

Species-typical lipids from the body surface and preputial glands of the sympatric voles Microtus montanus and M. pennsylvanicus : characterization and identification
by Clement Joseph Welsh

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biological Sciences
Montana State University
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Abstract:
Olfactory signals play a role in species recognition within communities of small mammals. It is possible that olfactory cues facilitate species recognition and contribute to the reproductive isolation between the sympatric voles, Microtus montanus and M. pennsylvanicus. Chemical analyses of likely odor sources were undertaken in a search for pheromone-like compounds which possessed the potential to promote species recognition in the sympatric voles. Gas chromatography revealed species-typical lipid patterns from the body surface and the preputial glands of the wild-trapped sympatric voles. The preputial glands of M. montanus contain species-tynical lipids which are not found in the preputial glands of M. pennsylvanicus. Those species-typical lipids were identified as esters of branched-, saturated-, and unsaturated-C5 alcohols, and C16 and C17 fatty acids. This is the first description of such esters from rodent preputial glands. The preputial glands of castrated M. montanus treated with testosterone produce normal quantities of the species-typical esters, but the levels of those esters in untreated castrated voles, and castrated voles treated with estradiol are greatly diminished. Relative quantities of the species-typical esters vary in M. montanus trapped from different geographic regions. Two surface lipids from M. montanus, which were absent in M. pennyslvanicus, were characterized. The hip glands of M. montanus are not the primary source of the two characteristic surface lipids in that species. The results are discussed relative to the possibility that the species-typical esters act as species recognition cues for the sympatric voles.

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A thesis submitted in partial fulfillment
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Doctor of Philosophy

in

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APPROVAL

of a thesis submitted by

Clement Joseph Welsh

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English, usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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FRONTISPIECE

"Almost always there was another mountain beyond..."

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ABSTRACT

Olfactory signals play a role in species recognition within communities of small mammals. It is possible that olfactory cues facilitate species recognition and contribute to the reproductive isolation between the sympatric voles, Microtus montanus and M. pennsylvanicus. Chemical analyses of likely odor sources were undertaken in a search for pheromone-like compounds which possessed the potential to promote species recognition in the sympatric voles. Gas chromatography revealed species-typical lipid patterns from the body surface and the preputial glands of the wild-trapped sympatric voles. The preputial glands of M. montanus contain species-typical lipids which are not found in the preputial glands of M. pennsylvanicus. Those species-typical lipids were identified as esters of branched-, saturated-, and unsaturated-C_5 alcohols, and C_{16} and C_{17} fatty acids. This is the first description of such esters from rodent preputial glands. The preputial glands of castrated M. montanus treated with testosterone produce normal quantities of the species-typical esters, but the levels of those esters in untreated castrated voles, and castrated voles treated with estradiol are greatly diminished. Relative quantities of the species-typical esters vary in M. montanus trapped from different geographic regions. Two surface lipids from M. montanus, which were absent in M. pennsylvanicus, were characterized. The hip glands of M. montanus are not the primary source of the two characteristic surface lipids in that species. The results are discussed relative to the possibility that the species-typical esters act as species recognition cues for the sympatric voles.

INTRODUCTION

General Background

Chemical communication plays an integral role in the physiology and sociobiology of animals. Based on extensive research of rodents and ungulates, there can be no doubt that chemical communication systems are important in the modification of mammalian physiology (Aron 1979; Eisenberg and Kleiman 1972; Bronson 1979) and behavior (Johnston 1983; Stoddart 1974; Mykytowycz 1974). The recent interest in mammalian chemical communication has perhaps been stimulated by the anticipation of using odor cues for behavioral modification of pest species and for better exploitation of animal husbandry. But despite an increased experimental interest, large gaps in our understanding of mammalian chemical communication remain.

Mammalian chemical communication is a relatively new area of study, and much of the terminology suggested for describing the active compounds has not been widely accepted. I will adhere to the definitions presented by Albone (1984). This outline of terms eliminates excessive "definition categories" and sets rather broad, inclusive boundaries. Several of the words defined by Albone are important for the logic and readability of this manuscript

and will be described in more detail.

"Semiochemicals" are "... compounds or groups of compounds carrying information or otherwise mediating interactions between organisms in the shared environment." (Albone 1984). Appropriate semiochemical synonyms include "chemical communicant", "chemical signal", and "chemosignal". While semiochemical is an inclusive term, a "pheromone", by definition, is more restrictive. Pheromone refers "... to semiochemical interactions between organisms of the same species." (Albone 1984). This pheromone concept is particularly useful for describing the chemical communication discussed in this document; thus "pheromone" as defined above is used frequently. Albone (1984) outlines two general subdivisions of pheromones; these are called "primer" and "releaser" pheromones. The primer pheromones elicit a long-term, usually irreversible physiological response, whereas releaser pheromones promote a shorter-acting, usually reversible, behavioral modification.

The advent of modern studies on mammalian chemical communication systems began in the late 1950's with discoveries of laboratory rodent priming pheromones. In 1956, Whitten described estrus synchrony in caged female rats exposed to male rat odors. Bruce (1960) characterized the "strange male pregnancy interruption" phenonema (the Bruce effect). In these studies, volatile chemical signals

were implicated as the active agents. Newer evidence also indicates the presence of non-volatile rodent chemosignals (Wysocki and Wellington 1980; Meredith et al. 1980; Beauchamp et al. 1980).

Generally, the rodent priming pheromones have been the target of the most intensive research; hence, it is the primer associated phenomena which have been better characterized (Bronson 1979). Despite an abundance of descriptive reports, the mechanisms by which mammalian pheromones act are poorly understood, and there exist only a few reports in which mammalian pheromones have been isolated and identified. Notable among those are descriptions of the lactone (Brownlee et al. 1969), and the acid/alcohol/ester mixture (Muller-Schwarz et al. 1974) that affect social interactions in black-tailed deer and pronghorn, respectively. Additionally, there is evidence that dimethyl disulfide acts as a sex attractant for hamsters (Singer et al. 1976), phenylacetic acid is a scent marking pheromone in gerbils (Thiessen et al. 1974), and that butyrates are used by tamarins for species and subspecies recognition (Epple et al. 1979).

Species Recognition and Odor Cues

Godfrey (1958) and Moore (1965) were among the first to present experimental evidence suggesting rodent odors could facilitate conspecific mate choices. Since then,

Hawes (1976), Nevo et al. (1976) and Perrigo and Bronson (1983) have provided supporting evidence for the role of pheromones in rodent species recognition systems. These authors have also suggested that by directing conspecific mate choices, rodent odor cues could act as reproductive isolating mechanisms.

Reproductive isolation is a fundamental element of the species definition. Within communities where closely related species co-exist, the various devices which prevent hybridization are critically important for the maintenance of genotypic integrity. Since reproductive isolating mechanisms reduce energy spent courting heterospecific mates, the characters of a species which serve to safeguard reproductive isolation are susceptible to improvement by natural selection. For many vertebrates this selective pressure has resulted in a "character divergence" of reproductive isolating mechanisms that is demonstrated by elaborate courtship behaviors and conspicuous secondary sexual characteristics. But many rodents lack elaborate premating displays, and exaggerated species dimorphism is uncommon. In these animals olfaction is a primary sensory modality, and it is likely that odor cues have evolved as mechanisms for species discrimination and are important as reproductive isolating mechanisms.

Statement of the Problem

Although the significance of rodent pheromones for reproductive isolation has received only limited study, available evidence suggests that chemosignals influence mate choices among rodents and therefore could play an important role in reproductive isolation. Investigations described here are aimed at answering the question; "Do odor sources from the male voles Microtus montanus and Microtus pennsylvanicus produce volatile lipid compounds which could act as chemosignals facilitating species identity during homo- and heterospecific encounters?" The investigations are centered on lipids from two odor sources, the preputial glands and the skin surface. An intriguing body of evidence suggests the likelihood of pheromonal species recognition and implicates the preputial glands and the skin surface in pheromone production. That evidence is summarized as follows.

Rationale on Which the Project Was Developed

The voles M. montanus and M. pennsylvanicus are morphologically similar; the taxonomic distinction is based on a single dental characteristic. In M. pennsylvanicus a tooth loop on the posterio-lingual surface of the second upper molar is characteristic of the species; that tooth loop is absent in M. montanus. The sympatric nature of these voles in the Bozeman, MT, area has been demonstrated

(Hodgson 1972; and Douglass 1976).

Despite the morphological similarities, the voles show striking differences in chromosome number; in M. pennsylvanicus 2n = 46; for M. montanus 2n = 24 (Hsu and Bernischke 1973). These vast genetic differences underscore the potential difficulty of natural hybridization. Experimental evidence against natural hybridization is derived from tests with laboratory vole colonies (Gray et al. 1977) which have shown that when maintained only as heterospecific pairs, the voles will mate, but produce no offspring. This finding suggests the probability of a premating isolating mechanism that would eliminate heterospecific matings in natural, sympatric populations.

Rodents possess a keen olfactory sensory ability. This acuity is demonstrated by the capability to use odor cues for recognition of individuals (Bowers and Alexander 1967; Yamaguchi et al. 1981), clan (Godfrey 1958), and species (Moore 1965). (See Muller-Schwarze 1974 for a review.) Perrigo and Bronson (1983) have presented evidence which suggests that olfactory cues can vary between different populations of the same species, and conspecific voles collected from geographically separated populations display different scent gland component profiles (Stoddart et al. 1975). Cross fostering tests have demonstrated the importance of the "olfactory

environment" in the developmental processes; a notable study on microtines (McDonald and Forslund 1978) revealed mate choices were influenced by olfactory cues present during the weanling stage. Additionally, it logically follows that nocturnal animals possessing a well developed olfactory system might rely heavily on chemical communication.

Microtines also show pheromone responses that have been reported in laboratory rodents. Among these are pheromones produced by male voles that increase the likelihood of successful reproduction in M. montanus (Berger and Negus 1982) and accelerate puberty in juvenile female M. pennsylvanicus (Baddaloo and Clulow 1980). Several microtine species, including M. montanus (Stehn and Jannett 1981) and M. pennsylvanicus (Clulow and Langford 1971; Mallory and Clulow 1977), demonstate the Bruce effect.

Summarizing, the voles possess several components of an exploitable system for studying mammalian chemical communication and species recognition. An olfactory signal promoting species recognition is plausible when considering the importance of odor signals for rodent recognition and the habits and habitat of voles. The animals are sympatric, morphologically similar and demonstrate pheromone mediated responses; they differ genetically and do not hybridize.

The Odor Sources

The preputial glands are paired accessory sex glands found in all murid and in many cricetid rodents. The glands are holocrine, and primarily produce lipids which are presumed to function as pheromones (Brown and Williams 1972). Experiments on laboratory rodents have shown preputial glands are androgen dependent: the preputial glands of male rodents are much larger than the glands of females. (See Brown and Williams 1972 for an extensive review of rodent preputial glands). Bronson (1976) and Christiansen et al. (1978) have suggested that preputial gland products are released in connection with urinary marking. Recent evidence supports that suggestion (Brinck and Hoffmeyer 1984).

Sexual attractant responses to rodent preputial gland chemical signals are well established. Gawienowski et al. (1975) have demonstrated a female olfactory preference for the preputial gland odors from male laboratory rats. The active preputial gland components were ether extractable lipids (Gawienowski et al. 1975). A similar phenomenon has been demonstrated in Clethrionomys glareolus (Brinck and Hoffmeyer 1984). Additionally, Bronson and Caroom (1971) showed that male mouse preputial glands, and lipid extracts of the preputial gland, possess strong attractive properties. Studies on odors of female laboratory rodents have also revealed attractant qualities of the preputial

glands (Lucas et al. 1982; Hayashi 1979). Netto and Pederson (1976) have suggested a preputial gland component is responsible for estrus induction in Microtus ochrogaster.

