

## ANALYSIS OF SECRETIONS FROM SCENT-PRODUCING GLANDS OF BRUSHTAIL POSSUM (*Trichosorus vulpecula* Kerr)

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**Abstract**—A characterization at a molecular level of the chemical composition of the secretions of the two pairs of paracloacal glands and of the sternal sebum of the brushtail possum (*Trichosorus vulpecula* Kerr) has been undertaken with a view to evaluating the potential of volatile "pheromone" components as species-specific attractants for use in novel baiting systems. Particular attention has been given to the respective fatty acid fractions produced by chemical hydrolysis, since these are believed to be the products of postemission microbial degradation (fermentation) of the secretions. In all instances, the highly complex distribution of the constituents present in these organic components of the secretions were shown to be virtually identical in adult males and females. A unique suite of low-molecular-weight branched-chain carboxylic acids has been shown to be produced by chemical degradation of the holocrine (oil-secreting) gland secretion. This odor signature is suggested to function as a unique "scenting-the-habitat" pheromone that might act as an attractant to all members of the species.

**Key Words**—Paracloacal glands, fatty acids, mammalian pheromones, possum, *Trichosorus vulpecula* Kerr.

### INTRODUCTION

The herbivorous brushtail possum (*Trichosorus vulpecula* Kerr) was introduced to New Zealand from Australia late in the 19th century, primarily with a view to starting a fur and skin industry. In the absence of diseases and predators the possum population proliferated to the point where it is now widespread through-

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out the country. Agricultural authorities now consider the possum to be a major pest in native forest and horticultural areas and, most particularly, in pastoral areas where it is the known vector for the transmission of bovine Tb to cattle and deer.

Present strategies designed to manage populations of the possum have focused principally upon the use of the poison sodium monofluoroacetate (Tenate). Arguments against the undeniably cost-effective use of this poison center around the lack of species-specificity, there being apparently deleterious effects upon both endemic and exotic wildlife (Morgan, 1982). Ecologists also recognize that a significant proportion of the possum population is poison-shy, that is, the animals can detect the presence of the poison in a bait.

With the purpose of seeking methods of circumventing either or both of these phenomena, we have undertaken a study of the chemical compositions of secretions of several glands of the possum with the ultimate expectation of developing a synthetic "pheromone"-based attractant for incorporation into the baiting system.

There have been many studies devoted to the identification and use of pheromones in insect mating disruption, and these studies have led to the development of successful population suppression strategies (Kydonieus and Beroza, 1982; Ridgway et al., 1990). However, studies that have addressed the nature and function of chemical signals in mammalian systems have revealed them to be much more complex by comparison (Albone, 1984). A few reports of the use of animal odors in animal population control studies have been published. For the greater part, these address the use of kairomones as "predator" (carnivore) odors in herbivore pest control (Sullivan and Crump, 1984; Sullivan et al., 1988, 1990a,b; Müller-Schwarz, 1990; Boag and Mlotkiewicz, 1991) and demonstrate the obvious potential that such repellency strategies have in certain situations. On the other hand, the use of mammalian pheromones as conspecific attractants in field situations has, to our knowledge, only recently been evaluated with mustelids and shown to have some potential as a species-specific (bait) attractant (Clapperton and Woolhouse, 1991).

Because the nature of the possum problem predicates the utilization of an approach based upon attractancy, we have undertaken an investigation of the organic constituents of secretions from the two pairs of paracloacal glands that have been characterized as oil-secreting (holocrine) and as cell-secreting (apocrine) glands (Russell, 1987) and also from the sternum region, within which is a modified sebaceous gland (Tyndale-Biscoe, 1975). Explanations of possum behavior elicited by each of the three gland secretions have been suggested (Bolliger and Hardy, 1944; Bolliger and Whitten, 1948; Thomson and Pears, 1962; Tyndale-Biscoe, 1975) but direct evidence of the importance of scent(s) in the wild has not been established. Field observations indicate that the holocrine secretion is delivered in copious quantities when the possum is aroused

