tralian National University) sugar secondary standard, with a ^{14}C activity of 150.8 ± 0.2 pMC (*16*), averaged 149.7 pMC with an SD of 3.0 pMC (number of samples, $n = 62$). The other two secondary standards ($n = 31$, and 49) also had accuracies within the precisions of the measurements. Duplicate sputter targets from the graphites of 23 CH_4 samples and single sputter targets from 14 CH_4 samples were remeasured on widely different occasions. These measurements had an SD of 1.4 pMC.

15. J. S. Vogel, D. E. Nelson, J. R. Southon, *Radiocarbon* **29**, 323 (1987); J. S. Vogel, J. R. Southon, D. E. Nelson, *Nucl. Instrum. Methods* **29B**, 50 (1987); D. E. Nelson, J. S. Vogel, J. R. Southon, T. A. Brown, *Radiocarbon* **28**, 215 (1986); J. S. Vogel, D. E. Nelson, J. R. Southon, *ibid.*, in press.
16. L. A. Currie and H. A. Polach, *Radiocarbon* **22**, 933 (1980).
17. The $^{13}C/^{12}C$ ratio is expressed in the well-known δ notation; see H. Craig, *Geochim. Cosmochim Acta* **3**, 53 (1953). Standardization was obtained from NBS-16 and NBS-17 standards. A control sample of −48.9 per mil was commonly included in the sample batches, and the SD of these measurements was 0.07 per mil. To check on the extremely light values, we also processed and measured CH_4 samples, which are used in the isotopic analysis of natural gases, for which the $\delta^{13}C$ values have been determined in an interlaboratory comparison study (*18*). From a series of measurements we determined the stable isotope fractionation of the sample separation and combustion steps. Both processes fractionate isotopes, resulting in lighter $\delta^{13}C$ and lower yields (Y), according to $\epsilon = [\delta^{13}C(Y) - \delta^{13}C(Y = 1)]/\ln Y$, with an enrichment factor ϵ of 7.5 per mil for the separation process, and of 2.2 per mil for the combustion process. As the measured yields for individual samples were generally high, the resulting corrections applied to the results were small; 22 samples were processed and analyzed in duplicate or triplicate, and the average SD of these data sets was 0.23 per mil. We therefore adopted an experimental uncertainty of ±0.2 per mil for all individual samples. No correction was required for N_2O as it was completely removed from the samples.
18. We measured an average $\delta^{13}C$ of −29.0 ± 0.1 per mil ($n = 2$), −44.4 ± 0.2 per mil ($n = 4$), and −72.0 ± 0.6 per mil ($n = 2$), respectively, for natural gas standards NGS-1A, NGS-2A, and NGS-3A, in good agreement with data in an intercomparison study [G. Hut, *Stable Isotope Reference Samples for Geochemical and Hydrological Investigations (IAEA Rep.*, International Atomic Energy Agency, Vienna, 1987).
19. Any errors associated with the compressor were investigated by compressing an already collected sample into a second tank, with the assumption that this incremental sampling would reveal any changes introduced by this procedure. We also compared samples separated from outside air directly piped into the separation train to samples simultaneously collected by compression. The results from these tests, which were conducted throughout the period of study, showed that the CH_4 concentration ratio for twice-compressed versus once-compressed samples was 1.003 ± 0.003 ($n = 5$), and the ratio for $\delta^{13}C$ was 0.998 ± 0.004 ($n = 4$), which indicates that the sampling process produced no statistically significant alteration. However, the concentration of ^{14}C was found to be lowered in the twice compressed samples by an average of 1.7 ± 0.6 pMC ($n = 4$). We do not understand how to reconcile these findings, but have applied such a correction to all the ^{14}C data. The combustion blank was determined intermittently between samples with UHP CH_4 (natural gas). The average contamination due to combustion was about 0.7 pMC ($n = 6$). We tested the separation procedure blank periodically by processing spiked air samples (mixture of ambient air and UHP methane, with a total CH_4 concentration of 22.5 ± 0.2 ppmv). The average blank from the separation procedure was about 0.9 pMC ($n = 10$). All the data were corrected for the contamination as measured. In addition, we have verified the experimental uncertainties, which were between ±0.9 and ±2.0 pMC for the individual samples. Nine samples were processed (entire procedure and measurement) in duplicate or triplicate and yielded an average SD of 1.4 pMC. Thus we conclude that the SD, including components for processing and analytical error for ^{14}C, was 1.5 pMC and that our total possible systematic error was of similar value. These tests demonstrate that the employed techniques produce accurate results, even though the sample size used (0.1 to 0.5 mg of carbon) was small.