The attractive function and weight of preputial glands are affected by social factors and circulating hormones. Several investigators have presented evidence indicating dominant mice and dominant Clethrionomys glareolus possess larger preputial glands than subordinants. (Benton et al. 1978; Huckelbridge et al. 1972; Gustafsson et al. 1980; Lloyd 1971). Early endocrinological studies have demonstrated the androgen sensitivity of preputial glands (Brown and Williams 1972; Ebling 1977). Recent studies implicate the regulatory role of gonadal hormones on the production of preputial gland attractants. Based on evidence from behavioral assays, female laboratory rats prefer the odor of preputial extracts collected from intact rats to that from castrated rats (Gawienowski et al. 1975). Similarly, estradiol therapy increases the attractiveness of preputial gland odors from ovariectomized female rats (Lucas et al. 1982; Thody and Dijkstra 1978).

The species-typical nature of laboratory rat and mouse preputial gland lipids has been demonstrated (Sansone and Hamilton 1967). Also, the identification and androgen induction of alkyl acetates in mouse preputial glands has been described (Spener et al. 1969; Sansone-Bazzono et al.

1972). At that time alkyl acetates had been identified as insect pheromones but had not been reported in vertebrates (Spener et al. 1969). Subsequent to those studies, Mukherjea (1977) showed that, when treated with exogenous testosterone, rat preputial glands increased biosynthesis of all lipid classes, and the effect was most noticable in the fatty alcohol and steroid classes. In specific investigations on the pheromonal properties of preputial glands, investigators found alkyl acetates in rat preputial glands and showed that female rats were attracted to synthetic alkyl acetates (Stacewicz-Sapuntzakis and Gawienowski 1977). More recently, hexadecyl acetate has been found in Clethrionomys glareolus preputial glands (Brinck and Hoffmeyer, 1984). The hexadecyl acetate was shown to have attractive properties and to be androgen dependent.

The sebaceous glands of mammalian skin produce sebum which is the primary component of the surface lipids. A large part of sebum is a mixture of relatively non-polar lipids, primarily produced through de novo biosynthesis in the sebaceous glands (Nikkari 1974). Several investigators have noted the novelty and the complex nature of the mammalian surface lipids (Grigor 1977; Nicolaides 1974; Kuksis 1978; Nikkari 1974; Downing 1976; see Albone 1984 for a general review of mammalian skin lipid chemistry). The complexity of the skin lipid film, paired with the

occurrence of many unusual lipids provide mammals with a unique surface coating. Analytical studies of sebum have demonstrated species-characteristic surface oil compositions for several common laboratory animals (Wheatly and James 1957). The species-typical surface lipid patterns from various mammals have been reviewed by Nikkari (1974), and subsequent to that study, Sokolov (1982) reported species-specific skin lipid patterns from 26 mammal species. Speculating on the function of sebaceous glands, several authors (Nicolaides 1974; Mykytowycz 1970; Stoddart et al. 1975; Albone 1984) suggest sebum lipids are odorous substances acting as olfactory messages.

Many mammals also possess an array of specialized cutaneous glands. The wide occurrence of the specialized glands may indicate their importance, but in most cases the function of these specialized skin patches has not been unequivocally demonstrated. The specialized skin glands have been shown to be associated with marking behaviors (Mykytowycz 1970; Wolff and Johnson 1979; MacIsaac 1977; Albone 1984) and are generally thought to act as scent glands. Ebling (1972) states the "specialized glands undoubtedly produce pheromones". This was found to be true in the pronghorn and black-tailed deer in which bioactive olfactory signals emanate from specialized sebaceous glands (Muller-Schwarze et al. 1974; Brownlee et al. 1969).

Numerous studies have demonstrated the hormone sensitivity of the sebaceous glands and have noted the stimulatory effect of testosterone on the sebaceous glands (Ebling 1963; Ebling 1972; Ebling 1977). Ebling (1977) summarized the available information relevant to the function of sebaceous glands and stated "There is general agreement that testosterone increases the size of the sebaceous glands and stimulates sebum production".

Only limited information concerning hormonal control of the specialized skin glands is available. However, rodent studies addressing that question indicate that testosterone plays a regulatory role (Clark 1975; Mitchell 1965; Doty and Kart 1972; Stoddart 1972). Data gathered by field mammalogists also support the role of testosterone in the development of specialized skin glands. Very often, field study reports note the modified sebaceous regions in adult males are more prominent during breeding seasons when the glands are presumably under the influence of maximum testosterone levels (Hawes 1976; Muller-Schwarze 1983; Sokolov 1982; Groves and Keller 1984; Stoddart et al. 1975; Albone 1984; Mykytowycz 1970). Jannett (1975, 1978) has added support for the role of testosterone in the development of specialized skin glands by inducing growth with exogenous androgens.

Quay (1968) has presented a systematic review of the posteriolateral sebaceous glands in microtine rodents and

reported that M. montanus possess hip glands while M. pennsylvanicus do not. Within M. montanus, prominant hip glands are more common in older mature males (Quay 1968; Groves and Keller 1984).

A Working Model

Integrating the information presented above allows construction of a working model for describing pheromone facilitated reproductive isolation. This model was formulated to limit the direction of the pheromone analyses and the following hypothetical characteristics are used as guidelines which focused the analyses on compounds which were the most likely species recognition cues. First, the pheromones should be unique for a given species. Second, the pheromones should be found in or on reproductively active individuals. Thirdly, it is predicted that such a chemosignal will consist of volatile components. Finally, available evidence suggests the pheromone will exist at maximal levels in or on sexually mature male voles. If the characteristics of the proposed hypothetical pheromone model are valid, the skin lipids and the preputial glands appear to be logical places to search for an expected species recognition pheromone that could facilitate reproductive isolation. The search for a species recognition pheromone will be primarily concerned with volatile, species-typical compounds from the preputial

glands and body surface of sexually mature male voles.

Statement of Objectives

The analytical investigations of this study were designed to work within the limitations outlined under the "Working Model". With those guidelines in mind, four objectives were established. The first is to search for, and characterize species-typical lipids from the preputial glands and body surface of the male voles, *M. montanus* and *M. pennsylvanicus*. The second objective is the identification of the species-specific lipids. Thirdly, hormonal studies will examine the endocrine regulation of the species-typical lipids. The final objective is to investigate the geographic variation of the species-typical lipids.

EXPERIMENTAL PROCEDURES

Animals, Trapping and Housing

Wild *M. montanus* (MTMm) and *M. pennsylvanicus* (MTMp) were live trapped from two sympatric populations during the summer breeding season from Gallatin and Madison counties, Montana. The rodents were transported back to the laboratory where they were housed one animal per cage under natural lighting conditions, and supplied with Nutrena feeds (Cargill) rabbit chow and water *ad libitum*. Sawdust shavings and cotton balls were provided for bedding material. A three day laboratory acclimation period allowed the voles to groom themselves and remove any trap related debris prior to collection of surface lipids. Other wild rodents examined included live trapped *Peromyscus maniculatus*, *Clethrionomys gapperi* and *Microtus longicaudus*. Laboratory stock mice (CD-1) and Holtzman albino rats (Madison, WI) were used for interspecific lipid comparisons studies. Wild voles classed as "adult" animals met the following criteria: 1) visible tubular development in the caudal epididymis (Boonstra and Youson 1982; Christiansen et al. 1978), 2) scrotal testis and 3) body weight greater than 25 g.

Voles used for geographic variation and hip gland

investigations were furnished by other investigators. Dr. Carol Rowsemitt, (Biology Department, University of Utah) and Dr. Terry Horton, (present address: Dept. of Neurobiology and Physiology, Northwestern University) provided M. montanus from a laboratory colony maintained at the University of Utah. The original laboratory stocks were wild trapped from Jackson Hole, Wyoming. (Subsequent wild stocks were trapped near Pinedale, WY.) Laboratory housing of the "Utah voles" (UTMm) has been described (Rowsemitt and Berger 1983; Rowsemitt et al. 1982). (Details of housing conditions for specific experiments are described later.)

Preputial glands from wild M. montanus trapped in Teton National Park, Wyoming (WYMm) were a gift from Dr. R. J. Jannette, Jr. (Museum of Science, St. Paul, MN). These animals were killed the same day as trapped and preputial glands were excised and handled as described in the "Lipid Collection, Extraction, and Storage" section of the "Experimental Procedures".

Lipid Collection, Extraction and Storage

Voles were killed by an overdose of ether vapor or by cervical dislocation. Preputial glands were excised and frozen in 3 ml H_2O at -20° C until extracted. For lipid extraction, the preputial glands were thawed and macerated in a tissue grinder containing 3 ml H_2O. The aqueous

portion was decanted and the remaining tissue shreds were further macerated in two 3.2 ml volumes of methanol and then in two 3.2 ml volumes of chloroform. The aqueous and organic volumes were combined and the lipids extracted into an organic phase as described by Bligh and Dyer (1959). Brain, liver, kidney, muscle, and adipose tissues were treated in a similar manner.

Body surface lipids were collected by immersing the posterior half of freshly killed voles into a chloroform/methanol (2:1, v:v) solvent mixture. The body surface lipids were then partitioned into the organic phase of the extraction system described by Bligh and Dyer (1959). All lipids collected from the extractions were concentrated under a stream of nitrogen and stored in chloroform/methanol (2:1, v:v) at -20° C, in a nitrogen atmosphere.

Thin-layer Chromatography

Preparative thin-layer chromatography (TLC) on 250µm silica layers (Baker precoated, Si250 TLC plates from J. T. Baker Chemical Co., Phillipsburg, NJ) provided separation of the major lipids, with Hexane:diethyl ether:acetic acid (60/40/1,v:v:v) as the solvent system. Preparative TLC with hexane:diethylether (95/10, v:v) was used for further resolution of the neutral lipids. Simultaneous TLC of authentic standards adjacent to the natural lipids provided

R_f guidelines which directed collection of the separated lipids. For general lipid class separation, authentic standards were visualized using iodine vapor, and corresponding TLC adsorbent bands containing natural lipids were scraped from the TLC plate. The vole lipids were eluted from the adsorbent with chloroform:methanol (2/1, v:v). After characterizing the TLC system resolving the neutral lipids, the TLC plates were routinely sprayed with a rhodamine 6C solution (1 mg/ml in H_2O:ETOH; 1/1, v:v) and natural lipid bands were visualized under UV light. Neutral lipid areas were scraped from the plate and eluted from the adsorbent with hexane. (See Touchstone 1982 for a general description of preparative TLC.)

Gas Chromatography

Gas chromatography (GC) of crude total lipid samples and lipid fractions collected from TLC plates employed Varian 3700 and 1400 instruments equipped with flame ionization detectors. A Hewlett Packard 3380A integrator furnished GC "peak area" values. Chromatographic separations for routine analysis were accomplished with several columns including: (1) a 2 mm ID 2 m glass column packed with 5% SE-30 on Gas Chrom - Z, 100/120; (2) a 2 mm ID 2 m glass column packed with 3% SE-30 on Supelcoport 100/120; (3) a 30 m, Durabond, DB-1 (0.250 µm film; 0.25 mm ID) capillary column; and (4) a 30 m, Durabond, DB-225

(0.25 µm film; 0.25 mm ID) capillary column. Both capillary columns were purchased from J and W Scientific, Inc. [Rancho Cordova, CA]. Details of GC parameters are given in the figure legends. Separation of neutral esters by preparative gas chromatography was accomplished using a Varian 3700 gas chromatograph equipped with a thermal conductivity detector. A 1.25 m, 6 mm ID, stainless steel column packed with 5% SE-52 on Chromosorb 100/120 was used for separation of ester fractions. The chromatography was carried out at an isothermal column temperature of 260° C.

High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) on a 5µm Adsorbosphere silica column (25 cm by 4.6 mm ID; Applied Science, Deerfield, IL) coated with $AgNO_3$ (Heath and Sonnet, 1980) was used to separate saturated and unsaturated esters. Elution with 100% Toluene delivered by a Waters Associates [Milford, MA] M-6000A pump at 1 ml/min resolved the different ester classes. A Waters Associates 401 differential refractometer detector was used to monitor compound elution.