by handling, fighting, or other stimuli and that the cell-containing material is secreted more or less continuously in the urine and feces, but not in quantity when the animal is excited (Thomson and Pears, 1962). Males and females were observed to show a number of behavioral responses to the holocrine scent of males, in particular, that were interpreted as being important in defense. By virtue of its presence in the urine, the cell secretion was considered to be important in recognition of sex and in territorial marking. These authors also found that the sternum gland sebum elicited no marked responses from either males or females of the species when compared to the holocrine scent. However, more recent studies of an anecdotal nature indicate that this secretion is implicated in defining social status and marking (Morgan, personal communication). Bioassays of this secretion have also been shown to elicit pronounced olfactory investigation in other conspecifics (Biggins, 1979). In this latter work, all major lipid classes were shown by thin layer chromatography (TLC) to be represented in the paracloacal gland secretions, which were both more complex than those present in the sternal hair extracts. The putative presence of low-molecular-weight alcohols in the holocrine oil was suggested as the major class responsible for the musky odor, although no bioassays on these secretions were conducted. As the results of preliminary pen bioassays for conspecific attractancy of the ether-soluble materials recovered from each set of paracloacal glands of both males and females revealed no enhanced behavioral activity (Innes and Frampton, 1991), we elected to further pursue the possibility that a unique "odor signature" with potentially attractive characteristics might be generated after emission as a result of (aerobic) microbial degradation of the glandular secretions. The significance of such fermentation products in chemical recognition among conspecifics of other mammalian species has been addressed by a number of researchers (Gorman, 1976; Albone and Perry, 1976; Albone, 1983; Gorman and Trowbridge, 1989), and fatty acids have been shown to be predominant in the *in situ* volatiles generated anaerobically within the sacs of many carnivore species. Whereas microorganisms have been observed in the sacs of carnivores (Albone, 1984), none has apparently been observed within that of the possum (Cowan, personal communication).

Herein we describe a preliminary chemical characterization of each of the gland secretions and, more particularly, of the respective fatty acid profiles produced by saponification that are expected to reflect those generated by bacterial fermentation in the wild.

#### METHODS AND MATERIALS

Holocrine (oil-producing) and apocrine (cell-producing) glands were excised from freshly killed wild adult possums and immediately frozen prior to subsequent manipulation. The volumes of the contents of each member of the pair

of glands were virtually identical and were expressed after puncturing the glands and pooled (10–20 sets) into diethyl ether (ca. 50 ml). The ethereal suspension was stirred vigorously at ambient temperature until finely dispersed, the ether decanted, and the operation repeated. The combined ether phase was filtered and concentrated by slow distillation (<35°C); the final traces of solvent were then removed in a stream of argon to furnish the lipid fractions. Those lipids recovered from the holocrine glands of adult males and females were consistently ~30% by weight of the secretion and the ether-insoluble residues (obtained after lyophilization) were ~5% by weight. Similarly recoveries from the apocrine glands were ~5% lipid and ~10% residue. Sternum gland secretions were isolated from solutions in ether, as described above, and obtained as waxy solids by swabbing the pigmented chest/hair regions of the same animals. Swabbings from contiguous chest/hair areas were also taken and the lipids isolated similarly.

Saponification of each lipid specimen was carried out using 2.0 M potassium hydroxide in 50% aqueous ethanol, and the hydrolysis products were isolated by standard procedures. Fatty acids were esterified with boron trifluoride-methanol prior to chromatographic analysis on either an HP5890II GC equipped with a 25-m HP Ultra 2 capillary column operating in the split mode at either (1), 50° (1 min) then 5°/min to 280°C (3 min) or (2), 40° (1 min) then 8°/min to 340°C (36.5 min) prior to flame ionization (FID) or mass selective detection (MSD) and analysis. Tetratriacontane (*R*, 32.4 min, system 2) was employed as an internal reference. TLC was performed on aluminum-backed silica plates (0.25 mm Merck) using standard solvent systems (Belitz and Grosch, 1986).