20. D. R. Blake and F. S. Rowland, *J. Atmos. Chem.* **4**, 43 (1986).
21. R. Nydal, K. Lovseth, F. H. Skogseth, *Radiocarbon* **22**, 626 (1980); I. Levin *et al.*, *ibid.* **27**, 1, (1985).
22. H. Doerr and K. O. Muennich, *ibid.* **28**, 338 (1986); P. Becker and H. W. Scharpenseel, *ibid.*, p. 383 (1986); D. D. Harkness, A. F. Harrison, P. J. Bacon, *ibid.*, p. 328 (1986).
23. W. Weiss *et al.*, *Nucl. Instrum. Methods* **B 17**, 571 (1986); W. Weiss *et al.*, *J. Geophys. Res.* **88**, 8574 (1983); A. Sittkus and H. Stockburger, *Naturwissenschaften* **63**, 272 (1976).
24. World Meteorological Organization (WMO), Global Ozone Research and Monitoring Project, *Atmospheric Ozone 1985* (*Rep. 16*, WMO, Washington, DC, 1987).
25. M. J. Whiticar, E. Faber, M. Schoell, *Geochim. Cosmochim. Acta* **50**, 693 (1986).
26. R. J. Cicerone and R. S. Oremland, *Global Biogeochem. Cyc.* **2**, 299 (1988).
27. I. Fung, personal communication.
28. J. A. Davidson *et al.*, *J. Geophys. Res.* **92**, 2195 (1987).
29. *World Nuclear Handbook 1989* (Reed, New York, 1988), pp. 36–37.
30. C. Kunz, *Health Phys.* **49**, 25 (1985).
31. L. Howles, *Nucl. Eng. Int.* **32**, 18 (1987).
32. *IAEA News Features 1* (IAEA, Vienna, Austria, April 1988).
33. I. Levin, personal communication.
34. D. H. Ehhalt and H. U. Schmidt, *Pageoph.* **116**, 452 (1978).
35. D. C. Lowe *et al.*, *Nature* **332**, 522 (1988).
36. Methane concentration was determined by gas chromatography with flame ionization defector on at least four splits of each sample on at least two different dates against a series of NBS or NBS–traceable standards covering the entire concentration range. Samples with ≥ 250 ppmv of CH_4 were measured with a small-volume TC detector. For atmospheric concentrations the average SD was 0.57%. This does not include a systematic error from the gas standards (±1% relative error).
37. M. Stuiver and H. A. Polach, *Radiocarbon* **19**, 355 (1977).
38. This work was supported by National Aeronautics and Space Administration grant (NAGW-843). We thank Trans Freight Lines and the officers and crews of the MVs *TFL Liberty*, *TFL Freedom*, *TFL Independence*, and *TFL Franklin* for transatlantic passages for sampling, and T. Yoshinari, S. R. Piotrowicz, P. Quay, M. Koehn, A. Herczeg, J. Yavitt, G. Lang, J. Samson, S. Barnard, D. Carpenter, the Smithsonian Tropical Research Institute, the Churchill Northern Studies Centre (Churchill, Manitoba, Canada), D. Carpenter, the Matheson Company, and the Rice Research Station (Crowley, Louisiana), for samples and support in the field. M. Schoell provided natural gas standards. We thank N. Blair, R. Cicerone, P. Crutzen, D. Ehhalt, H. Doerr, I. Fung, S. King, C. Kunz, I. Levin, C. Martens, E. Matthews, K. O. Muennich, E. Nelson, P. Quay, U. Schmidt, W. Rheeburg, M. Rubin, M. Schoell, C. Stevens, M. Wolin, and J. Yavitt for valuable discussions.

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Sex Pheromones in Snakes

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The majority of pheromones identified to date are insect pheromones, which are volatile in nature. Identification of nonvolatile pheromones have been relatively rare, especially in vertebrates. Male and female garter snakes use pheromones to mediate sexual behavior. The female sex attractiveness pheromone of the Canadian red-sided garter snake, *Thamnophis sirtalis parietalis*, consists of a novel series of nonvolatile saturated and monounsaturated long-chain methyl ketones, whereas the male sex recognition pheromone contains squalene. These compounds were isolated, identified, and partially synthesized, and field tests show them to be biologically active.