Gas Chromatography-Mass Spectrometry

Gas chromatography-Mass spectrometry (GC-MS) was performed on selected compounds. Electron impact mass spectra were obtained using a VG Instruments MM16 and 7070E

mass spectrometers. A Varian 3700 gas chromatograph equipped with a 30 m Durabond DB-5 (0.250 μm film, 0.25 mm ID) capillary column [J and W Scientific, Inc.] was routinely programed from 170 to 250° C at 2° C/min to provide natural ester resolution prior to introduction into the ion source. A Supelcowax 10 (30 m 0.25 μm film, 0.25 mm ID) capillary column (Supelco; Bellefonte, PA) was used for certain free alcohol analyses. For GC-MS analysis, methyl esters were chromatographed on the DB-5 column programed from 140 to 220° C at 2° C/min. Alcohols investigated by GC-MS were chromatographed (DB-5 column) at 25° C, isothermal for 5 minutes, then programed at 5° C/min. High resolution mass spectrometry was accomplished using the VG instruments 7070E mass spectrometer. All spectra were obtained at a 70 eV ionization energy.

Nuclear Magnetic Resonance Spectrometry

Nuclear magnetic resonance (NMR) spectra were obtained in a Fourier transform mode on a Bruker WM-250 MHz NMR spectrometer. Lipid samples were analyzed in deuterated chloroform (99.8%) [Stohler Isotope Chemicals, Waltham, MA]. Chemical shifts were measured using $CHCl_3$ as an internal standard.

Ester Synthesis

Hexadecanoic acid [Sigma; St. Louis, MO], (Z)-9-

hexadecenoic acid [Sigma], and 15-methyl-hexadecanoic acid [Accurate Chemical Co.; Westbury, NY] were converted to acid chlorides by their reaction with thionyl chloride (50° C, 1 hr) [Aldrich; Milwaukee, WI]. Exposure to reduced pressure removed excess thionyl chloride from the acid chlorides. Synthetic esters were prepared by reacting acid chlorides with alcohols (50° C, 45 min) from the following list. 2-Methyl-1-propanol, 3-methyl-1-butanol and 2-methyl-1-butanol, were purchased from J. T. Baker Chemical Co. 3-Methyl-2-buten-1-ol was furnished by Aldrich [Milwaukee, WI]. The synthetic esters were collected from the reaction mixture by extraction into hexane and purified over a column of Bio-sil A. (Bio-Rad Laboratories; Richmond, CA). 2-methyl-(E)-2-buten-1-ol was synthesized by reducing 2-methyl-(E)-2-butenoic acid [Aldrich] with lithium aluminum hydride and was esterified as described above. Fatty acid methyl esters (FAME) of the vole lipids were produced by reacting the natural esters with Meth-Prep II [Applied Science, Inc., Deerfield, IL]. The methyl ester of (E)-9-hexadecanoic acid was purchased from Sigma. Other FAME standards were purchased from Sulpelco [Bellefonte, PA] and Applied Science [Deerfield, IL]. The synthetic esters were used as analytical standards for GC and GC-MS investigations.

Ozonolysis

For location of double bonds, unsaturated lipids were subjected to ozonolysis as described by Beroza and Bierl (1967). Gas chromatography was used to compare the ozonolysis products with aldehyde standards. The chromatography was achieved on a 2 m 2 mm ID glass column packed with 3% Silar 5 CP. The column temperature was held at 60° C for 5 min and then programed to 250° C at 10° C/min and held at 250° C for 21 min.

Saponification

Free alcohols from the natural esters were analyzed after microsaponification. Approximately 20 µg of the natural ester was added to a capillary tube (one end sealed) and dried under nitrogen. 10 µl of 5% KOH in methanol was added and the open end of the capillary tube was heat-sealed. The saponification reaction mixture was heated at 45° C for 30 min. The capillary tube was opened and 10 µl H_2O and 10 µl methylene chloride were added. After thorough mixing, the mixture was centrifuged to speed separation to a bi-phasic mixture. Subsequent GC showed approximately 40% of the theoretical alcohol mass was recovered in the methylene chloride layer. The free alcohols were analyzed by GC and GC-MS.

Hydrogenation

Microhydrogenation, over palladium catalyst, of approximately 20 µg of the natural esters was performed as described by Parliment (1973). The saturated esters were analyzed by GC and GC-MS.

Hip Gland Experiments

Twelve mature male "Utah voles" (UTMm) which possessed obvious hip glands were selected for these studies. These animals were proven breeders and prior to the experiment were housed with at least one adult female. For comparisons of hip gland lipids, total surface lipids and "control area" skin lipids, the male voles were arbitrarily separated into two groups of six and manipulated as follows. In the first group, the hip glands were surgically excised, mascerated and the lipids extracted by the method of Bligh and Dyer (1959). After the removal of the hip glands, surface lipids were removed by immersion into chloroform/methanol (2:1, V:V) and the lipids collected as previously described. For the second group of voles, a "control" rump skin area (located between the two hip glands) was excised and lipids extracted as described above. Surface lipids were collected after removal of the rump skin patch.

Hormonal Manipulations

Utah voles (UTMm) were manipulated for hormonal control tests according to the following protocols. One group of mature males (> 3 months old) was castrated and six weeks later given exogenous hormones by surgically implanting (intraperitoneal) silastic capsules (Dow-Corning; Dimethylpolysiloxane, 20mm x 1.02mm ID x 2.16mm OD) containing the following hormones (purchased from Sigma): Testosterone, 5 α-dihydrotestosterone and 17 β-estradiol. The 20mm silastic tubing implants containing testosterone maintain a blood testosterone level of approximately 8 ng/ml (Dr. C. Rowsemitt, U. of Utah; personal communication). That blood testosterone titer is roughly 70% of the diurnal maximum found in M. montanus (Rowsemitt and Berger 1983). While carrying the silastic implants the voles were housed one per cage. Six weeks after given hormone implants, the voles were killed and the preputial glands excised and lipids extracted as described above. A second group of animals was used as "untreated controls". In addition to voles castrated after 3 months of age, juvenile male voles were castrated at 15 days old and received no further treatments. The juvenile castrates were killed and preputial glands excised at 75 days old.

Histological Preparations

Tissues were fixed in 4% neutral buffered formalin,

dehydrated through graded alcohols (70%, 95%, 100% ethanol), cleared with xylene, and embedded in paraffin. Tissue sections (5 μm) were cut and stained with a routine hematoxylin and eosin procedure.

Statistical Analysis

Several measured parameters appeared to have non-normal distributions, and unequal variances were found for some group comparisons. Non-parametric statistical tests were used for certain analyses. Kruskal-Wallis tests were used for multiple group comparisons and Mann-Whitney U tests were used for pair-wise comparisons. In addition, 1 way ANOVA and LSD (least significant differences by Student's T test) analyses were used for other data sets. The mathematical calculations were performed by the MSUSTAT Statistics Package computer program developed by Dr. R. E. Lund at Montana State University (Copyright 1983).

SURFACE LIPIDS

Preliminary Characterization

Surface lipids from mature male M. montanus (N=14), M. pennsylvanicus (N=13), Microtus longicaudus (N=2), Clethrionomys gapperi (N=2), Peromyscus maniculatus (N=4), and laboratory mice (CD-1; N=6) were resolved by gas chromatography (Figure 1). Although the chromatograms illustrated are from one individual of each species, the lipid patterns are characteristic of the species tested.

The chromatograms of M. montanus and M. pennsylvanicus were the result of injecting 20 µg of total surface lipid onto the SE-30 (5%) column (detection limit = 25-30 ng). The presence of two prominent peaks in the skin lipids of M. montanus, which are absent in M. pennsylvanicus, is obvious. These two M. montanus peaks are found at approximate retention times of 20 and 23 minutes. For the sake of brevity, those components are referred to as the "20min peak" and the "23min peak". A third major peak at a retention time of about 15 minutes ("15min peak") is present in the surface lipid profiles of both M. montanus and M. pennsylvanicus and also appears in the other species examined.

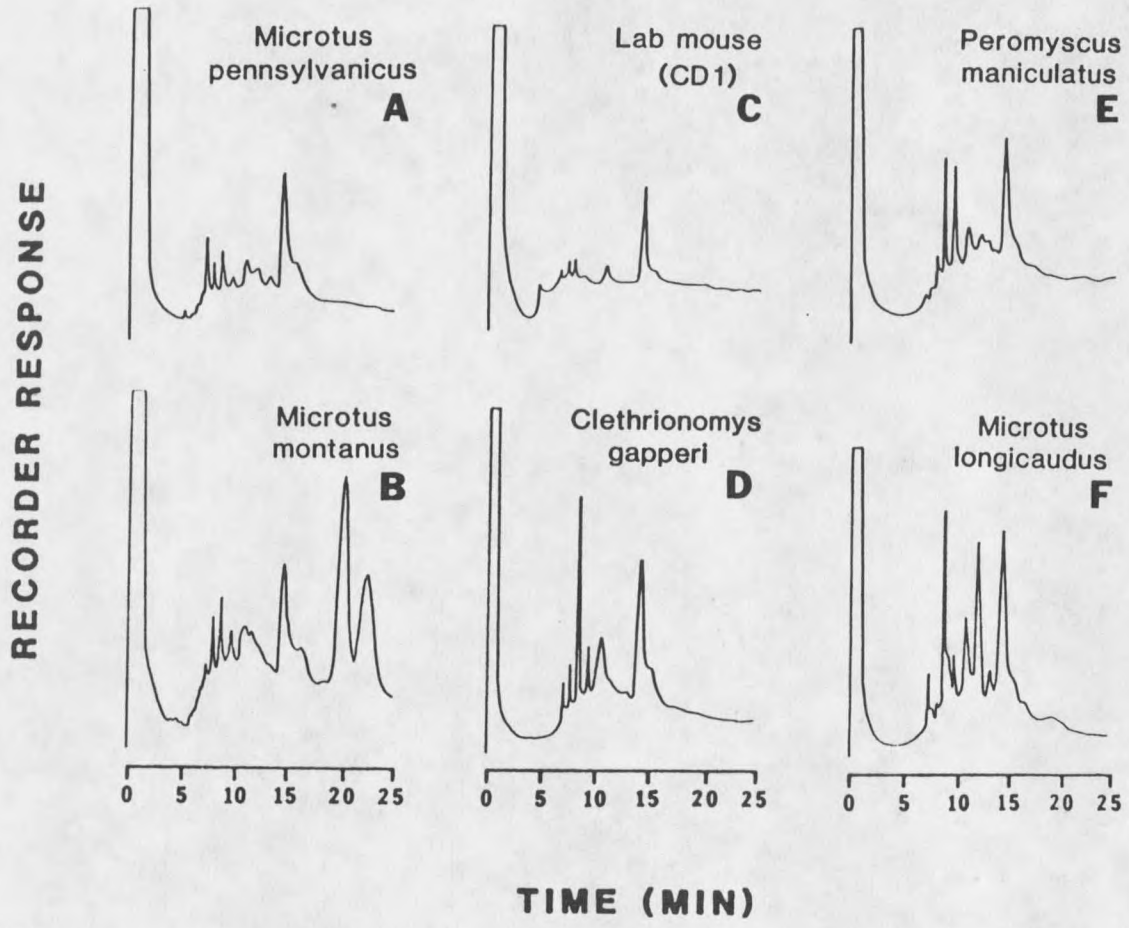


Figure 1. Surface lipid gas chromatograms from various rodents. The chromatograms were obtained on a 2 m, 2 mm ID, glass column packed with 5% SE-30; column temperature was programed from 180 to 295° C at 15° C/min.

The surface lipid chromatograms (Figure 1) demonstrate the species-characteristic lipid patterns from M. montanus and M. pennsylvanicus. Additionally, the chromatograms show that surface lipid species specificity also occurs in several other rodent genera. The finding of species-typical lipid patterns is similar to the species characteristic surface lipid profiles reported from a number of mammalian species (Sokolov 1982; Nikkari 1974; Wheatly and James 1957). The biological significance of such results is not known, but the extent of the surface lipid diversity leads to speculation that unique surface lipid mixtures are common in mammals. That those unique lipids may act as odor cues is an interesting possibility.

