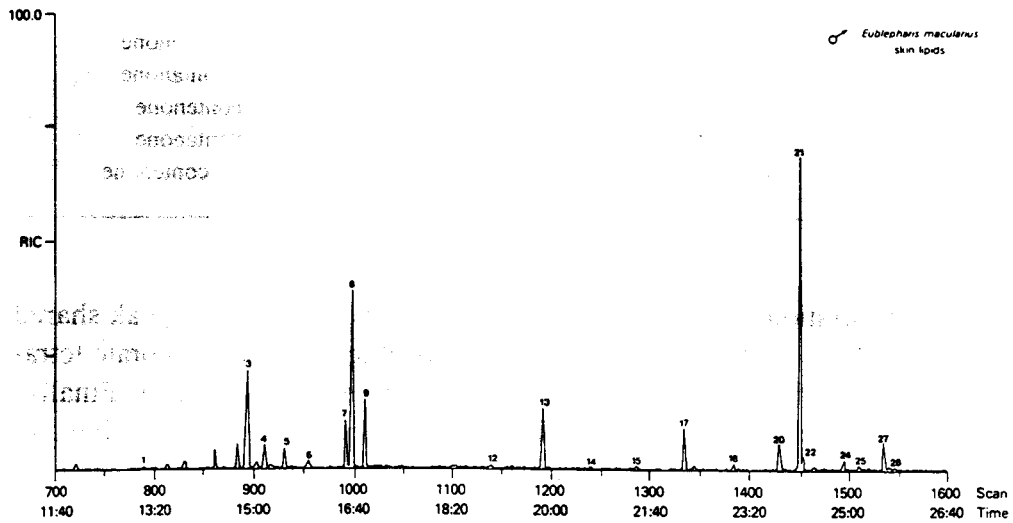
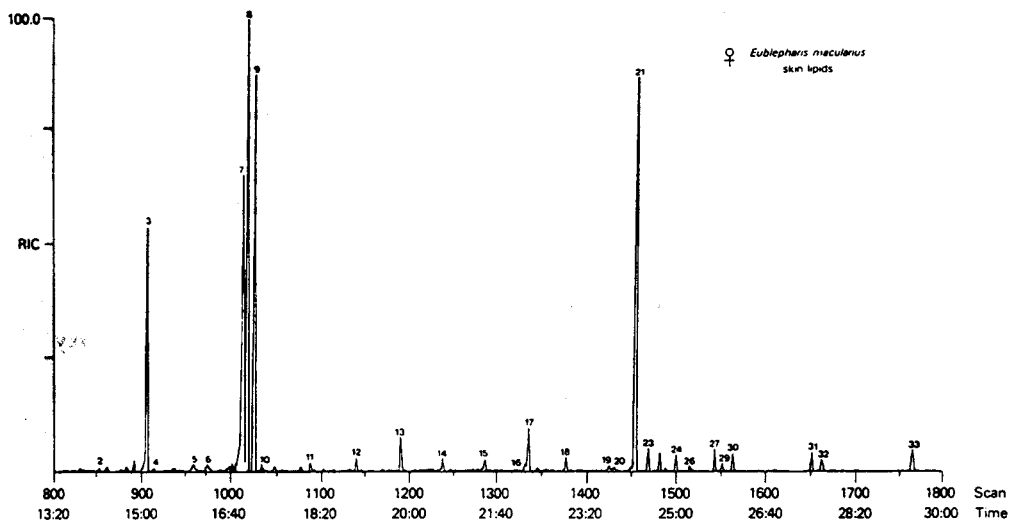


FIG. 2. Gas chromatograms of female and male leopard geckoes. The numbered peaks are identified in Table 1. Retention time is in minutes.

