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9. The present experiments were not the first to suggest that the cortical projections of geniculate layers 4 and 5 were "eccentric." In an earlier study, HRP was injected into layers III, IV, and V of the striate cortex; labeled cells were found in a column through all six layers of the GL [D. Raczkowski and I. T. Diamond, *Brain Res.* **144**, 383 (1978)]. But the width of the column was markedly less in layers 4 and 5. This earlier finding probably reflects the fact that the HRP entered the projections from GL layers 4 and 5 before these projections had arborized or spread to the limits they eventually attain in cortical layer I.
10. There is also evidence from anterograde degeneration studies in the monkey that the parvocellular layers of GL project to cortical layer IV with a much sparser projection to cortical layer I [D. H. Hubel and T. N. Wiesel, *J. Comp. Neurol.* **146**, 421 (1972); J. S. Lund, *ibid.* **147**, 455 (1973)]. We cannot tell from the available results whether the projections to layer I arise from one or more than one of the layers of GL; perhaps the projections to layer I originate from the smallest cells in all of the parvocellular layers. In any case our experience suggests that the intensity of the projection to layer I has probably been underestimated.
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Sex Pheromone in the Dog

Abstract. *Methyl* p-*hydroxybenzoate has been identified in the vaginal secretions of female dogs in estrus. When small amounts of this compound were applied to the vulvas of anestrous or spayed females, males placed with these females became sexually aroused and attempted to mount them.*

It is commonly assumed that female dogs give off a special odor or odors when they go into heat. This odor apparently attracts male dogs to the vicinity of the female and initiates courtship. While there is considerable ethological and veterinary data on *Canid* sex behavior and its hormonal regulation, the strict specification of the odor cues given off by a female dog during proestrus and estrus has not been achieved (*1*). In fact the source (or sources) of the odorants involved in male attraction and excitation has not been well defined. It is not clear from the work of others whether the odors derive from the vagina, the urine, both, or from some other less likely anatomical region. The purpose of this report is to describe studies indicating that the odor of a specific compound found in the vaginas of female dogs during proestrus and estrus has the capacity to incite male dogs to sexually approach and attempt to mount totally anestrous females whose vulvas have been treated with synthetic pheromone. This behavior persists despite resistance on the part of the females.

The studies were conducted as follows. Three unspayed 3-year-old sexually experienced and multiparous beagles were monitored cytologically and behaviorally as they passed through their estrous cycles. Two dogs went into heat spontaneously. Another was hormonally induced with two 1-mg injections of estradiol cypionate (given intramuscularly in oil vehicle) spaced 48 hours apart. Two vaginal smears were taken daily from each dog, as well as a urine sample. The vaginal cells were collected with sterile cotton applicators that had been defatted with chloroform and methanol. After some of the vaginal material had been applied to a microscope slide, the cotton tip of the applicator was sealed in a small vial and kept frozen until it was used as a source of vaginal odorant during chromatographic analyses. On a daily basis, each female was paired with an adult male dog, and a record was kept of the behavioral activity during a 15-minute interaction with the male. In this way, it was possible to accumulate a chronological record of changes in vaginal cytology associated with estrus and correlate these with changes in frequency of specific behavioral acts exhibited by both the males and females as the females passed from anestrus to proestrus through estrus and into metestrus. At the same time gas chromatographic and mass spectrometric (GC-MS) analyses could be performed on the odors generated by the daily samples of vaginal secretion and urine collected from each female. Thus, we accumulated cytological, behavioral, and GC-MS data on each of the three dogs at all four stages of their estrous cycles. Since each dog went into proestrus at different times, the cycles of the three dogs were staggered over a total period of about 55 days. Vaginal smears, urine, and behavioral data were collected on all three dogs over the entire 55-day period.

Smears were obtained by gently inserting a defatted, sterile, saline-soaked cotton applicator through a glass speculum into the vagina. The use of the speculum allowed us to get a deep vaginal smear and to avoid contamination of the smear by the cellular debris that normally collects along the labia. Only cervical smears can be histologically interpreted with accuracy (*2*). The two swabs were rolled over a microscope slide and then placed in solvent-cleaned vials and frozen until the time of analysis. The slides were stained with Wright's stain by an automated stainer. They were examined under low and high power with a light microscope. Both the hormonally induced and naturally cycling dogs exhibited the classical pattern of cellular changes in vaginal smears associated with estrus. The smears of the two naturally cycling females remained highly cornified for about 8 days, once full cornification was observed. The smears of the hormonally induced female remained highly cornified for at least 17 days. This female remained behaviorally receptive for a concomitantly longer period of time.

