DOCTOR OF PHILOSOPHY

An Investigation of Psoroptes ovis, the Sheep Scab Mite, with a View to Developing an in vitro Feeding System.

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An Investigation of *Psoroptes ovis*, the Sheep Scab Mite, with a View to Developing an *in vitro* Feeding System.

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University of Wales, Bangor

and the Central Veterinary Laboratory, New Haw, Surrey.

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Do not offer to the Lord the blind, the injured or the maimed or anything with warts or festering or running sores. Do not place any of these on the altar as an offering made to the Lord by fire.

LEVITICUS xxii, 22.

Acknowledgements.

This project would have been a far harder task without the valuable guidance, technical help and support of many colleagues and friends.

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This thesis is dedicated to

my brother, Simon John Mathieson,

who was killed on the 24th October 1995.

Summary.

The sheep scab mite, *Psoroptes ovis,* is the causative agent of a disease with significant economical implications and causes severe disease which can be life-threatening if untreated. Research into this ectoparasite is restricted by the expense and practical difficulties entailed with artificially infesting sheep- currently necessary due to the lack of a suitable animal model or an artificial system for culturing the mites off-host. This thesis is an investigation into many aspects of the mites' biology and ecology which were poorly understood but crucial for the development of an *in vitro* culture system.

Investigations into the mites' natural environment and the environmental conditions that produce optimum survival off-host are presented. The nature of the mites' diet was investigated initially by SDS-PAGE and Western blotting followed by light microscopy and immunohistological techniques. With this information several *in vitro* feeding devices were developed and some preliminary investigations into mite survival off-host on a variety of fluid diets were conducted. The structure and ultrastructure of the mites' digestive system was investigated and compared to other related acarines. These studies revealed that the mites' digestive system contained a significant population of bacteria which were isolated and cultured in a separate study. The potential importance of these bacteria is fully discussed.

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Chapter 1.

General Introduction.

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General Introduction.

Psoroptes ovis (Acari: Psoroptidae) is the causative agent of sheep scab or sheep mange and is present throughout the United Kingdom, Europe, the USA, South America and many other countries, approximately 149 in all (Kirkwood, 1986). This astigmatid mite is a non-burrowing, obligate ectoparasite and completes its entire life-cycle at the level of the epidermis at the base of the fleece of its ovine host. The adult female mites are the largest life stage and measure approximately 700μm in length [Fig. 1]. *P. ovis* are found on a range of domestic animals including cattle, rabbits and goats.

Sheep scab is one of the oldest known diseases of sheep, which was recognised by classical authors such as Cato and Virgil and also, it appears, has reference made to it in the Bible (see introductory quotation). Despite the mite being visible to the naked eye its role as the cause of sheep scab was not determined until 1809 by G. H. Waltz, a German veterinary surgeon (Downing, 1936).

The global distribution of the disease has changed minimally over the years. It appeared to have been eradicated in various countries from time to time, only to be reintroduced some time later. It was successfully eradicated from Australia in 1878, after a previous failed attempt in 1870, by shooting and burning all infested sheep and anything they had contact with. The disease has been reported to have been eradicated in Norway, New Zealand, Canada and Denmark between the end of the 19th and early 20th centuries (references in Kirkwood, 1986). In other countries including Germany, Hungary, Brazil and the UK, there were times when the disease had been eradicated but later there have been resurgences, largely due to infested sheep being reintroduced from other locations. In Britain the disease was successfully eradicated in 1952 but was

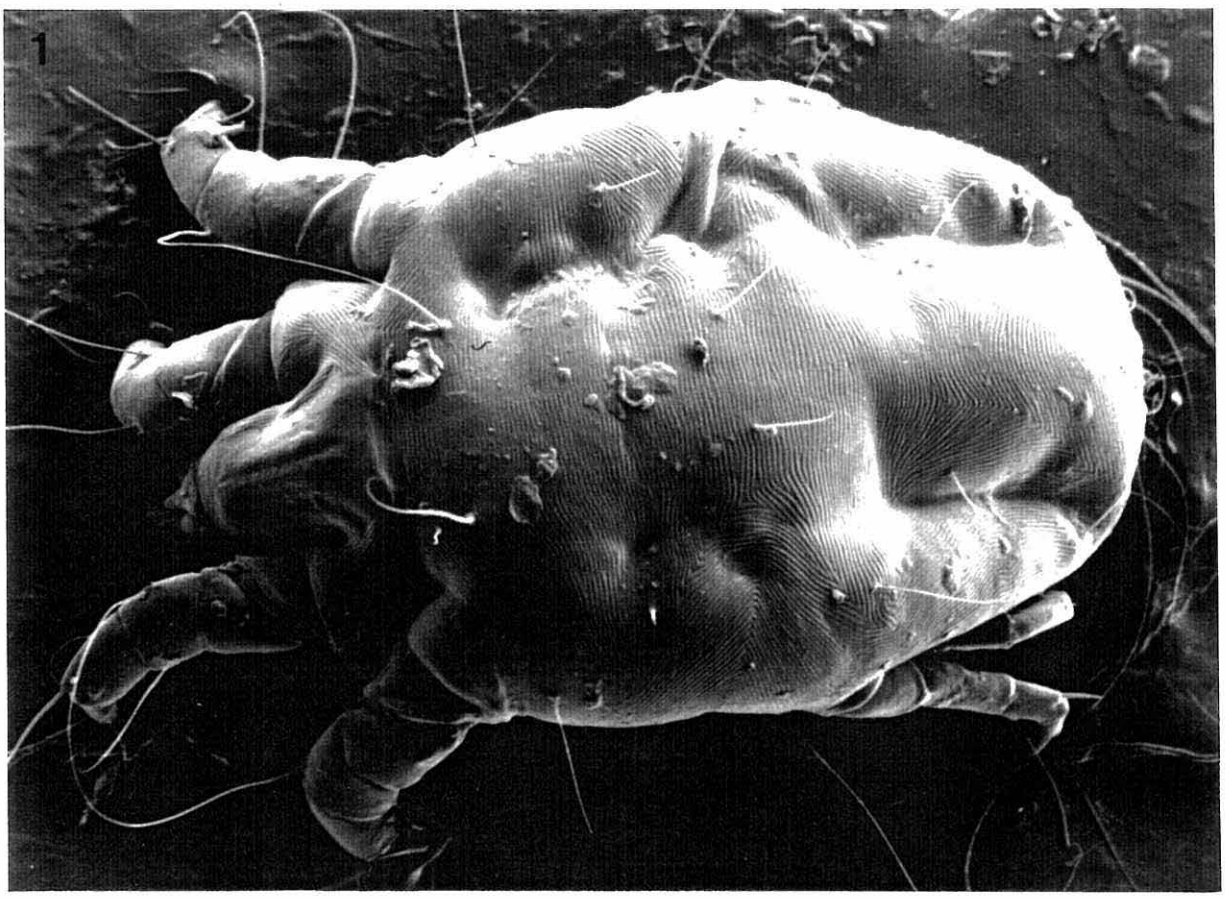

Figure 1. Dorsal view of an adult female *Psoroptes ovis* (Acari: Psoroptidae), total length approximately 700μm.

reintroduced in 1973. However, more recent views of the taxonomic relations of mites (see below) cast doubts on the likelihood of eradication ever having been achieved.

The life history of the mites has been studied by a number of authors which produced some early confusion concerning the duration of each stage, the longevity of adult females and the number of eggs they are capable of producing (see Sweatman, 1958 for a more in-depth discussion). It is now accepted that the mites moult through five stages of development; the egg, larva, protonymph, deutonymph and adult and that the male and female cycles consist of the same number of stages. The egg and larva show no sexual dimorphism, but the sex of the remaining stages is discernible. Adult males form attachment pairs with female deutonymphs and occasionally protonymphs but insemination is not believed to take place before the female finally moults to become an adult (Guillot and Wright, 1983). The above cycle, suggested by Sweatman (1958), should specifically refer to the male cycle. He suggested that the terminology for the last two stages in the female cycle should be pubescent female and ovigerous female respectively. The entire life-cycle from egg to adult takes 14-19 days and each female can lay between 40 and 100 eggs in approximately 2 weeks and live for an average of 40 days (Tarry, 1974; Sweatman, 1958; Bates, 1991). Although is must be emphasised that much of the work that produced these results was carried out off-host, it is believed likely that the results presented above do reflect the situation found in a natural infestation.

