

# Microanatomy and bacterial flora of the perineal glands of the North American porcupine

U. Roze, K.T. Leung, E. Nix, G. Burton, and D.M. Chapman

**Abstract:** The perineal glands of the porcupine, *Erethizon dorsatum* (L., 1758), are sexually dimorphic, paired pockets sprouting osmetrichial hairs. They lie between the anus and urethra, lateral to the midline, amid a sebaceous glandular expanse. In their active state, the glandular pockets secrete an amber substance with a terpenoid odor. When inactive, the glands produce no stain or odor. In males, activation of the glands is associated with fully descended testes. The glandular pockets yield a microbiota (“microflora”) in both their active and inactive states. We hypothesize that the active-state microflora transforms a sebaceous secretion into a pheromonally active product that is disseminated by anal dragging. The glandular microflora was characterized by gas chromatography of bacterial fatty acid methyl esters (GC-FAME) and polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE) of 16S ribosomal RNA gene fragments of bacteria. PCR-DGGE results showed the resulting bacteria profiles were the same in both sexes, but differed between the active and inactive states. Active-state microfloras were dominated by members of the Actinobacteria and showed greater coefficients of similarity than inactive-state microfloras. The microflora of individual animals changed with time and with secretory state. We argue for a reproductive role for the activated perineal glands.

**Résumé :** Les glandes périnéales du porc-épic, *Erethizon dorsatum* (L., 1758), consistent en une paire de poches sexuellement dimorphes d’où émergent des poils osmétrichiaux. Elles se situent entre l’anus et l’urètre, de part et d’autre de la ligne médiane dans une zone glandulaire sébacée. En état d’activité, les poches glandulaires sécrètent une substance ambrée à odeur de terpène. Lorsqu’elles sont inactives, les glandes ne produisent ni substance colorée, ni odeur. Chez les mâles, l’activation des glandes est associée à la descente complète des testicules. Les poches glandulaires contiennent une microflore tant dans leur état actif qu’inactif. Nous formulons l’hypothèse selon laquelle la microflore durant la période d’activité transforme la sécrétion sébacée en un produit qui agit comme phéromone et qui est disséminé par marquage anal. Nous avons caractérisé la microflore des glandes par la chromatographie en phase gazeuse des esters méthyliques des acides gras des bactéries (GC-FAME) et par l’amplification en chaîne par polymérase et l’électrophorèse sur gel en gradient dénaturant (PCR-DGGE) des fragments géniques 16S de l’ARN ribosomique. Les résultats de PCR-DGGE montrent que les profils bactériens obtenus sont les mêmes chez les deux sexes, mais qu’ils diffèrent entre les états actif et inactif. Durant la phase active, la microflore est dominée par des membres des actinobactéries et possède des coefficients de similarité plus élevés que la microflore de la phase non active. La microflore des individus change avec le temps et la phase de la sécrétion. Nous formulons des arguments qui appuient le rôle des glandes périnéales actives durant la reproduction.

[Traduit par la Rédaction]

## Introduction

Most mammals have a keen olfactory sense and produce odiferous secretions in the skin, intestines, kidney, and other sites. Microbial action can alter these secretions (Brown 1979). The objectives of this study are to describe the anatomical and histological structure of the perineal glandular areas and surrounding perineum of the porcupine, *Erethizon dorsatum* (L., 1758), and to characterize the bacterial community (“flora”) in the pocket perineal glands by the gas chromatography – fatty acid methyl ester (GC-FAME) and

polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE) methods during the active (secretory) and inactive states of the glands. We discuss advantages and disadvantages of bacterial analysis by the two methods, and also discuss possible behavioral functions of the glands.

The perineal glands of the porcupine have not been described before. Pocock (1922) described the external genitalia of 18 species of New and Old World hystricomorph rodents. He found perineal glands (which he called “anal glands” following Tullberg 1899) in 11 of the species, but failed to find them in *E. dorsatum* (his Fig. 26G). He stated

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that some of the specimens were museum skins, where visualization of the glands is problematic. His account of the perineal region of the North American porcupine lacks histological detail, and in his drawing the vagina is mislabeled. Likewise, these glands are not mentioned by Woods (1973) in his species account of the porcupine.

The scantily haired perineal region of hystricomorphs is surrounded by a skin ridge that is oval in the porcupine but less regular in shape in other hystricomorphs. In the porcupine this ridge runs from anterior to the urethra to a point caudal to the anus. This coincides with the perineum. Most of the central area is crammed with sebaceous glands. This glandular expanse has a lateral pair of invaginations (or pockets), each provided with a wick of protruding hairs. These invaginations will be referred to as the pocket perineal glands.

In mammals, where studied, the perineal glands play a role in agonistic encounters, in courtship behaviors, and in individual recognition (Kunkel and Kunkel 1964; Beauchamp 1974; Berüter et al. 1974; Macdonald et al. 1984). The glands may be sexually dimorphic, depending on the species. Shadle et al. (1946) noted anal dragging in captive porcupines, more commonly in males than females. In the related species *Coendou prehensilis* (L., 1758), Roberts et al. (1985) described frequent anal dragging, with males dragging 24 times more frequently than females. Similar observations have been made in other hystricomorph rodents such as *Cavia porcellus* (L., 1758) (Berüter et al. 1974) and *Hydrochoerus hydrochaeris* (L., 1766) (Macdonald et al. 1984). In the latter, odor dispersal is enhanced by detachable osmetrichial hairs, which are modified to hold secretions by a flaring of the cuticular scales.

Albone et al. (1977) and Wellington et al. (1979) showed supporting chemical and behavioral evidence that the invaginated skin glands of the perineum of some rodents, such as the wild male guinea pig, housed a bacterial flora that modified a parent secretion to give differentiation between the animals. Despite the intriguing roles of bacterial inhabitants on the perineal glands of animals, little is known about the community structure and seasonal dynamics of bacterial flora on the pocket perineal glands of the North American porcupine.

