STUDIES OF THE MICROENVIRONMENT AND MICROFLORA OF THE CANINE EXTERNAL EAR CANAL

by

HUI-PI HUANG

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Department of Veterinary Medicine
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AUTHOR'S DECLARATION

The work presented in this thesis was carried out by the author in the following locations: the Department of Veterinary Pathology and the Department of Veterinary Medicine, University of Glasgow Veterinary School, and the Department of Biochemistry, University of Glasgow.

Signed: Hui-pi Huang

Date: 25th June 1993

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SUMMARY

The aims of this study were to investigate aspects of the aural microenvironment in dogs. The aural microflora, histological features of the aural integument, and biochemical components of cerumen from healthy canine ears and those with otitis externa were studied. Interactions between canine cerumen and one member of the resident aural flora, *Malassezia pachydermatis* were also investigated.

Gram-positive, coagulase-negative cocci, and *M. pachydermatis* were the most common resident flora found in 52 healthy canine external ears. Microscopic examination of cytological smears from cerumen and microbiological culture indicated that these organisms were present in low numbers. Gram-positive, coagulase-positive staphylococci, Gram-negative rods, and *M. pachydermatis* were isolated most frequently from 27 canine ears affected by otitis externa. Eighty percent of these inflamed ears were associated with microbial overgrowth. The numbers of microorganisms found in cerumen cytological smears appear to be correlated to the growth density of microbial colonies on culture plates.

In an anatomical survey of the external ear canal, 40 canine ears were examined. The average length of the cartilaginous part of these canals was 5.3 cm. The diameter at the most proximal end of the annular cartilage averaged 0.5 cm; at the proximal end of the auricular cartilage the mean diameter was 0.7 cm; at the distal extremity of the ear canal, the average diameter was 4.8 cm.

Morphometric stereology was used to evaluate histological features of 28 clinically normal and 15 otitic canine ears at four anatomical levels. Marked variation was found in the distribution of sebaceous and apocrine glands in the aural integument in healthy ears and those with otitis. The area occupied by sebaceous glands increased gradually from the proximal to the distal extremity of the ear canal, whilst the area occupied by apocrine glands decreased gradually from the proximal to the distal parts of the ear. This same pattern of gland distribution was found in otitic ears. The areas occupied by sebaceous, apocrine and hair follicle tissue were significantly increased in otitic ears compared to healthy ears.

Immunoglobulin A (IgA), immunoglobulin G (IgG), and immunoglobulin M (IgM) were identified in canine cerumen using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Immunoglobulin quantitation was carried out by enzyme-linked immunosorbent assay. Immunoglobulin G was the predominant antibody in cerumen. The mean proportions of IgA and IgG in cerumen differed significantly between 13 otitic ears and 30 normal canine ears.

Lipid accounted for 50% of the wet matter in 36 cerumen samples from healthy canine ears. The lipid content of 12 otitic ears was 25%, significantly less than in the normal ears. Thin layer- and gas liquid chromatography were used to analyse cerumen lipid and fatty acid composition. Marked variations were found between individuals. In 30 samples from healthy ears, two thirds of the fatty acid composition comprised those with a chain length of 16 or 17 carbons, both saturated and unsaturated. Whereas in 12 cerumen samples from otitic ears, fatty acids with chain length of 17 or 18 carbons accounted for two thirds of the composition. The presence of antimicrobial unsaturated fatty acids, oleic (C18:1) and linoleic (C18:2) in canine cerumen was confirmed by gas chromatography - mass spectrum.

Two phenotypes of the yeast *M. pachydermatis* were found. These two phenotypes also showed differences in fatty acid composition. Oleic and linoleic acids, which were found in canine cerumen, were shown to have mycostatic effects on the growth of a strain of *M. pachydermatis*.

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ABBREVIATIONS

AEC	= amino-9-ethyl carbazole
cm	= centimetre
C.V.	= coefficient of variation
^{o}C	= degrees Celsius
DTT	= 2, 3-dihydroxybutane-1, 4-dithiol (Dithioerythritol)
E.C.L.	= equivalent chain length
ELISA	= enzyme linked-immunosorbent assay
F.A.M.E.	= fatty acid methyl ester
G.C. - M.S.	= gas chromatography and mass-spectrometry
G.L.C.	= gas liquid chromatography
$\geq$	= greater than or equal to
G	= force of gravity
HPF	= high-powered field
HRP	= horseradish peroxidase
HCl	= hydrochloric acid
IgA	= immunoglobulin A
IgE	= immunoglobulin E
IgG	= immunoglobulin G
IgM	= immunoglobulin M
Kd	= kilodalton
Kg	= kilogram
Kv	= kilovolt

L	= left
≤	= less than or equal to
l	= litre
M*	= mass
M/e	= mass/charge ratio
Max.	= maximum
m	= metre
ug	= microgram
ul	= microlitre
um	= micrometre
mA	= milliampere
mg	= milligram
ml	= millilitre
mm	= millimetre
mmol	= millimole
Min.	= minimum
min	= minute
M	= molarity
mol	= mole
nm	= nanometre
nm	= nanomole
N	= normality
no.	= number
n	= number of cases
%	= percent
PBST	= phosphate buffered saline tween
P	= probability

PNP	= product of nitrophenol palmitate
RAD	= rabbit-anti-dog
R	= regression coefficient
R	= right
Sec	= second
NaCl	= sodium chloride
SDS-PAGE	= sodium dodecyl sulfate-polyacrylamide gel electrophoresis
S.D.	= standard deviation
TEMED	= tetra-ethyl-methyl-enediamine
TMB	= tetramethyl benzidine
Q1	= the 25th percentile
Q3	= the 75th percentile
T.L.C	= thin layer chromatography
Tra	= trace
TBS	= tris buffered saline
v/v	= volume by volume
w/v	= weight by volume
w/w	= weight by weight

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To my parents

CHAPTER I

OTITIS EXTERNA
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THE MICROENVIRONMENT OF THE CANINE EXTERNAL EAR:
AN INTRODUCTION AND REVIEW

1.1 Introduction

Canine otitis externa, inflammation of the ear canal, is a descriptive term without implying any underlying cause (Fraser, Withers, and Spreull, 1961; Evans and Jemmett, 1978; Grono, 1980; Griffin, 1981; Woody and Fox, 1986a; Hayes, Pickle, and Wilson, 1987; August, 1988; Muller, Kirk, and Scott, 1989; Griffin, 1990; Little, 1990). Otitis externa is important because it is common. Moreover, many cases are extremely difficult to cure (McGinnis and England, 1949; Witter, 1949; Fraser *et al*, 1961; Fraser, 1965; Grono, 1969a; Fraser, Gregor, McKenzie, Spreull and Withers, 1970; Baxter and Lawler, 1972; Pugh, Evans and Hendy, 1974; Rose, 1976a; Grono, 1980; Harvey, 1980; Griffin, 1981; Woody and Fox, 1986a; Hayes *et al*, 1987; August, 1988). The condition appears to have a complicated and multifactorial aetiology. Otitis externa seems to result from a combination of dynamic changes affecting the anatomical, physiological and microbiological status of the external ear canal. Patients are usually treated symptomatically, but the problem frequently relapses (Evans and Jemmett, 1978; Woody and Fox, 1986a; August, 1988; Muller *et al*, 1989).

Despite the frequency of canine otitis externa and its importance to the small animal practitioner, only some aspects of this condition have been the subject of serious research, such as microbiology, classification, and treatment. Investigations concerned with the fundamental physiology of the canine external ear canal and the pathophysiology of otitis externa have been few and incomplete.

1.2 Anatomy and micro-anatomy of the external ear canal of dogs

The canine external ear consists of two parts, the pinna (auricle) and the external ear canal. The external ear canal originates proximally at the tympanic membrane and forms an air-filled passageway connecting to the exterior. The canal is supported by the annular and auricular cartilages. The annular cartilage, which lies proximally, connects with the auditory process of the petrous temporal bone and forms a narrow canal; more distally the proximal part of the auricular cartilage forms a funnel-shaped passage connecting the annular cartilage to the exterior. The ear canal may also be divided into the horizontal and the vertical ear canal according to their relative positions. Modified skin lines the inner surface of the ear canal. The distal part of the large auricular cartilage, which supports the pinna, is invested on both sides with skin (Miller and Wither, 1942; Getty, Foust, Presley, and Miller, 1956; Fraser *et al*, 1970).

Histologically, the structure of the aural integument closely resembles that of the pinna except that, in most breeds, hairs are fewer and do not extend throughout the ear canal. This skin is supported by connective tissue (Fraser, 1961d; Fernando, 1966). Witter (1949) stated that only sebaceous glands were apparent in normal aural integument. However, Nielsen (1953), Fraser (1961d), and Fernando (1966) reported that both sebaceous and apocrine (eccrine) glands were located in the integument.

Further details of anatomical and histological features of the canine external ear canal are discussed elsewhere in this thesis (see Chapter II).

1.3 Skin surface as a micro-ecosystem

It is known that healthy skin maintains certain bacterial and fungal populations in relatively stable numbers. These microorganisms are called the resident flora (Price, 1938; McEwan Jenkinson, 1993). The

constituents of the skin emulsion formed by the secretion of apocrine (eccrine) and sebaceous glands, such as lipids, carbohydrates, and nitrogen sources, act as nutrients for cutaneous flora (Usher, 1928; Arnold, Gustafson, Hull, Montgomery, and Singer, 1930; Pillsbury and Rebell, 1952; Cove, Holland, and Cunliffe, 1980; Holland, 1993; McEwan Jenkinson, 1993). These cutaneous microorganisms are subject to population change in response to microenvironmental variables such as humidity, hydration, temperature, hydrogen ion concentration, availability of nutrients, and stimulation or inhibition by other members of the cutaneous flora (Marples, and Kligman, 1969; Bibel and LeBrun, 1975; Noble, 1975; Kligman, Leyden, and McGinley, 1976; Aly, Shirley, Cunico, and Maibach, 1978; Youssef, Wyborn, Holt, Noble, and Clayton, 1978; Noble, Lloyd, and Appiah, 1980; Faergemann, Aly, Wilson, and Maibach, 1983; Hartmann, 1983; Lappin-Scott, Rogers, Adlard, Holt, and Noble, 1985; Allaker, Lloyd, and Noble, 1989; Allaker, Lloyd, and Smith, 1988; Allaker, Lloyd, and Lamport, 1990; Holland, 1993; McBride, 1993; Allaker and Noble, 1993).

The surface of the aural integument in dogs is inhabited by a variety of microorganisms (Joshua, 1958; Fraser, 1961a; Fraser, 1961b; Fraser, 1965; Grono and Frost, 1969; Fraser *et al*, 1970; Baxter, 1976; Marshall, Harris and Horne, 1974; Gedek, Brutzel, Gerlach, Netzer, Rocken. Unger, and Symoens, 1979; McCarthy and Kelly, 1982; Chengappa, Maddux, and Greer, 1983; Uchida, Nakade, and Kitazawa, 1990). Cerumen (earwax), a skin emulsion overlying the aural integument, is composed partly of the secretion of apocrine glands, and partly of desquamated keratinized cells (Chiang, Gessert, Lowry, and Senturia, 1956; Fernando, 1967; Okuda, Bingham, Stoney, and Hawke, 1991), and is thought to be capable of regulating the cutaneous flora. It has been shown that human cerumen has bactericidal and antifungal activities (Burtenshaw, 1938; Burtenshaw,

1942; Chai and Chai, 1980; Stone and Fulghum, 1984; Megarry, Pett, Scarlett, Teh, Zeigler, and Canter, 1988). However, canine cerumen was reported to enhance the growth of the yeast, *Malassezia pachydermatis*, one of the organisms resident on the canine aural integument (Gabal, 1988).

1.4 Otitis externa

Otitis externa, the most common disease of the canine ear canal, is very frequently encountered in small animal practice (McGinnis and England, 1949; Witter, 1949; Fraser *et al*, 1961; Fraser, 1965; Grono, 1969a; Fraser *et al*, 1970; Baxter and Lawler, 1972; Pugh *et al*, 1974; Rose, 1976a; Grono, 1980; Harvey, 1980; Griffin, 1981; Woody and Fox, 1986a; Hayes *et al*, 1987; August, 1988).

Prevalence / incidence

The prevalence of canine otitis externa has been reported to vary from 2% to as high as 25% of the total cases admitted to veterinary practices, and affects from 16% to 20% of the total canine population (Grono, 1969a; Moltzen, 1969; Fraser *et al*, 1970; Priester, 1970; Baxter and Lawler, 1972; Sharma and Rhoades, 1975; Rose, 1976a; Grono, 1980; Baba, Fukata, and Saito, 1981; Anderson, 1983).

It is generally agreed that some breeds of dogs are more prone to otitis externa than others. Dogs with pendulous pinnas or hirsute ear canals appear to have a higher risk of developing this disease. Thus Spaniels, Retrievers, and Poodles have a higher prevalence than other breeds of dogs (Blaine, 1841; Mayhew, 1854; Woodroffe-Hill, 1892; McGinnis and England, 1949; Joshua, 1958; Fraser, 1961a; Fraser *et al*, 1961; Fraser, 1965; Fernando, 1966; Grono, 1969a; Fraser *et al*, 1970; Baxter and Lawler, 1972; Pugh *et al*, 1974; Sharma and Rhoades, 1975;

Rose, 1976a; Grono, 1980; Baba *et al*, 1981; August, 1986; Hayes *et al*, 1987; August, 1988; McKeever and Torres, 1988; Macy, 1989; Matthiesen and Scavelli, 1990).

Dogs from four to eight years of age have been found to have a greater prevalence of otitis externa (Grono, 1969a; Fraser *et al*, 1970; Pugh *et al*, 1974; Sharma and Rhoades *et al*, 1975; Grono, 1980). Young dogs less than one year old, and elderly dogs aged more than 13 years appear to have a lower prevalence (Fraser *et al*, 1970).

Male dogs have been reported to have a significantly higher prevalence of otitis externa (Grono, 1969a; Hayes *et al*, 1987). However, one of these reports appears to have been based on a biased sample (Grono, 1969a). No sex bias in the prevalence has been reported in other studies (Fraser *et al*, 1961; Fraser *et al*, 1970; Baxter and Lawler, 1972; Pugh *et al*, 1974; Sharma and Rhoades, 1975).

Seasonal variation in the incidence of otitis externa has been reported, but no significant differences in seasonal incidence were found in several studies (Grono, 1969a; Baxter and Lawler, 1972; Sharma and Rhoades, 1975). Hayes *et al* (1987) however, in a large study, found evidence that the incidence of otitis externa does seem to fluctuate in response to climatic variation. They showed that monthly variations in ambient temperature, rainfall, and relative humidity correlate with the frequency of new cases of otitis in dogs but that there appears to be a two month lag period between fluctuations in these climatic variables and change to the rate of otitis.

Aetiology

The factors which can cause canine otitis externa are numerous (August, 1986; August, 1988). Dynamic interactions between these may render the aetiology more complicated still (Evans and Jemmett, 1978).

All the factors that can initiate aural irritation and lead to the process of inflammation may contribute to the aetiology of otitis externa (Evans and Jemmett, 1978). Generally, the aetiology can be categorized into primary causes and predisposing factors (Fraser, 1961a; Fraser *et al*, 1961; Fraser, 1965; Fraser *et al*, 1970).

A higher incidence of otitis externa in certain breeds of dogs probably indicates that some aural conformational characteristics might predispose to this disease (Fraser *et al*, 1970; Griffin, 1981; Hayes *et al*, 1987). Pendulous carriage of the pinnae and hirsute ear canals may decrease ventilation, increase humidity, elevate temperature, and allow the accumulation of cerumen within the canal. These factors were generally thought to be responsible for the higher prevalence of otitis externa in such dogs (Blaine, 1841; Mayhew, 1854; Woodroffe-Hill, 1892; Saunders, 1914; McGinnis and England, 1949; Joshua, 1958; Fraser, 1961a; Fraser *et al*, 1961; Fraser, 1965; Fernando, 1966; Grono, 1969a; Fraser *et al*, 1970; Baxter and Lawler, 1972; Pugh *et al*, 1974; Sharma and Rhoades, 1975; Evans and Jemmett, 1978; Rose, 1978; Grono, 1980; August, 1986; Hayes *et al*, 1987; August, 1988; McKeever and Torres, 1988; Bruyette and Lorenz, 1993; McKeever, 1993). Dogs which swim regularly have been reported to have a higher prevalence of otitis externa, perhaps for the same reasons (Blaine, 1841; Woodroffe-Hill, 1892; Griffin, 1981; August, 1986; August, 1988; Muller *et al*, 1989; Griffin, 1990). However, there is only limited evidence which shows differences in humidity or temperature between different types of canine ears (Grono, 1970a; Grono, 1970c).

Breed predisposition to aural inflammation has also been associated with certain histological features. Breeds which exhibit a higher prevalence of otitis appear to possess more, and better developed, glandular tissues in their aural integument (Fernando, 1966; Stout-

Graham, Kainer, Whalen, and Macy, 1990). Increased production of cerumen and its retention in the ear canal have also been incriminated as a cause of canine otitis (Joshua, 1958; Evans and Jemmett, 1978; August, 1986; August, 1988; Bruyette and Lorenz, 1993). Canine cerumen has been demonstrated to enhance the growth of *Malassezia pachydermatis* (Gabal, 1988). Whereas, on the contrary, human cerumen has been shown to possess antimicrobial activities (Burtenshaw, 1938; Burtenshaw, 1942; Chai and Chai, 1980; Stone and Fulghum, 1984; Megarry *et al*, 1988). Deficiency in cerumen secretion can induce otitis externa in people (Senturia, 1950; Senturia and Marcus, 1952; Senturia and Carr, 1957; Browning, 1987). Inadequate protection of the aural integument by cerumen is believed to enhance microbial infections in the human ear canal (Senturia, 1950; Senturia and Marcus, 1952; Sophian and Senturia, 1955; Senturia and Carr, 1957; Baumann, Carr, and Senturia, 1961).

Most studies related to canine otitis externa have been concerned with bacteria and fungi (Joshua, 1959; Pugh *et al*, 1974; Woody and Fox, 1986b). Nevertheless, the role that microorganisms play in both human and canine otitis externa is still controversial (Perry and Nichols, 1956; Fraser, 1961a; Fraser, 1965; August, 1986; Browning, 1987; Mason, 1992). A variety of bacteria and fungi have been isolated from inflamed as well as healthy ears, such as Gram-positive cocci, Gram-negative rods, and yeasts (Farrag and Mahmoud, 1953; Fraser, 1961a; Fraser, 1961c; Fraser *et al*, 1961; Fraser, 1965; Fraser *et al*, 1970; Baxter and Lawler, 1972; Rose, 1976c; Rose, 1976d; Sinha, Mohapatra, and Kumar, 1976; Rycroft and Saben, 1977; Evans and Jemmett, 1978; Baba *et al*, 1981; Griffin, 1981; Gabal, 1988; Griffin, 1990; Mason, 1992). Few data suggest that these microorganisms are of primary aetiologic importance in canine otitis externa (Fraser, 1965; McKeever, 1993). Some researchers consider that bacteria and fungi are chiefly responsible for the onset of the disease,

whilst others believe that microbial infections are always secondary to some other factor(s). In humans, infectious otitis externa is generally thought to be induced by compromised host defence or secondary to a deranged micro-environment. It is considered unlikely that infectious agents alone can initiate such inflammation (Salvin and Lewis, 1946; Perry and Nichols, 1956; Wilson, Pulec, Minn, and Linthicum, 1971; Petrozzi and Warthan, 1974; Yust, Radiano, Tartakovsky, Tanay, Shanon, and Segal, 1980; Salit, Miller, Wigmore, and Smith, 1982; Gherini, Brackmann, and Bradley, 1986; Babiatzki and Sade, 1987; Bergbrant and Faergemann, 1989; Cohen, 1990; Strauss, 1990).

The ear mite (*Otodectes cynotis*) has been found frequently to be associated with canine otitis externa (Hering, 1834; cited by DeJong, 1891; Sewell, 1891; Kaufmann and Frost, 1949; Zepp, 1950; Grono, 1969b; Fraser *et al*, 1970; Rose, 1976e; Evans and Jemmett, 1978; Grono, 1980; Griffin, 1981; Woody and Fox, 1986a; August, 1988; Muller *et al*, 1989; Griffin, 1990; Bruyette and Lorenz, 1993). Grono (1969b) has shown that this mite alone could cause the disease. Reaginic hypersensitivity to *Otodectes cynotis* infestation has been reported in cats (Powell, Weisbroth, Roth, and Wilhelmsen, 1980). Other parasites, such as *Demodex canis*, *Sarcoptes scabiei*, *Notoedres cati*, fly bites (*Stomoxys calcitrans*) and ticks (*Otobius megnini*) have also been reported as causes of canine otitis externa (Griffin, 1981; Yazwinski, Pote, Tilley, Rodriguez, and Greenway, 1981; August, 1988; McKeever and Torres, 1988; Muller *et al*, 1989; Neer, 1990).

Generalized skin conditions have frequently been reported in association with canine otitis externa. Dermatological disorders may be present in up to 50% of cases (Fraser, 1961a; Griffin, 1991). The extension of a skin disorder to the external auditory meatus might be responsible for the initial lesion of otitis. Dogs affected by primary or

secondary seborrhoea, pyoderma and zinc-responsive dermatoses can develop otitis. Allergic dermatoses, such as atopy, contact dermatitis, and food hypersensitivity; and immune-mediated diseases including lupus erythematosus, the pemphigus complex, and proliferative eosinophilic dermatitis may also lead to canine otitis. Otitis externa can also be associated with endocrinopathies, such as hypothyroidism (Fraser, 1961a; Fraser, 1965; Evans and Jemmett, 1978; Griffin, 1981; Woody and Fox, 1986b; August, 1988; McKeever and Torres, 1988; Mason, Harvey, and Orsher, 1988; Macy, 1989; Muller *et al*, 1989; Griffin, 1990, Matthiesen and Scavelli, 1990; Sharp, 1990; White and Pomeroy, 1990; Neer, 1990; Griffin, 1991; Poulet, Valentine, and Scott, 1991; McKeever, 1993).

Dogs fed mainly fish, or an imbalanced diet leading, for example to vitamin A deficiency, may develop otitis externa (Mayhew, 1854; Griffin, 1990).

Foreign bodies in the ear, such as dirt, grass awns, and seeds may provoke otitis externa (Saunders, 1914; Fraser *et al*, 1970; Evans and Jemmett, 1978; Griffin, 1981; Brenann and Ihrke, 1983; McKeever and Torres, 1988; Griffin, 1990; Neer, 1990; McKeever, 1993). Trauma, for example contusion or fracture of the cartilage of the external ear canal, or iatrogenically induced damage, can frequently initiate inflammation in this site (Evans and Jemmett, 1978; Griffin, 1981; Janssens, 1982; McKeever and Torres, 1988; August, 1986; McKeever, 1993).

Numerous different tumours around or within the external ear canal may present with clinical signs of otitis externa (Griffin, 1981; Rogers, 1988; Little, Pearson, and Lane, 1989; Kirpensteijn, 1993). Inflammatory polyps ascending from the nasopharynx can extend and provoke further inflammation in the external ear canal (McKeever and Torres, 1988; Rogers, 1988).

In spite of a wealth of information about the possible causes of otitis externa in dogs, very little is known about their relative importance in pathogenesis (Fraser, 1965).

Histopathology

The histopathological changes found in ears affected by chronic otitis externa include: epithelial hyperplasia and enlargement of the apocrine glands (Nielsen, 1953; Fernando, 1967; Fraser, 1961d; Fraser *et al*, 1961; Fraser *et al*, 1970; Woody and Fox, 1986b; Roth, 1988; Macy, 1989; Muller *et al*, 1989; Stout-Graham *et al*, 1990). Changes to the sebaceous glands have remained controversial. This glandular tissue has been reported to be hyperplastic (Nielsen, 1953) or to become atrophic during the disease (Fraser, 1961d; Fernando, 1967). However, Stout-Graham *et al* (1990) reported that in a series of dogs with severe otitis the sebaceous glands of the horizontal ear canal had neither become atrophic nor enlarged.

Hyperactivity of the apocrine glands in diseased ears has been postulated to contribute to chronic otitis externa (Fraser, 1961d; Fernando, 1967). More and well developed adnexal gland tissue in the aural integument is thought to produce more cerumen, or perhaps retention of cerumen in the canal. This has been proposed to predispose to canine otitis externa (Joshua, 1958; Fernando, 1967; Evans and Jemmett, 1978; August, 1986; August, 1988).

Based on histochemical studies of aural epithelial glands in dogs, Fernando (1966; 1967) stated that hyperactive apocrine glands which secrete acidic substances become the main secretory gland in otitis. He argued that this cerumen would be acidic which could create unfavourable conditions for microbial infections. In humans, on the contrary, hyperplastic apocrine glands in otitic ears were proposed to result from

plugging of the gland ducts by hyperkeratosis rather than genuine hypersecretion. Integument deprived of secretion from the apocrine glands may be inadequately protected. If this inadequate protection is prolonged, microbial invasion might ensue (Senturia, 1950; Browning, 1987). Goffin (1963a; 1963b) and Grono (1970b) found that ears affected by otitis externa had a higher pH on the aural integument than healthy subjects in both humans and dogs. This alteration would favour bacterial overgrowth (Goffin, 1963a).

Histochemical and immunostaining studies of canine skin secretion suggest that apocrine (eccrine) and sebaceous glands not only provide nutrients to cutaneous flora, but also can regulate the flora by antimicrobial activities mediated by immunoglobulins (Fernando, 1966; Halliwell, 1973; Garthwaite, Lloyd, and Thomsett, 1983). These studies examined the skin and not the ear canal, whilst little information concerned with the composition of canine cerumen and its physiological functions is available. As long ago as 1955, Senturia, Sophian, Chiang, and Lowry suggested that a sound understanding of the pathogenesis, treatment, and prophylaxis of otitis externa should be based on biochemical investigations of cerumen to elucidate its physiological functions.

Clinical signs

Presenting signs reported most commonly by the owners of dogs with otitis externa are head shaking and scratching at the ears. Erythema and swelling of the lining of the aural canal, aural discharges (otorrhoea), desquamation of the aural epithelium, and various degrees of pain or pruritus, are the most frequent clinical signs found in association with otitis externa (Blaine, 1841; Mayhew, 1854; Saunders, 1914; Witter, 1949; Kaplan, 1951; Fraser *et al*, 1970; Baxter and Lawler, 1972; Pugh *et al*, 1974; Grono, 1980; Griffin, 1981; August, 1986; Woody and Fox, 1986a;

August, 1988; Muller *et al*, 1989; Griffin, 1990). Self-trauma, excoriations of the pinna and pinnal alopecia may result from this pruritus (Griffin, 1981; Woody and Fox, 1986a; Griffin, 1990). When the condition is complicated by bacterial or fungal infections, the aural discharge may become purulent and moist and develop a foul odour (Muller *et al*, 1989; Griffin, 1990). The apparent degree of discomfort exhibited by a dog with otitis externa does not always appear to bear a close relationship to the severity of other clinical signs (Fraser *et al*, 1970).

Chronic otitis externa is characterized by irreversible proliferation of the aural integument (Evans and Jemmett, 1978; Grono, 1980). This condition may progress to end-stage otitis, which may be defined as stenosis of the horizontal canal occurring together with chronic otitis externa and otitis media (Figure 1.4.1). Marked thickening of the aural integument with narrowing of the canal lumen is usually seen in such cases and can be accompanied by ossification of the cartilages of the ear (Grono, 1980; Woody and Fox, 1986a; Little, 1988; McKeever and Torres, 1988; Mason *et al*, 1988; Little and Lane, 1989; Muller *et al*, 1989; Beckman, Henry, and Cechner, 1990; Matthiesen and Scavelli, 1990; Little, Lane, and Pearson, 1991). Otitis media, inflammation of the middle ear cavity, is common in dogs with chronic and severe otitis externa (Spreull, 1964; Smeak and DeHoff, 1986; Little *et al*, 1991). Clinical signs of head-tilt, circling, falling to the affected side, nystagmus, ataxia and deafness are exhibited when the disease extends into the inner ear (Woody and Fox, 1986a; Beckman *et al*, 1990).

Dermatological disorders are frequently associated with canine otitis externa. Clinical signs of generalized immune-mediated dermatological diseases, seborrhoeic dermatitis, and pyoderma, may be present in dogs with otitis externa (Fraser, 1961a; Fraser, 1965; Evans and Jemmett, 1978; Griffin, 1981; August, 1988; Mason *et al*, 1988; Macy,

1989; Griffin, 1990, Matthiesen and Scavelli, 1990; Sharp, 1990; White and Pomeroy, 1990; Griffin, 1991).

Diagnosis

The accurate diagnosis of otitis externa and its cause(s) relies upon a complete history and thorough physical examination (Baxter and Lawler, 1972; Griffin, 1981; Rosser, 1988; Macy, 1989; Little, 1990; Neer, 1990; McKeever, 1993). Medical history, especially of dermatological disorders, endocrinopathies, and previous medication may help to suggest the aetiology (Fraser, 1961a; Rosser, 1988; McKeever and Richardson, 1988a; Macy, 1989). Physical examination must include general evaluation of body condition and a dermatological assessment. The clinical signs of skin disorders present in dogs with otitis externa may indicate underlying cause(s) and suggest treatment (Mason *et al*, 1988; Rosser, 1988; Macy, 1989; Muller *et al*, 1989).

Cytological examination of the aural discharge, together with an otoscopic examination can contribute to diagnosis (Griffin, 1981; August, 1986; Neer, 1990; McKeever, 1993). The colour of the aural discharge has been reported to bear some relationship to the bacterial or fungal flora present (Fraser *et al*, 1970; Griffin, 1981; August, 1986; Woody and Fox, 1986b; Chester, 1988; Macy, 1989). However, some researchers have reported that the correlation between the colour of the discharge and associated microorganisms was not close (Pugh *et al*, 1974; Evans and Jemmett, 1978). Cytological examination of the aural discharge may help to determine the infective agent(s), if any, that are present (Griffin, 1981; August, 1986; Woody and Fox, 1986b; Chickering, 1988; Rosser, 1988; Muller *et al*, 1989; Griffin, 1990; Neer, 1990; Mason, 1992). This technique usually does not establish a definitive causative diagnosis (Griffin, 1981; Chickering, 1988; Griffin, 1990).

Otoscopic examination of the external ear canal provides for direct inspection of tissue changes in the integument, debris, discharge and the tympanic membrane. The presence of foreign bodies and parasites may also be recognised (Fraser *et al*, 1970; Griffin, 1981; Woody and Fox, 1986a; McKeever and Richardson, 1988a; Mansfield, 1988; Rosser, 1988; Macy, 1989; Muller *et al*, 1989; Neer, 1990). In chronic cases of otitis however, otoscopy may be difficult due to the accumulation of exudate and debris in the canal, or even impossible due to thickening or hyperplasia of the aural integument. The tympanic membrane itself has usually been affected as a consequence of the otitis externa. It may be thickened or ruptured (Spreull, 1964; Moltzen, 1969; Fraser *et al*, 1970; Little, 1988; Little and Lane, 1989; Little *et al*, 1991). Tympanometry can be used for the assessment of the tympanic membrane in cases suspected of extending to involve the middle ear; however this technique is not widely available (Forsythe, 1985; Little, 1988; Little and Lane, 1989).

Microbiological culture and sensitivity tests may be performed when systemic or topical antibiotic therapy is envisaged, particularly for chronic cases. These techniques can only indicate a possible therapeutic regimen rather than provide a causative diagnosis (Griffin, 1981; Rosser, 1988; Griffin, 1990).

Where otitis media is suspected radiographic examination can be used to evaluate the condition of the middle ear (Fraser *et al*, 1970; Little, 1988; Rosser, 1988).

Biopsy of the ear canal is rarely performed due to the potential surgical complications and considerations of cosmetic appearance, unless a tumour is suspected (Rosser, 1988; Muller *et al*, 1989). Where biopsy is done histological evaluation of the pattern of inflammation may allow a specific diagnosis, such as an immune-mediated aetology (Rosser, 1988; Roth, 1988).

In humans, computerized tomography (CT scanning), technetium-99m scanning, gallium 67 scintigraphy, and magnetic resonance imaging are used in addition to clinical assessment of chronic or severe otitis externa. These techniques can allow visualization of ear structures which the standard otoscopic inspection does not provide. Extension of the disease process, and the severity of changes to the soft, cartilaginous, and bony tissues damaged by otitis externa can be determined and staged (Curtin, Wolfe, and May, 1982; Gold, Som, Lucente, Lawson, Mendelson, and Parisier, 1984; Smoker and Dolan, 1984; Chakeres, Kapila, and LaMasters, 1985; Gherini *et al*, 1986; Piwnica-Worms, Forman, and Tumeh, 1986; Timon and O'Dwyer, 1989; Vanneste, Casselman, Lemahieu, and Wilms, 1989; Ford and Courteney-Harris, 1990; Rubin, Curtin, Yu, and Kamerer, 1990; Uri, Gips, Front, Meyer, and Hardoff, 1991).

Treatment

Canine otitis externa should be treated medically. Successful treatment depends on accurate identification of underlying causes (Grono, 1980; Griffin, 1981; Bradley, 1988; Little, 1990; McKeever, 1993). The efficacy of medical treatment is likely to depend on a correct understanding of normal physiology of the aural integument and the pathogenesis of otitis externa (Senturia, 1950). Numerous papers have reviewed this medical treatment (Zepp, 1950; Fraser *et al*, 1965; Fraser *et al*, 1970; Pugh *et al*, 1974; Rose, 1976b; Rycroft and Saben, 1977; Harvey, 1980; Griffin, 1981; August, 1986; Woody and Fox, 1986a; Chester, 1988; McKeever and Richardson, 1988b; Mansfield, 1988; Wilcke, 1988; Macy, 1989; Muller *et al*, 1989; Studdert and Hughes, 1991; Bruyette and Lorenz, 1993; McKeever, 1993).

The initial management depends upon thorough but gentle cleansing of the ear canal to remove tissue debris (Zepp, 1950; Fraser *et al*, 1970; Pugh *et al*, 1974; Rose, 1976b; Rycroft and Saben, 1977; Harvey, 1980; August, 1986; Woody and Fox, 1986a; Chester, 1988; McKeever and Richardson, 1988b; Mansfield, 1988; Macy, 1989; Muller *et al*, 1989; Bruyette and Lorenz, 1993; McKeever, 1993). Cleansing with ceruminolytic agents is often suggested (Fraser *et al*, 1970; Griffin, 1981; Macy, 1989; Muller *et al*, 1989; Robinson and Hawke, 1989; Robinson, Hawke, Mackay, Ekem, and Stratis, 1989; Griffin, 1990; McKeever, 1993). Nevertheless, there are little objective data which indicate that commercial ceruminolytic preparations have any advantages over water or saline (Robinson and Hawke, 1989).

Topical or systemic administration of antibiotics and antimycotics is widely recommended and the choice of these for any particular case should, wherever possible, be predicated upon knowledge of the organisms involved and their sensitivity to these agents as well as an understanding of the drugs themselves (Fraser *et al*, 1961; Fraser *et al*, 1970; Pugh *et al*, 1974; Rose, 1976b; Chester, 1988; Muller *et al*, 1989; Studdert and Hughes, 1991). Topical acidifying agents have been used in the treatment of otitis externa as antibacterials (Chester, 1988; Wilcke, 1988). However, change in hydrogen ion concentration on the aural surface may be neutralized immediately by the buffering system of the aural integument (Grono, 1970b). Lavage solutions containing chelating agents, surfactants, and lysozyme have also been utilized with some success in this role (Blue, Wooley, and Eagon, 1974; Blue and Wooley, 1977; Wooley, Jones, Gilbert, and Shotts, 1983; Wooley, Jones, and Shotts, 1985; Wooley, Dickerson, and Engen, 1988).

The use of anti-inflammatory drugs for the treatment of otitis externa is controversial (Grono, 1980; Moriello, Fehrer-Sawyer, Meyer, and Feder,

1988; Macy, 1989; Meyer, Moriello, Feder, Fehrer-Sawyer, and Maxwell, 1990; Merchant and Caprile, 1991). Corticosteroids may induce systemic side effects and provoke bacterial overgrowth by blunting the defence mechanisms of the body against pathogens (Grono, 1980; Moriello *et al*, 1988; Meyer *et al*, 1990; Merchant and Caprile, 1991; Bruyette and Lorenz, 1993). On the other hand, the itch-scratch cycle (Halliwell, 1974; Martin, 1985; Denman, 1986; Wahlgren, Scheynius, and Hagermark, 1990) which characterises otitis externa must be broken if further tissue destruction is to be avoided; here corticosteroids are often very effective and are thus widely recommended by clinicians (August, 1986; Muller *et al*, 1989; Merchant and Caprile, 1991; Bruyette and Lorenz, 1993).

Antiparasitic agents should be used in cases of otitis externa which present with parasitic infestation (Rose, 1976e; Faulk and Schwirck, 1978; Pott and Riley, 1979; August, 1986; Chester, 1988; Macy, 1989; Muller *et al*, 1989; Bruyette and Lorenz, 1993; McKeever, 1993).

Most topical medications which are commercially available are combinations which contain some or all of the following: antibacterial, antifungal, antiparasitc, and anti-inflammatory agents (Griffin, 1981; Chester, 1988). Based on the complicated and multifactorial nature of otitis externa, such combined medications have been used symptomatically (Sampson, Bowen, Murphy, and Schneider, 1973; Rycroft and Saben, 1977; Evans and Jemmett, 1978; Griffin, 1990; Studdert and Hughes, 1991). Symptomatic treatment of otitis externa may result in an adequate therapeutic response in many cases, but the condition may not resolve or may resolve only temporarily (McKeever and Richardson, 1988a). The recurrence rate for canine otitis externa treated medically has been reported to range from 11% to 50% (Houdeshell and Hennessey, 1972; Sampson *et al*, 1973; Blue *et al*, 1974; Pugh *et al*, 1974; Rycroft and Saben, 1977; Studdert and Hughes, 1991). Chronic and nonresponsive

otitis externa often develops when the underlying causes are left uncovered and untreated (Chester, 1988).

Surgical treatments for otitis externa are limited to ablation techniques for the removal of irreversibly diseased tissue and other salvage measures which aim to combine debridement with reconstructive techniques to improve the aural environment. Several surgical techniques have been developed, such as Lacroix's technique (V-shaped resection of the cartilage on the lateral wall of the vertical ear canal), Zepp's technique (a more radical resection of the lateral wall of the vertical ear canal), vertical ear canal ablation, and total ear canal ablation together with, or without, bulla osteotomy (Schnelle, 1941; Zepp, 1949a; Zepp, 1949b; Tufvesson, 1955; Fraser *et al*, 1961; Fraser *et al*, 1970; Abramowicz, 1978; Rose, 1978; Grono, 1980; Siemering, 1980; Griffin, 1981; Gregory and Vasseur, 1983; Fox and Woody, 1986; Lane and Little 1986; Smeak and DeHoff, 1986; Tirgari and Pinniger, 1986; Bradley, 1988; Hobson, 1988; Mason *et al*, 1988; Tirgari, 1988; Beckman *et al*, 1990; Matthiesen, and Scavelli, 1990; Sharp, 1990; White and Pomeroy, 1990; Layton, 1993; Smeak and Kerpsack, 1993).

Unresolved dermatological disorders, irreversible or residual lesions, otitis media, formation of fistula, or incorrect patient selection for the chosen surgical technique may contribute to the failure of surgical treatment of otitis externa (Lane and Little, 1986; Smeak and DeHoff, 1986; Mason *et al*, 1988; Beckman *et al*, 1990; Matthiesen, and Scavelli, 1990; Sharp, 1990; White and Pomeroy, 1990).

1.5 Aims of this study

The aim of this study was to reach a fuller understanding of the canine external ear as an ecosystem. Various aspects, including the microflora, anatomy, and micro-anatomy of this site, the composition of its

secretions, and the effect these may have on the microflora were investigated. This general aim was rationalised into five discrete studies.

The aims of these studies were:

1. To survey the microbial flora of healthy canine ears and ears affected by otitis externa and to elucidate the relationship between these microorganisms and otitis externa.

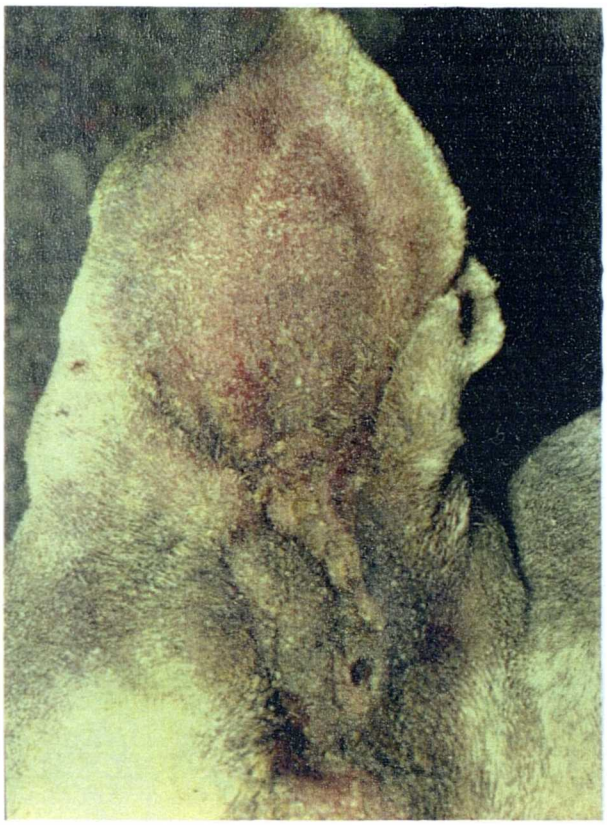
2. To analyse micro-anatomical features of the aural integument in both healthy ears and ears affected by otitis externa. A clearer understanding of anatomical and histological aspects of the ear canal should clarify the relevance of these characteristics to canine otitis externa.

3. To establish whether immunoglubulins are present in canine cerumen (earwax) and their relevance to both healthly and diseased ears in order more fully to characterise the defence mechanisms of the aural integument.

4. To analyse the lipid content of canine cerumen with a view to ascertaining the role(s) that lipids might play in the microenvironment of the canine ear.

5. To investigate whether components of canine cerumen can affect the resident aural flora in order more fully to understand the pathogenesis of otitis externa in dogs.

Figure 1.4.1 End-stage canine otitis. The external ear canal is occluded by irreversible proliferation of the aural skin.



CHAPTER II

CYTOLOGY AND MICROBIOLOGY OF THE EXTERNAL EAR CANAL IN DOGS

2.1 INTRODUCTION

Bacterial overgrowth has been found in association with many cases of otitis externa in dogs (Joshua, 1958). Numerous research publications and investigations which have addressed the aetiology of canine otitis externa have been concerned with these microorganisms (Jones, 1955; Joshua, 1958; Fraser, 1961a; Pugh *et al*, 1974; McCarthy and Kelly, 1982; Uchida, *et al*, 1990). It is however important to realise that the ear canals of most normal dogs harbour a variety of commensal and potentially pathogenic bacteria (Fraser, 1961a, August, 1988). These microorganisms, considered to belong to the resident flora, are usually found at a population density exceeding 1000/cm^2 (Merchant, 1990).

It is known that healthy skin elsewhere in the body also has a resident flora of bacteria and fungi, which is relatively stable in numbers (Price, 1938). The skin emulsion has been shown to support this resident flora. The constituents of the skin emulsion, such as lipids, carbohydrates, and nitrogen sources, act as nutrients for cutaneous flora (Usher, 1928; Arnold *et al*, 1930; Pillsbury and Rebell, 1952; Cove *et al*, 1980; Holland, 1993). The bacterial and fungal population is most abundant in the areas with a high density of sebaceous and sweat glands and high sebum secretion. This supports the theory that the skin surface emulsion serves as a culture medium for cutaneous flora (Kligman *et al*, 1976; Cove *et al*, 1980; Faergemann, Aly, and Maibach, 1983). Colonisation of the human skin with some elements of the resident flora appears to start when sebaceous glands become well developed (Matta, 1974; Faergemann and Fredriksson, 1980). Desiccation of the skin surface appears to be another factor which affects the microbial population (Rebell, Pillsbury, De Saint Phalle, and Ginsburg, 1950; Pillsbury and Rebell, 1952; Noble, 1975). A higher skin surface moisture induced by occlusion has been shown to

promote the growth of cutaneous microflora (Marples and Kligman, 1969; Bibel and LeBrun, 1975; Kligman *et al*, 1976; Aly *et al*, 1978; Faergemann *et al*, 1983; Hartmann, 1983).

Cutaneous microorganisms, such as coagulase-negative staphylococci, corynebacteria, and micrococci are capable of utilizing lipid in the skin surface emulsion and of liberating short-chain fatty acids. These short-chain fatty acids are thought to be responsible for the characteristic body odour of each individual (Marples, Kligman, Lantis, and Downing, 1970; Kligman *et al*, 1976; Lukacs, Korting, Ruckdeschel, and Ehret, 1991; Rennie, Gower, and Holland, 1991). Some members of the resident human cutaneous flora are also capable of regulating other resident microbes (Selwyn, 1975; Youssef *et al*, 1978; Noble *et al*, 1980; Lappin-Scott *et al*, 1985; Allaker *et al*, 1988; Allaker *et al*, 1989; Allaker *et al*, 1990), and by their metabolites (or secretion) prevent pathogenic agents from colonizing skin (Marsh and Selwyn, 1977; Nazzaro-Porro, 1987; Allaker and Noble, 1993).

It is important to evaluate the microflora inhabiting the healthy canine ear canal before assessing the abnormalities associated with, or caused by, microorganisms. A variety of bacteria have been isolated from healthy canine ears. Those most frequently found were coagulase-negative staphylococci, which have been isolated from 3% to 75% of canine ears in different studies (Fraser, 1961a; Fraser, 1961b; Marshall *et al*, 1974; McCarthy and Kelly, 1982). Coagulase-positive staphylococci have been found in 1.5% to 47.6% of healthy canine ear canals (Grono and Frost, 1969; Marshall *et al*, 1974; McCarthy and Kelly, 1982; Chengappa *et al*, 1983). Other Gram-positive cocci have also been reported, such as streptococci, recovered from 1.9% to 30% of healthy ears in dogs (Fraser, 1961a; Grono and Frost, 1969; Gedek *et al*, 1979; McCarthy and Kelly, 1982), micrococci, recovered from 1% to 30% of

healthy canine ears (Gedek *et al*, 1979; McCarthy and Kelly, 1982) and corynebacteria, isolated from 5% to 26% of normal canine ears. Gram positive rods, *Bacillus* species, were found in 21% to 74% of normal ears in dogs (Grono and Frost, 1969; Gedek *et al*, 1979; McCarthy and Kelly, 1982). Gram-negative rods are less common in healthy canine ears, they account for less than 3% of the bacteria isolated (Fraser, 1961a; Grono and Frost, 1969; Marshall *et al*, 1974; Gedek *et al*, 1979; McCarthy and Kelly, 1982; Chengappa *et al*, 1983). *Streptomyces* species, *Actinomyces* species, and *Propionibacterium* species have been recovered from healthy ears in dogs, but at low frequencies (Dickson and Love, 1983).

Yeasts are also members of the resident flora of normal canine ears (August, 1988). *Malassezia pachydermatis* is the most common yeast isolated from this habitat (Fraser, 1961b, Baxter, 1976; Gedek *et al*, 1979; McCarthy and Kelly, 1982; Uchida *et al*, 1990), and the prevalence of this organism has been reported to range from 2% to 96% (Fraser, 1961c; Smith, 1968; Marshall *et al*, 1974; Baxter, 1976; Sinha *et al*, 1976; Gedek *et al*, 1979; McCarthy and Kelly, 1982; Chengappa *et al*, 1983; Uchida *et al*, 1990). Candida species, and fungi, such as *Aspergillus* species, *Penicillium* species and *Rhizopus* species have all been isolated from healthy canine ears (Fraser, 1961c; Sinha *et al*, 1976; Gedek *et al*, 1979), but they are uncommon.

A variety of bacteria have been isolated from canine ears with otitis externa. Coagulase-positive staphylococci have been reported most frequently, in from 13% to 86% of cases (Fraser, 1961a; Grono and Frost, 1969; Baxter and Lawler, 1972; Marshall *et al*, 1974, Pugh *et al*, 1974; Sharma and Rhoades, 1975; Blue and Wooley, 1977; Gedek *et al*, 1979; Baba *et al*, 1981; McCarthy and Kelly, 1982; Chengappa *et al*, 1983; Mansfield, Boosinger and Attleberger, 1990; Uchida *et al*, 1990; Studdert and Hughes, 1991). Streptococci have been found in from 7% to 30% of

cases (Fraser, 1961a; Grono and Frost, 1969; Pugh *et al*, 1974; Blue and Wooley, 1977; Gedek *et al*, 1979; McCarthy and Kelly, 1982). *Corynebacterium* species, *Propionibacterium* species, and *Bacillus* species have also been isolated from canine ears with otitis externa, but they are less common than other Gram-positive cocci. *Pseudomonas* species have been found associated with from 5% to 35% of cases of otitis externa in dogs (Fraser, 1961a; Grono and Frost, 1969; Baxter and Lawler, 1972; Marshall *et al*, 1974; Sharma and Rhoades, 1975; Blue and Wooley, 1977; Gedek *et al*, 1979; Baba *et al*, 1981; McCarthy and Kelly, 1982); *Proteus* species have been isolated from 3% to 46% of dogs with otitis externa (Fraser, 1961a; Grono and Frost, 1969; Baxter and Lawler, 1972; Pugh *et al*, 1974; Blue and Wooley, 1977; Baba *et al*, 1981). Colifoms, such as *Escherichia coli*, have also been found to be involved in from 3% to 29% of cases (Fraser, 1961a; Baxter and Lawler, 1972; Blue and Wooley, 1977; Baba *et al*, 1981). *Enterobacter* species, *Pasteurella* species, *Bordetella* species, *Serratia* species, and *Actinomyces* species have also been isolated from inflamed canine ears (Grono and Frost, 1969; Baxter and Lawler, 1972; Sharma and Rhoades, 1975; Blue and Wooley, 1977; Dickson and Love, 1983; Uchida *et al*, 1990).

Malassezia pachydermatis is the yeast most frequently isolated from otitis externa in dogs (Fraser, 1961c; Baxter and Lawler, 1972; Sinha *et al*, 1976; Abou-Gabal, Chastain, and Hogle, 1979; Gedek *et al*, 1979; Uchida *et al*, 1990; Studdert and Hughes, 1991). The prevalence of this organism has been reported to vary from 12% to 80% in cases of this condition (Fraser, 1961b; Fraser, 1961c; Baxter and Lawler, 1972; Marshall *et al*, 1974; Pugh *et al*, 1974; Sharma and Rhoades, 1975; Sinha *et al*, 1976; Abou-Gabal *et al*, 1979; Gedek *et al*, 1979; Chengappa *et al*, 1983; Mansfield *et al*, 1990; Uchida *et al*, 1990; Studdert and Hughes, 1991). *Candida* species (Fraser, 1961c; Sharma and Rhoades, 1975; Blue

and Wooley, 1977; Gedek *et al*, 1979; McKellar, Rycroft, Anderson, and Love, 1990; Uchida *et al*, 1990) and *Cryptococcus* species (Pal, Ono, Goitsuka, and Hasegawa, 1990) have also been isolated from the inflamed ears of dogs. *Aspergillus* species, *Penicillium* species , and *Sporothrix* species have been found in association with otitis externa, but only at a very low frequency (Fraser, 1961c; Grono and Frost, 1969; Sharma and Rhoades, 1975; Sinha *et al*, 1976; Blue and Wooley, 1977; Dion and Speckmann, 1978; Studdert and Hughes, 1991).

Pathogenic agents, such as coagulase-positive staphylococci, haemolytic streptococci and Gram-negative rods are mainly isolated from otitic ears in dogs. These microorganisms in otitis externa were thought to play an important role in the pathogenesis (Jones, 1955; Joshua, 1958; Fraser, 1961a; Pugh *et al*, 1974; McCarthy and Kelly, 1982). According to an experiment carried out by Farrag and Mahmoud (1953), *Pseudomonas aeruginosa* alone could induce canine otitis externa. However, Salvin and Lewis (1946), Perry and Nichols (1956), and Grono (1969c) were unable to induce otitis externa in men and dogs using *Pseudomonas aeruginosa* alone; additional factors, such as an overhydrated skin (Salvin and Lewis, 1946; Hojyo-Tomoka, Marples, and Kligman, 1973), trauma, or ear mite infestation (Grono, 1969c) were required. Coagulase-positive staphylococci, haemolytic streptococci, and Gram-negative rods were unable to colonise normal, or unbroken human skin (Arnold *et al*, 1930; Burtenshaw, 1938; Rebell *et al*, 1950; Pillsbury and Rebell, 1952; Leyden, Stewart, and Kligman, 1980; Doroghazi, Nadol, Hyslop, Baker, and Alexlrod, 1981; Cole and Silverburg, 1986; Gregory and Schaffner, 1987; Kraus, Rehm, and Kinney, 1988; Zikk, Rapoport, Redianu, Shalit, and Himmelfarb, 1991). Primary overgrowth of these microorganisms rarely seems to start the process of bacterial skin infection (Hojyo-Tomoka *et al*, 1973; Hanifin and Homburger, 1986).