More than 50 years ago, Noble (*1*) showed that tongue-flicking behavior in male garter snakes serves to deliver nonvolatile sex pheromones sequestered on the female's dorsum to the male's vomeronasal organ. Unlike the majority of insect pheromones studied to date, garter snake pheromones are not produced by a discrete gland (*2*); rather, they are components of the integumental skin lipids found in all terrestrial vertebrates and similar to the cuticular lipids of insects that often also serve a pheromonal function (*3*). In garter snakes, if the pheromones are not present on the female's skin or the male is unable to perceive them, males will not exhibit courtship behavior (*1, 4*). Previous investigations (*2*) on the isolation and identification of the sex attractiveness pheromone indicated that the pheromone is chemically related to the yolking protein vitellogenin, or lipid-rich subunits of this molecule, and is sequestered in the female skin. Subsequent research (*5*) on the transport, immunoreactivity, and field tests of

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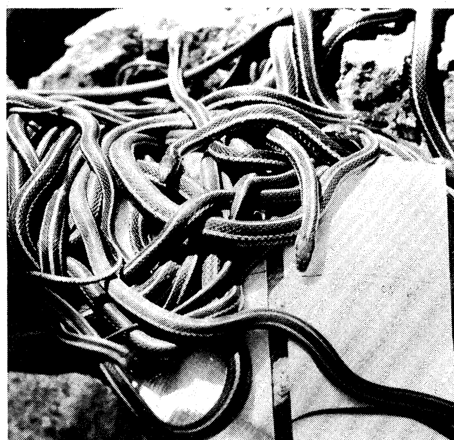

Fig. 1. Courting males exhibiting courtship behavior to chemical cues on the paper towel. Bioassays were conducted by pouring 1 ml of a solution of the extract (1.5 mg/ml) onto a paper towel and placing it in the den where courting male garter snakes had access to it. The number of total males responding with increased tongue-flick rates and chin-rubbing behavior for more than 30 seconds was counted for 5 minutes and compared to solvent controls. There were ten repetitions for each trial and all subsequent monitoring of materials followed this procedure.

circulating vitellogenin prevent equating the pheromone with vitellogenin. An alternative approach was therefore undertaken to study the attractiveness pheromone at the skin surface.

We have isolated, identified, and synthesized two classes of nonvolatile sex pheromones produced by female and male red-sided garter snakes, *Thamnophis sirtalis parietalis*. The female sex attractiveness pheromone consists of a series of previously undescribed long-chain (C_{29} to C_{37}) saturated and monounsaturated methyl ketones. The male sex recognition pheromone includes squalene and other unidentified skin lipid components. Although pheromonal communication is evident in all orders of Reptilia, this is to our knowledge the first identification of sex pheromones in that class.

During the spring mating season in Manitoba, Canada, male garter snakes emerge from underground hibernacula and aggregate by the thousands. Females emerge sporadically during the 4-week breeding season and are immediately surrounded by 10 to 100 males forming a "mating ball" (*6*). Courtship in the male is characterized by two behaviors: an increased tongue-flick rate and chin-rubbing behavior in which the male moves up and down the female's back, repeatedly rubbing his chin along her dorsal skin.

Adult, sexually attractive, unmated females (n = 18) and males (n = 24) were collected in the field near Narcisse, Manitoba, in 1986. The animals were killed with an overdose of brevital sodium and their skin lipids extracted with hexane (*7*). This extraction yielded an average of 38.4 mg of lipid per female and 8.4 mg per male. Comparison of male and female skin lipids showed clear qualitative and quantitative sex differences (*8*). Female extracts, but not male extracts, were attractive to sexually active, courting males in the bioassay (*9*).

Having thus determined that hexane extracts of female snakes contained the pheromone, we fractionated extracts of females on an alumina activity III column with hexane and ethyl ether as the mobile phase (*10*) generating 21 fractions. Only one fraction (fraction 5, 98% hexane:2% ethyl ether, 117.5 mg), elicited courtship behavior from males (Fig. 1).