Different strains of *Psoroptes* mites are found on sheep, horses, cattle, goats and rabbits. These mites are found both on the body and in the ears of their hosts. Sweatman (1958) believed that *P. cuniculi* from the animals' ears was a different species of *Psoroptes* to that found on the body of the hosts. This is doubtful since *P. ovis* from the body of sheep will also spread to the ears of their hosts and these body mites will readily infest the ears of rabbits also. Furthermore, cross-mating experiments with *P. cuniculi* from rabbits and *P. ovis* from cattle produce viable off-spring (Wright *et al.*, 1983). It is generally accepted that the mites found in the different locations and on

different hosts are the same species, merely different strains. This suggests it is unlikely *P. ovis* has ever been eradicated merely that the disease situation in sheep has been controlled from time to time.

A potentially dangerous situation then exists if it is possible for other animals to act as a reservoir of sheep scab. It appears though, that although it is possible to infest rabbits, cattle and goats with mites that have originated from sheep, the opposite infestation route is not readily successful: mites taken from the ears of rabbits, for example, fail to establish on the bodies of clean sheep although some of the mites do successfully migrate to the ears (Bates, 1991). None of these mites that are routinely found in the ears of infested sheep produce classical sheep scab when used to challenge acaricide-free sheep (Bates, 1991). It appears then that there are perhaps two variants of the same parasite: a benign ear mite and the classical sheep scab mite. The Sheep Scab (National Dip) Order 1990 states that *any Psoroptes* (or *Sarcoptes*) mite found on sheep constitutes sheep scab. This does not take into account the existence of benign ear mites. Before sheep scab was deregulated, farmers could face severe restriction due to mites being found on any of his animals when perhaps only the benign ear mite was found. The situation is further complicated by work carried out in the USA and repeated in the UK which demonstrated that although ivermectin killed 100% of *P. cuniculi* from infested rabbits, there was only 50% mortality when a population of *P. ovis* infesting rabbits' ears was challenged. These surviving mites could then be used to cause classical sheep scab on the body of sheep (Bates, 1991). It appears that populations of *P. ovis* can contain both the benign and the pathogenic variants and that ivermectin can select out the *P. ovis* component. It is probable that *P. cuniculi* and *P. ovis* are variants of the same species, and with time migrate to inhabit different ecological niches demonstrating their ecophenotypic variation.

An infestation of *P. ovis* generally follows one of two developmental paths. The disease either progresses acutely or chronically depending on the strain of mite, breed of sheep, immune status of the host and undoubtedly other variables that are poorly

understood. The major difference between these two manifestations is the speed with which the disease progresses, the degree of fleece loss and the perceived distress caused to the animals. Ignoring for the present the time course over which the disease develops, a typical infestation may proceed as follows: A small number of mites and/or eggs, perhaps as few as 3-5, are transferred onto a non-infested sheep. This may be by direct contact between an infested and non-infested animals or indirectly from sheep enclosures, trailers or scratching areas that have recently been used by at least one infested animal. Within two days small vesicles surrounded by areas of inflammation develop where the mites are found. These vesicles have a waxy appearance due to the production of a moist serous exudate. This is likely to be a combination of sebum produced in excess, and interstitial fluid leaking onto the skin surface, due to the increased vascular permeability associated with inflammation. After a further few days these zones of inflammation become confluent and their centres dry to form a crust or scab. The mites are found surrounding the dried scab in the moist periphery. After approximately a week, depending to a large extent on the virulence of the infesting strain, the lesion may be around $25cm^2$ containing several hundred mites. The irritation to the sheep steadily develops as the number of mites increases. Infested sheep are often seen nibbling irritating areas so severely they produce small haemorrhages, secondary bacterial infections often develop causing large subdermal abcesses. After 7 to 10 weeks the lesion may have spread over two thirds of the infested animals causing extreme discomfort and extensive fleece loss [Fig. 2] this can be a severe problem for hill sheep in the winter months as hypothermia is inevitable. The animals can become so preoccupied with relieving the irritation their food intake can be dramatically reduced, they become severely debilitated, exhausted due to constant scratching and under certain circumstances may die by failure to compete for food and hypothermia. Some animals experience violent epileptiform fits, often severely injuring themselves in the process. Fatalities are common. It has been demonstrated that infested sheep fail to gain weight at the rate their non-infested partners do which can herald serious financial losses for stock owners (Kirkwood, 1980). The damage the mites cause to skin and fleece is

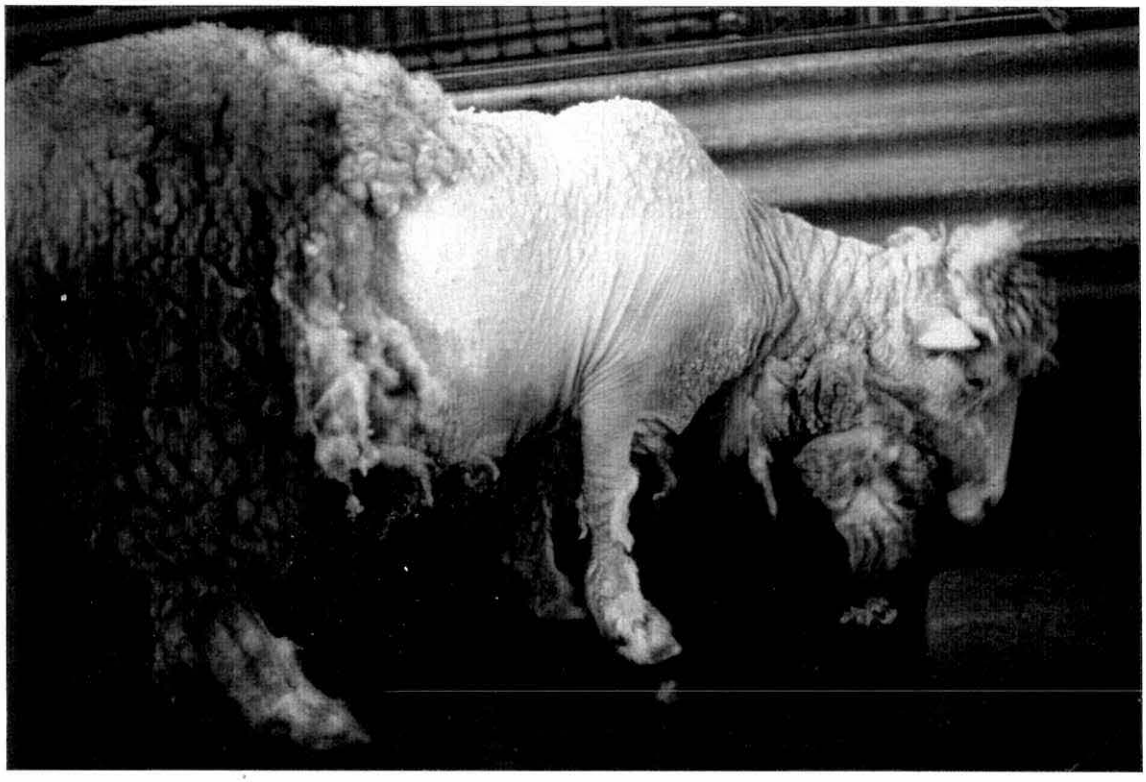

Figure 2. A ewe heavily infested with *Psoroptes ovis* showing characteristic signs of sheep scab. Although this sheep was experimentally infested it demonstrates the characteristic fleece loss (extensive in this case) often associated with the disease. This animal was showing signs of extreme exhaustion caused by constantly scratching to relieve the irritation provoked by the infestation. This animal recovered well after treatment in an organophosphate-based sheep dip.

significant. It has been suggested by the Farmers' Union of Wales that the sheep scab mite reduces the value of sheep skins by an estimated £13-20 million per annum. The poor quality of the fleece from infested animals must also contribute to the financial damage the disease causes. These financial implications, coupled with the distress the disease causes, makes sheep scab a very economically important disease which deserves to be the focus of a strict control campaign on welfare grounds alone.