Self-disinfection mechanisms have been proposed as intrinsic regulators of resident skin flora (Arnold *et al*, 1930). The cutaneous immune system (see Chapter IV) and antimicrobial fatty acids (see Chapters V and VI), are thought to regulate the cutaneous microflora. Once the microenvironment of the ear canal has been altered by a variety of disturbances, opportunistic infections are believed to exacerbate the inflammatory response within the ear (Fraser, 1961b; Woody and Fox, 1986b; August, 1988; Gabal, 1988; Mansfield *et al*, 1990).

Canine otitis externa seems to be characterised by a multifactorial aetiology (August, 1988). Culture of bacteria from otitic canine ears does not confirm that the organisms play a pathogenic role in the disease process (August, 1988). The wide variety of microorganisms isolated from healthy ears and ears with otitis externa suggests that otitis externa is not primarily caused by pathogenic microorganisms and that the aetiological role of the microorganisms present is uncertain (Dibb, 1991).

Cytological examination of cerumen ("earwax" or "otorrhoea") from ears with otitis externa can provide diagnostic information rapidly by revealing the identity of infective agents, if any, and the types of inflammatory cells present in the ear canal. Based on the recognition of microorganisms, this technique can help a clinician choose symptomatic treatment (Rausch and Skinner, 1978; Griffin, 1981; Chickering, 1988; Kowalski, 1988; Rosser, 1988; Cowell, Tyler, and Baldwin, 1989). Griffin and Rosenkrantz (1988, cited by Griffin, 1990) reported that cytology was more sensitive than microbiological culture in the detection of microorganisms. However, this technique usually cannot establish the cause(s) of otitis externa (Griffin, 1981). Microbial cultures and sensitivity tests can also be utilized to establish which antimicrobial agents are likely to be therapeutically useful particularly in cases of chronic or recurrent

otitis externa (Kowalski, 1988). Again, this technique may not reveal the cause.

It has been proposed that certain clinically useful information could be provided by visual inspection of the aural discharges from dogs with otitis externa. Some correlation between clinical signs and microbiological findings has been claimed by several researchers. Generally, brown or creamy discharge was supposed to be associated with staphylococcal or streptococcal infection (Griffin, 1981; Chester, 1988; Macy, 1989). According to these reports, pale yellow or green sticky exudate was usually found when Gram-negative bacteria, such as *Pseudomonas* species, *Proteus* species, or *E. coli* were present (Griffin, 1981; Chester, 1988; Macy, 1989). Dark brown to black, thick or waxy cerumen was associated with yeasts (Griffin, 1981; Woody and Fox, 1986b; Chester, 1988; Macy, 1989). However, contrary to these claims, Pugh *et al* (1974) reported that the predominant type of cerumen in their study was either dark brown or black in spite of the involvement of many different microorganisms.

The aims of this study were firstly to identify the resident flora in normal canine ear canals and those microorganisms involved in canine otitis externa. Secondly, the use of cerumen cytology and its relevance to microbiological culture were evaluated. Thirdly, the colour of the cerumen was noted and related to the aural microorganisms found in that sample in order to clarify the value of such observations to the clinician.

The results of cerumen cytological examination and microbial culture were compared on the basis of their sensitivity and specificity. Sensitivity was defined as the proportion of positive results found where the microorganism was present. Specificity was defined as the proportion of negative results found where the microorganism was absent (Fraser, 1986; Smith, 1991).

2.2 MATERIALS AND METHODS

Canine ears which appeared clinically healthy and belonging to dogs without an history of any ear disease were chosen as the healthy group for this study. Ears from dogs which were referred to the Department of Veterinary Surgery, University of Glasgow for the investigation and treatment of aural discomfort or histories of recurrent or chronic otitis externa were chosen as the otitic group.

Seventy nine cerumen samples from 79 ears of 41 dogs were obtained using sterile microbiological swabs (BioTrace Limited, Mid Glamorgan, Bridgend). The cerumen samples were taken from the distal end of those ear canals immediately proximal to the tubercle of the anthelix. The colour of these cerumen samples was recorded. Breed, sex, and age of these dogs are summarised in Table 2.2.1.

2.2.1 Cultures

Fifty two samples, one from each ear, from 26 dogs without an history of ear disease, and 27 samples from 27 diseased ears from 15 dogs with a history of recurrent or chronic otitis externa were studied. Breed, sex, and age of these dogs are shown in Tables 2.2.2 and 2.2.3.

Materials

1. Bacterial cultures

Seven percent (v/v) sheep blood agar plates were prepared in the Department of Veterinary Pathology (Sheep blood: Becton Dickinson Limited, Cowley, U.K.; Blood Agar Base No. 2: Oxoid, Unipath Limited, Hampshire, U.K.)

MacConkey agar plates (Oxoid, Unipath Limited, Hampshire, U.K.) and **Deoxyribonuclease (DNAase) plates** (Oxoid, Unipath Limited, Hampshire, U.K.) were also used throughout this study.

2. Fungal cultures

Sabouraud glucose-peptone agar plates were prepared locally in the Department of Veterinary Pathology, University of Glasgow, according to Pepin's method (1984). These Sabouraud agar plates contained 5 mg/100 ml chloramphenicol.

Urease Broth was also prepared in the Department of Veterinary Pathology (Oxoid, Unipath Limited, Hampshire, U.K.).

3. Isolate identification

API Staph, API Strep, API, 20E, API 20NE and API 20C AUX (API, BioMerieux UK Limited, Hampshire, U.K.) were used to confirm the identity of bacteria and yeasts obtained.

Procedures

Bacterial culture was performed by streaking the aural swabs directly onto 7% (v/v) sheep blood agar and MacConkey agar plates. These plates were then incubated at 37°C for 24 to 48 hours. The swabs were also streaked onto Sabouraud glucose-peptone agar plates and the plates were incubated at 37°C for 72 hours. The growth density of each isolate on culture medium was recorded.

The density of growth of the isolate on agar plates was scored from "-" to "+ + + +". If no bacterial or yeast colonies were recovered from the culture, the score was "-". Where microbial colonies were recovered on the first streak on the culture plate the density was scored as one plus (+); where microbial colonies grew on both the first and second streaks on the plate these were scored as two plus (+ +), and so on.

Bacterial colonies were identified presumptively by their colonial appearance and effect on the medium. Morphological characteristics of the bacteria in sample isolates were examined using Gram-stained smears. These were visualized under a microscope (Olympus CH-2,

Japan) by oil immersion and the identity of these isolates was confirmed by use of the API system. The pathogenicity of coagulase-positive staphylococci were confirmed by inoculation onto DNAase agar plates which were incubated at 37°C for a further 24 hours. Morphological characteristics of yeast isolates were examined using Gram's stained (Carter and Chengappa, 1991) smears visualized in the same way. These isolates were also incubated in urease broth at 37°C for a further 24 hours to identify *Malassezia* species. API 20C AUX was used to identified yeast genera. Identification of the isolates during this study was carried out in the routine diagnostic microbiology laboratory of the Department of Veterinary of Pathology, University of Glasgow.

2.2.2 Cytological examination

Materials

Forty five cerumen samples from 45 ears of 23 dogs (22 healthy ears from 11 dogs and 23 otitic ears from 12 dogs) were studied blind, i.e. cytological examination of cerumen samples was carried out without knowledge of the health status of the ears from which these swabs were recovered. Breed, sex, and age of the dogs are summarised in Table 2.2.4.

Procedures

1. Smear preparation

Cerumen samples were collected from the distal end of the ear canals proximal to the tubercle of the anthelix using sterile microbiological swabs. Smears were obtained by rolling the swabs onto clean flamed glass microscopic slides (Gold Star KTH-370, Chance Propper Limited, Warley, England) which were then allowed to air dry.

2. Staining

Gram's stain procedure was carried out according to the method described by Cater and Chengappa (1991).

3. Microscopic examination

Smears were rapidly scanned under low-power magnification (100x) using the microscope. The morphology of Gram-positive cocci, Gram-positive rods, Gram-negative rods, and yeasts were examined using a high power magnification (400x). The number of these bacteria and yeasts within one high-powered field (HPF) was counted. Based on the same morphological characteristics, i.e. Gram-positive coccus, Gram-negative rod, or Gram-positive peanut-shaped yeast, the smear was scored from "-" to "+ + +" for each microorganism. If no bacterium or yeast was seen in one HPF, then the smear was scored "-". One plus (+) was given to smears in which less than ten bacteria, or less than five yeasts, with the same morphological characteristics were identified per HPF. Two plus (+ +) was defined as ten to 100 bacteria of similar morphology, or five to 40 yeasts of similar morphology, counted within one HPF. If more than 100 bacteria with similar morphology or more than 40 peanut-shaped yeasts were counted per HPF, a score of three plus (+ + +) was allocated for each microorganism respectively.

2.2.3 The sensitivity and specificity of cytological examination and microbial cultures of canine cerumen samples

The reliability of cytological examination and microbial cultures was assessed by examining the sensitivity and the specificity of each method. The results of one technique were tested by comparison to the overall results, i.e. a combination of the results from both techniques. In the overall results, true positives were the sum total of cases in which specific microorganisms were found by either or both methods; whereas true

negatives were the number of cases in which the microorganism was not detected by either method.

Thus, the sensitivity and the specificity of the cytological smear technique were calculated by the following formula:

		Overall results	
		+	-
Cytological smear	+	*A*	*B*
	-	*C*	*D*

Sensitivity = $A/A+C$

Specificity = $D/B+D$

Similarly, the sensitivity and the specificity of results of bacterial and fungal cultures were calculated by the following formula:

		Overall results	
		+	-
Culture	+	*A*	*B*
	-	*C*	*D*

Sensitivity = $A/A+C$

Specificity = $D/B+D$

Table 2.2.1 Breed, Sex, and age (years) of 41 dogs used for cerumen colour study.

Breed	Sex	Age
Rottweiler	M	7
Labrador	M	12
Setter	M	9
Greyhound	F	3
Labrador	F	9
Boxer	F	6
Bull Terrier	F	5
Bull Terrier	F	2
Bull Terrier	F	3
Greyhound	F	6
Bull Terrier	M	4
Bull Terrier	F	2
Bull Terrier	F	5
Bull Terrier	M	2
Bull Terrier	M	5
Bull Terrier	F	3
Springer Spaniel	F	5
Bull Terrier	M	1
Bull Terrier	F	3
Bull Terrier	M	2
Cocker Spaniel	M	3
Springer Spaniel	F	12
Cocker Spaniel	F	8
Bull Terrier	F	3
Bull Terrier	M	5
Setter	M	1
Crossbreed	F	10
St. Bernard	M	8
Golden Retriever	M	7
Crossbreed	M	11
Rottweiler	F	4
Wire Haired Fox Terrier	M	9
Rottweiler	F	5
Yorkshire Terrier	M	9
German Shepherd Dog	M	5
Cairn Terrier	M	11
German Shepherd Dog	F	7
Rhodesian Ridgeback	M	7
Labrador	F	9
Crossbreed	M	13
German Shepherd Dog	F	6

F = female, M = male.

Table 2.2.2 Breed, sex, and age (years) of 26 dogs with normal ears used for the study of the microbiology of their external ear canals.

Breed	Sex	Age
Rottweiler	M	7
Labrador	M	12
Setter	M	9
Greyhound	F	3
Labrador	F	9
Boxer	F	6
Bull Terrier	F	5
Bull Terrier	F	2
Bull Terrier	F	3
Greyhound	F	6
Bull Terrier	M	4
Bull Terrier	F	2
Bull Terrier	F	5
Bull Terrier	M	2
Bull Terrier	M	5
Bull Terrier	F	3
Springer Spaniel	F	5
Bull Terrier	M	1
Bull Terrier	F	3
Bull Terrier	M	2
Cocker Spaniel	M	3
Springer Spaniel	F	12
Cocker Spaniel	F	8
Bull Terrier	F	3
Bull Terrier	M	5
Setter	M	1

F = female, M = male.

Table 2.2.3 Breed, sex, and age (years) of 15 dogs with recurrent or chronic otitis externa used for the external ear canal microbiology study.

Breed	Sex	Age
Crossbreed	F	10
St. Bernard	M	8
Golden Retriever	M	7
Crossbreed	M	11
Rottweiler	F	4
Wire Haired Fox Terrier	M	9
Rottweiler	F	5
Yorkshire Terrier	M	9
German Shepherd Dog	M	5
Cairn Terrier	M	11
German Shepherd Dog	F	7
Rhodesian Ridgeback	M	7
Labrador	F	9
Crossbreed	M	13
German Shepherd Dog	F	6

F=female, M=male.

Table 2.2.4 Breed, sex, and age (years) of 23 dogs used for cerumen cytological examination.

Breed	Sex	Age
Rottweiler	M	7
Labrador	M	12
Setter	M	9
Greyhound	F	3
Labrador	F	9
Boxer	F	6
Bull Terrier	F	5
Bull Terrier	F	2
Bull Terrier	F	3
Greyhound	F	6
Bull Terrier	M	4
Crossbreed	F	10
St. Bernard	M	8
Golden Retriever	M	7
Crossbreed	M	11
Rottweiler	F	4
Wire Haired Fox Terrier	M	9
Rottweiler	F	5
Yorkshire Terrier	M	9
German Shepherd Dog	M	5
Cairn Terrier	M	11
German Shepherd Dog	F	7
Rhodesian Ridgeback	M	7

F = female, M = male.

2.3 RESULTS

2.3.1 Microbial flora in canine external ear canals

Fifty two normal ears from 26 dogs were studied. Based on bacterial culture, 35% (18/52) of normal ears were colonised by coagulase-negative staphylococci, such as *Staphylococcus xylosus*, *Staphylococcus cohinii*, and *Staphylococcus hominis*. Coagulase-positive staphylococci, such as *Staphylococcus aureus* were also isolated from 12% (6/52) of healthy canine ears. Corynebacteria were recovered from 35% (18/52), and Micrococci were isolated from 29% of healthy ears. *Bacillus* species, which were the only Gram-positive rods isolated from healthy ears, were found in 12% (6/52). A coliform (Gram-negative rod) was isolated from one normal ear only (2%). *Malassezia pachydermatis* was the only yeast recovered from fungal culture, the organism was found in 25% (13/52) of normal canine ears (Table 2.3.1).

As for the microbial growth density on culture plates; nine cerumen samples from nine healthy ears showed no bacterial or yeast colonies on culture (-). The remaining 43 cerumen samples from 43 healthy ears each scored "+" for growth density on either bacterial or fungal culture.

The microbiology of twenty seven otitic ears from 15 dogs were studied. Forty four percent (12/27) of these ears were overgrown by coagulase-positive staphylococci, such as *S. aureus* and *S. intermedius.* Nineteen percent (5/27) of otitic ears were associated with haemolytic streptococci, such as *Streptococcus faecalis*. Corynebacteria were isolated from one otitic ear (4%). *Bacillus* species were also recovered from bacterial culture of one ear only (4%). Thirty seven percent (10/27) of cerumen swabs from otitic ears were associated with Gram-negative rods: *Pseudomonas aeruginosa* was isolated from five ears, *Escherichia coli* in three otitic ears, and *Proteus* species in two ears. *Malassezia*

pachydermatis was isolated from one third (9/27) of the otitic ears. This was the only yeast recovered (Table 2.3.1).

Nine cerumen samples exhibited low growth density (- to +) on culture plates. Six samples were scored "-" and three were scored "+". Eighteen samples showed high colony density (+ + to + + + +) recovered from bacterial culture. All eleven ears associated with Gram-negative rods had high bacterial densities (+ + + or + + + +) on culture plates. Seven out of nine fungal cultures of nine ears colonised by *M. pachydermatis*, had a high growth density (+ + + or + + + +).

2.3.2 Cytological examination

Forty five cerumen samples from 45 ears of 23 dogs were examined cytologically without knowledge of the health status of the ears concerned. Twenty two cerumen samples were collected from 22 healthy canine ears and 23 samples were collected from 23 ears with otitis externa. Cytological examination of one cerumen sample from one normal ear and one ear affected by otitis externa are shown in Figures 2.3.1 and 2.3.2. Gram-positive cocci were found in 37 ears either by cytological examination or bacterial culture. Cytological examination detected 31 cases (84%), whereas these cocci were recovered from only 59% (22/37) of bacterial cultures (Tables 2.3.2a and 2.3.2b, and Figure 2.3.3). The specificity of cytological examination and culture for detection of Gram-positive cocci were each 100%. Thirteen ears were colonised by Gram-negative rods, all were detected by cytological examination (sensitivity: 100%) but in only 9 cases (69%) were these bacteria grown on culture (Tables 2.3.3a and 2.3.3b, and Figure 2.3.4). The specificity of cytological examination and microbial culture in the detection of Gram-negative rods were each 100%. Yeasts were present in 22 ears. Cytological examination detected all 22 cases (sensitivity: 100%), whereas fungal

culture detected only 11/22 of the cases (sensitivity 50%) (Tables 2.3.4a and 2.3.4b, Figure 2.3.5). The specificity of cytological smear and fungal culture for detection of yeasts were, once again, each 100%. The results are summarised in Table 2.3.5.

Based both on cytological examination and microbial cultures, Gram-positive cocci were found in 90% (47/52) of the normal ears, Gram-positive rods were found in 12% (6/52). Only 2 ears (4%) were colonised by Gram-negative rods. Yeasts were found in 48% (25/52) of the normal ears (Table 2.3.6). Among these normal ears, 35% (18/52) were colonised by only one genus of microorganism, 40% (21/52) by two different genera of microorganisms, and in 15% (8/52) of the normal ears three different genera of microorganisms were present. Four different genera of microflora were isolated from 6% of normal ears (3/52). Two ears (4%) did not appear to contain any microorganism by either cytological examination or microbial cultures (Table 2.3.7).

In otitic ears, when both cytological examination and microbial culture results were combined, 67% (18/27) of cases were associated with Gram-positive cocci. *Bacillus* species (Gram-positive rods) were found in 11% (3/27) of the cases. Gram-negative rods were involved in 37% (10/27) of otitic ears. Fifty two percent (14/27) of the cases were associated with yeasts (Table 2.3.6). Among these otitic ears, no microorganisms were detected in three ears (11%) using cytological examination and culture. Three ears (11%) were colonised by only one genus of microorganism; 48% (13/27) of otitic ears were inhabited by two different genera of microorganisms; four otitic ears (15%) were colonised by three different genera of microorganisms, and in a further four otitic ears (15%) four different genera of microorganisms were present (Table 2.3.7).

The relationship between microbial numbers using cytological examination and microbial growth density on culture plates was also studied. Low counts of bacteria or yeasts from cytology (- or +) were usually associated with few or no bacterial or fungal colonies (- or +) recovered from the corresponding cultures. Culture plates with a higher density of microbial colonies (+ + to + + + +) usually had higher bacterial or yeast counts (+ + or + + +) in the corresponding cerumen cytology smear. However, two cerumen samples with high Gram-positive coccal counts (+ + +) and one cerumen sample with a high Gram-negative rod count (+ + +) showed few (+) or no colonies (-) in the corresponding bacterial cultures (Tables 2.3.8a, 2.3.8b, 2.3.9a, and 2.3.9b). One cerumen sample was negative for yeasts on fungal culture (-), but it contained a very high yeast count (+ + +) when examined by cytological smear (Tables 2.3.10a and 2.3.10b).

2.3.3 Colour of cerumen samples

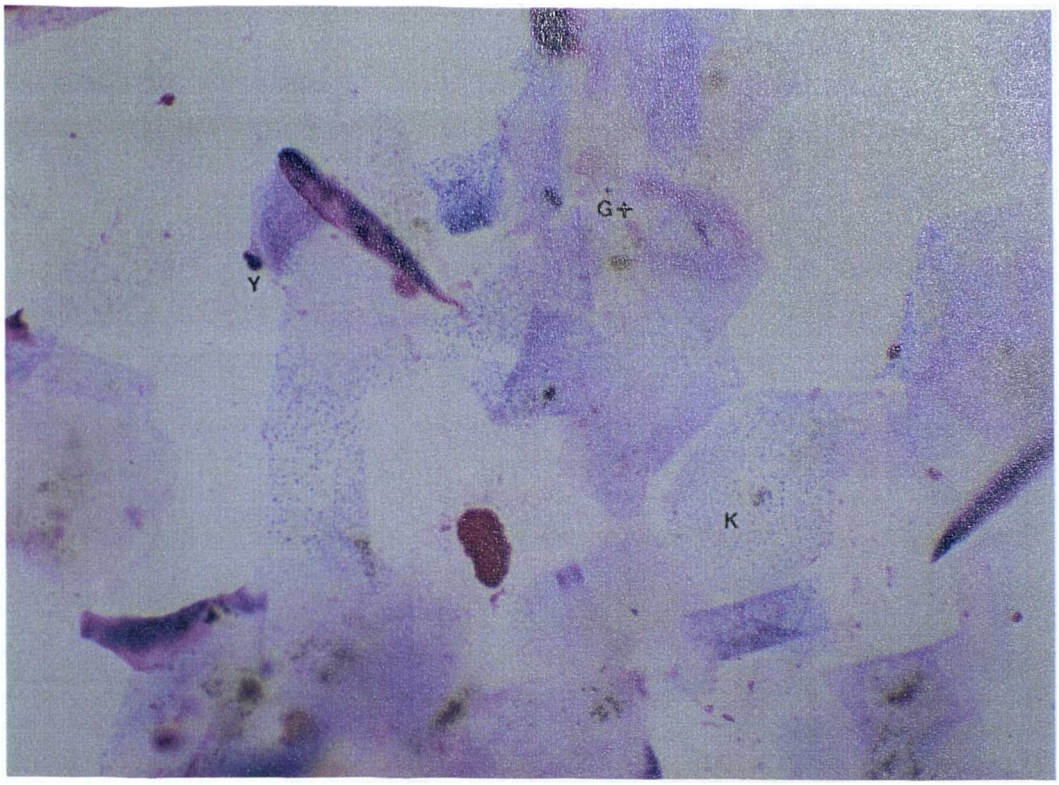
Canine cerumen samples were arbitrary categorised into two colour-types; either yellowish (including yellow and yellowish to mud coloured), or brown (including brown and brownish to black). A dry, brittle, or flaky cerumen consistency was found only in the yellow coloured cerumen only whilst a waxy or sticky consistency was found in both colour-types (Figure 2.3.6). The microorganisms found in the external ear canal were generally divided into four groups; Gram-positive cocci, Gram-positive rods, Gram-negative rods, and yeasts. Fifty three percent (36/68) of the ears from which Gram-positive cocci were recovered had brown coloured cerumen whilst 47% (32/68) of the ears had yellowish coloured cerumen. Of 13 ears containing Gram-negative rods 69% (9/13) exhibited brown cerumen and in 31% (4/13) the otorrhoea was yellowish in colour. Sixty seven percent of 33 ears (22/33) in which yeasts were found had

brown coloured cerumen, 33% (11/33) contained cerumen which was yellowish in colour (Table 2.3.11). In five ears, no microorganisms were found either by cytological examination or microbial cultures. Three of these contained cerumen with yellowish colour, and two ears had brown cerumen (Table 2.3.11).

Table 2.3.1 Resident flora isolated from 52 healthy normal ears and 27 otitic ears.

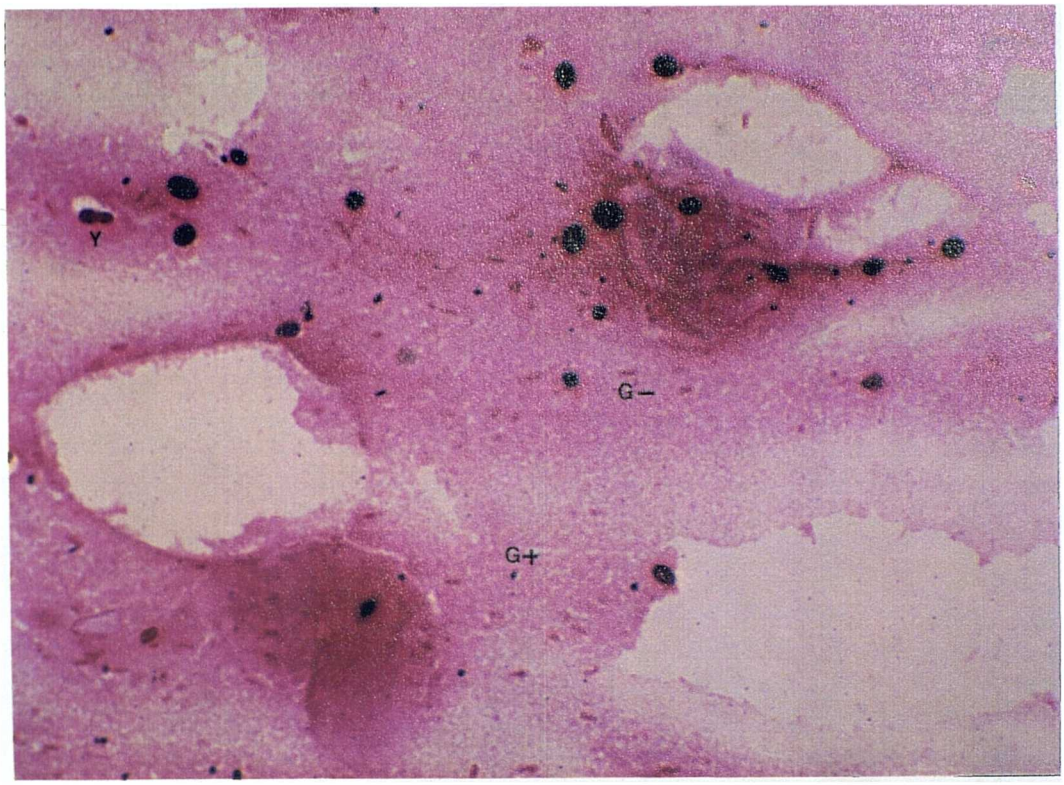
Resident flora	Normal ears	Otitic ears
Corynebacterium species	35% (18/52)	4% (1/27)
Coagulase-negative staphylococci	35% (18/52)	-
Coagulase-positive staphylococci	12% (6/52)	44% (12/27)
Micrococcus species	29% (15/52)	-
Streptococcus species	-	19% (5/27)
Bacillus species	12% (6/52)	4% (1/27)
Pseudomonas aeruginosa	-	19% (5/27)
Escherichia coli	2% (1/52)	11% (3/27)
Proteus species	-	7% (2/27)
Malassezia pachydermatis	25% (13/52)	33% (9/27)

Figure 2.3.1 Microscopic appearance of a cytological smear of cerumen from a normal canine ear (Gram, x120).



Many keratinocytes and sparse Gram-positive cocci (+) and yeasts (+) are present. K: keratinocyte; G+: Gram-positive coccus; Y: yeast.

Figure 2.3.2 Microscopic appearance of a cytological smear of cerumen from a canine ear affected by otitis externa (Gram, x1125).



Numerous Gram-positive cocci (+ + + +), Gram-negative rods (+ + + +), and yeasts (+ + + +) are present in this sample. G+: Gram-positive coccus; G-: Gram-negative rod; Y: yeast.

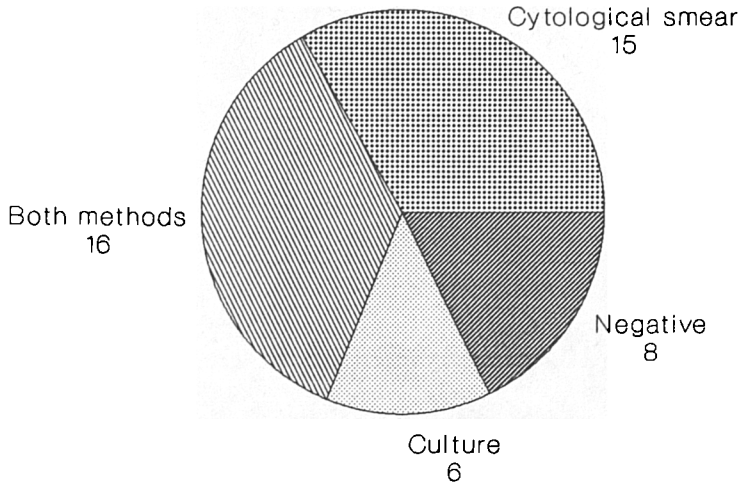
Table 2.3.2a The results of cytological examination in the detection of Gram-positive cocci from 45 cerumen samples (from 45 canine ears) compared to the overall results.

		Overall results		
		+	-	(Total)
Cytological	+	31	0	31
smear	-	6	8	14
(Total)		37	8	45

Table 2.3.2b The results of microbial culture in the detection of Gram-positive cocci from 45 cerumen samples (from 45 canine ears) compared to the overall results.

		Overall results		
		+	-	(Total)
Microbial	+	22	0	22
culture	-	15	8	23
(Total)		37	8	45

Figure 2.3.3 Comparison between the detection of Gram-positive cocci in cerumen samples by cytological smear and bacterial culture.



45 cerumen samples from 45 canine ears were studied. Gram-positive cocci were detected in 37 ears by either method.

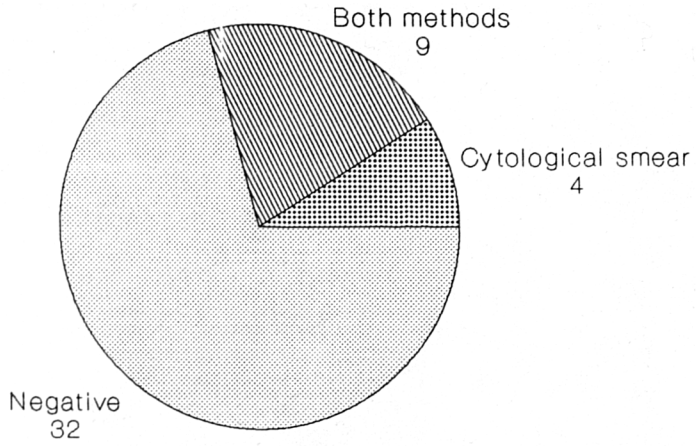
Table 2.3.3a The results of cytological examination in the detection of Gram-negative rods from 45 cerumen samples (from 45 canine ears), compared to the overall results.

		Overall results		
		+	-	(Total)
Cytological	+	13	0	13
smear	-	0	32	32
(Total)		13	32	45

Table 2.3.3b The results of microbiological culture in the detection of Gram-negative rods from 45 cerumen samples (from 45 canine ears), compared to the overall results.

		Overall results		
		+	-	(Total)
Microbial	+	9	0	9
culture	-	4	32	36
(Total)		13	32	45

Figure 2.3.4 Comparison between the detection of Gram-negative rods in cerumen samples by cytological smear and bacterial culture.



45 cerumen samples from 45 canine ears were studied. Gram-negative rods were detected in 13 ears by either method.

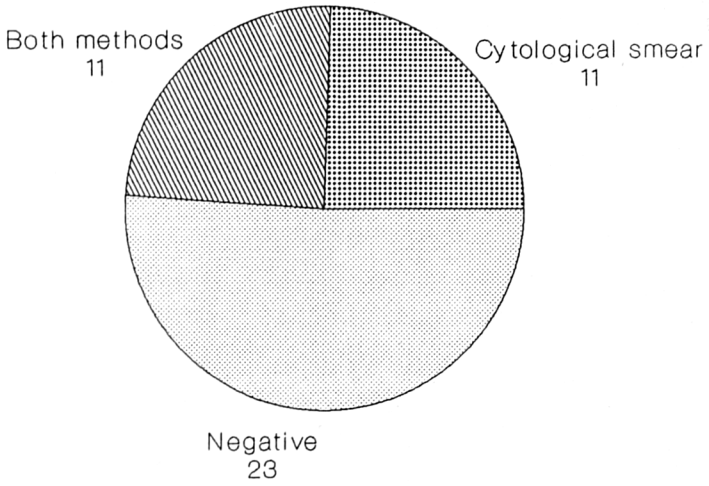
Table 2.3.4a The results of cytological examination in the detection of yeasts from 45 cerumen samples (from 45 canine ears), compared to the overall results.

		Overall results		
		+	-	(Total)
Cytological	+	22	0	22
smear	-	0	23	23
(Total)		22	23	45

Table 2.3.4b The results of fungal culture in the detection of yeasts from 45 cerumen samples (from 45 canine ears), compared to the overall results.

		Overall results		
		+	-	(Total)
Fungal	+	11	0	11
culture	-	11	23	34
(Total)		22	23	45

Figure 2.3.5 Comparison between the detection of yeasts in cerumen samples by cytological smear and fungal culture.



45 cerumen samples from 45 canine ears were studied. Yeasts were detected in 22 ears by either method.

Table 2.3.5 The sensitivity and specificity of cytological examination and culture in detecting the presence of Gram-positive cocci, Gram-negative rods, and yeasts in 45 cerumen samples from 45 canine ears.

	Cytological examination	Culture
Gram-positive cocci		
Sensitivity	84%	59%
Specificity	100%	100%
Gram-negative rods		
Sensitivity	100%	69%
Specificity	100%	100%
Yeasts		
Sensitivity	100%	50%
Specificity	100%	100%

Table 2.3.6 Prevalence of organisms in 52 healthy canine ears and 27 otitic canine ears based on both microbial cultures (Table 2.3.1) and cytological examination (Tables 2.3.2, 2.3.3, and 2.3.4).

Resident flora	Normal ears	Otitic ears
G (+) cocci	90% (47/52)	67% (18/27)
G (+) rods	12% (6/52)	11% (3/27)
G (-) rods	4% (2/52)	37% (10/27)
Yeasts	48% (25/52)	52% (14/27)

G (+) = Gram-positive; G (-) = Gram-negative

Table 2.3.7 Number of microbial genera found in 52 healthy canine ears and 27 otitic canine ears based on both cytological examination and microbial cultures.

Number of microbial genera found	Normal ears	Otitic ears
0	4% (2/52)	11% (3/27)
1	35% (18/52)	11% (3/27)
2	40% (21/52)	48% (13/27)
3	15% (8/52)	15% (4/27)
4	6% (3/52)	15% (4/27)

Table 2.3.8a The relationship between Gram-positive coccal numbers found on cytological examination and density of Gram-positive cocci isolated from bacterial culture (45 cerumen samples from 45 canine ears).

		Cytological smear				
G (+) cocci per HPF		- (0)	+ (<10)	++ (10-100)	+++ (>100)	Total
	-	8	9	5	1	23
C	+	6	7	1	1	15
plates	++	0	0	2	0	2
	+++	0	0	0	0	0
	++++	0	0	1	4	5
(Total)		14	16	9	6	45

C plates = culture plates; G (+) = Gram-positive

HPF = higher power field

Table 2.3.8b Summary of results from Table 2.3.8a.

	Cytological smear		
G (+) cocci per HPF	**- or + (0-10)**	**++ (10-100)**	**+++ (>100)**
- or +	30/30 (100%)	6/9 (67%)	2/6 (33%)
Culture			
++ to ++++	0 (0%)	3/9 (33%)	4/6 (67%)

G (+) = Gram-positive

HPF = higher power field

Table 2.3.9a The relationship between the numbers of Gram-negative rods found on cytological examination and the density of Gram-negative rods on bacterial culture (45 cerumen samples from 45 canine ears).

		Cytological smear				
G (-) rods per HPF		**-** **(0)**	**+** **(<10)**	**++** **(10-100)**	**+++** **>100**	Total
	-	32	2	1	1	36
C	+	0	1	0	0	1
plates	++	0	0	0	0	0
	+++	0	0	0	2	2
	++++	0	0	0	6	6
(Total)		32	3	1	9	45

C plates = culture plates; G(-) = Gram-negative

HPF = higher power field

Table 2.3.9b Summary of results from Table 2.3.9a.

	Cytological smear		
G (-) rod per HPF	**- or + (0-10)**	**++ (10-100)**	**+++ (>100)**
- or +	35/35 (100%)	1/1 (100%)	1/9 (11%)
Culture			
++ to ++++	0 (0%)	0 (0%)	8/9 (89%)

G (-) = Gram-negative

HPF = higher power field

Table 2.3.10a The relationship between yeast numbers found on cytological examination and the density of fungal culture (45 cerumen samples from 45 canine ears).

		Cytological smear				
Yeast per HPF		- (0)	+ (<5)	++ (5-40)	+++ >40	Total
	-	23	9	1	1	34
C	+	0	3	2	0	5
plates	++	0	1	0	1	2
	+++	0	0	0	0	0
	++++	0	1	1	2	4
(Total)		23	14	4	4	45

C plates = culture plates

HPF = higher power field

Table 2.3.10b Summary of results from Table 2.3.10a.

		Cytological smear		
Yeast per HPF		**- or + (0-5)**	**++ (5-40)**	**+++ (>40)**
Fungal culture	- or +	35/37 (95%)	3/4 (75%)	1/4 (25%)
	++ to ++++	2/37 (6%)	1/4 (25%)	3/4 (75%)

HPF = higher power field

Figure 2.3.6 Canine cerumen colours: yellow and brown.

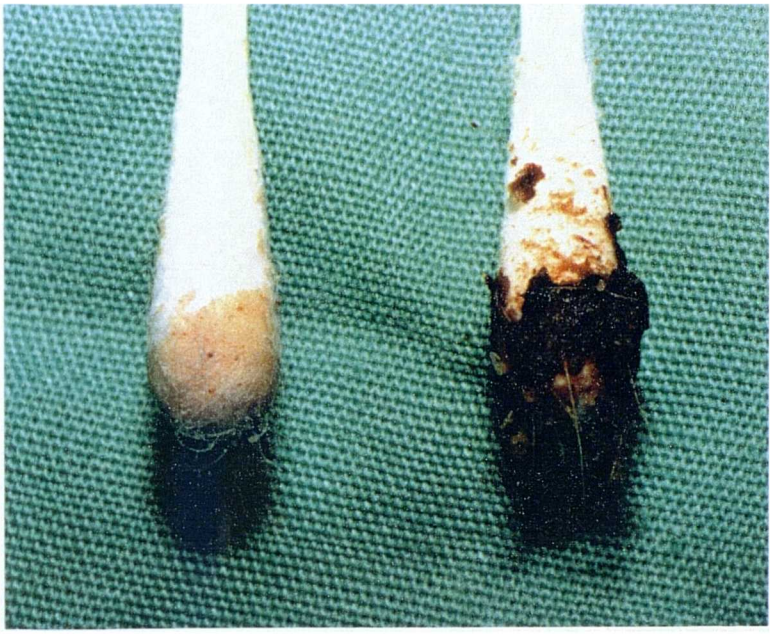


Table 2.3.11 Relationship between cerumen colour and associated microorganisms of 79 ears from 41 dogs.

	Cerumen colour	
	Brown	Yellowish
G (+)	53% (36/68)	47% (32/68)
G (-)	69% (9/13)	31% (4/13)
Yeast	67% (22/33)	33% (11/33)
No organism	40% (2/5)	60% (3/5)

G (+) = Gram-positive cocci, G (-) = Gram-negative rods

Many ears contained more than one microorganism (see Table 2.3.7).

2.4 DISCUSSION

The microbiology of 52 normal and 27 otitic canine ears was studied. Gram-positive cocci were isolated from 90% of normal canine ears. The majority of these bacteria were coagulase-negative staphylococci, corynebacteria, and micrococci. In each of these normal ears, small numbers of these cocci were found on the culture plates (- to +). Coagulase-negative staphylococci, such as *S. xylosus*, *S. cohnii*, and *S. hominis* have been regarded as belonging to the normal cutaneous flora of swine, cattle, horses, and the laboratory mouse (Shimizu, Ozaki, Kawano, Saitoh, and Kimura, 1992). Coagulase-positive staphylococci and Gram-negative rods were also isolated from normal ears with a low frequency and small numbers were present on culture plates. Coagulase-positive staphylococci have been isolated from the hair coat of 90% of clinically normal dogs (White, Ihrke, Stannard, Sousa, Reinke, Rosser, and Jang, 1983). These bacteria are regarded as transient flora, which lie free on the skin surface without attachment to the skin (Price, 1938), rather than as resident flora (Allaker, Lloyd, and Simpson, 1992).

In otitic ears, Gram-positive cocci were isolated from 50%. The majority of these cocci were characterised by their haemolytic abilities. In 40% of otitic ears Gram-negative rods were isolated. In nearly 70% of the ears inhabited by Gram-positive cocci the organisms yielded profuse cultures (+ + to + + + +) on culture plates. In the ears colonised by Gram-negative rods, a profuse culture (+ + to + + + +) of the organism was isolated in all cases.

These results suggest that the composition of the healthy aural flora is substantially different from that found in disease where bacterial overgrowth contributes to the inflammatory processes of otitis externa. However, those pathogenic bacteria, such as coagulase-positive staphylococci, haemolytic streptococci, *Pseudomonas aeruginosa*, and

Escherichia coli will not normally establish on healthy or unbroken skin (Arnold *et al*, 1930; Rebell *et al*, 1950; Pillsbury and Rebell, 1952; Leyden *et al*, 1980; Doroghazi *et al*, 1981; Cole and Silverberg, 1986; Gregory and Schaffner, 1987; Kraus *et al*, 1988; Zikk *et al*, 1991). A disrupted microenvironment, such as a damaged or superhydrated epithelial surface, or an alteration to the surface pH has been thought to initiate bacterial overgrowth (Arnold *et al*, 1930; Jones and McLain, 1961; Goffin, 1963a; Goffin, 1963b; Marples and Kligman, 1969; Hojyo-Tomoka *et al*, 1973; Bibel and LeBrun, 1975; Noble, 1975; Kligman *et al*, 1976; Aly *et al*, 1978; Hartmann, 1983). Desiccation is generally believed to play a major role in inhibiting the multiplication of pathogenic bacteria on skin (Arnold *et al*, 1930; Rebell *et al*, 1950; Pillsbury and Rebell, 1952; Aly, Maibach, Shinefield, and Mandel, 1972; Hojyo-Tomoka *et al*, 1973). Occlusion can elevate the relative humidity of the skin from 20% to 75%, change skin pH from 5 to 7, and increase the skin bacterial and yeast population from around $2 \times 10^2/cm^2$ to $1 \times 10^6/cm^2$ (Aly *et al*, 1978; Faergemann *et al*, 1983; Hartmann, 1983). Grono (1970c) found that the mean relative humidity in canine ears affected by otitis externa was 88%. This was 10% higher than normal canine ears. High humidity seems to favour the growth of pathogenic bacteria in the ear canals. According to other investigations (Grono, 1970b), the average canine aural skin pH was 6.1, with a range from pH 4.6 to 7.2. On aural skin affected by chronic otitis externa, average pH was 6.9, with a range from pH 5 to 9. When the aural skin has been damaged by the process of inflammation, the skin pH might have reached an equilibrium with the interstitial fluid, since the final pH was close to the body pH (Aly *et al*, 1978). No correlation could be demonstrated between human skin pH and bacterial count (Jones and McLain, 1961; Noble, 1968). Based on Goffin's studies in human subjects (1963a; 1963b), the optimal pH for the growth of most pathogenic bacteria

is pH 7.2 to 7.6. Infection was associated with a higher skin pH. Moreover, the optimal pH for *Pseudomonas* proteolytic and elastolytic activities is at pH 8.0. A higher skin pH, associated with bacterial infection, may provide optimal conditions for bacterial enzyme activity (Hall, Callaway, Tindall, Durham, and Smith, 1968). Other bacterial enzymes should not be overlooked when those bacteria are present in large numbers, such enzymes include the fibrinolysin, collagenase, hemolysins, keratinase, lecithinase and lipase of *Pseudomonas aeruginosa*; deoxyribonuclease (DNAase), hemolysins, esterase, lipases, and lysozyme of staphylococci; and DNAase, hemolysins, hyaluronidase, and protease of streptococci (Hall *et al*, 1968; Smith and Willett, 1968; Holt, 1972; Carter and Chengappa, 1991; Bailey and Redpath, 1992). These enzymes could cause direct damage to host tissue and exacerbate the inflammatory condition (Carter and Chengappa, 1991; Zikk *et al*, 1991). This may be one reason why antibiotics are effective in the management of otitis externa associated with bacterial overgrowth. However, bacterial overgrowth cannot usually occur in the absence of a primary cause, such as underlying skin disease. Dermatological disorders are well known to be involved in the development of many cases of otitis externa in dogs (Fraser, 1961a; Fraser, 1965; August, 1988; Griffin, 1991).

In the present study, 10% of normal ears were colonised by a low growth density (- to +) of pathogenic bacteria. This result agrees with most other studies concerned with the microbiology of the canine ear. It has been reported that pathogenic bacteria, namely coagulase-positive staphylococci and *Pseudomonas aeruginosa* were isolated from the external ear canals of 80% of children without a history of otitis externa (Brook, 1981). In another study (Ostfeld, Segal, Segal, and Bogokovski, 1983), 39% of external ear canals of newborn infants were colonised by coagulase-negative staphylococci, coagulase-positive staphylococci were

isolated from 23%, and Gram-negative rods were found in 22%. The type of delivery and duration of hospitalisation had a significant influence on the pattern of bacterial colonisation. The aural microbiology of neonatal puppies may also be influenced by their growing environment. A long term study of the changing population dynamics of the canine aural canal might perhaps contribute to our understanding of the roles played by bacteria and fungi in canine otitis externa.

Bacteria must attach to colonise skin. This adherence of bacteria and host epithelial cells is thought to be between the adhesion of the microbial cell wall and specific receptors on the epithelial cell. Epithelial cells in various anatomical sites have different receptors (Bibel, Aly, Shinefield, Maibach, and Strauss, 1982; Feingold, 1986), whilst *Staphylococcus aureus*, Streptococci, *E. coli*, and *Pseudomonas aeruginosa* do not usually colonise normal skin. These findings support the concept that bacteria are selected by host epithelial cells (Leyden *et al*, 1980; Abraham, Beachey, and Simpson, 1983; Cole and Silverberg, 1986). This may explain why only 10% of ears were colonised by these pathogenic bacteria. When the skin is afflicted by a disorder, the dermal receptors for these pathogenic bacteria may be uncovered (Abraham *et al*, 1983). Adherence between Gram-negative rods and epithelial cells may be interfered with by antibiotics or specific antibody (Hyslop, 1971; Eisenstein, Ofek, and Beachey, 1979).

In addition to epidermal receptors, skin surface immunoglobulins are also responsible for regulation of the cutaneous flora by neutralising microorganisms (Halliwell and Gorman, 1989). It has been documented that immunoglobulin deficiency is one contributor to infectious otitis externa in humans (Ichimura, Hoshino, Yano, and Nozue, 1983; Hanifin and Homburger, 1986). In order to understand the possible triggers for microbial overgrowth, knowledge of the relationship between immune

status and associated microorganisms is required. An evaluation of local immunoglobulin levels in the ear canal is described in Chapter IV.

Pseudomonas aeruginosa is often associated with "malignant externa otitis" in humans. This condition, a chronic end-stage otitis is characterised by granulation, cellulitis, chondritis, and often osteomyelitis of the temporal bone (Perry and Nichols, 1956; Meltzer and Kelemen, 1959; Chandler, 1968; Petrozzi and Warthan, 1974; Yust *et al*, 1980; Salit *et al*, 1982; Gherini *et al*, 1986; Babiatzki and Sade, 1987; Rubin and Yu, 1988; Strauss, 1990). Most cases of malignant external otitis occur in diabetic patients or patients with chronic diseases where host defence is compromised (Wilson *et al*, 1971; Petrozzi and Warthan, 1974; Yust *et al*, 1980; Gherini *et al*, 1986; Babiatzki and Sade, 1987; Castro, Robinson. Klein, and Geimeier, 1990; Cohen, 1990; Strauss, 1990). Staphylococci have also been isolated from cases of this condition in humans (Keay and Murray, 1988). The exact pathogenesis of this infection however, is not known. The relationship between aural microflora and host immunity deserves further study to elucidate the behaviour of aural microorganisms and their relevance to canine otitis externa. Human cerumen has been shown not only to prevent penetration of the epidermis by microorganisms, but also to possess antimicrobial activities (Burtenshaw, 1942; Baumann *et al*, 1961; Chai and Chai, 1980). Elsewhere in this study the physiological functions of canine cerumen and their relevance to aural microorganisms are addressed more fully (Chapters IV and V).

Malassezia pachydermatis was isolated by culture from 25% of normal and 33% of inflamed canine ears. *Malassezia pachydermatis* appears to belong to the resident flora of canine ears. Whether *M. pachydermatis* is a pathogen is currently the subject of controversy, although several studies indicate that this yeast may be a major cause of canine otitis externa (Sinha *et al*, 1976; Abou-Gabal and Fagerland, 1979;

Gedek *et al*, 1979). The role of *M. pachydermatis* in canine otitis externa cannot be ascertained from the present study. *Malassezia* species appear to proliferate after cutaneous disorders, such as skin occlusion or seborrhoeic dermatitis (Faergemann and Fredriksson, 1981; Faergemann *et al*, 1983; Plant, Rosenkrantz, and Griffin, 1992). By analogy with other microorganisms, the overgrowth of *M. pachydermatis* must be induced by a disrupted microenvironment or other unknown factors. An understanding of the microenvironment of ear canal and its influence on this yeast may help to elucidate the role of *M. pachydermatis* in canine otitis externa. Further studies concerning *M. pachydermatis* and the effects of canine cerumen on this yeast will be reported elsewhere in this thesis (Chapter VI).

In the present study, cerumen cytological examination has been shown to be more sensitive in the detection of aural microorganisms than microbial culture. Similar results were reported by Griffin and Rosenkrantz (1988, cited by Griffin, 1990). Cytological smears provide a quick method to detect microorganisms, and four categories can be recognized; Gram-positive cocci, Gram-positive rods, Gram-negative rods, and Gram-positive peanut-shaped yeasts. In otitic ears, the Gram-positive cocci will usually be pathogenic, i.e. coagulase-positive staphylococci or haemolytic streptococci; nearly 95% of the Gram-negative rods in the present study of otitic ears were pathogenic, i.e. *Pseudomonas aeruginosa*, or *E. coli*.

In the present study, the microbial numbers seen on a cytological smear of cerumen and microbial culture appear to be correlated. Based on health status, cultures from normal canine ears exhibited a low growth density (- to +), and the corresponding cerumen cytological smears contained low microbial numbers (- to +) as well. Contrariwise, over two thirds of the otitic ears had high growth density (+ + to + + + +) on culture plates and contained high microbial numbers (+ + or + + +) in the

corresponding cytological smears. A similar correlation has been reported in cytological quantitation of *M. pachydermatis* numbers on skin from normal dogs and dogs affected by seborrhoeic dermatitis (Plant *et al*, 1992). Together with a clinical evaluation indicating otitis externa, these techniques could be used as an index of the severity of bacterial or fungal overgrowth in otitis and may help a clinician to select an appropriate antibiotic when microbiological culture is not available.

The combination of the results of cerumen cytological examination and microbial culture reveals that four otitic cerumen samples could not be correlated to each other using these techniques. Each of these four samples contained large numbers of organisms (+ + +) when examined by cytological smears, but few or no (+ or -) colonies were recovered from culture. Possible explanations for these results might indicate; inactivation of microorganisms by antibodies or antimicrobial lysozymes in cerumen, or a previous antibiotic treatment. An uneven mixture of cerumen during the aural epithelial migration (Johnson, Hawke, and Berger, 1984; Johnson and Hawke, 1986; Youngs, Hawke, Ekem, and Stratis, 1988; Broekaert, 1990; Weinberger, Hawke, Clark, and Warren, 1990; Smelt, Stoney, Weinberger, and Hawke, 1991) may cause accumulation of microorganisms in cerumen. Technical problems in culture plate inoculation can alter the results of microbial growth density. These findings may indicate that the factors which can promote microbial growth *in vivo* and *in vitro* are complicated.

The relationship between cerumen colour and microbiological findings was studied. Canine cerumen colour was divided into two major categories, yellow and brown. These two colours were distributed almost evenly among these 79 cerumen samples. Dry, brittle, or flaky consistency was only found in the yellowish cerumen, but waxy, sticky consistency was recognized in both major colour-types of cerumen. No

correlation was found between the colour of cerumen and their microflora. This finding contradicts the suggestion that cerumen colour is a reasonably good indication of its microflora as has been claimed by several researchers (Griffin, 1981; August, 1986; Woody and Fox, 1986b; Chester, 1988; Macy, 1989). The concept that colour may indicate microflora is given credence by the knowledge that *Pseudomonas aeruginosa* produces pyocyanin, which imparts a greenish colour to exudates (Hojyo-Tomoka *et al*, 1973; Carter and Chengappa, 1991). In the present study, 80% of otitic ears contained two, or more than two, microorganisms in their external ear canals. This observation reflects the fact that mixed microbial colonisation is the rule in the natural environment (Marsh and Selwyn, 1977). The colour of cerumen could not indicate the associated microorganisms if the ears were affected by a combination of different agents. An increased concentration of cholesterol and the products of lipid peroxidation may be associated with the darker brown colour (Petrakis, Miike, King, Lee, Mason, and Chang-Lee, 1988). It is known that human cerumen occurs in two phenotypically distinct forms: dry and wet. Wet cerumen is light tan to dark brown and has a sticky consistency, whereas dry cerumen ranges from yellowish grey to tan colour and is scaly, brittle, or flaky in nature (Matsunaga, 1962; Petrakis, Molohon, and Tepper, 1967; Ibraimov, 1991). Whether the colour and consistency of canine cerumen is also determined by genetics or other undefined factors, is unclear. Further study of the patterns of canine cerumen colour, consistency and heritability remains to be done.

