

CHARACTERIZATION, SYNTHESIS, AND BEHAVIORAL
RESPONSES TO SEX ATTRACTIVENESS PHEROMONES
OF RED-SIDED GARTER SNAKES (*Thamnophis sirtalis*
parietalis)

ROBERT T. MASON,¹ TAPPEY H. JONES,¹ HENRY M. FALES,¹
LEWIS K. PANNELL,² and DAVID CREWS³

¹Laboratory of Chemistry
National Heart, Lung, and Blood Institute
Bethesda, Maryland 20892

²Laboratory of Bioorganic Chemistry
National Institute of Diabetes and Digestive and Kidney Diseases
Bethesda, Maryland 20892

³Institute of Reproductive Biology
Department of Zoology
University of Texas at Austin
Austin, Texas 78712

(Received October 31, 1989; accepted February 5, 1990)

Abstract—The sex attractiveness pheromone of the red-sided garter snake, *Thamnophis sirtalis parietalis*, has been characterized as a mixture of 13 long-chain (C₂₉–C₃₇) saturated and monounsaturated methyl ketones. Samples of the major unsaturated ketones in the mixture, (*Z*)-24-tritriaconten-2-one (**7**) and (*Z*)-26-pentatriaconten-2-one (**10**), and their saturated analogs, 2-tritriacontanone (**8**) and 2-pentatriacontanone (**11**) were prepared by unambiguous synthesis. In field bioassay tests, male garter snakes were presented with natural and synthetic pheromone components both individually and as a mixture. Males exhibited courtship behavior to the synthetic compounds approximating the natural blend.

Key Words—Pheromones, methyl ketones, red-sided garter snake, *Thamnophis sirtalis parietalis*, sexual behavior, bioassay, Reptilia.

INTRODUCTION

The study of chemical messages or semiochemicals in vertebrates is difficult because animals rarely rely on a single sensory input. For instance, in nature and even in the laboratory many mammals do not rely exclusively on olfaction for the initiation or maintenance of normal sex behavior (Singer et al., 1976). In most vertebrates the odors functioning in recognition of species, group, individual, or physiological state may consist of dozens of compounds (Duvall, 1986). More confounding is that positive responses in bioassays frequently disappear before fractionation procedures reach the level of single compounds (Müller-Schwarze et al., 1986).

The chemical communication system of garter snakes (*Thamnophis*) is an ideal model for the study of vertebrate semiochemicals. The garter snake possesses a relatively simple suite of semiochemicals, and the behaviors measured in the bioassay are robust, simple to quantify, and unequivocal in that they are only observed in a reproductive context in response to pheromonal cues. In addition, the sensory mechanisms involved in reception and perception of chemical cues in garter snakes are confined to one well-understood system. Noble (1937) purported to show that both the olfactory and the vomeronasal system mediate the reception and response to pheromones in the garter snake. He demonstrated that certain chemical manipulations of the pheromone on the female's back could abolish courtship by sexually active males. He also devised a number of ingenious experiments to determine which sensory modality the males were using to recognize and court females. Auditory, visual, and tactile cues were eliminated, leading him to conclude that the tongue delivers chemicals to the vomeronasal organ in the roof of the mouth. More recent work by Kubie *et al.* (1978) demonstrated unequivocally that the vomeronasal organ is the only means by which the skin-derived pheromone cues can be perceived.

Unlike the olfactory system, the vomeronasal system is extremely sensitive to nonvolatile chemical stimuli. Sheffield et al. (1968) demonstrated that newborn garter snakes detect with their vomeronasal organs a component of earthworm washes of molecular weight greater than 5000 daltons. More recently, Wang et al. (1988) isolated an approximately 20,000-dalton protein from aqueous earthworm extracts which is a chemoattractant for garter snakes. Recent investigations by Burghardt et al. (1988) also have isolated components in earthworms and minnows that elicit feeding behavior from garter snakes. Chloroform-methanol extracts of earthworms yielded a high-molecular-weight (432,000) component as well as a previously unknown low-molecular-weight component between 1350 and 1800 daltons.

The red-sided garter snake (*Thamnophis sirtalis parietalis*) is the most northerly living reptile in North America (Logier and Toner, 1961). As such, it is constrained to hibernate up to eight months of the year in underground dens

or hibernacula. In Manitoba, Canada, males emerge in late April and several thousand aggregate at the den entrance. Over the next three to four weeks, females emerge singly or in small groups. Males search for sexually attractive females and are initially attracted to movement (Joy and Crews, 1988), but they must actually come into contact with a female before courtship begins.

Upon encountering an unmated female, the male presses his chin onto her dorsal skin and emits very short, rapid tongue flicks. At the same time he traverses up and down the female, turning back at her head and tail, coming to rest with his head resting behind hers. Once stationary, the male's body undulates in caudocephalic waves. Both chin-rubbing and caudocephalic waves are observed only during mating and are not associated with any other behaviors. Many males will court a female simultaneously, forming a "mating ball" of 10–100 males. However, only one male will mate with the female while the others disperse to seek other unmated females.

Similar studies by Andr n and his colleagues have shown that in other snakes reproductive behaviors also are elicited in response to skin-derived pheromones (Andr n, 1982, 1986). Courtship in the European adder, *Vipera berus*, occurs when males begin chin-rubbing and tongue-flicking the dorsal surfaces of females. He has demonstrated that pheromone cues are gathered by direct contact with the tongue and are sensed by means of the vomeronasal organ.

It is important to note that the sex attractiveness pheromone produced by female garter snakes serves to advertise the female's unmated status. It does not imply attraction of males from a distance in that the pheromone is known to be a contact pheromone. Many snakes are known to trail conspecifics and garter snakes are especially adept at following trails. Indeed, male garter snakes will preferentially trail females during the breeding season, presumably basing this behavior on the ability to follow cuticular cues left on the substrate (Noble, 1937; Ford, 1978, 1981). Whether these trailing pheromones are the same as the sex attractiveness pheromone remains to be seen.

In a previous report (Mason et al., 1989), we isolated a series of long-chain methyl ketones that serve as the sex attractiveness pheromones in this species. In addition, we described the initial characterization and synthesis of two saturated methyl ketones. In the current study, we present the complete characterization of the natural material as well as the synthesis and field testing of a synthetic mixture of both saturated and unsaturated methyl ketones.

METHODS AND MATERIALS

Chemical Analyses. Gas chromatographic analyses were performed on a Shimadzu GC-12A equipped with a 30-m \times 0.53-mm-ID column with a bonded DB-17 phase. [^1H]- and [^{13}C]NMR spectra were obtained with a Varian XL-

200 spectrometer from CDCl_3 solutions. Mass spectra were obtained using an LKB-9000 GC-MS fitted with a 30-m \times 0.53-mm-ID column with a bonded DB-17 phase, or a Finnigan model 4500 GC-MS fitted with a 30-m \times 0.32-mm-ID column with a bonded DB-1 phase. High-resolution mass spectra were obtained using a VG-7070F in the EI mode at an ionizing voltage of 70 eV. All boiling points and melting points are uncorrected.