TABLE 1. SKIN LIPID COMPOUNDS FROM HEXANE EXTRACTS OF LEOPARD GECKO

Peak	Mol. Wt.	Elemental formula	Chemical name
1	254	C ₁₈ H ₃₈	octadecane
2	268	C ₁₉ H ₄₀	nonadecane
3	256	C ₁₆ H ₃₂ O ₂	palmitic acid
4	282	C ₂₀ H ₄₂	icosane
5	270	C ₁₇ H ₃₄ O ₂	heptadecanoic acid
6	296	C ₂₁ H ₄₄	heneicosane
7	280	C ₁₈ H ₃₂ O ₂	linoleic acid
8	282	C ₁₈ H ₃₄ O ₂	oleic acid
9	284	C ₁₈ H ₃₆ O ₂	stearic acid
10	310	C ₂₂ H ₄₆	docosane
11	324	C ₂₃ H ₄₈	tricosane
12	338	C ₂₄ H ₅₀	tetracosane
13	352	C ₂₅ H ₅₂	pentacosane
14	366	C ₂₆ H ₅₄	hexacosane
15	380	C ₂₇ H ₅₆	heptacosane
16	394	C ₂₈ H ₅₈	octacosane
17	410	C ₃₀ H ₅₀	squalene
18	408	C ₂₉ H ₆₀	nonacosane
19	422	C ₃₀ H ₆₂	triacontane
20	400	C ₂₈ H ₄₈ O	cholest-5-en-3-methoxy
21	386	C ₂₇ H ₄₆ O	cholest-5-en-3β-ol
22	388	C ₂₇ H ₄₈ O	cholestanol
23	436	C ₃₁ H ₆₄	hentriacontane
24	400	C ₂₈ H ₄₈ O	ergost-5-en-3β-ol
25	412	C ₂₉ H ₄₈ O	stigmasterol
26	450	C ₃₂ H ₆₆	dotriacontane
27	414	C ₂₉ H ₅₀ O	stigmast-5-en-3β-ol
28	416	C ₂₉ H ₅₂ O	stigmastanol?
29	448	C ₃₁ H ₆₀ O	hentriacontenone
30	450	C ₃₁ H ₆₂ O	hentriacontanone
31	476	C ₃₃ H ₆₄ O	tritriacontenone
32	478	C ₃₃ H ₆₆ O	tritriacontanone
33	504	C ₃₅ H ₆₈ O	pentatriacontenone

terms of concentration in both males and females. The other major peak shared in common by both sexes is cholesterol, which is ubiquitous in vertebrate tetrapod epidermis. A minor component shared by both sexes is squalene. Finally, a series of straight-chain hydrocarbons was noted in both sexes at approximately equal levels.

Some differences appear in the skin lipids of higher mass. For example, both sexes have several steroids that are analogs of cholesterol including cam-

pesterol and sitosterol, as well as the methyl ether of cholesterol. However, males also possess cholestanol, stigmaterol, and stigmastanol, which do not appear in the females' skin lipids. Females, on the other hand, possess a series of long-chain saturated and monounsaturated methyl ketones that are not found in the male chromatograms.

DISCUSSION

The results of this study demonstrate that the behavioral responses of male leopard geckoes toward females differ over the course of the female's shedding cycle. We propose that chemicals in the skin of females normally detected by males become unavailable while the female is shedding. Males responded to these shedding females in the same way that they responded to males. Males attacked other males whether those males were shedding or not. *Eublepharis macularius* does not exhibit obvious sexual dimorphism so perhaps visual cues alone would not be sufficient for males to determine gender. Unlike some geckoes, *Eublepharis* does not use vocalization to any great degree. It seems evident from these data that if a male cannot ascertain a conspecific's gender by means of chemical cues, he behaves toward that individual as if it were a male and attacks it.

These observations and hypotheses are supported by similar behaviors observed in other reptiles. For instance, skin-derived chemical cues sequestered in female garter snake skin elicit courtship behavior from males (Noble, 1937; Crews, 1976; Kubie et al., 1978; Garstka and Crews, 1981; Mason and Crews, 1985, 1986). Additionally, among zoo workers it is almost axiomatic, although not essential, that snakes breed best soon after the female has shed her skin (Radcliffe and Murphy, 1983). These authors state that the frequency with which courtship and copulation follow skin shedding in snakes strongly suggests that there is a sex pheromone associated with the newly shed snake and skin that acts as a releaser of courtship.