CHAPTER III

ANATOMY AND MICRO-ANATOMY OF THE EXTERNAL EAR CANAL IN DOGS

3.1 INTRODUCTION

The unique anatomy of the ear has been incriminated as one of the factors predisposing to canine otitis externa (McGinnis and England, 1949; Sharma and Rhoades, 1975; Hayes *et al*, 1987). The canine external ear canal is supported by two cartilages, the annular cartilage and auricular cartilage. These cartilages are covered by modified skin (Miller and Witter, 1942; Getty *et al*, 1956). The canal is funnel-shaped and divided into vertical and horizontal portions according to their relative positions (Fraser *et al*, 1970). Getty *et al* (1956) stated that the length of the canine external ear canal was approximately two centimetres (cm), and its diameter was about five to ten millimetres (mm). Miller and Witter (1942) reported that the length of the annular cartilage in dogs was about one cm.

Among the anatomical characteristics, the conformation (Mayhew, 1854; Sharma and Rhoades 1975; Hayes *et al*, 1987) and density of hair in the external auditory meatus have been supposed to be responsible for the high incidence of otitis externa (Blaine, 1841; McGinnis and England, 1949; Fraser, 1961a; Fraser *et al*, 1961; Fraser, 1965; Fraser *et al*, 1970; Sharma and Rhoades, 1975; Evans and Jemmett, 1978; August, 1986; Hayes *et al*, 1987). In those studies, dogs with pendulous ear carriage and hirsute ear canals, such as the Cocker spaniel and the Poodle had a significantly increased incidence of otitis externa compared to dogs with other ear types. Several mechanisms might be involved. It has been found that a higher relative humidity and elevated temperature are associated with increased susceptibility to human skin infections with bacteria and fungi (Aly *et al*, 1978; Faergemann *et al*, 1983). The resident flora on human skin is denser in moister regions (Aly and Maibach, 1977), and bacteria survive longer on wet than dry human skin (Rebell *et al*, 1950). It has also been shown that under occlusion the relative humidity on human

skin increased from 20% to 75% (Hartmann, 1983); the population numbers of cutaneous flora also increased (Marples and Kligman, 1969; Aly *et al*, 1978; Faergemann *et al*, 1983; Hartmann, 1983). Occlusion increased the degree of hydration in the stratum corneum and elevated the water content on the skin surface, actions which are thought to promote the growth of microorganisms (Aly *et al*, 1978; Faergemann *et al*, 1983). Hayes *et al* postulated that in dogs with pendulous ears or hirsute ear canals, ventilation and convection in the external ear canal may be restricted. Thus the aural skin would have an increased susceptibility to bacterial and fungal infections. Grono (1970c) found that the relative humidity, 87% to 89%, in ears of dogs with otitis externa was higher than the relative humidity in normal canine ears, which was about 80%. Temperature of the aural environment might also be an important factor. Nevertheless, no temperature difference was found between healthy and otitic ears nor was a temperature difference found between different types of canine ear (Grono, 1970a).

Histologically, canine aural skin contains two types of secretory glands; the apocrine and sebaceous glands (Miller and Witter, 1942; Nielsen, 1953). Miller and Wither (1942) reported that sebaceous glands were numerous near the tympanic membrane. Whereas Fraser (1961d) and Fraser *et al* (1970) reported that active sebaceous glands were prominent and abundant in the distal part of the external ear canal. Miller and Witter (1942) stated that apocrine glands were not visible in normal canine aural integument. However, Nielsen (1953), Fraser (1961d), and Fraser *et al* (1970) have reported that apocrine glands are indeed present in dogs, situated in the deeper dermal layer below the sebaceous glands, and are more obvious in the peripheral third of the external ear canal.

At microscopic level, the long-haired breeds of dog have been found to have better developed sebaceous and apocrine gland tissue in

their ears than short-haired ones. This was thought to be related to the high incidence of otitis externa in long-haired breeds (Fernando, 1966). Quantitative analysis of glandular tissue in canine horizontal ear canals has been carried out. No significant difference was found in the total area of sebaceous gland tissue in the horizontal ear canal between those breeds which are, and are not, predisposed to otitis externa (Stout-Graham *et al*, 1990). In contrast to sebaceous glands, the total area occupied by apocrine glandular tissue was significantly higher in breeds predisposed to otitis externa, such as the Cocker spaniel and the Labrador retriever (Stout-Graham *et al*, 1990). These findings suggest that an increased mass of apocrine glandular tissue in the ear canal may predispose to otitis externa (Stout-Graham *et al*, 1990).

An aromatic retinoid (etretin) has been demonstrated to induce otitis externa in dogs in association with increased apocrine secretion in the external ear canal (Teelmann, 1986). This substance, given systemically, caused distension of apocrine glands and epidermal hyperplasia in canine ear canals; when a higher dose of retinoid was administered, inflammation was recognized accompanied by erosion of the epidermis, diffuse epidermal and dermal inflammatory cell infiltration, and morphological changes to the sebaceous glands. Based on these findings, Teelmann suggested that increased apocrine glandular secretion and epidermal hyperplasia within the external ear canal led to a narrowing of the auditory canal lumen. This in turn caused the humidity of the environment to rise. Subsequently secondary bacterial or fungal infection and cutaneous inflammation developed in the external ear canal. In effect, it was conjectured that increased secretory activity of apocrine glands in the ear canal might predispose to otitis externa.

Pathological changes found in otitis externa in dogs include hyperplasia of the epidermis, dermis, and hair follicles, which leads to

constriction of the lumen of the external ear canal (Miller and Witter, 1942; Witter, 1949; Fraser, 1961d; Stout-Graham *et al*, 1990). In chronic otitis, the apocrine glands were found to be dilated and cystic, and seemed to have become the major secretory organ (Nielsen, 1953; Fraser, 1961d; Fernando, 1967). The size of apocrine glands was correlated with the duration of the disease (Stout-Graham *et al*, 1990). Whether the dilation of the apocrine glands in chronic otitis externa is due to local inflammation, caused by plugging of the gland duct by excessive keratin (Fernando, 1967), or the glands are stimulated by the demands of physiological function, becoming hypersecretory (Fraser, 1961d), or other mechanisms are involved has not been fully elucidated. Sophian and Senturia (1955) have reported that the pathological changes in human apocrine glands during otitis externa involved only the secretory part, but not the duct of the gland. It seemed that compression of apocrine glands may result from oedema and infiltration of the duct.

Pathological changes in sebaceous glands were various. In otitis externa, Nielsen (1953) reported that sebaceous glands were hyperplastic. According to Fraser (1961d) and Fernando (1967), in the early stages of the disease, they were grossly enlarged and hypersecretory; as the disease progressed, they had become smaller and less active. However, Stout-Graham *et al* (1990) have reported that the sebaceous gland tissue was not reduced with the progression of otitis externa; these glands neither proliferated nor had they become atrophied.

The secretion of both types of adnexal gland has been investigated by the use of histochemical stains (Fernando, 1966). Sebaceous glands were found to secrete neutral lipids, a small amount of glycogen, and alkaline phosphatase. Immature sebaceous gland cells also contained phospholipids. On the other hand, the materials secreted by apocrine glands were phospholipids, protein-bound lipids, acid and alkaline

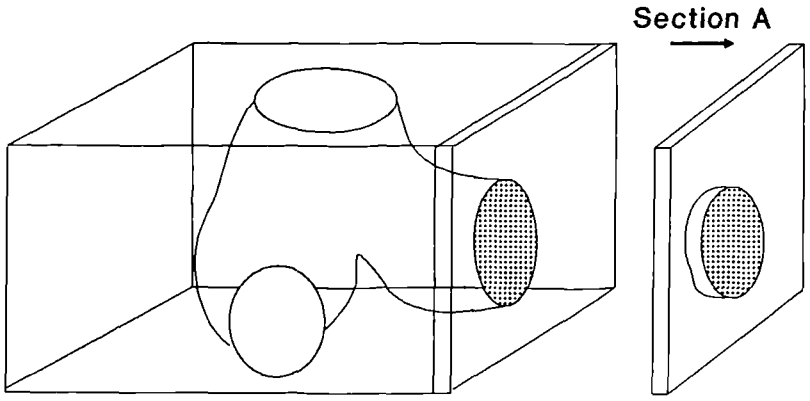
mucopolysaccharides, a diastase-resistant periodic acid and Schiff positive fraction, acid and alkaline phosphatase. Both sebaceous and apocrine glands are involved in the formation of cerumen. Morphological changes to these glands were shown to be associated with alterations in the nature of the cerumen in canine otitis externa (Fraser, 1961d; Fernando, 1966).

The purpose of the present study was to improve our knowledge of the gross anatomy of the normal ear canal, and to compare histological features of normal ear canals with those affected by chronic otitis externa. The histological features may prove relevant to the microbiology of these ears. Similarly, these studies complement those concerned with physiological aspects of cerumen reported elsewhere in this thesis.

The quantitative analysis of glandular tissue and hair follicles in each histological section was carried out by morphometric stereology. Morphometry is a quantitative approach to the description of structural features. Morphometric data were obtained by stereology, which is a body of mathematical methods allowing three-dimensional information on spatial structures to be derived from measurements made on two-dimensional sections. By stereological methods, the volume and the number of structural features of candidate tissue can be determined quantitatively. This technique divides the candidate tissue from a three-dimensional structure to a cut section (forming two dimensions). From the section, histological features can be quantified thus providing analytic data (Weibel, 1979a; Weibel, 1979b; Reid, 1980). A widely used stereological method for estimating the dimensions of a candidate tissue is based on the use of a graticule (a regular lattice of squares). The number of these squares enclosing the candidate tissue are divided by the total area (total squares) enclosing the relevant part of the section. A series of sections is evaluated to represent the three-dimensional size of candidate tissue

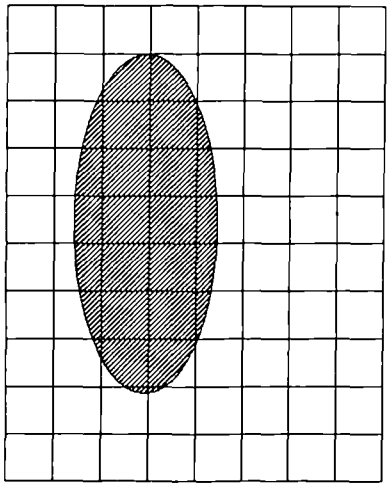
quantitatively. This method can be applied to histological sections using a microscope equipped with an eye-piece graticule (Weibel, 1979a; Reid, 1980). The principles of stereological estimation are illustrated in Figure 3.1.1.

Figure 3.1.1 The following diagrams illustrate the principles behind stereological analysis of sections of tissues.



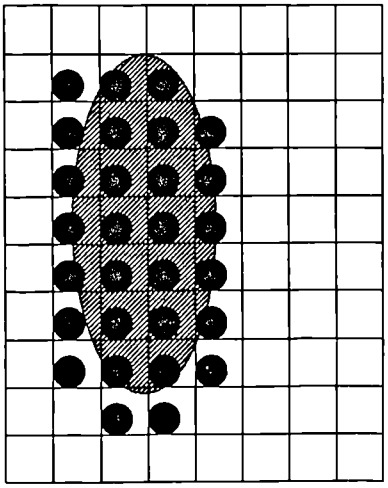
A. Cutting a section of the sample. A two dimensional image is created and a proportion of this section is composed of the candidate tissue.

Section A



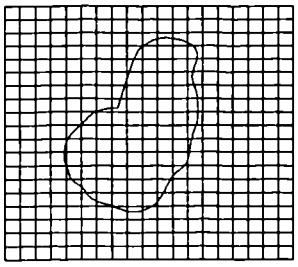
B. The area occupied by tissue is ready for estimation by using a microscope equipped with a graticule.

Section A

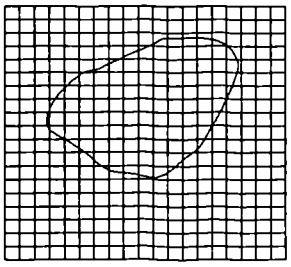


C. The area of the candidate tissue is derived by counting the squares occupied by that tissue. Note this method tends to lead to an over-estimation of small structures.

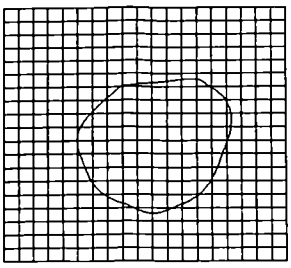
Section B



Section C



Section D



D. A representative series of two dimensional images are analysed. The data are collated and the overall results from the two-dimensional images give a mathematically dependable indication of the proportion of a three dimensional structure occupied by the candidate tissue.

3.2 MATERIALS AND METHODS

3.2.1 Anatomical survey of canine external ear canal

Materials

Forty normal ears from 20 fresh canine cadavers were used for this investigation. These canine cadavers were obtained from the Department of Veterinary Pathology, University of Glasgow. The breed, sex, and body weight of these 20 dogs are summarised in Table 3.2.1.

Methods

1. Dissection of the external ear canal

The cartilaginous part of the external ear canal was obtained from the fresh cadaver by dissection following a technique resembling the total ear canal ablation surgical procedure (Mason *et al*, 1988; Beckman *et al*, 1990). The external ear canal was separated into two parts, namely; the proximal part supported by the annular cartilage and the distal segment which contained the auricular cartilage (Figure 3.2.1).

2. The length of the cartilaginous external ear canal

A thread was passed though each segment of the external ear canal, the length of each part was estimated by measurement of the length of thread equal to the shortest length of the cartilage. The overall length of the cartilaginous external ear canal was the combined lengths of these two segments.

3. The internal diameter of the external ear canal

Three sites were selected for measurement. The first diameter was measured at the distal opening of the external ear canal rostrocaudally; the second diameter was measured at the proximal end of the auricular

cartilage dorsoventrally, and the last diameter was measured at the proximal end of the cartilaginous part of the auditory canal dorsoventrally (Figure 3.2.2). Because of the oval cross-sectional shape of the external ear canal, these measurements were based on the longest axis which could be determined at each site in each ear.

3.2.2 Histological evaluation of the external ear canal

Materials

The healthy group comprised 28 normal ears collected from 14 fresh canine cadavers provided by the Department of Veterinary Pathology, University of Glasgow. The breed and sex of these 14 dogs are summarised in Table 3.2.2.

The otitic group comprised 15 ears obtained from 13 dogs which were referred to the Department of Veterinary Surgery, University of Glasgow, for treatment of chronic and recurrent otitis externa. The breed, sex, and age from these animals is shown in Table 3.2.3.

Methods

1. Dissection of the external ear canal

In the healthy group, the cartilaginous part of the external ear canal was obtained from the fresh cadavers by dissection following a technique resembling the total ear canal ablation surgical procedure (Mason *et al*, 1988; Beckman *et al*, 1990).

The otitic ears were obtained by performing total ear canal ablation on dogs with chronic and recurrent otitis externa. In each case the diseased ear canal was considered to have become so altered as to be beyond the scope of medical therapy. Surgery was performed only when clinically justified and with the informed consent of the animal's owners.

The specimens were cut at four levels (Figure 3.2.3). The first level was at the widest part of the pinna, tissue specimens from this level were only available from the healthy group. The second level was sited immediately proximal to the tubercle of the anthelix. The third level was at the junction between the vertical and horizontal ear canal, and level four was at the most proximal extremity of the annular cartilage (Figure 3.2.3).

2. Specimen preparation for haematoxylin and eosin stained paraffin sections

The tissue was fixed for 24 hours in approximately ten times the tissue volume of 10% (v/v) neutral buffered formalin. This tissue was then wax embedded, sliced into thin sections (thickness 3-4 um) on a microtome, and stained with haematoxylin and eosin (Ross and Reith, 1985).

3. Morphometry

The total area occupied by sebaceous glands, total area occupied by apocrine glands, and the area occupied by hair follicle tissue within the limits of the enclosing cartilage and borders of the graticule were determined. The method used in this study was modified from the methods of Weibel (1979). Each section was examined under a microscope (Leitz, SM-LUX, Germany) equipped with an eye-piece graticule which contained 361 (19x19) squares (Figure 3.2.4). The slide was positioned so that the epidermis and enclosing cartilage in each section was orientated approximately parallel to the lines of the graticule. Six sites from each section, each exhibiting both an intact epidermis and enclosing cartilage beneath the integument were chosen for morphological measurement. The number of squares which contained each candidate tissue were then counted (Figure 3.2.5). The ratio of the

area occupied by the candidate tissues to the total area examined in six different sites in the section was deemed to represent the proportion of the candidate tissues within the soft tissue of the external ear canal at this level.

3.2.3 Statistical methods

In the study of the gross dimensions of the external ear canals, paired T-tests were used to examine whether there were statistically significant differences between the left and right ears. The data from the micro-anatomical survey were stored on a file on a personal computer (Viglen, U.K.). Paired T-tests were carried out using the Minitab statistical program. A five percent (5%), significance level was used throughout the study ($P \leq 0.05$).

Three way analysis of variance was used to search for significant differences in the morphometric data between the right and left ears, between the four anatomical levels, and significant interaction between the right and left ears and the four levels. The dogs in this study were treated as a random effect for this analysis. The data were stored in a Minitab file and analyzed using the GLIM statistic package on an ICL 3980 main-frame computer. In the case of significant difference between the four levels, subsequent analysis using Newman-Keuls multiple range test was carried out to determine significant differences between specific levels using a bespoke program run on the Minitab package installed on an IBM PS/2 50Z personal computer.

Analysis of variance using a repeated measures design was employed to determine significant differences between the otitic and normal ears, and between the three anatomical levels (2, 3, and 4). Significant interactions between data from the otitic and normal ears and the three levels were also searched for using the same technique. These

data were again stored in a Minitab file and analyzed using GLIM on an ICL 3980 main-frame computer. Once again in the case of significant differences between the three levels, subsequent analysis of the data was carried out utilizing Newman-Keuls multiple range test using the same bespoke program run on an IBM PS/2 50Z personal computer (U.K.).

Table 3.2.1 Breed, sex and body weight of 20 dogs with normal ear canals used for the anatomical survey of healthy ears.

Breed	Sex	Body weight (Kg)
1. German Shepherd Dog	M	36.0
2. Greyhound	M	37.1
3. German Shepherd Dog	F	32.3
4. German Shepherd Dog	F	21.0
5. Crossbreed	F	2.8
6. Miniature Poodle	F	9.2
7. Cocker Spaniel	F	18.0
8. Crossbreed	F	15.3
9. Labrador	F	32.2
10. Crossbreed	F	30.0
11. Crossbreed	M	21.0
12. German Shepherd Dog	M	23.2
13. Crossbreed	F	15.3
14. Crossbreed	M	31.7
15. Crossbreed	M	11.0
16. Crossbreed	F	5.0
17. Crossbreed	M	26.3
18. Bull Mastiff	F	52.0
19. Rottweiler	F	48.1
20. Crossbreed	M	14.0

Figure 3.2.1 Schematic diagram illustrating the major anatomical features of the canine external ear canal.

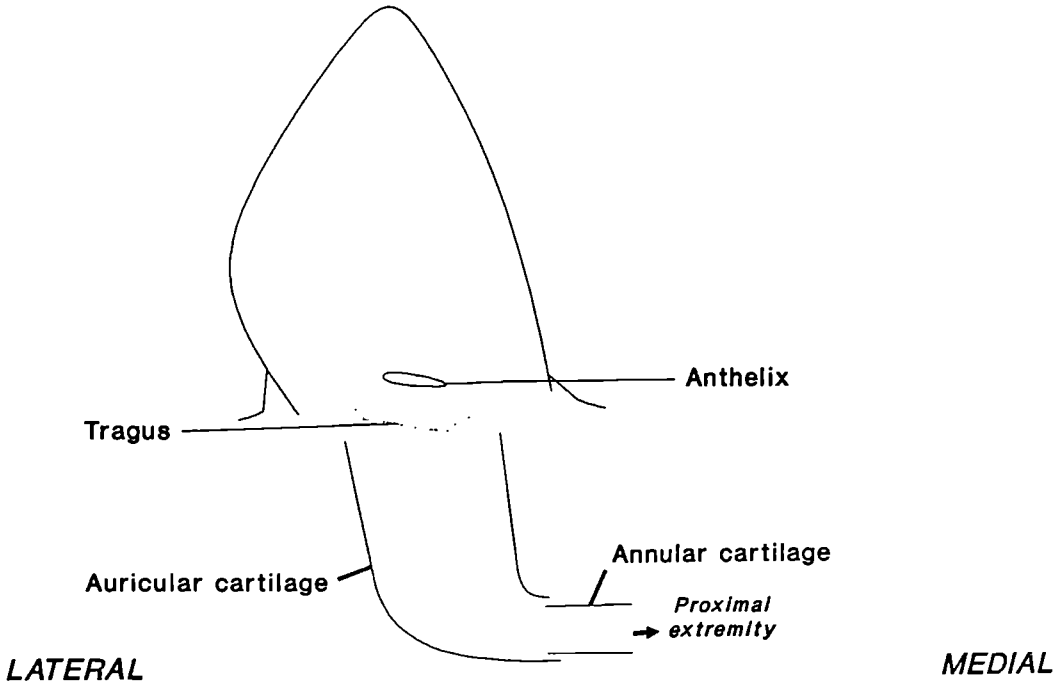


Figure 3.2.2 Schematic diagram to illustrate the approximate locations of the sites at which the diameter of the external ear canal was measured.

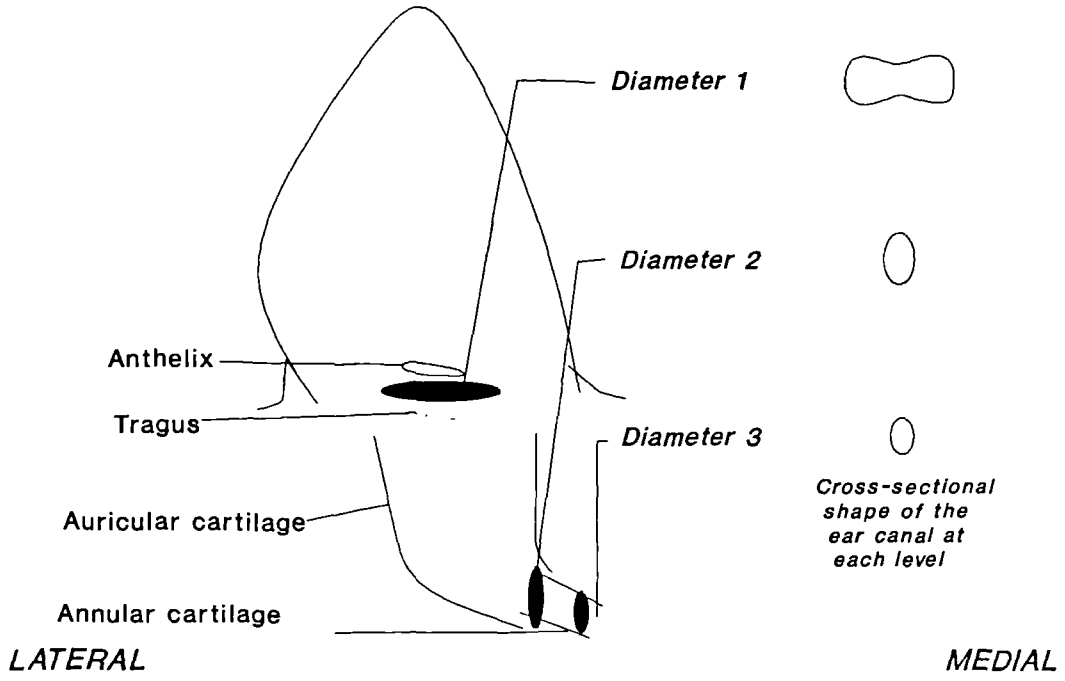


Table 3.2.2 Breed and sex of 14 dogs with normal ear canals used for morphometric evaluation of the healthy ear.

Breed	Sex
Boxer	M
German Shepherd Dog	F
German Shepherd Dog	F
Crossbreed	F
Cocker Spaniel	F
Great Dane	M
Crossbreed	F
Crossbreed	M
Crossbreed	M
Crossbreed	F
Crossbreed	M
Bull Mastiff	F
Rottweiler	F
Crossbreed	M

Table 3.2.3 Breed, age (years) and sex of 13 dogs with otitis externa used for morphometric evaluation of the chronically diseased ear.

Breed	Sex	Age
Labrador	M	6
German Shepherd Dog	F	9
Crossbreed	F	10
Labrador	M	13
St. Bernard	M	5
Golden Retriever	M	7
Rottweiler	F	4
Wire Haired Fox Terrier	M	9
Rottweiler	F	5
Yorkshire Terrier	M	9
Cairn Terrier	M	11
German Shepherd Dog	F	6
Sharpei	M	2

Figure 3.2.3 Schematic diagram illustrating the approximate locations of the four levels nominated for the morphometric evaluation.

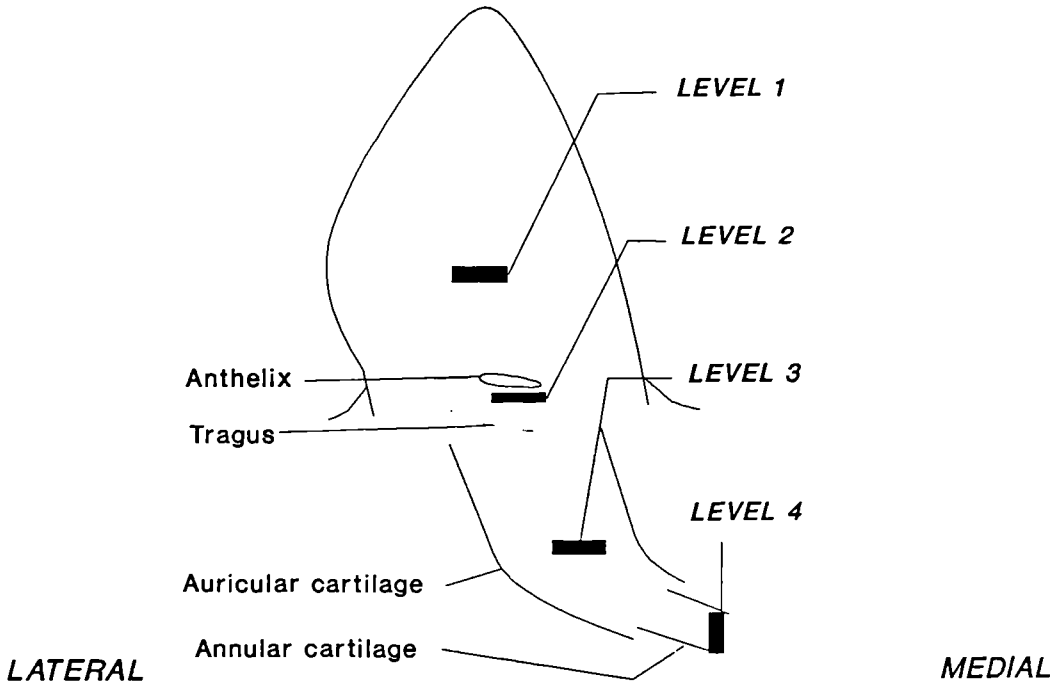


Figure 3.2.4 Schematic diagram to illustrate the eye-piece graticule under a microscope.

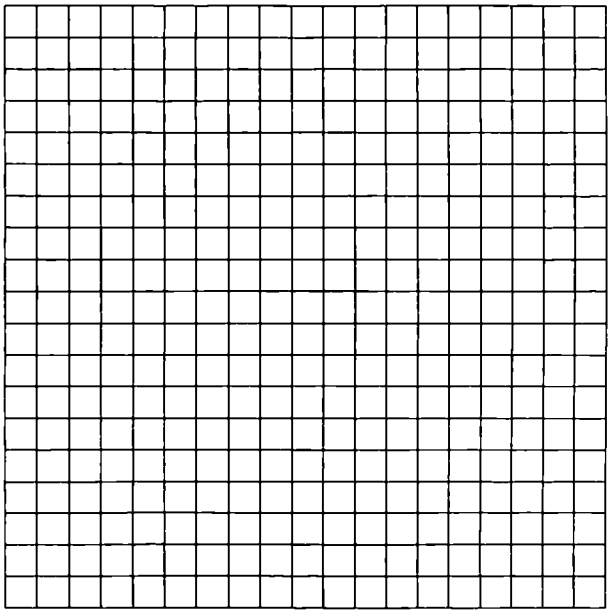
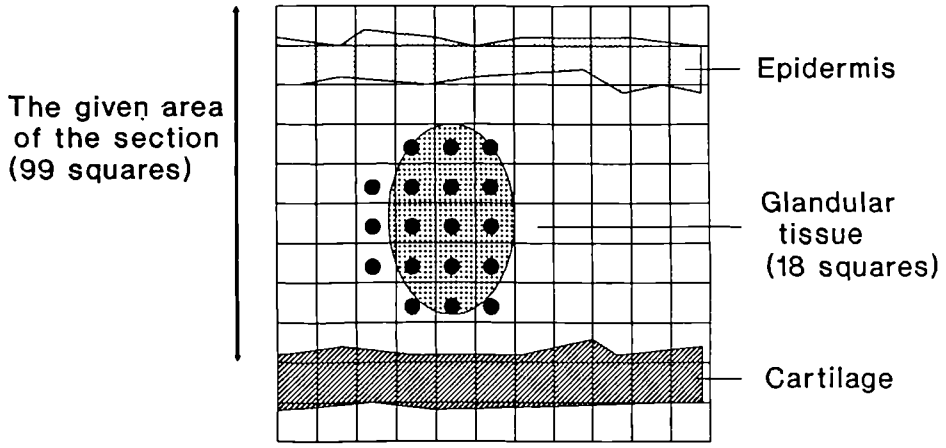


Figure 3.2.5 Schematic diagram to illustrate the collection of morphometric data.



Those squares of the graticule which contain any of the candidate tissue are counted; the total number of squares occupied by any of the given area (from the top of the epidermis to the cartilage) are also counted. Thus, the fraction of the area occupied by the candidate tissue can be estimated mathematically.

3.3 RESULTS

3.3.1 Length of the external ear canal

The average length of the segment contained within the annular cartilage from 40 healthy ears was 1.2 cm, with a range of 0.8 cm to 1.9 cm (S.D. = 0.2 cm). The average length of the segment supported by the auricular cartilage from 40 ears was 4.1 cm, with a range of 2.2 cm to 5.7 cm (S.D. = 0.9 cm). Thus the overall length of the external ear canal from these 40 ears was 5.3 cm, with a range of 3.0 cm to 7.0 cm (S.D. = 1.0 cm). These data are presented in Table 3.3.1. and summarised in Table 3.3.2 and Figure 3.3.1)

Statistically significant differences between the left and right ears were not found in these data when examined by paired T-test ($P>0.95$). A positive correlation between the overall length of the external ear canal and the body weight was found ($R=0.79$, $P<0.001$) (Figure 3.3.2).

3.3.2 Diameter of the external ear canal

The cross sectional shape of the external ear canal in dogs, varies along its length from the proximal end to the distal opening of the canal, the cavum conchae. Only the longest axis at each chosen site in the canal was measured. The maximal internal diameter at the distal end of the external ear canal was 5.8 cm on average, with a range from 2.1 cm to 7.9 cm (S.D. = 1.5 cm). The second diameter examined, at the proximal opening of the auricular cartilage, measured on average 0.7 cm, with a range from 0.3 cm to 1.0 cm (S.D. = 0.2 cm). The diameter at the most proximal opening of the cartilaginous part of the external ear canal measured 0.5 cm on average, with a range of 0.3 cm to 0.8 cm (S.D. = 0.1 cm). These data are presented in Table 3.3.3. and summarised in Table 3.3.4 and Figure 3.3.3.

No statistically significant differences were found in this study between the left and right ears (paired T-test ($P>0.55$). A positive correlation between the diameter of the distal end of the external ear canal and the body weight was found ($R=0.47$, $P<0.005$) (Figure 3.3.4).

3.3.3 Morphometric evaluation of the canine external ear canal

The area occupied by sebaceous gland tissue in the aural skin of the healthy dogs' ears at four different levels exhibited marked variation (Table 3.3.5). These data were consequently not normally distributed. The average proportions of sebaceous gland tissue in the integument of the ear canal at levels 1, 2, 3, and 4, were 4.1% (range 0.1%-18.6%), 10.5% (range 0.4%-69.5%), 8.1% (range 0.1%-22.1%), and 4.7% (range 0.4%-11.1%) respectively (Table 3.3.6). Sebaceous gland tissue as a proportion of the integument increased gradually from the proximal end of the ear canal (level 4), reaching a peak immediately proximal to the tubercle of the anthelix at level 2. The proportion then decreased again on the pinna (level 1) (Figure 3.3.5). The area occupied by sebaceous glands at level 2 was significantly greater than at level 1 and level 4 ($P<0.05$), but not significantly different from level 3. Although great variation was found between dogs, no statistically significant differences were found between the left and right ears ($P>0.05$).

In the dogs with otitis externa the area occupied by sebaceous gland tissue in the aural skin also showed a marked variation between dogs and at the three different levels of the ear canal (Table 3.3.7). These data were again not normally distributed but the average proportion occupied by sebaceous glands in these ears at levels 2, 3, and 4 were, 19.2% (range 1.0%-38.4%), 14.1% (range 1.3%-27.3%), and 5.4 (range 0.1%-16.4%) respectively (Table 3.3.8). The glands exhibited a similar pattern of distribution in otitic ears to those of healthy ears (Figure 3.3.5).

Level 2 contained significantly more sebaceous glandular tissue than the other two levels ($P<0.05$), and level 3 contained significantly more sebaceous tissue than level 4 ($P<0.05$). Otitic ears had a significantly greater proportion of sebaceous gland tissue than healthy ears at all three levels where a comparison could be made ($P<0.05$).

The area occupied by apocrine gland tissue in healthy canine external ear canals showed great variation between dogs and at the four levels examined (Table 3.3.9). The average proportion of the integument occupied by apocrine glandular tissue, and its range at levels 1, 2, 3, and 4 were: 1.4% (0.1%-5.4%), 2.2% (0.1%-5.6%), 2.6% (0.8%-6.1%), and 4.5% (0.6%-12.1%) respectively (Table 3.3.10). The proportion of apocrine glandular tissue decreased gradually from the proximal end of the external ear canal toward the pinna (Figure 3.3.6). At level 4 (close to the tympanic membrane), this proportion was significantly greater than at the other three levels ($P<0.05$) in these healthy ears. Although marked variation was found between ears, data from the right and left ears did not differ significantly from each other ($P>0.05$).

Similarly, distribution of the apocrine glands in otitic ears exhibited great variation between dogs and at three levels (Table 3.3.11). Once again these data were not normally distributed. The average proportion of the aural skin occupied by apocrine glandular tissue and its range in otitic ears, at levels 2, 3, and 4 were 10.1% (2.3%-36.2%), 12.2% (0.8%-27.6%), and 17.1% (2.5%-47.2%) respectively (Table 3.3.12). In canine ears affected by chronic otitis, as in health, the proportion of apocrine glandular tissue gradually decreased from the proximal end of the ear canal toward the outside (Figure 3.3.6). Level 4 contained significantly more apocrine gland tissue than level 3 and level 2 ($P<0.05$). In dogs with otitis the proportion of apocrine glandular tissue in the aural skin was significantly

greater than in healthy ears ($P<0.05$) at each of the three different levels where a comparison could be made.

The area occupied by hair follicles in the aural skin of healthy ears exhibited marked variation as well (Table 3.3.13). These data were not normally distributed. The average proportion of hair follicles in the integument of the ear canal at levels 1, 2, 3, and 4 were 3.6% (range 0.1%-30.9%), 1.4% (range 0.1%-6.0%), 2.5% (range 0.1%-19.4%), and 1.5% (range 0.1%-6.8%) respectively (Table 3.3.14). Follicular tissue was most abundant at levels 1 and 3 (Figure 3.3.7). However, no statistically significant differences were found between levels, or between the left and right ears ($P>0.05$).

In the dogs with otitis externa the proportion of the aural integument occupied by hair follicles showed great variation between dogs and at the three levels (Table 3.3.15). Again these data were not normally distributed. The proportion of aural skin occupied by hair follicles in these ears at levels 2, 3, and 4, were 4.2% (range 0.9%-13.6%), 4.7% (range 0.8%-11.6%), and 2.2% (range 0.1%-5.4%) respectively (Table 3.3.16). In otitis externa, follicles represented a significantly greater proportion of the aural integument at level 2 and 3 than at level 4 ($P<0.05$). Otitic ears contained a significantly greater proportion of hair follicle tissue than healthy ears at all three levels ($P<0.05$).

In healthy ears, a marked variation was found in the proportion of the aural integument occupied by connective tissue at the four levels examined (Table 3.3.17). These data were not normally distributed either. The average proportion of connective tissue in the integument of healthy ears at levels 1, 2, 3, and 4, and its range, were 91.0% (68.2%-98.3%), 85.9% (23.3%-95.9%), 86.8% (70.8%-97.8%) and 89.4% (72.4%-98.6%) respectively (Table 3.3.18 and Figure 3.3.8). Data from the left and right ears did not significantly differ from each other ($P>0.05$).

Similarly, the proportion of connective tissue in otitic ears exhibited great variation between these ears and these data were not normally distributed (Table 3.3.19). The average proportion of this skin occupied by connective tissue at levels 2, 3, and 4 were 66.5% (range 45.2%-93.3%), 69.0% (range 50.9%-89.3%), and 75.2% (range 47.7%-92.8%) respectively (Table 3.3.20). Level 4 contained significantly more connective tissue than level 3 and level 2 ($P<0.05$). In dogs with otitis externa the proportion of connective tissue was significantly less than in dogs with healthy ears at all three levels ($P<0.05$).

The histological features of an healthy canine external ear canal at the four anatomical levels and an otitic ear at three levels are shown in Figures 3.3.9 and 3.3.10.

Table 3.3.1 Length (centimetres) of the external auditory meati of 20 dogs with healthy ears.

	Length of the ear canal		Auricular cartilage		Annular cartilage	
Ear	R	L	R	L	R	L
1	6.2	6.7	1.0	1.4	5.2	5.3
2	5.4	5.1	1.3	1.1	4.1	4.0
3	6.9	6.4	1.4	1.3	5.5	5.1
4	5.8	5.8	0.8	0.8	5.0	5.0
5	3.2	3.0	0.8	0.8	2.4	2.2
6	3.6	3.6	1.0	1.0	2.6	2.6
7	4.2	4.6	1.0	1.1	3.2	3.5
8	4.7	4.3	1.0	1.0	3.7	3.3
9	5.3	5.0	1.1	1.1	4.2	3.9
10	6.3	6.8	1.1	1.1	5.2	5.7
11	4.9	5.2	0.9	0.9	4.0	4.3
12	5.0	5.3	1.3	1.3	3.7	4.0
13	5.0	5.2	1.0	1.0	4.0	4.2
14	5.3	5.3	1.3	1.3	4.0	4.0
15	4.8	4.5	1.3	1.3	3.5	3.2
16	4.7	4.7	1.2	1.1	3.5	3.6
17	5.8	5.4	1.2	1.1	4.6	4.3
18	6.6	7.0	1.8	1.9	4.8	5.1
19	6.0	6.0	1.3	1.2	4.7	4.8
20	5.3	5.2	1.2	1.3	4.1	3.9

R = right, L = left

Table 3.3.2. Summary and statistical analysis of the figures from Table 3.3.1.

Length (cm)	Mean	Range	S.D.
External ear canal	5.3	3.0-7.0	1.0
Auricular cartilage	4.1	2.2-5.7	0.9
Annular cartilage	1.2	0.8-1.9	0.2

S.D. = standard deviation

Figure 3.3.1 Measured length of the external ear canal and the cartilages (mean and range, centimeters).

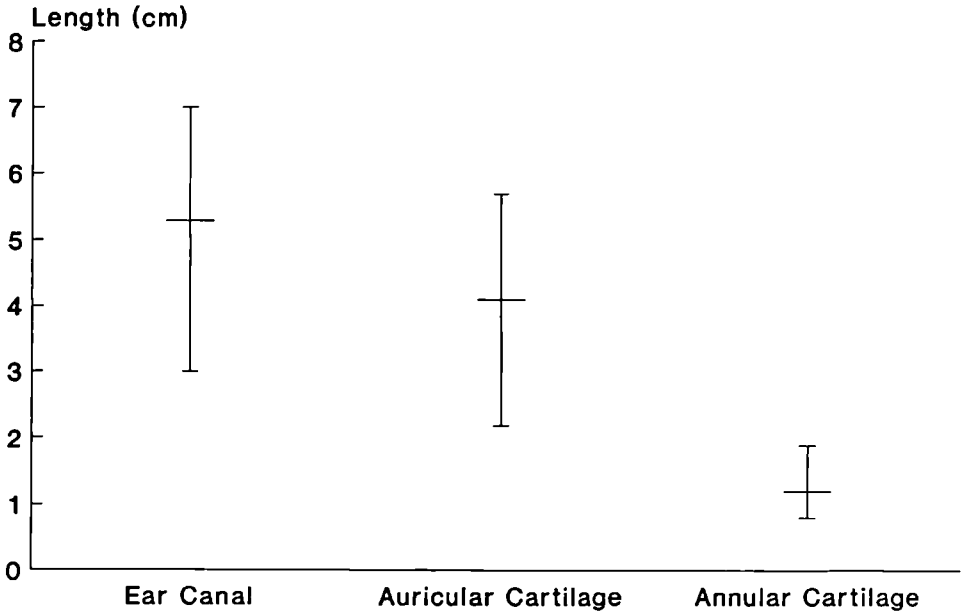
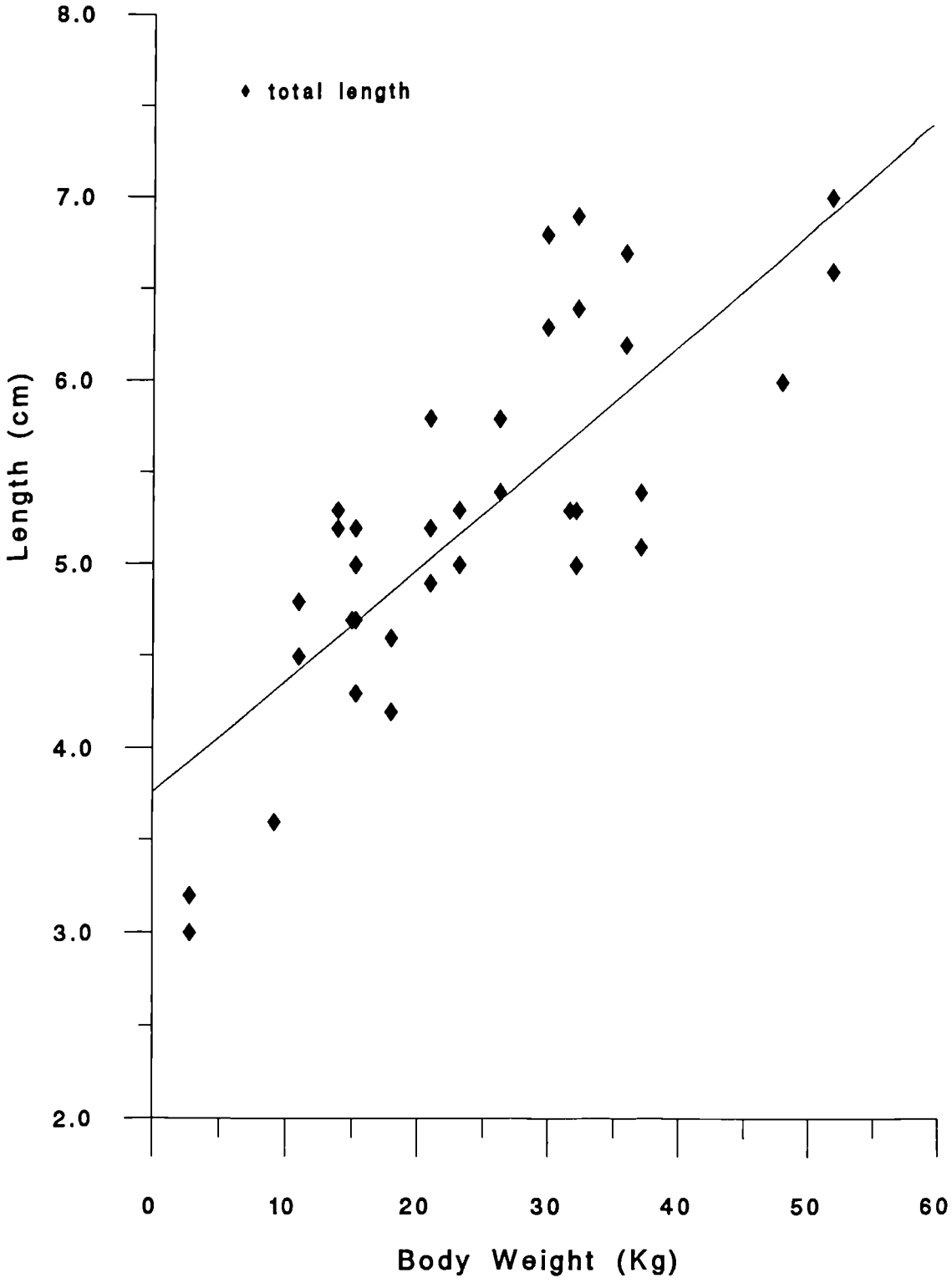


Figure 3.3.2 The relationship between body weight and the overall length of the cartilaginous part of the external ear based on 20 dogs.



The line shown is a regression line (R=0.49, P<0.001).

Table 3.3.3 Maximal diameter (centimetres) of the external auditory meati of 20 dogs with healthy ears at 3 different levels.

	Diameter 1		Diameter 2		Diameter 3	
Dog	R	L	R	L	R	L
1	4.2	4.5	0.9	0.8	0.8	0.8
2	4.4	3.9	0.7	0.8	0.7	0.8
3	5.5	5.1	0.9	1.0	0.7	0.6
4	5.0	5.0	0.9	0.9	0.9	0.7
5	2.1	2.2	0.6	0.6	0.4	0.4
6	2.6	2.6	0.7	0.6	0.5	0.5
7	6.6	7.4	0.7	0.7	0.4	0.4
8	5.3	5.0	0.7	0.8	0.5	0.5
9	6.7	6.5	0.8	0.6	0.3	0.3
10	6.7	6.7	0.8	0.9	0.6	0.6
11	6.3	6.7	0.8	0.9	0.6	0.6
12	6.7	7.0	0.7	0.7	0.3	0.5
13	5.6	6.8	0.7	0.7	0.4	0.5
14	7.8	6.5	0.6	0.4	0.6	0.4
15	6.3	5.5	0.4	0.3	0.5	0.4
16	6.5	6.5	0.6	0.6	0.5	0.5
17	7.1	7.0	0.6	0.7	0.5	0.6
18	7.5	7.5	0.5	0.8	0.3	0.5
19	7.1	7.9	0.5	0.8	0.4	0.5
20	6.5	6.0	0.5	0.6	0.3	0.3

R = right, L = left

Diameter 1 : the distal opening of the external ear canal

Diameter 2 : the proximal end of the auricular cartilage

Diameter 3 : the proximal end of the annular cartilage

Table 3.3.4 Summary and statistical analysis of the figures from Table 3.3.3.

Diameter (cm)	Mean	Range	S.D.
1	5.8	2.1-7.9	1.5
2	0.7	0.3-1.0	0.2
3	0.5	0.3-0.8	0.1

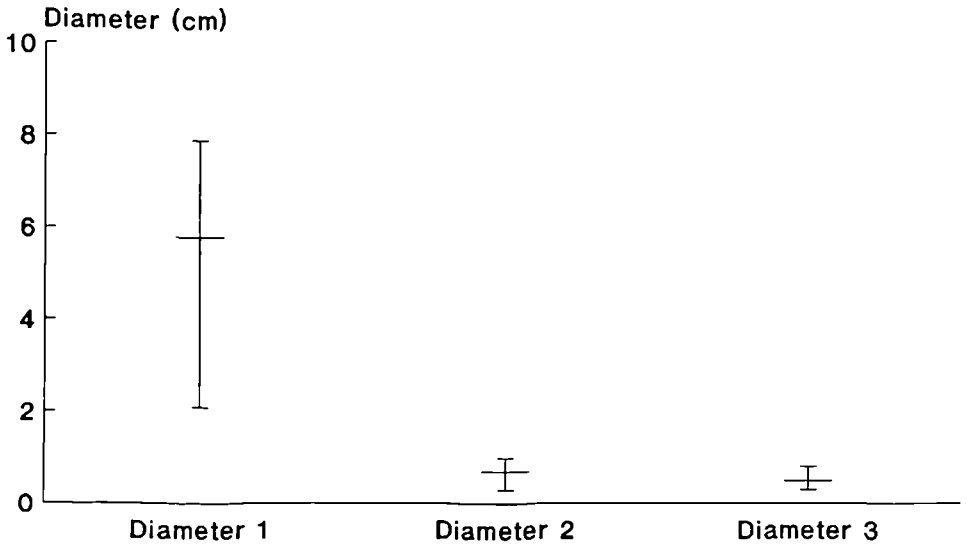
S.D. = standard deviation

Diameter 1 : the distal opening of the external ear canal

Diameter 2 : the proximal end of the auricular cartilage

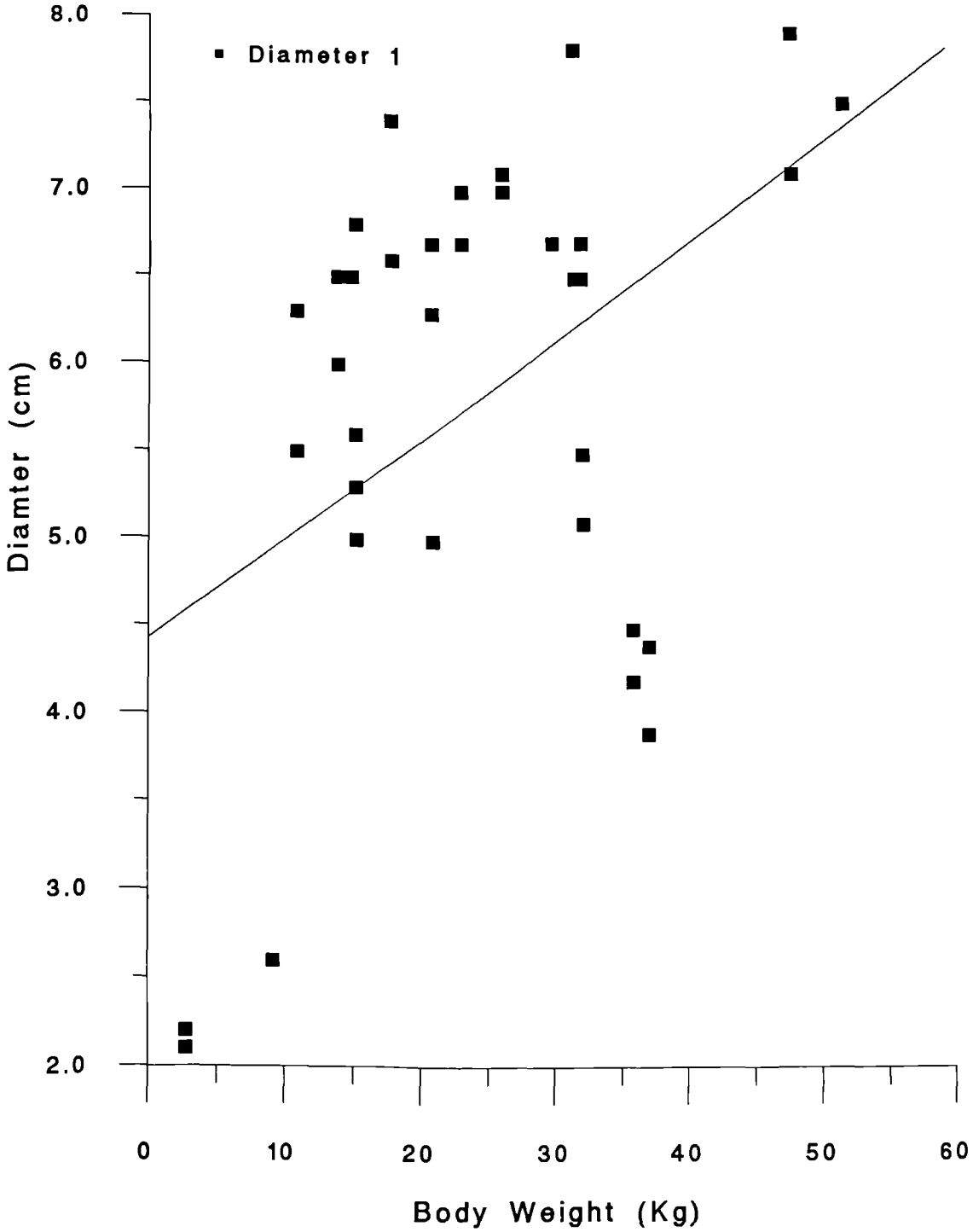
Diameter 3 : the proximal end of the annular cartilage

Figure 3.3.3 Measurements of the diameter of the external ear canal at three levels (mean and range, centimeters).