Preliminary characterization of the 20min and 23min peaks was accomplished by gas chromatography and preparative thin-layer chromatography paired with gas chromatography. Using TLC, the surface lipids of M. montanus were resolved into general lipid classes (Figure 2; Fractions A-D). Subsequent GC of specific TLC fractioned lipids revealed the 15min peak component is recovered from the "sterol" TLC fraction (Fraction C) whereas the 20min and 23min peaks are found in TLC Fraction A and hence are "neutral lipids". Further characterization compared retention times of the natural product lipids with a hydrocarbon standard. Gas chromatography showed that the 20min peak elutes from the SE-30 (5%) column approximately

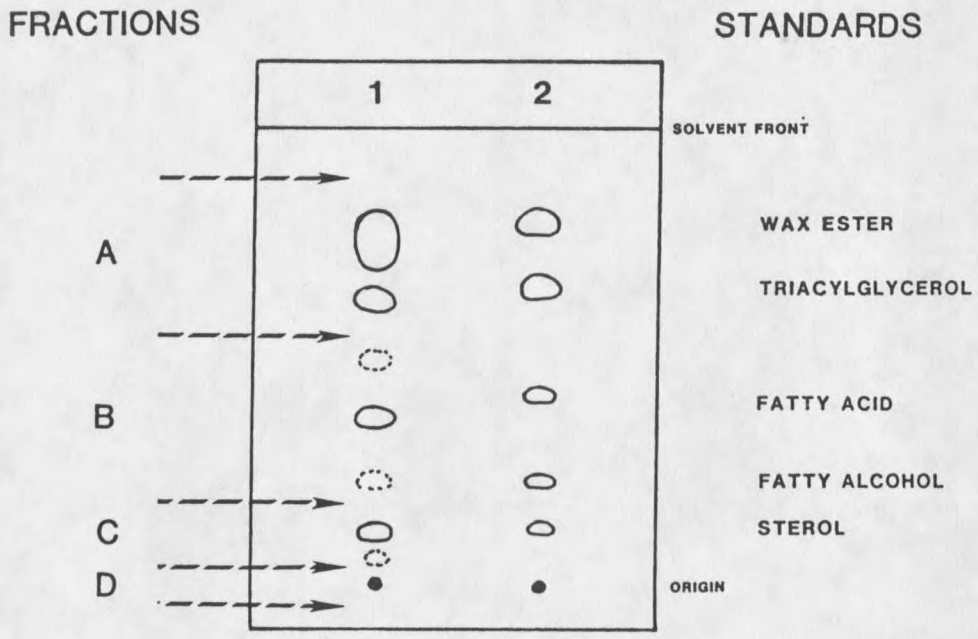


Figure 2. Illustration of thin-layer chromatographic resolution and fractionation of surface lipids from Microtus montanus. Lipid resolution was obtained on silica gel layers using hexane:diethylether:acetic acid (60/40/1; v:v:v). Lane 1 contains M. montanus total surface lipids; standards are in lane 2.

2 minutes later than a C_{32} straight chain hydrocarbon standard.

Gas chromatography on SE-30 generally separates compounds according to boiling points. The chromatographic behavior of the species-typical 20min and 23min peaks indicates that the characteristic lipids have boiling points higher than the C_{32} hydrocarbon standard. This evidence leads to the conclusion that the 20min and 23min peaks are relatively nonvolatile and probably do not act as volatile species recognition chemosignals; they may act as non-volatile signals received by the vomeronasal organ.

Hip Gland Lipids

Since M. montanus are reported to possess specialized sebaceous glands, the hip glands (Quay, 1968), it was of interest to investigate the contribution of the hip glands to the total surface lipid profile. These studies were directed by the hypothesis that the hip glands are the specialized source of the 20min and 23min peak lipids. If so, the relative percentage of the 20min peak from hip gland should exceed the relative percentage of the 20min peak found in the total surface lipids. This contention was tested by analyzing isolated hip gland lipids and by removing the hip glands, collecting the remaining surface lipids and estimating the relative abundance of the 20min peak component.

Studies on the hip glands used UTMm and MTMm which were maniupulated as described in the "Experimental Procedures". GC chromatograms (5% SE-30) of UTMm hip gland lipids, rump skin lipids, surface lipids (minus hip gland and rump skin lipids) and total surface lipids from wild M. montanus were compared. For those chromatogram comparisons, the area of the 20min peak was divided by the sum of the areas the 20min peak and the 15min peak and converted to percentages. The "% 20min peak" values were used as an estimate of the relative mass of the 20min peak within a given lipid sample. The % 20min peak values were analyzed by Mann-Whitney U tests to compare equality of treatment groups. Table 1 gives mean % 20min peak values for different treatment groups and summarizes the results of the treatment group comparisons.

Surface lipid comparisons (Table 1), indicate the species-typical surface lipid (the % 20min peak) exists at similar levels in MTMm and UTMm. Since total UTMm surface lipids were not included in the study, it is not known to what extent removing skin patches and exposing the body wall to the lipid collection solvents affects the surface lipids patterns in the UTMm. However, the % 20min peak values of partly skinned UTMm were similar to the total surface lipids of MTMm. Removing the hip glands vs. the rump skin patch did not significantly alter the remaining surface lipids that contribute to the % 20min peak value.

Table 1. Relative levels of a *Microtus montanus* species-typical lipid from specific surface lipid samples.

Surface lipid samples	Relative levels of a species-typical surface lipid[1]	Statistical similarities[2]
MTMm[3] total surface lipid	68±14 N=9	A
UTMm[4] surface lipid minus rump skin	56±17 N=5	A
UTMm surface lipid minus hip gland	58±19 N=5	A
UTMm isolated rump skin	31±10 N=5	B
UTMm isolated hip gland	20±5 N=5	B

1 Mean relative species-typical lipid (20min peak) quantities ± standard deviation; N=sample size. Areas under GC peaks were used to determine the relative quantities of an unidentified species-typical lipid.

2 Similar letters are given to denote similar surface lipid samples as determined by least significant difference by Student's T test of arcsin percentage transformations of the % 20 min peak values.

3 MTMm = wild *Microtus montanus* collected from areas near Bozeman, MT.

4 UTMm = *Microtus montanus* from a laboratory colony maintained at the University of Utah.

This finding suggests that, with respect to the 20min peak component, the specialized hip gland lipids are very similar to the "control area" rump skin lipids. Isolated hip glands contain relatively little 20min peak compounds and isolated hip gland lipids vs the rump skin patch lipids comparison indicates similar 20min peak levels in the two samples.

It appears the hip glands are not the primary source of the 20min peak compound. This conclusion is supported by evidence indicating; 1) removal of the hip gland vs the rump skin does not significantly affect the relative % 20min levels of the total surface lipids, 2) the isolated hip gland lipids do not contain relatively large quantities of the 20 min peak, and 3) 20min peak levels in the rump skin are similar to those in the hip glands. It is not known where the M. montanus species-typical 20min peak lipid originates.

Since relative measurements were used to evaluate surface lipid quantities, the absolute quantities of the 15min peak lipid in the total surface lipids are not known. Given only the relative quantities used in the studies of the "hip gland lipids", the role of the hip glands in production of the 15min peak is not clear. GC of surface lipids from several rodent species shows that a peak with a very similar GC behavior to the 15min peak is present in all rodents examined. Based solely on that GC evidence,

the 15min peak appears to be a common component of the surface lipids from several rodents, and because of that observation the 15min peak was not considered for further investigation.

In summary, the surface lipids of the examined rodents show species typical patterns. M. montanus surface lipids contain two prominent peaks not found in M. pennsylvanicus. Hip glands do not play a major role in the biosynthesis of the species-typical lipids found in the surface oil chromatograms of M. montanus. Due to the apparent low volatility of the M. montanus species-typical surface lipids, they were not subjected to further analysis.

PREPUTIAL GLANDS

Preliminary characterization

Gross morphological differences between the preputial glands of M. montanus and M. pennsylvanicus are readily apparent. The glands of the M. montanus closely resemble the superficial morphology of lab mouse preputial glands and possess a distinct vesicular appearance with a distal arrangement of bulbar enlargements filled with a clear, amber colored, viscous oil. In contrast, the M. pennsylvanicus preputial glands are minute and contain a thick, waxy secretion. (M. montanus (UTMm) preputial gland weight = 68.8 mg $\pm$ 27.4; N = 11; M. pennsylvanicus (MTMp) preputial gland weight = 9.8 mg $\pm$ 1.9, N = 10; $P < 0.002$, Mann-Whitney U test). Due to the small size and unusual appearance of the preputial glands in M. pennsylvanicus, the actual presence of preputial glands in that species was questionable. Subsequent examination of hematoxylin-eosine stained M. montanus preputial glands and the questioned tissues in M. pennsylvanicus revealed that the cell structures of the tissues from both species were identical. The cytological similarities indicated that the M. pennsylvanicus tissues were actually preputial glands.

Species-typical lipid profiles (Figure 3) were

revealed by gas chromatography of 10 µg of mature male preputial gland total lipids (3%, SE-30; detection limit=10 ng.) The chromatograms presented, although from the lipids of one individual, are characteristic of chromatograms from the 23 mature male M. montanus and the 14 mature male M. pennsylvanicus examined. The presence or absence of the species-characteristic peaks at a retention time of about 5 minutes ("5min peaks") was consistent within the two vole species. The species characteristic peaks (the 5min peaks) (Figure 3) were not found in brain, liver, kidney, muscle, or adipose tissues taken from mature male M. montanus. Additionally, the 5min peaks were not present in the preputial gland total lipids of mature male laboratory rats (Holtzman albino N=6) and mice (CD1, N=5).

A series of compounds (the 5min peaks) are prominent in the preputial gland lipid profile of M. montanus but are absent in M. pennsylvanicus (Figure 3). In an attempt to find trace levels of the M. montanus 5min peaks in M. pennsylvanicus, the preputial gland lipids of 6 individual M. pennsylvanicus were concentrated 5 fold and re-examined by gas chromatography. Five-minute peaks did not appear in the chromatograms of 50 µg of M. pennsylvanicus preputial gland total lipid.

In a preliminary chemical characterization of the M. montanus lipids, the chromatographic behavior of the 5min

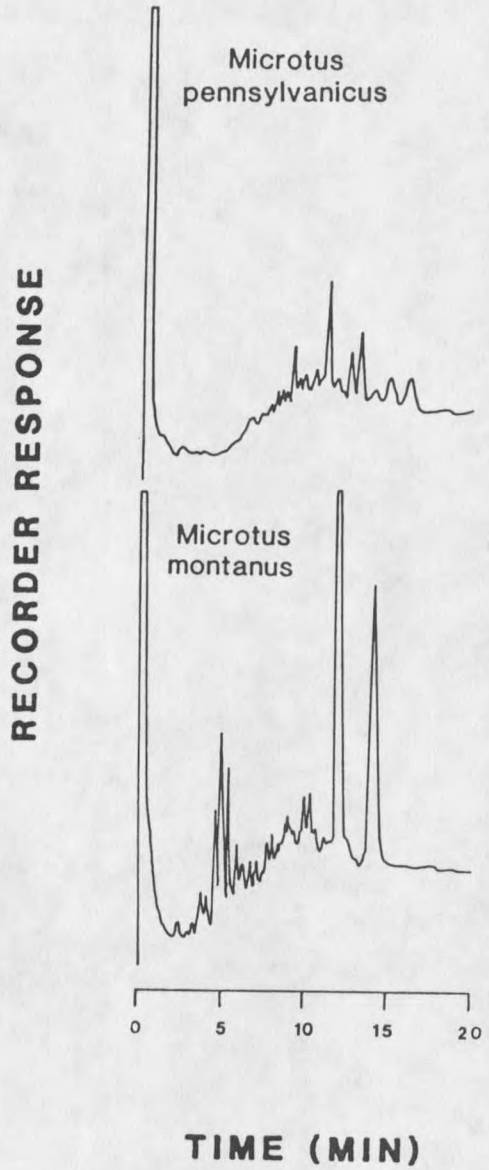


Figure 3. Gas chromatograms of total preputial gland lipids from Microtus montanus and Microtus pennsylvanicus. The chromatograms illustrate the resolution of total preputial gland lipids (10 µg) on a 2 m glass column (2 mm ID) packed with 3% SE-30; column temperature was programed from 180 to 295° C at 15° C/min.

peaks was compared to that of known standards. Gas chromatography (3% SE-30) of straight chain hydrocarbons showed the 5min peaks had retention times in the range shown by C_{22} and C_{23} hydrocarbon standards. Similar tests indicated that GC retention behavior of the 5min peaks was similar to C_{19} and C_{20} alcohols and acetates of C_{18} and C_{20} fatty alcohols. The M. montanus lipids which had retention times of approximately 13 and 15 minutes (Figure 3) eluted from the SE-30 column between C_{32} and C_{34} hydrocarbon standards.