Derivatization. A small sample (ca. 10 mg) of the natural methyl ketone fraction (Mason et al., 1989) was treated with dimethyldisulfide and a catalytic amount of iodine and after 12 hr, worked up in the usual manner (Buser et al., 1983). The resulting mixture contained bithiomethyl derivatives of the unsaturated ketones, **4**, **7**, **9**, **10**, **12**, and **13**, whose mass spectra had characteristic ions at $m/z = 58$ and 61 , as well as the following pairs of intense ions: **4** + $(\text{CH}_3\text{S})_2$: $m/z = 173, 369$; **7** + $(\text{CH}_3\text{S})_2$: $m/z = 173, 397$; **9** + $(\text{CH}_3\text{S})_2$: $m/z = 173, 411$; **10** + $(\text{CH}_2\text{S})_2$: $m/z = 173, 425$; **12** + $(\text{CH}_2\text{S})_2$: $m/z = 173, 439$; **13** + $(\text{CH}_2\text{S})_2$: $m/z = 173, 453$.

This mixture was taken up in pyridine (0.1 ml), treated with 10 mg of *O*-methylhydroxylamine hydrochloride, and heated to 100°C for 15 min. Analysis of the resulting mixture by GC-MS (oven programmed 180 – 320°C) revealed the presence of three late-eluting components in a 1:12:15 ratio with mass spectra indicative of the bithiomethyl-*O*-methoxime derivatives of **4**, **7**, and **10**, respectively: MS m/z (relative intensity) peak 1: 571(0.5, M+), 398(25), 174(10), 173(60), 100(25), 97(55), 95(30), 87(100), 83(60), 81(35), 71(30), 70(30), 69(65), 61(30), 57(90), 55(70), 43(65), 41(40). Peak 2: 599(10, M+), 552(7), 427(20), 426(61), 173(100), 172(27), 100(20), 97(36), 95(33), 87(96), 83(70), 81(36), 71(25), 70(30), 69(80), 61(43), 57(71), 55(85), 43(59), 41(35). Peak 3: 627(5, M+), 580(3), 455(7), 454(45), 173(100), 172(20), 100(15), 97(35), 95(30), 87(75), 83(65), 81(25), 70(25), 71(30), 69(80), 61(45), 57(75), 55(70), 43(45), 41(30).

Synthesis of 2-(13-Hydroxytridecyl)-2-methyldioxolane (14, N = 12). Ethyl acetoacetate (30 ml, 0.23 mol) was added to a rapidly stirred ethanolic solution of sodium ethoxide made of dissolving 5.0 g of sodium in 200 ml of absolute ethanol. After 0.5 hr, a solution containing 58.3 g (0.22 mol) of 12-bromodecanol (Kang et al., 1985) in 50 ml of ethanol was added dropwise, and the mixture was heated to reflux under anhydrous conditions overnight. The mixture was cooled, neutralized with a few drops of glacial acetic acid, and the solvent was removed under reduced pressure. The residue was taken up in ether, washed with water, and after removal of the solvent, the residue from the ether solution was stirred overnight with 150 ml of aqueous 10% NaOH. This mixture was carefully acidified with 50% H_2SO_4 and heated in a warm water bath until gas evolution ceased. Upon cooling, the mixture was extracted with ether, and after removal of the solvent, the residue from the ether solution was taken up in benzene, combined with 20 ml of ethylene glycol and 0.2 g of *p*-toluene-

sulfonic acid, and heated to reflux under a Dean-Stark trap for 4 hr until the rapid evolution of water ceased. The mixture was cooled, washed with saturated NaHCO_3 , dried over anhydrous MgSO_4 , filtered and distilled. Kugelrohr distillation provided 28.45 g (49% yield) of hydroxyketal **14** ($N = 12$), bp 160–170°C (0.35 mm Hg); [^1H]NMR $\delta = 3.9(4\text{H}, \text{m}), 3.6(2\text{H}, \text{t}, J = 6\text{Hz}), 2.1(1\text{H}, \text{br s}), 1.65\text{--}1.45(4\text{H}, \text{m}), 1.28(3\text{H}, \text{s}), 1.25(2\text{OH}, \text{m})$; [^{13}C]NMR $\delta = 110.22, 64.53(2\text{C}), 62.96, 39.19, 32.74, 29.83\text{--}29.13(8\text{C}), 25.70, 24.05, 23.85$; MS m/z (relative intensity) 271(9, $\text{M}^+ - 15$), 99(5), 88(5), 87(100), 69(5), 55(15), 43(30), 41(10); HRMS m/z 271.2276 ($\text{C}_{16}\text{H}_{31}\text{O}_3$, $\text{M}^+ - 15$, calcd. 271.2273).

Synthesis of (Z)-24-Tritriaconten-2-one (7). A well-stirred solution containing 2.0 g (7 mmol) of hydroxyketal **14** and 1 ml of pyridine in 10 ml of chloroform was cooled to 0°C and treated with 0.8 g of methanesulfonyl chloride. The resulting mixture was stirred for 1 hr at 0°C, and then treated with 30 ml of ether and 30 ml of saturated NaHCO_3 solution. The ether solution was separated, dried over anhydrous MgSO_4 , filtered, and the solvent removed in vacuo to 0.1 mm Hg. The fluffy white residue was taken up in anhydrous THF, filtered, and the solvent was removed in vacuo to 0.1 mm Hg to provide the unstable methanesulfonate ester **15** ($N = 12$); MS m/z (relative intensity) 349(0.5, $\text{M}^+ - 15$), 253(5), 99(8), 88(9), 87(100), 55(15), 43(20), 41(10). This material was taken up in 20 ml of anhydrous THF.

A solution of oleylmagnesium bromide, decanted from the reaction of 3 g of oleyl bromide with 1.0 g of magnesium in 50 ml of anhydrous THF, was cooled to -78°C under a nitrogen atmosphere and treated with the above THF solution of **15** ($N = 12$). After the addition of 4 ml of a freshly prepared 0.2 M solution of Li_2CuCl_4 in THF, the resulting blue–purple mixture was maintained at 0°C overnight. Following the addition of 3 ml of water, the mixture was stirred for 0.5 hr and the organic layer was decanted from the solids, cooled in an ice bath, and treated with 10 ml of water and 3 ml of 70% HClO_4 . After 3 hr, the mixture was carefully neutralized with solid NaHCO_3 , extracted with ether, and the ether solution was dried over anhydrous MgSO_4 . After filtration, the solvent was removed in vacuo and the residue was taken up in 20 ml of hot hexane, allowed to cool, and filtered again. The solvent was removed from the resulting mixture, and the residue was purified by chromatography over 200 g of activity III alumina (50:1 hexane–ether) to provide 1.32 g (40% yield) of **7**: mp 52–53°C; [^1H]NMR $\delta = 5.35(2\text{H}, \text{br t}), 2.41(2\text{H}, \text{t}, J = 7\text{Hz}), 2.17(3\text{H}, \text{s}), 2.04(4\text{H}, \text{m}), 1.65\text{--}1.2(50\text{H}, \text{m}), 0.88(3\text{H}, \text{br t})$; [^{13}C]NMR $\delta = 209.48, 130.13(2\text{C}), 44.06, 32.14, 30.01\text{--}29.55(22\text{C}), 27.45, 24.13, 22.91, 14.32$; MS m/z (relative intensity) 476(6, M^+), 461(1), 459(3), 458(12), 418(2), 125(25), 111(20), 109(18), 97(40), 96(33), 95(28), 85(11), 83(50), 82(30), 81(27), 71(60), 69(55), 59(35), 58(43), 57(44), 55(70), 43(100), 41(25); HRMS m/z 476.4944 ($\text{C}_{33}\text{H}_{64}\text{O}$, M^+ calcd. 476.4957).