The actual *cause* of the disease is not completely understood. In the past the clinical symptoms of the disease were attributed to the physical feeding activities of the mites (Shilston, 1915). It was proposed that the mites pierce the epidermis with chelicerae, physically disrupting the integrity of the epidermis causing inflammation, pruritus, serous exudate and so on. Although the mites do possess mouthparts that appear to be able to abrade or pierce tissue to some extent, recent scanning electron microscopy and *in vitro* feeding studies have demonstrated that the mites possess mouth-part structures that function simply by sucking liquid material without the need to first pierce the epidermis (Blake *et al.*, 1978; Deloach, 1984; Mathieson, unpublished). Also, given the variability in pathogenicity of different strains, straight feeding effects seem an unlikely cause of disease. The question of what stimulates the clinical symptoms then arises. Bates and Groves (1991) reported that even after treatment, despite 96% mite mortality, the scab lesion continued to progress at a rate comparable to untreated controls. This would suggest that the mites contain antigenically active material which contributes to the development of the disease and which can persist even following certain treatments. The material is very likely to be guanine, the major nitrogenous waste of acarines and arachnids. But once again the variability in pathogenicity of different strains suggests a constant element in secretion like guanine is unlikely to explain disease causation. Work conducted at the Central Veterinary Laboratory[1] has

[1] Central Veterinary Laboratory, New Haw, Surrey, UK.

demonstrated that guanine is a potential antigen- sheep injected intradermally with guanine demonstrate an immediate type sensitivity and pure guanine can be used as an antigenic reagent in the enzyme linked immunosorbent assay (ELISA) sero-diagnostic test for *P. ovis* (P. Bates, personal communication). It is quite widely accepted that the clinical signs of sheep scab are caused only in part, if at all, by the feeding activities of the mites and that the disease itself is a reaction to antigenic material produced by the mites combined with complications associated with secondary bacterial infection.

The picture is further complicated by the existence of several field strains of mites with varying degrees of pathogenicity, depending on their geographical origin. For example, four strains were compared by Bates (1991); these were from Norfolk, Cornwall, Wales and Dorset. The Dorset and Cornish mite strains produced a considerably more virulent disease than the Welsh strain or the now 31 year-old CVL strain producing a severe disease in a short period of time. The Welsh and Norfolk strains on the other hand, produced a much more chronic form of the disease. Some strains appeared to cause more serious disease in younger animals, other strains affected all ages equally. These differences are unlikely to be due to varying feeding activities or a variation in the antigenic material the mites produce, these one would expect to be constant throughout. The immune-status of the sheep is likely to be a contributing factor and perhaps an extrinsic factor, the involvement of different species of bacteria for instance (see Chapter 5).

In Britain approximately 50 million sheep are potentially at risk from scab. From 1984 until 1988 there was a compulsory dip order enforced by the Government as part of a national Sheep Scab Eradication Campaign. This legislation demanded the bi-annual dipping of the national flock in a MAFF approved sheep dip formulation. From 1989, following the steady reduction in the number of reported sheep scab cases, legislation dictated that a single yearly treatment would suffice. As many predicted, the number of reported case of scab increased over the following years and finally in July 1992 the compulsory dip order was removed completely and sheep scab was no longer a notifiable

disease. Anecdotal evidence would suggest that the number of cases of sheep scab and its prevalence has dramatically increase over the last three years (P. Bates, personal communication) but since the disease is no longer notifiable, the precise number of cases is unknown. The only circumstances under which a case could be reported to the Ministry is on welfare grounds.

The control of sheep scab is very effective if conducted properly using one of the three types of MAFF approved insecticides. These compounds are efficient at eradicating sheep scab with a single dipping and have a residual effect controlling against re-infection for at least three weeks. Two of the compounds are organophosphorus (OP) based- propentamphos and diazinon, the other is a synthetic pyrethroid- flumethrin. There are a number of scab-approved dips based on these compounds, the OP dips have the broadest spectrum of activity being effective against blowflies, lice, keds and sheep scab mites. Flumethrin based compounds are not effective against blowflies.

Organophosphates have been the subject of a heated debate concerning their implication in ill health suffered by stock owners and dipping contractors who routinely use these compounds. It is believed that in man OP poisoning is caused by organophosphates blocking the activity of cholinesterase which normally breaks down the neurotransmitter acetylcholine (Ach). This normally occurs once the neurotransmitter has bound to a post-synaptic nerve receptor and propagated the neural impulse. Since the Ach is not broken down it continues to stimulate a random flow of impulses along the associated nerve. This causes a variety of neurological symptoms depending on the regions of the nervous system affected. Effects such as muscle twitching, salivation, nausea and so on are common, but if breathing or circulatory centres are severely affected the results can be fatal (Swanston and Shaw, 1990).

The study of the disease and *P. ovis* is very restricted due to the lack of a suitable animal model. The absence of a practical *in vitro* system demands that any realistic work on the disease must be carried out artificially infested sheep. This not only creates a very

expensive and labour intensive practice but also subjects the experimental animals to obvious discomfort and suffering. There have been previous investigations into *in vitro* systems for other mite species, although the majority of these methods were developed for blood feeding mites. Crystal (1986) and Fletcher and Lancaster (1984) have both developed membrane feeding systems for the Northern Fowl Mite, *Ornithonyssus sylviarum* but unfortunately their methods and apparatus are not readily modifiable to the feeding behaviour of *P. ovis*.

There is a great need for research into *Psoroptes* spp. since preventing scab outbreaks and treating existing cases is an expensive, laborious and potentially harmful exercise for both the stock owner, due to OP poisoning, and the environment since controlling the disease involves the use of large quantities of insecticides that are disposed of in perhaps environmentally unsound ways. There is a great need therefore for developing other methods of control and treatment and hence the need for large numbers of mites to study. The benefits of being able to maintain and cultivate colonies of sheep scab mites off-host are obvious, but represent a long term goal which needs to be approached in stages. In this study the feeding activities of the mite and digestive functions have been studied in order that a rational approach could be taken in the development of an *in vitro* feeding system. Clearly the successful development of such a system will be a major step in the eventual development of an *in vitro* cultivation system and in addition will enable the off-host testing of a variety of potential control agents such as drugs, antibodies and potential vaccine candidates which can be incorporated in the mite's food.

Any *in vitro* system, whether it be a culture or a feeding system, must mimic to a certain extent the environment the organism experiences in its natural habitat. With this reasoning, it was proposed that the initial steps of this project should be to investigate the local environment (temperature and relative humidity) the mites experience naturally, to replicate these conditions *in vitro* and to further investigate the conditions most conducive to maximum mite survival off-host (see Chapter 2).

A further requirement is the necessity to provide the mites with a nutritionally complete diet to. With *P. ovis* there is much speculation as to what the mites actually feed on and what host-derived materials they come in contact with. This is not only important for the purposes of producing an artificial feeding system but also for the development of new control compounds. It is imperative to present the control agent to the mites via a suitable carrier- it would be a little myopic to develop a vaccine control strategy if the mites do not ingest significant quantities of host-derived antibodies. Therefore a large proportion of this thesis is dedicated to investigating the dietary needs of *P. ovis*, indirectly beginning in Chapter 3. The mites are likely to utilise a range of materials normally found in close proximity to the epidermis and others released during the infestation; sebum, serum, erythrocytes, bacteria, for example and other fluids associated with inflammation and increased vascular permeability concurrent with sheep scab. These materials are only *likely* to be ingested by the mites, there is no real conclusive information on the nature of the mites' diet. Information of this sort would not only be immensely useful for the production of an *in vitro* feeding system, for the development of novel control strategies, but also alleviate much speculation and assumptions made about the mites' normal diet.

The anatomy and ultrastructure of the mites' digestive system does not appear to have been previously studied and published. These two subject areas were investigated in turn (Chapter 3 and 4) in order to gain an understanding of the structure and function of the mites' digestive tract. This will help further studies on *P. ovis* in many ways. In particular it will help in the understanding of the dietary requirements of the mite, an area full of speculation. Accurate information on food requirements is essential for the development of an *in vitro* culture system for *P. ovis*. While conducting this work it became apparent that there was a significant population of bacteria present in much of the digestive system. The structure and distribution of these potentially extremely important microorganisms were further investigated during this TEM study and their isolation and characterisation was investigated in Chapter 5.

Chapters 6 and 7 concentrate on determining the dietary components of *P. ovis* using SDS-PAGE and Western blotting techniques, and immunohistological methods respectively. These studies were conducted in an attempt to demonstrate conclusively the nature and location of the mites' diet. As suggested previously, information of this nature is not only imperative for the production of an *in vitro* feeding system but also for the advancement of novel control procedures.

In the final experimental chapter, Chapter 8, the development and subsequent investigation of several artificial feeding devices incorporating a variety of experimental diets is discussed. The work was conducted intermittently throughout the course of the research, due to the irregular availability of mites, rather than in its logical place as a conclusive investigation following the conclusions of the other related chapters. The relevance of the study has not suffered as a consequence.