Gas chromatography on SE-30 generally resolves components of a mixture relative to boiling points. The 13min and 15min lipid peaks in the M. montanus preputial glands were, based on chromatographic behavior, judged to be relatively non-volatile. Comparisons of GC retention times suggest that the 5min peaks are at least "somewhat" volatile; that is, they possess characteristics which impart a volatility similar to C_{22} and C_{23} hydrocarbons and alkyl acetates with total carbon numbers of 20 and 22. From work on an unrelated mammal, Epple et al. (1979) described a mixture of butyrate esters with total carbon numbers ranging from 20 to 28 which act as chemical cues promoting species and subspecies recognition.

Defining the general lipid class to which the 5min peaks belong was accomplished by TLC. Total preputial gland lipids were fractioned by preparative TLC (Fractions

A-E) as illustrated in Figure 4. Specific lipids from discrete TLC adsorbent bands were collected and analyzed by GC. The gas chromatograms showed that the 5min peaks (R_f = 0.60; TLC Fraction C) migrate just below the wax ester standard (hexadecyl stearate; R_f = 0.68) but above the alkyl acetate standard (hexadecyl acetate; R_f = 0.40). The 13min and 15min peaks (R_f = 0.68; TLC Fraction B) co-chromatographed with the wax ester standard.

Hormonal Control Studies

Due to their species-typical nature and volatility, the 5min peak preputial gland lipids in M. montanus were subjected to further experimentation. From the previously presented "working pheromone model", it is expected that a pheromone promoting reproductive isolation would be found in or on reproductively active individuals. Logical speculation leads to the hypothesis that biosynthesis of such a pheromone would be regulated by gonadal hormones. To test that hypothesis male UTMm were castrated and given various hormone treatments as described in the "Experimental Procedures". The effects of those hormone treatments were assessed with regard to several parameters reflecting preputial gland activity. Subsequent to the treatment period, the voles were sacrificed, preputial glands excised and frozen in 3 ml H_2O, thawed and weighed wet. Preputial gland lipids were extracted and weighed.

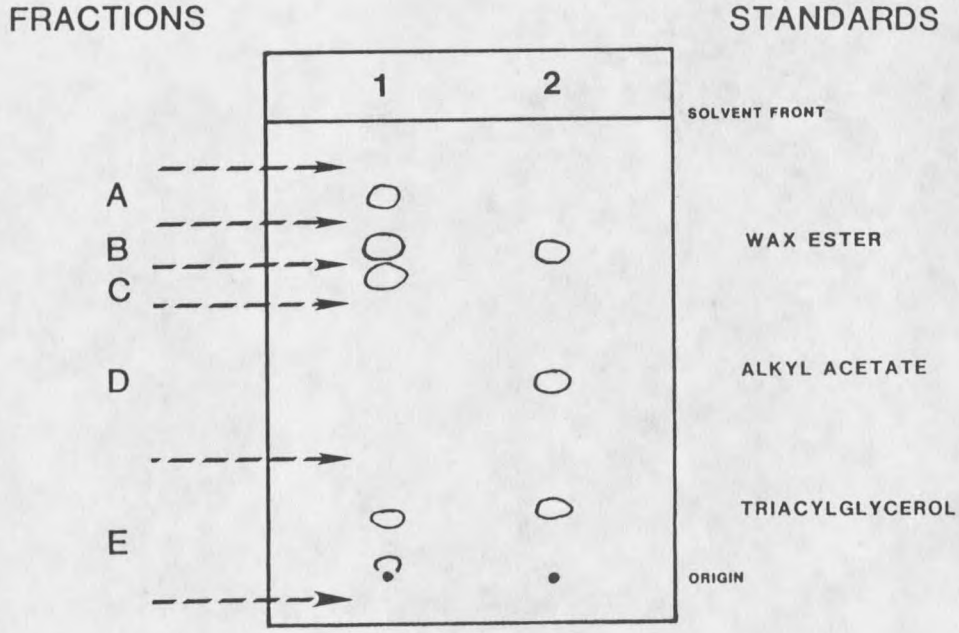


Figure 4. Illustration of thin-layer chromatographic resolution and fractionation of neutral lipids from the preputial glands of Microtus montanus. Lipids resolution was obtained on silica gel layers using hexane:diethylether (95/10; v:v). Lane 1 contains M. montanus total preputial gland lipids; standards are in lane 2.

From individual chromatograms the species-typical 5min peak areas (taken collectively) were quantified relative to a known quantity of a C_{32} hydrocarbon standard. Voles from all treatment groups had similar body weights ($P = 0.2042$; Kruskal-Wallis rank test for equality of groups).

Testosterone maintains the species-typical lipid (5min peaks) quantities, as well as preputial gland weights and total lipid quantities (Table 2). With testosterone therapy, the mean 5min peak levels from castrated voles are similar to untreated controls but are at least 10 times higher than those found with dihydrotestosterone or estradiol treatments. Comparison of testosterone vs estradiol treatments revealed that the mean 5min peak levels were roughly 40 times higher with testosterone treatment. In contrast, dihydrotestosterone is not as effective as testosterone for stimulating preputial gland growth and secretion. Of the preputial gland parameters measured, testosterone had its greatest effect on the preputial gland concentration of the 5min peak lipids.

These findings are consistent with earlier reports of androgen dependent biosynthesis of specific rodent preputial gland compounds (Brinck and Hoffmeyer 1984; Sansone-Bazzano et al. 1972; Mukherjea 1977). If the 5min peaks actually function as pheromones, these results are similar to the evidence that demonstrates production of preputial gland attractant odors is hormone sensitive

Table 2. Hormonal manipulations and male *Microtus montanus* preputial gland weights, preputial gland total lipids, and preputial gland species-typical lipids.[A]

Treatment[B] groups	Preputial gland weight (mg±SD)	Preputial gland total lipid (mg±SD)	Total preputial gland species-typical lipid (µg±SD)
Adult untreated control[C] N=7	71.5±24.4	27.8±22.0	225.2±129.9
Adult castrate Testosterone[C] N=7	56.9±25.7	21.8±13.9	238.1±180.4
Adult castrate Dihydrotestosterone N=6	21.8±13.5	5.2±4.2	19.7±20.0
Adult castrate Estradiol N=8	9.3±3.3	2.5±1.5	5.3±6.6
Juvenile castrate no further treatment N=8	8.2±5.4	4.6±2.7	15.0±16.3

A Values listed are means ± standard deviations.

B Exogenous hormone treatments for castrated voles were administered by silastic implants (20 mm x 1.02 mm ID x 2.16 mm OD) containing the various hormones. N=sample size.

C All values obtained from untreated controls and testosterone treated castrated voles were similar but were significantly different from all values of all other treatment groups (Mann-Whitney U tests, $P < 0.05$).

(Gawienowski et al. 1975, Brinck and Hoffmeyer 1984; Lucas et al. 1982). Additionally, the apparent testosterone maintenance of M. montanus preputial gland weight agrees with reports of a similar phenomena observed in laboratory rats and mice (Ebling 1977; Brown and Williams 1972). However, the finding of only a weak dihydrotestosterone action in M. montanus conflicts with studies suggesting dihydrotestosterone is the androgen which stimulates preputial gland activity in laboratory mice (Brain and Homady 1985) and rats (Ebling 1972). A vast difference between the 5min peak levels in testosterone and estrogen treated animals suggests that the 5min peak levels are greatly elevated in male M. montanus.

Geographic Variation Studies

To achieve better resolution of the 5min cluster of peaks (Figure 3), the 5min peak lipids were subjected to capillary column GC (DB-1). The resulting chromatograms revealed the 5min peaks were resolved into a series of 12 components which were numbered according to the scheme presented in Figure 5. Increased resolution (and increased complexity) of the species-typical 5min peak lipids afforded an opportunity to investigate the possibility of geographic variation within the 12 peak series.

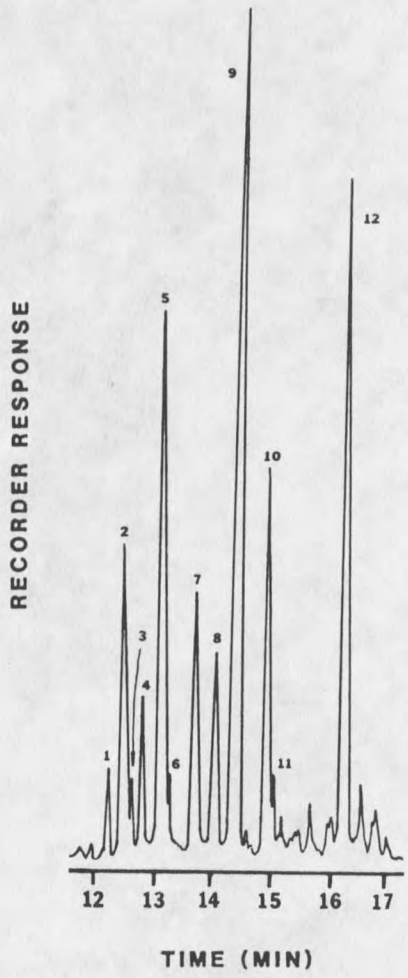


Figure 5. Capillary gas chromatography resolution of species-specific preputial gland lipids from Microtus montanus. The lipids were resolved on a 30 m 0.250 micron film, DB-1 fused silica capillary column; column temperature was programed from 170 to 250° C at 2° C/min.

For assessment of possible geographic variation, three preputial lipid samples were analyzed for each of MTMm, WYMm and UTMm; each sample consisted of the pooled preputial gland lipids from three individual mature male voles. (The qualitative GC and GC-MS behavior of the 5min peaks from UTMm, WYMm and MTMm were identical.) Relative peak areas from gas chromatograms provided estimates of geographic variation between the voles. In the MTMm, peaks 2+3 and 4 are larger than those same peaks in either UTMm or WYMm (Table 3). The same pattern is also found in the relative levels of peaks 7 and 8, where as the level of peak 5+6 is lower in MTMm.

Analytical studies of mammalian scent gland components have revealed the complex nature of odorous mixtures (Goodrich and Mykytowycz 1972; Stoddart et al. 1975, 1974; Eppel et al. 1979). Capillary column chromatography of the 5min peaks from M. montanus preputial glands shows the 5min peaks are composed of many different compounds. The 12 peak series of species-typical lipids has been found in three different populations of M. montanus. However, relative quantitative differences in the 5min peaks from voles collected from the different populations are apparent. The chromatogram variations between MTMm vs UTMm and WYMm are clearer than the differences between UTMm and WYMm. The distance separating the particular populations may account for that finding. While MTMm are

Table 3. Variation in gas chromatograms of species-typical lipids from the preputial glands of *Microtus montanus* from different geographic regions[1].