The active compounds in this fraction were identified by infrared (IR) (*11*), gas chromatography–mass spectrometry (GC-MS) (*12*), nuclear magnetic resonance (NMR) spectroscopy (*13*), and formation of derivatives. The GC-MS suggested the presence of a homologous series of saturated and monounsaturated methyl ketones (Fig. 2) and ^{1}H NMR confirmed this. The locations of the double bonds were determined from the mass spectra of the bis thiomethyl ether derivatives of the ketones (*14*) and their *N*-methoximes (*15*). Cleavage between the vicinal thiomethyl groups provided two characteristic ions for each olefinic compound, and the methoxime moiety permitted assignment of the carbonyl function to one of these fragments (*16*). Two major cleavage ions for each unsaturated compound allowed identification of the mixture as a homologous series of saturated and ω-9 cis-unsaturated methyl ketones.

Two of the major saturated peaks (422 and 450) were prepared (*17*) and found identical in all respects to the natural materials. The unsaturated ketones are novel compounds that are currently being synthesized. Subsequent to identifying these compounds in the extracts, we tested them in the bioassay. The synthetic ketones were tested with male snakes in the field along with the natural material and several other compounds identified in the garter snake skin lipids (Table 1). Although the results showed that male garter snakes responded to the synthetic methyl ketones at essentially the same level as the natural saturated methyl ketones, the natural unsaturated methyl ketones were significantly more effective in eliciting male courtship. Indeed, they were not significantly different from the entire mixture of naturally occurring methyl ketones.

Noble (*1*) showed that males do not normally court other males, presumably basing this discrimination on pheromonal cues. Recent work has suggested that a male sex recognition pheromone also exists which

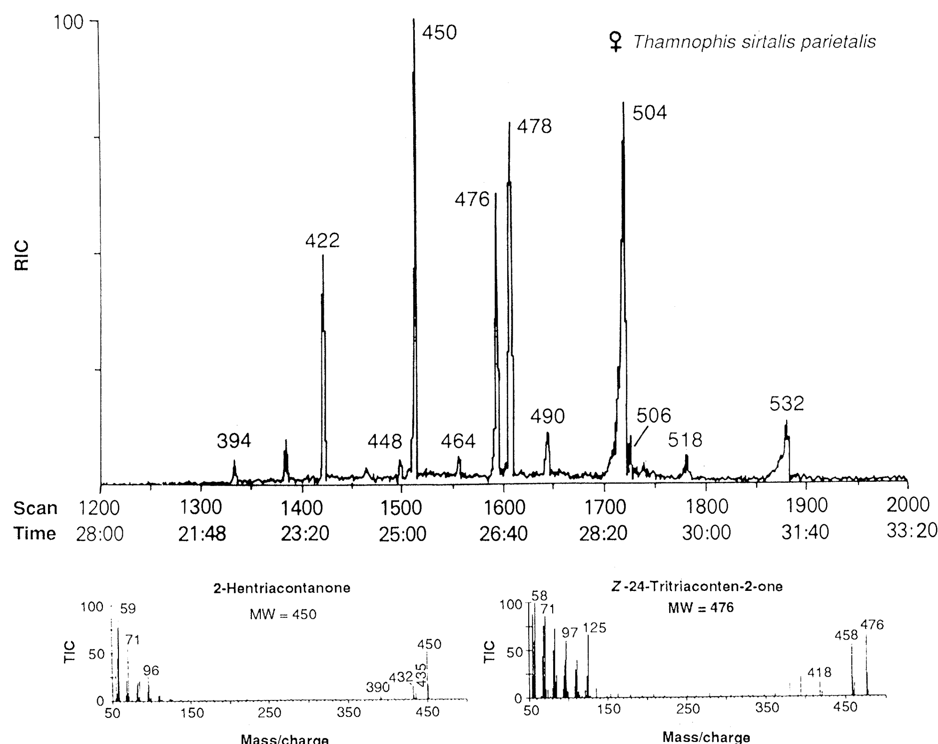

Fig. 2. Gas chromatogram of methyl ketones of female red-sided garter snakes, *Thamnophis sirtalis parietalis*. The numbers over the peaks are the molecular weights. The insets are mass spectra of a saturated methyl ketone and a monounsaturated methyl ketone. TIC, total ion chromatogram; RIC, reconstructed ion chromatogram.

identifies them to other courting males as unsuitable individuals to court (*18*). In the field, courting males investigate all individuals that they contact; most males are summarily ignored whereas females are instantly recognized and courted. There are two possible mechanisms by which males could recognize each other. One mechanism is that any individual that does not have the female-specific pheromones must be a male and is therefore not courted. Alternatively, males might possess specific chemical cues, not present in females, that identify them as males and thus inappropriate individuals to court. Evidence supports the second hypothesis: when hexane extracts of males were added to female extracts, male courtship ceased (*19*). This decline in courtship behavior was not due to habituation to the stimuli as males presented with fresh paper towels containing the female attractiveness pheromone resumed courtship promptly.