The traditional approach to identifying a bacterial community is to cultivate and isolate individual bacteria from a culture. We do that here and use the GC-FAME method to identify isolates by their fatty acid profiles. However, because of the interdependency of different bacteria and the lack of knowledge of specific growth requirements of bacteria in the natural environment, only a small portion (estimated 0.1% to 1%) of bacteria can be cultivated from most environmental samples (Amann et al. 1995; Muyzer 1999). The PCR-DGGE method is a non-cultivation approach to examining the population structure of bacterial communities (Muyzer and Smalla 1998); therefore, it circumvents the limitations of the traditional cultivation approach. The PCR-amplified 16S ribosomal RNA gene (i.e., 16S rDNA) fragments from various bacterial species in a community can be separated and analyzed by DGGE.

## Materials and methods

### Sample collection

For histology, one male (6.5 kg) and one female (4.5 kg) porcupine were collected as fresh (<6 h old) roadkills in western Greene County, New York. The perineal areas with underlying tissues were excised and fixed in three changes of phosphate-buffered 10% formalin. The samples were shipped in this fixative to Lakehead University for histological analysis.

For GC-FAME analyses, porcupines were live-trapped from 1996 to 1998 at a salt source in Greene County, New York (Roze 1984). The animals included six unique females (with one female sampled three times) and one male. Animals were briefly anesthetized with 10 mg/kg ketamine chloride by intramuscular injection (Ketaset, Fort Dodge Laboratories, Fort Dodge, Iowa). Perineal glands were sampled by gently twirling a sterile cotton swab in the perineal pockets. Glands in the active secretory phase imparted an orange color and terpenoid odor to the cotton swab; glands in the inactive state imparted no color or odor. After recovery, the animals were released at the site of capture. The swabs were stored at  $-20^{\circ}\text{C}$  for 1–7 days before culturing and GC-FAME analysis.

For PCR-DGGE analyses, animals were live-trapped, anesthetized, and sampled the same way. Five unique females and four males were sampled during 2002–2004, with one female sampled twice and one male sampled four times. Samples were kept at  $-20^{\circ}\text{C}$  for 1–7 days, then lyophilized and shipped to Lakehead University for analysis. No animal was sampled by both methods. Protocols for capture and handling of animals were approved by the Queens College Institutional Animal Care and Use Committee.

### Anatomical and histological examination

The skin and subcutis of the perineum were dissected out and fixed in phosphate-buffered formaldehyde. In the male, eight slabs were removed from around one side to observe any regional differences. Sections were also taken from the female, through the pocket.

Routine 10  $\mu\text{m}$  thick paraffin sections were stained in haemalum and Biebrich scarlet (Humason 1967). Thick (125  $\mu\text{m}$ ) sections (Chapman 1984) were dewaxed and stained for 15 min in Mayer's aluminum chloride – carminic acid (Lee 1950), briefly differentiated in 70% ethanol containing 4 drops of concentrated HCl per 100 mL, rinsed in 70% alcohol, dehydrated in an alcohol series (80%, 90%, 95%, and three changes of 100%), and finally cleared in xylene before mounting in balsam. These thick sections were best viewed under a dissecting microscope, which gave a three-dimensional appreciation of the sebaceous glands; unfortunately, the photographs did not retain the three-dimensional effect well and were omitted.

For microdissection of the pilosebaceous units, small pieces of skin were brought to 70% ethanol and then stained for 3 days in 70% alcohol saturated with Sudan IV, which demonstrates sebaceous glands. The pieces were washed in water and then cleared in glycerol through a graded series. Cover slips were sealed with Fant's resin.

Hairs destined for scanning electron microscopy were treated as follows: a wash in Sparkleen (Fisher Scientific;

0.5 g / 100 mL distilled water) was followed with washes in distilled water, then repeated washes in 100% ethanol, followed by a wash in xylene and overnight drying. Hairs were then mounted on double-sided sticky tape and gold-coated.

### GC-FAME

Individual cotton swabs were vortexed for 1 min in 5 mL of nutrient broth; 2 mL aliquots were plated on nutrient agar plates at 1:1, 1:100, and 1:10000 dilutions. Plates were incubated at 28 °C for 1 week. Individual colonies were then picked and quadrant-streaked on nutrient agar plates. Colonies from these plates (isolates) were stored on nutrient agar slants until analysis, which was performed at the Plant Pathology Laboratory at Auburn University, Auburn, Alabama.

For GC-FAME analysis (Sasser 1990), isolates were grown in trypticase soy broth at 28 °C. The culture was quadrant-streaked on trypticase soy agar. A colony was picked and placed in a 13 mm × 100 mm culture tube. The sample was then saponified with NaOH–MeOH, methylated with MeOH–HCl to form fatty acid methyl esters, and extracted into hexane – methyl *tert*-butyl ether, and the organic phase was subjected to gas chromatography analysis. The resultant FAME spectrum was then matched with a library containing over 60000 strains (Microbial ID, Inc., Newark, Delaware, USA).

### PCR-DGGE: DNA extraction and purification

The lyophilized (freeze-dried) swab sample taken from the porcupine's perineal pockets was transferred into a 1.5 mL Eppendorf tube containing 500 µL of sterile phosphate-buffered saline. The tube was then vortexed at maximum setting for 1 min to release the microflora into the sterile buffer. To extract DNA from the perineal microflora, 100 µL of the perineal swab suspension was mixed with an equal volume of Instagene DNA extraction matrix solution (Bio-Rad Laboratories Inc., Hercules, California, USA). The sample was then placed in a 100 °C water bath for 8 min and centrifuged at 14000g for 5 min. The crude DNA extract in the supernatant was transferred to another sterile 1.5 mL tube for further purification using an UltraClean DNA Purification Kit (MO BIO Laboratories, Solana Beach, California, USA). DNA from the crude DNA extract was purified by binding to a silica matrix provided in the Ultra-Clean DNA kit. The DNA–silica matrix was washed with buffered ethanol and the purified DNA was eluted from the silica by 25 µL of sterile deionized water as recommended by the manufacturer.