*Synthesis of Carboxylic Acids.* ( $\pm$ )-4-Methyl- and 5-methyl-hexanoic acids (caproic acids) were prepared by standard methods (Milburn and Truter, 1965) from *s*-butyl bromide and isobutyl bromide respectively, by sequential homologation firstly with cyanide ion and secondly with sodium diethylmalonate followed by hydrolytic decarboxylation. ( $\pm$ )-5-Methylheptanoic acid was prepared according to the general method via sequential two-carbon homologation of *s*-butylbromide, reported by Vogler and Chopard-dit-Jean (1960). 6-Methylheptanoic acid and ( $\pm$ )-6-methyl- and 7-methyl-octanoic acids respectively, were all prepared by the addition of the appropriate alkyl Grignard reagent to 2,3-dibromotetrahydropyran followed by a standard sequence of reduction and oxidation reactions (Crombie and Harper, 1950a,b). All of the above known carboxylic acids (see Figure 3 below) were purified by distillation under reduced pressure to give colorless liquids. The 300 MHz <sup>1</sup>H- and <sup>13</sup>C NMR data were entirely consistent with expectation. Samples of each were methyl-esterified as described above. Efforts are presently underway to establish unequivocally the stereochemistry of these anteiso acids.

## RESULTS

*Holocrine Gland Secretions.* In an attempt to establish the presence or absence of volatile organic compounds in the heavy odorous lipid fraction, a headspace sampling technique employing Tenax GC (followed by solvent elution rather than thermal desorption) was applied to ca. 1-g samples of oil and the eluted "volatiles" subjected to GC (Olafsdottir et al., 1985). By this technique insignificant amounts were recovered and no identifications were made. TLC analysis of the lipid material with reference to representative lipid standards confirmed the presence of most neutral lipid classes with triacyl glycerides predominating and capillary GC of the whole oils from both adult male (Figure 1) and female animals revealed essentially the same complex profiles.

When compared qualitatively to a selection of lipid standards (e.g., milk fat,  $C_{2:0}$ - $C_{18:0}$  cholesterol esters), holocrine lipids are seen to be characterized by the occurrence of low-molecular-weight triacylglycerides and by the occurrence of two pairs of relatively abundant components with retention times in the range 6-10 min. Diazomethylation experiments revealed (Figure 2) these to be saturated fatty acids and GC-MS (GC conditions 1, Methods) confirmed molecular weights corresponding to  $C_7$ - $C_9$  acids. Moreover, analytical methanolysis of the whole lipid under conditions that are known to leave free fatty acids unaffected (Bannon et al., 1982) produced the same suite of methyl esters as did diazomethylation.

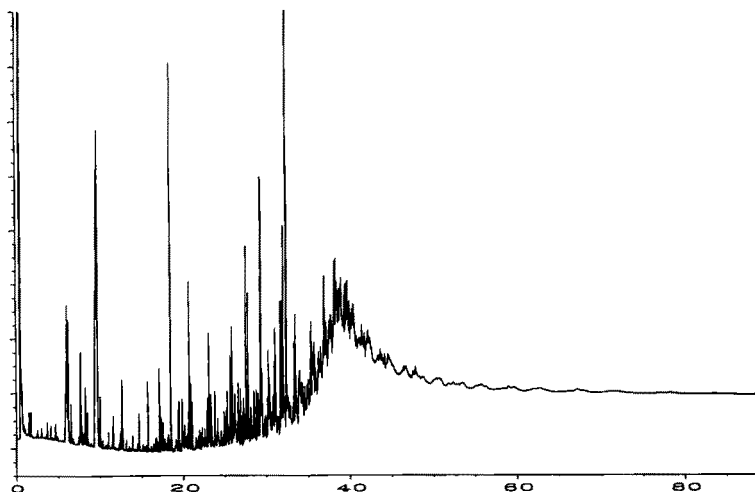


FIG. 1. Gas chromatogram (GC conditions 2, Methods) showing total neutral lipid profile from male (and female) holocrine anal glands.