The three females were tested with a male in outdoor runs (0.85 by 4 m). These were constructed of 2-m cyclone fence with smooth concrete floors and a metal sunroof. A set of eight fully adult male dogs varying in breed, size, age, and prior sexual experience were paired in a systematically rotating fashion with the females during the study. Three were purebred beagles, four were the product of beagle-spitz crosses, and one was a large German shepherd. Most tests were run between 8:00 a.m. and 3:00 p.m.

Each trial was conducted as follows. A female was placed randomly in one of seven clean runs. Almost invariably the female would urinate within 5 minutes of entry. One milliliter or more of urine was collected immediately with a pipette. If the female failed to urinate within 10 minutes, the test was initiated anyway. One of the eight males was brought to the testing area and introduced into the run containing the female. A timer was started and a written record of the male-female interaction was made for both the male and female; a standardized score sheet was used.

For the purposes of this report, a general summary of the pattern of behavioral changes observed is more appropriate than a detailed specification of the frequency data for each female and male in our study.

As a female passed from anestrus to proestrus, the frequency of her urination within the run during the interaction with the male rose sharply. Urination by the males also increased, as did ano-genital investigation of the female. Over the course of approximately 5 days, the female became increasingly solicitous toward the male—exhibiting a stereotypic bowing posture, turning rapidly in circles in front of the male, standing crosswise in front of him, backing into him, then standing still, and, finally, lordosing with tail averted. During this time, the males likewise became increasingly more interested in the females; urinated more frequently, often counterurinating literally on top of female urine deposits; became more persistent at ano-genital investigation and licking; exhibited the bow posture more frequently, and eventually attempted to mount the female more frequently, and more and more persistently. These behavioral changes are typical of the period of proestrus (*3*).

During proestrus, the female enticed the male but refused to let him mount and intromit. A major deterrent to mounting is barking. If the male is especially insistent, the female may growl or snap at the male. In contrast, during full estrus, the female becomes highly receptive and lordoses for the male with little or no courtship behavior and lets the male mount almost immediately. As a consequence, under the confined conditions of our tests, the frequency of most behavioral measures fell off sharply during estrus because the females were fully attractive and fully receptive at that time. For two of the dogs, this period of maximal receptivity lasted 3 days. The third dog, which was hormonally induced, was highly receptive for 4 days. After that she became selective and would allow only the five nonbeagle dogs to mount. She remained receptive to these dogs for 7 days.

During metestrus, the females lordose to some extent but do not allow the males to mount. Barking and snapping, which had waned during estrus, return, and the females gradually refuse to let the males mount or investigate them ano-genitally. Male interest and persistence wanes.

As a general summary, then, we observed a sudden increase in a variety of olfactory-related behaviors at the beginning of proestrus. Over a period of days these alter in frequency. The males were highly attracted a number of days before the females were willing to copulate, but their behavior was regulated by the females through postural and acoustic cues. Final sexual arousal or stimulation of the male may involve special olfactory signals emitted during courtship. The males remain attracted to the females into early metestrus, but the females refuse to fully lordose even though they may still solicit and present. Eventually, male-female interactions return to baseline behavior typical of anestrus.

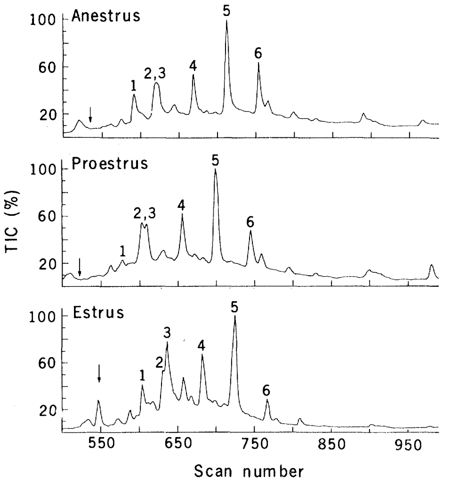

Fig. 1. Total ion current chromatograms of trimethylsilylated volatile components of beagle vaginal secretions. Chromatographic conditions are as described in text. Only those peaks eluting between 175° and 250°C are shown. Arrows indicate the retention time of the silylated derivative of methyl *p*-hydroxybenzoate. The numbered peaks are identified compounds with identical spectra (1, diethylphthalate; 2, unknown; 3, heptadecane; 4, unknown; 5, unknown; and 6, dibutylphthalate). The electron energy was 70 eV.