### PCR

PCR primers targeting the V3 region of 16S rDNA of eubacteria at nucleotide positions 341 to 534 were used to amplify the 16S rDNA fragments of the DNA samples extracted from the perineal swabs. The sequences of the forward and reverse primers were 5'-CGCCCGCCG-CGCCCGCGCCCGTCCCGCCGCCCCGCCCCGCCTAC-GGGAGGCAGCAG (primer 341-f-GC, GC clamp underlined) and 5'-ATTACCGCGGCTGCTGG (primer 534-r), respectively. The PCR assay was performed in a 25 µL PCR mixture containing 0.5 unit of *Taq* DNA polymerase (MBI Fermentas, Burlington, Ontario, Canada), 0.5 µL of each

25 µmol/L primer, 2.5 µL of 10× buffer without MgCl<sub>2</sub>, 3 µL of 25 mmol/L MgCl<sub>2</sub>, 2.5 µL of dNTP mix (0.2 mmol/L for each kind of nucleotide), 12.5 µL of deionized water, and 1 µL of DNA sample extracted from a perineal swab. The PCR mixtures were prepared aseptically and processed in a Hybaid PCR Sprint Thermal Cycler (Midwest Scientific, St. Louis, Missouri, USA). Amplification was performed under the following conditions: denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extending at 72 °C for 1 min. The DNA amplification cycle was performed 30 times and then followed by a final extension step at 72 °C for 10 min. The PCR amplification product was examined by electrophoresis in a 1% agarose gel. DNA was stained with ethidium bromide (0.2 µg/mL) and visualized under UV light. DNA concentrations were estimated with a ChemiGenius Bioimaging System and the GeneTools ver. 3.00.22 software program (Synoptic Ltd., Syngene division, Cambridge, UK).

### DGGE

DGGE was performed with a DCode Universal Mutation Detection System 16 cm × 16 cm 10% polyacrylamide gel (Bio-Rad, Hercules, California, USA) maintained at 60 °C in 7 L of Tris–acetate–EDTA buffer (TAE, 40 mmol/L Tris–acetate, 1 mmol/L EDTA, pH 8.0). Gradient gels were prepared with 25% and 65% denaturant (100% denaturant defined as 7 mol/L urea plus 40% (v/v) formamide). Each well was loaded with 0.5 to 1 µg of amplified DNA and gels were run at 35 V for 16 h. Gels were stained in 150 mL of TAE buffer combined with 15 µL of 10000× concentrated SYBR Green I (Sigma-Aldrich, St. Louis, Missouri, USA) for 30 min and destained in 150 mL of TAE for 15 min. Images were captured using a ChemiGenius Bioimaging System, which detected fluorescence from the SYBR Green I at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Band patterns were analyzed and compared using DNA Fingerprinting II Informatix Software (Bio-Rad, Hercules, California, USA), which provided dendrograms based on the percentage of similarity between banding patterns of the samples analyzed by the Dice correlation coefficient (Camu et al. 2007).

### Cloning of 16S rDNA fragments resolved by DGGE

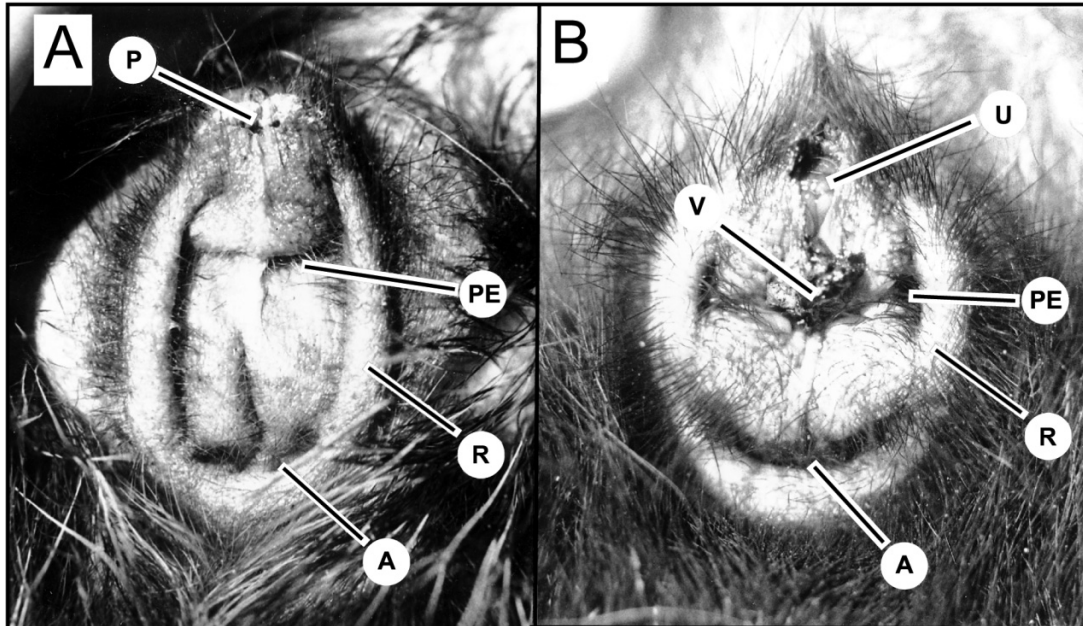
The major bands in the polyacrylamide gel were excised and rinsed with 1 mL of sterile deionized water. The gel bands were then crushed in 50 mL portions of sterile deionized water and stored at 4 °C overnight to allow the DNA to diffuse from the gel fragments. Three microlitres of the DNA extract were used for PCR as described earlier, except that the forward primer (primer 341-f) did not have a GC clamp. The PCR products were purified by a gel electrophoresis protocol (Leung and Topp 2001). The purified PCR-amplified DNA fragments were cloned into the pGEM-T Easy Cloning Vector (Promega, Madison, Wisconsin, USA) as described by the manufacturer for sequence analysis.

### Sequence analysis

Two clones from each DGGE band were randomly chosen for sequence analysis. The cloned PCR fragments were sequenced by an ABI PRISM 3100 automatic sequencer (Mox Laboratory, McMaster University, Hamilton, Ontario, Can-



**Fig. 1.** External view of the perineal region of an adult male (A) and adult female (B). Field of view approx. 7.5 cm × 9 cm. U, urethral opening; R, oval ridge; PE, perineal pocket; A, anus; P, penis (retracted); V, vaginal closure membrane. The female has recently given birth and her vaginal closure membrane is incompletely resealed.



ada) using a T7 primer (5'-TAATACGACTCACTA-TAGGG) targeting the T7 transcription initiation site of the pGEM-T Easy vector. Sequence alignments were performed using the DNAMAN software program (version 5.0; Lynnon BioSoft, Vaudreuil, Quebec, Canada). Sequences were compared with sequences in the GenBank database using the nucleotide–nucleotide BLAST program from the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>).