Synthesis of 2-Tritriacontanone (8). A solution containing 0.5 g (1.05

mmol) of **7** in 50 ml of hexane was hydrogenated over 47 mg of 5% palladium on carbon at 3 atm of pressure for 1 hr. Filtration of the resulting mixture through a short pad of Florisil and removal of the solvent in vacuo provide 0.47 g (94% yield) of ketone **8**: mp 79–80°C; MS m/z (relative intensity) 478(20, M^+), 463(4), 460(6), 420(1), 97(22), 96(18), 95(12), 85(27), 83(25), 82(20), 81(11), 71(65), 69(28), 59(100), 58(70), 57(55), 55(40), 43(95), 41(28); HRMS m/z 478.5099 ($C_{33}H_{66}O$, M^+ calcd. 478.5113).

Synthesis of 2-(15-Hydroxypentadecyl)-2-methyldioxolane 16 (N = 14). 14-Bromotetradecanol, 18.8 g (64 mmol) was carried through the steps described above for **14** (N = 12), to provide, after Kugelrohr distillation 8.6 g (43% yield) of **16**: bp 210–220°C (0.1 mm Hg); [1H]NMR δ = 3.92(4H, m), 3.62(2H, t, J = 7 Hz), 1.9(1H, br s), 1.7–1.5(4H, m), 1.3(3H, s), 1.26(24H, m); [^{13}C]NMR δ = 110.16, 64.49(2C), 62.82, 39.16, 32.74, 29.53(10C), 25.71, 24.02, 23.61; MS m/z (relative intensity) 299(9, $M^+ - 15$), 281(1), 99(3), 88(5), 87(100), 55(10), 43(20), 41(7); HRMS m/z 299.2581 ($C_{18}H_{35}O_3$, $M^+ - 15$ calcd. 299.2586).

Synthesis of (Z)-26-Pentatriaconten-2-one (10). The hydroxy ketal **16**, 2.1 g (6.7 mmol), was converted to its unstable methanesulfonate ester **17** (N = 14) in the manner described above: MS m/z (relative intensity) 377(4, $M^+ - 15$), 281(5), 99(3), 88(6), 87(100), 55(10), 43(18), 41(7). In the manner described above, this material was allowed to react with an excess of oleylmagnesium bromide in the presence of lithium tetrachlorocuprate to provide, after chromatography over activity III alumina, 1.26 g (39% yield) of **10**: mp 56–57°C; [1H]NMR δ = 5.45(2H, br t), 2.43(2H, t, J = 7 Hz), 2.17(3H, s), 2.04(4H, m), 1.65–1.2(54H, m), 0.92(3H, br t); [^{13}C]NMR δ = 209.49, 130.14(2C), 44.07, 32.15, 30.36–29.65(24C), 27.45, 24.14, 22.91, 14.32; MS m/z (relative intensity) 504(10, M^+), 489(2), 486(18), 446(3), 125(25), 111(2), 109(18), 97(44), 96(35), 95(30), 83(55), 82(35), 81(27), 71(60), 69(55), 67(25), 59(38), 58(45), 57(50), 55(70), 43(100), 41(25); HRMS m/z 504.5275 ($C_{35}H_{68}O$, M^+ , calcd. 504.5270).

Synthesis of 2-Pentatriacontanone (11). In the manner described above, a hexane solution of **10** was hydrogenated to provide 0.46 g (92% yield) of **11**: mp 80–82°C; MS m/z (relative intensity) 506(10, M^+), 491(1), 488(5), 448(1), 97(3), 96(20), 95(25), 85(33), 83(40), 82(25), 81(28), 71(95), 69(50), 59(75), 58(65), 57(70), 55(62), 43(100), 41(45); HRMS m/z 506.5428 ($C_{35}H_{70}O$, M^+ calcd. 506.5427).

Synthetic Mixtures for Bioassay. The average snake from these populations is 60 cm long and 3 cm in diameter (Gregory, 1974), thus the average dorsal surface area is 282 cm². Chromatography over Al₂O₃ of hexane extracted lipids from 18 snakes provided 117 mg of ketones or 6.5 mg/snake. Analysis by GC-MS revealed that 88.4% of the sample consists of compounds **2**, **5**, **7**, **8**, **10**, **11**, in a ratio of 4:7.5:6:6:5:1. Thus, one female snake has 0.67 mg **2**, 1.25

mg **5**, 1.0 mg **8**, 0.8 mg **7**, 1.0 mg **10**, and 0.16 mg **11**. Based on the synthetic material available, a mixture consisting of 70 mg **2**, 40 mg **5**, 100 mg **8**, 80 mg **7**, 100 mg **10**, and 20 mg **11** [100 female equivalents (FE)] in hexane was utilized as a stock solution for the bioassays. The major difference from the natural mixture is the smaller amount of the major saturated component (**5**) in the synthetic mixture due to the lack of synthetic **5** available, and the necessity of having a sufficient quantity of the mixtures for the bioassays.

A mixture approximating the remaining skin lipids was made up of 41% oleic acid, 16% palmitic acid, 20% cholesterol, and 20% tripalmitin in hexane. Using 38 mg as the average amount of total lipids per snake (Mason et al., 1987), 31.5 mg of this mixture is 1 FE of remaining skin lipids. This mixture, the mixture of ketones, and the individual ketones **7**, **8**, and **10**, were dissolved in hexane and diluted in terms of female equivalents as shown in Tables 2 and 3 below.

Field Bioassay. All behavioral tests were conducted in the field at Narcisse Community Pasture, Manitoba, Canada, during the spring emergence of the garter snakes in May 1989. Testing of individual saturated and unsaturated ketones **7**, **8**, and **10** was conducted with the same bioassay procedure used in previous studies (Mason and Crews, 1985; Mason et al., 1989) to facilitate direct comparisons of data. In these tests, varying concentrations (0.01–10 FE/ml) of individual synthetic methyl ketones in hexane were applied to an area 16 × 16 cm on paper towels. After applying the solutions to the paper towels, the paper towels were placed in the den where courting males had ready access to them. Untreated and hexane-soaked paper towels were used as controls. Males responding with tongue-flicking and chin-rubbing for 10 sec or more were scored as positive responders. Three replicate tests were conducted for each compound.