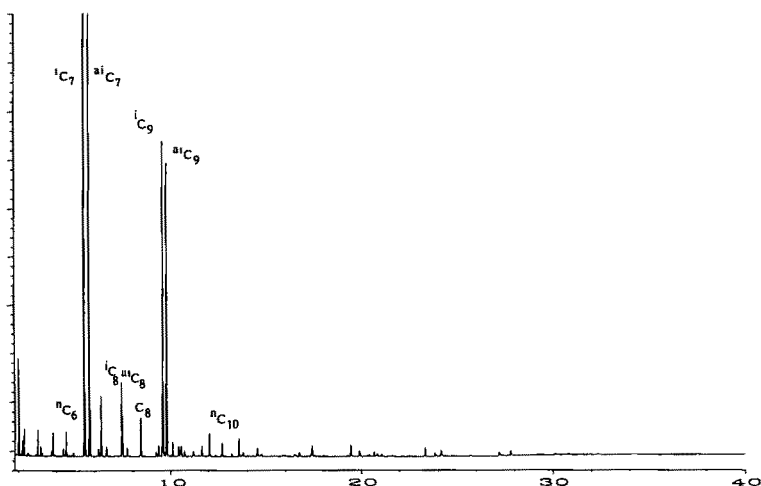


FIG. 2. Free fatty acid methyl ester profiles from diazomethylation of the male (and female) holocrine gland lipids.

Saponification and subsequent esterification of samples of both male and female holocrine lipids confirmed the above findings and revealed (Figure 3) also the presence of  $C_{14}$ ,  $C_{16}$ , and  $C_{18}$  fatty acids derived from the structural lipid component of the secretion. Further confirmation of the fact that these low-molecular-weight components were branched-chain acids stems, firstly from the chromatographic behavior of the corresponding methyl esters with respect to a standard *n*-alkanoic acid methyl ester cocktail and secondly, from an examination of (GC) MS data, which revealed prominent fragmentation attributable to the loss of  $C_3H_7$  for the more mobile iso series (denoted *i* in Figure 2) and to  $C_2H_5$  losses for the anteiso series (denoted *ai* in Figure 2), with respect to that of ester methoxyl loss (Abrahamsson et al., 1963). The presence as minor constituents only of esters derived from *n*-hexanoic, *n*-octanoic, and *n*-decanoic acids was also established (by coelution). That these abundant components are in fact the  $C_7$ ,  $C_8$ , and  $C_9$  iso and anteiso branched-chain fatty acids was established unequivocally by synthesis and methyl ester coelution experiments. Each of these compounds was synthesized by published procedures (Figure 4); the anteiso acids were obtained as racemates.

*Apocrine Gland Secretion.* TLC analyses of the odorless lipid fractions recovered from both male and female apocrine glands show essentially the full suite of neutral lipid classes, in which the triacylglycerides predominate. Capillary GC of both lipid extracts are complex but very similar at this level of resolution and analysis. Unlike the holocrine secretion, the apocrine lipids con-

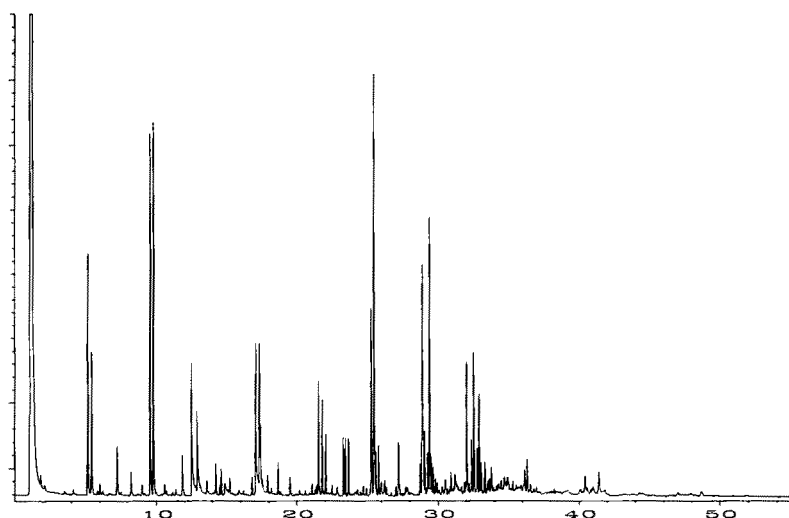


FIG. 3. Fatty acid methyl ester profile (GC conditions 2, Methods) from saponification of the male (and female) holocrine lipids.