Diameter 1: the distal opening of the canal
Diameter 2: the proximal end of the auricular cartilage
Diameter 3: the proximal end of the annular cartilage

Figure 3.3.4 The relationship between body weight and the diameter of the distal end of the external ear canal based on 20 dogs.



The line shown is a regression line ($R=0.47$, $P<0.05$).

Table 3.3.5 The proportion of each section occupied by sebaceous glands at four nominated locations in the aural skin of 28 healthy canine ears (%).

Ear	Level 1	Level 2	Level 3	Level 4
1	0.3	2.8	3.9	1.5
2	0.5	4.2	2.4	1.3
3	1.5	5.1	4.8	3.4
4	2.1	2.0	4.9	2.7
5	1.6	22.0	22.1	6.1
6	10.1	14.7	11.4	5.3
7	14.0	69.5	12.6	7.5
8	11.6	11.8	9.1	11.1
9	0.1	4.7	7.8	6.9
10	2.0	6.5	7.2	11.1
11	2.5	7.9	4.4	2.7
12	*	6.3	5.5	7.1
13	2.3	2.2	7.3	1.2
14	5.0	17.8	2.7	2.2
15	0.3	10.0	4.9	3.5
16	0.9	2.4	4.8	2.4
17	1.9	1.9	2.2	4.8
18	2.3	2.9	5.8	11.1
19	7.6	21.6	11.5	4.3
20	18.6	16.8	19.0	2.8
21	1.4	8.1	3.1	1.1
22	2.1	0.9	6.0	*
23	6.2	6.8	14.8	3.7
24	6.4	12.4	16.5	8.6
25	3.2	18.0	11.0	4.2
26	5.4	12.6	20.0	8.7
27	0.2	0.5	0.1	0.6
28	0.5	0.4	0.7	0.4

* = Missing value

These data are not normally distributed.

Level 1 : the widest part of the pinna

Level 2 : immediately proximal to the tubercle of the anthelix

Level 3 : the junction between the vertical and horizontal canal

Level 4 : the most proximal end of the annular cartilage

Table 3.3.6 Summary and statistical analysis of the figures from Table 3.3.5.

	Level 1	Level 2	Level 3	Level 4
Mean	4.1	10.5	8.1	4.7
Min.	0.1	0.4	0.1	0.4
Max.	18.6	69.5	22.1	11.1
Q1	0.9	2.5	4.1	3.3
Q3	5.3	14.1	11.4	7.1
Median	2.1	6.6	5.9	3.7
S.D.	4.7	13.3	6.0	3.3

Min. = minimum, Max. = maximum

Q1 = the 25th percentile; Q3 = the 75th percentile

S.D. = standard deviation

Data are percentages (%).

Table 3.3.7 The proportion of each section occupied by sebaceous glands at three nominated locations in the aural skin of 15 otitic canine ears (%).

Ear	Level 2	Level 3	Level 4
1	22.9	26.1	6.8
2	12.0	2.6	0.1
3	27.7	16.9	16.4
4	24.6	27.3	7.9
5	1.2	2.1	0.1
6	1.0	1.3	0.2
7	20.3	20.3	11.0
8	21.8	6.9	2.8
9	23.5	19.7	8.6
10	9.4	14.5	0.5
11	31.9	4.8	3.0
12	5.2	*	3.8
13	*	14.2	*
14	38.4	23.6	5.1
15	29.5	17.3	9.6

* = Missing value

These data are not normally distributed.

Level 2 : immediately proximal to the tubercle of the anthelix

Level 3 : the junction between the vertical and horizontal canal

Level 4 : the most proximal end of the annular cartilage

Table 3.3.8 Summary and statistical analysis of the figures from Table 3.3.7.

	Level 2	Level 3	Level 4
Mean	19.2	14.1	5.4
Min.	1.0	1.3	0.1
Max.	38.4	27.3	16.4
Q1	8.3	4.2	0.4
Q3	28.1	21.1	8.8
Median	22.3	15.7	4.4
S.D.	11.7	9.1	4.9

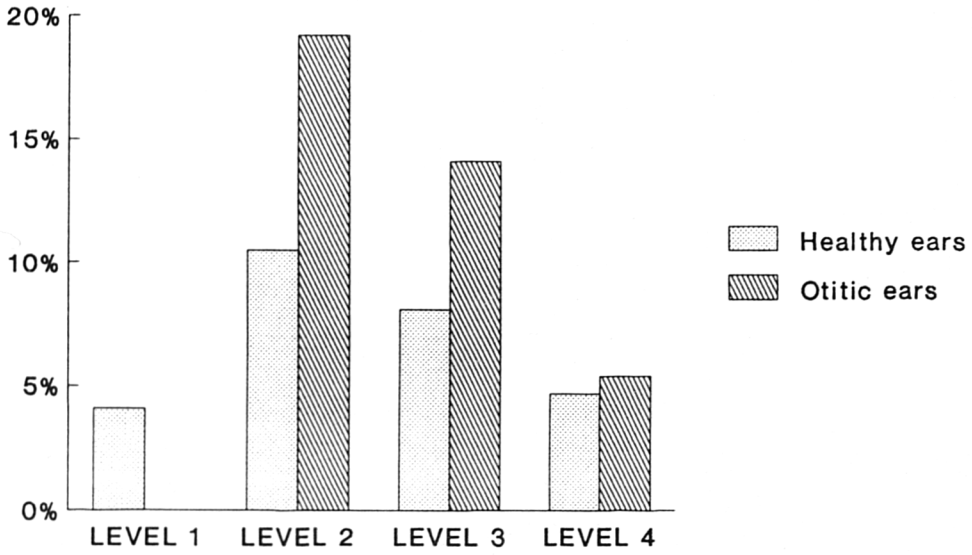
Min. = minimum, Max. = maximum

Q1 = the 25th percentile; Q3 = the 75th percentile

S.D. = standard deviation

Data are percentages (%).

Figure 3.3.5 The mean proportion of each section occupied by sebaceous glands in the aural skin at four anatomical levels in healthy ears and ears with otitis externa.



Data from level 1 was not available from ears with otitis externa.

Table 3.3.9 The proportion of each section occupied by apocrine glands at four nominated locations in the aural skin of 28 healthy canine ears (%).

Ear	Level 1	Level 2	Level 3	Level 4
1	1.1	3.8	1.9	2.0
2	0.2	1.9	1.4	1.6
3	0.7	1.4	3.7	3.9
4	0.9	0.6	3.2	2.8
5	1.0	0.6	4.0	3.0
6	1.7	0.8	1.8	2.7
7	0.3	5.1	2.3	1.8
8	2.2	3.5	1.4	2.3
9	0.8	0.1	2.9	12.1
10	1.0	2.0	1.8	9.7
11	0.4	0.4	3.0	2.8
12	*	2.1	2.3	1.8
13	0.9	1.0	1.1	2.6
14	1.4	1.0	1.3	2.5
15	0.1	1.3	0.8	2.7
16	0.1	0.7	2.3	8.5
17	0.1	3.0	1.4	1.5
18	1.2	5.1	3.2	2.9
19	1.4	4.5	2.1	4.3
20	5.4	5.6	3.8	5.4
21	1.5	0.8	4.2	3.2
22	1.4	2.1	2.5	*
23	0.5	1.0	2.0	8.7
24	1.1	0.8	4.7	11.0
25	3.4	2.2	6.1	5.7
26	1.8	2.2	3.7	7.6
27	2.2	4.1	2.1	7.2
28	5.0	3.8	2.0	0.6

* = Missing value

These data are not normally distributed.

Level 1 : the widest part of the pinna

Level 2 : immediately proximal to the tubercle of the anthelix

Level 3 : the junction between the vertical and horizontal canal

Level 4 : the most proximal end of the annular cartilage

Table 3.3.10 Summary and statistical analysis of the figures from Table 3.3.9.

	Level 1	Level 2	Level 3	Level 4
Mean	1.4	2.2	2.6	4.5
Min.	0.1	0.1	0.8	0.6
Max.	5.4	5.6	6.1	12.1
Q1	0.5	0.8	1.8	2.3
Q3	1.7	3.7	3.6	7.2
Median	1.1	2.0	2.3	2.9
S.D.	1.3	1.7	2.2	3.2

Min. = minimum, Max. = maximum

Q1 = the 25th percentile; Q3 = the 75th percentile

S.D. = standard deviation

Data are percentages (%).

Table 3.3.11 The proportion of each section occupied by apocrine glands at three nominated locations in the aural skin of 15 otitic canine ears (%).

Ear	Level 2	Level 3	Level 4
1	2.3	6.6	24.7
2	3.9	16.3	7.2
3	5.0	18.1	20.8
4	2.8	0.8	2.5
5	4.2	9.5	7.0
6	8.7	7.9	7.3
7	4.7	6.1	5.2
8	3.3	11.0	16.2
9	10.5	3.0	3.0
10	5.7	11.5	15.9
11	16.9	27.6	47.2
12	36.2	*	41.9
13	*	11.2	*
14	14.4	21.5	26.5
15	23.4	19.5	14.4

* = Missing value

These data are not normally distributed.

Level 2 : immediately proximal to the tubercle of the anthelix

Level 3 : the junction between the vertical and horizontal canal

Level 4 : the most proximal end of the annular cartilage

Table 3.3.12 Summary and statistical analysis of the figures from Table 3.3.11.

	Level 2	Level 3	Level 4
Mean	10.1	12.2	17.1
Min.	2.3	0.8	2.5
Max.	36.2	27.6	47.2
Q1	3.8	6.5	6.6
Q3	15.1	16.4	25.2
Median	5.4	11.1	15.1
S.D.	9.7	7.5	14.0

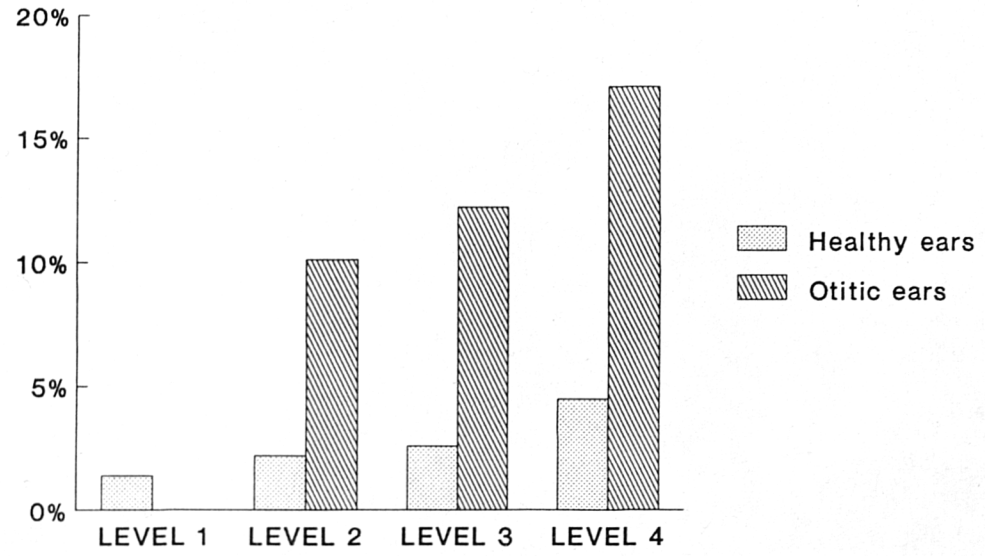
Min. = minimum, Max. = maximum

Q1 = the 25th percentile; Q3 = the 75th percentile

S.D. = standard deviation

Data are percentages (%).

Figure 3.3.6 The mean proportion of each section occupied by apocrine glands in the aural skin at four anatomical levels in healthy ears and ears with otitis externa.



Data from level 1 was not available from ears with otitis externa.

Table 3.3.13 The proportion of each section occupied by hair follicles at four nominated locations in the aural skin of 28 healthy canine ears (%).

Ear	Level 1	Level 2	Level 3	Level 4
1	6.3	1.9	2.2	0.9
2	8.5	1.7	1.1	1.2
3	0.5	0.8	0.4	0.2
4	0.1	1.7	0.8	0.1
5	0.5	0.2	3.1	1.0
6	2.5	1.4	0.8	0.9
7	1.3	2.1	2.3	1.2
8	2.5	1.1	1.9	2.5
9	30.9	3.5	6.6	5.8
10	16.9	6.0	19.4	6.8
11	1.2	1.0	0.6	0.3
12	*	2.0	0.5	0.4
13	2.4	0.9	0.2	0.3
14	0.5	0.4	0.2	0.1
15	1.3	0.5	0.9	0.1
16	1.0	1.0	1.6	1.5
17	1.2	0.1	1.0	0.6
18	2.4	0.6	1.2	1.0
19	1.1	1.1	4.9	2.1
20	1.9	1.7	4.3	2.1
21	0.8	1.7	0.9	1.7
22	2.1	2.4	0.4	*
23	2.0	0.9	3.0	2.1
24	0.6	0.9	3.4	2.8
25	2.0	0.6	1.8	1.5
26	3.0	2.2	3.3	1.6
27	1.3	0.9	0.1	0.4
28	1.3	0.5	2.2	0.5

* = Missing value

These data are not normally distributed.

Level 1 : the widest part of the pinna

Level 2 :immediately proximal to the tubercle of the anthelix

Level 3 : the junction between the vertical and horizontal canal

Level 4 : the most proximal end of the annular cartilage

Table 3.3.14 Summary and statistical analysis of the figures from Table 3.3.13.

	Level 1	Level 2	Level 3	Level 4
Mean	3.6	1.4	2.5	1.5
Min.	0.1	0.1	0.1	0.1
Max.	30.9	6.0	19.4	6.8
Q1	1.0	0.7	0.7	0.4
Q3	2.5	1.7	3.1	2.1
Median	1.3	1.1	1.4	1.0
S.D.	6.2	1.2	3.7	1.6

Min. = minimum, Max. = maximum

Q1 = the 25th percentile; Q3 = the 75th percentile

S.D. = standard deviation

Data are percentages (%).

Table 3.3.15 The proportion of each section occupied by hair follicles at three nominated locations in the aural skin of 15 otitic ears (%).

Ear	Level 2	Level 3	Level 4
1	1.3	1.3	5.1
2	0.9	4.9	0.1
3	1.8	0.8	2.2
4	1.3	3.3	2.1
5	1.3	5.7	0.6
6	3.9	1.6	1.3
7	13.6	8.1	2.7
8	10.2	5.4	2.7
9	8.1	4.9	5.4
10	3.4	6.4	0.1
11	4.4	3.8	2.1
12	4.1	*	3.9
13	*	11.6	*
14	4.2	4.1	0.4
15	2.0	3.5	2.6

* = Missing value

These data are not normally distributed.

Level 2 : immediately proximal to the tubercle of the anthelix

Level 3 : the junction between the vertical and horizontal canal

Level 4 : the most proximal end of the annular cartilage

Table 3.3.16 Summary and statistical analysis of the figures from Table 3.3.15.

	Level 2	Level 3	Level 4
Mean	4.2	4.7	2.2
Min.	0.9	0.8	0.1
Max.	13.6	11.6	5.4
Q1	1.3	2.9	0.6
Q3	5.3	5.9	3.1
Median	3.7	4.5	2.2
S.D.	3.8	2.8	1.7

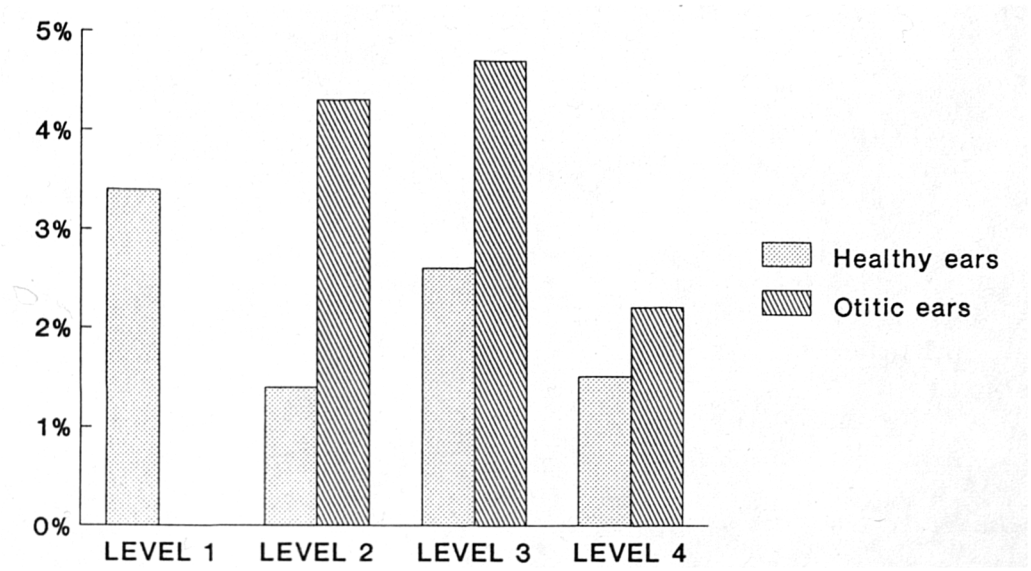
Min. = minimum, Max. = maximum

Q1 = the 25th percentile; Q3 = the 75th percentile

S.D. = standard deviation

Data are percentages (%).

Figure 3.3.7 The mean proportion of each section occupied by hair follicles in aural skin at four anatomical levels in healthy ears and ears with otitis externa.



Data from level 1 was not available from ears with otitis externa.

Table 3.3.17 The proportion of each section occupied by connective tissue at four nominated locations in the aural skin of 28 healthy canine ears (%).

Ear	Level 1	Level 2	Level 3	Level 4
1	92.9	91.8	92.0	95.7
2	90.7	92.2	95.1	95.9
3	97.3	92.8	91.1	92.5
4	97.0	95.8	91.2	94.5
5	96.9	77.2	70.8	89.9
6	85.7	83.2	86.1	91.1
7	84.4	23.3	82.8	89.5
8	83.7	83.5	87.7	84.2
9	68.2	91.7	82.7	75.3
10	80.1	85.4	71.6	72.4
11	96.0	90.7	92.0	94.2
12	*	89.6	91.7	90.7
13	94.5	95.9	91.4	96.0
14	93.1	80.8	95.8	95.3
15	98.3	88.2	93.5	93.0
16	98.1	95.9	91.3	87.6
17	96.9	95.1	95.4	93.1
18	94.2	91.3	89.8	85.0
19	89.9	72.7	81.6	89.3
20	74.1	76.0	72.9	89.7
21	96.4	89.4	91.8	94.1
22	94.4	94.6	91.1	*
23	91.3	91.3	80.3	85.6
24	91.9	86.0	75.5	77.6
25	91.5	79.3	81.2	88.6
26	89.9	83.1	73.0	82.2
27	96.4	94.5	97.8	91.8
28	93.1	95.4	95.2	98.6

* = Missing value

These data are not normally distributed.

Level 1 : the widest part of the pinna

Level 2 : immediately proximal to the tubercle of the anthelix

Level 3 : the junction between the vertical and horizontal canal

Level 4 : the most proximal end of the annular cartilage

Table 3.3.18 Summary and statistical analysis of the figures from Table 3.3.17.

	Level 1	Level 2	Level 3	Level 4
Mean	91.0	85.9	86.8	89.4
Min.	68.2	23.3	70.8	72.4
Max.	98.3	95.9	97.8	98.6
Q1	89.9	83.1	81.3	85.6
Q3	96.4	94.1	92.0	94.2
Median	93.1	90.1	91.1	90.7
S.D.	7.4	13.9	8.1	6.5

Min. = minimum, Max. = maximum

Q1 = the 25th percentile; Q3 = the 75th percentile

S.D. = standard deviation

Data are percentages (%).

Table 3.3.19 The proportion of each section occupied by connective tissue from three nominated locations in the aural skin of 15 otitic ears (%).

Ear	Level 2	Level 3	Level 4
1	73.5	66.0	63.4
2	83.2	76.2	92.8
3	65.5	64.2	60.5
4	71.3	68.7	87.5
5	93.3	82.6	92.2
6	86.4	89.3	91.2
7	61.4	65.5	81.1
8	64.7	76.8	78.3
9	57.9	72.3	83.0
10	81.5	67.6	83.5
11	46.9	63.8	47.7
12	54.5	*	50.5
13	*	63.0	*
14	46.0	50.9	68.0
15	45.2	59.8	73.4

* = Missing value

These data are not normally distributed.

Level 2 : immediately proximal to the tubercle of the anthelix

Level 3 : the junction between the vertical and horizontal canal

Level 4 : the most proximal end of the annular cartilage

Table 3.3.20 Summary and statistical analysis of the figures from Table 3.3.19.

	Level 2	Level 3	Level 4
Mean	66.5	69.0	75.2
Min.	45.2	50.9	47.7
Max.	93.3	89.3	92.8
Q1	52.6	63.6	62.7
Q3	81.9	76.4	88.4
Median	65.1	66.8	79.7
S.D.	16.0	9.8	15.1

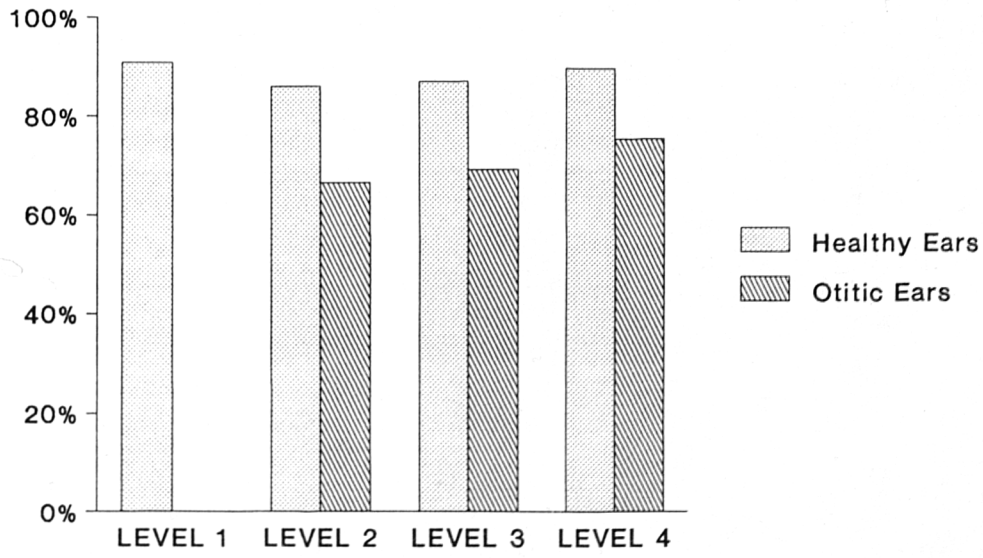
Min. = minimum, Max. = maximum

Q1 = the 25th percentile; Q3 = the 75th percentile

S.D. = standard deviation

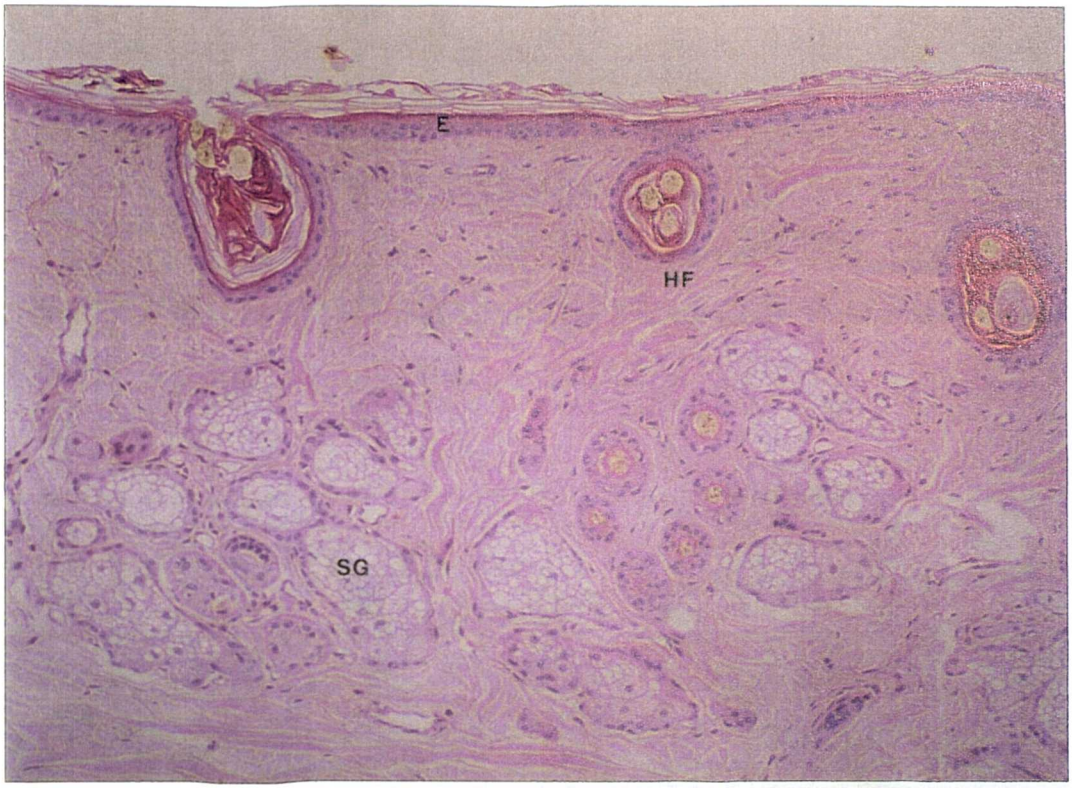
Data are percentages (%).

Figure 3.3.8 The mean proportion of each section occupied by connective tissue in the aural skin at four anatomical levels in healthy ears and ears with otitis externa.



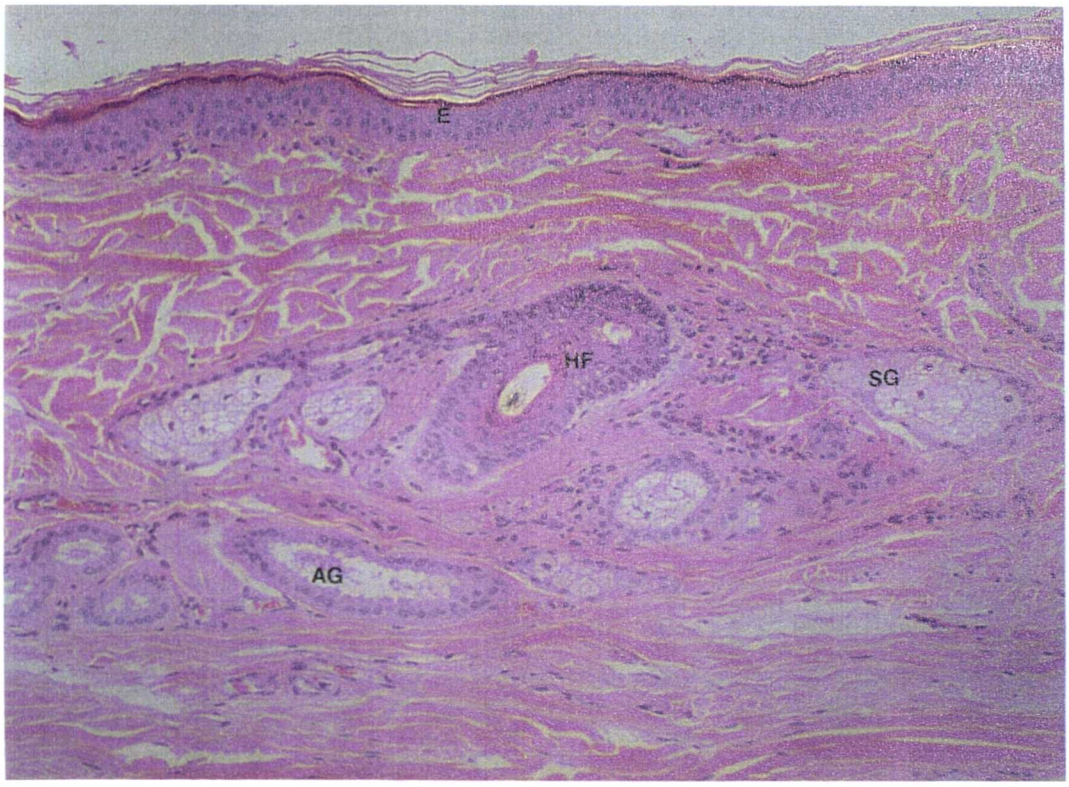
Data from level 1 was not available from ears with otitis externa.

Figure 3.3.9a The histological features of an healthy canine external ear at the widest part of the pinna (level 1) (Haematoxylin and Eosin, x60).



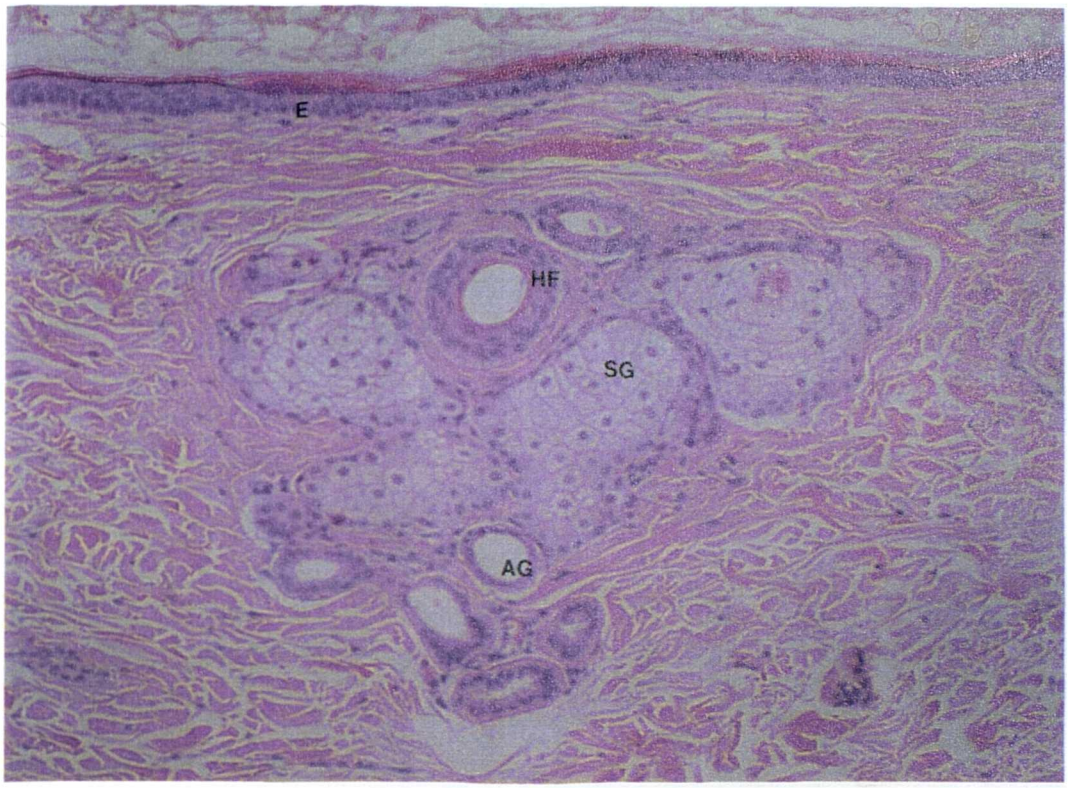
E: epidermis; HF: hair follicle; SG: sebaceous gland.

Figure 3.3.9b The histological features of an healthy canine external ear immediately proximal to the tubercle of the anthelix (level 2) (Haematoxylin and Eosin, x60).



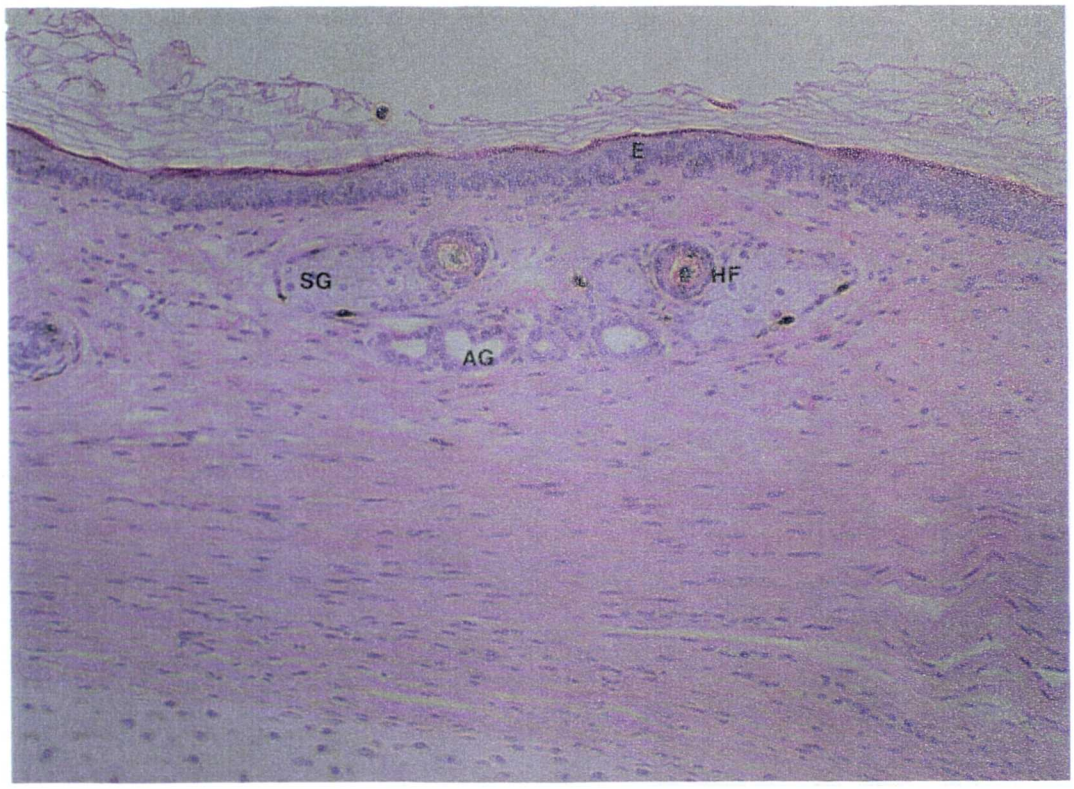
AG: apocrine gland; E: epidermis; HF: hair follicle; SG: sebaceous gland.

Figure 3.3.9c The histological features of an healthy canine external ear at the junction between the vertical and horizontal ear canal (level 3) (Haematoxylin and Eosin, x60).



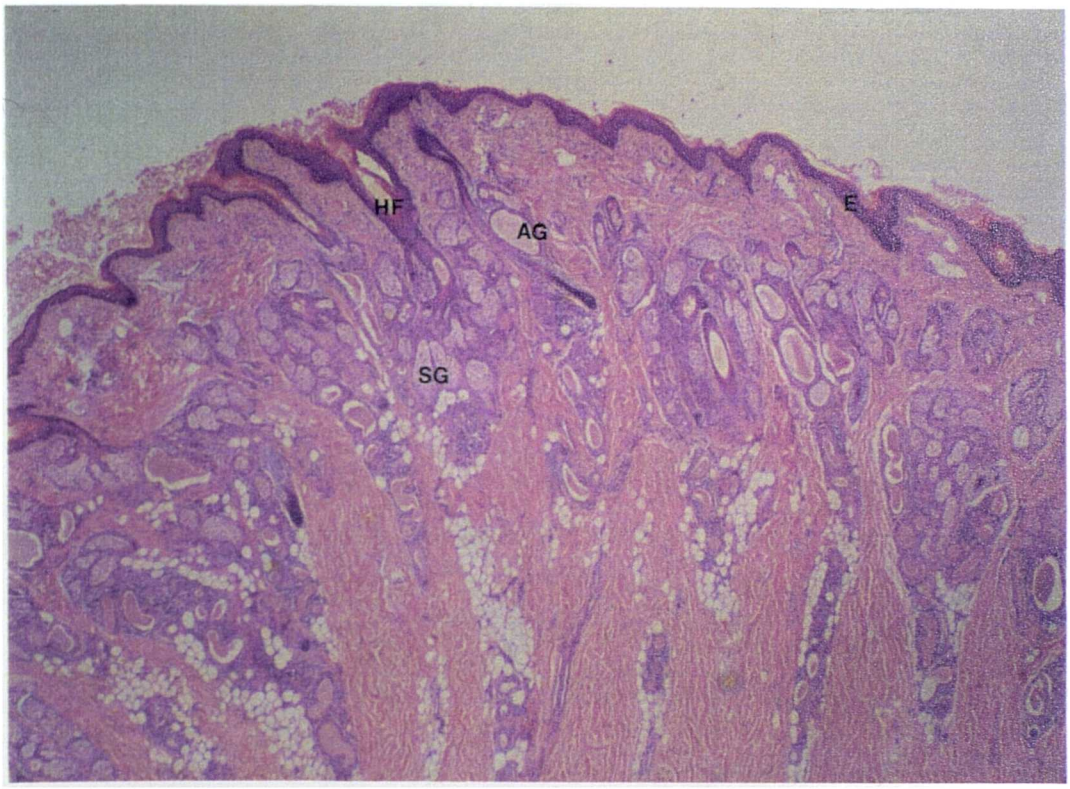
AG: apocrine gland; E: epidermis; HF: hair follicle; SG: sebaceous gland.

Figure 3.3.9d The histological features of an healthy canine external ear at the most proximal extremity of the annular cartilage (level 4) (Haematoxylin and Eosin, x60).



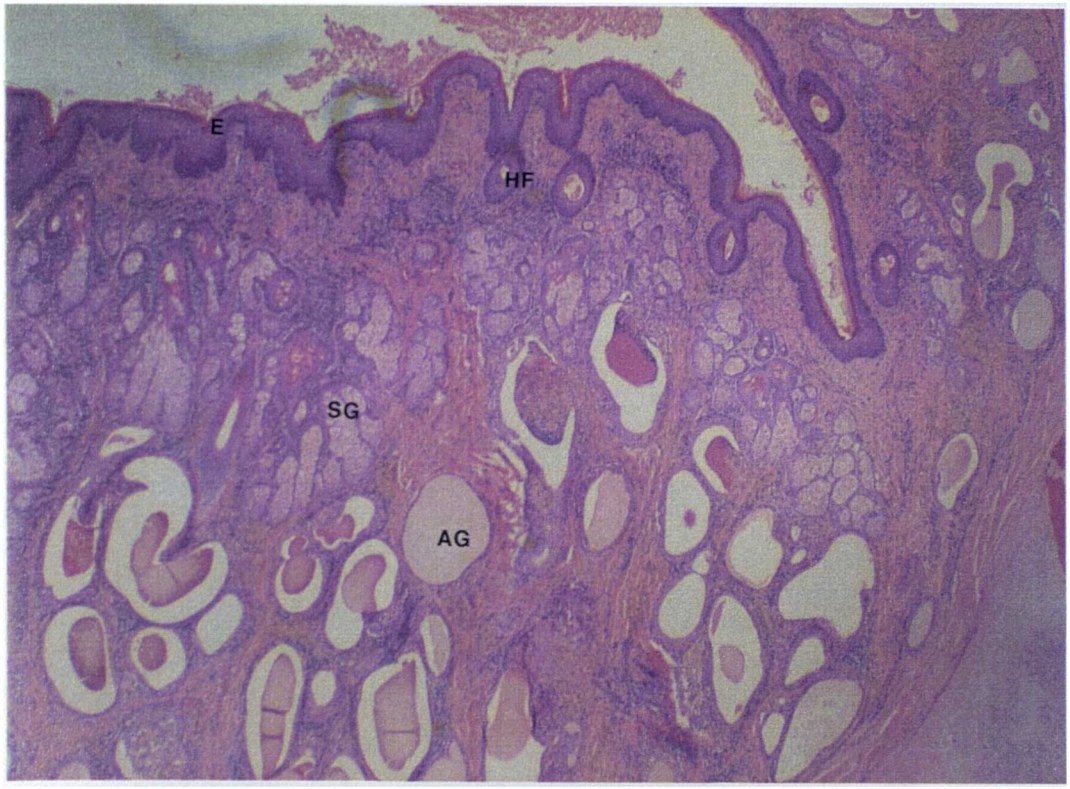
AG: apocrine gland; E: epidermis: HF: hair follicle; SG: sebaceous gland.

Figure 3.3.10a The histological features of an otitic canine ear immediately proximal to the tubercle of the anthelix (level 2) (Haematoxylin and Eosin, x12).



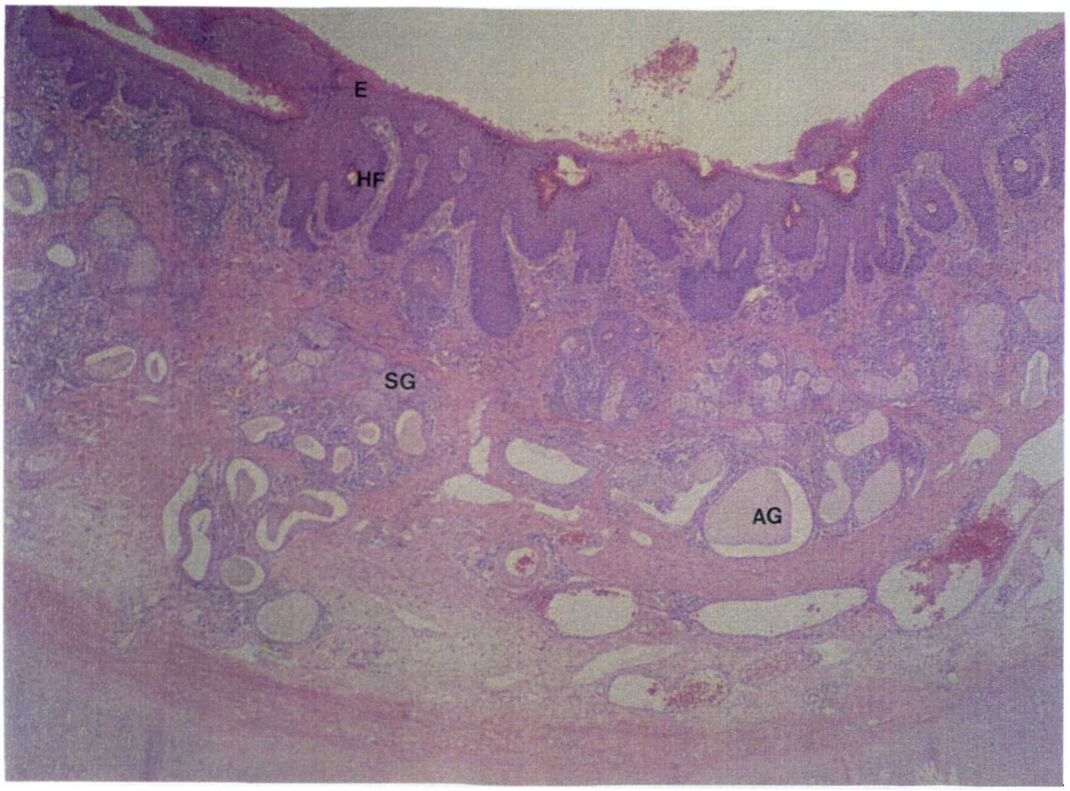
AG: apocrine gland; E: epidermis; HF: hair follicle; SG: sebaceous gland.

Figure 3.3.10b The histological features of an otitic canine ear at the junction between the vertical and horizontal ear canal (level 3) (Haematoxylin and Eosin, x12).



AG: apocrine gland; E: epidermis; HF: hair follicle; SG: sebaceous gland.

Figure 3.3.10c The histological features of an otitic canine ear at the most proximal extremity of the annular cartilage (level 4) (Haematoxylin and Eosin, x12).



AG: apocrine gland; E: epidermis; HF: hair follicle; SG: sebaceous gland.

3.4 DISCUSSION

In the present study the overall length of the cartilaginous part of 40 canine external ears averaged 5.3 cm. This result differs substantially from the value of 2 cm reported by Getty *et al* (1956). On average the segment supported by the annular cartilage measured 1.2 cm in length, Miller and Witter (1942) reported similar data. The average diameter of these ears was 0.5 cm at the proximal end of the horizontal ear canal, and more distally, at the proximal end of auricular cartilage, 0.7 cm. At the distal opening of the ear canal, the maximal diameter measured 5.8 cm on average.

The external ear canal is a funnel shaped tube and it forms a narrow neck at the annular cartilage. It is likely that poor ventilation, convection and radiation of heat may also be minimal at the narrow part of the horizontal canal. The internal volume of the canal shows a positive correlation with body weight in dogs (Forsythe, 1985).

The histological features of the aural skin of 28 normal ears from 14 dogs were studied by morphometric measurement. The method chosen for the present study was square-counting. Square-counting is an easy and straightforward method to collect morphometric data (Weibel, 1979a). However, it tends to lack accuracy. This method leads to an over-estimation of the size of small areas, whilst the size of large areas of candidate tissue are under-estimated. This inaccuracy can be corrected for by using a finer scale of graticule or by point-counting (Weibel, 1979a; Reid, 1980). In the present study only one graticule size was used and the magnification of the tissue was kept constant (100x). Thus, despite the inaccuracy of the method, tissues from different sites in the ear canal and different individuals can be compared with a high degree of confidence

that those differences found are real and significant. No further effort was made to correct for the inaccuracy of the method.

The proportion of sebaceous gland tissue in healthy aural skin showed a marked variation between ears and at the four anatomical levels. Variation between dogs has also been reported by Fraser (1961d). In the normal ears, the proportion of sebaceous gland tissue increased gradually from the proximal end of the ear canal, reaching a peak at the tubercle of the anthelix. The proportion decreased again on the pinna. These findings resemble the report by Fraser (1961d) that sebaceous glands were most prominent and numerous in the more distal part of the ear canal. However, in contrast, Miller and Witter (1942) reported that sebaceous glands reached a maximum number near the tympanic membrane.

Apocrine glandular tissue was seen in the aural skin at each of the four levels, although the proportion of these glands exhibited great variation between dogs. These findings do not agree with Miller and Witter (1942) who reported that no apocrine glands were visible in the normal canine aural integument. The proportion of apocrine gland tissue decreased gradually from the proximal end of the external ear canal towards the pinna. This observation contradicts the study by Fraser *et al* (1970); they reported that ceruminous glands were very prominent and numerous in the more peripheral parts of the external ear canal.

The distribution of the hair follicular tissue showed variation between healthy ears and the four levels, but this variation between site was not statistically significant. Follicular tissue was most abundant at levels 1 and 3, that is on the medial aspect of the pinna, and at the proximal end of the vertical canal.

The proportions of sebaceous and apocrine glands in the present study differ from the results reported by other researchers (Miller and

Wither, 1942; Fraser, 1961d; Fraser *et al*, 1970). This may be due to differences between individual dogs. Smaller observation sets could also lead to different results. None of the descriptive histological reports stated the number of ears used for their study, nor were the anatomical sites chosen for these observations precisely described (Miller and Witter, 1942; Fraser, 1961d; Fraser *et al*, 1970). Descriptive data (Miller and Witter, 1942; Fraser, 1961d; Fraser *et al*, 1970) rather than morphometric quantitation of histological features cannot provide a mathematically dependable comparison between sites or individuals. In the present study of normal ears the glands show significant differences in distribution in the aural integument between the four anatomical levels. Marked variation in the proportion of these glands between individuals have been reported by Fraser (1961d), and were also found in the present study.

The phenomenon of outward epidermal migration from the tympanic membrane along the external auditory meatus is well known in many mammalian species, although no data are available for dogs. This physiological phenomenon is regarded as a "self-cleansing mechanism" (Johnson *et al*, 1984; Johnson and Hawke, 1986; Youngs *et al*, 1988; Broekaert, 1990; Weinberger *et al*, 1990; Smelt *et al*, 1991). It is known that skin surface lipids are mainly secreted by sebaceous glands (Nielsen, 1953; Greene, Downing, Pochi, and Strauss, 1970; McEwan Jenkinson and Lloyd, 1979; Lloyd, Dick, and McEwan Jenkinson, 1979b; Lloyd and Garthwaite, 1982; McEwan Jenkinson, 1993). Apocrine glands on the other hand, seem to secrete modified intercellular fluid onto the skin surface (Smith, 1890; Kerr and Snow, 1983; Robertshaw, 1991) although no data are available to confirm the secretion of canine aural apocrine glands . Based on the morphological data from the present study, the ratio of apocrine to sebaceous glandular tissue decreases from the proximal end to the distal opening of the aural canal. This finding perhaps

suggests that cerumen with a more aqueous nature may be formed in the deeper part of the canal. Such cerumen may be most conducive to epidermal migration from the deep ear canal towards its external opening. The lower ratio of apocrine to sebaceous glandular tissue at the external opening of the ear canal, might suggest that cerumen with a higher lipid content is secreted at the opening of the canal. Lipid probably fulfills water repellent and antibacterial roles on the aural integument (Holland, 1993). According to Dickson and Love (1983), 50% of the horizontal ear canals of clinically normal dogs are not inhabited by microorganisms.

In otitic canine ears, the pattern of sebaceous gland distribution was similar to normal ears. The proportion of these glands again exhibited marked variation between ears. Otitic ears had a significantly greater proportion of sebaceous glands than healthy ears at each of three levels where they could be compared. Nielsen (1953) reported that sebaceous glands became hyperplastic in otitis externa. However, the findings in the present study contradict reports by some other researchers. Fraser (1961d) and Fernando (1967) reported that sebaceous glands enlarge early in the course of this disease, but later became less active and reduced in size. Stout-Graham *et al* (1990) stated that in the horizontal ear canal in dogs these glands did not change in size during otitis. However, in their study, no comparison was reported concerning the area occupied by sebaceous glands compared to the total area of enclosed tissue in otitic ears. They simply reported a significant decrease in the ratio of sebaceous to apocrine glands and that this resulted from an increase in the apocrine glandular tissue rather any change in the sebaceous glands. That finding again may be an artefact due to the marked variation between individuals and the sampling size adopted by Stout-Graham *et al* (1990).

Apocrine glands also exhibited marked variation between otitic ears in dogs. The same pattern of apocrine gland distribution was found in

both otitic and normal ears. These glands increased in density deep in the canal with the maximal number near the tympanic membrane. The proportion of apocrine glands in otitic ears was significantly greater than healthy ears at all the three levels. This result was expected. According to other reports, these glands increase in size and become hyperplastic, or cystic during the process of chronic disease (Miller and Witter, 1942; Nielsen, 1983; Fraser, 1961d, Fernando, 1966; Fernando, 1967; Stout-Graham *et al*, 1990).

In the present study, otitic ears had a significantly greater proportion of hair follicular tissue than healthy ears. The area of hair follicle tissue does not necessarily indicate the relative abundance of hairs themselves in the external ear canal. On the bovine skin, microflora were found in the hair infundibula, and the intercellular spaces. These bacteria and yeasts were generally found in mixed microcolonies closely associated with sebaceous lipids (Lloyd, Dick, and McEwan Jenkinson, 1979a; McEwan Jenkinson, 1993). Larger hair follicles may be formed as a result of follicular hyperplasia, a consequence of the inflammatory process or due to microbial overgrowth.

The density of hair in the external auditory meatus has been incriminated as a contributing factor to the high incidence of otitis externa in certain breeds of dog (Blaine, 1841; McGinnis and England, 1949; Fraser, 1961a; Fraser *et al*, 1961; Fraser, 1965; Fraser *et al*, 1970; Sharma and Rhoades, 1975; Evans and Jemmett, 1978; August, 1986; Hayes *et al*, 1987) However, in the present study, no further evidence was found to either support or refute this hypothesis.

Cerumen is formed by secretions of glands in the aural integument together with cell debris from the epidermis (Fernando, 1966; Fernando, 1967; Bortz, Wertz and Downing, 1990). Pathological changes in secretory tissues could alter this composition. The presumed natural

barrier to water and microorganisms afforded by cerumen might be a critical factor in the pathogenesis of otitis externa. The health status of the external ear canal depends on the functional integrity of all structures within this area. When this homeostasis is broken by any of them, for instance due to an underlying dermatological disorder, otitis externa may result.

According to the study of McGinley, Webster, Ruggieri, and Leyden (1980), cutaneous flora on human skin are affected by the glands. Regional variations in the cutaneous flora are thought to be influenced by the composition of the local skin emulsion (Greene *et al*, 1970; Kligman *et al*, 1976; McGinley *et al*, 1980; Midgley, 1989; Holland, 1993). The changes in the proportions of both glands in otitis externa could vary the composition of cerumen, thereby affecting resident flora. In the present study, most healthy canine ears were inhabited by Gram-positive, coagulase-negative cocci, whilst 85% of the ears affected by otitis externa were associated with Gram-positive, coagulase-positive cocci, and Gram-negative rods (see Chapter II Results). To understand the roles of bacteria or yeasts in this condition, the composition of cerumen in ears with and without otitis externa may be important. Understanding cerumen composition would help to elucidate the pathogenesis of canine otitis externa.

Microbial overgrowth can be induced by a change in microclimate of an ecosystem (Bibel and LeBrun, 1975; Aly *et al*, 1978; Faergemann *et al*, 1983) (Chapter II). Grono (1970c) has reported that the relative humidity in otitic ears was 10% higher than in normal ears in dogs. Apocrine gland tissue is more abundant in otitic ears (see above) and this could be related to a higher relative humidity in otitic ears, if a larger area of gland produces more, or more aqueous, secretion. An elevated skin surface moisture might then promote growth of pathogenic microbes

(Hojyo-Tomoka *et al*, 1973; Aly *et al*, 1978; Hartmann, 1983) (see Chapter II, Discussion).