The results of this experiment indicate that some compound or compounds are present in the skin of male garter snakes that prevent courtship. Hexane washes of males were fractionated by column chromatography as described earlier. Interestingly, fraction 2 contained a single compound (squalene) that appeared on both thin-layer chromatography plates and GC-MS data on males but not females. Males courted female hexane-extracted skin lipids but significantly fewer males courted female extracts in the presence of squalene. However, the diminution of male courtship response was not as great as the response elicited by the whole male skin lipid extract. Thus, we conclude that squalene is one component of the male sex recognition system. Other components remain to be identified.

Table 1. Field bioassay results. Each compound was tested in ten trials with ten snakes per trial; different snakes were used for all compounds and between trials of the same compound. Of chief interest, the synthetic saturated methyl ketones elicited significantly greater courtship behavior than the control substance ($P < 0.012$), indicating that an active component of the pheromone has indeed been identified and produced. The natural compounds (all in hexane) indicate the range of response that was obtained. In particular, the unsaturated methyl ketones of fraction 5 elicited a significantly stronger response than the saturated methyl ketones of fraction 5 ($P < 0.006$), whereas the responses to saturated methyl ketones, whether synthetic or natural, were not significantly different ($P \geq 0.5$). Tests involved randomization of the data with Bonferroni corrections, treating the score for each trial (not each snake) as a datum. The trial values were, for each compound: fraction 5 (6, 8, 10, 9, 9, 10, 7, 9, 10, 10); saturated methyl ketone (2, 1, 0, 2, 1, 2, 0, 1, 2, 3); unsaturated methyl ketone (5, 4, 8, 9, 8, 9, 10, 5, 7, 7); synthetic saturated methyl ketones (2, 1, 4, 2, 1, 0, 0, 3, 2, 2); all other values were zero. Each value represents the number of male snakes (out of ten animals) exhibiting courtship behavior in response to the compound.

Compound tested	Males responding (%)
Natural compounds	
Fraction 5	88
Saturated methyl ketones of fraction 5	14
Unsaturated methyl ketones of fraction 5	72
Fractions 1-4 and 6-21	0
Synthetic compounds	
Synthetic methyl ketones	17
Control (hexane)	0
Cholesterol	0
Palmitic acid	0

There exists in the red-sided garter snake a natural experiment that allows us to test the hypothesis that sex-typical sex pheromones exist. There are a small subset of males in wild populations that are courted by other males as if they were females. These female mimics or she-males (*20*) confuse other courting males and thus gain a decided advantage in the highly competitive mating balls. If what we have found in males and females is correct, she-males provide a good model for contrasting the pheromone systems of males and females. Hexane washes from she-males elicited chin-rubbing behavior from males and analyses showed that the methyl ketones of she-males were proportionally different from those of both males and females. However, and perhaps more importantly, squalene was absent from the skin lipid components of she-males, thus rendering them more similar to females in their skin lipid chemistry than to males.

Methyl ketones have long been known to be active as pheromones in mammals (*21*) and insects (*22, 23*), as well as alarm and defensive secretions (*24*). However, reports of such long-chain methyl ketones are rare. Long-chain methyl ketones have been described as an allomone in the fruits of *Evodia hupehensis* (*25*), as well as a potential pheromone in the German cockroach *Blattella germanica* (*26*). Long-chain methyl ketones have been described in snake skin lipids (*27*). However, there were no behavioral data pertaining to possible semiochemical function in these studies. Interestingly, the unsaturated methyl ketones in the latter study all had double bonds in the ω-7 position, whereas we find the double bonds all in the ω-9 position, suggesting that they are oleic acid– rather than palmitoleic acid–derived (*23*).