In order to test for possible synergistic effects of ketones working as a mixture as found naturally, the synthetic ketones available were tested together in proportion to their relative abundance in the snake skin. All ketones were tested both alone and with the lipid.

As for the individual ketones above, the synthetic mixture of ketones was tested at varying concentrations (0.001–1 FE/ml). Three behaviors were monitored and scored in response to the synthetic pheromone mix. Duration of courtship was the number of seconds courting males exhibited courtship behavior in response to the test compound. Total tongue-flicks elicited by a given mixture were counted for the duration of a 1-min test. Finally, the tongue-flick rate was recorded as tongue-flicks per minute. For each concentration of the synthetic pheromone mix a total of 10 different males were observed.

Statistics. Physical parameters of male courtship response to each experimental group were compared by one-way analysis of variance ANOVA (SAS Institute Inc., 1985). Responses to ketone mixes both with and without lipids

were analyzed by two-way ANOVA with concentration of ketones and presence or absence of lipid as factors. Unplanned comparisons between means were identified by Duncan's multiple-range test. A simple linear regression analysis was conducted on the courtship data to determine the predictability of courtship versus concentration of the synthetic pheromone mix. Courtship responses were normalized by log transformation before statistical analysis. Alpha was set at 0.05 for all statistical tests.

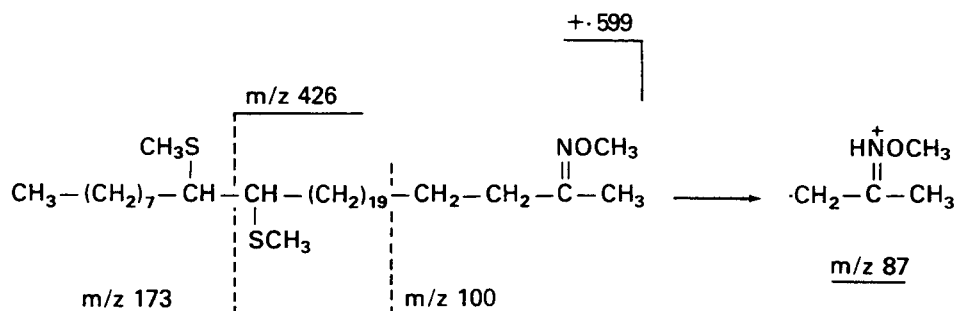
RESULTS

The skin lipids of female *Thamnophis sirtalis parietalis* were extracted and fractionated as previously described (Mason et al., 1989). Initial spectroscopic examination of the bioactive fraction revealed the presence of methyl ketones (IR 1712 cm^{-1} ; [^1H]NMR, $\delta = 2.10$, singlet, CH_3CO and 2.38, triplet, CH_2COCH_3) along with unconjugated *Z* double bonds (IR 718 cm^{-1} ; [^1H]NMR $\delta = 5.32$) (Mason et al., 1989).

Complete GC-MS analysis of the ketone fraction revealed the presence of 13 methyl ketones (**1-13**, Table 1). Seven of these [2-heptacosanone (**1**), 2-nonacosanone (**2**), 2-triacontanone (**3**), 2-hentriacontanone (**5**), 2-dotriacontanone (**6**), 2-tritriacontanone (**8**), 2-pentatriacontanone (**11**)] had mass spectra in accord with saturated methyl ketones, showing a molecular ion, and losses of 15, 18, and 60 as well as an intense ion at $m/z = 58$ and a base peak at $m/z = 59$ (Sharkey et al, 1956). The molecular ions and fragmentation patterns of the remaining ketones indicated monounsaturated methyl ketones. To determine the double-bond position, the mixture was subjected to bithiomethylation (Buser et al., 1983). The mass spectra of the derivatives showed two abundant ions resulting from cleavage between vicinal thiomethyl groups. One of these ions in each derivative, $m/z = 173$, indicated the double bond to be the same distance ($\omega-9$) from the end of the molecule distal to the ketone. To establish the relationship between the carbonyl and olefinic functions, the derivatized mixture was treated with *O*-methylhydroxylamine hydrochloride. Subsequent GC-MS analysis provided readily discernible mass spectra for the bithiomethyl-*O*-methyloxime derivatives of **4**, **7**, and **10**. Figure 1 depicts the mass spectral fragmentation pattern of the bithiomethyl methoxime derivative of **7**. Fragments at $m/z = 87$ and 100 are the McLafferty rearrangement and α -cleavage ions characteristic of methyl ketone *O*-methyloximes (Budzikiewicz et al., 1967), and the ions at $m/z = 426$ and 173 result from cleavage between the vicinal thiomethyl groups. Since the nitrogen of the methoxime is incorporated in the larger, even-mass fragment ion, **7** is (*Z*)-24-tritriaconten-2-one. The unchanged ion at $m/z = 173$ common to all the unsaturated ketone dimethyl disulfide derivatives permits the assignment of **4** as (*Z*)-22-hentriaconten-2-one,

TABLE 1. GC-MS ANALYSIS OF METHYL KETONES FROM FEMALE *T. sirtalis parietalis*

Compound	R _f (min)	Relative %	MS: m/z (relative intensity) Characteristic Ions
1	16:29	1.24	394(25, M+), 379(6), 376(14), 334(15), 109(5), 97(11), 96(10), 85(10), 71(45), 59(100), 58(60), 57(30)
2	17:49	11.4	422(55, M+), 407(13), 404(20), 362(11), 96(30), 85(25), 71(60), 59(100), 58(80), 57(45), 55(30)
3	18:45	0.74	436(10, M+), 421(3), 418(4), 376(2), 96(23), 85(23), 71(50), 59(100), 58(75), 57(40), 55(27)
4	18:55	1.24	448(6, M+), 433(2), 430(12), 125(2), 111(30), 109(45), 97(63), 96(56), 83(78), 71(80), 69(77), 59(60), 58(60), 57(73), 55(100)
5	19:08	22.8	450(8, M+), 435(3), 432(3), 390(5), 96(15), 85(11), 83(15), 71(40), 59(100), 58(75), 57(45), 55(30)
6	19:45	1.48	464(20, M+), 449(9), 446(11), 85(50), 71(55), 59(65), 58(100), 57(50), 55(55)
7	20:22	14.4	476(7, M+), 458(16), 418(5), 125(23), 123(17), 111(24), 109(35), 97(67), 96(55), 95(57), 83(84), 71(70), 69(90), 59(60), 58(70), 57(65), 55(100)
8	20:32	17.9	478(35, M+), 463(11), 460(25), 418(30), 96(11), 83(11), 71(40), 59(100), 58(54), 57(25), 55(15)
9	21:03	2.72	490(5, M+), 475(6), 472(12), 432(4), 125(16), 123(17), 109(41), 97(75), 83(94), 69(95), 59(62), 58(74), 57(75), 55(100)
10	22:00	18.9	504(8, M+), 489(3), 486(17), 446(5), 125(24), 123(18), 111(28), 109(30), 97(67), 96(55), 95(50), 83(86), 71(70), 69(86), 59(57), 58(64), 57(68), 55(100)
11	22:04	2.48	506(5, M+), 488(7), 446(3), 109(20), 97(35), 83(57), 71(57), 59(100), 58(68), 57(65), 55(58)
12	22:32	1.48	518(7, M+), 500(9), 458(3), 125(17), 109(32), 97(66), 83(77), 69(80), 59(63), 58(64), 57(75), 55(100)
13	23:26	3.22	532(6, M+), 514(13), 474(4), 125(22), 109(30), 97(70), 83(96), 69(92), 59(68), 58(64), 57(70), 55(100)