Hyperplastic apocrine glands in otitis externa show similar histopathological patterns in both dogs and humans (Senturia, 1950; Sophian and Senturia, 1954; Sophian and Senturia, 1955; Senturia and Carr, 1957). Based on histochemical staining, Fernando (1967) has stated that apocrine glands mainly secrete acidic materials. According to this worker, apocrine glands become the major secretory organs during chronic otitis externa (Fernando, 1966). He has postulated that hyperplasia of apocrine glands in otitic ears would alter the hydrogen ion concentration in cerumen; cerumen in chronic otitis should be acidic and inhibit the growth of microbes (Fernando, 1966; 1967). In humans however, cystic or hyperplastic apocrine glands are generally thought to result from poor drainage from the glands by plugging of their ducts or increased fibrous tissue in skin. The cerumen in this condition would be less acidic and less protective (Senturia, 1950; Sophian and Senturia, 1954). Goffin (1963a; 1963b) and Grono (1970b) have reported that in ears affected by chronic otitis in both humans and dogs, aural skin pH is in fact elevated. Although significant changes were found in the proportions of sebaceous and apocrine glands in the aural integument affected by otitis externa, changes in the epidermis, e.g., epidermal hyperplasia, may also account for changes in cerumen pH since epidermis also participates in the formation of cerumen (Bortz *et al*, 1990). When aural skin is damaged by the inflammatory process, leakage of transudate or exudate may bring local aural pH into equilibrium with the interstitial fluid (Aly *et al*, 1978). Aural pH may thus be altered from slightly acidic to near neutral as inflammation progresses. Higher environmental pH promotes the growth of pathogenic bacteria (Goffin, 1963a), and the actions of bacterial enzymes (Hall *et al*, 1968). These findings indicate that the

pathophysiology of bacterial overgrowth in otitis externa is complicated. In the present study, morphometric evaluation of the pathological changes in otitic ears alone cannot elucidate the role that secretory glands play in the pathogenesis of canine otitis externa. Studies concerned with the pathophysiology of these glands would be essential to clarify their significance in otitis in both dogs and humans.

Epidermal hyperplasia and excessive keratin were noticed in most otitic ears in the present study. Senturia (1950) has postulated that induced apocrine sweat would lead to prolonged exposure to humidity and poor evaporation within the ear canal. The stratum corneum would then become softened and swollen. Abnormal cornification and hyperkeratosis would ensue. Growth of pathogenic bacteria would be promoted by the changed environment and epidermal inflammation would be induced by bacterial or fungal infection. Otitic ears may have excessive keratin because of mal-cornification and the pathological cycle outlined by Senturia (1950).

Abnormally high levels of keratinocyte cytokines are thought to be responsible for the cutaneous pathological cycle (McKenzie and Sauder, 1990). Injury to the skin with accompanying infection leads to release of these cytokines. Such cytokines are involved in the initiation of inflammation, the regulation of epidermal homeostasis, and also maintain the growth of keratinocytes. Proliferation of keratinocytes could be induced when these cytokines are not at normal physiological levels. Anastasia and Conley (1977) have postulated that regeneration of epidermal cells requires oxidation of fatty acids as the energy source. Oxidation of epidermal fatty acids may also be involved in proliferation of epidermis. It seems that the process of inflammation could be initiated by several causes, and its consequences would also be affected by different intrinsic factors. Investigations concerning the metabolism of the aural

epidermis might help to elucidate the pathogenesis of otitis externa and its pathology in detail. Based on such knowledge, more specific treatments for canine otitis might be developed in future.

CHAPTER IV

IDENTIFICATION AND QUANTITATION OF CANINE CERUMEN IMMUNOGLOBULIN

4.1 INTRODUCTION

A high incidence of microbial overgrowth is found in canine otitis externa (see Chapter II). Increased adherence of pathogenic bacteria to epidermal cells is thought to be essential to promote bacterial overgrowth (Leyden *et al*, 1980; Abraham *et al*, 1983; Cole and Silverberg, 1986). On the other hand, defective host defence mechanisms may also be involved in the pathogenesis of bacterial infections on human and canine skin (Ichimura *et al*, 1983; Hanifin and Homburger, 1986; Moroff, Hurvitz, Peterson, Saunders, and Noone, 1986). In humans, increased susceptibility to pyogenic infections has been associated with selective immunoglobulin subclass deficiency (Sorensen and Nielsen, 1988). Compromised host defence is well known to contribute to "malignant external otitis" in people (Wilson *et al*, 1971; Petrozzi and Warthan, 1974; Yust *et al*, 1980; Gherini *et al*, 1986; Babiatzki and Sade, 1987; Castro *et al*, 1990; Cohen, 1990; Strauss, 1990). The hypersensitivity states of certain dermatological disorders are also caused by abnormal immune responsiveness. Thus, significantly high levels of immunoglobulin G (IgG) and Immunoglobulin E (IgE) are found in dogs and human patients affected by atopic dermatitis (Barratt, Turner, and Johansson, 1971; Walsh, Richards, Douglas, and Blumenthal, 1981; Falanga, Campbell, Leyden, and Douglas, 1985; Gabrielsen and Brandtzaeg, 1985; Husby, Larsen, Ahlstedt, and Svehag, 1986; Halliwell and Gorman, 1989). Atopic dermatitis is frequently associated with otitis externa in the dog. More than 50% of dogs afflicted by atopic dermatitis have been reported to have otitis externa (Griffin, 1991).

Locally synthesized immunoglobulins are the first specific defence preventing bacteria or fungi from attaching to secretory surfaces (Page and Remington, 1967; Williams and Gibbons. 1972; Metze, Kersten,

Jurecka, and Gebhart, 1991; Stenfors and Raisanen, 1991a; Stenfors and Raisanen, 1991b). The secretory immunoglobulins of the secretory surface attach to antigens on the surface of microorganisms and thus modify their adhesive and infectious properties (Metze *et al*, 1991; Stenfors and Raisanen, 1991a; Stenfors and Raisanen, 1991b). Similar antibodies can neutralise bacterial toxins, opsonize microorganisms, enhance phagocytosis by macrophages, and lyse the microorganisms in the presence of complement (Kramer, 1978; Halliwell and Gorman, 1989). The same immunological pathways however, can result in tissue damage, for which the term hypersensitivity is used. Atopic dermatitis is an example of an inappropriate antibody response to environmental allergens (Halliwell and Gorman, 1989).

The presence of IgA, IgG and IgM in human sweat and sebaceous glands has been confirmed by immunohistochemical techniques (Tourville, Adler, Bienestock, and Tomasi, 1969; Tomasi, 1972; Kawai, Kawai, Konishi, Kishimoto, Kanemitsu, Kamei, Obayashi, Okada, and Konishi, 1991; Metze, Jurecka, Gebhart, Schmidt, Mainitz, and Niebauer, 1988; Metze *et al*, 1991). Human sweat and cerumen have also been found to contain IgA, IgG, and IgE (Page and Remington, 1967; Petrakis, Doherty, Lee, Smith, and Page, 1971; Forstrom, Goldyne, and Winkelmann, 1975). These findings indicate that secretory immunoglobulins in sebum, sweat, and cerumen may play an important role in protective functions on the body surface (Page and Remington, 1967; Petrakis *et al*, 1971; Metze *et al*, 1988). A significantly higher level of IgE in human sweat from patients with dermatitis also suggests that defective immunoregulation may be involved in certain human cutaneous disorders (Forstrom *et al*, 1975).

Secretory immunoglobulins have been identified and quantified in various external secretions of several other species (Reynolds and

Johnson, 1970; Franz and Corthier, 1981; Yamada, Tomoda, and Usui, 1984; Halliwell and Gorman, 1989). Variations between different secretions and different species have also been reported. Immunoglobulin G is the predominant antibody in colostrum in dogs and cats; IgG is also a major antibody in canine and porcine faeces. Immunoglobulin A forms the major proportion in intestinal secretions, tears, and saliva of cats, sheep, cattle, and horses (Reynolds and Johnson, 1970; Franz and Corthier, 1981; Yamada *et al*, 1984; Halliwell and Gorman, 1989). Differences between secretory units (glands) are generally thought to be responsible for these variations between secretions and species (Tourville *et al*, 1969). The demonstration of IgA, IgG, IgM, and IgE in apocrine and sebaceous glands, and hair follicles in canine, bovine and ovine integument (Halliwell, 1973; Lloyd, McEwan Jenkinson, and Mabon, 1979; Garthwaite *et al*, 1983) indicates that the skin surface possesses specific antimicrobial antibodies. Raised levels of IgG in bovine skin washings were found after intradermal vaccination with *Dermatophilus congolensis*; this indicates that the skin can mount an immune response to cutaneous infections (Lloyd and McEwan Jenkinson, 1981; Lloyd, McEwan Jenkinson, Miller, and Sykes, 1987). A cutaneous secretory immune system has also been demonstrated in fish (Saint Louis-Cormier, Osterland, and Anderson, 1984; Lobb, 1987). Dysfunction of the secretory immune system may promote overgrowth of cutaneous flora (Moroff *et al*, 1986). An understanding of this local defence mechanism is of importance to the elucidation of the pathogenesis of certain cutaneous disorders especially those conditions frequently associated with microbial infections.

Canine otitis externa is characterised by multifactorial aetiology (Chapter I). In spite of numerous publications, many of them concerned with infectious agents (Chapter II), the defence mechanisms of the

external ear canal in dogs are poorly understood. Knowledge of the aural secretory immune system is essential to realise the pathogenesis of otitis externa. Thus, the aims of this study were firstly to identify whether immunoglobulins are present in cerumen samples from healthy canine ears; secondly, to explore aural immune status and its relevance to both healthy and otitic ears in dogs.

The methods used in this study were sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, and enzyme-linked immunosorbent assay (ELISA). The SDS-PAGE was used for separation and elution of protein from cerumen samples. After this procedure, the recognition of immunoglobulins was carried out by Western blotting. This method transfers the cerumen proteins to nitrocellulose paper where their identity could be determined using specific antiserum for canine immunoglobulin. ELISA tests were used to quantitate antibody levels (Harrington, 1990).

4.2 MATERIALS AND METHODS

4.2.1 Source of canine cerumen samples

Cerumen samples were collected from both healthy canine ears and ears with otitis externa. Samples were obtained from dogs provided by a local kennel, or referred to the Department Veterinary Medicine, University of Glasgow Veterinary School for different illnesses, but without an history or signs of any ear disease, and this material was deemed to be healthy cerumen. Cerumen samples from dogs which were referred to the Department of Veterinary Surgery, University of Glasgow for investigation and treatment of chronic and recurrent otitis externa were deemed to be otitic cerumen samples.

4.2.2 Sample collection

The cerumen samples for absolute immunoglobulin quantitation (see 4.2.5.A below) were collected from eight ears of four Greyhounds (three female and one male, aged from 6 to 14 years). Ten microgram samples were gently collected from each ear using a curette. These samples were stored at -20°C until analysed.

Further cerumen samples were collected from 30 healthy ears of 15 dogs and 13 diseased ears of 8 dogs with otitis externa using sterile microbiological swabs (BioTrace Limited, Mid Glamorgan, Bridgend). Cerumen has a very viscid nature and, as a result, it is difficult to accurately divide samples on swabs into precise quantities for subsequent analysis. These cerumen samples were assayed to reveal the proportion of different immunoglobulin classes in the composition (see 4.2.5.B below). Once again, these samples were stored at -20°C until analysed.

The breed, sex and age (years) of these 15 healthy dogs and 8 dogs with otitis are summarised in Tables 4.2.1 and 4.2.2. These cerumen samples were also submitted for microbiological culture (see Chapter II).

4.2.3 Cerumen preparation

Cerumen lipid and water were removed by chloroform : methanol (2:1) (Folch, Lees, and Stanley, 1957) and the sample was then centrifuged at 1,500 g for 10 minutes. After discharging the supernatant, the sediments were dried in a fume cupboard overnight. The dried deposits were soaked and homogenized in 400 ul 1:3 diluted stacking gel buffer. These prepared samples were used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, and enzyme-linked immunosorbent assay (ELISA).

4.2.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Cerumen proteins were separated on the gel, and then identified by Western blotting (Harrington, 1990). The method for this study was modified from Mostofa (1989).

Materials

Separating gel : 6.66 ml 10% acrylamide, 5 ml 1.5 M Tris-HCl, 0.2 ml 10% SDS, 0.01 ml TEMED, and 8 ml distiled water. One hundred microlitres 10% (w/v) ammonium persulfate was added just prior to use.

Stacking gel : 1.34 ml 4% acrylamide, 2.5 ml 0.5 M Tris-HCl, 0.1 ml 10% SDS, 0.004 ml TEMED, and 6 ml distiled water. Fifty microlitres 10% (w/v) ammonium persulphate was added just prior to use.

Treatment buffer : 0.125 M Tris-HCl 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol, 1% DTT.

Tank buffer : 0.025 M Tris, 0.192 M glycine, and 0.1% SDS at pH 8.3.

Transfer buffer : 0.025 M Tris, 0.192 M glycine, and 0.05% (v/v) ethanol.

Blocking protein : 5% (w/v) dried milk (Marvel skimmed milk powder).

Tris buffered saline (TBS) : 0.05 M Tris-HCl, 0.05% (v/v) Tween 20, and 0.15 M NaCl at pH 7.4.

Solvent for the first and secondary antiserum : One percent (w/v) dried milk in TBS

The first antiserum : rabbit anti-dog (RAD)/IgA, IgG, IgM, and albumin (Nordic Immunological Laboratories, The Netherlands)

The secondary antiserum : horseradish peroxidase (HRP)-antirabbit IgG (Scottish Antibody Production Unit, Scotland).

Substrate : 20 mg of amino-9-ethyl carbazole (AEC) dissolved in 2.5 ml of dimethyl formamide 2.5 ml, and mixed with 47.5 ml of 0.05 mol/l acetate buffer, 25 ul of 30% (v/v) hydrogen peroxide was added just before use.

Equipment

Electrophoresis apparatus; Mighty Small II SE 250 (Hoefer Scientific Instruments, San Francisco) and Power Supply; EPS 500/400 (Pharmacia, Fine Chemicals, Sweden)

Protein transfer apparatus; The Genie (Idea Scientific Company, U.S.A.); power was supplied by a Sigma 6 Battery Charger.

A shaker (Luckhan Recipro-shake, Luckhan Ltd, Sussex, England) was required after the proteins had been transferred onto the nitrocellulose papers.

Procedures

The SDS-PAGE gel was made by firstly pipetting the separating gel into the gel holder. This was covered by a few drops of isopropanol. After the separating gel had polymerised the isopropanol was poured off and

stacking gel was pipetted on the top. Then the comb was inserted into the gel.

The electrophoresis equipment was set up and the tank was filled with the tank buffer. Twenty microlitres of prepared sample diluted with treatment buffer at 1:1 ratio and heated in a boiling water bath for 5 minutes were loaded into a well in the gel. Electrophoresis was carried out at 40 mA for 60 minutes.

Protein blotting was the next step; nitrocellulose paper and gels were soaked in a dish which contained transfer buffer for 10 minutes. Protein transfer was then carried out using the transfer apparatus at 12 mA for 60 minutes.

Immunoglobulin identification was carried out by incubating the transferred nitrocellulose papers in 5% (w/v) milk in TBS at room temperature on a shaker overnight. The first antiserum, RAD/IgA, IgG, and IgM 1:50 diluted by 1% (w/v) milk in TBS, was added for 60 minutes incubation, then washed with 1% (w/v) milk in TBS three times. The secondary antiserum, HRP-anti rabbit IgG 1:500 diluted by 1% (w/v) milk in TBS was added. Washing was then repeated three times. The substrate was added and incubated for 15 minutes. Finally, the procedure was stopped by suspending the nitrocellulose papers in distiled water, and drying them between sheets of filter paper.

4.2.5 Enzyme-linked immunosorbent assay (ELISA)

The method used in this study was modified from that of Mostofa (1989).

Materials

Standard immunoglobulins : Canine IgA, IgG, and IgM (ICN Biochemicals Limited, Eagle House, Bucks, UK)

Control plasma : a pooled sample from 17 dogs which were patients in the Veterinary Hospital of the University Glasgow Veterinary School (unaffected by otitis externa). Zero point four ml of plasma from each animal was harvested from routine clinical biochemistry samples submitted to the Department of Clinical Veterinary Biochemistry.

Coating buffer : 0.035 M sodium bicarbonate and 0.014 M sodium carbonate buffer at pH 9.6.

Phosphate buffered saline Tween (PBST) : 0.002 M sodium di-hydrogen orthophosphate, 0.0075 M di-sodium hydrogen orthophosphate, and 0.15 M sodium chloride at pH 7.4.

The first and secondary antiserum were identical to those described above.

The substrate : 25 ml of 0.1 mol/l acetate buffer at pH 5.5, 400 ul of 0.6% tetramethyl benzidine (TMB), and 100 ul of 1% hydrogen peroxide.

Equipment

An ELISA reader; Titertek Multiskan Plus (Labsystems & Row Laboratories, France). This was connected to a computer (IBM PC) running an ELISA reading programme "Chromo-scan". A shaker (Luckhan Recipro-shake, Luckhan Ltd, Sussex, England) was also utilized in the study.

Procedures

A. Absolute immunoglobulin quantitation

The standard immunoglobulins were series (two-fold) diluted using coating buffer. Two hundred microlitres of each diluted standard immunoglobulin 0.5% (v/v) were added to a microtitre plate in duplicate. Coating buffer was used as the zero standard.

Two hundred microlitres of coating buffer which contained 0.5% (v/v) of prepared sample were added to a microtitre plate in duplicate. The plate was incubated on a shaker, at 4°C, overnight.

The plate was washed using 300 ul PBST per well three times and incubated in 200 ul 2% milk in PBST for 60 minutes. The washing procedure was then repeated. The first antiserum was added using 200 ul of a 1:5000 dilution in PBST and incubated at room temperature for 2 hours, then the washing procedure was repeated. The secondary antiserum was added using 200 ul of a 1:10000 dilution in PBST and incubated at room temperature for 50 minutes The washing procedure was repeated again. Two hundred microlitres of the substrate was added and incubated at 37°C for 15-30 minutes. This was followed by adding 50 ul 2M sulphuric acid to stop the process. The absorbance in the ELISA wells was read at 450 nm on an ELISA plate reader.

B. Composition of canine cerumen immunoglobulins

The standard and the samples were prepared as described above. Coating buffer was used as the zero standard.

Corresponding plasma samples from those dogs with otitis were also diluted (two-fold) with coating buffer. Two hundred microlitres of the diluted plasma sample 0.5% (v/v) were added to the same plate in duplicate.

The control plasma (a pooled sample form 17 dogs unaffected by otitis) was also diluted (two-fold) with coating buffer. Again, 200 ul of diluted control plasma sample 0.5% (v/v) were added to the same plate in duplicate.

The plate was incubated on a shaker, at 4°C, overnight. The remaining steps of this procedure were the same as those described above (4.2.5.A).

4.2.6 Assay validation

Specificity

The specificity of an assay is defined as the ability of a method to determine solely the analyte(s) it purports to measure (Fraser, 1986; Kelley, Kleiss, and Brief, 1988). The specificity of the antisera were investigated using agarose immunoelectrophoresis gels.

The agarose gel was made by dissolving 0.12 g agarose (Type II-A, Sigma Chemical Company Ltd, Poole, Dorset, England) in 12 ml barbituric buffer at pH 8.6 (0.02 M sodium barbituric acid and 0.0043 M barbituric acid). The gel was poured onto Gelbond film (Sigma Chemical Company, Poole Dorset, England). After the gel solidified, wells and troughs were made using a mold.

Twenty microlitres of the pooled plasma standard and 20 ul of 1% bromophenol blue were added to each of the wells in the gel. The electrophoresis tank was filled with barbituric buffer. Electrophoresis was carried out using the apparatus (LKB Multiphor II Horizontal Electrophoresis System, LKB Produkter AB, Sweden) supplied with LKB 2103 Power Supply (LKB Produkter AB, Sweden) at 0.025 KV, 20 mA for about 90 minutes.

One hundred microlitres of the first antiserum (RAD/IgA, RAD/IgG, and RAD/IgM) at 1:10 dilution (with barbituric buffer) were added to each of the troughs in the gel. The gel was incubated at 0°C for 18 hours, and was soaked in saline for another 12 hours. The gel was then compressed between sheets of filter paper and soaked in distiled water for 1 hour. This compression step was repeated.

Finally, the gel was stained using 0.125% coomassie blue (in 50% methanol and 10% acetic acid) for 15 minutes. A destain solution, which contained 7% acetic acid and 5% methanol, was applied for 30 minutes.

Using this technique a single band of antigen-antibody precipitation indicates acceptable specificity of the antiserum.

Limit of detection

The limit of detection of a method is the smallest result which can be distinguished from a true "zero standard" (Fraser, 1986; Kelley *et al*, 1988). In the present study, the zero standard for ELISA tests contained no analyte (Fuentes-Arderiu, 1992; Kelley, Ratliff, and Nenadic, 1992). The limit of detection of the assay was defined as the measured concentration of analyte at 2 standard deviations from the mean of the zero standard.

Accuracy / recovery studies

Accuracy indicates how well results of the assay agree with the exact amount of the analyte in the sample (Fraser, 1986; Kelley *et al*, 1988). The accuracy of the ELISA assay was determined by the recovery of immunoglobulin added to a sample (Fraser, 1986; Kelley *et al*, 1988; Kelly *et al*, 1992). This procedure was carried out by adding known quantities of the analyte (purified immunoglobulin) to an actual sample (cerumen). Five tests were carried out on a single sample; one had nothing added to it; the second, third, fourth, and fifth had known, increasing quantities of the analyte added to them. By measuring the analyte levels in each of these, the percentage recovery of the analyte could thus be determined (Kelley *et al*, 1992).

Precision

The precision of the ELISA assay was determined by calculating the within (intra) assay and between (inter) assay coefficients of variation (McLaren, Lillywhite, and Au, 1981; Fraser, 1986; Kelley *et al*, 1988).

In the present study intra-assay precision was derived by calculating the differences between 10 pairs of results within one assay run for each of the three ELISA assays (IgA, IgG, and IgM). The standard deviation (S.D.) of these differences was calculated as follows (Fraser, 1986):

$$S.D. = \frac{(\text{differences between pairs})^2}{2x10}$$

The mean of these pairs was found and the coefficient of variation (C.V.) was calculated using the formula:

$$C.V. = \frac{S.D.}{Mean} \times \frac{100}{1}$$

These data were calculated for each set of assays.

The inter-assay precision was determined as follows. Two cerumen samples were assayed on five separate occasions and the mean and standard deviation of these results was obtained. The inter-assay coefficient of variation was calculated using the conventional formula shown above.

4.2.7 Statistical methods

The data concerned with immunoglobulin quantitation were stored on a file on a personal computer (Viglen, UK). In the study of the proportion of immunoglobulin classes in the cerumen from healthy ears, paired-T tests were used to examine whether there were statistically significant differences between the left and right ears. In the same study, Mann-Whitney tests (U test) were used to look for significant differences between healthy ears and ears affected by otitis externa Paired-T tests and Mann-Whitney tests were carried out using the Minitab statistical

program. A five percent (5%) significance level was used throughout the study.

Table 4.2.1 Breed, sex and age (years) of 15 dogs with 30 normal ears used for the study of cerumen immunoglobulin composition.

Breed	Sex	age
Bull Terrier	F	2
Bull Terrier	F	5
Bull Terrier	M	2
Bull Terrier	M	5
Bull Terrier	F	3
Springer Spaniel	F	5
Bull Terrier	M	1
Bull Terrier	F	3
Bull Terrier	M	2
Cocker Spaniel	M	3
Springer Spaniel	F	12
Cocker Spaniel	F	8
Bull Terrier	F	3
Bull Terrier	M	5
Setter	M	1

Table 4.2.2 Breed, sex and age (years) of eight dogs with 13 ears affected by otitis externa used for the study of cerumen immunoglobulin composition.

Breed	Sex	Age
Rottweiler	F	4
Yorkshire Terrier	M	9
Fox Terrier	M	9
Rottweiler	M	5
German Shepherd Dog	F	7
German Shepherd Dog	M	5
Cairn Terrier	M	11
German Shepherd Dog	F	6

4.3 RESULTS

4.3.1 Assay validation

Specificity

The result of agarose immunoelectrophoresis yielded a single line of precipitation between the antiserum for each immunoglobulin class (RAD /IgA, RAD/IgG, and RAD/IgM) and the pooled plasma standard in the gel (Figure 4.3.1).

Limit of detection

In the present study, the concentration of analyte 2 S.D. above the mean of the zero standard was less than 0.001% of the pooled plasma standard for each assay.

Accuracy / recovery studies

The percentages of the added immunoglobulin recovered were no less than 84% or greater than 116% for all antibody classes. These results are summarised in Table 4.3.1.

Precision

The coefficients of variation of the intra-assay and inter-assay precision tests on each ELISA assay were less than 20% (Tables 4.3.2, 4.3.3a, 4.3.3b, and 4.3.3c). These results were considered to be acceptable (Kelley *et al*, 1992).

Figure 4.3.1 The results of agarose immunoelectrophoresis of canine immunoglobulins. The single band of each antigen-antibody precipitation is shown.

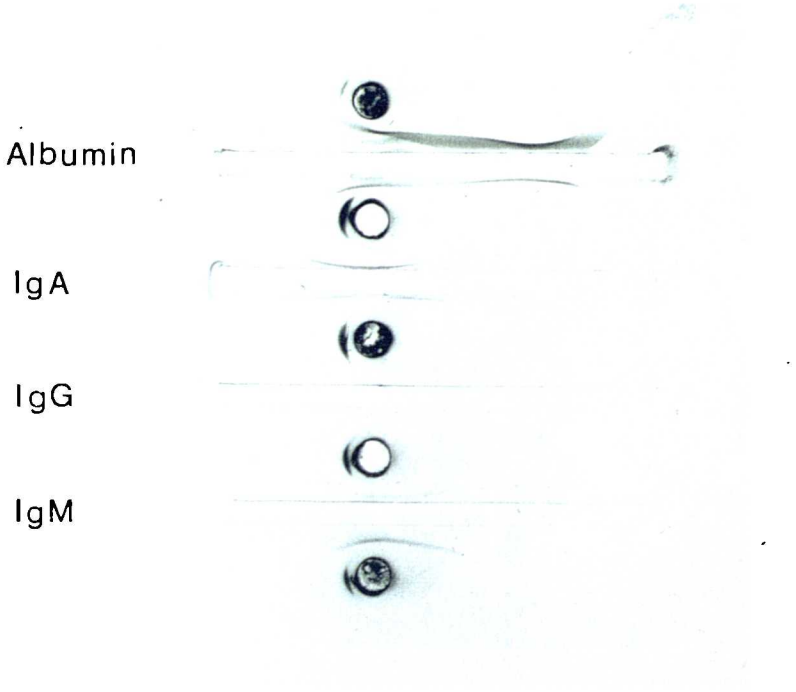


Table 4.3.1 Accuracy / recovery studies. Results of the enzyme-linked immunosorbent assay (ELISA) tests measuring the addition of known quantities of immunoglobulin to cerumen samples.

	Actual concentration added (ug/100ml)	**Recovered concentration** (ug/100ml)	**Recovered Percentage** (%)
IgA			
	0.46	0.39	85
	0.91	1.05	115
	1.92	1.63	84
	3.75	3.51	94
IgG			
	0.44	0.39	89
	0.88	0.79	86
	1.76	1.58	90
	3.51	3.25	93
IgM			
	0.48	0.55	115
	0.97	1.13	116
	1.94	1.75	90
	3.88	4.21	108

Table 4.3.2 The intra-assay coefficient of variation (%) of the enzyme-linked immunosorbent assay (ELISA) assays for immunoglobulins in cerumen.

Assay	Healthy[1] group	Healthy[2] group	Otitic[3] group
IgA	13.26	9.38	4.78
IgG	5.19	6.85	7.39
IgM	7.36	9.12	8.29

[1]Healthy group: absolute immunoglobulin concentration study (see section 4.3.3).

[2]Healthy and [3]otitic groups: cerumen immunoglobulin composition studies (see sections 4.3.4 and 4.3.5).

Table 4.3.3a The inter-assay coefficients of variation (%) of the immunoglobulin A enzyme-linked immunosorbent assay (ELISA) based on two cerumen samples tested five times in separate assay runs.

	Immunoglobulin A (ug/100ml)	
	Sample 1	Sample 2
	1.26	2.16
	2.10	2.64
	1.74	2.16
	1.80	2.10
	1.50	2.22
Mean	1.68	2.26
S.D.	0.32	0.22
C.V.	19.0%	9.7%

S.D. = standard deviation; C.V. = Coefficient of variation

Table 4.3.3b The inter-assay coefficients of variation (%) of the immunoglobulin G enzyme-linked immunosorbent assay (ELISA) based on two cerumen samples tested five times in separate assay runs.

	Immunoglobulin G (ug/100ml)	
	Sample 1	Sample 2
	1.81	2.46
	2.26	2.81
	1.71	3.05
	1.93	2.41
	2.14	2.78
Mean	1.97	2.70
S.D.	0.23	0.27
C.V.	11.2%	10.0%

S.D. = standard deviation; C.V. = Coefficient of variation

Table 4.3.3c The inter-assay coefficients of variation (%) of the immunoglobulin M enzyme-linked immunosorbent assay (ELISA) based on two cerumen samples tested five times in separate assay runs.

	Immunoglobulin M (ug/100ml)	
	Sample 1	Sample 2
	0.22	0.34
	0.25	0.40
	0.28	0.35
	0.23	0.33
	0.28	0.39
Mean	0.25	0.36
S.D.	0.03	0.03
C.V.	12.0%	8.3%

S.D. = standard deviation; C.V. = Coefficient of variation

4.3.2 Identification of canine cerumen immunoglobulin(s)

Immunoglobulin A, IgG, and IgM were identified in canine cerumen samples using SDS-PAGE and Western blotting (Figures 4.3.2, 4.3.3a, 4.3.3b, 4.3.3c).

4.3.3 Canine cerumen immunoglobulin quantitation

Eight cerumen samples from eight healthy ears of four Greyhounds were investigated for the presence of immunoglobulin classes. The concentration of IgG was the highest among these immunoglobulins. The mean concentration of IgG was 2.42 ug/100ml (S.D. = 0.16). The mean concentrations of IgA and IgM were 1.56 ug/100ml (S.D. = 0.68) and 0.36 ug/100ml (S.D. = 0.22) respectively (Table 4.3.4).

The proportion of these immunoglobulin classes in cerumen immunoglobulin showed marked variation between samples (Table 4.3.5). The mean proportion of IgA, IgG, and IgM of these eight cerumen samples were 34.8%, 57.3% and 7.8% respectively.

4.3.4 Cerumen immunoglobulin composition in samples from healthy canine ears

Difficulties encountered in the collection of samples and the viscid nature of cerumen meant that only a tiny amount of each sample (less than 100 ug) was obtained using microbiological swabs, these were too small to be divided into the desired weight for accurate protein quantitation. Thus, for these 30 cerumen samples from healthy ears, valid quantitation of protein content on an absolute scale could not be accomplished. Instead the immunoglobulin composition was studied. However, immunoglobulin composition in the present study included IgA, IgG, and IgM only. The data which indicate the proportion of the different

immunoglobulin classes in each cerumen sample are presented in Table 4.3.6.

Marked variation between individual samples was noticed (Table 4.3.6). The mean proportions of IgA, IgG, and IgM in this group (n=30) were 38.4%, 53.9%, and 7.6% respectively. Although a marked variation of the proportions of immunoglobulin was noticed in these cerumen samples from healthy ears, no statistical difference was found between the right and left ears for the three different immunoglobulin classes (IgA, P=0.41; IgG, P=0.55, IgM, P=0.39).

No statistically significant differences were found between the two healthy groups of cerumen samples in the relative abundance of these three immunoglobulin classes (n=8, section 4.3.3 above and n=30, section 4.3.4) (IgA, P=0.90, IgG, P=0.98, IgM, P=0.71).

4.3.5 Cerumen immunoglobulin composition in samples from otitic canine ears

The proportion of IgA, IgG, and IgM in 13 cerumen samples from 13 ears affected by otitis externa also showed marked variation between individual samples (Table 4.3.7). The mean proportions of IgA, IgG, and IgM were 7.4%, 82.6%, and 9.9% respectively.

The proportion of IgA in these cerumen samples from ears affected by otitis externa was significantly lower than in those cerumen samples from ears which were normal (sections 4.3.3 and 4.3.4) ($P<0.005$); whilst, the fraction of IgG in cerumen samples from otitic ears was significantly greater than in those from normal dogs. However, the proportion of IgM in these samples from otitic ears was not significantly different from the proportion of IgM in cerumen samples from healthy dogs (P=0.78).

The data concerning with immunoglobulin composition are summarised in Tables 4.3.8a, 4.3.8b, and 4.3.8c.

4.3.6 The concentration of immunoglobulin in the pooled control plasma

The concentration IgA, IgG, and IgM of the pooled plasma control (n=17) was measured. The concentrations of each immunoglobulin were: IgA, 117 mg/100 ml; IgG, 1143 mg/100 ml; and IgM 178 mg/100 ml. Thus the proportion of these immunoglobulins in the pooled control plasma was: IgA, 8.1%; IgG, 79.4%; and IgM, 12.3%.

4.3.7 Immunoglobulin composition in the plasma of dogs with otitis

The immunoglobulin compositions of plasma samples (n=6) corresponding to dogs with otitis for which cerumen samples were investigated, were also assayed. The mean concentration of IgA, IgG, and IgM in these plasma samples was 18 mg/100 ml, 2164 mg/100 ml, and 271 mg/100 ml respectively. Thus the proportion of these immunoglobulins was: IgA, 0.7%; IgG, 88.3%; and IgM, 10.8%. The concentration of these plasma immunoglobulins are presented in Table 4.3.9. No statistical evaluation was made of this information because of the small size of the data set.

Figure 4.3.2 The cerumen proteins revealed by sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Figures shown are molecular weights of the proteins (kilodaltons).

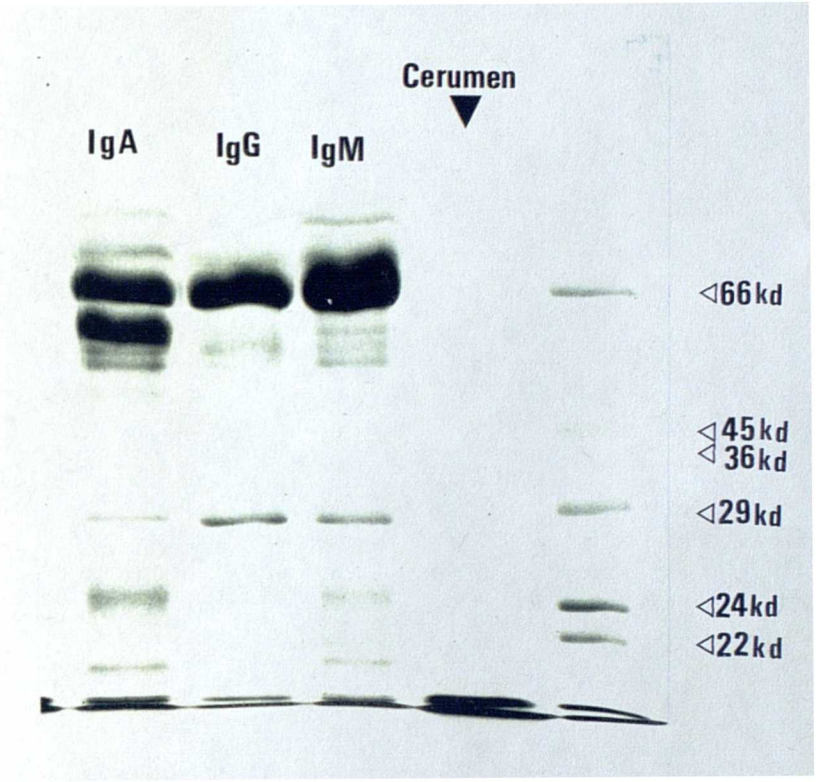


Figure 4.3.3a The identification of immunoglobulin A in canine cerumen using sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and Western-blotting. Figures shown are molecular weights of the proteins (kilodalton).

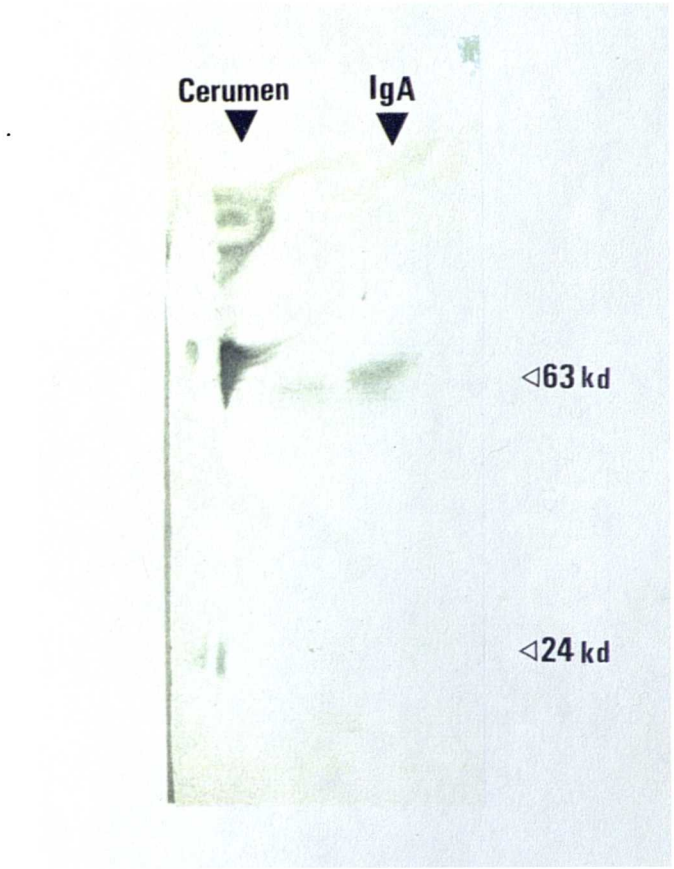


Figure 4.3.3b The identification of immunoglobulin G in canine cerumen using sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and Western blotting. Figures shown are molecular weights of the proteins (kilodalton).

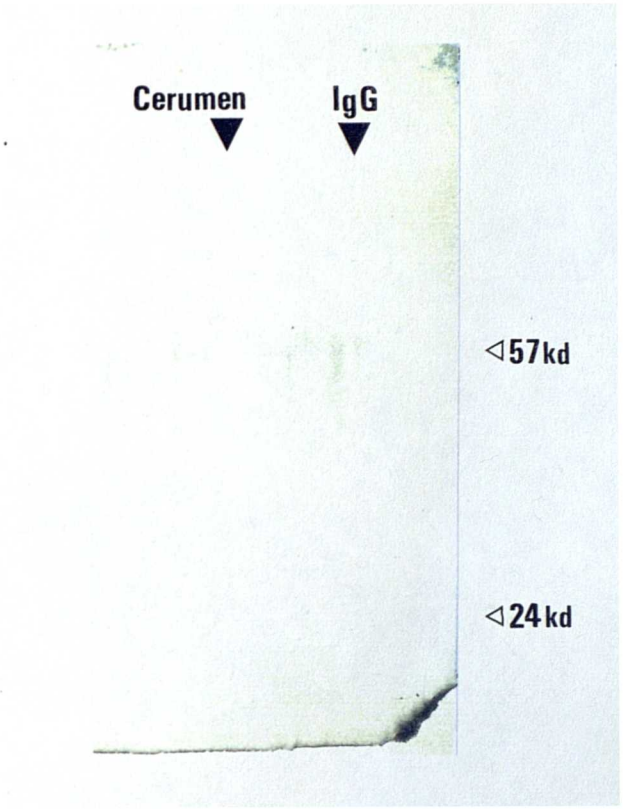


Figure 4.3.3c The identification of immunoglobulin M in canine cerumen using sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and Western blotting. Figures shown are molecular weights of the proteins (kilodalton).

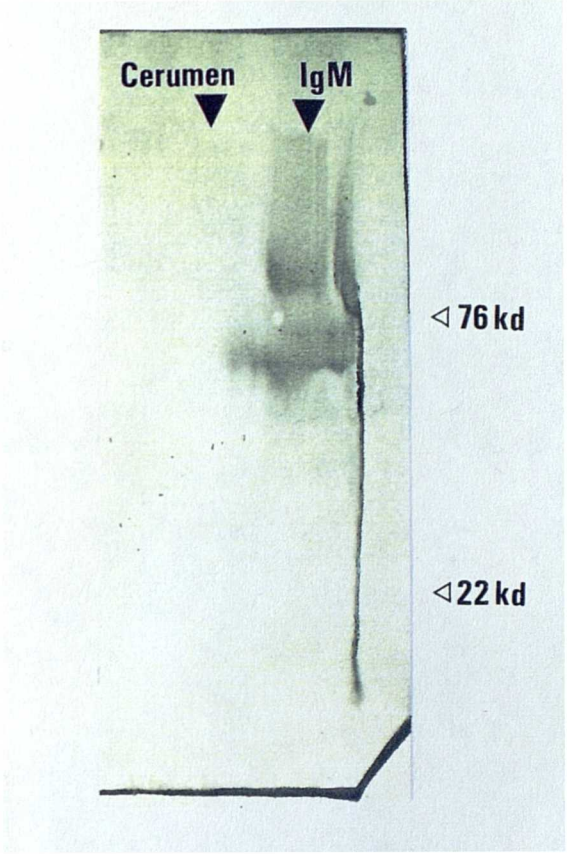


Table 4.3.4 The results from immunoglobulin quantitation of eight cerumen samples from healthy canine ears (ug/100 ml).

Ear	IgA	IgG	IgM
1	0.98	2.38	0.45
2	1.43	2.33	0.10
3	1.23	2.55	0.40
4	1.03	2.30	0.05
5	2.85	2.38	0.50
6	2.38	2.58	0.45
7	1.13	2.20	0.20
8	1.50	2.68	0.70
Mean	1.56	2.42	0.36
S.D.	0.68	0.16	0.22
Median	1.33	2.37	0.43

Table 4.3.5 The immunoglobulin composition calculated from Table 4.3.4 (% by weight).

Ear	IgA	IgG	IgM
1	25.6	62.5	11.8
2	37.0	60.3	2.5
3	29.3	61.0	9.5
4	30.3	68.1	1.4
5	49.7	41.4	8.7
6	43.9	47.6	8.3
7	31.9	62.4	5.6
8	30.7	54.8	14.3

Data are not normally distributed.

Table 4.3.6 The cerumen immunoglobulin composition of 30 samples from 30 healthy canine ears (% by weight).

Ear	IgA	IgG	IgM
1	14.2	79.6	6.1
2	33.4	63.5	3.0
3	24.6	70.9	4.4
4	47.1	47.8	4.9
5	24.4	65.3	10.1
6	67.3	29.5	3.0
7	16.5	76.4	6.9
8	44.6	52.1	3.2
9	25.7	65.5	8.7
10	5.5	81.7	12.6
11	36.4	54.9	8.5
12	58.5	35.8	5.5
13	62.5	31.7	5.6
14	23.8	67.5	8.5
15	37.3	47.7	14.8
16	3.3	92.7	3.8
17	43.8	51.5	4.6
18	5.3	87.5	7.1
19	34.2	61.0	4.7
20	30.0	58.9	10.9
21	68.0	25.3	6.5
22	45.4	49.4	5.1
23	55.0	37.2	7.7
24	85.0	7.7	7.2
25	7.4	74.9	17.4
26	86.2	6.7	6.9
27	7.6	74.9	17.4
28	69.8	24.1	5.9
29	62.7	29.2	7.9
30	26.0	64.9	9.0

Data are not normally distributed.

Table 4.3.7 The cerumen immunoglobulin composition of 13 samples from 13 otitic canine ears (% by weight).

Ear	IgA	IgG	IgM
1	6.7	81.1	12.1
2	1.9	94.1	3.8
3	2.9	91.5	5.4
4	2.0	90.7	7.2
5	3.0	88.9	8.0
6	2.3	94.2	3.4
7	8.1	82.4	9.4
8	8.1	79.5	12.3
9	1.3	98.3	0.3
10	2.2	90.5	7.1
11	8.2	84.4	7.3
12	44.3	10.1	45.5
13	5.1	87.8	6.9

Data are not normally distributed.

Table 4.3.8a Descriptive statistics showing the proportion of immunoglobulin A as a percentage of the total cerumen immunoglobulin from the data in Tables 4.3.5, 4.3.6, and 4.3.7 (% by weight).

	[1]Normal group (n=8)	[2]Normal group (n=30)	[3]Otitic group (n=13)
Mean	34.8	38.4	7.4
S.D.	8.2	23.9	11.4
Median	31.3	35.3	3.0
Q1	29.6	22.0	2.1
Q3	42.2	59.5	8.1

Q1 = the 25th percentile, Q3 = the 75th percentile

[1]Normal group: absolute immunoglobulin concentration study (see section 4.3.3).

[2]Normal and [3]otitic groups: cerumen immunoglobulin composition studies (see sections 4.3.4 and 4.3.5).

Table 4.3.8b Descriptive statistics showing the proportion of immunoglobulin G as a percentage of the total cerumen immunoglobulin from the data in Tables 4.3.5, 4.3.6, and 4.3.7 (% by weight).

	[1]Normal group (n=8)	[2]Normal group (n=30)	[3]Otitic group (n=13)
Mean	57.3	53.9	82.6
S.D.	8.8	22.7	22.4
Median	60.7	56.9	88.9
Q1	49.4	34.8	4.6
Q3	62.4	71.9	10.7

Q1 = the 25th percentile, Q3 = the 75th percentile

[1]Normal group: absolute immunoglobulin concentration study (see section 4.3.3).

[2]Normal and [3]otitic groups: cerumen immunoglobulin composition studies (see sections 4.3.4 and 4.3.5).

Table 4.3.8c Descriptive statistics showing the proportion of immunoglobulin M as a percentage of the total cerumen immunoglobulin from the data in Tables 4.3.5, 4.3.6, and 4.3.7 (% by weight).

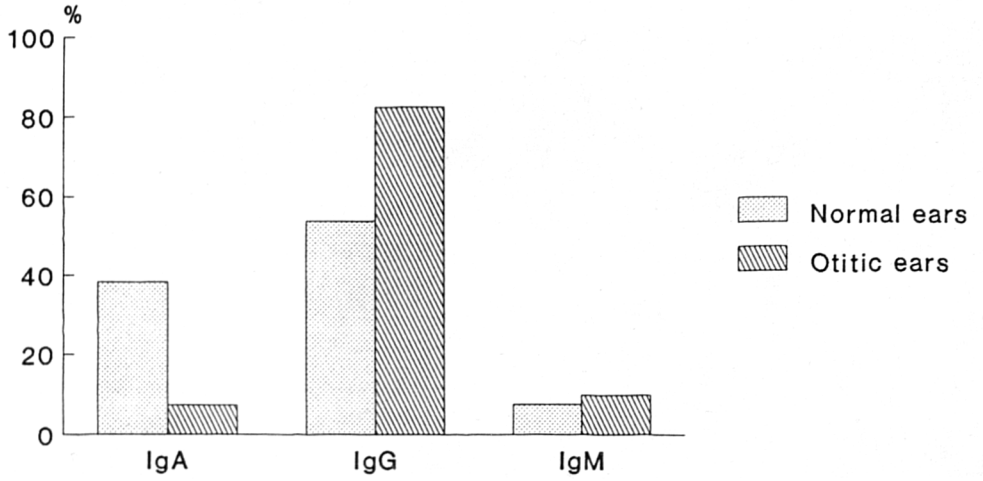
	[1]Normal group (n=8)	[2]Normal group (n=30)	[3]Otitic group (n=13)
Mean	7.8	7.6	9.9
S.D.	4.3	3.8	11.1
Median	8.5	6.9	7.2
Q1	3.3	4.9	4.6
Q3	11.2	8.8	10.7

Q1 = the 25th percentile, Q3 = the 75th percentile

[1]Normal group: absolute immunoglobulin concentration study (see section 4.3.3).

[2]Normal and [3]otitic groups: cerumen immunoglobulin composition studies (see sections 4.3.4 and 4.3.5).

Figure 4.3.4 Comparison between the mean proportions of immunoglobulin A, immunoglobulin G, and immunoglobulin M in cerumen from healthy canine ears and those with canine otitis externa.



The results for normal ears are pooled data from sections 4.3.3 and 4.3.4.

Table 4.3.9 The concentration of immunoglobulins in plasma (mg/100 ml) from 6 dogs affected by otitis externa and the corresponding ears for which data are tabulated in Table 4.3.7.

Ear	IgA	IgG	IgM
2, 3	13	1463	170
4, 5	15	1792	233
6, 7	14	1901	193
8	11	2084	233
9, 10	26	2633	393
12, 13	30	3108	404
Mean	18	2164	271
Proportion (mean, %)	0.7	88.3	10.8
Pooled control plasma	117	1143	178
proportion (%)	8.1	79.4	12.3

4.4 DISCUSSION

In the present study, IgA, IgG, and IgM have been demonstrated in canine cerumen. These immunoglobulins have also been found on the skin surface of humans and cattle and in human cerumen (Page and Remington, 1967; Petrakis *et al*, 1971; Williams and Gibbons. 1972; Forstrom *et al*, 1975; Lloyd, Mabon, and McEwan Jenkinson, 1977). These immunoglobulins are thought to be secreted by the glandular tissues of the aural integument (Main and Lim, 1976). It has been shown that apocrine and sebaceous glands in canine skin contain immunoglobulins (Halliwell, 1973; Garthwaite *et al*, 1983). Based on the histological features of the integument of the canine ear canal, the aural skin is regarded as a modified part of the skin which covers the rest of the body surface (Fraser, 1961a). No essential difference has been found between the apocrine and sebaceous glands distributed in different parts of the canine skin by histochemical staining (Nielsen, 1953) so that the apocrine and sebaceous glands of the aural integument may be capable of secreting these immunoglobulins into cerumen. On the other hand, no studies of the aural integument appear to have been published to support this proposed mechanism.

In the present study, marked variation in the proportion of canine cerumen IgA, IgG, and IgM was found in individual samples from healthy ears. This result could be due to the marked variation in the distribution of glands in the aural integument (see Chapter II). However, IgG was the predominant immunoglobulin in canine cerumen from healthy ears. A similar finding has been reported in human sweat, although the concentration of canine cerumen IgG (mean 2.42 ug/ 100 ml) was strikingly low comparing to that in human sweat (mean 92 ug/100 ml) (Page and Remington, 1967). The major antibody found in bovine skin

washings however, was IgA (Lloyd and McEwan Jenkinson, 1981). In the present study, the average proportion of IgA in cerumen from healthy ears was 37.6% (pooled data from two healthy groups). The proportion of IgA in cerumen was greater than that in pooled control plasma (8.1%). This elevation in the IgA fraction in canine cerumen compared to the plasma suggests that IgA may have an important role to play in cutaneous host defence. Page and Remington (1967) have reported similar results for human sweat.

The proportions of IgA and IgG in cerumen from otitic ears differed significantly from healthy ones. The proportion of IgA was significantly decreased in cerumen from inflamed ears; whilst the fraction of IgG was significantly raised in the otitic group. As in the healthy ear however IgG was the abundant antibody on the aural surface when it was affected by otitis externa. In ears affected by otitis externa, pathological changes to the secretory organs of the aural integument (Chapter III) may alter the composition of cerumen as well as the ratios between immunoglobulins. Changes in cerumen immunoglobulin ratios could also be caused by leakage of immunoglobulins through transudation or exudation due to increased permeability of the blood vessels or direct damage to the integument. Cutaneous inflammation usually causes changes in blood vessel permeability (Mason and Lloyd, 1990).

It has been reported by Lloyd and McEwan Jenkinson (1981) and Lloyd *et al* (1987) that an increased concentration of IgG2 was found in cattle vaccinated intradermally with *Dermatophilus congolensis*. These workers have also reported that antibodies of IgA, IgG1, and IgM specific to *D. congolensis* were increased on the skin surface. In human otitis media, the middle ear effusion has been found to contain an increased level of IgG (Sorensen and Nielsen, 1988). Increased IgG concentration has been documented in faecal samples from adult pigs affected by

diarrhoea due to swine dysentery (Elazhary, Tremblay, Lagace, and Roy, 1973; Franz and Corthier, 1981). The main functions of IgG are to promote lysis of bacteria, to opsonize bacteria by activating the complement system, and to neutralize toxins (Stokes and Bourne, 1989). In the present study, over 80% of the otitic ears were affected by bacterial overgrowth, and the proportion of IgG in cerumen from these otitic ears was significantly greater than in cerumen from normal ears. This finding suggests that IgG plays a role in the defence of the canine aural integument against microbial overgrowth.