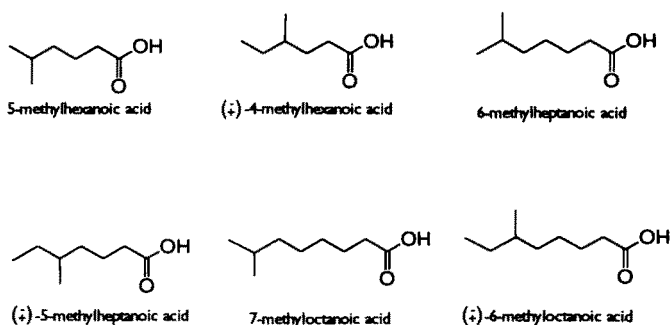


FIG. 4. Iso and anteiso aliphatic carboxylic acids shown to be present as "free fatty acid" and as acylglyceride in the male and female holocrine secretions. All have been authenticated by synthesis.

tain (Figure 5) larger amounts of regular triacylglycerides (*R*, 40–75 min) typical of animal tissue fats (Davy et al., 1983).

In accord with expectation, saponification and esterification of the fatty acids recovered from both adult male and adult female apocrine lipids clearly showed (Figure 6) the suite of  $C_{10:0}$ ,  $C_{12:0}$ ,  $C_{14:0}$ ,  $C_{16:0}$ ,  $C_{18:1\omega6}$ ,  $C_{18:2\omega6}$ ,  $C_{18:1\omega9}$ ,  $C_{18:3\omega3}$ , and  $C_{18:0}$  acids in which  $C_{16:0}$  and  $C_{18:1\omega9}$  predominate.

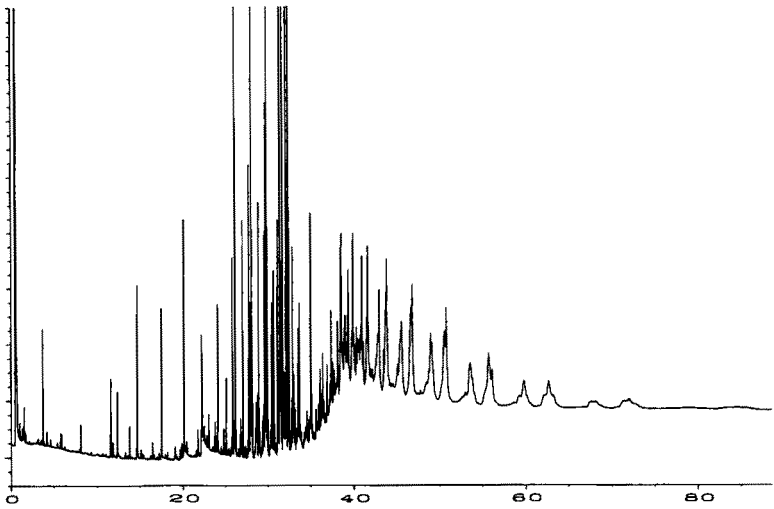


FIG. 5. Gas chromatogram (GC conditions 2, Methods) showing total neutral lipid profile from male (and female) apocrine glands.