## Results

### Anatomical and histological structure of the porcupine perineum

The perineum is the region from the pubic symphysis to the base of the tail; laterally, it extends to the ischial tuberosities. In the porcupine this area is demarcated by an oval ridge of skin. The pocket perineal glands are paired shallow perineal cutaneous depressions lying in a sparsely haired region between and lateral to the anal and urethral openings. In males, the pocket openings form slits oriented perpendicularly to the ano-urethral axis (Fig. 1A). In females (Fig. 1B), where a vaginal closure membrane typically lies between the urethra and the anus, the pocket openings lie parallel and lateral to the ano-urethral axis.

In both sexes, tufts of a long type of hair emerge from the pockets. These distally tapering hairs measure between 29 and 160  $\mu\text{m}$  in diameter at the skin's surface. They are firmly anchored and carry a surface film, which makes them cling to glass. Scanning electron microscope examination shows increased scale separation and waviness in pocket hairs, even in proximal hair shaft regions, suggesting osmetrichial specialization (Fig. 2A). The pocket hairs average 13.7 mm in length, not significantly different from sur-

rounding hairs (Fig. 2B). They range from amber to black in color.

Microscopy revealed a thick layer of sebaceous glands not only in the pockets but also laterally reaching the summit of the skin ridges; cranially, the glands stop before the urethra, while caudally they end near the anus. A few millimetres lateral to the oval skin ridge (Fig. 1), the large sebaceous glands go through a quick transition to the usual skin of the belly.

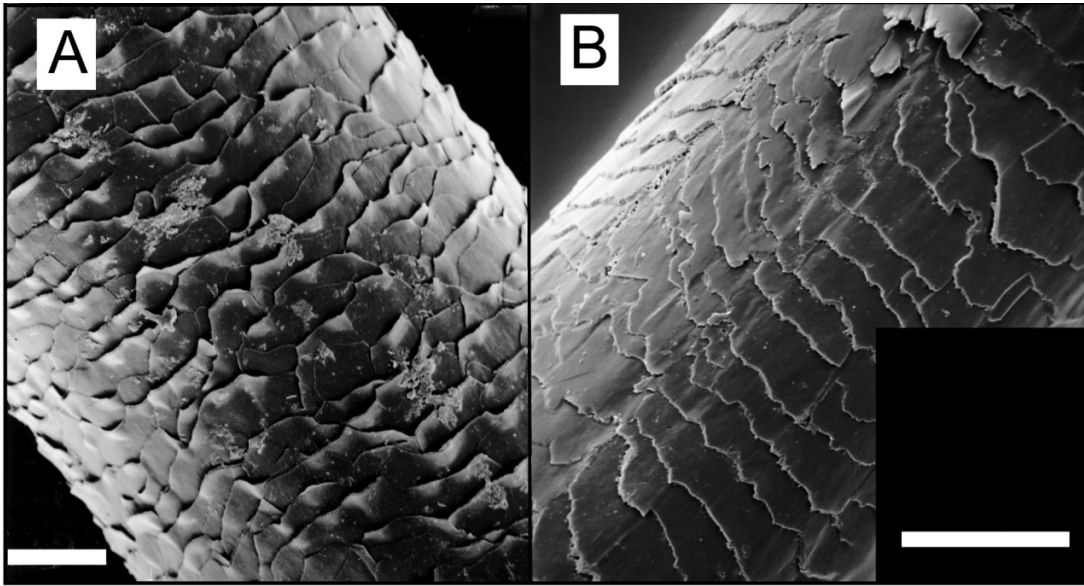
In both sexes, sections show that these contiguous pilosebaceous units take up almost the whole of the subcutis (Fig. 3). Microdissection reveals more convincingly that these are large and small pilosebaceous units. In the large type of pilosebaceous unit (Fig. 4A), the sebaceous glands enter the piliary canal at different levels and from different directions. The glandular offshoots divide and are in turn lobulated. In the small type of pilosebaceous unit (Fig. 4B), the fine companion hairs measure about 5–29  $\mu\text{m}$  in diameter. There is no qualitative difference between the sexes in the histology of the pocket perineal glands.

The unpigmented glandular perineal epidermis measures 80  $\mu\text{m}$  in thickness in the female and 70–120  $\mu\text{m}$  in the male. The thickness of the glandular skin and subcutis measures 3.0 mm in the female and 3.2 mm in the male. A 200  $\mu\text{m}$  layer of collagenous connective tissue separates the glands from the deeper fascia with its adipose tissue lobules.