The ratios of IgA : IgG : IgM in canine faeces have been reported to be 3.4:5:1(Reynolds and Johnson, 1970). The ratios of IgA- : IgG- : IgM-coated cells in canine colonic mucosa is about 2.2:1.6:1.0 (Roth, Walton, and Leib, 1992). Similar data have been reported by Hart (1973). These values differ from the ratios of immunoglobulins in canine serum (0.8:7.4:1.0) (Halliwell and Gorman, 1989), and indicate that this mucosal surface has its own secretory immunity. Studies of IgA quantitation in tears and serum from dogs also indicated that a low IgA concentration in serum was not associated with a significant decrease in the local IgA level (Ginel, Novales, Lozano, Molleda, and Lopez, 1993). In the present study, there are differences in the fractions of IgA, IgG, and IgM in cerumen from normal ears compared to the pooled standard plasma. Moreover, the fractions of IgA, IgG, and IgM in cerumen from otitic ears and plasma samples from the same animals differ substantially. These findings suggest that the aural skin has its own secretory immune system and that this system is at least in part responsible for local defence.

One of the most important functions of IgA on the skin is to inhibit microbial adherence to the epidermis, thus it can limit the colonization of the skin surface by microorganisms (Williams and Gibbons, 1972; Stenfors and Raisanen, 1991b). In human suppurative otitis media associated with

Pseudomonas infection, a large amount of immunoglobulin-coated *Staphylococcus aureus* was found in the middle ear effusion, whilst immunoglobulin-coated *Pseudomonas aeruginosa* was only present in small numbers. This finding strongly suggests that immunoglobulin-coating of bacteria plays a key role in the eradication of bacterial infection associated with otitis media (Stenfors and Raisanen, 1991a, 1991b, 1992). In the present study, 80% of ears affected by otitis externa were found to be associated with overgrowth by pathogenic bacteria. A strikingly lower fraction of IgA was found in both cerumen and the corresponding plasma from dogs affected by otitis externa. A low fraction of IgA in the cerumen of dogs with otitis compared to normal individuals might be a factor in canine otitis externa associated with bacterial overgrowth. However, low plasma IgA level might not indicate a true immunodeficiency state (Day and Penhale, 1988; Ginel *et al*, 1993). Low plasma IgA could be related to either decreased production or increased removal of IgA from the blood (Whitbread, Batt, and Garthwaite, 1984; Moroff *et al*, 1986; Day and Penhale, 1988).

In the present study, increased IgG levels were found in cerumen samples from ears affected by otitis externa. This might be a compensation for a lowered IgA fraction (Whitbread *et al*, 1984). The precise role of canine cerumen immunoglobulins in regulating resident and pathogenic aural flora deserves more study exploring specific immunoglobulins directed against particular bacteria or fungi. The prevention of aural infection by vaccination might thus become theoretically possible.

Immunoglobulins are the first specific defence mechanism of the skin surface (Stenfors and Raisanen, 1991a). The epidermis also represents a component of this local immune system. Keratinocytes of the epidermis prevent microorganisms or toxins from freely penetrating

tissues. They are also immunocompetent (Baadsgaard and Wang, 1991). Cellular immunity in the epidermis has been shown to play a key role against dermatophytosis in human skin (Tagami, Kudoh, and Takematsu, 1989). The defence mechanism relies on all members of this system. If the secretory immunity of the aural integument is defective this may be balanced by other parts of the local cutaneous mechanisms against microbial invasion. Vigorous cellular epidermal inflammation in the face of bacterial infections might compensate for defects in local humoral immunity. However, neutrophilic migration induced by chemotaxis during the inflammatory process may enhance epidermal proliferation (Tagami *et*, 1989). Significantly higher levels of certain cytokines of keratinocytes have been found to be associated with the skin of patients affected by proliferative dermatitis (McKenzie and Sauder, 1990). In the present study, a lower fraction of IgA in cerumen samples from ears affected by otitis externa was found. Deficiency in the secretory immunity of the aural integument might be responsible for the epidermal hyperplasia found in otitic ears. A study addressing specific immunity in the canine external ear canal might contribute to our understanding of the pathogenesis of otitis externa. Knowledge of the interactions between aural immune regulators and their relevance to microorganisms might help clinicians to prevent and control otitis externa.

CHAPTER V

CANINE CERUMEN LIPID ANALYSIS

5.1 INTRODUCTION

It has been postulated that lipids in human cerumen may act as a mechanical and chemical barrier and prevent microorganisms from penetrating the upper layers of the aural skin (Baumann *et al*, 1961). By analogy it seems likely that lipids in canine cerumen may fulfill similar biological functions. Deficiency in these lipids has been thought to be a factor contributing to the pathogenesis of certain types of external otitis in humans (Senturia, 1950; Sophian and Senturia, 1955).

A knowledge of the composition of cerumen lipids is required if the relationship between the physiological role of cerumen and otitis externa is to be understood (Senturia, 1950; Senturia *et al*, 1955; Chiang, Lowry, and Senturia, 1957).

Lipids make up the largest component of human cerumen (Bortz *et al*, 1990). It has been reported that lipids account for twice the weight of the protein in human cerumen (Berzelius, cited by Petrequin, 1869). The reported lipid content of human cerumen varies from about 20% (Nakashima, 1933; Akobjanoff, Carruthers, and Senturia, 1954; Kataura and Kataura, 1967; Gershbein, Broder, and Sheladia, 1980) to 67% or more of crude weight (Chiang, Lowry, and Senturia, 1955, Chiang *et al*, 1957). No significant differences in the lipid content by sex (Chiang *et al*, 1955; Cipriani, Taborelli, Gaddia, Melagrana, and Rebora, 1990) or between the left and right ears have been found (Cipriani *et al*, 1990). A small variation in lipid content with age has been reported; according to this observation (Chiang *et al*, 1955), adults have a slightly lower average lipid content than children because of ageing effects on the activities of the sebaceous glands (Yamamoto, Serizawa, Ito, and Sato, 1987). Chiang *et al* (1957) have reported that in humans significant differences were found between the lipid content of fresh cerumen samples and those samples

which had been present in the ear canal for more than 24 hours (casual cerumen). The average lipid content of casual cerumen samples was 36.8%, with a range of 13% to 64%, compared to an average lipid content of 67% in fresh human cerumen samples.

The composition of human cerumen lipid has been determined in several studies. Fatty acids and cholesterol are generally agreed to be the major lipids by most researchers (Nakashima, 1933; Chiang *et al*, 1956; Gershbein *et al*, 1980; Bortz *et al*, 1990; Okuda *et al*, 1991). However, the proportion of cholesterol in human cerumen showed a great variation in these studies; it varied from as low as 9% to as high as 86% of the lipid. The free fatty acid fraction comprised about a quarter of the cerumen lipid (Nakashima, 1933; Chiang *et al*, 1956; Gershbein *et al*, 1980; Bortz *et al*, 1990; Okuda *et al*, 1991). Other lipids, such as ceramides, squalene, cholesterol esters, wax esters, triglycerides, and phospholipids have also been identified in human cerumen (Akobjanoff *et al*, 1954; Bortz *et al*, 1990; Okuda *et al*, 1991).

Using more sophisticated techniques, the lipid composition has been analyzed in greater detail. Human cerumen fatty acids contain 12 to 26 carbon atoms and are straight, branched, saturated or unsaturated (Haahti, Nikkari, and Koshinen, 1960; Kataura and Kataura, 1967; Gershbein *et al*, 1980; Harvey, 1989; Osborne and Baty, 1990). Fatty acids with chain lengths of 14, 16, 18, and 20 carbon atoms are the major fatty acids in human cerumen (Haahti *et al*, 1960; Kataura and Kataura, 1967; Harvey, 1989; Osborne and Baty, 1990). The presence of these fatty acids appeared to be very variable. The percentage (w/w) of fatty acids was also spread widely; C14:0 comprised from less than 1% to 4%; C16:0 varied from 1% to 25%; and C18:0 varied between 6% and 25%. C18:1 comprised from 1% to more than 25% whilst the percentage of C18:2 varied between 4% to 20%. C20:4 comprised from less than 1% to

more than 18% of human cerumen fatty acids (Kataura and Kataura, 1967; Harvey, 1989; Osborne and Baty, 1990). Moreover, a marked variation in the content of other fatty acids which form a minor proportion of human cerumen fatty acid was also found in these studies (Haahti *et al*, 1960; Harvey, 1989).

These strikingly heterogeneous results could be due to differences in the extraction procedures employed; various chloroform - methanol mixtures , and ethyl acetate were used as extracting solvents in these different studies (Harvey, 1989; Bortz *et al*, 1990; Osborne and Baty, 1990). Different derivatisation methods for analysis may also have caused variation in the results; transesterification with HCl in methanol, methylation with diazomethane, saponification in triethylene glycol, and other methods were used for the preparation of either fatty acids or their methyl esters (Haahti *et al*, 1960; Osborne and Baty, 1990; Okuda *et al*, 1991). The apparatus for analysis and its limitations may also affect the results; thin layer chromatography, gas liquid chromatography, infrared spectroscopy, and gas chromatography - mass spectroscopy were employed in producing these various data (Haahti *et al*, 1960; Kataura and Kataura, 1967; Harvey, 1989; Bortz *et al*, 1990; Okuda *et al*, 1991). However, even with these possible sources of variation due to methodology, it seems likely that cerumen shows a remarkably variable fatty acid composition.

The lipid components are essential to several physiological functions of human cerumen in the external auditory meatus. The nature of lipids gives the aural skin an hydrophobic protection (Nikkari, 1974). The antimicrobial activities of human cerumen are thought to be related to these hydrophobic properties (Chai and Chai, 1980). It has also been proposed that the fatty acids of human cerumen are mainly responsible for its bactericidal activity (Burtenshaw, 1942; Baumann *et al*, 1961). It is known that some polyunsaturated fatty acids are antibacterial and

cytotoxic (Gutteridge, Lamport, and Dormandy, 1974; Knapp and Melly, 1986); polyunsaturated fatty acids with a chain length of 18 carbon atoms (Butcher, King, and Dyke, 1976; Gutteridge, Lamport, and Dormandy, 1976; Lacey and Lord, 1981), are regarded as self-disinfectant on both the human skin (Burtenshaw, 1942; Kanai and Kondo, 1979) and human aural skin (Burtenshaw, 1938, Burtenshaw, 1942). Other biological functions of fatty acids, such as linoleic acid, linolenic acid, and arachidonic acid are to act as precursors of pro-inflammatory and immunoregulating mediators (Wilkinson, 1972; Chapkin, Ziboh, Marcelo, and Voorhees, 1986). Linoleic acid and arachidonic acid have also been shown to restore impaired barrier function of skin by topical and systemic administration (Prottey, 1976; Prottey, 1977; Elias, Brown, and Ziboh, 1980). Their ability to regulate barrier function indicates that these fatty acids may modulate inflammatory processes which require the production of prostaglandins (Goldyne, 1975; Fogh, Herlin, and Kragballe, 1989).

The studies of Kellum (1968) and Kligman, Wheatley, and Mills (1970) have shown that short-chain fatty acids with chain lengths from 2 to 12 carbon atoms, are highly irritant and comedogenic to human skin. Human body odour has been assumed to be the odour of short-chain fatty acids released by the metabolism of skin surface lipids by cutaneous bacteria (Shelley, Hurley, and Nichols, 1953; Lukacs *et al*, 1991).

The importance of human skin surface lipid as a nutrient reservoir is shown by a significant correlation between the populations of cutaneous propionibacteria and the total lipid on the skin surface (McGinley *et al*, 1980; Leyden and McGinley, 1982; Bergbrant and Faergemann, 1989; Holland, 1993). The expression of lipolytic activities by the resident flora on human skin (Reisner, Silver, Puhvel, and Sternberg, 1968; Smith and Willett, 1968) may be essential to allow the use of skin lipid as a nutrient.

Free fatty acids found in human skin lipids may be produced by bacterial lipases (Scheimann, Knox, Sher, and Rothman, 1960).

Because of the complicated roles postulated for human cerumen fatty acids, differences between normal and diseased ears have been studied (Osborne and Baty, 1990). These authors found a slightly lower ratio of total saturated fatty acid to unsaturated fatty acid in cerumen samples from otitic ears, but the difference was not significant. Whether the changes in cerumen lipid composition were due to the inflammatory process (Ruzicka, Simmet, Peskar, and Ring, 1986), microorganisms (Scheimann *et al*, 1960), or pathogenic changes in the glands which are the source of these (Fernando, 1966; Fernando, 1967; Greene *et al*, 1970) (see Chapter III), remains unclear.

The author of this thesis is not aware of any extensive publications contributing to knowledge concerning canine cerumen lipids. The functions that canine cerumen lipids fulfill in the physiology of the external ear of the dog are unknown. The role these lipids play in canine otitis externa is also not understood in spite of the widespread occurrence of this condition (see Chapter I).

The aim of this study was to explore canine cerumen lipid composition and its relationships to canine otitis externa.

Lipids are a chemically heterogeneous group of substances having in common the property of insolublity in water, but solubility in non-polar solvents, such as chloroform and methanol (Gurr and Harwood, 1991). The first step in these analyses is the isolation of lipid from tissue by extraction with non-polar solvents, and the removal of non-lipid materials from these extracts (Christie, 1982). Single classes of lipid, such as triacylglycerols, cholesterol, cholesterol esters, wax esters etc., can be isolated from thin layer chromatography (T.L.C.) which differentiate lipids according to the polarity of the constituent parts. These lipids can then be

identified by their chromatographic behaviour relative to that of authentic standards and by the use of specific spray reagents (Christie, 1982).

Free fatty acids are the products of lipolytic activity from bacteria or yeasts. By the action of lipases, these microorganisms are capable of utilising triglycerides in the skin surface emulsion, thus liberating free fatty acids (Kellum, 1968; Reisner *et al*, 1968; Kligman *et al*, 1970; Weary, 1970; Cove *et al*, 1980; McGinley *et al*, 1980; Lukacs *et al*, 1991).

Gas lipid chromatography (G.L.C.) is used to determine the fatty acid composition of the total lipids in the mixture. The lipids are first converted to their methyl ester derivatives by an appropriate procedure for G.L.C. analysis.

Combined gas chromatography - mass spectrum (G.C. - M.S.) provides accurate molecular weights of, and allows the detection of double bonds in, the fatty acids. It can also allow identification of other functional groups (Gurr and Harwood, 1991). The criteria for the identification of saturated fatty acids are characterised by the molecular weight of the parent ion which gives the molecular weight of the fatty acid, together with presence of the peaks with the mass of the parent ion minus 31 and parent ion minus 43 (M-31 and M-43) which are characteristic for the loss of a methyl group ($-OCH_3$) from the methyl ester and the loss, by a complicated rearrangement, of the second, third and fourth carbon atoms (-C2, -C3, and -C4) of the fatty acid chain respectively. In addition, the peaks at mass/charge ratio (M/e) = 74 and M/e = 87 are characteristic ions produced during fragmentation of fatty acid methyl esters (McLafferty, 1959).

5.2 MATERIALS AND METHODS

5.2.1 Source of canine cerumen samples

Cerumen samples were collected from both healthy canine ears and ears with otitis externa. Samples were obtained from dogs provided by a local kennel, or referred to the Department of Veterinary Medicine, University of Glasgow Veterinary School for different illnesses, but without a history or signs of any ear disease, and this material was deemed to be healthy cerumen. Cerumen samples from dogs which were referred to the Department of Veterinary Surgery, for investigation and treatment of chronic and recurrent otitis externa were deemed to be otitic cerumen samples.

5.2.2 Sample collection

Cerumen samples were collected using sterile microbiological swabs (BioTrace Limited, Mid Glamorgan, Bridgend) from external ear canals. These samples were stored at -20°C until analysed.

5.2.3. The percentage lipid content in cerumen samples

Thirty six cerumen samples from 20 dogs with normal ears and 15 cerumen samples from 8 dogs with otitis externa were investigated. Breed, sex, and age of these dogs are summarised in tables 5.2.1. and 5.2.2. The cerumen samples were weighed and lipid was extracted by the method of Folch *et al* (1957) by placing the sample in a stoppered tube with 3 ml chloroform : methanol 2:1 (v/v). The sample was vigorously shaken with the extracting solvent and then the extract was filtered though a filter paper. The extraction and filtration steps were repeated three times on the residue. The combined extracts were dried under a stream of

nitrogen at 40°C in a fume cupboard. The lipid residue was then weighed so that the lipid content of the cerumen sample could be calculated.

5.2.4 Thin layer chromatography (T. L. C.)

Materials

Sixteen cerumen samples from the 11 dogs with normal ears were examined by T.L.C. Breed, sex and age of these 11 dogs are summarised in table 5.2.3. The lipid residue was dissolved in 0.2 ml chloroform.
The T.L.C. plates were prepared using Silica Gel H60 (Merck, Damstadt, Germany) (See below procedure 1).

Standard lipids : cholesterol, cholesterol esters, oleic acid, monoglyceride, diglyceride, triglyceride, phosphatidyl choline, phosphatidyl ethanolamine, lecithin, and sphinomyelin were obtained from Sigma Chemical Company (Poole, Dorset, England). Oleoyl stearate was synthesised in the Department of Biochemistry, University of Glasgow.

Developing solvents:

Standard solvent: a mixture of 150 ml petroleum spirit (boiling point: 40-60°C), 50 ml diethyl ether , and 1 ml acetic acid was prepared. This solvent was used to separate waxes, cholesterol and its esters, triglycerides, and fatty acids.

Polar solvent: a mixture of 130 ml chloroform, 50 ml methanol, and 8 ml distiled water was prepared. This solvent was used for separation of phospholipids.

The T.L.C. solvent tank

Stains: iodine and 0.4% ninhydrin (v/v) in n-butanol spray

Procedures

1. Preparation of thin layer chromatography plates

Thirty grams of silica gel and 60 ml distiled water were mixed thoroughly and the suspension was spread evenly onto 6 glass plates at a thickness of 0.5 mm. The plates were kept at room temperature for 1 hour to allow setting and then heated at 105°C for at least 30 minutes to dry.

2. Loading of cerumen lipid and the standards

One hundred microlitres of a solution of cerumen lipid dissolved in 0.2 ml chloroform and 100 ul of solutions of the standards (about 0.1% v/v) were placed along an horizontal line 3 cm above the bottom of the plate.

3. Developing

The loaded plate was developed in a T.L.C. tank with the relevant solvents at room temperature for 60-90 minutes. The plate was then removed and dried in air in a fume cupboard.

4. Staining

The lipids were located by placing the plate in an iodine tank for 15 minutes. Lipids appeared as brown spots on the plates. Plates developed using the polar solvent were then stained with the 0.4% ninhydrin (v/v) in n-butanol spray to detect the presence of amino-phospholipids. After spraying, the plates were heated at 105°C until spots of purple-red colour appeared.

5.2.5 Gas liquid chromatography (G. L. C.)

Materials

Thirty cerumen samples from 15 dogs with healthy ears and 12 cerumen samples from 8 dogs with chronic and recurrent otitis externa (same group as for lipid content study in Table 5.2.2) were analysed in the study. Breed, sex, and age of these dogs are summarised in Tables 5.2.4

and 5.2.2. These samples were also submitted for microbial culture (see Chapter II)

A pooled skin surface lipid sample and a pooled subcutaneous adipose tissue sample were also collected from three canine cadavers provided by the Department of Veterinary Pathology, University of Glasgow.

Standards : myristic acid (14:0), pentadecanoic acid (15:0), palmitoleic acid (16:1), palmitic acid (16:0), margaric acid (17:0), *r*-linolenic acid methyl ester (18:3), linoleic acid methyl ester (18:2), *a*-linolenic acid methyl ester (18:3), oleic acid methyl ester (18:1), stearic acid (18:0), nonadecanoic acid (19:0), and arachidonic acid methyl ester (20:4). All were 99% pure analytic grade and were obtained from Sigma Chemical Company (Poole, Dorset, England).

Two percent (v/v) sulphuric acid in methanol was used for transesterification, and diethyl ether was used to extract fatty acid methyl esters (F.A.M.E.) after transesterification.

Equipment

Gas liquid chromatography was carried out using a Perkin-Elmer 8420 gas chromatograph fitted with a Supelco (Supelco Inc., Bellefonte, USA) 30 m SBP-1 bonded phase column (0.25 um film thickness) with nitrogen as carrier gas.

Procedures

1. Preparation of cerumen free fatty acids

The cerumen free fatty acids were separated from other cerumen lipids by T. L. C. using the standard solvent. The area of the silica gel containing the free fatty acids was scraped off and 2 ml chloroform was

used to elute the fatty acids from the silica gel. The suspension was filtered and the chloroform solvent removed by drying under a stream of nitrogen in a fume cupboard. The fatty acid residue was then ready for transesterification.

2. Preparation of skin surface lipid

Pooled skin surface lipid was obtained by rubbing cotton swabs which had been soaked in chloroform : methanol 2:1 (v/v) on the skin of three fresh dog cadavers. This skin had not been clipped free of hair. The skin lipids were then extracted from the swabs by the method of Folch *et al* (1957).

3. Preparation of lipids of subcutaneous adipose tissue

Three grams of subcutaneous adipose tissue were collected from each of the same three dog cadavers. The lipids were extracted from a mixture of these samples by the method of Folch *et al* (1957).

4. Preparation of bacterial lipids

Samples of the resident flora on canine aural skin were isolated from healthy and diseased ears. The strains of bacteria were purified by subculture. The following bacteria were isolated from the canine external ear during this study (see Chapter II); *Corynebacterium* species, *Staphylococcus xylosus*, *Staphylococcus aureus*, *Staphylococcus intermedius*, *Streptococcus faecalis*, and *Pseudomonas aeruginosa* .

Lipids were extracted by the method of Folch *et al* (1957) from 3-5 colonies of each purified bacterial subculture.

5. Transesterification

The fatty acid fraction and the cerumen lipid residue were placed in stoppered tubes with 2 ml 2% (v/v) sulphuric acid in methanol and heated at 50°C for 2 hours. Three ml diethyl ether and 5 ml distiled water were then added and the tube was well shaken. The upper diethyl ether layer was collected. The extraction was then repeated, using a second aliquot

of diethyl ether. The combined upper layers were neutralised by shaking with 2 ml 0.05% sodium bicarbonate. The ether layer was then dried with anhydrous sodium sulphate. Finally, diethyl ether was evaporated under a stream of nitrogen in a fume cupboard. The fatty acid methyl ester (F.A.M.E.) residue was dissolved in 100 ul toluene ready for G.L.C.

Standard free fatty acids, the pooled sample of skin surface lipid, the pooled sample of subcutaneous adipose tissue, and the bacterial lipids were transesterified by the same procedure.

6. Gas Liquid Chromatography

The G.L.C. was carried out at 200°C isothermally for 40 minutes. A calibration graph of the chain length of saturated straight chain F.A.M.E. against the log of the retention time was prepared and this was used to determine the equivalent chain length (E.C.L.) of any component whose identity was in doubt (Gurr and Harwood, 1991). Fatty acids were identified by comparison of their retention times with those of the standards. In the case of fatty acid components which could not be identified with certainty, they were designated by their equivalent chain length (E.C.L.); this is the number of carbon atoms which they would contain, if they were straight chain saturated fatty acids (Christie 1982).

5.2.6 Gas chromatography and mass - spectrometry (G.C. - M.S.)

Structures of the odd-numbered and the C_{18} unsaturated fatty acid methyl esters were confirmed in two cerumen samples from healthy canine ears by gas chromatography - mass spectrometry (G.C. - M.S.) with a VG Analytical model 70-250 S chromatograph-mass spectrometer fitted with a Chrompack CP-SIL 5CB 25 m x 0.32 mm column (Chromack Ltd) for the analysis of fatty acid methyl esters. The M.S. source temperature was 240°C and the electron ionization potential was 35 eV.

5.2.7 Statistical methods

Differences between the lipid content of cerumen samples from right and left ears in an individual were investigated by the Paired T-test. The two-sample Mann-Whitney test was used to explore differences in lipid content and fatty acid compositions between cerumen from normal ears and those with otitis externa. The data were analysed using a statistical software package "Minitab" (Minitab Inc., USA) on an IBM (PS 2 50 Z) personal computer.

Table 5.2.1 Breed, sex and age (years) of 20 dogs with 36 normal ears used for total cerumen lipid content study.

Breed	Sex	age
Bull Terrier	F	2
Bull Terrier	F	5
Bull Terrier	M	2
Bull Terrier	M	5
Bull Terrier	F	3
Springer Spaniel	F	5
Bull Terrier	M	1
Bull Terrier	F	3
Bull Terrier	M	2
Cocker Spaniel	M	3
Springer Spaniel	F	12
Cocker Spaniel	F	8
Bull Terrier	F	3
Bull Terrier	M	5
Setter	M	1
Golden Retriever	M	7
Boxer	F	6
Greyhound	F	3
Samoyed	M	1
Cavalier King Charles Spaniel	M	3

Table 5.2.2 Breed, sex and age (years) of eight dogs with 12 ears affected by otitis externa used for total cerumen lipid content and cerumen fatty acid analysis by gas liquid chromatography studies.

Breed	Sex	Age
Rottweiler	F	4
Yorkshire Terrier	M	9
Wire Haired Fox Terrier	M	9
Rottweiler	M	5
German Shepherd Dog	F	7
German Shepherd Dog	M	5
Cairn Terrier	M	11
German Shepherd Dog	F	6

Table 5.2.3 Breed, sex and age (years) of 11 dogs with 16 normal ears used for cerumen lipid analysis by thin layer chromatography.

Breed	Sex	Age
Crossbreed	F	2
Greyhound	M	14
Greyhound	F	10
Greyhound	F	9
Greyhound	F	6
Greyhound	F	4
Golden Retriever	M	7
Boxer	F	6
Greyhound	F	3
Samoyed	M	1
Cavalier King Charles Spaniel	M	3

Table 5.2.4 Breed, sex and age (years) of 15 dogs with 30 healthy ears used in the analysis of cerumen by gas liquid chromatography.

Breed	Sex	Age
Bull Terrier	F	2
Bull Terrier	F	5
Bull Terrier	M	2
Bull Terrier	M	5
Bull Terrier	F	3
Springer Spaniel	F	5
Bull Terrier	M	1
Bull Terrier	F	3
Bull Terrier	M	2
Cocker Spaniel	M	3
Springer Spaniel	F	12
Cocker Spaniel	F	8
Bull Terrier	F	3
Bull Terrier	M	5
Setter	M	1

5.3 RESULTS

5.3.1 The total lipid content of canine cerumen

Cerumen samples from 36 healthy ears from 20 dogs and 12 otitic ears from 8 dogs were analysed. These cerumen samples had very variable lipid contents, as can be seen from the results in Table 5.3.1.

In the healthy ears, the range of total lipid content was from 18.2% to 92.6% (w/w) (Table 5.3.1); in some cases, a similar lipid content was found in both ears from the same dog (dog 1 and 3), but prominent differences between right and left ears could also be found, e.g., dog 5. Nevertheless, there was no statistically significant difference (paired-T test) between cerumen lipid content of right and left ears in these healthy ears ($P=0.28$).

Similarly, great variation in lipid content was found in the otitic ears (Table 5.3.2.). However, the average lipid content in the diseased ears, 24.4% (S.D. = 23.9%), was significantly lower ($P=0.003$) than that in the healthy ears, 49.7% (S.D. = 22.10%) (Mann-Whitney test).

5.3.2 The lipid composition of canine cerumen

Sixteen cerumen samples from 16 healthy ears from 11 dogs were used in this study by T.L.C. The cerumen lipids found from T.L.C. using two different developing solvents were: cholesterol, cholesterol esters, free fatty acids, fatty aldehydes, waxes, triglycerides, lecithin, and sphingomyelin. The assessment for lipid in a sample was based on visual inspection of the T.L.C. plate and this could mean that small amounts of lipids were not detected. The frequency at which each lipid appeared in cerumen samples was: cholesterol 100% (16/16); cholesterol esters 93.8% (15/16); free fatty acids 93.8% (15/16); fatty aldehydes 93.8%

(15/16); waxes 93.8% (15/16); triglycerides 68.8% (11/16); lecithin 56.3% (9/16); and sphingomyelin 18.8% (3/16)

5.3.3 The fatty acid composition of canine cerumen

There were small differences in the percentage composition of F.A.M.E. between the free fatty acid fraction of cerumen isolated by T.L.C. and that of pooled cerumen investigated by G.L.C. (Table 5.3.3). The fatty acid composition of whole cerumen lipid is similar to those reported later, and only a simplified composition is given in this Table.

The cerumen lipid fatty acid compositions of 30 healthy ears from 15 dogs, and 12 otitic ears from 8 dogs, were investigated by G.L.C. The fatty acid composition of cerumen was very complicated; more than 60 detectable components were found in each cerumen sample (Figure 5.3.1). The fatty acids found appeared to contain between 14 carbon atoms and at least 20 carbon atoms. The composition from the right and left ears of the same dog could show both quantitative and qualitative differences. Based on all these analyses, 31 components (each greater than 1% by weight of F.A.M.E. of the sample analysed) were deemed to be the major fatty acids found in canine cerumen and these are detailed in Table 5.3.4. The fatty acids which contained 16 and 17 carbon atoms were the major fatty acids of cerumen from healthy ears (Table 5.3.5 and Figure 5.3.2) and they comprised about 70% of the total. More than one third (w/w) of the fatty acids in healthy ears were those containing 17 carbon atoms, both saturated and unsaturated. The fatty acids with a chain length of 16 carbon atoms accounted for about one third of the total fatty acid in cerumen samples from healthy canine ears. There were many minor components in cerumen (less than 0.1% by weight of F.A.M.E. of each sample), but these are not reported.

In the diseased ears, the fatty acids with 18 carbon atoms, were most abundant in the cerumen samples, comprising more than one third (w/w) of the total fatty acid; whereas the fatty acids which contained 17 carbon atoms formed the second largest group. These comprised about one quarter of the total fatty acids in the cerumen samples from otitic ears (Table 5.3.5 and Figure 5.3.2).

In cerumen from diseased ears, the unsaturated C_{16} and C_{17} fatty acids were found in lower proportion than in healthy ears (Table 5.3.4). Significant differences were found between healthy and otitic ears in total C_{16} fatty acids ($P=0.006$) and C_{17} fatty acids ($P=0.03$) (Mann-Whitney test). Unsaturated C_{18} fatty acids which contained 3, or fewer double bonds, and stearic acid (18:0) were increased in the otitic ears (Table 5.3.4 and Figure 5.3.3). The total proportion of C_{18} fatty acids was significantly increased in the diseased ears ($P=0.003$) compared to cerumen from healthy ears (Table 5.3.5) (Mann-Whitney test). The unsaturated fatty acids that contained 20 carbon atoms, especially arachidonic acid (20:4), were also significantly elevated in the otitic ears ($P=0.04$) (Mann-Whitney test) (Table 5.3.4), when compared to cerumen from healthy ears.

The ratio of total saturated fatty acids to total unsaturated fatty acids in the healthy group was 0.19:1, with a range of 0.02-2.57:1; whereas the ratio of total saturated fatty acids to unsaturated fatty acids in the otitic ears was 0.29:1, with a range of 0.01-0.81:1. The ratio of saturated to total unsaturated C_{18} fatty acids was 0.02:1 in the normal ears, compared to 0.32:1 in the diseased ears (Figure 5.3.4). The increased ratio of stearic acid to the C_{18} unsaturated fatty acids in diseased ears was due to the greater increase in stearic acid than that of the total C_{18} unsaturated fatty acids in cerumen from these ears (Table 5.3.4). The ratio of saturated to unsaturated fatty acid C_{16} F.A.M.E. was increased in otitic ears because of a decreased content of unsaturated C_{16} fatty acids (Table 5.3.4 and

Figure 5.3.4). The ratio of saturated C_{17} fatty acids to the unsaturated C_{17} fatty acids was similar in both groups (Figure 5.3.4).

The results of fatty acid composition of those cerumen samples are summarised in Tables 5.3.6 and 5.3.7.

5.3.4 The fatty acid composition of skin surface lipid and the subcutaneous adipose tissue of dogs

The fatty acids of pooled skin surface lipid from three dog cadavers mainly comprised those with chain lengths of 16, 17, and 18 carbon atoms. Fatty acids with 16 carbon atoms were the most abundant components and accounted for about one third of the total. C_{17} fatty acids were the second biggest proportion accounting for about 20% of the total fatty acid. Oleic acid (C18:1) formed about 17% of the total fatty acid of skin surface lipid. Among the fatty acids identified on the canine skin surface, even-numbered fatty acids accounted for about 54% and odd-numbered about 46% (Table 5.3.8).

The fatty acids of subcutaneous adipose tissue were almost exclusively even-numbered fatty acids, about 98% of the F.A.M.E. Those containing 18 carbon atoms accounted for more than 60% of the total. Among these, oleic acid was the major individual fatty acid found in this tissue (Table 5.3.8).

5.3.5 The fatty acid composition of the bacteria on the aural skin

Results of microbiological culture of 30 cerumen samples from 30 normal ears and 12 cerumen samples from 12 ears affected by otitis externa are shown in Tables 5.3.9 and 5.3.10 (see Chapter II).

The resident flora commonly found in healthy dog ears in this study were *Corynebacterium* species and *Staphylococcus xylosus*. The major fatty acid in both these isolates was palmitic acid (16:0) and both species

had other fatty acids in common. *Staphylococcus xylosus* contained a fatty acid of E.C.L. 14.32 which was not detected in the corynebacteria (Table 5.3.11a).

Staphylococcus aureus and *Staphylococcus intermedius* were isolated from otitic ears. *Staphylococcus aureus* and *S. intermedius* also had palmitic acid as their major fatty acid, but only *S. intermedius* contained the fatty acid with E.C.L. 14.32 found in *S. xylosus* (Table 5.3.11b).

Streptococcus faecalis, which was also found in otitic ears contained palmitic acid as its major component and shared other components with the *Staphylococcus* species, but it did not contain the fatty acid of E.C.L. 14.32 (Table 5.3.11b).

Pseudomonas aeruginosa, found in otitic ears, had a very different fatty acid composition from all the above species of bacteria. It contained two fatty acids of E.C.L. 15.64 and 17.71. which were not detected in either canine cerumen samples or the other bacteria isolated (Table 5.3.12).

5.3.6 The identification of fatty acids by gas chromatography and mass - spectrometry

The composition of canine cerumen fatty acid was very complicated, more than 50 compounds were detected in each single sample from normal ears (Figure 5.3.5). Fatty acids containing chain lengths from 12 to 26 carbon atoms were identified by this technique (Figure 5.3.5).

The presence of odd-numbered fatty acids and linoleic (C18:2) and oleic acids (18:1) in canine cerumen samples were confirmed from mass spectra obtained.

The even and odd numbered fatty acids identified by G.L.C. were confirmed by G.C. - M.S. Saturated C_{15} is characterised by a parent ion

peak (M^*) at mass 256; peaks at M^*-31 (mass 225) and M^*-43 (mass 213), and also peaks at mass 74 and mass 87 (Figure 5.3.6a). Saturated C_{16} and C_{17} fatty acids are also characterised by peaks at mass of 74 and 84, but with different parent ion (M^*) peaks and different M^*-31 and M^*-43 peaks. For saturated C_{16} fatty acid, the parent ion peak is M^*=270, M^*-31 is mass 239, and M^*-43 is mass 227 (Figure 5.3.6b); whereas parent ion (M^*) peak for C_{17} saturated fatty acid is mass 284, with M^*-31 (mass 253) and M^*-43 (mass 241) (Figure 5.3.6c).

The identity of linoleic (C18:2) and oleic (18:1) acids, which were found in most cerumen samples from both healthy and diseased ears, were also confirmed by this technique. The identity of linoleic acid is proven by a parent ion (M^*) peak at mass 294, which is a mass of saturated C18 fatty acid (M=298) minus four (M^*=298-4=294) because of formation of two double bonds, and M^*-31 (mass 263) and M^*-43 (mass 251) (Figure 5.3.6d). Oleic acid is identified by mass-spectrum which shows a parent ion (M) peak at M=296 (Figure 5.3.6e) (McLafferty, 1959; see introduction).

There were other compounds that were not fatty acid methyl esters in the cerumen samples which were also transesterified during the derivatisation for the G.L.C. method used (Figure 5.3.5 and Figure 5.3.7). However, these minor components have been included as calculated in the results of G.L.C. as if they were fatty acid methyl esters reported in equivalent chain length (E.C.L.).

More than 13 hours were required for one single cerumen sample to be analysed by gas chromatography-mass spectrum (G.C. - M.S.). Limited time was available for G.C. - M.S. Consequently, not all samples could be analysed by this method, and therefore, all identifications of G.L.C. are represented as equivalent chain length (Table 5.3.6), except where G.L.C. identification was confirmed by G.C. - M.S.

Table 5.3.1 The percentage lipid content of cerumen from 36 healthy dog ears (Table 5.2.1).

	Cerumen lipid (% by weight)	
Dog	**Right ear**	**Left ear**
1	73.6	72.5
2	18.2	29.3
3	66.7	72.7
4	58.8	-
5	25.9	92.6
6	65.9	42.1
7	72.7	36.8
8	23.3	69.0
9	48.6	75.0
10	29.4	50.0
11	22.7	28.6
12	22.4	46.2
13	66.7	-
14	28.0	23.1
15	77.3	75.7
16	58.3	-
17	64.3	25.0
18	55.1	45.7
19	70.0	-
20	21.4	35.0
Mean	48.5	51.2
S.D.	21.6	21.9

Cerumen lipids combined both right and left ears

Mean 49.7 Range 18.2-92.6 S.D. 21.5

Table 5.3.2 The percentage lipid content of cerumen from 12 dog ears affected by chronic otitis externa (Table 5.2.2).

Ear	**Cerumen lipid (% by weight)**
1	19.0
2	16.0
3	4.3
4	5.0
5	15.9
6	12.0
7	4.3
8	69.6
9	63.9
10	53.6
11	13.3
12	39.3
Mean	24.4
Range	4.3-69.6
S.D.	23.9

Table 5.3.3 The composition (% by weight of fatty acid methyl ester) of the free fatty acid fraction isolated by thin layer chromatography and the fatty acid composition of total lipid in a pooled cerumen sample.

Fatty acid [E.C.L.]	**% by weight of fatty acid methyl ester**	
	Free Fatty acid fraction	**Total cerumen lipid**
[C 14.67]	15.5	18.8
[C 16.50]	12.5	13.9
[C 16.61]	23.5	21.9
C18:1	1.5	1.3
[C 18.55]	12.2	11.2
[C 18.78]	11.4	9.7
[C 19.85]	11.1	10.9

Figure 5.3.1 Chromatogram of components of a cerumen sample from an healthy ear by gas liquid chromatography.

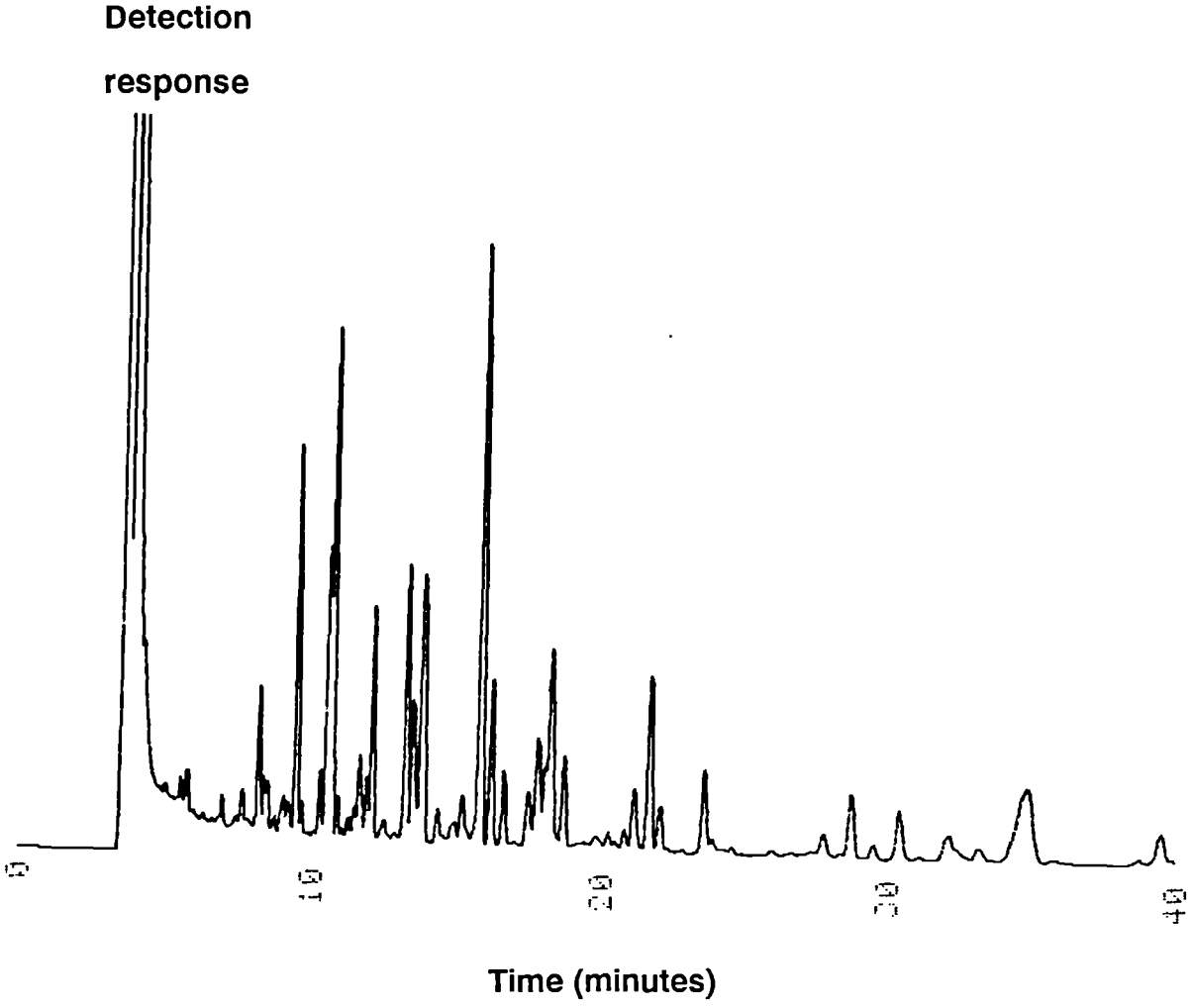


Table 5.3.4 Descriptive statistics of the fatty acid composition (% by weight of fatty acid methyl ester) of the cerumen samples from 30 healthy ears and 12 ears with otitis externa. From raw data displayed in Tables 5.3.6 and 5.3.7.

	% by weight of fatty acid methyl ester					
Fatty acid	*Healthy*			*Otitic*		
[E.C.L.]	Mean	Range	S.D.	Mean	Range	S.D.
C_{14} Fatty acid						
14:0	0.5	Tra	0.1	0.3	Tra	0.3
C_{15} Fatty acids						
[C 14.67]	4.7	Tra-15.4	3.8	3.8	Tra-11.8	4.4
15:0	2.0	Tra-27.1	5.2	1.4	Tra- 6.7	2.4
C_{16} Fatty acids						
[C 15.01]	4.9	Tra-10.7	4.1	2.0	Tra- 9.0	3.2
[C 15.06]	12.8	Tra-22.0	5.7	3.8	Tra-18.6	5.8
[C 15.31]	2.0	Tra- 7.6	1.9	4.8	Tra-12.7	4.7
16:1	4.3	Tra-17.7	4.3	2.5	Tra-18.2	5.3
[C 15.83]	5.8	Tra-34.5	6.6	4.4	Tra-13.4	6.0
16:0	4.1	Tra-32.1	6.6	5.5	Tra-17.0	6.9
C_{17} Fatty acids						
[C 16.01]	8.8	Tra-22.5	5.1	8.8	Tra-18.2	6.8
[C 16.50]	17.7	Tra-31.1	8.0	9.0	Tra-17.1	6.0
[C 16.61]	3.7	Tra- 8.2	3.3	2.8	Tra- 9.5	3.6
[C 16.70]	2.7	Tra-36.8	8.2	3.1	Tra-13.3	4.3
17:0	4.1	Tra-32.2	6.3	3.2	Tra- 7.4	3.5
C_{18} Fatty acids						
[C 17.01]	6.6	Tra-11.4	3.7	2.9	Tra-12.9	4.3
[C 17.12]	1.2	Tra-12.0	2.6	0.7	Tra- 4.8	1.3
***r* 18:3**	0.4	Tra	0.2	1.4	Tra-11.2	3.3
18:2	1.0	Tra- 9.6	2.2	6.9	Tra-22.1	6.3
***a* 18:3**	0.9	Tra-14.3	2.6	0.8	Tra- 9.0	2.6
18:1	3.0	Tra-15.7	4.6	11.8	Tra-43.4	11.1
[C 17.92]	4.3	Tra-20.6	4.9	3.3	Tra-12.1	4.2
18:0	0.3	Tra	0.3	8.8	Tra-28.2	9.2
C_{19} Fatty acids						
[C 18.08]	1.6	Tra-45.7	8.3	0.2	Tra	0.3
[C 18.55]	2.4	Tra-11.8	4.1	2.1	Tra-11.8	3.8
[C 18.74]	0.9	Tra-23.5	4.3	0.8	Tra- 5.1	1.8
[C 18.78]	0.1	Tra- 3.7	0.1	0.1	Tra	0.2
[C 18.95]	1.6	Tra-27.4	5.6	0.1	Tra	0.2
19:0	0.5	Tra-18.5	1.0	0.3	Tra	0.3
C_{20} Fatty acids						
20:4	0.5	Tra- 4.4	0.8	2.9	Tra-18.4	5.5
[C 19.65]	0.1	Tra- 2.7	0.5	0.5	Tra- 4.8	1.4
[C 19.84]	1.2	Tra-11.6	3.1	1.9	Tra-12.7	3.9

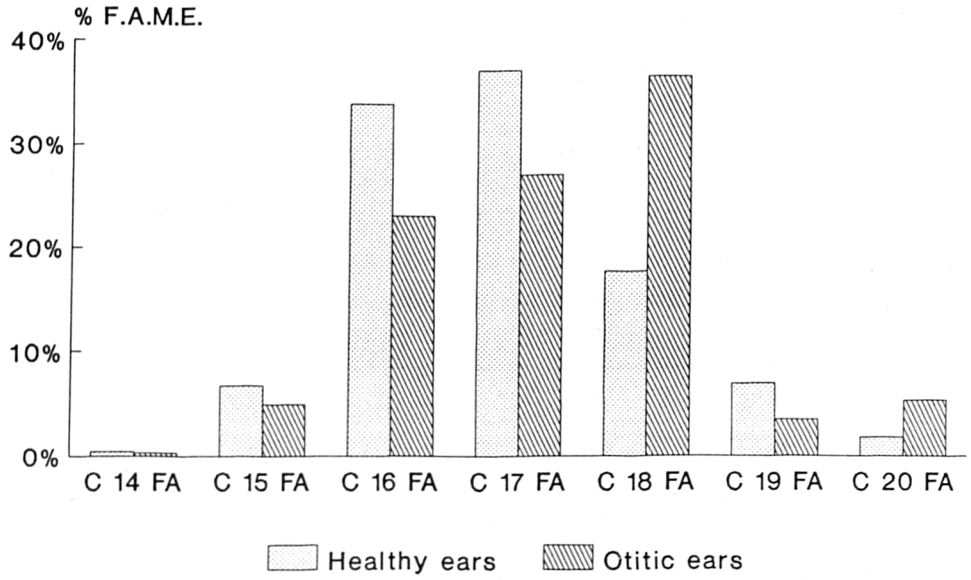
Tra = trace, 0.1-1%

Table 5.3.5 Summary of data in Table 5.3.4. Comparision of the classes, by carbon chain length, of fatty acid in cerumen samples from healthy ears and ears with otitis externa (% by weight of fatty acid methyl ester).

	% by weight of fatty acid methyl ester	
Fatty acid	Mean Healthy ears	Mean Otitic ears
C_{14} fatty acid	0.5	0.3
C_{15} fatty acids	6.8	4.9
*C_{16} fatty acids	33.8	23.0
*C_{17} fatty acids	37.0	26.9
*C_{18} fatty acids	17.7	36.4
C_{19} fatty acids	6.9	3.5
*C_{20} fatty acids	1.8	5.3

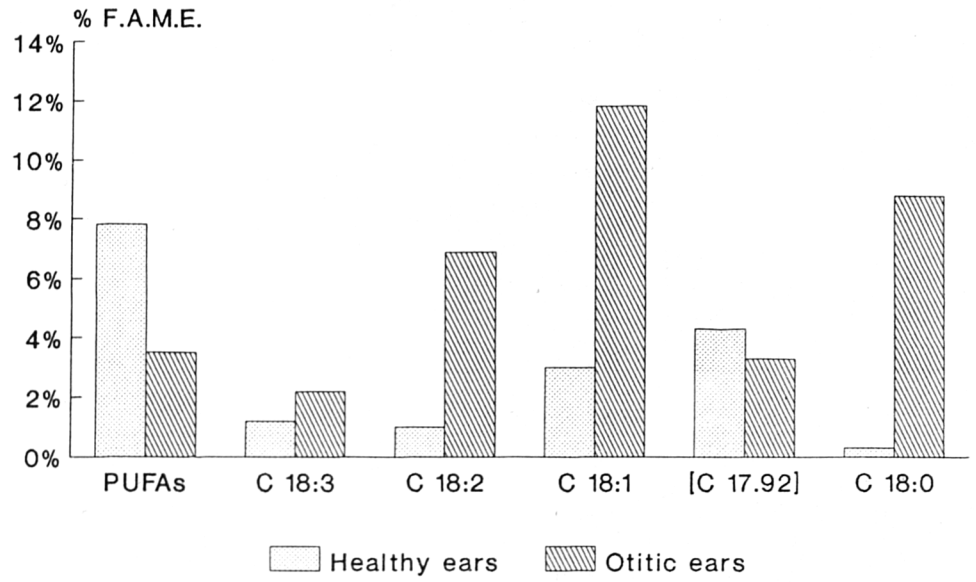
* indicates statistically significant differences between healthy and otitic ears.

Figure 5.3.2. The mean fatty acid composition of canine cerumen samples from 30 healthy and 12 otitic ears.



F.A.M.E. = fatty acid methyl ester

Figure 5.3.3. A comparison of mean C18 fatty acid contents in 30 healthy and 12 otitic ears.



PUFAs = polyunsaturated fatty acids

Figure 5.3.4. Mean ratios of saturated to unsaturated fatty acid in the fatty acid composition of 30 healthy and 12 otitic ears.

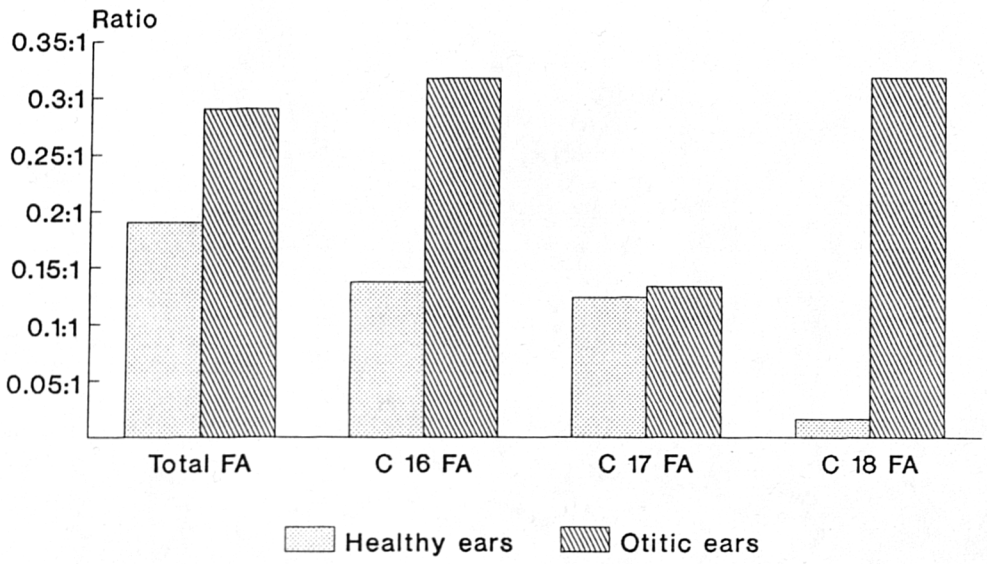


Table 5.3.6 The fatty acid composition (% by weight of fatty acid methyl ester) of cerumen samples from healthy canine ears.