Our study also shows that squalene and other components in male skin surface lipids are acting as male sex recognition pheromones in male garter snakes. Squalene is a major component of the surface lipids of man whereas it occurs only in traces, if at all, in other animals (*28*). However, rat preputial gland is known to produce significant quantities of squalene (*29*). The odor of preputial glands of male rodents is attractive to the opposite sex and also leads to aggressive behavior among males (*30*).

We do not yet know how general these results are in other reptiles. However, it may be hypothesized that variation in the chain length and position and geometry of the double bonds of these methyl ketones may impart species-specific information to courting male garter snakes.

REFERENCES AND NOTES

1. G. K. Noble, *Bull. Am. Mus. Nat. Hist.* **73**, 673 (1937).
2. W. R. Garstka and D. Crews, *Science* **214**, 681 (1981).
3. D. G. Ahern and D. T. Downing, *Lipids* **9**, 8 (1973); N. Nicolaides, *Science* **186**, 19 (1974); E. Sondheimer and J. B. Simeone, Eds. *Chemical Ecology* (Academic Press, New York, 1970); M. Beroza, Ed., *Chemicals Controlling Insect Behavior* (Academic Press, New York, 1970); J. Vandenbergh, Ed., *Pheromones and Reproduction in Mammals* (Academic Press, New York, 1983); W. J. Bell and R. T. Cardé, *Chemical Ecology of Insects* (Sinauer, Sunderland, MA, 1984).
4. M. Halpern, in *Advances in Vertebrate Neuroethology*, J. P. Ewert, R. R. Capranica, D. J. Ingle, Ed. (Plenum, New York, 1983), pp. 141–176; J. L. Kubie, A. Vagvolgyi, M. Halpern, *J. Comp. Physiol. Psychol.* **92**, 627 (1978).
5. W. R. Garstka and D. Crews, in *Chemical Signals in Vertebrates: Ecology, Evolution, and Comparative Biology*, D. Duvall, D. Müller-Schwarze, R. M. Silverstein, Eds. (Plenum, New York, 1986), vol. 4, pp. 253–255; R. T. Mason and D. Crews, unpublished results.
6. P. T. Gregory, *Can. J. Zool.* **52**, 1063 (1974); D. Crews and W. Garstka, *Sci. Am.* **247**, 158 (November 1982).
7. Animals were killed with an overdose of brevital sodium. Each animal was then placed dorsal side down in a 500-ml glass beaker with 5 to 10 ml of hexane. Care was taken so that the head and tail did not touch the solvent so as to avoid contamination by bodily fluids. The extracted lipids were pooled and the solvent evaporated on a rotovaporator at 40°C. The resulting viscous semisolid was taken up in fresh hexane and stored in amber glass vials with teflon caps at −20°C.
8. R. T. Mason, J. W. Chinn, D. Crews, *Comp. Biochem. Physiol.* **87B**, 999 (1987).
9. Male garter snakes will respond to novel stimuli as well as to pheromones with a dramatically increased tongue-flick rate. Chin-rubbing behavior, in which the male presses his head onto the female's dorsum and traverses up and down her body, is only observed in a mating context. Thus, it is an unambiguous indicator of the presence of female pheromone.
10. Alumina activity III (Sigma) (100 g) were loaded onto a 35 by 2.5 cm glass column with a stopcock. Fractionation was accomplished by elution with three 100-ml volumes of hexane and three 100-ml volumes of succeeding solutions of 2% diethyl ether and 98% hexane, 4% ether:96% hexane, 8% ether:92% hexane, and so on.
11. The infrared (IR) spectra were obtained on a Perkin-Elmer model 3600 IR data station. Approximately 5 μl of sample in chloroform were applied to a NaCl microcavity cell, scanned from 600 to 4000 cm^{-1}, and referenced against either $CHCl_3$ or air. The IR spectrum showed absorption at 3000 to 2850 cm^{-1} (CH_2), 1712 cm^{-1} (C=O), 1468 to 1460 cm^{-1} (CH_3CO), and 718 cm^{-1} (the C–H stretch of cis–CH=CH–).
12. Capillary GC-MS analyses were performed on a