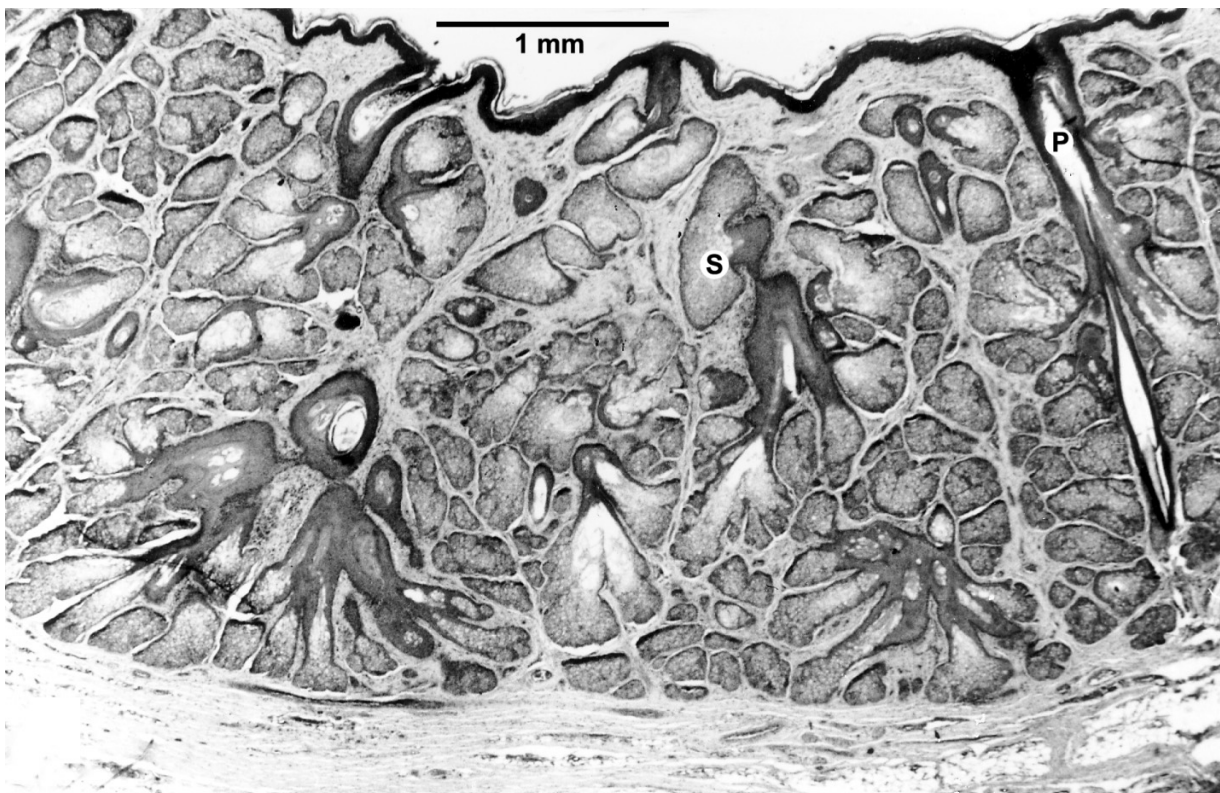
### Pocket perineal gland physiology

Of 25 males captured from 1996 through 2004 (of which only 5 were sampled for GC-FAME or PCR-DGGE analysis), 14 had fully descended testes along with perineal glandular secretory activity, 10 had undescended testes and no secretory activity, and 1 had undescended testes along with

**Fig. 2.** Scanning electron microscope views of perineal pocket hair from adult female (A) and control hair from exposed perineum of same (B). Bars = 25  $\mu$ m.



**Fig. 3.** Ten micrometre section of perineum of 6.5 kg adult male showing large pilosebaceous units taking up most of the dermis. P, piliary canal; S, sebaceous gland.



perineal secretory activity. The correlation between descended testes and perineal secretory activity is highly significant ( $P < 0.001$ , Fisher's exact test).

**GC-FAME**

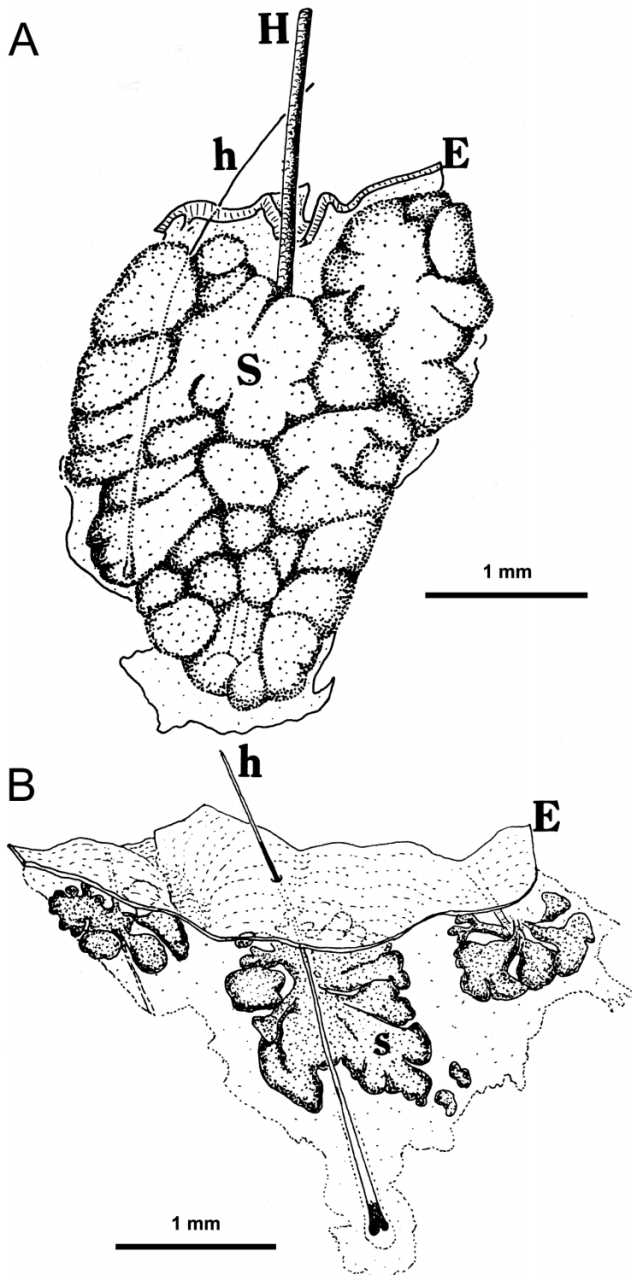
Seventeen isolates from nine samples (representing seven unique animals) showed 13 bacterial populations and 3

yeasts; 1 isolate failed to grow (Table 1). The most common bacterial genus identified (8 of 13 isolates) was *Staphylococcus*. Other genera identified included *Corynebacterium* (2 isolates), *Rhodococcus* (1), and *Micrococcus* (1). Among the yeasts, genera identified included *Cryptococcus* (2 isolates) and *Candida* or *Pichia* (1 isolate).

Five of nine samples yielded multiple isolates. However,



**Fig. 4.** Microdissections. (A) A large pilosebaceous unit. (B) Small pilosebaceous units, which fit between the larger variety. Sudan IV stain. H, large hair; h, fine hair; E, epidermis; S, sebaceous gland.



the true microfloral diversity of the perineal pockets is certainly higher, since multiple colonies were picked from a single plate only if they showed obvious differences in color or morphology. The GC-FAME data were used qualitatively, with no statistical analyses attempted.

One female (Sq) was sampled three times over an 8-month period to see whether her bacterial flora remained constant. The three sampling events yielded three different floras, with only *S. hemolyticus* common to all.