GC peak[2]	*Microtus montanus* source		
	Bozeman Montana (wild)	Grand Teton National Park (wild)	University of Utah (lab colony)
1	4 ± 2	4 ± 3	7 ± 2
2 + 3*	12 ± 2	6 ± 2	3 ± 3
4*	7 ± 2	4 ± 0.7	3 ± 0.5
5 + 6*	15 ± 1	22 ± 2	23 ± 3
7*	7 ± 0.7	1 ± 1	1 ± 0.3
8*	5 ± 1	0.7 ± 0.4	1 ± 0.5
9	16 ± 4	22 ± 8	15 ± 1
10 + 11**	14 ± 0.3	21 ± 12	22 ± 3
12	18 ± 2	21 ± 5	22 ± 5

1 Values listed are means of chromatogram peaks from three different pooled samples for each of the three sources; the numbers represent percentage of a particular peak relative to the total area, ± standard deviation.

2 Numbered peaks from capallary column GC chromatograms are listed. For the purpose of this study the areas under peaks 2 and 3; 5 and 6; and 10 and 12 were calculated as one peak.

* Surface lipid peak areas in voles collected from different locations are significantly different ($P<0.05$; 1 way ANOVA).

** Large differences in variances precluded statistical analysis by 1 way ANOVA.

separated from the other vole populations by approximately 150 miles, (including the continental divide), the WYMm and the original UTMm trap sites were separated by less than 15 miles. Differences in Arvicola terrestris scent gland compounds analyzed by gas chromatography are suggested to be the result of genetic variability between populations (Stoddart et al. 1975). Preliminary evidence suggesting that a chemosignal divergence can occur with geographically separated populations has been presented by Perrigo and Bronson (1983).

The complex nature of mammalian odor formulations provides an available means of varying chemosignal quality; alterations in compound structures or levels of one or more components are possible means of changing odor quality and hence the signal information. Such differences in the ratios of odor components have been suggested as a mechanism by which pheromones provide signals for gender recognition (Sokolov 1982), individual recognition (Gorman 1976), and species and subspecies recognition (Epple et al. 1979). With regard to species recognition in tamarins (Epple et al. 1979), variations in relative abundance of compounds are thought to contribute to differences in the information content of scent marks. The quantitative differences found in the 5min peaks from the geographically separated vole populations may represent a divergence of scent signals.

At this point the evidence concerning the species-typical lipids (5min peaks) from the preputial glands of M. montanus is in agreement with the postulated criteria of tne "working pheromone model". Of the rodent species tested, the 5min peaks are found only in M. montanus. The species-typical lipids are androgen sensitive. Those lipids are found in male voles with normal testosterone levels and are greatly diminished in castrated voles and in castrated voles treated with estradiol. Preliminary characterization of the 5min peaks suggests those compounds are within a volatility range shared by other mammalian scent gland compounds which carry species recognition information. Additionally, the species-typical lipids have been found in three different M. montanus populations, and results from geographic variation studies suggest differences in quantities of certain species-typical lipids from the different vole populations.

Given the possible pheromonal nature of the 5min peak lipids, it was decided that a more rigorous chemical analysis was warranted. The analyses that follow provide structural identifications for the 5min peak lipids. GC evidence suggests that the 13min and 15min peaks do not possess the molecular qualities of a volatile chemosignal. Hence those lipids were not subjected to additional analyses.

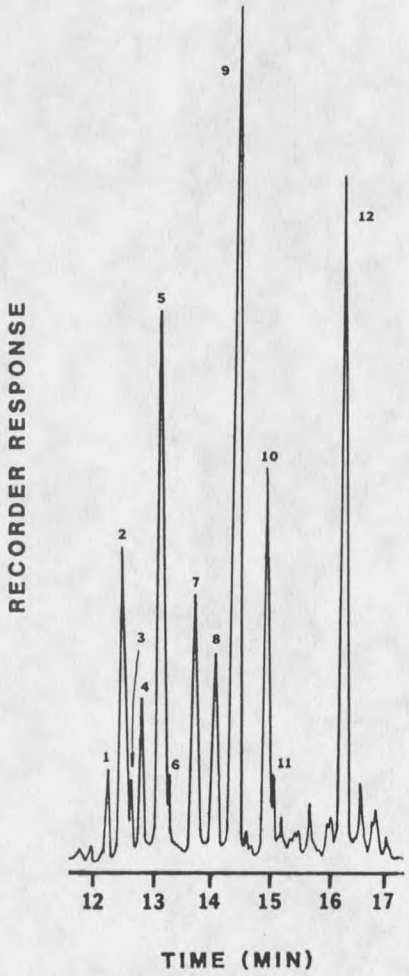
STRUCTURE ANALYSIS

Preliminary Characterization

The species-typical lipids (5min peaks) have the TLC mobility of esters (Figure 4). High resolution mass spectrometry provided elemental analyses of molecular ions and revealed molecular formulas (Table 4) which support the suspected ester structures. Additionally, the high resolution GC-MS revealed the elemental composition of ions which are characteristic of esters containing C_{16} fatty acids ($\underline{m}/\underline{z}$ = 256 = $C_{16}H_{32}O_2$) and C_{17} fatty acids ($\underline{m}/\underline{z}$ = 270 = $C_{17}H_{34}O_2$). Unsaturated fatty acids as well as saturated and unsaturated C_5 alcohols were also suggested by the mass spectra (Table 4). (See "Appendix A" for supplementary mass spectral data.)

The GC-MS evidence indicates the species-typical lipids are a complex series of esters which apparently differ by degree of unsaturation and the presence or location of one carbon atom. To simplify analyses of the complicated 12 component mixture, the species-typical lipids were fractioned by preparative GC. Although this technique did not isolate 12 individual components, it did resolve peaks 1-6 (GC Fraction A) from peaks 7-11 (GC Fraction B) and isolated peak 12 (GC Fraction C). The

Table 4. Structural evidence based on GC-MS and high resolution GC-MS of the species-typical lipids from the preputial glands of M. montanus.



GC peak	Molecular weight	Molecular formula	Number of alkyl double bonds
1	326	$C_{21}H_{42}O_2$	0
2	324	$C_{21}H_{40}O_2$	1
3	324	$C_{21}H_{40}O_2$	1
4	324	$C_{21}H_{40}O_2$	1
5	326	$C_{21}H_{42}O_2$	0
6	326	$C_{21}H_{42}O_2$	0
7	322	$C_{21}H_{38}O_2$	2
8	322	$C_{21}H_{38}O_2$	2
9	324	$C_{21}H_{42}O_2$	1
10	340	$C_{22}H_{44}O_2$	0
11	340	$C_{22}H_{44}O_2$	0
12	338	$C_{22}H_{42}O_2$	1

isolation of peak 12 afforded an opportunity to use an analytical technique (NMR) which is not well suited for analysis of complex mixtures.

To further simplify the analytical approach, the esters contained in each GC fraction were hydrolyzed and the resulting fatty acids (analyzed as FAMEs) and the free alcohols (obtained after saponification) were analyzed. GC (on DB-1 unless otherwise stated) and GC-MS (using DB-5 unless noted) were used to confirm the actual structures of the hydrolysis products as well as alcohol and acid reaction products. Further analyses provided the information necessary to make structure assignments for five major alcohols and the three most abundant fatty acids (Table 5). The relative abundance of the identified alcohols and acids in particular GC fractions later provided clues to the most likely combinations of acids and alcohols in the natural product esters.

The species-typical lipids contain two pairs of isomeric C_5 alcohols (Table 5). Initial GC retention comparisons indicated the presence of branched alcohols. For the saturated alcohols, GC-MS was diagnostic for the 3-methyl-and 2-methyl-1-butanols. The gas chromatographic behavior of standard alcohols was identical to that of the saturated alcohols that were identified. Although analyses of unsaturated alcohol mixtures was clouded by the fact that the GC techniques (DB-1, DB-5, DB-225, and

Table 5. Hydrolysis products of the species-typical esters from the preputial glands of Microtus montanus.

Structure Assignments		Analytical Evidence[1]					
						Standard Matches	
Alcohols	GC Fraction[2]	GC	GC-MS	NMR	Ozonolysis	GC	GC-MS
2-methyl-1-propanol	A		+				
3-methyl-1-butanol	A* B*	+	+			+	+
2-methyl-1-butanol	A B	+	+			+	+
2-methyl-2-buten-1-ol	B* C*	+	+	+		+	+
3-methyl-2-buten-1-ol	C	+	+	+		+	+
Acids							
(Z)-9-hexadecenoic acid	A B	+	+		+	+	+
hexadecanoic acid	A* B*	+	+			+	+
15-methylhexadecanoic acid	B* C*	+	+	+		+	+

1 The evidence from particular analytical techniques (indicated with a "+") supports the given structure. A "+" under "Standard Matches" indicates the natural product was identical to the GC or GC-MS behavior of a known standard of the designated alcohol or acid.

2 The letters indicate the particular GC fractions in which specific acids or alcohols were found. An asterisk indicates the major alcohols or acids in a given GC fraction.

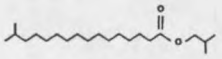
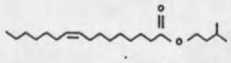
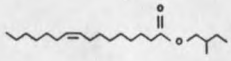
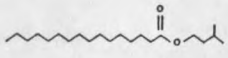
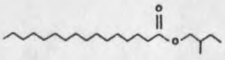
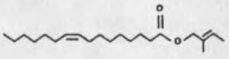
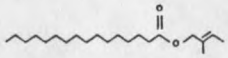
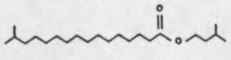
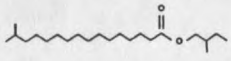
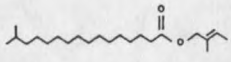
Suplelcowax) failed to resolve the suspected unsaturated alcohol isomers, NMR analysis (of peak 12) indicated the presence of two unsaturated alcohols which were identified as 2-methyl- and 3-methyl-2-buten-1-ol. The characteristic alcohol resonances (2-methyl-2-buten-1-ol: singlet, 2 H, δ4.44; quartet, J = 7 Hz, 1 H, δ5.54; 3-methyl-2-buten-1-ol: doublet, J = 7 Hz, 2 H, δ4.49; triplet, J = 7 Hz, 1 H, δ5.32) were identical to standard esters containing 2-methyl-(E)-2-buten-1-ol and 3-methyl-2-buten-1-ol. (See "Appendix B" for supplemental NMR data.) All other double bond positions are eliminated by the characteristic NMR resonances. The NMR spectra also revealed that in peak 12, 2-methyl-2-buten-1-ol is the predominant unsaturated alcohol. Although the mass spectra of the unsaturated alcohols are almost identical, the spectra do support the alcohol structures indicated by NMR. The mass spectrum of peak 1 suggested the presence of a C_4 alcohol (M^+-56 and M^+-57). The structure of 2-methyl-1-propanol was indicated by analyses of complete esters which are described later under "peak 1".

The species-typical esters contain three major fatty acids. Hexadecanoic acid was characterized by GC and GC-MS. (Z)-9-Hexadecenoic acid is the most abundant unsaturated fatty acid and that structure is supported by GC and GC-MS data. The position of the double bond was determined by analysis of ozonolysis products and the

double bond configuration was confirmed by comparing the GC behavior (DB-225) of (Z)- and (E)-9-hexadecenoic acid standards. 15-Methylhexadecanoic acid was identified by GC, GC-MS, and NMR. A branched C_{17} fatty acid was suggested by initial GC retention comparions. The fatty acid "iso" configuration was obvious in the NMR spectrum of peak 12 (doublet, J = 7 Hz, 6 H, δ0.084) and was confirmed by decoupling experiments. GC retention comparisons of known standards also confirmed the 15-methylhexadecanoic acid and eleminated the possibility of heptadecanoic acid or 14-methylhexadecanoic acid.