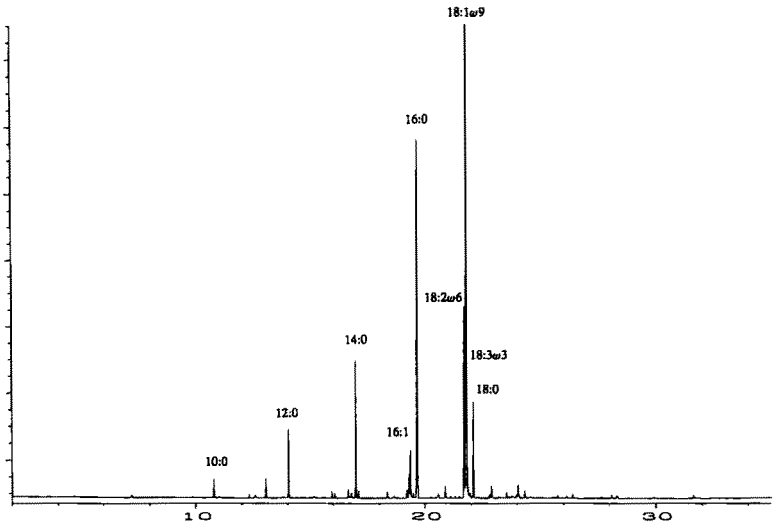


FIG. 6. Fatty Acid methyl ester profile (GC conditions 2, Methods) from saponification of the male (and female) apocrine gland lipids.



*Sternum Lipids.* An examination of the respective pairs of the waxy yellow sternum and contiguous-skin lipids recovered from both adult male (Figures 7 and 8) and adult female animals indicates little compositional variation in the relative abundances of the lipid components between the sexes and between those recovered from the easily recognized sternum and surrounding skin regions of each animal. TLC analysis suggests that the dominant lipid classes are those corresponding to the steryl/wax ester class; triacylglycerides are undetectable under these conditions. The predominant suite of components ( $R_t = 42\text{--}55$  min) is believed to be representative of the wax ester (rather than the steryl ester) class. Authentic  $C_{16:0}$ ,  $C_{18:1}$ ,  $C_{18:0}$  cholesteryl esters coelute with those species indicated with an asterisk in Figure 7.

Saponification and esterification of each of the pairs of lipids reveals, not surprisingly, virtually identical fatty acid profiles (Figure 9, for example). With respect to the whole sternum lipid profile (Figure 7), this finding suggests that the wax esters are perhaps representatives of a less common lipid class such as the diester group, that is based upon an  $\alpha$ -hydroxyacid or an alkane-1,2-diol (Nicolaidis, 1974; Albone, 1984). This is inferred also from a comparison of the gas chromatogram of the neutral lipids from orange roughy oil, a marine wax ester lipid class that is known to comprise regular linear esters derived from  $C_{16:1}\text{--}C_{22:1}$  acids and  $C_{18:1}\text{--}C_{24:1}$  alcohols and that elute under these conditions in the  $R_t = 35\text{--}41$  min region (Buisson et al., 1982).

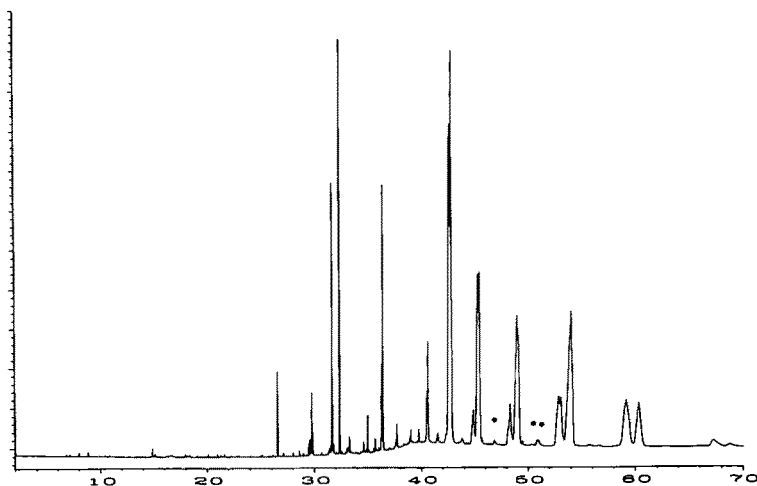


FIG. 7. Gas chromatogram (GC conditions 2, Methods) showing total lipid profile from male (and female) sternal integument (asterisks indicate  $R_t$  for cholesteryl esters  $C_{16:0}$ ,  $C_{18:1}$ , and  $C_{18:0}$ ).