#### PCR-DGGE: Bacterial community in the porcupine perineal pockets

The DGGE analysis (Table 2) revealed that the perineal

pockets of the porcupines were colonized by complex bacterial communities (visualized in Fig. 5). The DNA bands in Fig. 5 represent various bacterial species, or “phylotypes”, that possess phylogenetic differences in their 16S ribosomal RNA gene sequences. For example, the sample from female 5271 in Fig. 5 (lane 5) consists of six dominant 16S ribosomal DNA fragments. On the other hand, some samples were dominated by only one or two major bacterial phylotype(s) and numerous minor phylotypes represented by the faint DNA bands and smearing in the profile, such as swabs from animals B, 412, and 510a (lanes 4, 6, and 7, respectively). These minor bands were not studied further. However, the relatively few major DNA bands (bands A, B, C, F, H, J, and K) indicated that the perineal bacterial communities were dominated by a relatively small number of bacterial phylotypes.

Cluster analysis separated the samples into two clusters, designated I and II (Fig. 6) (Camu et al. 2007). With the exception of animal 922, all the perineal samples in cluster II were collected during the active state of the glands. The bacterial communities in cluster II shared a similarity of 50%–75%. Samples collected during the inactive state of the glands appeared in cluster I and were composed of more diverse bacterial communities. The perineal bacterial communities of both the male and female porcupines could be found in each of the two clusters, indicating little distinction between the perineal bacterial communities of the female and male porcupines.

The major DGGE bands (A–K) were cloned, sequenced, and matched with sequences in the GenBank database (Table 3). With various degrees of similarity, the six major phylotypes, bands A, B, C, F, H, and K, corresponded to known organisms in the GenBank database: uncultured compost bacterium (95%), *Staphylococcus* sp. (99%), *Enterococcus* sp. (100%), *Corynebacterium kroppenstedtii* (95%), *Corynebacterium atypicalis* (94%), and *Aerococcus sanguinicola* (95%), respectively. Bands G, I, and J were identical to bands C, A, and F, respectively.

Bands D and E were artifacts (i.e., heteroduplexes) of bands A and F. Lanes 2, 3, 4, 5, 6, and 7 in Fig. 7 show the PCR-amplified products of DNA extracted from bands B, C, D, E, A, and F of the DGGE gel in Fig. 5, respectively. Amplification of bands B, C, A, and F produced DNA fragments that were identical to their respective template DNA samples. However, repeated attempts to amplify bands D and E always produced a tetraplex banding pattern represented in lanes 4 and 5 of Fig. 7, respectively. This indicates that bands D and E were composed of a mismatch of two similar single-stranded DNA (i.e., a heteroduplex), each originating from a strand of DNA from bands A and F (Speksnijder et al. 2001).

## Discussion

### Anatomy

Perhaps because of the anatomical inconspicuousness and the absence of odor perceptible without a cotton-swab probe, the pocket perineal glands of the porcupine had not been previously described in anatomical, histological, and functional detail.

It is of interest to think that a slight change in the flat ge-

**Table 1.** Sampling information for GC-FAME samples.

No. <sup>a</sup>	ID <sup>b</sup>	Mass (kg)	Sex and age <sup>c</sup>	Phase <sup>d</sup>	Date <sup>e</sup>	Closest match <sup>f</sup>
1	Mid	2.9	F(j)	?	2/11/96	<i>Staphylococcus hemolyticus</i>
2	Spr	5.8	M(a)	+	17/5/97	<i>S. hemolyticus</i>
3	Spr	5.8	M(a)	+	17/5/97	<i>Rhodococcus rhodochrous</i>
4	Spr	5.8	M(a)	+	17/5/97	<i>Cryptococcus albidus</i> (yeast)
5	Spr	5.8	M(a)	+	17/5/97	<i>Micrococcus luteus</i>
6	Pal	5.9	F(a)	–	24/5/97	<i>S. aureus/epidermidis</i>
7	Sq a	7.2	F(a)	+	14/6/97	<i>S. hemolyticus</i>
8	Sq a	7.2	F(a)	+	14/6/97	<i>S. aureus</i>
9	Sq a	7.2	F(a)	+	14/6/97	<i>Corynebacterium ammoniagenes</i>
10	Jul	4.6	F(a)	–	3/7/97	<i>Candida bertae</i> or <i>Pichia dispora</i> (yeast)
11	Nos	7.5	F(a)	+	6/9/97	No growth
12	Nos	7.5	F(a)	+	6/9/97	<i>C. albidus</i> var. (yeast)
13	Sq b	7.3	F(a)	+	28/9/97	<i>S. hemolyticus</i>
14	Sq c	6.9	F(a)	+	13/1/98	<i>S. hemolyticus</i>
15	Sq c	6.9	F(a)	+	13/1/98	<i>Corynebacterium xerosis</i>
16	L7B	1.9	F(j)	+	22/2/98	<i>S. hemolyticus</i>
17	L7B	1.9	F(j)	+	22/2/98	<i>S. epidermidis</i>

<sup>a</sup>Individual bacterial or fungal isolate.

<sup>b</sup>Porcupine sampled.

<sup>c</sup>M, male; F, female; a, adult (≥2 years old); j, juvenile (<1 year old).

<sup>d</sup>Secretory phase: +, active; –, inactive.

<sup>e</sup>Date sampled, D/M/Y.

<sup>f</sup>Best match between fatty acid profile of isolate and sample library (Microbial ID, Inc., Newark, Delaware, USA).

**Table 2.** Sampling information for PCR-DGGE samples.

Lane <sup>a</sup>	ID <sup>b</sup>	Mass (kg)	Sex and age <sup>c</sup>	Phase <sup>d</sup>	Date <sup>e</sup>	Major bands <sup>f</sup>
4	B	4.5	F(a)	–	1/6/02	A
5	5271	5.0	F(a)	+	13/7/02	B, C, D, E, A, F
6	412	4.6	M(a)	–	8/6/02	G, F
7	510a	3.1	M(sa)	–	10/5/02	B, H
8	53	5.8	M(a)	+	3/5/02	D, E, I, J
9	922	5.4	F(a)	–	22/9/02	D, E, I, J
10	510b	4.8	M(a)	+	25/5/03	D, E, I, J
11	75a	6.5	F(a)	+	7/6/03	K, I, J
12	5253	5.1	M(a)	+	25/5/03	D, E, I, J
13	510d	6.3	M(a)	–	28/5/04	C, K, J
14	510c	5.2	M(a)	–	17/4/04	K, J
15	F	4.8	F(a)	–	24/5/04	E, J
16	75b	6.6	F(a)	+	15/5/04	D, E, I, J

<sup>a</sup>Lane numbers of the porcupine perineal samples in Fig. 5.

