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

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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APPROVAL

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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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TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

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.

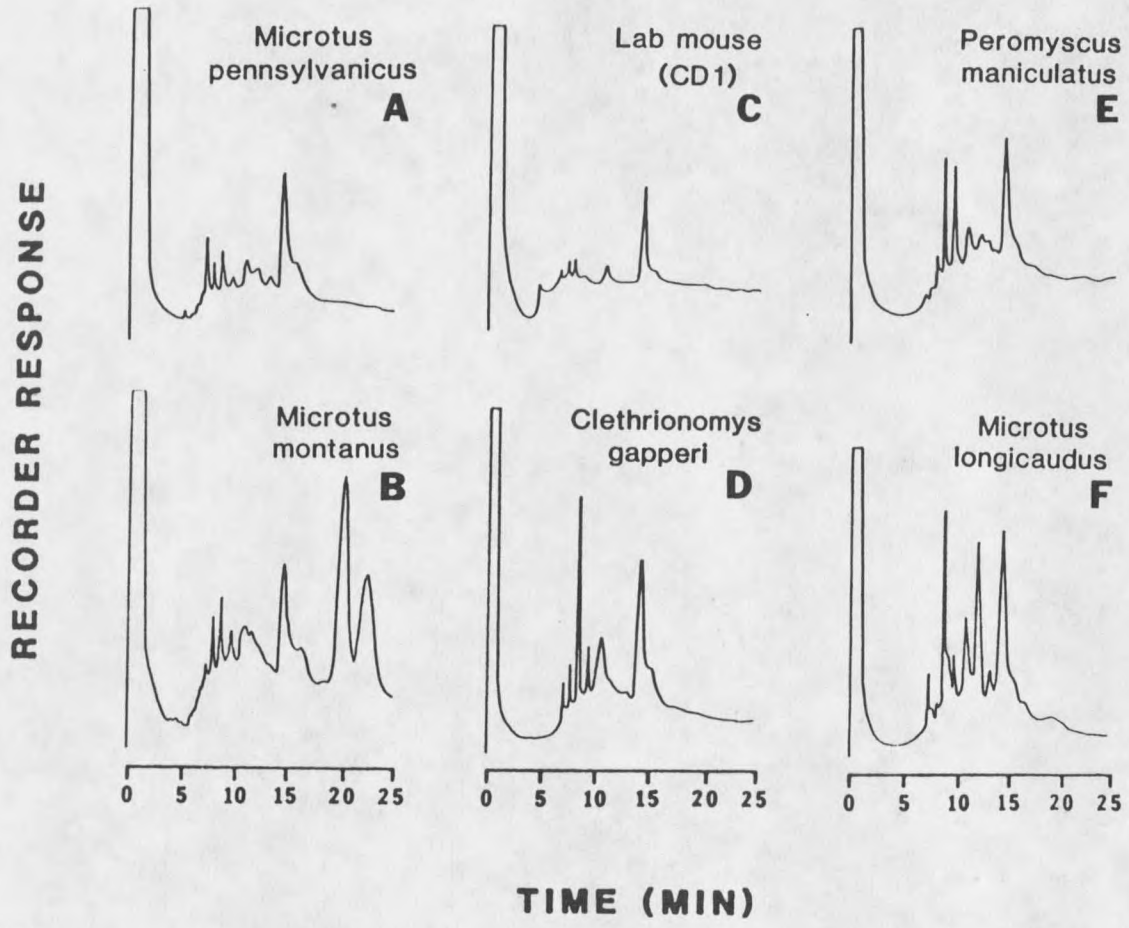

Figure 1. Surface lipid gas chromatograms from various rodents. The chromatograms were obtained on a 2 m, 2 mm ID, glass column packed with 5% SE-30; column temperature was programed from 180 to 295° C at 15° C/min.

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

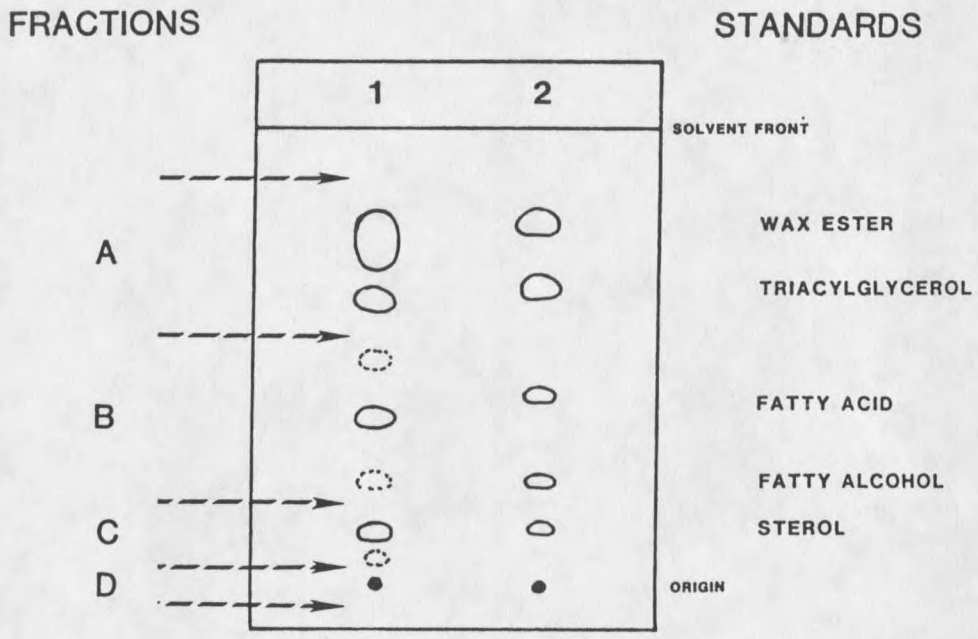

Figure 2. Illustration of thin-layer chromatographic resolution and fractionation of surface lipids from Microtus montanus. Lipid resolution was obtained on silica gel layers using hexane:diethylether:acetic acid (60/40/1; v:v:v). Lane 1 contains M. montanus total surface lipids; standards are in lane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