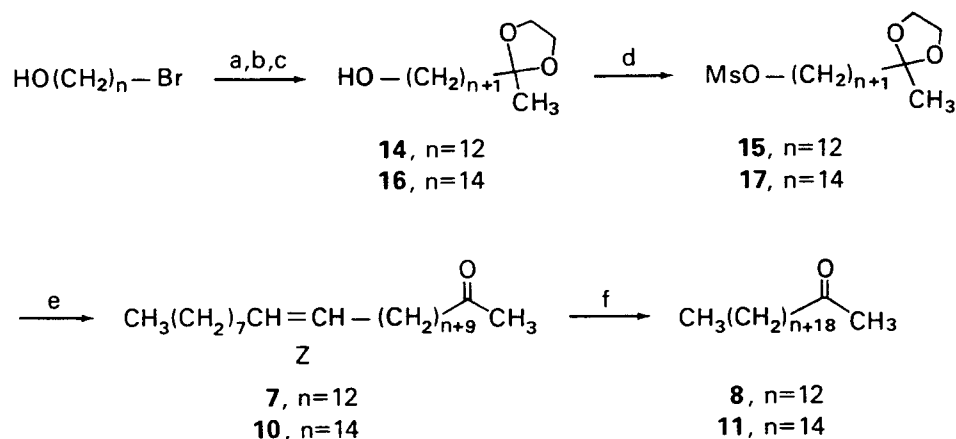
FIG. 1. Suggested interpretation of the MS of the bistiomethyl-*O*-methyloxime derivative of 7.

9 as (*Z*)-25-tetratriaconten-2-one, **10** as (*Z*)-26-pentatriaconten-2-one, **12** as (*Z*)-27-hexatriaconten-2-one, and **13** as (*Z*)-28-heptatriaconten-2-one.

Since **7** and **10** are the major unsaturated ketones in the mixture, their synthesis was undertaken to provide them as well as their saturated analogs **8** and **11** for subsequent bioassays.

The preparations of **7** and **10** were based on the copper-catalyzed coupling of the Grignard reagent prepared from oleyl bromide (Kocienski et al., 1977) with the appropriate methanesulfonate (Scheme 1). Ethyl acetoacetate was condensed with 12-bromododecanol (Kang et al., 1985), to provide, after routine saponification, decarboxylation, and ketalization, the hydroxy ketal **14** in moderate yield. The unstable methanesulfonate ester **15** was prepared in the usual manner and carefully dried. The condensation of this methanesulfonate with oleylmagnesium bromide in the presence of lithium tetrachlorocuprate (Fouquet and Schlosser, 1974) furnished the unsaturated ketone **7**, which could be isolated in gram quantities by simple chromatography over activity III alumina. In the same way, **10** was prepared from 14-bromotetradecanol (Valicenti et al., 1985). Samples of the saturated ketones **8** and **11** were obtained by hydrogenation of **7** and **10**, respectively, over a 5% Pd/C catalyst.

Behavior. Behavioral responses of sexually active male garter snakes to individual synthetic methyl ketones **7**, **8**, and **10** are shown in Table 2. It appears that males will respond to unsaturated methyl ketones at lower concentrations than required to elicit courtship from individual saturated ketones. These results follow the general trend demonstrated in an earlier study with isolated natural saturated and unsaturated methyl ketones (Mason et al., 1989). Males did not respond to the ketones at all at concentrations less than 1 FE, indicating that the concentrations of ketones on females are near the threshold of the males' ability to detect them. Interestingly, partially dried hexane controls, as com-



SCHEME 1. (a) $\text{CH}_3\text{COCH}_2\text{CO}_2\text{Et}$, EtONa; (b) OH^- , H^+ ; (c) $(\text{CH}_2\text{OH})_2$, ptsa; (d) MsCl, Py; (e) oleylMgBr, Li_2CuCl_4 ; (f) Pd/C, H_2 .

TABLE 2. BEHAVIORAL RESPONSES TO INDIVIDUAL SYNTHETIC METHYL KETONES^a

	Solvent blank	Concentration (FE/ml)				
		0.01	0.1	1	2	10
C ₃₃	0 ^b	0	0	0	1.3 ± 0.88	3.67 ± 0.33
C _{33:1}	0	0	0	0.33 ± 0.33	3 ± 1.15	2 ± 0
C _{35:1}	0	0	0	1.33 ± 0.88	3.3 ± 1.76	2.67 ± 1.2

^aTests conducted at Narcisse Community Pasture, Manitoba, Canada, May 10–12, 1989. Results of triplicate test. Testing was monitored for 1 hr/day. Those males that responded with increased tongue-flicking and chin rubbing for 10 sec or more were given a positive score.

^bNumber of males responding with courtship (mean ± SE).

pared to untreated paper towels, proved very aversive to males. Thus, not only did males respond positively to the ketones, but they also overcame a strong aversion to the hexane in which the ketones were dissolved. Males never demonstrated any interest in the lipid controls.

Results of courtship tests with the synthetic ketone mixture alone were compared to the same mixture including the supplemental lipids. A two-way ANOVA for the duration of courtship data indicated that ketones plus lipids elicited significantly more courtship than ketones alone [$F(5,94) = 6.25, P < 0.001$]. Similarly, the total number of tongue-flicks elicited also differed significantly [$F(5,94) = 27.40, P < 0.001$]. Thus, we conclude that the additional lipids may be serving as a matrix that makes the long-chain methyl ketones more readily available for males. Because these ketones are a contact pheromone, the lipids may be facilitating the physical transfer of chemical cues from the female's back to the male's vomeronasal organ.