<sup>b</sup>Individual porcupine.

<sup>c</sup>M, male; F, female; sa, subadult (1–2 years old); a, adult (≥2 years old).

<sup>d</sup>Secretory phase: +, active; –, inactive.

<sup>e</sup>Date of sample collection, D/M/Y.

<sup>f</sup>Major visible bands (major phylotypes) excised and studied.

ometry of the glandular perineum to form pockets could provide an abrasion-protected chamber for the microbial modification of sebum that would prevent the sebum from being soon rubbed off as presumably happens on the flat region of the perineum.

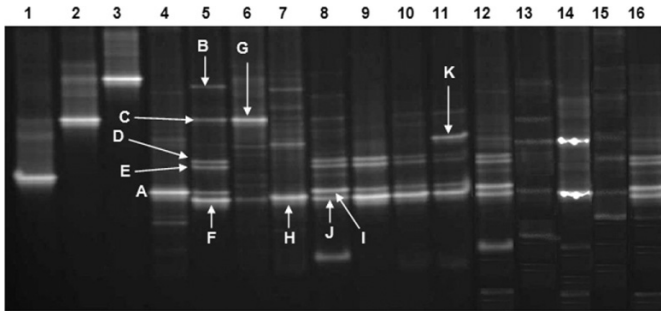
Anal dragging is typically more pronounced in male than female hystricomorphs, and since the porcupine appears to fit this pattern, one might have expected evidence for a sexual dimorphism in histology. Aside from the parallel versus perpendicular orientation of gland openings, no clear-cut qualitative difference between the sexes was observed in the

present study, though quantitative differences may be present.

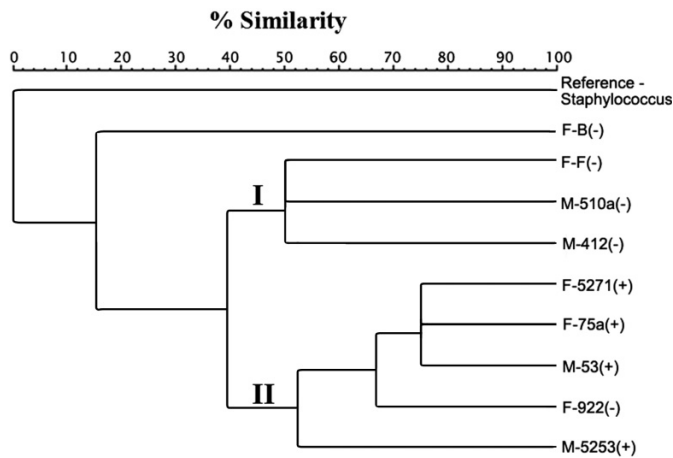
### Bacterial flora

Zechman et al. (1984) showed that bacteria grew on the perineal gland secretions of cavies (*Cavia aperea* Erxleben, 1777). Male wild cavies preferred the perineal gland secretions that were colonized by bacteria over the freshly obtained perineal secretions, indicating that perineal bacteria were responsible for cavy perineal scent marks. Furthermore, 90% of the cavy perineal bacterial population was

**Fig. 5.** DGGE profiles of the amplified 16S rDNA fragments of the bacterial communities recovered from the porcupine perineal pockets. Lanes 1, 2, and 3 are 16S rDNA reference fragments of *Escherichia coli*, *Enterococcus faecalis*, and *Staphylococcus epidermidis*, respectively. Lanes 4–15 represent animals B(-), 5271(+), 412(-), 510a(-), 53(+), 922(-), 510b(+), 75a(+), 5253(+), 510d(-), 510c(-), F(-), and 75b(+), respectively (+, active secretory phase; -, inactive secretory phase). Bands A–K represent some of the major bacterial phylotypes of the perineal bacterial samples.



**Fig. 6.** Relatedness of the perineal bacterial communities of the porcupine samples based on a cluster analysis of their DGGE profiles. This analysis did not use the repeated samples from male 510 and female 75. A *Staphylococcus epidermidis* 16S rDNA reference fragment was used as an outgroup of the analysis. The prefixes F and M represent female and male, respectively.



Gram-positive coryneform bacteria and the rest was made up of alpha and gamma hemolytic streptococci. Unfortunately, the dynamics of the perineal bacterial community structure were not examined.

In this study, we have shown that the central transverse band of the perineal region of the porcupine is glandular, indicating a rich source of metabolic substrates for bacterial growth, presumably mainly in the pockets. With the GC-FAME and PCR-DGGE methods, we confirmed that like the perineal bacterial population of cavies, the perineal bacterial community of the porcupine is dominated by a few major bacterial phylotypes. However, the DGGE profiles also showed that the major perineal bacterial phylotypes co-existed with numerous minor phylotypes. (The GC-FAME technique as used here gives no indication of relative abundances.)

The PCR-DGGE technique identified the major perineal

bacterial genus of active-state glands as *Corynebacterium* (Tables 2 and 3, Fig. 5). The uncultivable bacterial phylotype TA9 is also closely related to *Corynebacterium* species based on its 16S rDNA sequence (Dees and Ghiorse 2001). *Corynebacterium* is a member of the bacterial phylum Actinobacteria (Stackenbrandt et al. 1997). Three of four bacterial genera identified by GC-FAME (*Corynebacterium*, *Micrococcus*, *Rhodococcus*) are members of the Actinobacteria. This bacterial group is characterized by Gram-positive staining, a high DNA G+C content, and great versatility of its metabolic pathways (Willey et al. 2008).

Examples of actinobacterial metabolic versatility include conversion of odorless human axillary secretions into malodorous metabolites (Zeng et al. 1992), production of pheromones in humans (Gower et al. 1986; Kohl et al. 2001), generation of antibiotics and antibiotic precursors, and decomposition of complex organic molecules as well as hydrocarbons and aromatic compounds. There is evidence that the yeast genus *Cryptococcus*, revealed by the GC-FAME technique, has similar metabolic plasticity (Botes et al. 2005). Actinobacteria and *Cryptococcus* species are typically found in the soil but are also found on human skin. By virtue of their metabolic plasticity, and because they make up the dominant perineal gland bacterial phylotypes during the pocket glands' active state, the actinobacteria and *Cryptococcus* species are good candidates for the role of converting sebaceous precursors into pheromonally active end products.