Finnigan-MAT 4023 quadropole mass spectrometer. The capillary column was a fused silica 4m BP1 (0.5-μm film, SGE). The conditions of the GC-MS were as follows: injector temperature at 290°C, source at 150°C, electron multiplier at −2200 volts. Temperature program was set at 45°C for 1 minute ramping to 290°C at 10°C per minute.
13. The NMR spectra were collected on a Varian 360-MHz spectrometer ($CDCl_3$, TMS standard, δ=chemical shift in parts per million) 0.85, triplet (CH_3), 1.24, broad singlet (CH_2-CH_2), 1.54, triplet ($CH_2CH_2COCH_3$), 1.88, quartet (CH_2CH=CH), 2.10, singlet (CH_3C=O), 2.38, triplet (CH_2COCH_3), 5.32, triplet (CH=CH).
14. A hexane solution (25 μl) containing 500 ng of material was treated with 50 μl of dimethyl disulfide and 5 μl of 0.06% iodine in ethyl ether and stirred overnight at room temperature. The sample was diluted with 200 μl of hexane and 50 μl of an aqueous solution of sodium thiosulfate (5%) stopped the reaction. The sample was reduced under nitrogen and analyzed directly by GC-MS.
15. To 100 μl of pyridine and 10 mg of *O*-methylhydroxylamine hydrochloride were added 500 μl of the dimethyl disulfide derivative of the methyl ketones in ether. The mixture was heated at 100°C for 15 minutes and analyzed by GC-MS directly.
16. For example, the bis thiomethyl derivative of the compound with molecular weight = 476 yielded two major ions in electron impact–mass spectrometry (EI/MS) at masses 397 and 173; its methoxime yielded a parent ion at mass-to-charge ratio of 599.
17. Methyl ketones were synthesized by reacting 100 mg of octadocosanoic acid (0.235 m*M*) or mellissic acid (0.221 m*M*) in hexane with methyl lithium (1.4*M*) under nitrogen overnight. The reaction was quenched with H_2O, extracted in ether and dried over $MgSO_4$.
18. A. Vagvolgyi and M. Halpern, *Can. J. Zool.* **61**, 1171 (1983).
19. Hexane extracts (20 ml) of the skin lipids of males and females were presented in a sequential choice design. Female skin lipids were placed on a paper towel and the percentage of males that courted in 5 minutes noted. Male skin lipids were then added on top of the female's skin lipids. A final test of female skin lipids alone shows no lasting inhibition of courting males. Twenty males were used in each experiment.
20. R. T. Mason and D. Crews, *Nature* **316**, 59 (1985).
21. E. S. Albone, *Mammalian Semiochemistry: The Investigation of Chemical Signals Between Mammals* (Wiley, Chichester, 1984).
22. N. F. Hadley, *The Adaptive Role of Lipids in Biological Systems* (Wiley-Interscience, New York, 1985).
23. F. W. Forney and A. J. Markovetz, *J. Lipid Res.* **12**, 383 (1971).
24. M. S. Blum, *Chemical Defenses of Arthropods* (Academic Press, New York, 1981).
25. M. Gellért *et al.*, *Herba Hungarica* **24**, 53 (1985).
26. R. Nishida *et al.*, *Experientia* **30**, 978 (1974); T. Sato *et al.*, *Agr. Biol. Chem.* **40**, 391 (1976).
27. F. M. Schell and P. J. Weldon, *Agr. Biol. Chem.* **49**, 3597 (1985); D. G. Ahern and D. T. Downing, *Lipids* **9**, 8 (1973).
28. N. Nicolaides, H. C. Fu, G. R. Rice, *J. Invest. Derm.* **51**, 83 (1968).
29. N. Nicolaides, *J. Am. Oil Chem. Soc.* **42**, 691 (1965).
30. R. Mykytowycz and B. S. Goodrich, *J. Invest. Derm.* **62**, 124 (1974).
31. Supported by an NIMH predoctoral fellowship NIMH NRSA 09310 to R.T.M. and NIMH Research Scientist Award 00135, NICHHD 16687, and a Texas Higher Education Coordinating Board Advanced Research Program grant to D.C. The authors wish to thank M. Shoesmith and B. Koonz of the Manitoba Department of Natural Resources, Wildlife Branch, for permits and many efforts on our behalf. We thank E. Sokoloski for obtaining the NMR spectra; J. Bull assisted in the statistical analyses and critical reading of the manuscript; J. Whittier, M. Mendonca, A. Tousignant, R. Krohmer, S. Macmillan, S. Miller, T. Spande, L. Tsai, and Q.-L. Pu assisted in the field and in discussion of ideas.