To date we can only report on the analysis of the vaginal secretion samples. Our efforts to isolate and identify compounds were directed at analyzing the volatile components of the vaginal samples rather than vaginal secretions per se (*4*).

Numerous samples were analyzed from anestrous, proestrous, estrous, and metestrous points in each of the female's cycles. The chromatograms resulting from our odor trapping and on-column derivatizing procedures were moderately complex, yielding approximately 25 peaks in sufficient quantity to integrate and scan with the mass spectrometer. Three representative chromatograms from females at anestrous, proestrous, and estrous points in their cycles are given in Fig. 1.

Some differences between animals were observed in chromatographic profiles but intra-animal profiles remained quite similar. In general, chromatograms from proestrous and estrous samples contained quantitatively larger peaks. More important, some unique peaks were noted in the chromatograms generated from samples on the days of maximal behavioral receptivity. These peaks were not present at any other time. We concentrated on the interpretation of the mass spectra of these peaks. One of these compounds exhibited intense fragment ions at mass to charge (*m/e*) 224(M), 209(M-15), 193(M-31), 177, 149, 135(M-89), 91, 89, and 73. The mass spectral fragmentation and chromatographic characteristics of this compound were identical to those of the trimethylsilyl ether of methyl *p*-hydroxybenzoate. In view of the silylation step in the separation of these vaginal odorants, it may be concluded that the natural product is methyl *p*-hydroxybenzoate.

Having identified methyl *p*-hydroxybenzoate as a component of vaginal odor at the time of full estrus, we decided to test for behavioral responses to the pure compound. The tests were conducted as follows. A female was brought to one of the runs. A male was brought to the area and introduced into the pen of the female. The pair was allowed to interact for 5 to 7 minutes. The female was then taken from the run and out of sight of the male. A small amount of methyl *p*-hydroxybenzoate (Aldrich) was absorbed on the tip of a saline-soaked cotton applicator. The cotton applicator was then inserted into the vagina and rubbed over the vulvar area. The female was then reintroduced into the pen containing the male. During inclement weather, it was necessary to conduct some of the tests indoors. The procedure for testing remained identical, but the indoor runs differed in dimension from the outdoor runs (1.83 by 3.6 m). The same three females used throughout the experiment were used in these tests. Eleven male dogs were used, including most of the males used previously.

The results were unequivocal. In no case did the males exhibit overt sex behavior during the 5 to 7 minutes when initially paired with the female. In 18 of 21 trials conducted intermittently over a period of 6 weeks, the males attempted to mount the female after reentry. The males exhibited intense ano-genital investigation of the female, the sexual bowing posture, frequent urination, and clear attempts to mount. In some tests overt erections were observed. The females were unwilling to stand or lordose and generally avoided the males. In some cases where the male was very persistent, they barked and snapped at the male to discourage him. Positive responses were also elicited when the compound was applied to a spayed German shepherd, an anestrous dalmatian (house pet), and two other beagles not used in previous behavioral tests. We have also run six trials with each of the following compounds: 4-hydroxy-3-methoxybenzaldehyde (Baker), methyl salicylate (Rush and Hebble), *p*-hydroxybenzoic acid (Sigma), and ethyl *p*-hydroxybenzoate (Aldrich). Application of all these compounds to the vulvar area of the females caused increased ano-genital investigation of the females. This effect waned rapidly, however, and did not lead to sexual behavior or mounting. In two of six trials with propyl *p*-hydroxybenzoate (Aldrich), the males attempted to mount the female. In each trial there were two attempts to mount, but in both trials male interest waned within 10 minutes.