In contrast to the Actinobacteria, *Staphylococcus* species are known as normal skin components with little metabolic plasticity. They show up as minor phylotypes in PCR-DGGE; their frequent occurrence in GC-FAME samples may reflect their easy growth in culture. The PCR-DGGE and GC-FAME techniques may thus provide complementary information. PCR-DGGE gives an indication of bacterial community diversity and relative abundances of the various phylotypes. It also reveals unculturable forms such as TA9. GC-FAME reveals yeasts as well as bacteria.

In our study, PCR-DGGE showed a high degree of diversity within and between the perineal bacterial populations during the inactive stage. The bacterial community structures became less diverse during the active stage, sharing more than 65% similarity. The only sample that does not fit the pattern is that from female 922. Despite having inactive glands at the time of capture, this female showed two double bands (D, E, I, and J) typical of the active state. One possible explanation is that she had only recently activated her pocket glands, attracting the active-stage microflora but failing to accumulate sufficient pheromonal product to discolor the cotton swab. Most females activate their pocket glands in August and September (data not shown). Female 922 was sampled in September.

### Physiology

In males, perineal gland activity is associated with fully descended testes. Testicular descent in males is androgen-dependent (Frey et al. 1983; Raivio et al. 2003). Androgens are implicated in secretory control of the sebaceous glands in males as well as females (Pochi and Strauss 1974; Thody and Shuster 1989). Thus in males at least, there appears to be a host-mediated control of the commensal microflora.



**Table 3.** Identity of the major bacterial phlotypes of PCR-DGGE samples.

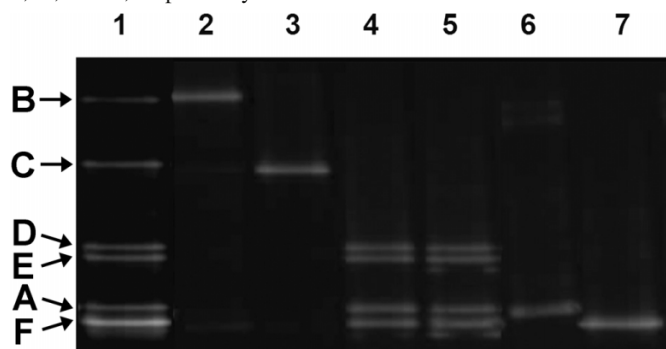
Band designation <sup>a</sup>	Closest relative strain <sup>b</sup>	% similarity to the closest relative <sup>c</sup>	GenBank acc. No. of the closest relative
A	Uncultured bacterium TA9 (a compost bacterium)	95	AF252314
B	<i>Staphylococcus</i> sp. (typical microflora of skin)	99	AF322002.1
C	<i>Enterococcus</i> sp. (typical fecal bacterium of animals)	100	AY269871.1
F	<i>Corynebacterium kroppenstedtii</i>	95	Y10077
G	<i>Enterococcus</i> sp. (same as C)	100	AY269871.1
H	<i>Corynebacterium atypicalis</i> (clinical isolate from human)	94	AJ441057
I	Uncultured bacterium TA9 (same as A)	95	AF252314
J	<i>Corynebacterium kroppenstedtii</i> (same as F)	95	Y10077
K	<i>Aerococcus sanguinicola</i>	95	AF318316.1

<sup>a</sup>Band designations correspond to bands identified in Fig. 5. Bands D and E are artifacts (i.e., heteroduplexes) of bands A and F, and are not listed in this table.

<sup>b</sup>The closest relative strains were identified from the GenBank database using the BLAST program.

<sup>c</sup>% similarity is the DNA sequence similarity between the 16S rDNA fragment of a DGGE band and its closest relative strain in the GenBank database.

**Fig. 7.** DGGE of the PCR-amplified products obtained from DNA bands excised from the DGGE profiles of the perineal samples. Lane 1 represents the DGGE profile of animal 5271(+). Lanes 2, 3, 4, 5, 6, and 7 represent amplified DNA products of bands B, C, D, E, A, and F, respectively.



Control of perineal gland activity in females requires further clarification.

### Behavior

Some of the behavioral roles ascribed to perineal glands in other mammals, namely mediation of agonistic encounters and individual recognition, do not appear valid in the porcupine. While males with descended testes (and perineal secretory activity) may engage in battles during the mating season (Sweitzer and Berger 1996; Sweitzer 2003; Roze 2009), there is no comparable association between agonistic activity and perineal glandular secretion in females.

Likewise, the perineal glands of porcupines are unlikely to serve in individual recognition. This is because the perineal flora of individuals can change with time (Fig. 5: lanes 7, 10, 13, and 14 represent the same male sampled at four different time periods, and lanes 11 and 16 represent the same female sampled at two different time periods). Moreover, the great overlap of bacterial flora between individual porcupines, and the limited number of major phlotypes found, suggest that the glands are not used for individual recognition in these animals.

Rather, the association of gland activity with descended testes, and hence elevated testosterone levels, in males sug-

gests the glands play some role in reproduction. A similar role in females is suggested by numerous observations during the fall mating period that a male guarding a female in a tree canopy carefully sniffs the place on the branch where the female had been resting (Roze 2009). However, the behavioral role of porcupine pocket perineal glands requires further study. The anal dragging used to deploy the glandular product has been observed almost entirely under captive conditions (Shadle et al. 1946). Normal movements of the porcupine over tree branches would in most cases obscure any anal dragging.

A better understanding of the ecological role of these glands will require further studies of captive as well as free-ranging porcupines.

To summarize, we have described the anatomy and histology of the perineal glands of the North American porcupine. We have shown that the glands cycle between active and inactive states. Because activity requires elevated testosterone levels in males, we propose the glands play a role in reproductive activities. We show by two independent methods (GC-FAME and PCR-DGGE) that the glandular pockets are colonized by a microflora whose composition changes between the active and inactive states. The active-state microflora is characterized by the presence of Actinobacteria, a metabolically versatile group. We propose that the active-state microflora transforms a sebaceous substrate into a pheromonally active product.

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