This thesis has been structured with each experimental chapter beginning with a brief Summary of the experimental aims and conclusions. A full Introduction follows which is proceeded by a Materials and Methods section followed by the Results and a full Discussion of the results and related work. In the last experimental chapter the Results and Discussion sections are combined to facilitate the presentation and discussion of the results. Finally, a brief General Discussion completes the work by summarising the main conclusions from each chapter and outlines future endeavours worthy of immediate attention.

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Chapter 2.

Determining the Conditions for Optimum Survival of *Psoroptes ovis* Off-Host.

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Determining the Conditions for Optimum Survival of *Psoroptes ovis* Off-Host.

Summary.

The skin and core temperatures of naive and *Psoroptes ovis* infested sheep were investigated to determine the conditions the mites are likely to experience at the skin surface during an infestation. It was found that although the core temperature significantly rose, an elevated skin temperature could not be demonstrated.

Groups of adult female *Psoroptes ovis* mites were incubated off-host under a range of temperatures and relative humidities to determine the conditions conducive to optimal mite survival off-host and unfed. Of the conditions investigated, 82% relative humidity at 33°C appeared to produce significantly better survival than the other conditions investigated.

Introduction.

As an ectoparasite, *Psoroptes ovis* has specifically evolved and adapted to take advantage of a particular ecological niche that its chosen host supplies and maintains. If we are to have any success in producing an artificial cultivation system, it is imperative that we understand at least the key components of this environment. Free-living mites are, by definition, far more independent than their parasitic cousins and more able to maintain their water balance under a wider range of environmental conditions. *P. ovis* and other parasitic animals are more sensitive to being removed from their natural environment and manipulated in an *in vitro* situation. Consequently this study concentrates specifically on the relative humidity (RH) and the temperature found at the mite's habitat, so they can be simulated in an *in vitro* system.

P. ovis mites, like other Acari, must maintain their body-water content within a physiologically determined range. They ingest water while feeding and absorb water both actively and passively from sub-saturated air via their cuticle (Knülle, 1967; Wharton, 1974). They are also likely to oxidise organic nutrients to help in the maintenance of water balance (Arlian *et al.*, 1988). Body-water will be lost through transpiration, respiration, defecation, copulation and possibly while feeding, depending on the mite's feeding behaviour (Arlian and Veselica, 1979; Wharton, 1974). *P. ovis* mites lack salivary glands and so water loss through feeding is perhaps less likely than in mites that secrete saliva to aid ingestion and digestion.

The lowest value of relative humidity that allows the maintenance of water balance is known as the Critical Equilibrium Humidity (CEH) (Gaede and Knülle, 1987) or Critical Equilibrium Activity (CEA) (Wharton, 1974; Arlian and Wharton, 1974). Although it is not necessary for our purposes to determine exactly the CEH for *P. ovis,* we do need to discover roughly the threshold of relative humidity above which the mites can sustain their water balance. The approximate CEH can be determined in several ways. Gaede and Knülle (1987) used a very sensitive microbalance to monitor the weight fluctuations of groups of mites incubated at a range of sub-saturated relative

humidities, while studying the CEH of the feather mite *Proctophyllodes troncatus*. Arlian *et al.* (1988) investigated water balance of scabies mites, *Sarcoptes scabiei,* using tritium labelled water as did Seethaler *et al.* (1979) with the house mite *Glycyphagus domesticus.* Here, a simpler set of experiments are used to investigate the environment the mites experience *on-host*, and to determine the environmental conditions most conducive to mite survival *off-host*.

O'Brien *et al.* (1994) have also studied the survival (and retention of infectivity) of *P. ovis* mites off-host. They were interested in determining how long mites and eggs would remain infective off-host in pieces of fleece in sheep enclosures, trailers and other places likely to accumulate pieces of fleece and mites, where they could be passed on to other sheep. It will be interesting to compare the results of their study with the data from this investigation.

Materials and Methods.

Direct Skin Measurements.

Two methods of investigation were employed. Initially, temperature recordings were taken from three CVL strain mite-infested Dorset Horn Cross ewes maintained in semi-covered enclosures at the Central Veterinary Laboratory. Surface skin temperatures were recorded using an electronic thermocouple (Logitech® Thermocouple LT21) at 15cm intervals around the edge of the lesion and also across the lesion in both antero-posterior and lateral directions. The fleece was quickly parted to determine the location of the lesion edge and the thermocouple was then pushed through the fleece to the epidermis with minimal disruption to the micro-climate. The device was left in place for a few seconds until the temperature reading became constant. This procedure was repeated 15cm further round the lesion edge and repeated until the lesion circumference had been mapped. The dorsal and lateral readings were then taken. Three readings were taken from each location at approximately the same time of day on two consecutive days and the means calculated. Temperatures were recorded from corresponding areas on three *P. ovis*-naive control sheep for comparison.

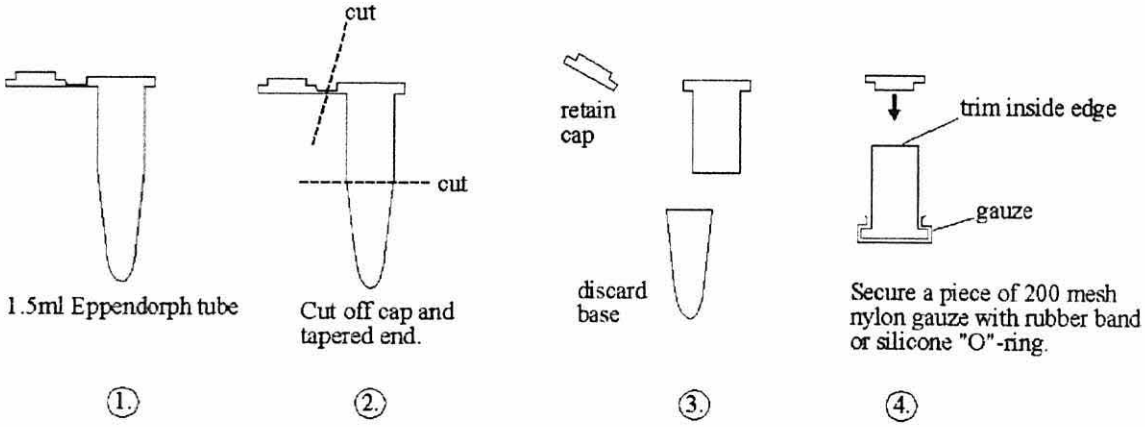
An attempt was made to record the relative humidity within the fleece around the lesion edge from experimental animals and in corresponding positions on control sheep using a Grant Squirrel Meter/Logger [model number SQ32-3U/1L] and its associated relative humidity probe. Rectal core temperatures were taken from both groups using a standard medical thermometer and ambient air temperatures and relative humidity were recorded using the Grant Meter.

Survival Off-Host Trials.

A series of trials were conducted over several months to determine the environmental conditions (temperature and relative humidity) that produce optimal mite survival off-host by monitoring the survival of groups of mites incubated under a range of temperatures and relative humidities.

18 adult female mites were used for each experimental condition examined. Usually three different conditions were investigated simultaneously, thus approximately 54 adult female mites were required for each trial. Sufficient numbers of CVL strain mites were removed from infested sheep by careful scraping around the lesion edge with the broad side of a mounted needle. Adult females were separated from males and larval stages, counted and transferred into modified Eppendorph tubes (incubation cages) [Fig. 1]. Occasionally the mites needed to be washed in warm phosphate buffered saline (PBS) (approximately 30°C) to remove excess host-derived exudate from their cuticles. This material could serve as a nutritional source for the mites and as the mites were required to be un-fed during the trial, it was often necessary to remove the exudate. The experimental mites were divided randomly into three groups of six mites for each condition and transferred into the incubation cages.

Figure 1. Procedures for the construction of the Eppendorph incubation cages.



The tapered end and cap of a 1.5ml Eppendorph were cleanly cut off. A small scalpel blade was used to shave the inner edge of the remaining tube where the tapered end had been. Enough plastic was trimmed from the edge to allow the cap to be used as a seal in this new position. A piece of approximately 100 mesh nylon gauze was secured to the original top aperture using a silicone "O-ring", rubber band etc. This creates a secure incubation chamber for the mites which is assumed to allow the free movement of water vapour.