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Pulmonary Blood Flow Regulation in an Aquatic Snake

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Regulation of pulmonary blood flow was studied during voluntary diving in the aquatic file snake, *Acrochordus granulatus*. Measurements of pressure and blood flow in pulmonary and systemic vessels indicate that blood flow completely bypasses the lung for significant periods during prolonged and quiescent submergence (greater than 30 minutes). When the lung is ventilated, pulmonary blood flow increases to 36 milliliters per minute per kilogram of body mass (measured in the anterior pulmonary artery), and the cardiac output largely bypasses the systemic circulation. These reciprocating patterns of preferential blood flow reflect inverse relations between flow and vascular resistance, with the result that systemic and pulmonary arterial pressures remain virtually constant throughout repetitive dive cycles. Neuropharmacological studies of freely diving snakes and isolated, perfused lung preparations show that pulmonary blood flow is regulated by an interplay of adrenergic vasodilatation and cholinergic vasoconstriction within the densely innervated lung vasculature. The patterns of blood circulation shown by diving *Acrochordus* reflect an unusual lability of intracardiac shunts.

IN AMPHIBIANS AND NON-CROCODILIAN reptiles, a single ventricle allows redistribution of cardiac output between systemic and pulmonary vessels by way of central cardiovascular shunts. Such shunts are especially pronounced in aquatic species in which perfusion of the lung closely matches its ventilation (*1*). Although patterns of cardiovascular shunts are documented in several diverse species, knowledge of their controlling mechanisms is rudimentary. We investigated the regulation of pulmonary blood flow as it relates to diving behavior in the aquatic file snake, *Acrochordus granulatus* (*2*). We report that both sympathetic and parasympathetic components of the autonomic nervous system play interactive roles in regulating pulmonary blood flow in precise correspondence with ventilation and intracardiac shunts. Although the sympathetic (adrenergic) innervation of pulmonary vascular beds has been studied in mammals, little is known concerning a possible antagonistic interplay of parasympathetic and sympathetic nerves as occurs in the heart (*3*). Such activity in *Acrochordus* profoundly changes pulmonary perfusion and the magnitude of intracardiac shunts during repetitive dive cycles.

The species we studied is particularly interesting because of its adaptations to prolonged submergence in shallow, aquatic habitats. These include low metabolic rate, comparatively large volume and oxygen capacity of blood, large pulmonary oxygen stores, high-affinity *p*H-sensitive hemoglobin, and cutaneous gas exchange (*4*). File snakes possess sufficient oxygen stores to sustain aerobic dives for 1.5 to 2 hours (minimally), whereas some individuals can remain submerged for as long as 3 to 5 hours (*4*). As in all three species of the genus and family, typical breathing episodes consist of two to four breaths spaced over several minutes, during which lung gases reequilibrate with air, CO_2 stores are released, and the blood is saturated with oxygen (*5*).

The most prominent cardiovascular event associated with the ventilatory period is a large increase of pulmonary blood flow ($\dot{Q}_p$) attributable to tachycardia, a decrease in pulmonary vascular resistance and a left to right shunt of the ventricular outflow (Fig. 1). Ventilatory tachycardia typically entails four- to sevenfold increases of heart rate over submergence values (mean maximum rate ± SD = 25.7 ± 3.3 beats per minute; n = 47 dives in seven animals) (Fig. 1). However, the increase of $\dot{Q}_p$ during ventilation entails a net left to right cardiac shunt as well, for systemic blood flow ($\dot{Q}_s$) measured in the dorsal aorta, carotid artery, or either aortic arch does not mirror the increases of $\dot{Q}_p$ during ventilation. More usually, $\dot{Q}_p$ and $\dot{Q}_s$ change in a reciprocating manner, and changes of stroke flow in pulmonary and systemic vessels are inversely related during ventilatory episodes as well as during diving when the cardiac output shifts predominantly to the systemic circuit (Fig. 1). Characteristically, $\dot{Q}_p$ increases more than tenfold while $\dot{Q}_s$ approaches zero during ventilation (*6*). Thus, the major fraction of ventricular outflow is directed to the lung during ventilatory episodes.

Both systemic and pulmonary arterial pressures are relatively constant throughout dive cycles, except for occasional, small changes of pulmonary pressure which are coincident with ventilatory tachycardia and

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