There have been numerous discussions regarding the chemical complexity of higher animal chemical communication systems (*5*). Specialized scent glands are common in vertebrates, but the odor profiles generated by the secretions of these glands are highly complex. Often they consist of hundreds of compounds in varying concentrations (*6*). Our own chromatographic analysis of odors produced by beagle vaginal secretions show that these too are highly complex. There can be little doubt that there is a large amount of information stored in the olfactory signatures of these secretions. The question is whether animals have a need to use all of this information in all cases.

In the case of the release of sexual behavior in the dog, it is premature to speculate. There are many other components of the vaginal odor that we have not yet identified and tested for behavioral effects. Some of these odorants may be active alone or as synergists to methyl *p*-hydroxybenzoate. The fact still remains, however, that a single synthetic compound will release sexual behavior identical to that released by an estrous female dog. It is possible that some odorants may be far more important than others as signal carriers in scent.

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7. This work was supported by grant 7816256 from the National Science Foundation. We thank Dr. S. Badalak for assistance with the interpretation of vaginal smears and P. Winters for help in data collection. Journal paper 7075 from the Purdue Agricultural Experiment Station.

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Postmating Reproductive Isolation in *Pseudophryne* and the Evolutionary Significance of Hybrid Zones

Abstract. *Hybrid zones involving* Pseudophryne *(Anura: Leptodactylidae) provide direct evidence for the operation of postmating reproductive isolating mechanisms in animals in a natural situation. Significant introgression is prevented by hybrid inviability but there is no evidence for the reinforcement of premating isolating mechanisms. These parapatric hybrid zones are of unknown etiology, but are interpreted as being relatively old and persistent.*

When two distinct populations with imperfect isolating mechanisms come together, it is generally believed that they will either fuse completely or diverge to become more perfectly isolated (*1*). A third possible outcome is that some hybrid zones are relatively ancient and are at equilibrium (*2*). Factors preventing fusion or reinforcement of isolating mechanisms in such cases are seldom known but assumed to involve an interplay between selection against hybrids, and the influx of genes from adjacent homospecific populations (*3*). There is considerable evidence for postmating isolation derived from artificial hybridization (in groups where these experiments are feasible), but there are few data on their operation in nature. In the frog genus *Pseudophryne*, I found evidence of hybrid inviability in nature and identified some factors involved in long-term maintenance of narrow hybrid zones (*4*). I conclude from this study that the evolutionary significance of other similar hybrid zones now requires reassessment. Many such zones, traditionally interpreted as resulting from secondary contact following a period when the taxa were differentiating in separate refugia (*5*), may have evolved in situ by parapatric differentiation (*6*).

Pseudophryne semimarmorata, *P. bibroni*, and *P. dendyi* are small terrestrial frogs with essentially allopatric ranges in southeast Australia (Fig. 1A). Despite species-specific differences in adult coloration, the three taxa are very similar in anatomy, karyotype, size, breeding season and site, common male vocalizations, pre- and postmating behavior, reproductive rates, mating system, and pattern and rates of embryonic and larval development (*4, 7*).

Except where unfavorable habitats prevent contact, the northern borders of *P. semimarmorata* are marked by narrow zones of parapatric hybridization (*8*) with *P. bibroni* in the west and *P. dendyi* in the east (Fig. 1A). I mapped these contacts on the basis of five diagnostic features of adult coloration and used a hybrid index to identify hybrids (*4, 9*). Interactions between *P. semimarmorata* and *P. bibroni* (four study areas), and between *P. semimarmorata* and *P. dendyi* (three study areas) were found to be similar. A transect of the hybrid zone near Wallan, 50 km north of Melbourne is typical; the morphological transition zone is less than 9 km wide and 80 percent of the change in mean hybrid index occurs in the central 3 km (Figs. 1B and 2A). Intrasample variance in hybrid index is zero in allopatry and at the edge of the zone, and maximal in the center of the zone ($\sigma = 3.4$). The diversity of hybrid phenotypes suggests that hybridization is not restricted and that backcrossing to both parental species is occurring. Both *P. semimarmorata* and *P. bibroni* have fixed alternate alleles of heart lactate dehydrogenase (LDH) (*10*). Independent association ($P = .79$) of LDH allozymes with various parental and intermediate morphotypes suggests that there is ran-