The cages and mites were suspended above saturated salt solutions (with an excess of salt) (Wiston and Bates, 1960), in 17 x 11.5 x 6cm sandwich boxes (Stewart

Plastics, crystal boxes, type 145) to produce the desired relative humidities and held in incubators at the experimental temperature. Saturated potassium chloride (KCL) was used to produce 82% RH; saturated sodium hydrogen sulphate ($NaHSO_4$) created 50% RH and distilled water was used for 100% RH. The experimental conditions were monitored using the Grant Squirrel Meter.

The incubation cages were removed from the incubators and sandwich boxes at least twice daily to inspect and record the number of live mites. Mites were considered "alive" if they demonstrated active mobility of at least four of their eight legs. Dead mites were not removed to minimise disruption to those remaining alive.

One-way Analysis of Variance (ANOVA) tests or Student's t-tests were performed on the data collected to test for significance.

Results.

Direct Skin Measurements.

Figure 2 demonstrates the mean skin-surface temperatures from the individual control and experimental animals. No significant difference was found between the skin temperatures of any of the three infested sheep and the three non-infested sheep.

Figure 3 demonstrates the pooled mean skin-surface temperatures from the three infested and three control animals. Again, no significant difference ($p=0.43$) between the skin temperatures of the two groups.

The rectal core temperature histograms [Figs. 4 and 5] indicates that infestation leads to a highly significant ($p<0.005$) elevated core temperature following statistical analysis.

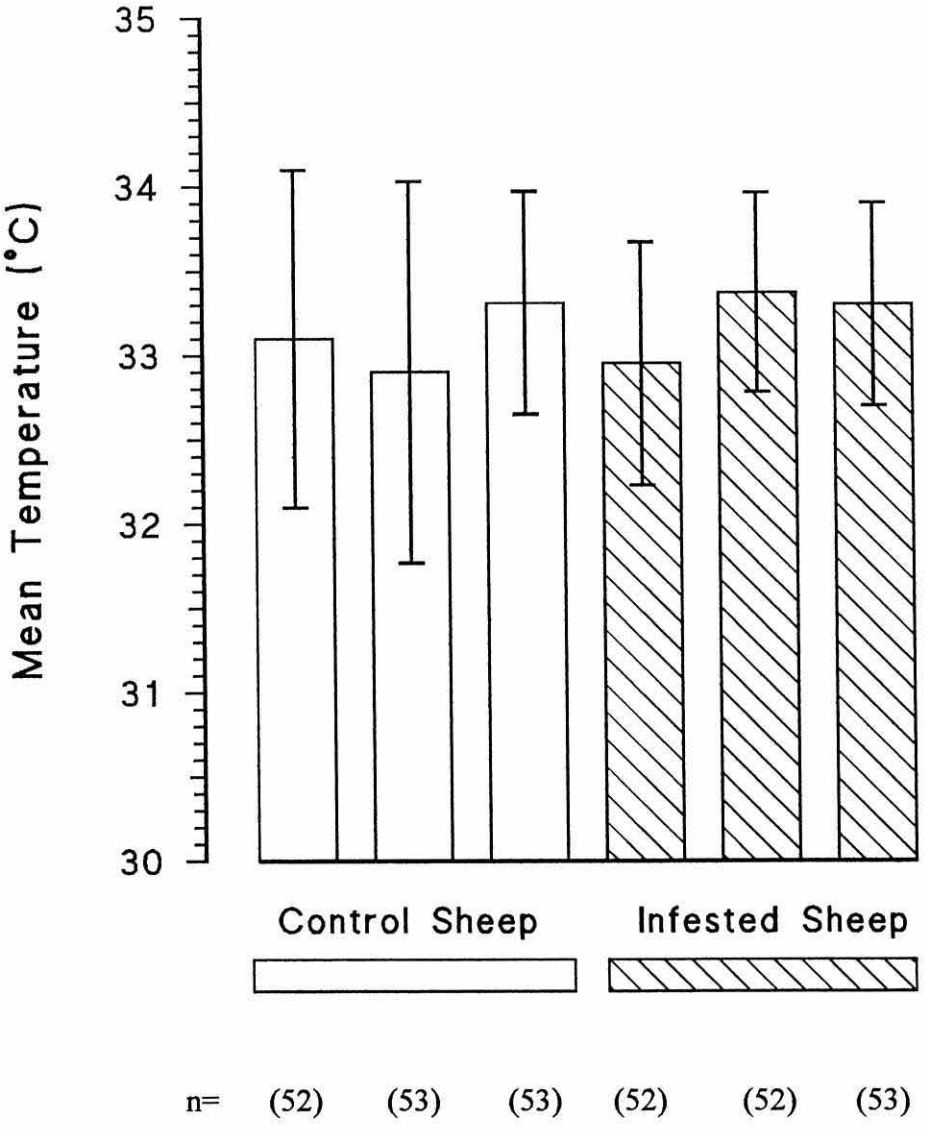
No significant differences were found between either the mean air temperatures ($p=0.22$) or the mean relative humidities ($p=0.64$) recorded from the control and infested sheep enclosures used [Fig. 6].

Survival Off-Host Trials.

The results from our survival trials will be presented in three forms. Firstly, the data will be displayed using the Mean Maximum Survival Time each experimental condition produced. Secondly, the time in incubation taken for 50% of the mites in each sample to die, here termed the LT50 values, under the various conditions was calculated and presented. Finally, the data from each individual maximum survival trial will be presented in line graph form demonstrating how each experimental condition affected the mite sample's survival in each individual trial.

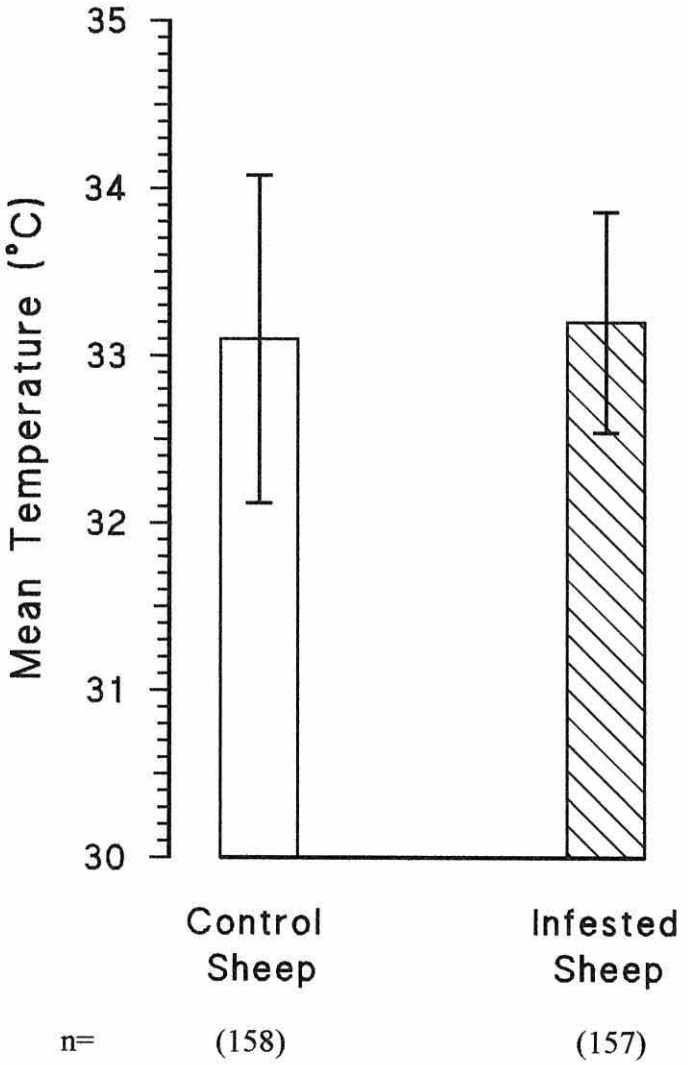
Figure 7. shows the mean maximum survival time each experimental condition produced. The conditions are presented in a descending order of effectiveness, i.e. 82% RH at

Figure 2. Mean Skin-Surface Temperatures of three control sheep and three experimental sheep infested with *Psoroptes ovis.*