Fatty acid [E.C.L.]	% by weight of fatty acid methyl ester					
	Dog 1		Dog 2		Dog 3	
Ear	R	L	R	L	R	L
14:0	-	Tra	Tra	Tra	Tra	Tra
[C 14.67]	Tra	4.70	8.43	Tra	Tra	Tra
15:0	6.74	9.96	Tra	27.13	5.67	Tra
[C 15.01]	Tra	Tra	8.16	Tra	5.03	Tra
[C 15.06]	13.99	13.91	14.57	Tra	10.02	Tra
[C 15.31]	Tra	1.55	Tra	Tra	Tra	1.30
16:1	12.68	5.95	7.96	Tra	6.13	Tra
[C 15.83]	9.25	4.80	8.64	Tra	13.91	34.46
16:0	Tra	Tra	4.61	Tra	Tra	Tra
[C 16.01]	5.38	4.87	10.34	Tra	9.58	Tra
[C 16.50]	18.77	29.96	22.69	14.47	12.15	Tra
[C 16.61]	Tra	6.61	8.4	Tra	Tra	-
[C 16.70]	-	-	Tra	Tra	Tra	Tra
17:0	Tra	5.62	Tra	32.17	-	-
[C 17.01]	6.73	10.07	6.33	Tra	7.01	Tra
[C 17.12]	-	Tra	Tra	Tra	Tra	Tra
***r* 18:3**	Tra	Tra	-	-	-	Tra
18:2	7.35	Tra	-	Tra	-	-
***a* 18:3**	14.33	Tra	Tra	Tra	-	-
18:1	Tra	Tra	Tra	-	10.67	15.68
[C 17.92]	4.89	Tra	-	-	Tra	Tra
18:0	-	Tra	-	-	Tra	Tra
[C 18.08]	-	-	-	-	-	45.73
[C 18.55]	-	-	Tra	Tra	10.52	Tra
[C 18.74]	-	Tra	Tra	5.11	-	-
[C 18.78]	-	-	-	3.66	-	-
[C 18.95]	-	Tra	-	-	Tra	-
19:0	-	-	-	18.48	-	-
20:4	Tra	Tra	-	-	Tra	Tra
[C 19.65]	-	-	-	-	Tra	2.65
[C 19.84]	-	-	-	-	Tra	-

R = right, L = left

Tra = trace, 0.1-1%

Table 5.3.6 The fatty acid composition (% by weight of methyl ester) of cerumen samples from healthy canine ears.

Fatty acid [E.C.L.]	% by weight of fatty acid methyl ester					
	Dog 4		Dog 5		Dog 6	
Ear	R	L	R	L	R	L
14:0	Tra	Tra	-	Tra	Tra	Tra
[C 14.67]	Tra	Tra	Tra	3.73	11.96	8.45
15:0	Tra	Tra	Tra	Tra	Tra	Tra
[C 15.01]	Tra	8.86	Tra	7.00	8.83	11.52
[C 15.06]	6.88	21.98	10.43	14.45	17.43	20.97
[C 15.33]	Tra	6.66	Tra	2.51	Tra	Tra
16:1	Tra	8.26	5.73	3.38	7.20	5.55
[C 15.83]	4.43	6.57	8.08	6.83	8.05	4.58
16:0	Tra	Tra	Tra	Tra	Tra	6.77
[C 16.01]	5.72	8.06	12.55	8.51	10.53	12.21
[C 16.50]	5.14	12.14	18.85	31.13	17.07	13.41
[C 16.61]	Tra	Tra	5.46	7.33	Tra	5.65
[C 16.70]	27.56	-	Tra	Tra	Tra	Tra
17:0	Tra	8.05	Tra	Tra	Tra	Tra
[C 17.01]	4.16	11.35	5.04	6.67	7.49	10.02
[C 17.12]	Tra	-	Tra	Tra	Tra	-
***r* 18:3**	Tra	Tra	Tra	Tra	Tra	Tra
18:2	-	Tra	Tra	Tra	Tra	Tra
***a* 18:3**	Tra	Tra	Tra	Tra	Tra	Tra
18:1	5.45	Tra	5.60	-	6.23	Tra
[C 17.92]	6.11	8.23	5.76	4.89	5.40	Tra
18:0	Tra	Tra	Tra	Tra	-	-
[C 18.08]	Tra	-	-	-	-	-
[C 18.55]	5.26	Tra	-	-	-	-
[C 18.74]	-	-	23.47	Tra	-	-
[C 18.78]	-	-	-	-	-	-
[C 18.95]	27.38	-	-	-	Tra	-
19:0	Tra	Tra	Tra	Tra	-	Tra
20:4	Tra	Tra	-	-	Tra	Tra
[C 19.65]	-	-	-	-	-	-
[C 19.84]	-	-	-	-	-	-

R = right, L = left

Tra = trace, 0.1-1%

Table 5.3.6 The fatty acid composition (% by weight of fatty acid methyl ester) of cerumen samples from healthy canine ears.

Fatty acid [E.C.L.]	% by weight of fatty acid methyl ester					
	Dog 7		Dog 8		Dog 9	
Ear	R	L	R	L	R	L
14:0	Tra	Tra	Tra	Tra	Tra	Tra
[C 14.67]	8.58	8.26	2.02	1.87	3.39	4.04
15:0	Tra	Tra	Tra	Tra	Tra	Tra
[C 15.01]	7.31	6.99	9.45	Tra	6.49	7.94
[C 15.06]	16.30	15.77	19.74	6.31	15.17	13.84
[C 15.31]	1.22	1.03	2.41	1.01	2.52	3.21
16:1	5.90	5.69	Tra	-	-	-
[C 15.83]	7.80	8.64	6.18	4.95	-	-
16:0	Tra	-	3.00	Tra	32.05	8.13
[C 16.01]	7.95	6.62	6.89	4.10	2.97	17.68
[C 16.50]	25.09	26.10	26.22	9.18	11.18	16.67
[C 16.61]	7.78	4.98	8.29	3.50	Tra	-
[C 16.70]	-	-	-	36.75	Tra	-
17:0	4.94	4.36	Tra	-	4.40	5.59
[C 17.01]	7.55	11.26	10.12	3.47	7.19	10.18
[C 17.12]	-	-	-	-	-	-
***r* 18:3**	Tra	Tra	-	-	Tra	-
18:2	-	Tra	-	Tra	Tra	Tra
***a* 18:3**	Tra	Tra	Tra	-	Tra	Tra
18:1	Tra	Tra	Tra	-	7.66	3.35
[C 17.92]	Tra	Tra	5.76	13.18	7.06	6.49
18:0	-	-	Tra	Tra	-	-
[C 18.08]	-	-	-	-	-	-
[C 18.55]	-	-	Tra	14.19	Tra	Tra
[C 18.74]	-	-	-	-	-	-
[C 18.78]	-	-	-	-	-	-
[C 18.95]	-	-	Tra	-	-	-
19:0	Tra	Tra	-	Tra	Tra	Tra
20:4	Tra	Tra	Tra	Tra	Tra	Tra
[C 19.65]	-	-	-	-	-	-
[C 19.84]	-	-	-	-	-	-

R = right, L = left

Tra = trace, 0.1-1%

Table 5.3.6 The fatty acid composition (% by weight of fatty acid methyl ester) of cerumen samples from healthy canine ears.

Fatty acid [E.C.L.]	% by weight of fatty acid methyl ester					
	Dog 10		Dog 11		Dog 12	
Ear	R	L	R	L	R	L
14:0	Tra	Tra	Tra	Tra	Tra	Tra
[C 14.67]	5.19	4.23	15.39	3.19	9.44	6.63
15:0	Tra	Tra	Tra	Tra	Tra	Tra
[C 15.01]	8.13	8.18	Tra	Tra	10.69	Tra
[C 15.06]	12.76	13.62	9.53	Tra	16.16	10.95
[C 15.31]	2.49	3.03	-	-	Tra	2.01
16:1	7.91	5.00	9.66	3.32	Tra	Tra
[C 15.83]	Tra	Tra	Tra	-	Tra	-
16:0	5.79	5.12	15.02	10.28	8.49	9.96
[C 16.01]	7.07	12.69	9.39	22.50	9.87	7.35
[C 16.50]	29.32	25.60	6.91	6.80	9.03	16.56
[C 16.61]	7.16	7.94	Tra	3.05	Tra	8.18
[C 16.70]	-	-	Tra	-	-	-
17:0	4.20	6.87	Tra	Tra	Tra	Tra
[C 17.01]	7.87	7.44	-	-	10.09	8.86
[C 17.12]	-	-	Tra	Tra	6.02	Tra
***r* 18:3**	Tra	Tra	Tra	Tra	Tra	Tra
18:2	-	-	5.32	9.56	Tra	Tra
a 18:3 Tra	Tra	-	-	Tra	Tra	
18:1	Tra	Tra	13.35	12.91	Tra	Tra
[C 17.92]	Tra	Tra	9.38	20.61	Tra	Tra
18:0	Tra	Tra	Tra	Tra	Tra	Tra
[C 18.08]	-	-	-	-	-	-
[C 15.55]	-	-	-	3.83	8.32	6.69
[C 18.74]	-	-	-	-	-	Tra
[C 18.78]	-	-	-	-	-	-
[C 18.95]	-	-	Tra	-	-	15.29
19:0	Tra	Tra	5.76	Tra	Tra	Tra
20:4	Tra	Tra	-	4.42	Tra	-
[C 19.65]	-	-	-	-	-	-
[C 19.84]	-	-	-	-	11.61	6.90

R = right, L = left

Tra = trace, 0.1-1%

Table 5.3.6 The fatty acid composition (% by weight of fatty acid methyl ester) of cerumen samples from healthy canine ears.

Fatty acid [E.C.L.]	% by weight of fatty acid methyl ester					
	Dog 13		Dog 14		Dog 15	
Ear	R	L	R	L	R	L
14:0	Tra	Tra	Tra	Tra	Tra	Tra
[C 14.67]	2.48	2.69	7.12	4.23	5.78	5.82
15:0	Tra	Tra	Tra	Tra	Tra	Tra
[C 15.01]	7.35	8.13	10.71	Tra	Tra	Tra
[C 15.06]	17.13	14.36	19.20	16.89	8.51	9.09
[C 15.31]	2.26	2.01	3.64	4.67	5.09	7.63
16:1	17.73	9.05	Tra	Tra	1.02	Tra
[C 15.83]	7.91	Tra	4.67	5.88	7.67	7.01
16:0	Tra	Tra	Tra	Tra	2.01	2.04
[C 16.01]	4.40	10.39	9.50	21.23	7.75	8.12
[C 16.50]	17.45	15.15	23.06	21.09	27.01	21.77
[C 16.61]	Tra	2.51	7.96	7.96	Tra	Tra
[C 16.70]	5.86	6.30	-	Tra	-	-
17:0	7.33	7.20	Tra	4.51	11.31	8.94
[C 17.01]	9.98	10.53	8.89	8.35	Tra	Tra
[C 17.12]	Tra	11.95	4.46	5.01	Tra	Tra
***r* 18:3**	Tra	-	Tra	Tra	Tra	Tra
18:2	Tra	-	Tra	Tra	Tra	Tra
***a* 18:3**	Tra	Tra	Tra	-	Tra	Tra
18:1	-	Tra	Tra	Tra	Tra	Tra
[C 17.92]	Tra	-	-	Tra	8.43	9.23
18:0	Tra	-	-	-	Tra	-
[C 18.08]	-	Tra	-	-	-	-
[C 18.55]	-	-	-	-	7.20	11.77
[C 18.74]	-	-	-	-	-	-
[C 18.78]	-	-	Tra	-	-	-
[C 18.95]	Tra	-	Tra	-	-	-
19:0	Tra	Tra	Tra	-	Tra	Tra
20:4	-	Tra	-	Tra	-	-
[C 19.65]	-	-	-	-	-	-
[C 19.84]	-	Tra	Tra	-	8.00	8.42

R = right, L = left

Tra = trace, 0.1-1%

Table 5.3.7 The fatty acid composition (% by weight of fatty acid methyl ester) of cerumen samples from otitic canine ears.

Fatty acid [E.C.L.]	% by weight of fatty acid methyl ester					
Ear	1	2	3	4	5	6
14:0	Tra	Tra	-	Tra	Tra	Tra
[C 14.67]	2.73	Tra	9.81	Tra	5.91	Tra
15:0	Tra	6.47	Tra	6.71	Tra	Tra
[C 15.01]	7.99	Tra	8.97	-	Tra	Tra
[C 15.06]	8.26	9.69	18.55	Tra	Tra	Tra
[C 15.31]	2.43	Tra	6.06	11.13	12.65	Tra
16:1	Tra	Tra	Tra	6.99	18.23	Tra
[C 15.83]	14.88	Tra	Tra	Tra	Tra	Tra
16:0	-	6.90	Tra	Tra	Tra	17.03
[C 16.01]	18.20	7.35	9.22	15.21	Tra	Tra
[C 16.50]	7.93	11.97	14.07	4.86	10.70	4.33
[C 16.61]	Tra	Tra	-	-	Tra	-
[C 16.70]	Tra	Tra	Tra	13.28	7.56	Tra
17:0	Tra	Tra	7.37	9.08	3.88	Tra
[C 17.01]	6.12	8.31	12.90	-	Tra	Tra
[C 17.12]	-	-	Tra	-	Tra	Tra
***r* 18:3**	-	Tra	Tra	-	11.24	-
18:2	9.88	10.17	-	Tra	Tra	7.33
***a* 18:3**	8.96	-	Tra	-	-	-
18:1	Tra	11.44	6.07	-	13.45	13.39
[C 17.92]	12.07	-	Tra	8.82	Tra	5.91
18:0	-	13.25	-	11.92	11.26	17.91
[C 18.08]	Tra	-	-	Tra	-	-
[C 18.55]	-	Tra	-	11.83	-	5.96
[C 18.74]	-	-	-	Tra	5.09	-
[C 18.78]	-	-	-	-	-	Tra
[C 18.95]	-	-	-	-	Tra	-
19:0	-	-	-	Tra	Tra	Tra
20:4	Tra	Tra	-	Tra	Tra	18.35
[C 19.65]	-	Tra	-	-	-	4.80
[C 19.84]	-	Tra	-	Tra	-	Tra

Tra = trace, 0.1-1%

Table 5.3.7 The fatty acid composition (% by weight of fatty acid methyl ester) of cerumen samples from otitic canine ears.

Fatty acid [E.C.L.]	% by weight of fatty acid methyl ester					
Ear	7	8	9	10	11	12
14:0	Tra	-	-	-	-	Tra
[C 14.67]	Tra	-	Tra	11.82	-	8.86
15:0	-	-	Tra	Tra	Tra	Tra
[C 15.01]	Tra	-	Tra	-	3.88	Tra
[C 15.06]	-	-	Tra	Tra	6.37	Tra
[C 15.31]	5.54	-	Tra	6.26	Tra	10.95
16:1	Tra	Tra	Tra	-	Tra	Tra
[C 15.83]	Tra	12.49	7.87	13.35	Tra	Tra
16:0	16.72	1.68	-	15.11	16.93	Tra
[C 16.01]	14.00	1.45	17.12	12.24	8.83	Tra
[C 16.50]	9.17	2.20	19.99	5.65	Tra	17.01
[C 16.61]	6.59	1.96	5.09	9.52	Tra	8.64
[C 16.70]	-	Tra	6.74	6.30	Tra	Tra
17:0	-	7.13	Tra	7.09	Tra	Tra
[C 17.01]	-	Tra	-	-	4.91	Tra
[C 17.12]	-	-	Tra	Tra	4.83	Tra
***r* 18:3**	Tra	3.77	-	-	-	-
18:2	7.01	22.11	8.40	-	7.50	9.77
***a* 18:3**	-	-	-	-	-	
18:1	11.10	43.40	12.91	6.00	13.10	9.82
[C 17.92]	Tra	3.91	Tra	6.83	-	-
18:0	14.84	-	-	-	28.22	7.76
[C 18.08]	-	-	Tra	-	-	Tra
[C 18.55]	Tra	-	5.85	-	-	-
[C 18.74]	-	-	-	-	-	4.17
[C 18.78]	-	-	-	-	-	Tra
[C 18.95]	-	-	Tra	-	-	Tra
19:0	Tra	Tra	-	-	Tra	-
20:4	7.63	-	Tra	-	5.22	Tra
[C 19.65]	Tra	Tra	-	-	-	-
[C 19.84]	-	Tra	12.70	Tra	-	8.09

Tra = trace, 0.1-1%

Table 5.3.8 The fatty acid composition (% by weight of fatty acid methyl ester) of the pooled skin surface lipid and the pooled subcutaneous adipose tissue of three dogs.

	% by weight of fatty acid methyl ester	
Fatty acid [E.C.L.]	Skin surface lipid	Adipose tissue
14:0	-	2.4
[C 14.31]	7.6	-
[C 14.83]	10.3	-
[C 15.40]	14.7	-
16:1	-	-
16:0	19.4	12.1
[C 16.23]	20.2	-
[C 16.61]	-	1.1
18:2	3.0	-
a 18:3	-	9.7
18:1	16.6	50.5
18:0	-	6.1
[C 18.15]	8.2	-

Table 5.3.9 Microbiological findings from 30 healthy canine ears from 15 dogs (Table 5.2.4), whose cerumen lipids were analysed by gas liquid chromatography.

Ear	Microbiological findings
1	Coagulase-negative staphylococci Corynebacteria Micrococci
2	Coagulase-positive staphylococci
3	Coagulase-positive staphylococci Corynebacteria Micrococci *Malassezia pachydermatis*
4	Coagulase-positive staphylococci Corynebacteria *Malassezia pachydermatis*
5	Coagulase-negative staphylococci Corynebacteria Micrococci
6	Coagulase-negative staphylococci Corynebacteria
7	Coagulase-positive staphylococci Corynebacteria
8	Coagulase-positive staphylococci Corynebacteria
9	Corynebacteria Micrococci
10	Corynebacteria Micrococci
11	Negative
12	Bacilli
13	Coagulase-negative staphylococci Corynebacteria *Malassezia pachydermatis*
14	Coagulase-negative staphylococci

Continued overleaf

Continued from previous page

15 Micrococci

16 Coagulase-negative staphylococci
Micrococci

17 Coagulase-negative staphylococci
Bacilli

18 Corynebacteria
Bacilli

19 Coagulase-negative staphylococci
Corynebacteria
Micrococci
Malassezia pachydermatis

20 Coagulase-negative staphylococci
Corynebacteria
Micrococci
Malassezia pachydermatis

21 Micrococci

22 Coagulase-negative staphylococci
Micrococci

23 Coagulase-negative staphylococci
Corynebacteria
Malassezia pachydermatis

24 Coagulase-negative staphylococci
Corynebacteria
Malassezia pachydermatis

25 Bacilli

26 Coagulase-negative staphylococci

27 Coagulase-negative staphylococci
Malassezia pachydermatis

28 Coagulase-negative staphylococci
Bacilli

29 Corynebacteria

30 Corynebacteria
Malassezia pachydermatis

Table 5.3.10 Microbiological findings from 12 otitic ears (Table 5.2.2) used for cerumen analysis by gas liquid chromatography.

Ear	Microbiological findings
1	*Pseudomonas aeruginosa*
2	Corynebacteria *Staphylococcus aureus* *Streptococcus faecalis*
3	Negative
4	*Staphylococcus aureus* *Streptococcus faecalis*
5	*Staphylococcus aureus* *Streptococcus faecalis*
6	*Pseudomonas aeruginosa* Streptococci
7	*Pseudomonas aeruginosa* Streptococci
8	Negative
9	*Malassezia pachydermatis*
10	*Malassezia pachydermatis*
11	Negative
12	Staphylococci *Malassezia pachydermatis*

Table 5.3.11a The fatty acid composition (% by weight of fatty acid methyl ester) of Gram-positive cocci isolated from healthy ear canals in dogs during this study.

	% by weight of fatty acid methyl ester	
Fatty acid [E.C.L.]	*Corynebacterium* species	*Staphylococcus xylosus*
[C 14.32]	-	8.1
[C 15.06]	13.2	29.9
[C 15.83]	11.5	9.5
16:0	51.7	37.7
18:1	6.7	4.8
[C 17.92]	16.8	10.4

Table 5.3.11b The fatty acid composition (% by weight of fatty acid methyl ester) of Gram-positive cocci isolated from otitic canine ears during this study.

	% by weight of fatty acid methyl ester		
Fatty acid [E.C.L.]	*Staph. aureus*	*Staph. intermedius*	*Strept. faecalis*
[C 14.32]	-	17.1	-
[C 15.06]	29.7	16.2	24.6
[C 15.83]	10.0	10.6	11.7
16:0	41.0	30.0	43.9
18:1	8.9	7.2	75.1
[C 17.92]	9.7	18.8	9.5

Table 5.3.12 The fatty acid composition (% by weight of fatty acid methyl ester) of *Pseudomonas aeruginosa* isolated from one otitic canine ear during this study.

Fatty acid [E.C.L.]	Fatty acid methyl ester (% by weight)
[C 15.18]	7.5
[C 15.64]	6.2
[C 15.87]	28.7
[C 16.01]	16.9
[C 17.71]	27.7
18:0	4.9
19:0	3.4

Figure 5.3.5 Chromatogram of components of canine cerumen from an healthy ear by gas chromatography and mass - spectrometry. Figures shown are peak numbers.

Peak 174: C14:0 saturated fatty acid

Peak 198: a mixture (not a single fatty acid)

Peak 206: C15:0 saturated fatty acid

Peak 222: C16:0 saturated fatty acid (palmitic)

Peak 271: C17:0 saturated fatty acid (margaric)

Peak 285: C18:0 saturated fatty acid (stearic)

Peak 299: C18:2 linoleic acid

Peak 301: C18:1 oleic acid

Peak 310: C18:0 saturated fatty acid (stearic)

Peak 328: C19:0 saturated fatty acid

Peak 356: C20:0 saturated fatty acid

Peak 382: C21:0 saturated fatty acid

Peak 408: C22:0 saturated fatty acid (behenic)

Peak 432: C23:0 saturated fatty acid

Peak 464: C24:0 saturated fatty acid (ligoceric)

Peak 479: C25:0 saturated fatty acid

Peak 509: C26:0 saturated fatty acid

Figure 5.3.5 Chromatogram of components of one canine cerumen sample from a healthy ear by gas chromatography and mass-spectrometry. Figures shown are peak numbers.

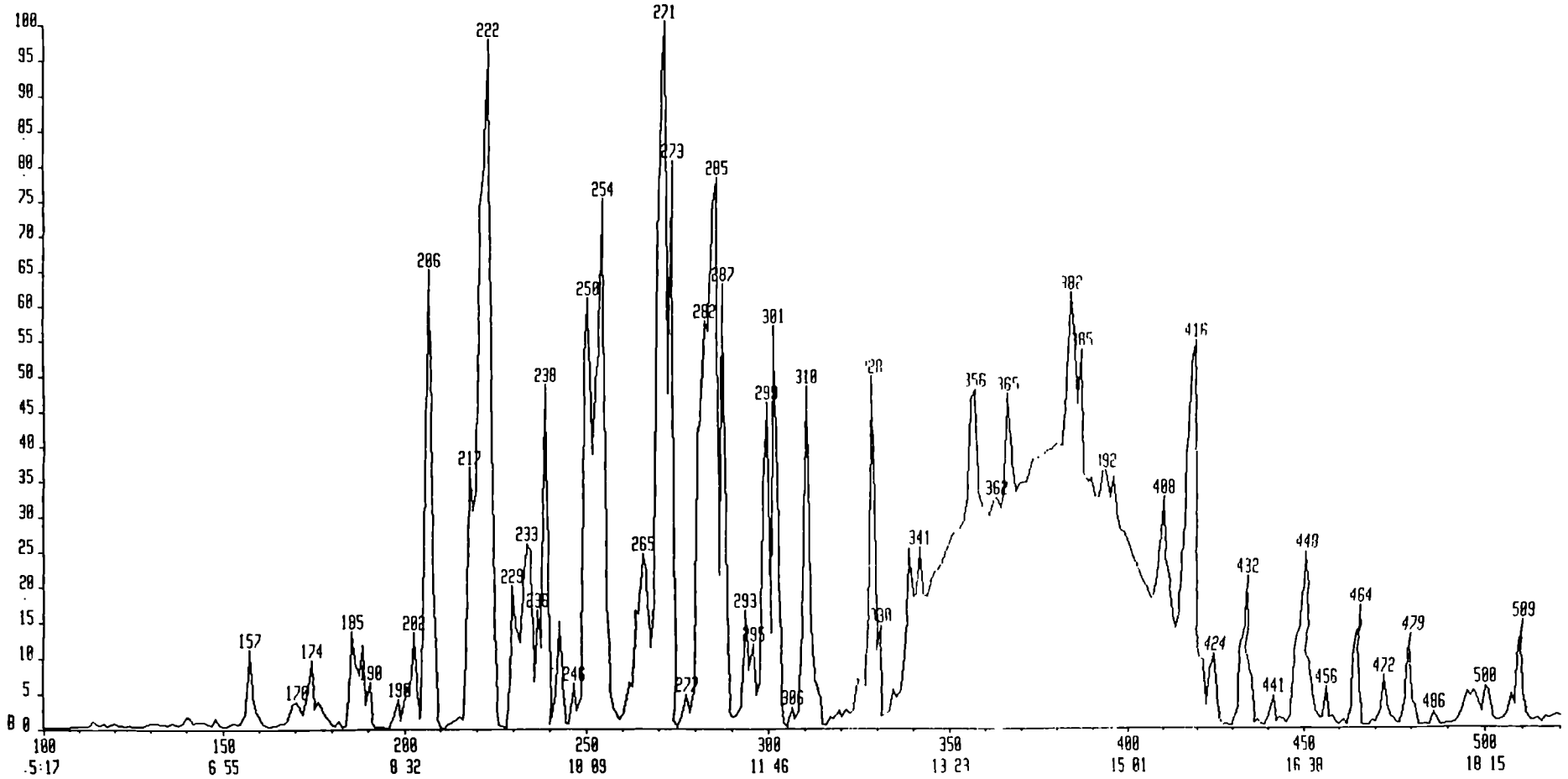


Figure 5.3.6a Mass spectrum of C 15:0 saturated fatty acid (peak 206) from figure 5.3.5.

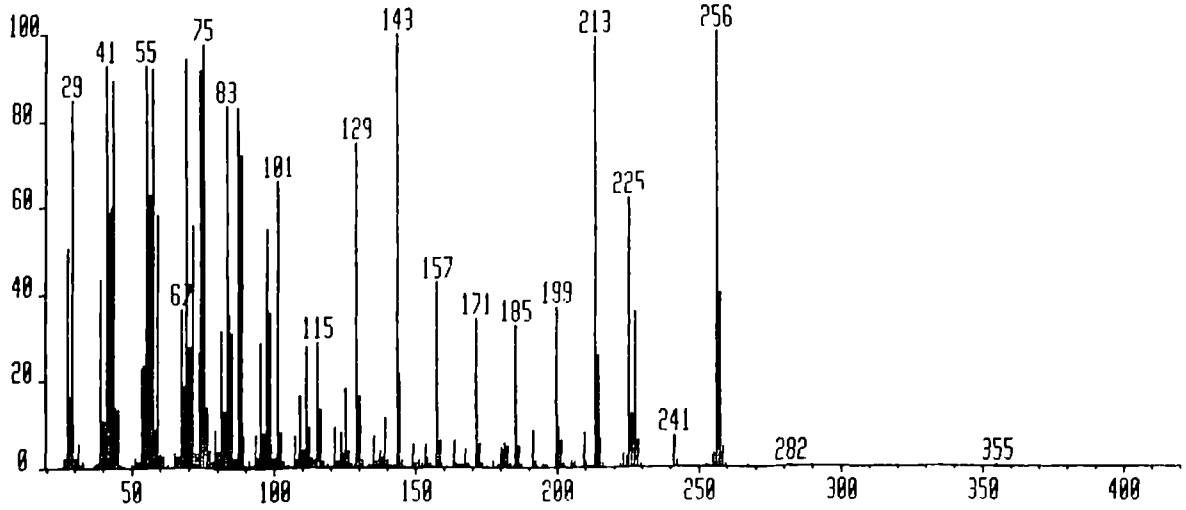


Figure 5.3.6b Mass spectrum of C16:0 saturated fatty acid (peak 222) from figure 5.3.5.

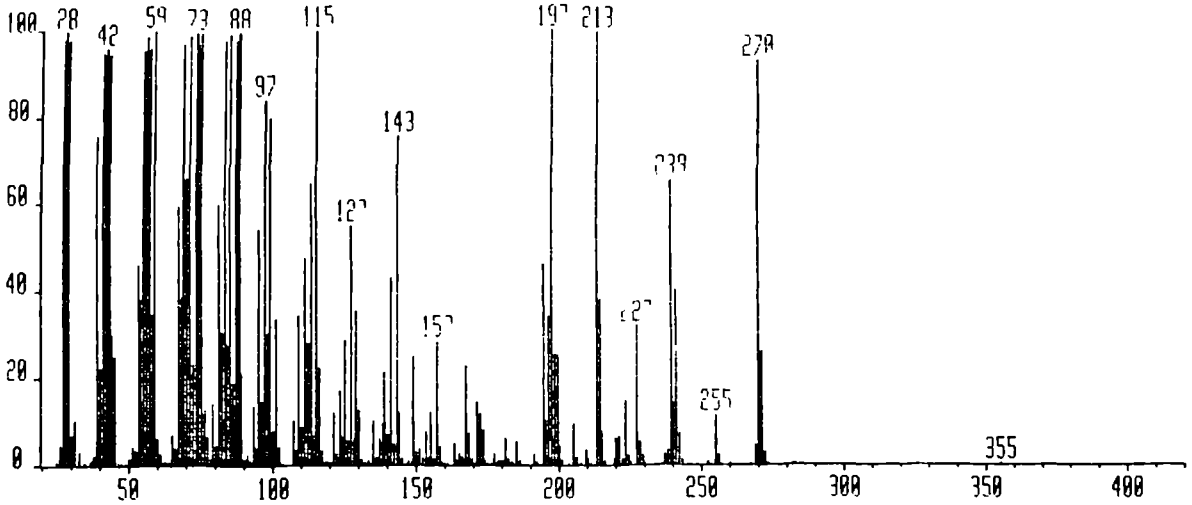


Figure 5.3.6c Mass spectrum of C17:0 saturated fatty acid (peak 271) from figure 5.3.5.

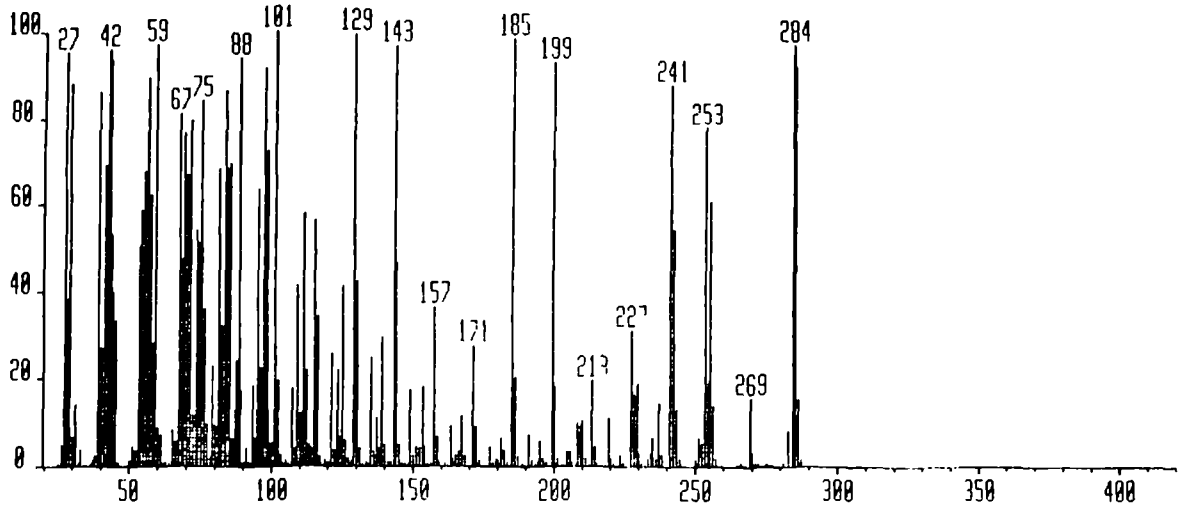


Figure 5.3.6d Mass spectrum of linoleic acid (18:2) (peak 299) from figure 5.3.5.

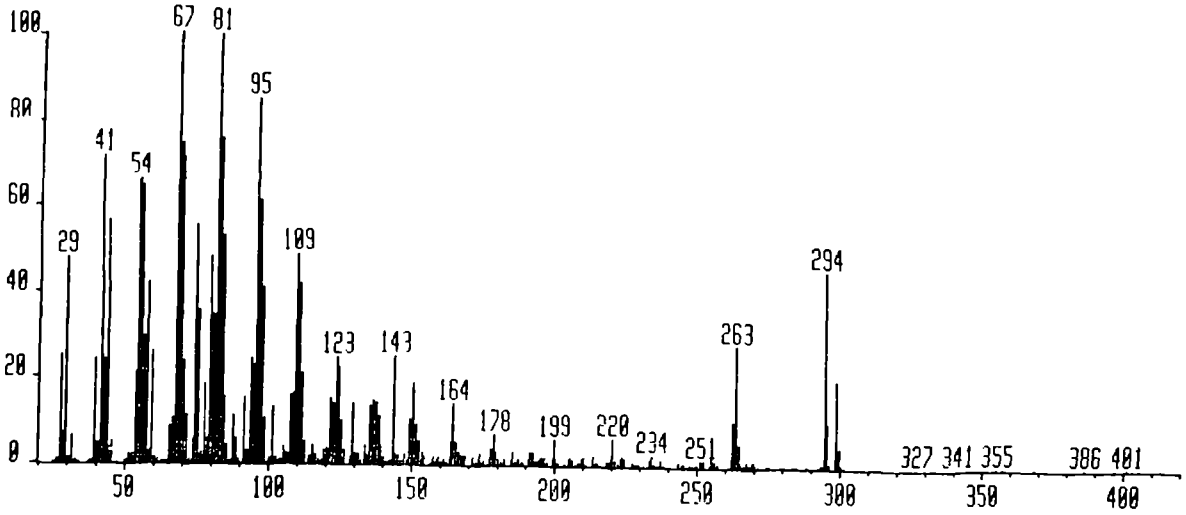


Figure 5.3.6e Mass spectrum of oleic acid (18:1) (peak 301) from figure 5.3.5.

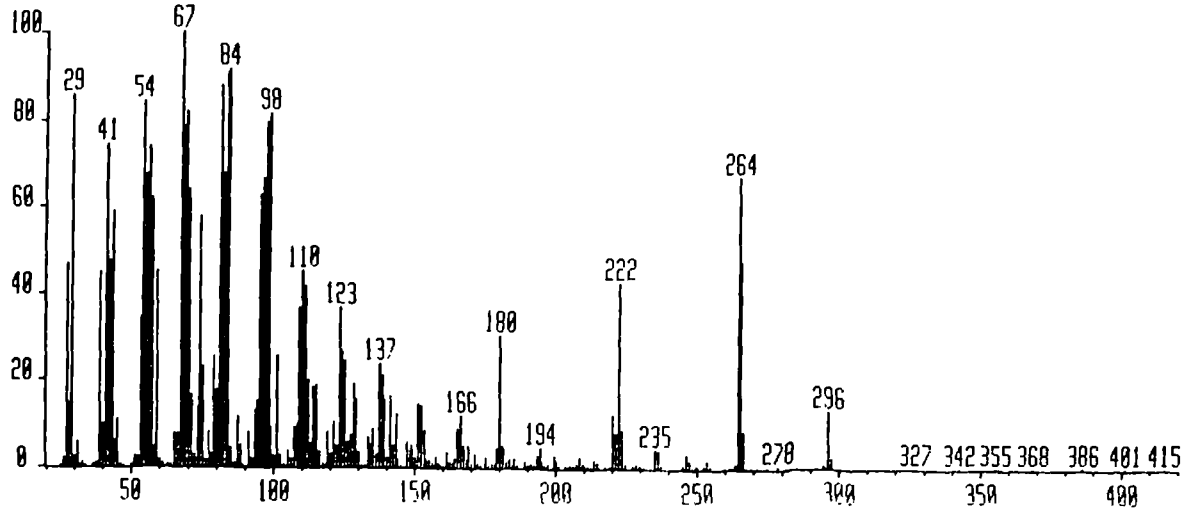
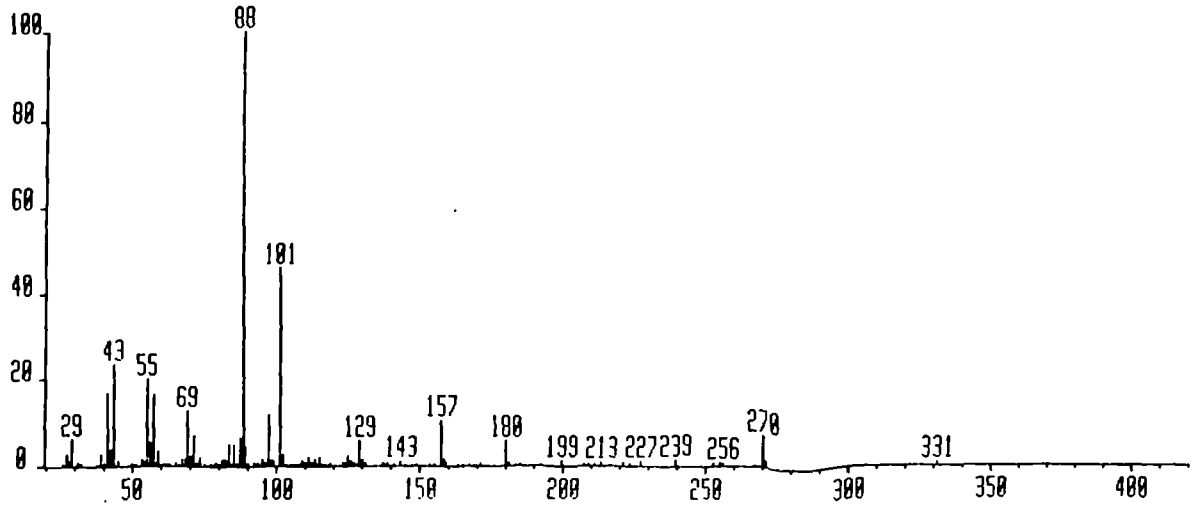


Figure 5.3.7 Mass spectrum shows the compound that is not a single fatty acid (peak 198) from figure 5.3.5.



5.4 DISCUSSION

Lipids accounted for 50% on average, and up to 92.6% of the total weight of the cerumen samples from healthy canine ears. These findings are similar to the average lipid content of human cerumen which has been reported to range from 20% to as high as an average of 67% by weight (Nakashima, 1933; Chiang *et al*, 1955, Chiang *et al*, 1957; Kataura and Kataura, 1967; Gershbein *et al*, 1980). A marked variation in lipid content was found in the cerumen samples analysed in this study, but no statistically significant difference was found between the right and left ears in the normal group. These findings resemble those of Cipriani *et al* in humans (1990). Several factors may contribute to the variation between individuals. Greene *et al* (1970) have found that the densitiy of sebaceous glands in human skin affected the amount of skin surface lipid. The results in Chapter III, indicate that the density of sebaceous glandular tissue in canine aural skin varied substantially between dogs. This could lead to different amounts of lipid being found on the aural skin of different dogs. The ratio of sebaceous glandular tissue to apocrine glandular tissue in the aural skin also differed between dogs (Chapter III; Stout-Graham *et al*, 1990). This could produce lipids of different composition in cerumen from different dogs (Fernando, 1966; Fernando, 1967; Bortz *et al*, 1990; Okuda *et al*, 1991). The lipid content in cerumen may also be affected by the relative rates of two physiological processes, lipid delivery from the adnexal glands and exfoliation of the epithelium of the aural integument (Chiang *et al*, 1957), which show variation between individuals. In humans, it has been reported that the longer that the cerumen is present in the external ear canal, the higher the degree of oxidation of the lipids. As a result of this oxidation, some lipids cannot be extracted for analysis, this leads to arteficially lower lipid levels being recorded from casual

human cerumen samples (Chiang *et al*, 1957). No information concerning lipid oxidation was available for this study, however the degree of oxidation of the cerumen lipids probably varies, consequently the variations in lipid yield in these canine cerumen samples may also be a reflection of various degrees of oxidation.

Similarly, a scattered range of lipid content was obtained from the otitic ears, but the lipid yield in these ears was significantly lower. This may be due to pathological changes in the glands which are responsible for the formation of cerumen (Fernando, 1966; Fernando, 1967; Okuda *et al*, 1991). The apocrine glands are thought to be obligatory for maintaining the consistency of the earwax (Fraser, 1961), even though they may contribute little in the way of lipids to cerumen. Lipids excreted by sebaceous glands and lipids from the epidermis comprised a larger portion of human cerumen lipids (Bortz *et al*, 1990). During the process of otitis externa, the apocrine glands become very hyperplastic and cystic, they are then supposed to be the main secretory organ (Fraser, 1961; Fernando, 1967; Stout-Graham *et al*, 1990), whilst the morphological changes to the sebaceous glands could vary from hypertrophic (Chapter III) to atrophic (Fraser, 1961; Fernando, 1967; Stout-Graham *et al*, 1990). Such changes in these secretory tissues, may cause changes in cerumen lipid content, giving a generally lower lipid yield and great variation in lipid content in ears with external otitis. Damage to the epithelium of the aural skin caused by otitis externa may also lead to disruption of the normal epidermal barrier. Thus, leakage of transudate or exudate may alter the consistency, lipid content and composition of the cerumen.

The lipid classes found in canine cerumen included, cholesterol, free fatty acids, cholesterol esters, waxes, fatty aldehydes, and triglycerides as detected by T.L.C., but all these constituents were not found in all samples. These lipids have been reported in human cerumen

(Kataura and Kataura, 1967; Gershbein *et al*, 1980; Bortz *et al*, 1990; Okuda *et al*, 1991). Phospholipids, lecithin and sphingomyelin, were also detected in canine cerumen samples but less frequently. These lipids are also not consistently present in human cerumen (Akobjanoff *et al*, 1954).

At low total lipid, a lower concentration of an individual lipid class may escape detection. Analyses which have been based on cerumen samples from individual people indicated that the lipid content of human cerumen differs between individuals (Akobjanoff *et al*, 1954; Chiang *et al*, 1955, Chiang *et al*, 1956; Chiang *et al*, 1957; Gershbein *et al*, 1980; Cipriani *et al*, 1990). This may cause a varying frequency of detection of different lipid classes (Gershbein *et al*, 1980; Cipriani *et al*, 1990). On the other hand, studies carried out on pooled human cerumen would not detect individual differences (Kataura and Kataura, 1967; Bortz *et al*, 1991). The detection of an individual lipid class will be very dependent on either the number of individual samples analysed or the individuals used to produce the pooled sample.

The fatty acid fraction of healthy canine cerumen comprised more than 60 compounds as detectable by G.L.C. Chain length ranged from 14 to at least 20 carbon atoms. The C_{17} fatty acids, the largest proportion, usually accounted for more than one third of the total fatty acid. Fatty acids containing 16 carbon atoms comprised about one third of the fatty acids in the healthy ears. These findings differ from the fatty acid composition of human cerumen, which was characterised mainly by C_{16}, C_{18}, and C_{20} fatty acids (Haahti *et al*, 1960; Gershbein *et al*, 1980; Harvey, 1989), with odd numbered fatty acids such as C_{17} only being present in trace amounts.

Thirty one fatty acids appeared to represent the major fatty acids of healthy canine cerumen. Distinct differences, both qualitative and quantitative, in fatty acid composition were found between individual

samples. Such complicated variations in fatty acid composition were not unexpected; a wide range of lipid content with a differing frequency of respective lipids may explain the marked variation in cerumen fatty acid composition.

The fatty acids of cerumen from otitic ears predominantly comprised those with chain lengths of 17 and 18 carbon atoms; C_{18} fatty acids accounted for more than one third, whereas C_{17} fatty acids comprised about a quarter of the fatty acids in cerumen from otitic ears. Qualitative and quantitative variations in the fatty acids in cerumen between individual samples were also observed in the otitic ears. The total C_{16} unsaturated fatty acid was significantly lower in otitic ears than that in healthy ears, which made the total C_{16} fatty acid significantly lower in the otitic group. Due to the lower level of C_{17} unsaturated fatty acids in the otitic group, a significantly lower proportion of total C_{17} fatty acids was found in otitic ears.

In contrast to the decreased proportions of C_{16} and C_{17} fatty acids, the fatty acids containing 18 carbon atoms were significantly elevated in the otitic group. Arachidonic acid, was also increased in most otitic ears. An elevated arachidonic acid content in human skin has been reported in many inflammatory dermatoses (Ziboh and Chapkin, 1988). Increased levels of precursors of pro-inflammatory and immunomodulating mediators, such as polyunsaturated C_{18} fatty acids and arachidonic acid, might be involved in the pathogenesis of cutaneous inflammation (Chapkin *et al*, 1986; Ruzicka *et al*, 1986; Schafer and Kragballe, 1991). On the other hand, polyunsaturated C_{18} fatty acid and arachidonic acids have been shown to restore the barrier function of epidermis in human and rat skin either by systemic or topical administration (Prottey, 1977; Elias *et al*, 1980). Increased levels of these

unsaturated fatty acids on the aural skin of otitic ears might help to restore the normal physiological barrier subsequent to damage to this integument.

Ratios of total saturated to unsaturated fatty acid were not significantly different between healthy and otitic ears although a higher ratio was found in the otitic ears. This finding was similar to that found by Osborne and Baty studying human ears (1990) but their results were not significantly different either.

It has been proposed that malfunction of the cutaneous barrier, associated with defects in lipid metabolism, contributes to the pathogenesis of certain cutaneous inflammatory conditions of human skin (Elias and Brown, 1978; Schaffer and Kragballe, 1991). Whether the disorders in lipid metabolism are involved in producing the different fatty acid compositions of cerumen in the otitic ears is not clear. The lipid metabolism of aural skin affected by otitis externa requires further investigation.

In this study, no correlation could be found between the fatty acid composition of the cerumen sample and the bacteria found in the corresponding ears.

Corynebacterium species and *Staphylococcus xylosus* were commonly isolated from healthy ears in this study. The major fatty acid of these isolates was palmitic acid, which was also generally present in canine cerumen samples. However, ears which were colonized by corynebacteria could show no detectable palmitic acid in the corresponding cerumen samples, e.g., left ear of dog 7; contrariwise the ear, from which no microorganisms were isolated, i.e., the right ear of dog 6, contained palmitic acid in the corresponding cerumen sample. Thus, it is unlikely that palmitic acid from corynebacteria or *S. xylosus* represents a significant source of the palmitic fatty acid found in canine cerumen.

Gram-positive cocci, such *as Staphylococcus aureus*, *Staphylococcus intermedius*, and *Streptococcus faecalis* were isolated from otitic ears in this study. These bacteria also shared a major fatty acid, palmitic acid. Otitic ears which were not colonized by these pathogenic cocci contained palmitic acid in cerumen, e.g., ear 11. Again, it is unlikely that palmitic acid from these Gram-positive cocci is a significant source of the palmitic acid found in canine cerumen.

Pseudomonas aeruginosa was also isolated from otitic ears in this study, but it had a very different fatty acid composition from the Gram-positive cocci mentioned above; two fatty acids with E.C.L. 15.64 and 17.71 were detected in this organism, which were not identified in any of the canine cerumen samples, including ears infected with this microorganism.

These findings suggest that cerumen fatty acids were mainly produced endogenously by the local glandular tissue and the epidermis, and that the fatty acids from bacteria found in ears are not a significant source of the fatty acids in cerumen.

It has been proposed that the free fatty acids on the human skin surface are released by lipase activity of the cutaneous bacteria (Scheimann *et al*, 1960; Shalita, 1974; Cove *et al*, 1980) and a lipase in human skin (Jimenez-Ascota, Planas, and Penneys, 1990; Holland, 1993). A positive correlation between the number of the cutaneous bacteria and the production rate of free fatty acid has also been reported (Cove *et al*, 1980). However, little quantitative variation in the fatty acid composition between the free fatty acid fraction and the total fatty acid of canine cerumen lipid was found in this study. The possibility that lipolytic actvities of the resident flora in canine external ear canal alter free fatty acid levels cannot be excluded.

One important role for skin surface lipids is to maintain and regulate the cutaneous flora (Pillsbury and Rebell, 1952; Roth and James, 1989). A positive correlation between the population of cutaneous *Propionibacterium acnes* and sebaceous lipid excretion has been found in humans (McGinley *et al*, 1980; Leyden and McGinley, 1982). Lipolytic activity by human cutaneous microorganisms, such as *Corynebacterium*, *Micrococcus*, *Propoinibacterium*, *Staphylococcus*, *Malassezia*, and *Candida* (Reisner *et al*, 1968; Smith and Willet, 1968; Cove *et al*, 1980; McGinley *et al*, 1980; Faergemann, 1986; Bailey and Redpath, 1992), might explain why these microorganisms colonise the human skin surface where the growth medium is rich in lipids (Jimenez-Acosta, Planas, and Penneys, 1989; Holland, 1993). On the other hand, the bactericidal activity of human skin lipids can regulate the resident flora (Burthenshaw, 1942; Pillsbury and Rebell, 1952). Unsaturated fatty acids are thought to be responsible for this antibacterial action (Burthenshaw, 1942; Pillsbury and Rebell, 1952; Butcher *et al*, 1976; Lacey and Lord, 1981). Unsaturated C_{18} fatty acids are fatty acids commonly found on the human skin surface which exhibit strong growth-inhibiting activity on bacteria (Butcher *et al*, 1976; Kanai and Kondo, 1979; Lacey and Lord, 1981). It has been postulated that these bactericidal effects are associated with the autoxidation of the double bonds contained in unsaturated fatty acids (Gutteridge *et al*, 1974). Similarly, the lipid of human cerumen has been shown to possess bactericidal effects on the resident flora in the human external auditory meatus (Baumann *et al*, 1961). G.C.-M.S. in this study indicated the presence of linoleic and oleic acids in canine cerumen, which suggests that canine cerumen may also regulate the resident flora on the aural integument due to the presence of antibacterial unsaturated fatty acids.

The fatty acid composition of the canine skin surface was also investigated. It was similar to that of canine cerumen; fatty acids with chain lengths of 16, 17, and 18 carbon atoms, both saturated and unsaturated, predominated. Resemblance between the fatty acid composition of skin and cerumen has also been demonstrated in humans (Haahti *et al*, 1960). This is probably a reflection of the fact that canine and human skin, and aural skin, are histologically and physiologically similar (see Chapter III).

The fatty acid composition of canine skin surface lipid revealed in this study differs from the results reported by Sharaf, Clark, and Downing (1977). They indicated that canine skin fatty acid was mainly comprised of components with 20, 21, and 22 carbon atoms. However, their research was based on a single sample and may have been influenced by a wide variety of individual factors (as discussed above). Nevertheless, the limitations of the methods used for analysis should also be considered (Nicolaides, Apon, and Wong, 1976). The fatty acid composition reported in the present study was within a range of C_{12} to C_{26}, but this range may not be the actual limits. Fatty acids with less than 9 carbon atoms are very volatile and difficult to detect without resorting to special methods.

Subcutaneous adipose tissue fatty acid comprised mainly C_{18} fatty acids. This finding was similar to the study of Wheatley and Sher (1961). In their research on canine adipose lipids, it was reported that oleic acid comprised nearly 50% of the total fatty acid; palmitic acid and unsaturated C_{18} fatty acid were the next most abundant constituents.

Nearly 50% of the fatty acids in canine cerumen from healthy ears and the skin surface, and more than one third of the total fatty acid in canine cerumen from otitic ears, were odd numbered fatty acids. Fatty acids which contained 17 carbon atoms were the most abundant odd numbered fatty acids found. In canine cerumen samples from healthy

ears, from inflamed ears, and on the healthy skin surface, they accounted for more than one third, one quarter, and one fifth of the fatty acid composition, respectively. The presence of these odd numbered fatty acids was confirmed by the G.C.-M.S. These findings contradict the claim that odd numbered fatty acids are uniquely found in human sebaceous lipids (Steward and Downing, 1985). The functions and origins of these odd numbered fatty acids remain to be explored in detail.

Lipids play an important part in biological structures whose purpose is to provide a barrier to protect organisms against their environment. The importance of lipids in such barriers lies in their ability to exclude water and reduce the entry of harmful materials or microorganisms (Gurr and Harwood, 1991). Although the cerumen lipid composition showed a marked variation between dogs, its viscosity and hydrophobic properties are maintained by a combination of lipid classes rather than a single constituent. Individual constituents may nevertheless possess important characteristic properties and these might fulfill other physiological functions (Gurr and Harwood, 1991).

CHAPTER VI

STUDIES OF

Malassezia pachydermatis

AND ITS RELEVANCE TO CANINE OTITIS EXTERNA

6.1 INTRODUCTION

In dogs, *Malassezia pachydermatis* is commonly found in both healthy ears and ears with otitis externa (Sharma and Rhoades 1975; Rausch and Skinner, 1978; Gedek *et al*, 1979; Abou-Gabal *et al*, 1979; see Chapter II). It has been considered to belong to the normal resident flora of the aural skin (Fraser, 1961b; Baxter 1976; Uchida *et al*, 1990). This yeast formerly called *Pityrosporum pachydermatis* or *Pityrosporum canis* belongs to the family *Cryptococcaceae*. The genus *Malassezia* comprises two species, *M. furfur* and *M. pachydermatis* (Midgley, 1989). *Malassezia furfur* is found in association with various human skin conditions, such as comedo (Weary, 1970), tinea versicolor, seborrhoeic dermatitis, and dandruff (Roth and James, 1989); *M. pachydermatis* was first isolated from the inflamed skin of an Indian rhinoceros (Weidman, 1925), and has since been found in healthy and otitic canine ears (Manktelow, 1960; Fraser 1961c; Smith, 1968; Sharma and Rhoades, 1975; Sinha *et al*, 1976; Abou-Gabal and Fargland 1979; Gedek *et al*, 1979), several canine skin conditions (Dufait, 1983; Scott and Miller 1989; Evans 1991; Mason and Evans 1991; Mason 1992; Plant *et al*, 1992), and feline ears (Baxter 1976).