Perhaps the closest approximation to the behaviors observed in this study occur in the European adder, *Vipera berus* (Andren, 1982, 1986). The males in this species are aggressive and fight for access to unmated females. Vernal courtship behavior begins only when sexually active males shed their skin. In the adder, males actively tongue-flick chemical cues from shed females and initiate courtship behavior. When a male encounters another shed male, he begins combat behavior. Interestingly, in the adder, if a recently shed male encounters a nonshed male or female, he will ignore them. Andren (1982) concluded that male European adders must obtain chemical information from a conspecific's skin in order to initiate courtship or combat behavior.

Anecdotal observations suggest that diverse reptilian groups use sex pher-

omones, but few aspects of their chemistry are known. Only two studies have investigated the chemical constituents of skin lipids in any lizard, and these did not focus on intraspecific differences between the sexes. Roberts and Lillywhite (1980) isolated skin lipids from the green iguana (*Iguana iguana*) by thin-layer chromatography (TLC). They identified lipids in several classes, including fatty acids, sterol esters, cholesterol, wax esters, and others. The other study was an extensive survey of the shed skins of 23 species of lizards by TLC and demonstrated the great variability in the composition of skin lipids in lizards (Weldon and Bagnall, 1987). These authors conclude that more details are needed on the chemical structures of these lipids, many of which could not be determined by TLC techniques.

The present study identifies several lipids suggested in previous studies of saurian skin lipids, thus verifying their presence in lizard skin. Cholesterol is a common constituent in all vertebrate skin and seems to be present in all of the reptiles examined thus far. Fatty acids are also commonly found in the skin lipid profiles of vertebrates including reptiles (Ahern and Downing, 1973; Weldon and Bagnall, 1987; Mason et al., 1987). For example, fatty acids have been demonstrated to serve as pheromones in four species of tortoise (*Gopherus*). Rose (1970) identified several fatty acids in the mental glands of male tortoises. He applied either a control solution of solvents or a mixture of fatty acids to the head of a plaster tortoise model. Females responded by approaching the models and head-bobbing, a display indicating courtship. Males responded by ramming the models—clearly an aggressive response.

The presence of straight-chain hydrocarbons in the skin lipids of both male and female geckoes is an interesting finding. Hydrocarbons were identified in the indigo snake, *Drymarchon corais*, but were thought to be contaminants (Ahern and Downing, 1973). However, evidence of hydrocarbons was found in a TLC survey of skin lipids in lizards by Weldon and Bagnall (1987). These authors concluded that the hydrocarbons indicated in their study were not contaminants and that hydrocarbons may occur commonly among squamates. We agree that the hydrocarbons identified in the present study are indeed components of the skin lipid profiles of these lizards. The possible function of these integumental hydrocarbons warrants further study.

Other possible sources of semiochemicals in these reptiles include the sex differences found in the steroids and methyl ketones. Although the steroids identified here are not sex steroids (androgens or estrogens and progestins), they may still impart information about the sex of the individual. Finally, the methyl ketones found in the females' skin lipids are known to occur in female snakes. Ahern and Downing (1973) and Schell and Weldon (1985) identified methyl ketones in the skin lipids of a female indigo snake, *Drymarchon corais*, while Mason (1987) has identified them in the skin lipids of female garter snakes, *Thamnophis sirtalis parietalis*, where they serve as sex attractiveness pheromones (Mason, 1987).

Both Maderson (1986) and Graves et al. (1986) have suggested that reptilian skin lipids have been protoadapted or exapted to serve as semiochemicals or pheromones. It is now widely acknowledged that skin lipids originally evolved in reptiles to serve as a water-retention mechanism. Over the course of evolutionary time, males and females of a given species have acquired the ability to recognize and respond differentially to integumental chemical cues of conspecifics. It has been demonstrated in this study and a previous study (Mason et al., 1987) that clear sex differences exist in skin lipids, which would be a necessary condition for this mechanism of sex recognition to occur.

The results of this work and the previously cited studies pose some questions on the evolution of skin-derived pheromones in reptiles. In the case of sex recognition pheromones, do males of various species of reptile cue in on a set of specific components of the female's skin lipids such as fatty acids, steroids, or methyl ketones? If this is generally the case, as has been demonstrated in a few species, how could such a mechanism evolve?

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