Given the indicated molecular weights of the species-typical esters and the identified acids and alcohols, all likely esters were synthesized from known acids and alcohols. The GC and GC-MS behavior of the analytical ester standards served as a template which guided structure assignments of the natural esters. HPLC analyses suggested the presence or absence of unsaturated fatty acids in the preputial gland esters. In addition, hydrogenation of the natural esters resulted in identifiable saturated products that provided further evidence for molecular arrangements. The structures of the complete esters are described below and a summary of the structure assignments is given in Table 6.

Table 6. Structure assignments for the species-typical lipids from the preputial glands of *Microtus montanus*.

GC peak	Structure	Compound Name	Standard[1] Matches GC	Standard[1] Matches GC-MS
1		2-methylpropyl 15-methylhexadecanoate	+	+
2		3-methylbutyl (Z)-9-hexadecenoate	+	+
3		2-methylbutyl (Z)-9-hexadecenoate	+	+
4				
5		3-methylbutyl hexadecanoate	+	+
6		2-methylbutyl hexadecanoate	+	+
7		2-methylbutenyl (Z)-9-hexadecenoate[2]	+	+
8				
9		2-methylbutenyl[3] hexadecanoate	+	+
10		3-methylbutyl 15-methylhexadecanoate	+	+
11		2-methylbutyl 15-methylhexadecanoate	+	+
12		2-methylbutenyl 15-methylhexadecanoate[4] and	+	+
		3-methylbutenyl 15-methylhexadecanoate	+	+

[1] A "+" indicates GC or GC-MS behavior which was identical to authentic standards.

[2] 3-methylbutenyl (Z)-9-hexadeconoate is also a possibility. The alcohol is suspected to contain a "trans" double bond (see peak 12).

[3] 3-methylbutenyl hexadecanoate is also a possibility. A "trans" double bond is suggested (see peak 12).

[4] 2-methylbutenyl 15-methylhexadecanoate accounts for ~70% of the area under peak 12. Available evidence indicates a "trans" double bond.

Peaks 2 and 3

Peaks 2 and 3 are isomers and were identified as 3-methylbutyl (Z)-9-hexadecenoate and 2-methylbutyl (Z)-9-hexadecenoate, respectively. The GC and GC-MS behavior of the identified esters was identical to synthetic esters. GC, GC-MS and HPLC indicated an unsaturated, C_{16} fatty acid (for both peaks) and mass spectra of the free alcohols was diagostic. Hydrogenation products of peaks 2 and 3 were identical to peaks 5 and 6.

Peaks 5 and 6

Peaks 5 and 6 are isomers and were identified as 3-methylbutyl hexadecanoate (peak 5) and 2-methylbutyl hexadecanoate (peak 6). GC and GC-MS of natural lipid FAMEs confirmed the hexadecanoic acid structure and mass spectra of free alcohols were diagnostic for 3-methyl-1-butanol and 2-methyl-1-butanol. GC and GC-MS of peaks 5 and 6 were identical to the respective 3-methyl- and 2-methylbutyl hexadecanoates.

Peak 12

2-Methyl-2-butenyl 15-methylhexadecanoate and 3-methyl-2-butenyl 15-methylhexadecanoate were identified as peak 12 components. GC-MS and HPLC suggested a saturated C_{17} acid. NMR Spectra and GC confirmed the 15-methylhexadecanoic acid structure. 2-Methyl-2-buten-1-ol

and 3-methyl-2-buten-1-ol structures were indicated by the NMR spectra. GC and GC-MS data support the structure of those alcohol isomers. The GC-MS ion at $\underline{m}/\underline{z}$ = 86 from peak 12 was diagnostic for 2-methyl-2-butenyl 15-methylhexadecanoate. An inability to resolve esters of 2-methyl- and 3-methyl-2-buten-1-ol as well as the free alcohols, leaves NMR as the positive indicator that peak 12 also contains 3-methyl-2-butenyl 15-methylhexadecanoate.

In addition to the esters identified, peak 12 also contains several minor components (~10% of the total peak 12 area). With regard to possible esters which fit the peak 12 GC and GC-MS behavior, those compounds are unexplained. Among those unexplained compounds are: 3-methyl-1-butanol, heptadecanoic acid, 14-methylhexadecanoic acid and two C_6 unsaturated alcohols. All likely synthetic esters of those questioned components plus the isomeric alcohols and 15-methylhexadecanoic acid previously found under peak 12 were, based on GC retention times, not possible contributors to peak 12.

Peaks 10 and 11

Peaks 10 and 11 are isomers and were identified as 3-methylbutyl 15-methylhexadecanoate and 2-methylbutyl 15-methylhexadecanoate, respectively. 2-Methyl- and 3-methyl-1-butanol were confirmed by GC-MS of free alcohols. Peaks 10 and 11 are the hydrogenation products

of peak 12 and 15-methylhexadecanoic acid demonstrates a GC behavior that is identical to the fatty acid of peaks 10 and 11. With respect to GC and GC-MS behavior, peak 10 was identical to 3-methylbutyl 15-methylhexadecanoate and peak 11 was identical to 2-methylbutyl 15-methylhexadecanoate.

Peak 9

Peak 9 contains 2-methyl-2-butenyl hexadecanoate. HPLC and GC-MS indicated a saturated fatty acid and an unsaturated alcohol. Hexadecanoic acid was characterized by GC and GC-MS. The synthetic ester of 2-methyl-2-butenyl hexadecanoate exhibits a diagnostic mass spectral ion at m/z = 86 which is almost absent in the 3-methyl isomeric ester. The ion at m/z = 86 in the peak 9 mass spectrum verifies the presence of 2-methyl-2-butenyl hexadecanoate. Due to the inability to resolve mixtures of suspected alcohols and hexadecanoic acid esters of those alcohols, 3-methyl-2-butenyl hexadecanoate is left as a possible peak 9 component.

Peak 7

2-Methyl-2-butenyl (Z)-9-hexadecenoate is a component of peak 7. Two carbon-carbon double bonds were indicated by GC-MS and HPLC revealed an unsaturated fatty acid. (Z)-9-Hexadecenoic acid was characterized by GC and ozonolysis. 2-Methyl- and 3-methyl-2-buten-1-ols were possibilities

suggested by GC and GC-MS. The peak 7 ion at $\underline{m}/\underline{z}$ = 86 was diagnostic for 2-methyl-2-butenyl (Z)-9-hexadecanoic acid. Suspected alcohols and esters of those alcohols were not resolved by GC, therefore, 3-methy-2-butenyl (Z)-9-hexadecanoate remains as a possible peak 7 ester.

Peak 1

Peak one was identified as 2-methyl-1-propyl 15-methylhexadecanoate. Although 2-methyl-1-propanol was not recoved in free alcohol samples, the mass spectrum was diagnostic for a C_4 alcohol. GC of possible esters showed that, based on retention behavior, the 1-butanol and 2-butanol esters of 15-methylhexadecanoic acid were not possibilities. The GC and GC-MS behavior of peak 1 was identical to 2-methyl-1-propyl 15-methylhexadecanoate.

Peaks 4 and 8

The analytical techniques used could not provide definitive structure assignments for peaks 4 and 8. However several inferences about those peaks were obtained.

The peak 4 ester possesses an unsaturated C_{16} fatty acid and a saturated alcohol. From GC retention comparisons, the fatty acid is not (Z)- or (E)-9-hexadecenoic acid. Hydrogenation products of peak 4 are peaks 5 and 6. This evidence suggested structural similarities between peaks 4, 5, and 6.

For peak 8, GC, GC-MS, and HPLC indicate the ester contains an unsaturated C_{16} fatty acid and an unsaturated alcohol. Based on GC behavior, the fatty acid is not (Z)- or (E)-9-hexadecanoic acid. Peaks 5 and 6 are the reduction products of peak 8. This information indicates the peak 8 ester shares structural similarities to either or both of peaks 5 and 6. The presence of an ion at m/z = 86 in the peak 8 mass spectrum indicates the presence of 2-methyl-2-buten-1-ol.

The preputial glands of mature male M. montanus contain novel species-typical esters. Those esters account for approximately 1% of the preputial gland total lipid and are composed of 2-methyl- and 3-methyl-1-butanols and 2-methyl- and 3-methyl-2-buten-1-ols and C_{16} and C_{17} fatty acids. This report is the first to describe esters containing saturated and unsaturated C_5 alcohols in preputial glands. Using GC retention comparison techniques, methyl branched fatty acids from laboratory mouse preputial glands (Sansone and Hamilton 1967; Snyder and Blank 1969) have been suggested. The structure of the 15-methylhexadecanoic acid reported here has been characterized by GC, GC-MS and NMR.

The uniqueness and physical properties of the species-typical preputial gland esters suggest they may serve as volatile chemical signals. The molecular weights of the species-typical esters from M. montanus range from 322 to

340; all compounds have total carbon numbers of either 21 or 22. Esters of butyric acid ranging from 21 to 28 total carbons (molecular weight range = 310 - 410) have been described (Epple et al. 1979) as chemical signals that convey species and subspecies information. Another study has demonstrated rodent olfactory responses to esters containing 22 carbon atoms (Stacewicz-Sapuntzakis and Gawienowski 1977). In terms of general compound class, total carbon numbers, and molecular weights, the novel esters from the M. montanus preputial glands are similar to previously described odorous compounds which demonstrate attractive properties.

The novel preputial gland compounds described are primarily esters of three different acids and five different alcohols. The compound identifications show 3-methyl-1-butanol is the most abundant saturated alcohol while 2-methyl-2-buten-1-ol is the predominant unsaturated alcohol. This finding suggests a degree of specificity in the ester biosynthesis. The significance of that specificity is unknown.

The configuration of the double bond in 2-methyl-2-buten-1-ol has not been unequivocally established. However, the NMR spectra of the "Z" and "E" isomers theoretically exhibit different chemical shifts for the resonance from the olefinic hydrogen (Silverstein et al. 1981). The NMR resonances of the natural product alcohol

and those same resonances from a standard ester containing 2-methy-(E)-2-buten-1-ol were identical in every detail. That evidence strongly supports the "E" configuration for the double bond of the natural product 2-methyl-1-buten-1-ol.

Peaks 4 and 8 are unidentified. From all analytical indications, the fatty acid of those peaks is an unsaturated C_{16} acid. That fatty acid is not (E)-or (Z)-9-hexadecanoic acid. It is possible that the fatty acid of the peak 4 and 8 esters is a double bond positional isomer of (Z)-9-hexadecanoic acid. The ozonolysis results are consistent with the possibility of a double bond at carbon 12 or higher. From the pattern seen with the identified alcohols, speculation leads to the suggestion that peak 4 contains 3-methyl-1-butanol and mass spectrometry suggests peak 8 contains 2-methyl-2-buten-1-ol.

The inability to find the 2-methyl-2-propanol in the free alcohol samples may be the result of a combination of problems. First, the volatility and water solubility of the C_4 alcohol may require special techniques to trap the alcohol after saponification. Second, 2-methyl-1-propanol represents approximately 5% of the total alcohols. Due to the high volatility of the alcohol, it is probable that significant amounts of the C_4 alcohol were lost during sample manipulations. Such losses could leave the remaining alcohol at levels below detection limits of the

analytical instrumentation.

It has been theorized (Wilson and Bossert 1963) that through evolution, the chemical properties of a pheromone are manipulated to maximize effectiveness for a given function. Speculation on the relationship between pheromone functions vs. molecular size follows a path of predicting "persistence" or "fade-out" qualities of signaling chemicals relative to expected functions. A hypothetical pheromone should be composed of the most efficient formulation of "persistant" vs. "diffusion" qualities: the relative proportion of those qualities will depend on the pheromone function. It follows that chemosignals mediating short-lived phenomena, for instance alarm responses, should possess a readily diffusible, highly volatile, nature. On the other hand, in order to maximize the communicative efficiency of the sender, home range marking signals should be more persistent. This persistence allows an animal to advertise a signal over a long distance for a long period of time and requires only minimal re-deposition to insure constant signal emission.