The responses of males to the synthetic ketone mixture plus lipids are listed in Table 3. In each case, the one-way ANOVAs for duration of courtship, total tongue-flicks, and tongue-flick rate were significantly different between concentrations. However, these ANOVAs included the two controls of solvent and lipids, which never elicited any courtship. Likewise, the natural material was almost always 5–10 times greater in level of response. Thus, an ANOVA was calculated on just the five different concentrations of synthetic mixtures. The duration of courtship was found to be significantly different between concentrations [$F(4,45) = 12.12, P < 0.001$] while total tongue-flicks were also significantly different [$F(4,45) = 11.47, P < 0.001$]. However, when the natural material and controls are factored out of the tongue-flick frequency data, there are no significant differences between the five concentrations [$F(4,45) = 2.46, P < 0.05$]. Thus, it appears that although males will respond differentially with tongue-flicks and duration of courtship response, they do not alter their tongue-flick rate as the concentration of material changes.

TABLE 3. SCORES (DURATION, NUMBER, OR FREQUENCY) OF MALE COURTSHIP RESPONSES TO SYNTHETIC PHEROMONE MIX AND CONTROLS^a

	Hexane	Lipids	Natural (1 FE)	FE/ml (mean \pm SE, N = 10)					ANOVA
				1	0.5	0.1	0.01	0.001	
Duration of courtship (sec)	0a	0a	110.6 \pm 16.4b	20.1 \pm 3.84c	8.7 \pm 1.3c	6.2 \pm 1.02c	4.9 \pm 0.48c	3.8 \pm 0.53c	F(7, 72) = 39.06, P < 0.001
Total tongue-flicks elicited	0a	0a	136.8 \pm 4.65b	27.8 \pm 5.97c	12.6 \pm 1.45c, d	8.5 \pm 1.37d	6.1 \pm 0.89d	3.3 \pm 0.4d	F(7, 72) = 267.9, P < 0.001
Tongue-flick frequency (tongue-flicks/min)	0a	0a	142.1 \pm 2.52b	107.8 \pm 11.51b, c	83.4 \pm 9.53c	75.9 \pm 10.24c	70.5 \pm 9.22c	70.4 \pm 8.91	F(7, 72) = 38.1, P < 0.001

^aLetters common in a row denote mean values that do not differ significantly (Duncan's multiple-range test).

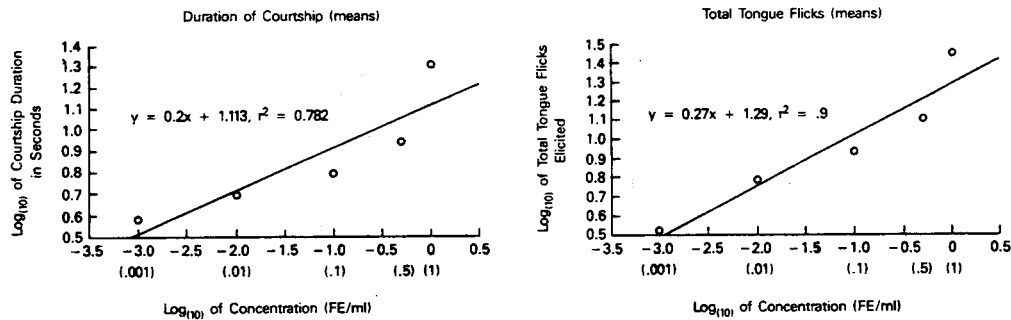


FIG. 2 Responses of courting male *Thamnophis sirtalis parietalis* towards five different concentrations of synthetic pheromone mix. Values represent the duration of courtship in seconds (left panel) and the total number of tongue-flicks elicited towards the synthetic mix (right panel). Each datum represents the mean of 10 observations.

Finally, we plotted the five concentrations of ketones plus lipid mixture as the log of concentration versus the log of response for the duration of courtship data and total tongue-flicks elicited and performed a simple linear regression analysis. The results are shown in Figure 2. The straight line fitted to the data in both cases was significant accounting for 78% of the variance in the former and 90% of the variance in the latter.

DISCUSSION

Beginning with studies by Noble and his colleagues in the 1930s, there are nearly 50 years of data documenting, in squamate reptiles, specialized behaviors of touching the conspecifics' skin with the tongue and then placing the tongue tip close to, if not in, the buccal opening of the Jacobson's or vomeronasal organ (Maderson, 1986). Graves et al. (1986) and Maderson (1986) have proposed that the integument of all extant amniotes, and probably that of their ancestors, is or was to varying degrees an uncontrolled semiochemical source.

All higher vertebrates possess corneous tissues that contain intra- and extracellular lipids that affect the waterproofing of the integument. These lipids have been shown to impede transcutaneous water loss in several species of squamate reptiles (Roberts and Lillywhite, 1980, 1983; Lillywhite and Maderson, 1982; Baeyens and Rountree, 1983; Burken et al., 1985a). Indeed, many investigators have identified lipids in the skin secretions of snakes (Jackson and Sharaway, 1978; Tsumita et al., 1979; Birkby et al., 1982; Burken et al., 1985b; Mason et al., 1987). Principle among these are studies by Ahern and Downing (1974) and Schell and Weldon (1985), who first identified long-chain methyl ketones in the skin of snakes.

There is now a growing body of evidence that strongly suggests that the lipid molecules within or beneath epidermal tissues "seep" through to the skin

surface (Garstka and Crews, 1981; Garstka et al., 1982; Maderson, 1986). In birds, lipids are excreted from epidermal cells undergoing keratinization (Menon et al., 1981). These lipids then appear to be extruded to the skin surface through pores (Purton, 1986). Similar pores have been described on the dorsal surface of snakes (Stille, 1987; Chiasson and Lowe, 1989; Chiasson et al., 1989). Chiasson et al. (1989) suggest that these pores in reptiles serve as the surface opening by which lipids produced in the epidermis reach the skin surface. In this model, the lipid semiochemicals would be derived from those lipids that serve as the integumental water barrier. Thus, the integumental lipids would have been protoadapted (Gans, 1979) or exapted (Gould and Vrba, 1982) to serve an additional role, that of semiochemical communication.

Although females may engage in deep breathing during courtship, which may aid in expressing the skin pheromone, the "passive" secretion of semiochemicals in snakes is supported by numerous field observations of courtship in garter snakes. The response to the female attractiveness pheromone by courting males is very robust. Males are not uncommonly observed courting and even mating with females that have been dead for several days (Aleksiuk and Gregory, 1974; Garstka et al., 1982). Thus, the female does not need to actively secrete the attractiveness pheromone, and it seems to remain intact on her skin surface unaltered for several days. These observations demonstrate not only the powerful and long-term effect of these skin lipids as pheromones, but also point out the fact that, in garter snakes, the female's behavior is not essential for eliciting male sexual behavior.

The fact that skin surface lipids are serving as semiochemicals merits comment. Both the number of different products and their relatively high molecular weight may, after cursory examination, argue against their usefulness as species-specific semiochemicals. In the early years of insect semiochemical research, a pheromone was usually reported as a single compound. The concept of "one insect-one specific compound" became strongly implanted among researchers in the developing pheromone field (Hadley, 1985). Field trials on these single compounds often proved to elicit a weaker response than a multicomponent mixture. Moreover, the purer the compound, the more disappointing the results (Silverstein, 1977). Our results show a similar trend.