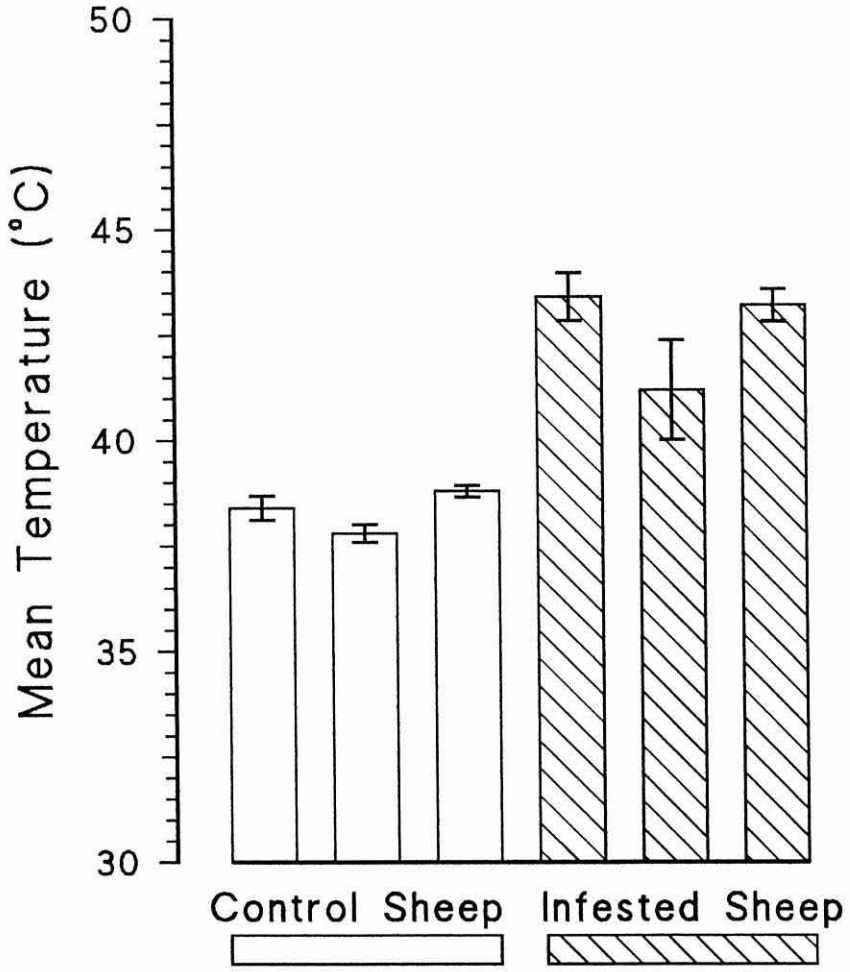
No significant differences were found between any permutation of control and infested-sheep mean skin-temperature following one way analysis of variance (ANOVA). Standard Deviations (± SD) are included, Sample Numbers (n) are in parentheses.

Figure 3. Pooled Mean Skin Surface Temperatures of the three control and three *Psoroptes ovis* infested sheep.



No significant difference (p=0.43) was found between the pooled mean skin temperature of the three control and three mite-infested animals following Student's t-test. The mean skin temperature of control sheep was 33.2 ±1.21°C (SD) and infested sheep 33.3 ± 1.23°C. The number of temperature readings taken from the control and infested animals were 158 and 157 respectively. Standard Deviations are shown, Sample Numbers (n) are in parentheses.

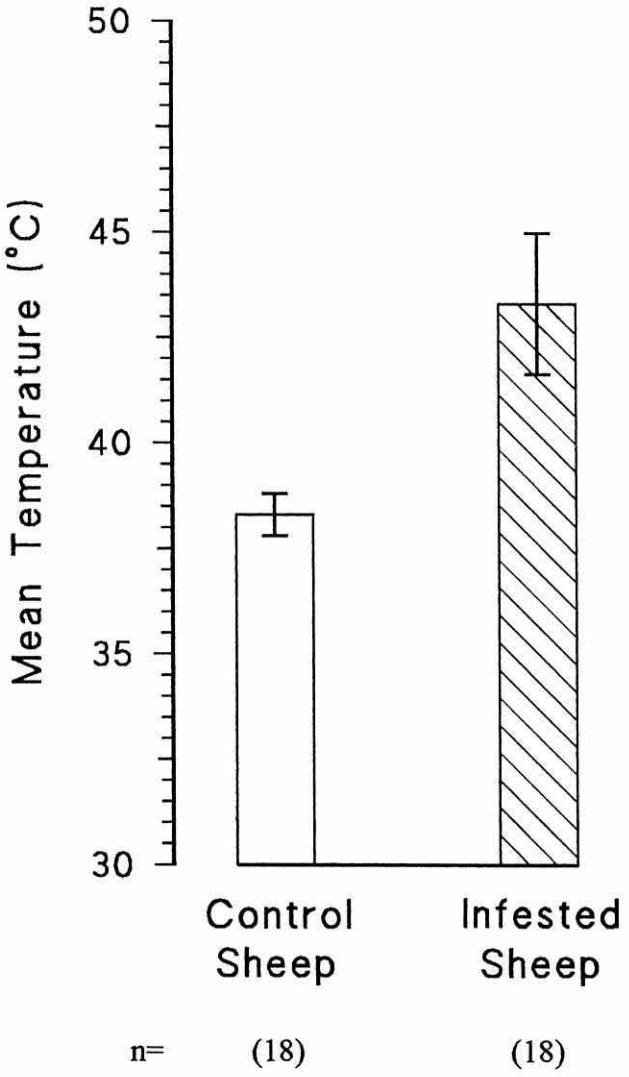
Figure 4. Mean Rectal Core Temperatures of the three control and three *Psoroptes ovis* infested sheep.



n= (6) (6) (6) (6) (6) (6)

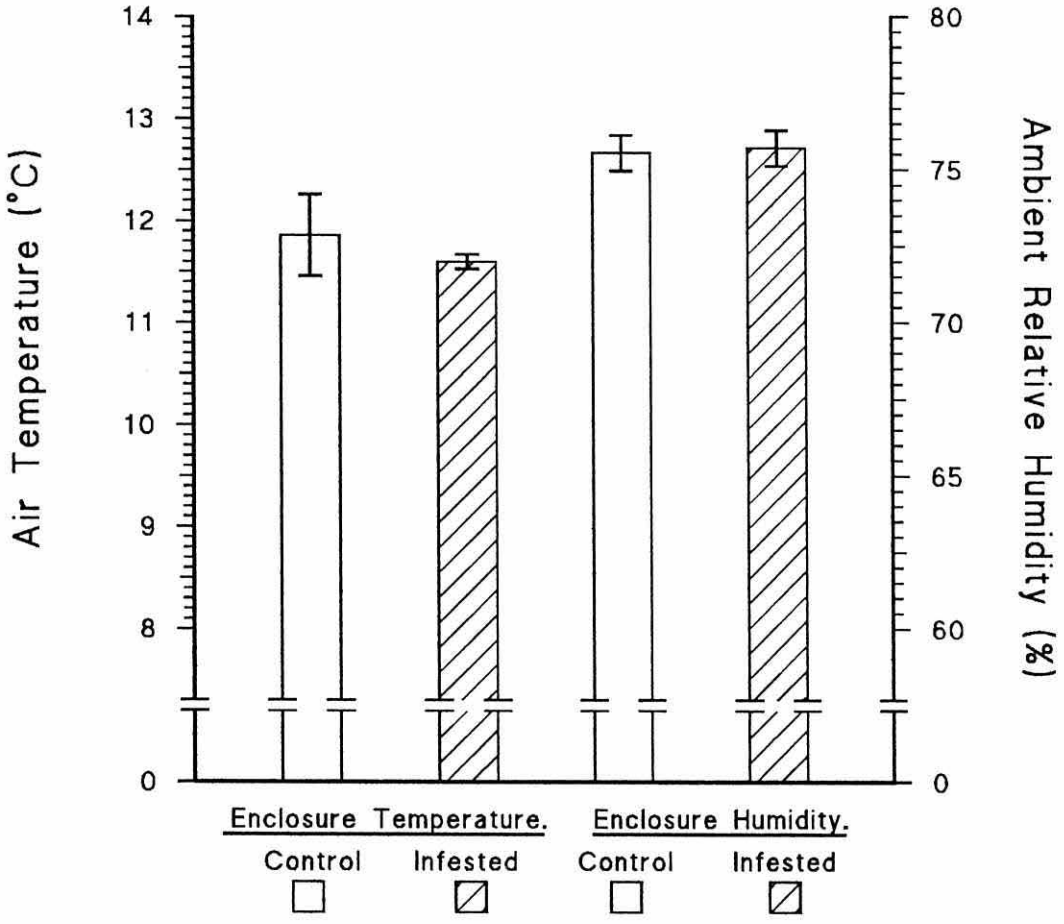
Following One-way ANOVA calculations significant differences were observed between all permutations of the mean control and the infested sheep core temperatures investigated. The individual pair with the closest means still were significantly different ($p<0.02$), the other pairs had at least this level of significance. Standard Deviations are shown, Sample Numbers (n) are in parentheses.