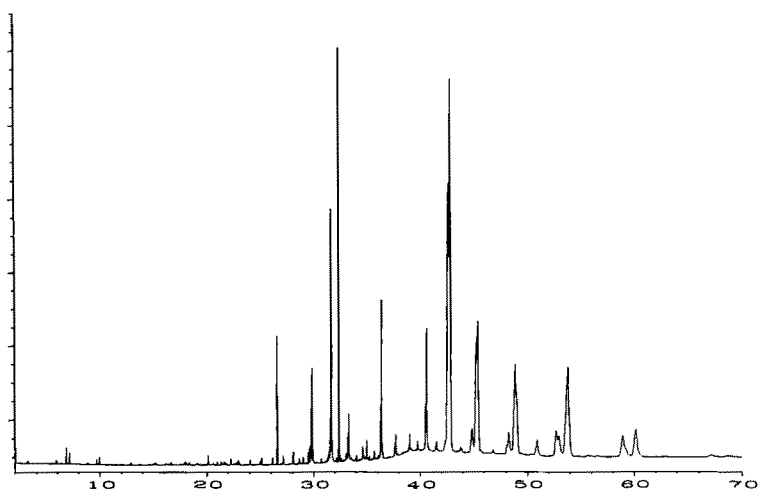


FIG. 8. Gas chromatogram (GC conditions 2, Methods) showing total lipid profile from male (and female) nonsternal skin regions.

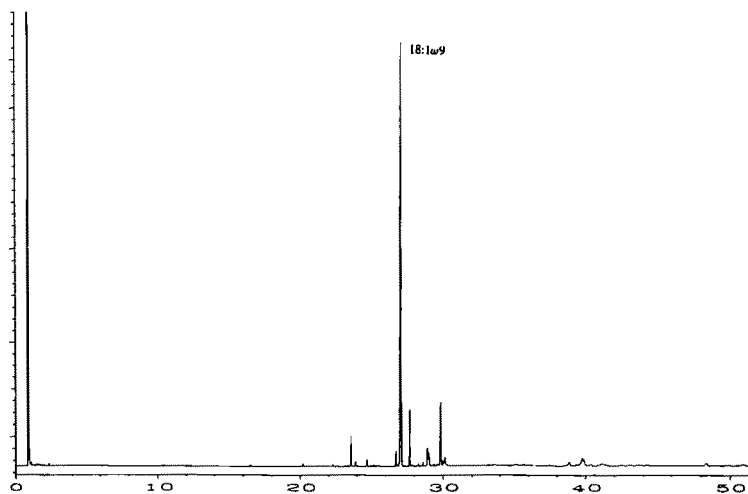


FIG. 9. Fatty acid methyl ester profile (GC conditions 2, Methods) from saponification of the male (and female) sternal and nonsternal lipids.

## DISCUSSION

Our results confirm the presence of a diversity of lipids in the holocrine, apocrine, and sternum gland secretions, a finding that is not inconsistent with Christie's (1983) statement that lipids from different tissues of an animal can vary markedly in structure and that such differences reflect likely differences in function of the tissues.

No multivariate analysis has been undertaken in this initial attempt to delineate significant differences between adult male and adult female lipid secretions which are chemically extremely complex. However, the striking compositional similarities in distributions of components in each of the pairs of gland secretions and their respective fatty acids, at this level of analysis suggests that "odors" derived from these secretions do not play a significant role in specific communication between the sexes.

The presentation of whole ether-soluble gland materials, recovered from each of the holocrine (oil) and apocrine (cell) glands, to possums elicited no significant behavioral change or olfactory investigation (Innes and Frampton, 1991). Therefore, we reasoned that the chemical signal would more than likely be due not to an in situ volatile, but rather to a signal generated as a result of postemission bacterial degradation of one or another of the secretions. As fatty acids have been shown to be the principal odorous components produced by bacterial fermentation of gland secretions, we reasoned that a simple chemical saponification of the three possum gland secretions would be a reasonable mimic of this process.