The first definitive demonstration of a multicomponent attractant pheromone was provided by Silverstein et al. (1966), who showed that the pheromone produced by the male bark beetle, *Ips paraconfusus*, which attracts both males and females, was a mixture of three terpene alcohols, none of which was particularly active by itself in the field. Subsequent studies in widely ranging taxa have shown that, although there are pheromones that consist of a single active compound, multicomponent systems or pheromone blends predominate. Indeed, most vertebrate odors functioning in recognition of species, group, individual, or physiological state consist of dozens of compounds (Duvall, 1986).

Another idea that has become entrenched over the years is that all pheromones must be somewhat volatile. Bossert and Wilson (1963) proposed a set

of theoretical constraints on the chemical properties of pheromones. At this time, virtually all pheromones known were those of insects. They predicted that the majority of pheromones should contain between 5 and 20 carbons and have molecular weights between 80 and 300. The major constraints in Bossert and Wilson's model were the volatility and diffusability of molecules. However, these predictions pertaining to the expected size of pheromone molecules were made at a time when the number of these compounds and the knowledge of their chemistry was quite limited. The subsequent discovery in phylogenetically diverse organisms of numerous semiochemicals that are waterborne, gustatory, or cutaneous in nature has laid to rest the idea held for so long that pheromones are highly volatile, low-molecular-weight compounds (Hadley, 1985).

General Conclusions. We have demonstrated that a series of synthetic saturated and monounsaturated methyl ketones elicit courtship behavior from male red-sided garter snakes. As in an earlier study (Mason et al., 1989), we find that the natural methyl ketones from the female are more attractive to males. In part this is due probably to the fact that only the major components of the methyl ketone series were tested.

Acknowledgments—We thank Dr. R. J. Highet of the Laboratory of Chemistry, NHLBI, for the NMR spectra and Noel Whittaker of the Laboratory of Bioorganic Chemistry, NIDDK, for the high-resolution mass spectra. We gratefully acknowledge Bill Koonz and Merlin Shoemith of the Wildlife Branch, Manitoba Department of Natural Resources for permits and their many efforts on our behalf through the course of these studies. We also thank Joan Whittier and Mary Mendonca for helpful discussions. We acknowledge the Texas Higher Education Coordinating Board Advanced Research Program Grant 2DC.

REFERENCES

- AHERN, D.G., and DOWNING, D.T. 1974. Skin lipids of the Florida indigo snake. *Lipids* 9:8-14.
- ALEKSIUK, M., and GREGORY, P. 1974. Regulation of seasonal mating behavior in *Thamnophis sirtalis parietalis*. *Copeia* 1976:170-178.
- ANDRÉN, C. 1982. The role of the vomeronasal organs in the reproductive behavior of the adder, *Vipera berus*. *Copeia* 1982:148-157.
- ANDRÉN, C. 1986. Courtship, mating and agonistic behaviour in a free-living population of adders, *Vipera berus* (L.). *Amphibia-Reptilia* 7:353-383.
- BAEYENS, D.A., and ROUNTREE, R.L. 1983. A comparative study of evaporative water loss and epidermal permeability in an arboreal snake, *Opheodrys aestivus*, and a semi-aquatic snake, *Nerodia rhombifera*. *Comp. Biochem. Physiol.* 76A:301-304.
- BIRKBY, C.S., WERTZ, P.W., and DOWNING, D.T. 1982. The polar lipids from keratinized tissues of some vertebrates. *Comp. Biochem. Physiol.* 73B:239-242.
- BOSSERT, W.H., and WILSON, E.O. 1963. The analysis of olfactory communication among animals. *J. Theor. Biol.* 5:443-469.
- BUDZIKIEWICZ, H., DJERASSI, C., and WILLIAMS, D.H. 1967. *Mass Spectrometry of Organic Compounds*. Holden-Day, San Francisco. pp 368-372.
- BURGHARDT, G.M., GOSS, S.E., and SCHELL, F.M. 1988. Comparison of earthworm- and fish-derived chemicals eliciting prey attack by garter snakes (*Thamnophis*). *J. Chem. Ecol.* 14:855-881.
- BURKEN, R.R., WERTZ, P.W., and DOWNING, D.T. 1985a. The effect of lipids on transepidermal water permeation in snakes. *Comp. Biochem. Physiol.* 81A:213-216.

- BURKEN, R.R., WERTZ, P.W., and DOWNING, D.T. 1985b. A survey of polar and nonpolar lipids extracted from snake skin. *Comp. Biochem. Physiol.* 81B:315-318.
- BUSER, H.-R., ARN, H., GUERIN, P., and RAWSCHER, S. 1983. Determination of double-bond position in monounsaturated acetates by mass spectrometry of dimethyl disulfide adducts. *Anal. Chem.* 55:818-822.
- CHIASSON, R.B., and LOWE, C.H. 1989. Ultrastructural scale patterns in *Nerodia* and *Thamnophis*. *J. Herpetol.* 23:109-118.
- CHIASSON, R.B., BENTLEY, D.L., and LOWE, C.H. 1989. Scale morphology in *Agkistrodon* and closely related *Crotaline* genera. *Herpetologica* 45:430-438.
- DUVALL, D. 1986. A new question of pheromones: Aspects of possible chemical signalling and reception in the mammal-like reptiles, pp. 219-238, in N. Hotton III, P.D. MacLean, J.J. Roth, and E.C. Roth (eds.). *The Ecology and Biology of Mammal-like Reptiles*. Smithsonian Institution Press, Washington, D.C.
- FORD, N.B. 1978. Evidence for species-specificity of pheromone trails in two species of sympatric garter snake, *Thamnophis* (Colubridae). *Southwest. Nat.* 26:385-388.
- FORD, N.B. 1981. Seasonality of pheromone trails of sympatric and allopatric garter snakes. *Copeia* 1982:10-13.
- FOUQUET, G., and SCHLOSSER, M. 1974. Improved carbon-carbon linking by controlled copper catalysis. *Angew. Chem. Int. Ed.* 13:82-83.
- GANS, C. 1979. Momentarily excessive construction as the basis for protoadaptation. *Evolution* 33:227-233.
- GARSTKA, W., and CREWS, D. 1981. Female sex pheromone in the skin and circulation of a garter snake. *Science* 214:681-683.
- GARSTKA, W., CAMAZINE, B., and CREWS, D. 1982. Interactions of behavior and physiology during the annual reproductive cycle of the red-sided garter snake (*Thamnophis sirtalis parietalis*). *Herpetologica* 38:104-123.
- GOULD, S.J., and E.S. VRBA. 1982. Exaptation—a missing term in the science of form. *Paleobiology* 8:4-15.
- GRAVES, B.M., DUVALL, D., KING, M.B., LINDSTEDT, S.L., and GERN, W.A. 1986. Initial den location by neonatal prairie rattlesnakes: Functions, causes, and natural history in chemical ecology, pp. 285-304, in D. Duvall, D. Müller-Schwarze, and R.M. Silverstein (eds.). *Chemical Signals in Vertebrates IV. Ecology, Evolution, and Comparative Biology*. Plenum Press, New York.
- GREGORY, P.T. 1974. Patterns of spring emergence of the red-sided garter snake, *Thamnophis sirtalis parietalis* in the Interlake region of Manitoba. *Can. J. Zool.* 52:1063-1069.
- HADLEY, N.F. 1985. *The Adaptive Role of Lipids in Biological Systems*. Wiley-Interscience, New York.
- JACKSON, M.K., and SHARAWAY, M. 1978. Lipids and cholesterol clefts in the lacunar cells of snake skin. *Anat. Rec.* 190:41-46.
- JOY, J.E., and CREWS, D. 1988. Male mating success in red-sided garter snakes: Size is not important. *Anim. Behav.* 36:1839-1841.
- KANG, S.-K., KIM, W.-S., and MOON, B.-H. 1985. An effective method for the preparation of ω -bromoalkanols from α,ω -diols. *Synthesis* 1985:1161-1162.
- KOCIENSKI, P.J., CERNIGLIARO, G., and FELDSTEIN, G. 1977. (\pm)-Methyl *n*-tetradeca-*trans*-2,4,5-trienoate, an allenic ester produced by the male dried bean beetle *Acanthoscelides obtectus* (Say). *J. Org. Chem.* 42:353-355.
- KUBIE, J.L., VAGVOLGYI, A., and HALPERN, M. 1978. The roles of the vomeronasal and olfactory systems in the courtship behavior of male snakes. *J. Comp. Physiol. Psychol.* 92:627-641.
- LILLYWHITE, H.B., and MADERSON, P.F.A. 1982. Skin structure and permeability, pp. 397-442, in C. Gans and F.H. Pough (eds.). *Biology of the Reptilia*, Vol. 12, *Physiological Ecology*. Academic Press, New York.