Figure 5. Pooled Mean Rectal Core Temperatures of the three control and three *Psoroptes ovis* infested sheep.



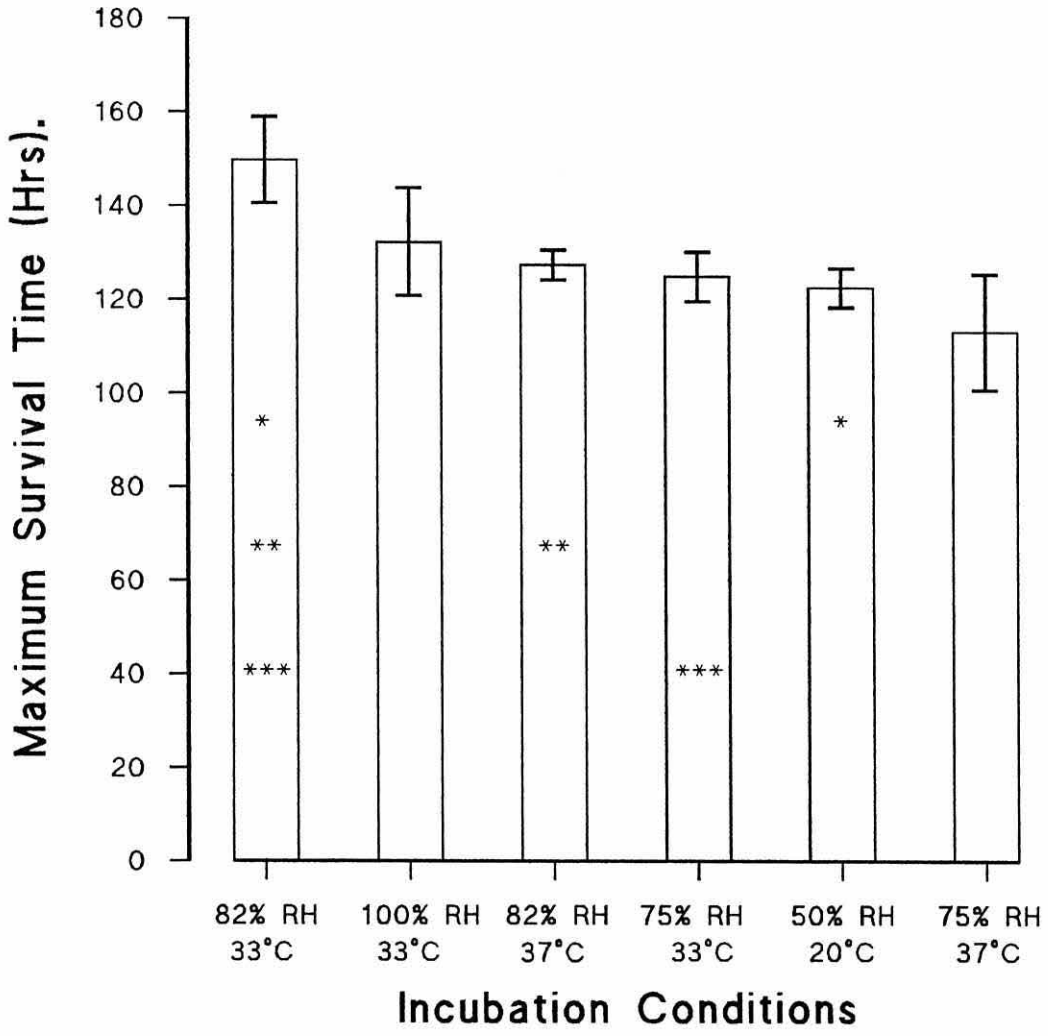
Student's t-tests were performed on the control and infested sheep core temperature means. Results suggested that an infestation of *P. ovis* produces a significantly elevated core temperature ($p<0.005$). The mean pooled core temperature of control sheep was $38.3^{o}C \pm 0.5$ compared to $43.3^{o}C \pm 1.67$ for the infested sheep. Standard Deviations are shown and Sample Numbers (n) are in parentheses.

Figure 6. A comparison of the mean ambient air-temperatures and mean relative humidities recorded in the enclosures used to house the control and *Psoroptes ovis* infested sheep.



No significant differences were found between the mean ambient air temperatures ($p=0.22$) or the mean ambient relative humidities ($p=0.64$) in the control and experimental sheep enclosures after performing Student's t-tests. The mean air temperatures were 11.9°C ±0.40 and 11.6°C ±0.07 for the control and experimental enclosures respectively and the mean relative humidities were 75.5% ±0.58 and 75.7% ± 0.26. Standard Deviations shown and the numbers in parentheses are Sample Numbers (n).

Figure 7. Mean Maximum Survival Times of *Psoroptes ovis* mites incubated under various temperatures and relative humidities.



n= (13) (9) (3) (4) (16) (3)

One-way ANOVA calculations were carried out on all Maximum Survival mean combinations possible. Those conditions that were significantly different from each other are annotated with asterisks (*). Bars with similar symbols are significantly different from each other at the level shown below. No other significance between pairs was observed. Standard Deviations are shown, Sample Numbers (n) are in parentheses. (* $p<0.02$ ** $p<0.04$ *** $p<0.04$)

33°C, in our trials, produced the best survival times, while 75% RH at 37°C produced the fastest mortality rates under the conditions investigated. The histogram indicates the favourability of the other conditions to mite survival in ranked order. The ANOVA tests that were performed on the data suggested that several of the experimental conditions produce significantly better survival times than others. Table 1. presents the significant findings.

Table 1. Mean Maximum Survival times ±Standard Deviations of mites incubated under various environmental conditions. The incubation conditions are ranked in order of compatibility to mite survival.

Incubation Condition	Mean Maximum Survival ±SD (in hours)		Significant Differences
82% RH at 33°C	149.7 ± 9.2	n=13	* ** ***
100% RH at 33°C	132.2 ± 11.5	n=9	
82% RH at 37°C	127.3 ± 3.2	n=3	**
75% RH at 33°C	124.8 ± 5.3	n=4	***
50% RH at 20°C	122.4 ± 4.2	n=16	*
75% RH at 37°C	113.0 ± 12.3	n=3	

Incubation conditions with similar symbols are significantly different with the probability values: * $p<0.02$ ** $p<0.04$ *** $p<0.04$

Below is summary of the significant results:

- 82% RH at 33°C was more favourable than 82% RH at 37°C. $p<0.04$
- 82% RH at 33°C was more favourable than 75% RH at 33°C. $p<0.04$
- 82% RH at 33°C was more favourable than 50% RH at 20°C. $p<0.02$

No other significant differences were found between the Maximum Survival results produced by the various experimental conditions used in this investigation.

As with the Maximum Survival information, the LT50 data has been presented in descending order favouring mite survival [Fig. 8]. Again 82% RH at 33°C produced the

conditions for optimum survival, compared to the other conditions we investigated, and 100% RH at 33°C produced the second most favourable to mite survival. See Fig. 8 for a graphical description of all the mean LT50 results in ranked order favouring mite survival. Table 2. presents the data of the LT50 investigations and statistical significance following One-way ANOVA analysis.

Table 2. Mean LT50 times ±Standard Deviations of mites incubated under various environmental conditions. The incubation conditions are ranked in order of compatibility to mite survival.