Our findings of the existence of a predominant suite of low-molecular-weight branched-chain free fatty acids (Figure 2) in the copious holocrine secretions and in the hydrolysates of the same secretions recovered from both adult males and adult females can perhaps be taken as evidence that the odor profile described by this mixture is unique to the possum and that it might serve as a pheromone to regularly "scent the habitat" to aid colonization, for example. Moreover the presence of these fatty acids in the whole gland secretion (Figure 1) suggests that anaerobic microorganisms are indeed likely to be present within these sacs. The reported occurrence of fatty acids and of lipids containing them in this molecular weight range is unusual in mammalian secretions (Albone, 1984). To our knowledge there are only two other reports of the occurrence of branched-chain fatty acids in this range in marking fluids of other animals. The first of these describes a suite of 4-ethyl-substituted octanoic, decanoic, dodecanoic, and tetradecanoic acids as components of goat scent, the first member of which is believed to be responsible for the characteristic odor of the male goat (Sugiyama et al., 1981). In the marking fluid of the tiger, a suite comprising 14 low-molecular-weight saturated fatty acids was identified that included *n*-

and iso-pairs of hexanoic, heptanoic, octanoic and nonanoic acids (Sarkar and Brachmachary, 1991).

The fatty acid distributions in the much less copious apocrine (cell) gland secretions of adult males and adult females are again virtually identical (Figure 5) as are those derived from the sternal and nonsternal secretions of both sexes. All are characterized by the presence of fatty acids regularly associated with animal fats (Davey et al., 1983). The apocrine acids are comprised of a suite of these acids in the  $C_{10:0}$ - $C_{18:0}$  range, and the sternal secretion yields only  $C_{18:1}$  as the predominant component. Based upon the volumes of the holocrine (oil) gland secretion and the obviously greater volatility of the fatty acid component derived from it, it is tempting to speculate that this secretion rather than the apocrine secretion, as suggested by Tyndale-Biscoe (1975), is used more regularly, perhaps in response to fright or alarm. This suggestion is also consistent with the observations of Thomson and Pears (1962).

A previous (and hitherto unpublished) study has demonstrated a behavioral response by adult male possums, manifested as olfactory investigation and scent-marking activity, to the representation of a highly polar lipid fraction derived from the (male) sternal sebum (Biggins, 1979). This so-called lipid fraction was said to be characteristically pigmented with a rufus coloration that was similar to the staining uniquely associated with the sternal hair in adult male possums. The work was not developed further, and no suggestion was advanced as to the lipid class to which the bioactive material(s) belonged. While we have not yet undertaken bioassays with sebum lipids, the fact that the profiles of sternal and nonsternal lipids from both males and females are identical suggests that the positive bioassay response observed by Biggins could be due to other components, which are unlikely to be free low-molecular-weight alcohols. Based upon the observed compositional similarities of the ether extracts of these secretions and of the fatty acids derived from them, it would appear that these constituents, either alone or in concert, are unlikely to be uniquely responsible for signaling social status unless they are associated perhaps with a discrimination between adults and juveniles.

It is recognized however that there is the probability that the whole secretion as discharged from whatever gland contains in its aqueous component proteinaceous (and possibly polysaccharide) material and enzymes that might effect the chemistry necessary to generate an odor signal. Previous studies with scent sacs of the otter, *Lutra lutra*, have indeed revealed the presence of both protein and mucopolysaccharide in addition to sebaceous gland lipid (Gorman et al., 1978).

Because the short-chain volatile fatty acids from the holocrine paracloacal glands are uncommon in the animal world and have a distinctive animal odor, it is possible that, when cocktailed, they may be utilized to attract possums and ultimately to be used in conjunction with poisoned bait in order to better control populations of possums within localized areas.

## SUMMARY

1. The lipids from the sternum, apocrine, and holocrine glands of the brushtail possum are identical in both male and female animals. These lipids therefore appear to have no function in sexual attraction.

2. The lipid classes from each of the three glands are distinctly different.

3. The three glands can be easily differentiated by the profile of fatty acids obtained by hydrolysis of the total lipid fraction.

4. The volume of oil from the holocrine gland and the volatility of the short chain fatty acids in this secretion suggest that it may function as a source of pheromone by both male and female animals to perhaps "scent the habitat" and to signal alarm.

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