- LOGIER, E.B.S., and TONER, G.C. 1961. Check list of amphibians and reptiles in Canada and Alaska. Royal Ontario Museum Contribution No. 53. University of Toronto Press, Toronto.
- MADERSON, P.F.A. 1986. The tetrapod epidermis: A system protoadapted as a semiochemical source, pp. 13-26, in D. Duvall, D. Müller-Schwarze, and R.M. Silverstein (eds.). *Chemical Signals in Vertebrates, IV, Ecology, Evolution, and Comparative Biology*. Plenum Press, New York.
- MASON, R.T., and CREWS, D. 1985. Female mimicry in garter snakes. *Nature* 316:59-60.
- MASON, R.T., CHINN, J.W., and CREWS, D. 1987. Sex and seasonal differences in the skin lipids of garter snakes. *Comp. Biochem. Physiol.* 87B:999-1003.
- MASON, R.T., FALES, H.M., JONES, T.H., PANNELL, L.K., CHINN, J.W., and CREWS, D. 1989. Sex pheromones in snakes. *Science* 245:290-293.
- MENON, G.K., AGGARWAL, S.K., and LUCAS, A.M. 1981. Evidence for the holocrine nature of lipid secretion by avian epidermal cell: A histochemical and fine structural study of rictus and uropygial gland. *J. Morphol.* 167:185-199.
- MÜLLER-SCHWARZE, D., MOREHOUSE, L., CORRADI, R., ZHAO, C.-H., and SILVERSTEIN, R.M. 1986. Odor images: Responses of beavers to castoreum fractions, pp. 561-570, in D. Duvall, D. Müller-Schwarze, and R.M. Silverstein (eds.). *Chemical Signals in Vertebrates, Vol. IV, Ecology, Evolution, and Comparative Biology*. Plenum Press, New York.
- NOBLE, G.K. 1937. The sense organs involved in the courtship of *Storeria*, *Thamnophis*, and other snakes. *Bull. Am. Mus. Nat. Hist.* 73:673-725.
- PURTON, M.D. 1986. Skin surface topography in the domestic fowl and Japanese quail. *Br. Vet. J.* 142:446-452.
- ROBERTS, J.B., and LILLYWHITE, H.B. 1980. Lipid barrier to water exchange in reptile epidermis. *Science* 207:1077-1079.
- ROBERTS, J.B., and LILLYWHITE, H.B. 1983. Lipids and the permeability of epidermis from snakes. *J. Exp. Zool.* 228:1-9.
- SAS Institute Inc. 1985. SAS User's Guide: Statistics, Version 5 Edition. Cary, North Carolina. 1290 pp.
- SCHELL, F.M., and WELDON, P.J. 1985. ¹³C-NMR analysis of snake skin lipids. *Agric. Biol. Chem.* 49:3597-3600.
- SHARKEY, A.G., SHULTZ, J.L. and FRIEDEL, R.A. 1956. Mass spectra of ketones. *Anal. Chem.* 28:934-940.
- SHEFFIELD, L.P., LAW, J.M., and BURGHARDT, G.M. 1968. On the nature of chemical food sign stimuli for newborn garter snakes. *Commun. Behav. Biol.* 2:7-12.
- SILVERSTEIN, R.M. 1977. Complexity, diversity, and specificity of behavior-modifying chemicals: Examples mainly from Coleoptera and Hymenoptera, pp. 231-251, in H.H. Shorey and J.J. McKelvey, Jr. (eds.). *Chemical Control of Insect Behavior*. Wiley-Interscience, New York.
- SILVERSTEIN, R.M., RODIN, J.O., and WOOD, D.L. 1966. Sex attractant in frass produced by male *Ips confusus* in Ponderosa pine. *Science* 154:509-510.
- SINGER, A.G., AGOSTA, W.C., O'CONNELL, R.J., PFAFFMAN, C., BOWEN, D.V., and FIELD, F.H. 1976. Dimethyl disulfide: An attractant pheromone in hamster vaginal secretion. *Science* 191:948-950.
- STILLE, B. 1987. Dorsal scale microdermatoglyphics and rattlesnake (*Crotalus* and *Sistrurus*) phylogeny (Reptilia: Viperidae: Crotalinae). *Herpetologica* 43:98-104.
- TSUMITA, T., NIWA, T., SHIMOYAMA, Y., and TOMITA, H. 1979. Fatty acid composition of skin tissues of snakes. *Snake* 11:19-21.
- VALICENTI, A.J., PUSCH, F.J., and HOLMAN, R.T. 1985. Synthesis of octadecenoic acids and [1-¹⁴C]-labeled isomers of octadecanoic acids. *Lipids* 20:234-242.
- WANG, D., CHEN, P., JIANG, X.C., and HALPERN, M. 1988. Isolation from earthworms of a proteinaceous chemoattractant to garter snakes. *Arch. Biochem. Biophys.* 267:459-466.
-