The persistence design feature of pheromones is not without limitations: volatility is correlated with molecular weight in a given homologous series. As molecular weight (and persistence) increases, eventually vapor phase existence of a molecule becomes impossible. The upper practical volatility limit then is an important

design factor for pheromones. Wilson and Bossert (1963) suggested that, due to physiochemical characteristics restricting volatility of large compounds, the upper-limit molecular weights of volatile pheromones would fall in the range of 300 to 400. The compounds which facilitate species and subspecies recognition in tamarins (Epple et al. 1979) span a molecular weight range of 310 to 410. The M. montanus species-typical preputial gland lipids have molecular weights ranging from 322 to 340. The tamarin species recognition cues as well as the identified M. montanus preputial gland esters possess molecular weights in the range of what might be predicted for a species recognition pheromone.

Diversity and complexity of scent gland compounds is common in mammalian chemical communication systems (Goodrich and Myktowycz 1972; Stoddart et al. 1975; Stoddart 1974; Epple et al. 1979; see Beauchamp et al. 1976 for a review); if the 5min peaks of M. montanus preputial glands are pheromones, they certainly fit that pattern of complex odor formulations. The occurrence of at least 12 compounds which differ by the position or presence of only one carbon is an indication of the complexity of the M. montanus species-typical lipids. Finding two compounds under peak 12 demonstrates the similarity of certain compounds. It is apparent that given a few predominant alcohols and fatty acids, the vole preputial glands produce

esters in a manner that maximizes end-product diversity.

Given this information, and the previously described compound identifications, it is appropriate to reconsider Table 2 and the relative differences in ester quantity found in M. montanus collected from different geographic areas. Those comparisons (Table 2) suggest MTMm preputial glands contain higher concentrations of peaks 2, 3, 7, and 8 than do UTMm or WYMm. Those esters contain unsaturated fatty acids. Although the significance of those unsaturated esters is unknown, it is possible that an increase in unsaturated esters affects the odor quality of the species typical esters.

This work represents the initial work aimed at identifying a rodent species recognition pheromone. Novel esters from the preputial glands of M. montanus have been identified and those esters are similar to the esters which function as species recognition cues in tamarins. From the results of experiments conducted for this study, there is no reason to believe the novel esters of the M. montanus preputial glands can not act as species-recognition pheromones.

FURTHER CONSIDERATIONS

Given the results described in this report, it is important to bioassay the lipids from the preputial glands of the adult male voles. Relatively simple two-choice preference tests could provide information on the ability of female voles to distinguish between con- and heterspecific male preputial gland odors. Those bioassays should test female preferences for conspecific preputial gland lipids, and should test specific preputial gland lipid fractions. Those bioassays could also provide information with which to evaluate the bioactivity of the species-typical esters from the preputial glands of M. montanus. Extending the analyses described in this communication to include M. montantus and M. pennsylvanicus from allopatric populations may give some insight on possible character divergence of species-typical odors in the sympatric voles. Additionally, similar studies on other sympatric rodents may provide evidence which supports the role of preputial glands in production of species recognition pheromones.

Once bioactive substances have been found, the active components could be mixed with heterospecific odor substances and female behavoral responses to the conspecific/heterospecific odor mixture assayed. The

results of such experiments may find attractive responses which were similar to those exhibited when the females were presented with only conspecific odor stimuli. If so, a stronger argument could be made for the existence of a species recognition pheromone.

If the species-typical esters are bioactive, a logical "next step" would be the bioassay of the synthetic esters used as analytical standards in this study. Such assays would permit evaluation of the relative importance of a single or a few active esters from the complete species-typical ester mixture. Subsequent studies might also consider the possibility that complete esters serve as "propheromones". Perhaps esterases release free alcohols and fatty acids which then function as a pheromone(s). Behavioral assays of synthetic alcohol and acid mixtures or a mixture of alcohols, acids, and the corresponding esters could shed light on the possible role of free alcohols and acids in the induction of pheromonal responses.

Our understanding of the mechanisms of the hormonal regulation of lipogenesis in preputial glands is at a very primitive level. Comparative investigations could provide baseline informating on species differences in androgenic control of lipogenesis and pheromone production in preputial glands. The results described in this report and another preputial gland investigation (Brain and Homaday 1985) suggest that Mus musculus (lab mice), M. montanus

and M. pennsylvanicus represent three preputial gland systems which are regulated by different mechanisms. Studies on the preputial glands of the different species could center on: (1) the mechanisms by which testosterone is converted to dihydrotestosterone; (2) the interaction of intracellular androgens with specific receptors, and nuclear components, and; (3) the resulting biosynthetic effect of the active hormone(s).

The biosynthesis and esterification of the C_5 alcohols might also provide the basis of some interesting studies. Perhaps the C_5 alcohols are reduction products of C_5 acids. If so, it is possible the acid analog of 3-methyl-1-butanol is the "branched-starter" for biosynthesis of the "iso" C_{17} fatty acid. Speculation on the biosynthesis of 3-methyl-2-buten-1-ol leads to other intriguing biosynthetic problems. It is possible that the alcohol is derived from isoprene or intermediates of isoprene biosynthesis.

The extension of analyses reported here to other species, as well as females of those species, could solidify the role of pheromonal species recognition. However, only through the continued cooperative efforts of biologists and chemists will such studies be realized. Hopefully, collaborative efforts will be stimulated by the many interesting and challenging questions concerning a very basic understanding of mammalian chemical communication.

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APPENDICES

APPENDIX A

MASS SPECTROMETRY

Table 7. Mass spectral fragmentation patterns of species-typical lipids from the preputial glands of *Microtus montanus*.

GC Peak	Key Mass Fragments[1]
1	M^+ = 326(6) 41(49), 43(84), 55(41), 56(71), 57(100), 60(18), 69(22), 70(23), 71(22), 73(11), 83(11), 85(6), 227(4), 253(3), 270(15), 271(21), 272(4)
2	M^+ = 324(5) 43(38), 54(4), 55(9), 69(26), 71(100), 82(13), 83(40), 84(12), 97(19), 98(17), 111(12),112(2), 125(9), 135(7), 153(6), 217(5), 236(33), 237(27)
3	M^+ = 324(2) 43(69), 54(13), 69(54), 71(100), 82(16), 83(54), 84(20), 97(28), 98(18), 111(12), 112(26), 125(12), 135(10), 153(6), 217(4), 236(23), 237(30)
4	M^+ = 324(4) 41(34), 43(94), 54(12), 55(59), 57(10), 67(7), 69(27), 70(41), 71(100), 83(15), 84(11), 97(15), 111(7), 152(5), 153(3), 236(15), 237(19), 281(2)
5	M^+ = 326(3) 41(1), 43(37), 55(12), 57(6), 67(1), 70(100), 71(44), 73(3), 87(2), 111(2), 239(7), 240(2), 256(4), 257(5), 283(1)
6	M^+ = 326(2) 41(23), 43(52), 55(24), 57(21), 67(4), 70(100), 71(52), 73(9), 87(5), 111(4), 239(16), 240(3), 256(12), 257(5)
7	M^+ = 322(0.4) 41(60), 43(23), 53(9), 55(51), 57(14), 67(27), 68(20), 69(100), 70(11), 71(7), 83(34), 86(5), 95(16), 97(25), 111(15), 137(7), 153(4), 217(6), 235(11), 237(4), 252(4), 253(6)
8	M^+ = 322(0.5) 41(58), 43(22), 54(9), 55(64), 57(13), 67(26), 68(20), 69(100), 70(13), 71(10), 83(37), 86(4), 95(16), 97(30), 111(17), 137(7), 153(4), 217(5), 235(10), 237(3), 252(4), 253(5)
9	M^+ = 324(5) 41(60), 43(51), 53(9), 55(26), 57(52), 67(28), 68(100), 69(74), 70(16), 71(36), 85(27), 86(30), 95(8), 239(30), 240(6), 257(10), 258(3)
10	M^+ = 340(3) 41(13), 43(42), 55(15), 57(14), 67(2), 70(100), 71(59), 87(3), 95(2), 97(5), 111(3), 129(3), 253(3), 270(5), 271(4)
11	M^+ = 340(2) 41(18), 43(30), 55(17), 57(27), 67(2), 70(100), 71(57), 87(7), 95(3), 97(8), 111(2), 129(5), 253(7), 270(13), 271(8)
12	M^+ = 338(6) 41(58), 43(57), 53(8), 55(29), 67(20), 68(100), 69(87), 70(17), 71(30), 85(27), 86(38), 95(11), 97(12), 253(11), 269(3), 270(2), 271(13), 272(3)

[1] Mass fragment lists are not complete but contain characteristic and diagnostic ions. Values listed are m/z (relative intensity). Base peaks (100% relative intensity) are underlined.

APPENDIX B

NUCLEAR MAGNETIC RESONANCE SPECTROMETRY

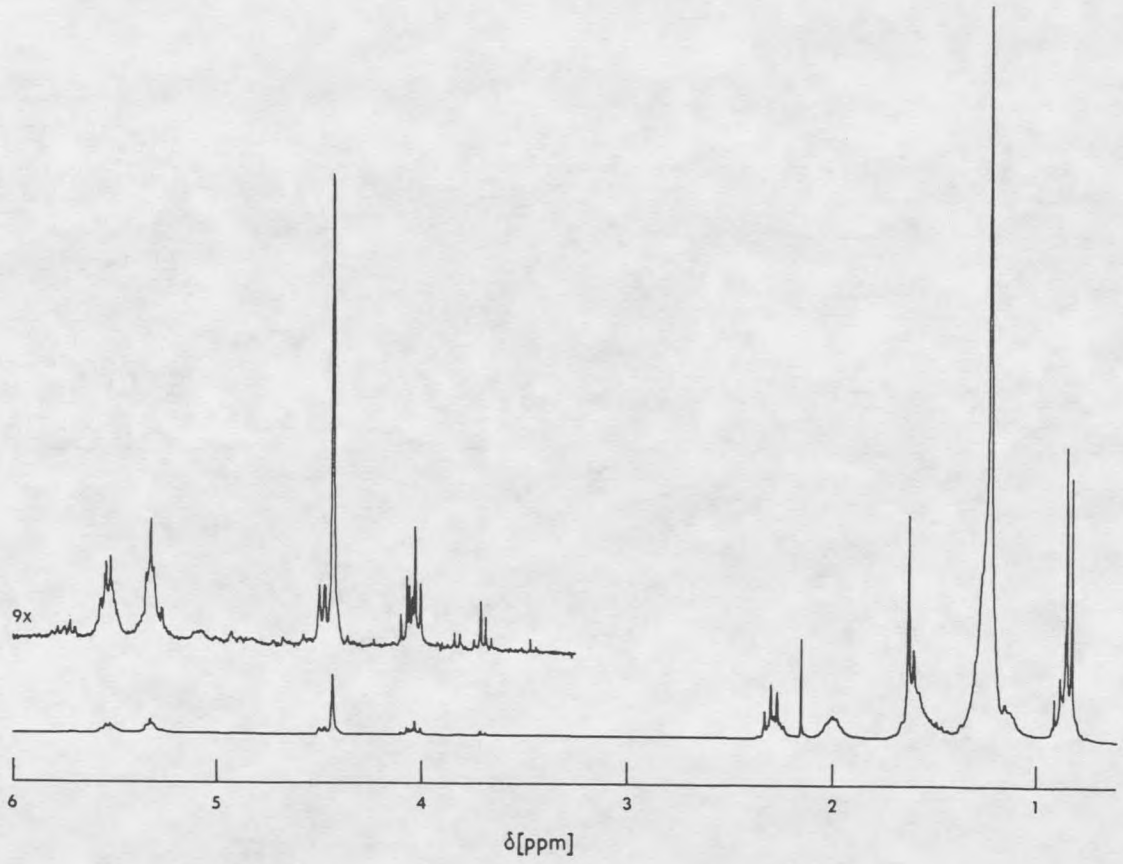


Figure 6. Nuclear magnetic resonance spectrum of gas chromatography fraction C. Peak 12 accounts for ~ 90% of the fraction C mixture. Fraction C was resolved by preparative gas chromatography.

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