Recently, emphasis has been placed on the importance of this yeast in cases of canine otitis externa (Abou-Gabal and Fagerland 1979; Mason and Evans 1991). The pathogenic role of *M. pachydermatis* in canine otitis externa is not clear (Gedek *et al*, 1979; Lloyd, 1993a). However, studies by Sinha *et al* (1976), Abou-Gabal *et al* (1979), and Mansfield *et al* (1990) have shown that *M. pachydermatis* might be a major cause of canine external otitis.

The role of environmental factors and in particular cerumen and cerumen lipids in regulating the growth of *M. pachydermatis* are unclear and controversial. *Malassezia pachydermatis* has been distinguished from

M. furfur on the grounds that it, unlike *M. furfur*, does not require exogenous lipid for growth (Fraser, 1961b; Kockova-Kratochvilova, Ladzianska, and Bucko, 1987). However Gabal (1988), who reported that canine cerumen stimulates the growth of *M. pachydermatis*, suggested that this stimulation of growth was due to the presence of various lipid substrates in cerumen. Speculations concerning the possible effects of cerumen and its lipids on the growth of *M. pachydermatis* are complicated by reports of antibacterial and antifungal activity in human cerumen and human skin surface lipids (Nicolaides, 1974; Aly, Maibach, Rahman, Shinefield, and Mandel, 1975; Chai and Chai, 1980; Stone and Fulghum, 1984; Megarry *et al*, 1988; Miller, Aly, Shinefield, and Elias, 1988; Lloyd, 1993b). The lipid layer on the human aural skin surface may contribute to host defence as proposed by Burtenshaw (1942) and Baumann *et al* (1961), whilst fatty acids are thought to be responsible for the antimicrobial effect of human cerumen (Burtenshaw, 1942; Baumann *et al*, 1961). Nevertheless, the effects of such environmental factors on *M. pachydermatis* have not been established. The requirements of this yeast for growth are unclear and controversial.

The aims of this study were to explore the effects of canine cerumen lipids on the growth characteristics of *M. pachydermatis*. The relationship between growth of *M. pachydermatis* and such microenvironmental factors could be important in the aetiology of canine otitis externa.

6.2 MATERIALS AND METHODS

6.2.1 Isolation of strains of *Malassezia pachydermatis*

Ten strains of *M. pachydermatis* were obtained by streaking direct-swabs from inflamed ear canals and inflamed skin directly on to Sabouraud agar plates (see Chapter II) and incubating these at 37°C for 72 hours. *Malassezia pachydermatis* was identified by its colonial morphology on culture and by microscopical examination of smears from colonies. The large-colony forming strains isolated were also tested using the API system (API 20C AUX, BioMerieux UK Limited, Hampshire, U.K.) to confirm that the yeasts isolated could not to be assigned to other yeast genera, such as *Candida*, *Cryptococcus*, *Rhodotorula*, *Trichosporon*, or *Rhodosporidium*. The urease test was also used for the identification of *M. pachydermatis*; *M. pachydermatis* is urease-positive, whereas *Candida* is urease-negative. It proved impossible to use such tests on the few small-colony forming strains isolated due to their rapid loss of viability *in vitro*.

6.2.2 Measurement of growth rate in the presence of fatty acids in liquid culture

Strain 5, a large-colony forming strain of *M. pachydermatis* isolated in this study was used (Table 6.3.1.).

Materials

Sabouraud-Dextrose medium (Oxoid, Unipath Limited, Hampshire, U.K.), Brij-35, margaric acid (17:0), stearic acid (18:0), oleic acid (18:1), and linoleic aicd (18:2) were required. The effect of the detergent, Brij-35, was to create an homogenous suspension of the liquid culture by dispersion of the cells of the yeast colony; this allowed the growth rate of the yeast to be measured by optical density.

Equipment

A spectrophotometer (Philips PU 8680 Spectrophotometer) was used for determination of the optical density of yeast cultures in the growth experiment.

Procedures

A loopful of yeast from a colony was inoculated into 100 ml of Sabouraud-Dextrose medium in a 250 ml Ehrlenmeyer flask and incubated at 37°C for 72 hours on an orbital shaker. Three ml of this culture was used to inoculate flasks of Sabouraud-Dextrose medium containing 0.2% (w/v) Brij-35 and the fatty acids to be tested. Fatty acids were added to the medium just prior to inoculation. Growth rate was followed by determination of the optical density of the culture at 500 nm using the spectrophotometer.

6.2.3 Measurement of yeast protein yield as an indicator of growth in the presence of fatty acids in liquid culture

This step was carried out on samples of culture obtained 72 hours after inoculation of the culture. The method used in this study was modified from the method of Lowry, Rosbrough, Farr, and Randall (1951).

Materials

Reagent A: 2% (w/v) sodium carbonate in 0.1 N sodium hydroxide

Reagent B: 1% (w/v) cupric sulphate pentahydrate

Reagent C: 2% (w/v) sodium potassium tartrate

Folin and Ciocalten's phenol reagent

The standard was bovine serum albumin (Sigma Chemical Company Limited, Dorset, U.K.).

Procedures

Five ml samples were aseptically removed from the culture and centrifuged at 1,500 g for 10 minutes. The pellets so formed were resuspended in 1ml distilled water.

An aliquot of the yeast suspension containing about 100 ug (wet matter) was made up to 1 ml with distilled water. Four ml of alkaline copper reagent (a fresh mixture of 100 ml reagent A, 1 ml reagent B, and 1 ml reagent C) was then added and incubated at room temperature for 10 minutes. Folin and Ciocalten's phenol reagent was added and the solution was then incubated at room temperature for a further 30 minutes. The absorbance at 660 nm was then determined using the spectrophotometer. Protein yield was estimated by comparison of optical density against a standard curve.

6.2.4 The fatty acid composition of *Malassezia pachydermatis*

The fatty acid composition was determined on samples of culture obtained 72 hours after inoculation of the culture.

Materials and equipment

The methyl esters of myristic (14:0), palmitic (16:0), margaric (17:0), Stearic (18:0), oleic (18:1), and linoleic (18:2) acid (Sigma Chemical Company Limited, Dorset, U.K.) were used as standards for G.L.C.(see Chapter V) The chemicals for transesterification and the equipment for G.L.C. were as described in Chapter V.

Procedures

Pellets from cultures, obtained by centrifugation as described above, were washed with distilled water before carrying out lipid extraction

by the method of Folch *et al* (1957). Lipid extracted in this way was subject to transesterification by the method of Christie (1982). The procedures for lipid extraction and transesterification were as described in Chapter V. The G.L.C. analysis was carried out isothermally at 200°C with nitrogen as the carrier gas (see G.L.C. section in Chapter V).

6.2.5 Lipase assay

The lipase activity of strain 5 was determined by the production of 4-nitrophenol from 4-nitrophenyl palmitate (Paznokas and Kaplan, 1977).

Materials

Sabouraud-Dextrose medium (Oxoid, Unipath Limited, Hampshire, U.K.), Tween 20, Tween 40, and Tween 85 (BDH Limited, Glasgow, U.K.) were used as the culture base and culture supplement. The supplement, Tween 20, consists mostly of short-chain fatty acid; 50% lauric acid (12:0) balanced primarily by myristic (14:0), palmitic (16:0), and stearic (18:0) acids. Tween 40 consists of 90% palmitic acid whilst the remaining 10% is stearic acid. Tween 85 contains 70% oleic acid (18:1), the balance being composed of elaidic (18:1), linoleic (18:2), and palmitic acids. The chemicals used for the measurement of growth of the yeast were as described above. The buffer for sonication was 0.05 M potassium phosphate, pH 7.5. The substrate, 4-nitrophenyl palmitate was initially dissolved in 1 ml dimethyl formamide and then dispersed in 1% (w/w) Triton X-100 in 0.05 M Tris-HCl pH 8.5. This gave a final concentration of 0.032 M.

Equipment

The equipment required consisted of an orbital shaker, spectrophotometer (Philips PU 8680) and spectrophotometer 4050 UV/Visible (LRB, Ultraspec II)

Procedures

A loopful of yeast from a colony was inoculated into 100 ml of Sabouraud-Dextrose medium in a 250 ml Ehrlenmeyer flask and grown for 72 hours at 37°C on an orbital shaker. Five ml of this culture was inoculated into Sabouraud-Dextrose medium containing 0.1% (v/v) Tween 20, or 0.1% (v/v) Tween 40, or 0.1% (v/v) Tween 85. The cultures were incubated for 27 hours at 37°C on an orbital shaker. The growth rate measurement was carried out as described above.

Samples (5 ml) of culture were harvested as described above, and the pellets were resuspended in 5 ml 0.05 M KH_2PO_4 buffer and sonicated. One hundred ul of the sonicated sample was added to one ml of the substrate solution and incubated at 37°C for four minutes. The production of nitrophenol palmitate, was determined using a spectrophotometer, measuring the absorbance at 400 nm. The amount of nitrophenol produced was determined as a measure of lipase activity.

6.3 RESULTS

6.3.1 Isolation and characteristics of *Malassezia pachydermatis*

Ten *M. pachydermatis* strains were isolated from canine external ear canals and skin. Two types of this organism were found. Both comprised cells with the characteristic "peanut-shaped" morphology of *M. pachydermatis* (Figure 6.3.1). The commonest isolate was characterised by ease of growth on Sabouraud agar and the formation of large colonies, about 3 mm diameter, after 72 hours growth. A second type, which was isolated less often, formed small colonies, measuring only about 1 mm diameter after 72 hours growth, proved very difficult to subculture and became non-viable after two passages. A single isolate showed characteristics intermediate between these two types, changing from small to large-colony type after the second passage. The sources and types of colony of *M. pachydermatis* isolated are shown in Table 6.3.1.

6.3.2 The fatty acid composition of *Malassezia pachydermatis*

The fatty acid composition of six large-colony isolates, one small-colony isolate, and one intermediate isolate were analysed. Different types of isolate had different fatty acid compositions. The fatty acid composition of strains of the large colony type were relatively constant. Palmitic acid (16:0) 24.9%, linoleic acid (18:2) 35.6%, oleic acid (18:1) 27.5%, and stearic acid (18:0) 12.0%, were the major fatty acids (Table 6.3.2).

The one small-colony type (strain 3) analysed showed a much reduced content of linoleic acid compared to the large colony type and oleic acid was not detected. This strain also contained several unidentified fatty acids (Table 6.3.3).

The fatty acid composition of the one intermediate type isolated showed some similarities with that of the large type, having a relatively high

content of palmitic and linoleic acids, but it had a relatively low oleic acid content (Table 6.3.2).

6.3.3 Effects of exogenous fatty acids on the growth of the large colony type of *Malassezia pachydermatis*

Saturated fatty acids, margaric (17:0) and stearic (18:0) acids, at concentrations between 0.005 and 0.1% (w/v) did not cause large changes in the growth rates or growth yields of the large colony type (Table 6.3.4 and Figures 6.3.2a and 6.3.2b). Oleic (18:1) and linoleic (18:2) acids, the unsaturated fatty acids, caused a large reduction in growth rates at concentrations between 0.005 and 0.1% (v/v) (Table 6.3.4 and Figures 6.3.3a and 6.3.3b), but the growth yields in terms of protein, were not greatly changed (Table 6.3.5).

6.3.4 Effect of the inclusion of fatty acids in the growth medium on the fatty acid composition of the large colony type of *Malssezia pachydermatis* (strain 5)

The culture without additional Brij-35 failed to produce a constant absorbance for reproducible results when growth rate was determined by optical density. In the absence of this detergent the yeast grew in a pellet form and, as result, a reliable growth rate for *M. pachydermatis* could not be obtained in the absence of Brij-35. However, a change in fatty acid composition was caused by Brij-35, compared to the fatty acid composition of the yeast grown in the absence of the detergent. In the yeast grown with Brij-35, palmitic acid (43.9%) was increased, but linoleic acid (20.5%) was decreased (Tables 6.3.2 and 6.3.6).

Increasing the concentrations of saturated fatty acids, margaric (17:0), and stearic (18:0), in the medium increased the relative amount of that fatty acid found in the yeast. There were parallel decreases in all the

other fatty acids normally present in the yeast as the percentage of the added fatty acid increased (Table 6.3.6).

On the other hand, the effects of the addition of unsaturated fatty acids, oleic (18:1) and linoleic (18:2) to the medium were complicated. Supplementation with both these unsaturated fatty acids led to the appearance of a "new" fatty acid in the yeast which had an equivalent chain length of 16.01. Exogenous linoleic acid resulted in a large reduction in the palmitic acid content and an increase in the stearic acid content of the yeast. The percentage of palmitic acid decreased to less than 5%. Stearic acid became the major saturated acid present, reaching about 60% of the total fatty acids at all three concentrations of added linoleic acid. Added linoleic acid generally reduced the relative amount of oleic acid in the yeast, but the effect was not consistent. The lowest concentration of supplemented linoleic acid led to a reduction in its content in the yeast. The higher concentrations caused a small increase in this fatty acid from this low level. In the presence of exogenous oleic acid, the percentage of palmitic (16:0) and linoleic acids were decreased at the highest concentration of oleic acid added. The lowest concentration of oleic acid supplementation reduced the relative amount of that fatty acid found in the yeast; whilst the highest concentration of added oleic acid increased its content in the yeast (Table 6.3.6).

6.3.5 Lipase activity

Lipase was detected in sonicated samples of the yeast (strain 5) and also in the culture supernatant. A higher lipase activity was detected from the yeast that was grown on the media supplemented with Tweens (Table 6.3.7).

Figure 6.3.1 The "peanut-shaped" morphology of *Malassezia pachydermatis* (strain 5) under a microscope with oil immersion (Gram, 1125x).

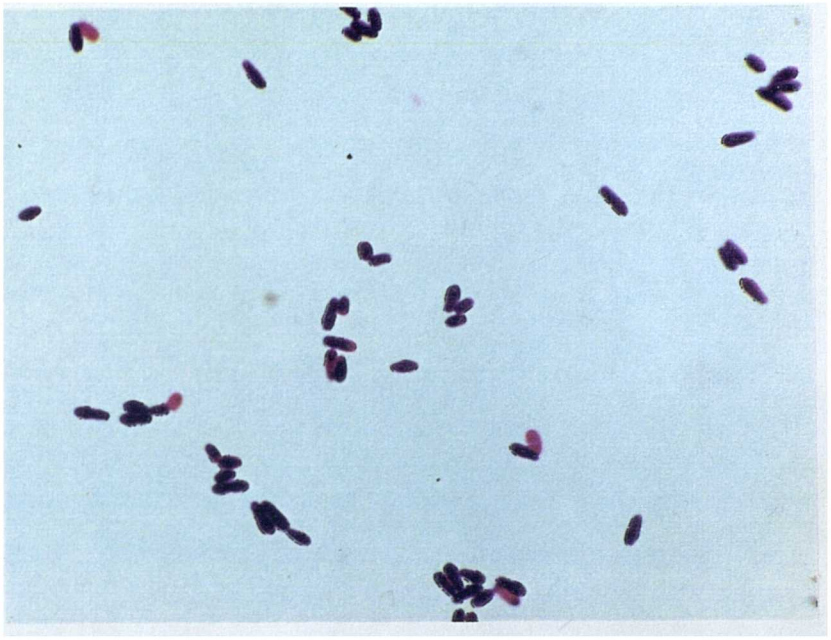


Table 6.3.1 The source and colonial morphology of the *Malassezia pachydermatis* strains isolated.

Strain	Source	Colonial morphology
1	skin	large
2	skin	large
3	skin	small
4	ear	small
5	skin	large
6	skin	large
7	skin	large
8	ear	intermediate
9	ear	large
10	ear	large

Table 6.3.2 The fatty acid composition (% by weight of fatty acid methyl ester) of six strains of the large-colony type of *Malassezia pachydermatis* and the single strain which exhibited intermediate morphology.

	% by weight of F.A.M.E.			
Strain	C16:0 palmitic	C18:2 linoleic	C18:1 oleic	C18:0 stearic
2	22.69	35.74	29.32	12.36
5	27.73	35.44	25.44	11.40
6	25.06	32.79	34.00	7.84
7	24.53	31.30	24.19	19.87
9	24.36	38.64	25.16	11.81
10	24.93	39.50	26.64	8.94
Mean	24.88	35.57	27.46	12.04
S.D.	1.63	3.19	3.66	4.22
8 (intermediate)	34.29	44.04	12.74	9.01

F.A.M.E. = fatty acid methyl ester

Table 6.3.3 The fatty acid composition (% by weight of fatty acid methyl ester) of the one strain (3) of small-colony type *Malassezia pachydermatis* investigated.

Fatty acid [E.C.L.]	**% by weight of F.A.M.E.**
C14:0	12.64
[C14.70]	6.28
[C15.20]	7.69
C16:0	21.10
[C16.01]	9.34
[C16.16]	8.77
C18:2	11.70
C18:1	-
C18:0	13.50
[C19.93]	8.87

E.C.L. = equivalent chain length

F.A.M.E. = Fatty acid methyl ester

Table 6.3.4 Mean generation time (hours) of *Malassezia pachydermatis* (strain 5) growing in Sabouraud-Dextrose media supplemented with different fatty acids.

	Concentration of added fatty acid			
Fatty acid	0%	0.005%	0.01%	0.1%
Control (0.2% Brij-35)	3.72 3.63 3.39 3.36			
C17:0		3.97 3.65	3.98 3.91	3.80 3.66
C18:0		3.41 3.46	3.67 3.85	3.48 3.22
C18:2		6.35 4.05	6.13 8.84	9.36 8.62
C18:1		3.71 4.38	5.39 5.23	7.02 8.35

Assays were performed in duplicate except for the control where n=4.

Figure 6.3.2a Growth curves of the yeast in Sabouraud-Dextrose medium with added margaric acid.

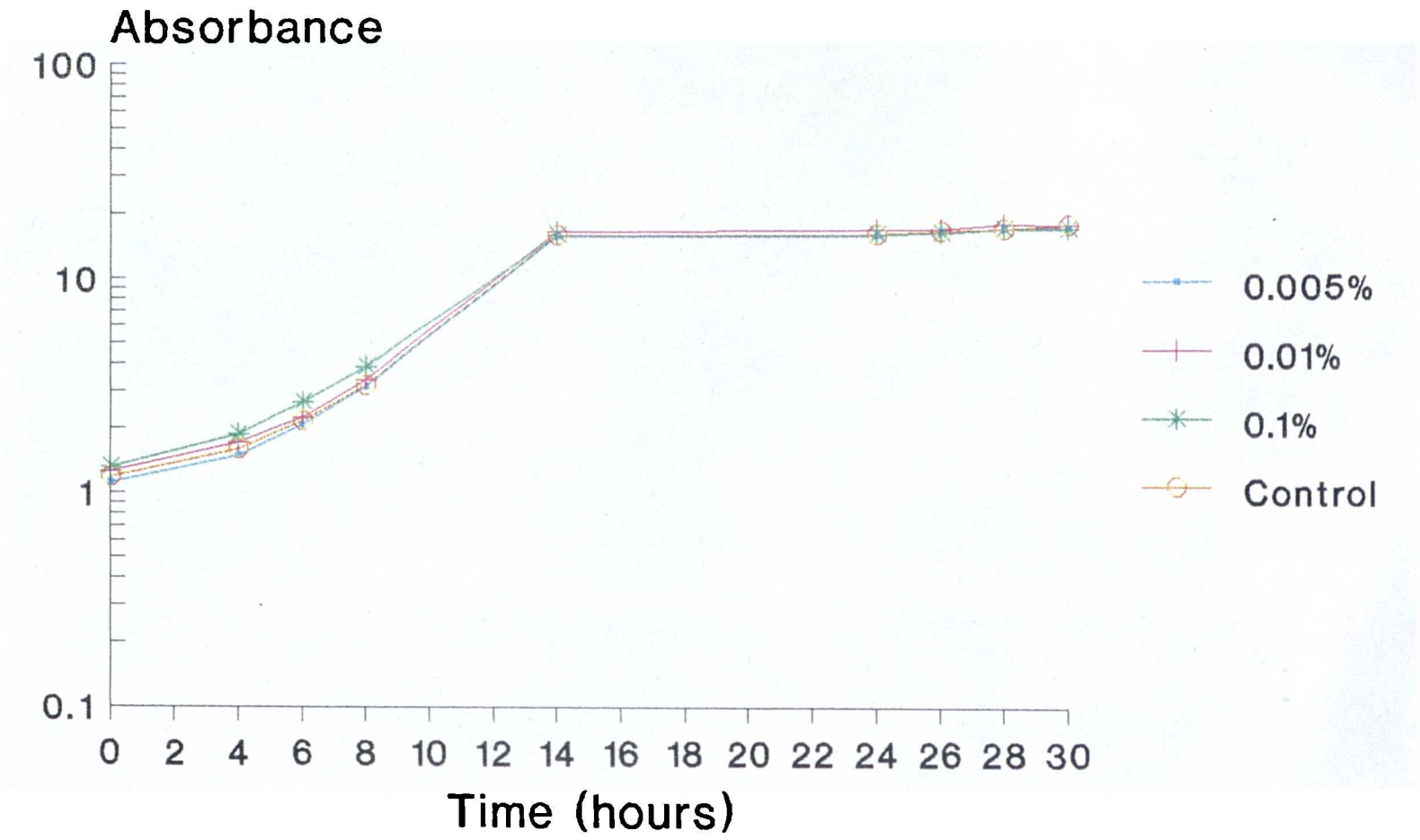


Figure 6.3.2b Growth curves of the yeast in Sabouraud-Dextrose medium with added stearic acid.

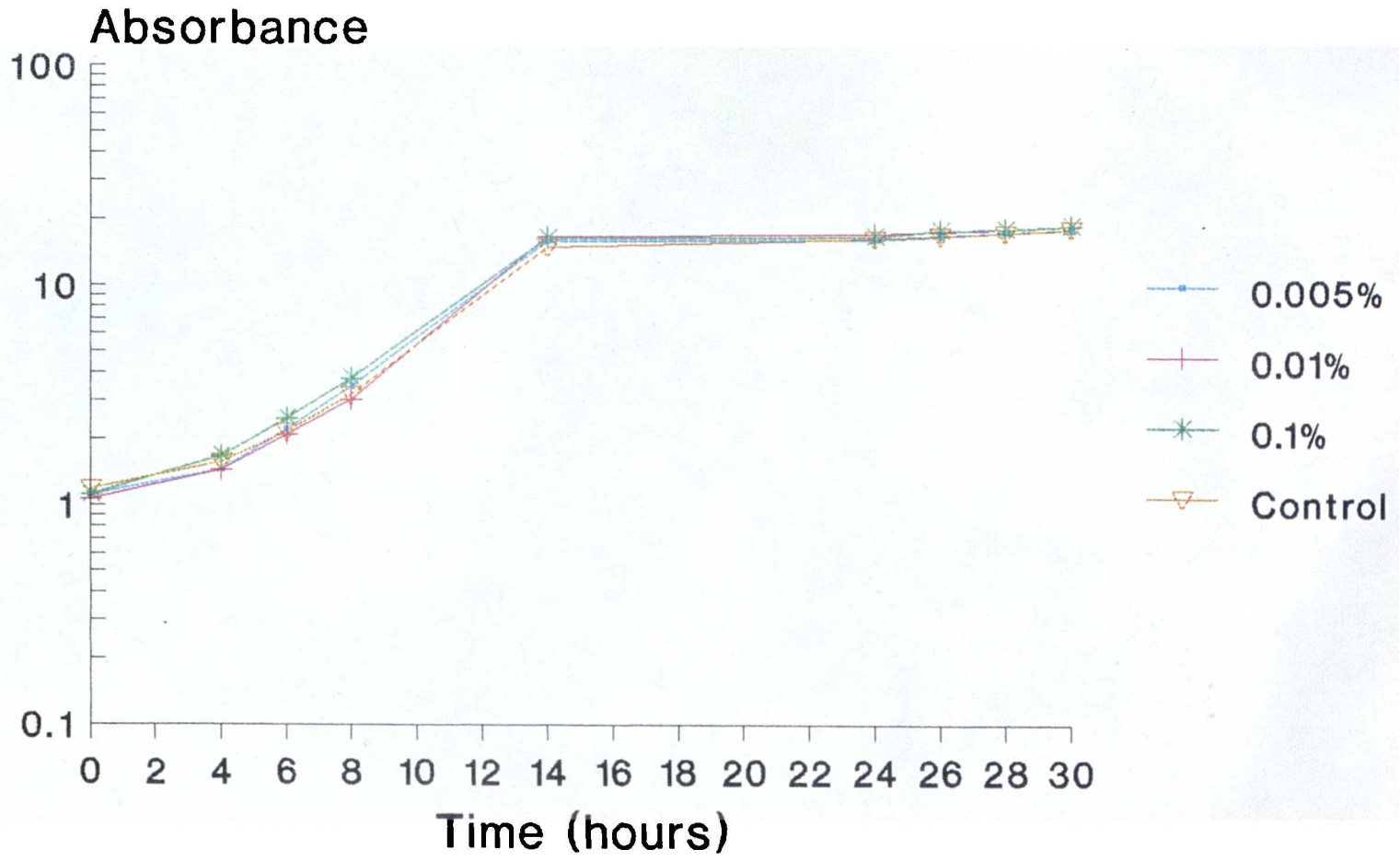


Figure 6.3.3a Growth curves of the yeast in Sabouraud-Dextrose medium with added linoleic acid.

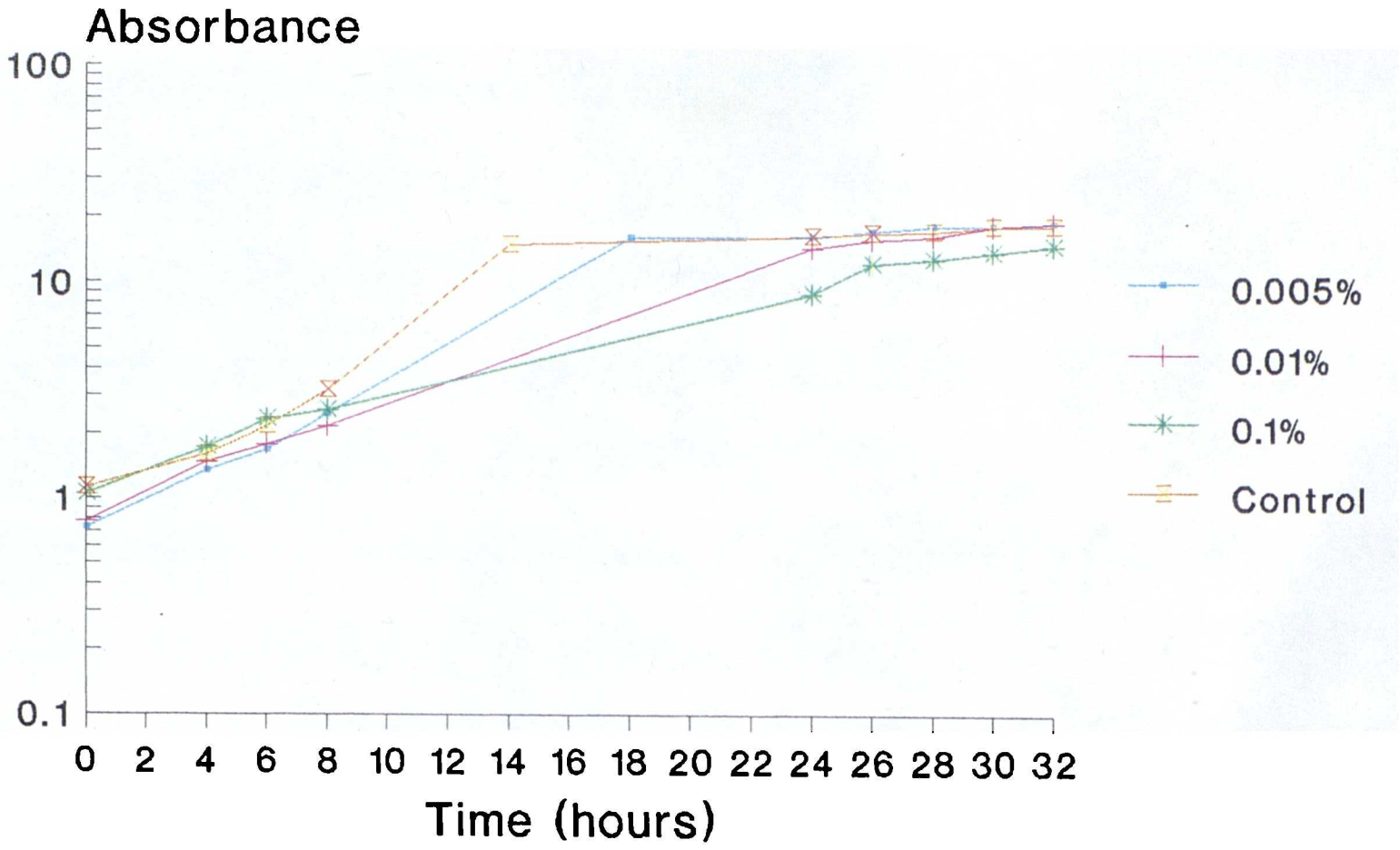


Figure 6.3.3b Growth curves of the yeast in Sabouraud-Dextrose medium with added oleic acid.

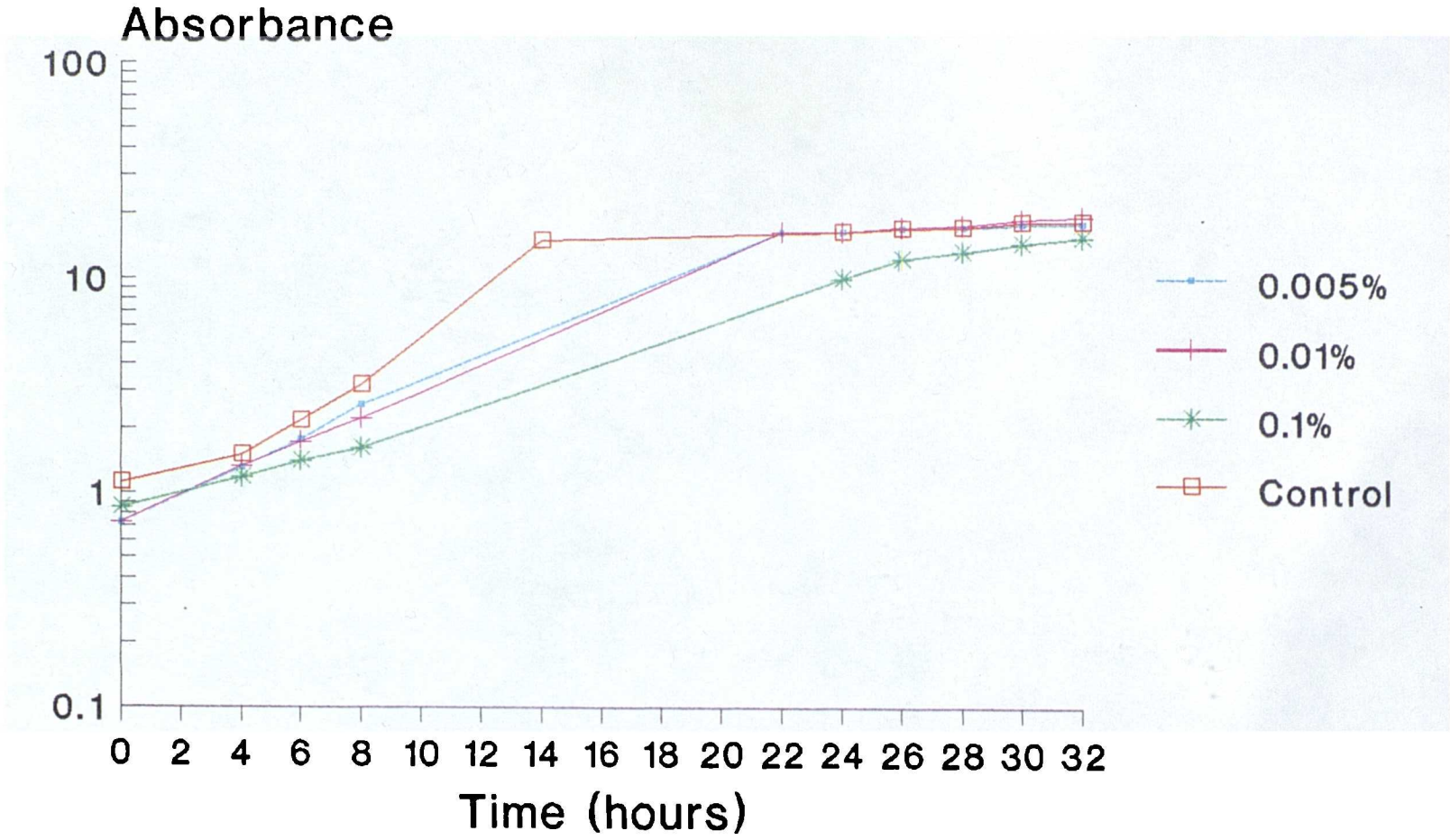


Table 6.3.5 Final growth yield (mg yeast protein/ 100 ml) of *Malassezia pachydermatis* (strain 5) grown in Sabouraud-Dextrose media supplemented with different fatty acids.

	Concentration of added fatty acid			
Fatty acid	0%	0.005%	0.01%	0.1%
Control (0.2% Brij-35)	31.0			
C17:0		29.8	32.5	29.6
C18:0		29.7	36.0	28.6
C18:2		28.2	30.0	30.8
C18:1		31.0	31.0	30.1

Table 6.3.6 Fatty acid composition (% by weight of fatty acid methyl ester) of *Malassezia pachydermatis* (strain 5) grown in Sabouraud-Dextrose medium with supplemented fatty acids.

	% by weight of F.A.M.E.					
Fatty acid supplement	C16:0	NFA*	C17:0	C18:2	C18:1	C18:0
C17:0						
0.005%	17.93	-	20.93	16.30	22.18	21.95
0.01%	14.88	-	43.52	9.82	14.28	17.02
0.1%	1.62	-	89.72	3.08	3.67	1.07
C18:0						
0.005%	18.52	-	-	17.39	23.00	41.10
0.01%	16.38	-	-	20.57	21.63	42.42
0.1%	7.13	-	-	2.81	3.54	86.24
C18:2						
0.005%	4.41	20.44	-	4.59	6.46	64.10
0.01%	3.29	5.08	-	8.96	23.19	59.19
0.1%	2.96	8.79	-	12.41	18.15	57.69
C18:1						
0.005%	20.36	25.04	-	15.48	19.28	19.39
0.01%	21.93	20.77	-	21.64	21.62	14.02
0.1%	10.55	32.14	-	9.61	31.68	16.02
Control						
0.2% Brij-35	43.93	-	-	20.54	26.49	8.75

*NFA = New fatty acid with equivalent chain length 16.01

F.A.M.E. = fatty acid methyl acid

Table 6.3.7 The lipase activity of *Malassezia pachydermatis* grown in Sabouraud-Dextrose medium supplemented with fatty acid rich media (PNP nmole/mg protein^{-1} min^{-1}).

Added fatty acid rich medium	nmole *PNP mg protein^{-1} min^{-1}
Tween 20	11.3
Tween 40	9.9
Tween 85	11.2
Control	7.1

*PNP = product of nitrophenol palmitate

6.4 DISCUSSION

Malassezia pachydermatis is one of many agents in the multifactorial aetiology of canine external otitis (Gedek *et al*, 1979). Recently this microorganism has been incriminated as a major cause of canine otitis externa (Gabal, 1988) and has also been implicated in cases of canine dermatitis (Scott and Miller 1989; Evans 1991; Mason and Evans 1991; Mason 1992; Plant *et al*, 1992). In the present study, *M. pachydermatis* was isolated from both inflamed ears and skin. Different colony sizes of *M. pachydermatis* were found among these isolates. The colony size of the yeast allowed easy differentiation into two phenotypes, one forming large colonies and another which formed small colonies. Under microscopical examination, both phenotypes of *M. pachydermatis* had the characteristic peanut-shaped appearance described for this yeast (Gabal 1988). These two phenotypes also showed differences in fatty acid composition. A strain which had changed from a small colony into large colony phenotype after a second passage on Sabouraud medium, had a fatty acid composition that could not be categorised into the pattern of fatty acids found in either the large- or the small-colony strains. In this study, only three strains of small colony type *M pachydermatis* were isolated, and each of these strains were not viable after two passages *in vitro*. It is possible that the environmental conditions of the external canal or skin surface are more favourable to the survival of the small colony type. This may deserve further study.

The exact trigger which causes *M. pachydermatis* to become pathogenic is not clear. A change in the microclimate of the ear canal, such as an increase in moisture content or excessive accumulation of cerumen (Gabal 1988; Mansfield *et al*, 1990; Mason and Evans 1991), or defects in host-defence mechanisms (Scott and Miller 1989), have all been

proposed as triggers. Growth-inhibitory activity of human cerumen against *Candida* species has been demonstrated by Megarry *et al* (1988). In contrast to these results, Gabal (1988) has shown the ability of canine cerumen to promote the growth of M. *pachydermatis*. In that study, there was no investigation of the composition of the cerumen nor of the effective concentration of cerumen used, particularly of its lipid components. Because the factors in the cerumen that promoted the growth of *M. pachydermatis* were not identified, it is difficult to reconcile this finding with other studies which have shown bactericidal and mycocidal effects of the fatty acid components of cerumen (Chai and Chai 1980; Megarry *et al*, 1988). Changes in the composition of cerumen or skin lipids might play a role in promoting or inhibiting the growth of *M. pachydermatis*. Previous work in this study (Chapter V) indicated that margaric, stearic, oleic and linoleic acids are common fatty acids in cerumen lipids. Margaric and stearic acids, saturated fatty acids containing odd and even numbers of carbon atoms respectively, did not change the mean generation time or the final growth yield of the yeast. The lipid analysis of the yeast indicated that *M. pachydermatis* had incorporated these exogenous fatty acids and this changed the lipid composition of the yeast itself. Among the fatty acids of canine cerumen, oleic and linoleic acids have been found in other studies to have antibacterial activity (Gutteridge *et al*, 1974; Knapp and Melly, 1986). They are fatty acids commonly found on human skin which exhibit strong growth-inhibitory activity on microorganisms (Kanai and Kondo 1979). The increased mean generation times without change in the final growth yields in the present studies caused by addition of these unsaturated fatty acids indicates that these are mycostatic not mycocidal for *M. pachydermatis*. No data are available to indicate the concentration of fatty acids in solution on the ear canal surface. Thus, it is difficult to say

whether or not the unsaturated fatty acids of cerumen act as mycostatic agents *in vivo*.

It has been proposed that the bactericidal or bacteriostatic activity of unsaturated fatty acids is either mediated by a peroxidative process involving hydrogen peroxide and bacterial iron (Knapp and Melly 1986), or an autoxidation process involving only the unsaturated fatty acids (Gutteridge *et al* 1974). The target attacked by the fatty acid may also be the respiratory enzyme associated with the cytoplasmic membrane in bacteria (Kanai and Kondo 1979). The change in the fatty acid composition of *M. pachydermatis* lipids caused by exogenous unsaturated fatty acids may contribute to the mycostatic effects of these fatty acids. The unexpected effect of exogenous fatty acids in reducing the amount of unsaturated fatty acid in the yeast may be part of the mycostatic action. It is known that biological membrane lipid fluidity, which is controlled by the ratio of saturated to unsaturated fatty acids present in membrane lipid, is critical to normal cell function (Cullis and Hope 1985). Thus, the changes caused by exogenous unsaturated fatty acids may lead to changes in membrane fluidity which slow growth.

The increased lipase activity of the yeast grown in the presence of fatty acid (Tweens) may be part of the adaptation of *M. pachydermatis* to an altered environment. A new fatty acid, with an equivalent chain length of 16.01 was found in yeast incubated in Sabouraud medium with unsaturated fatty acids. This new fatty acid may have been generated by the action of lipoxygenases, which have been reported in *Malassezia* species and are capable of transforming unsaturated fatty acids into unusual fatty acids (Nazzaro-Porro and Passi, 1978; Nazzaro-Porro, Passi, Picardo, Mercantini, and Breathnach, 1986). Nazzaro-Porro (1987) found that lipoxygenases of *Malassezia* species are capable of oxidizing oleic acid into azelaic acid (C_9 dicarboxylic acid) which has antimicrobial activity

against *Staphylococcus* . The presence of lipase indicates that *M. pachydermatis* has the potential to degrade cerumen lipid. Consequences of the lipolytic action of *M. pachydermatis* might be to cause changes to the microenvironment as the lipase liberates antimicrobial fatty acids from the cerumen lipids, and the lipoxygenase oxidises these into materials cytotoxic to other resident flora (Nazzaro-Porro, 1987). The lipase also may produce short-chain fatty acids from cerumen lipids; short-chain fatty acids (C_8-C_{12}) irritate skin (Kellum, 1968; Weary, 1970). In conclusion this yeast might participate in modulating canine aural microbial flora, but it may also be a potential pathogen. Further studies concerned with the metabolites of *M pachydermatis* produced *in vitro* and *in vivo* would be useful in exploring its role as a pathogen in canine otitis externa.

CHAPTER VII

CONCLUSIONS

7.1 CONCLUSIONS

Some aspects of the microenvironment of the canine external ear canal were investigated in the present study. The cartilaginous part of the canine external ear canal measured 5.3 cm in length on average in a series of 20 dogs. This measurement differs widely from the value of 2 cm reported by Getty *et al* (1956). The canine externa ear is funnel shaped and forms a narrow neck at its most proximal part. It is likely to be poorly ventilated as a result of this conformation, and this may predispose the aural skin to microbial overgrowth.

The aural skin of dogs shows some similarities to the skin elsewhere on the body surface. Morphometric evaluation of histological features of this integument revealed that the density and distribution of sebaceous and apocrine gland tissue exhibits marked variation between individuals. Nevertheless, general patterns were observed; sebaceous tissue increases gradually from the proximal to the distal parts of the ear canal, whilst apocrine gland tissue by contrast decreases. This combination of secretory glands, together with the epidermal migration which is known to occur on the tympanic membrane and in the osseous ear canal, might facilitate movement of cerumen outwards (a "self cleansing mechanism") (Johnson *et al*, 1984; Johnson and Hawke, 1986; Youngs *et al*, 1988; Weinberger *et al*, 1990; Smelt *et al*, 1991). By analogy with epidermal turnover, which is thought to participate in regulating cutaneous microflora (McEwan Jenkinson, 1993), this physical process might restrict microbial colonisation of the aural skin surface. However, investigations to examine the nature and consistency of the secretions of the aural sebaceous and apocrine glands themselves in the dog have not been carried out to support this hypothesis. Detailed studies concerning the nature and mode of secretion of these glands might elucidate whether

the patterns of gland distribution in the aural skin have physiological consequences, and what these are.

The healthy canine aural surface is colonised by a variety of microorganisms. A similar phenomenon has been reported on the skin surface of many mammalian species, including man (Lloyd, 1993a; McEwan Jenkinson, 1993). The resident flora in the canine external ear are predominantly Gram-positive, coagulase-negative cocci, and the yeast, *Malassezia pachydermatis*. These microorganisms are present in low numbers as exhibited by the sparsity of these on cerumen cytological examination and the low growth density found on microbiological culture of cerumen from healthy ears.

Two biochemical investigations of canine cerumen were undertaken in the present study. Firstly, identification and quantitation of immunoglobulins were carried out. The antibody classes, immunoglobulin A (IgA), immunoglobulin G (IgG), and immunoglobulin M (IgM) were detected in canine cerumen. Immunoglobulin G was most predominant in cerumen from healthy dogs. Immunoglobulin A and IgG have also been demonstrated in human cerumen (Petrakis *et al*, 1971). Secretory immunoglobulins are generally believed to contribute to local host defence on the skin surface (Tourville *et al*, 1969; Tomasi, 1972; Main and Lim, 1976; Metze *et al*, 1988; Kawai *et al*, 1991; Metze *et al*, 1991). These findings indicate that canine cerumen may possess specific antimicrobial activity.

The second biochemical investigation in the present study showed that lipids are a major component of canine cerumen; they account for 50% of the wet matter of cerumen on average, although a wide variation of the lipid content was found (ranging from 18% to 93%). Eight lipid classes were identified in cerumen. Cholesterol was the only lipid class present in all cerumen samples analysed. Marked variation in the cerumen lipid

content may be a result of the great variation in the distribution of secretory glands in the aural skin amongst individuals. Canine cerumen fatty acid composition was studied in detail; quantitative and qualitative variation in these components was also found. Generally, saturated and unsaturated fatty acids with carbon chain lengths of 16 or 17, together account for 70% of the fatty acids of cerumen. No correlation could be found between cerumen fatty acids and fatty acids from the microflora of these ears. This result indicates that the fatty acids in cerumen are produced endogenously. However, the possibility that cerumen fatty acids are altered by lipolysis or other activities of the resident flora cannot be excluded.

The identity of the fatty acids in canine cerumen was confirmed by gas chromatography and mass spectrometry. The antimicrobial unsaturated fatty acids (Butcher *et al*, 1976; Kanai and Kondo, 1979; Lacey and Lord, 1981), oleic (C18:1) and linoleic (C18:2) acids, demonstrated a mycostatic effect on the growth of *Malassezia pachydermatis in vitro*. This finding supports the view that canine cerumen may be capable of regulating the resident flora *in vivo*.

Several factors are generally thought to contribute to the low density of the resident flora on the aural skin surface of healthy dogs (Scott and Miller, 1989; Mason, 1992). In the present study, at least two constituents of cerumen, antimicrobial unsaturated fatty acids and immunoglobulins (IgA, IgG, and IgM) were found to be important. The presence of these components in canine cerumen may serve to create a biochemical barrier preventing microbial invasion of the aural integument. However, the functions of other biochemical components of canine cerumen are poorly understood. In order to define more clearly the physical and chemical functions of cerumen, studies might address such issues as the degree of hydration, the concentration, activities and effects

of inorganic ions, microbial nutrient sources including vitamins, and the partial pressures of gases such as carbon dioxide and oxygen.

In the present study, hyperplastic sebaceous and apocrine glands were found in the aural integument of dogs with otitis externa. However, the pathological changes in the aural integument are non-specific and cannot confirm the underlying aetiology of canine otitis externa. The hyperplastic appearance of sebaceous and apocrine glands may be a consequence of the inflammatory process.

Microbial overgrowth is a common finding in canine otitis externa. The microorganisms found in association with canine otitis most frequently were Gram-positive, coagulase-positive staphylococci, Gram-positive, haemolytic streptococci, Gram-negative rods, and *M. pachydermatis*. Differences in the microbial species and the numbers of these in canine ears affected by otitis compared to the normal microflora indicate that these microorganisms may be related to the pathological process. However, on healthy skin, cutaneous flora seems to be regulated by host defence mechanisms (McEwan Jenkinson, 1993). It is likely that where microbial overgrowth occurs this is induced by alterations intrinsic to the host.

In the present study, the proportion of IgA in cerumen from canine ears affected by otitis externa was significantly lower than in cerumen from healthy ears. Immunoglobulin deficiency is a well known predisposing factor to malignant external otitis externa in humans (Wilson *et al*, 1971; Petrozzi and Warthan, 1974; Yust *et al*, 1980; Gherini *et al*, 1986; Babiatzki and Sade, 1987; Castro *et al*, 1990; Cohen, 1990; Strauss, 1990). This phenomenon might either be induced by microbial overgrowth or may be a consequence of it. Low levels of IgA could arise either from decreased production or excessive consumption (Whitbread *et al*, 1984; Moroff *et al*, 1986; Day and Penhale, 1988). Moreover, increased levels of IgG may be

a compensation for IgA deficiency or might be induced by microbial overgrowth (Whitbread *et al*, 1984). The antibodies in cerumen deserve further study to clarify the specific functions of these and whether a true deficiency of immunoglobulin contributes to canine otitis externa. More specific treatment for otitis externa might thus be developed in the future.

Microbial colonisation of the aural surface may also fluctuate as a result of alteration to secretions on the skin surface. The availability of nutrients, microbial inhibitors, and physical factors, such as skin surface pH and moisture may be related to dynamic changes in the secretory glands. In the study of histopathological morphology, the hyperplastic appearance of these glands does not necessarily indicate their secretory activities or the nature of the secretion. Significant differences in cerumen fatty acid composition and the relative abundance of immunoglobulin classes in cerumen from otitic ears compared to healthy ears may suggest that these alterations to cerumen are related to the morphological changes in the adnexal glands. However, at present the physiology of the sebaceous and apocrine glands of the canine aural integument is not completely understood. The pathophysiological effects of visibly hyperplastic secretory glands in canine otitis externa will remain obscure until the dynamic pathophysiology of these glands is investigated more fully.

In the study of cerumen fatty acid composition, fatty acids with a carbon chain length of 18 or 20 were significantly increased in cerumen from ears affected by otitis. The meaning of this biochemical change in cerumen lipid content is unclear but it might be a consequence of the inflammatory process of the integument. These fatty acids are precursors of pro-inflammatory and immunomodulating mediators (Chapkin *et al*, 1986; Ruzicka *et al*, 1986; Schafer and Kragballe, 1991). On the other hand, these fatty acids are also involved in restoration of the barrier

function of the epidermis (Prottey, 1977; Elias and Brown, 1978; Elias *et al*, 1980). Significantly elevated levels of unsaturated C18 and C20 fatty acids in cerumen from ears affected by otitis might indicate that their role lies in restoration of the physiological barrier of the epidermis. However, further research into the metabolism of the canine aural integument is required to elucidate the pathophysiological role of cerumen in otitis externa.

Malassezia pachydermatis differs from other members of the resident flora of the canine aural integument in that large numbers of this yeast were also found frequently in association with canine otitis. Two phenotypes of *M. pachydermatis* were isolated during this study. These two phenotypes also exhibit differences in their cell membrane fatty acid composition. In the present study, the factors required for growth of the small colony-type *M. pachydermatis in vivo* and *in vitro* have not been defined.

Overgrowth of *M. pachydermatis* in both canine otitis and dermatitis is generally believed to exacerbate the local inflammation (Mason, 1992). The condition that triggers the overgrowth of this yeast and its pathogenesis is still unclear. In the present study, *M. pachydermatis* was shown to possess lipase activity. Greater lipase activity was induced by the presence of fatty acids known to be found in canine cerumen. This finding suggests that *M. pachydermatis* is capable of utilizing cutaneous lipids. Further studies to investigate the interactions between this yeast and cerumen, such as other enzyme activities, the metabolites of this microorganism, and the interactions of its metabolites with other resident flora, would be helpful to understand the pathological role that this yeast may play in canine otitis externa.

From the present study, cerumen cytological examination appears to be a useful index of microbial identity (Gram's categories) and microbial overgrowth in cases of canine otitis externa. Together with a clinical

evaluation of the patient, this simple investigation can help a clinician to select appropriate antibiotic treatment. However, this method alone cannot establish the pathogenesis of otitis.

Cerumen colour has been used by clinicians as a rule of thumb to give an indication of the microorganisms associated with otitis externa (Griffin, 1981; August, 1986; Woody and Fox, 1986b; Chester, 1988; Macy, 1989). In the present study, cerumen was classified into two arbitrary colour types; yellow and brown. No correlation could be found between cerumen colour and the microorganisms present. Moreover, the colour of cerumen could not indicate the associated microflora if the ears are colonised by more than one microorganism, a situation which is very commonly found in both healthy and otitic ears.

In the present study, only certain aspects of the aural microenvironment were investigated. Availability of nutrients and optimal physical factors are generally believed to be necessary for microorganisms to colonise the skin surface (Marples and Kligman, 1969; Bibel and LeBrun, 1975; Noble, 1975; Kligman *et al*, 1976; Aly *et al*, 1978; Cove *et al*, 1980; Faergemann *et al*, 1983; McBride, 1993). The skin surface secretions provide a source of organic and inorganic substances for cutaneous microbes (Usher, 1928; Arnold *et al*, 1930; Pillsbury and Rebell, 1952; Cove *et al*, 1980; Holland, 1993). On the other hand, these microorganisms also seem to be selected by factors intrinsic to the host (Arnold *et al*, 1930; Burtenshaw, 1938; Burtenshaw, 1942; Chai and Chai, 1980; Stone and Fulghum, 1984; Megarry *et al*, 1988; McEwan Jenkinson, 1993). Cerumen fatty acids and immunoglobulin are the only two host defence mechanisms to have been explored in this thesis. The homeostasis of the aural microenvironment is unlikely to depend on these two regulators alone. Other cerumen lipids and proteins may fulfill other physiological functions, such as contributing to the physical barrier, or as

stimulants to the growth of certain microorganisms. Knowledge concerning the aetiology, treatment, and prophylaxis of microbial overgrowth and its relationship to otitis externa remains sparse. Further research into both the physical and chemical factors modulating the microenvironment of the canine external ear canal should address this issue.

The understanding of canine otitis externa, its treatment and prophylaxis require a comprehensive knowledge of the physiology of the aural integument. The physical aspects of the external ear canal also require exploration to clarify precisely how conformation is implicated in the high prevelance of otitis externa found in certain breeds of dog.

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