Incubation Condition	Mean LT50 times ±SD (in hours)		Significant Differences
82% RH at 33°C	77.4 ± 4.4	n=13	*
100% RH at 33°C	62.4 ± 6.5	n=9	
82% RH at 37°C	69.0 ± 2.1	n=3	
50% RH at 20°C	65.8 ± 3.4	n=16	*
75% RH at 33°C	64.2 ± 6.0	n=4	
75% RH at 37°C	75.3 ± 10.7	n=3	

Incubation conditions with similar symbols are significantly different with the probability values: * $p<0.05$

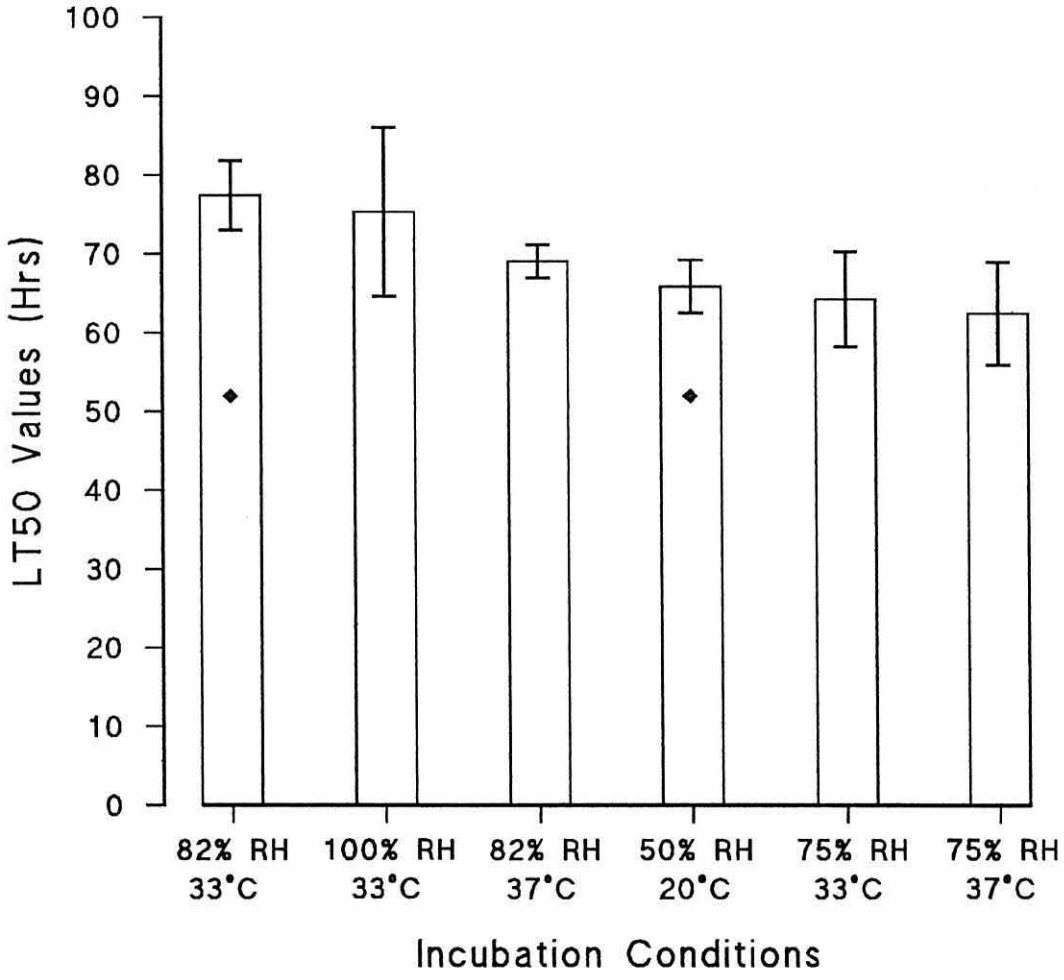
Below is a summary of the significant difference found:

- 82% RH at 33°C was more favourable than 50% RH at 20°C. $p<0.05$

No other significant differences were found between the LT50 results produced by the various experimental conditions used in this investigation.

The data from each individual maximum survival trial are presented from Figs. 9-24. The survival of mites incubated under three different environmental conditions were compared one at a time and replicated between three and nine times. Looking at the individual figures it is noticeable that in 13 of the 16 graphs, the 82% RH environment produced better survival than the other conditions. In the other trials though there was little difference between the various conditions on survival times [Figs. 10, 12, and 14].

Figure 8. Mean incubation times resulting in 50% mortality (LT50) of *P. ovis* mites incubated under various temperatures and relative humidities.



n= (13) (9) (3) (16) (4) (3)

A One-way ANOVA test was performed on the data. The significant differences between conditions revealed are annotated with solid diamonds (♦). Bars with similar symbols are significantly different from each other at the level shown below. No other significant differences were observed between pairs. Standard Deviation bars are shown, numbers in parentheses are the Sample Number from each trial. (♦ $p<0.05$)

In summary, no significantly elevated skin-surface temperature was demonstrated in infested sheep in this study ($p<0.43$), contrary to what we might expect. There was a highly significantly ($p<0.005$) elevated rectal core temperature in the infested sheep. There was no significant difference between the temperature and relative humidity recorded from the control and experimental enclosures used to accommodate the sheep.

Technical difficulties with the Grant Relative Humidity Probe prevented any reportable information from being gathered. See Discussion section for more details.

The environmental conditions investigated that produced maximum mite longevity was 82% RH at 33°C followed by 100% RH at 33°C, when looking at Maximum Survival times off-host, and if we consider the LT50 data, again we see an identical picture.

Figures 9-11. Individual Mite Survival Off-Host profiles for the incubation conditions: 82% RH at 37°C; 75% RH at 37°C and 50% RH at 20°C.

Figure 9.

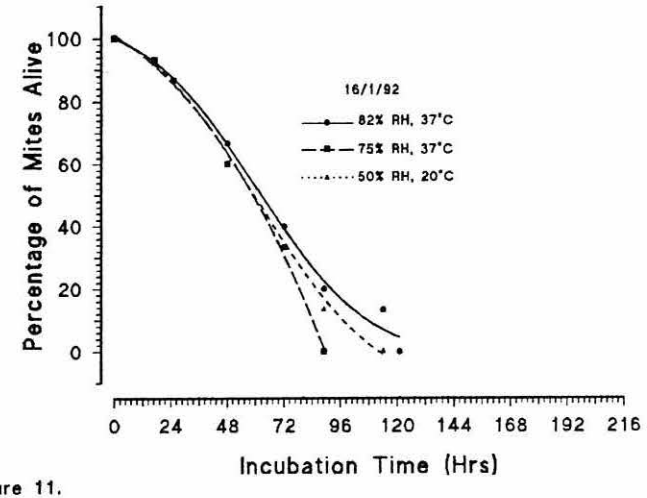


Figure 10.

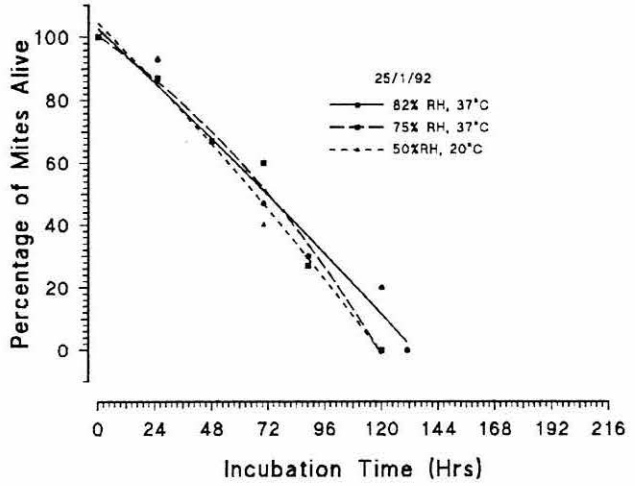
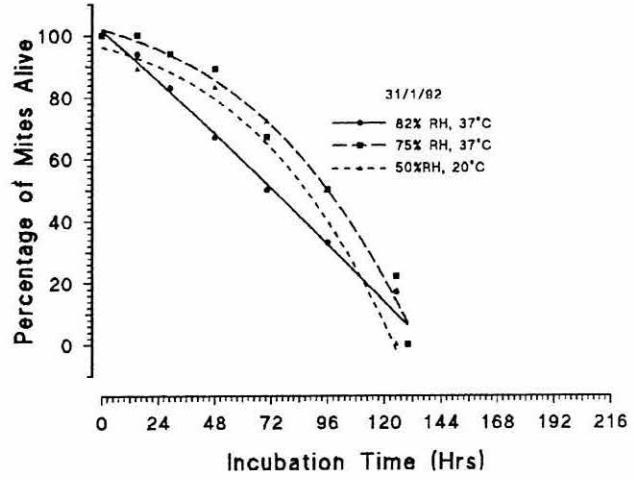


Figure 11.



Figures 12-15. Individual Mite Survival Off-Host profiles for the incubation conditions: 82% RH at 33°C; 75% RH at 33°C and 50% RH at 20°C.

Figure 12.

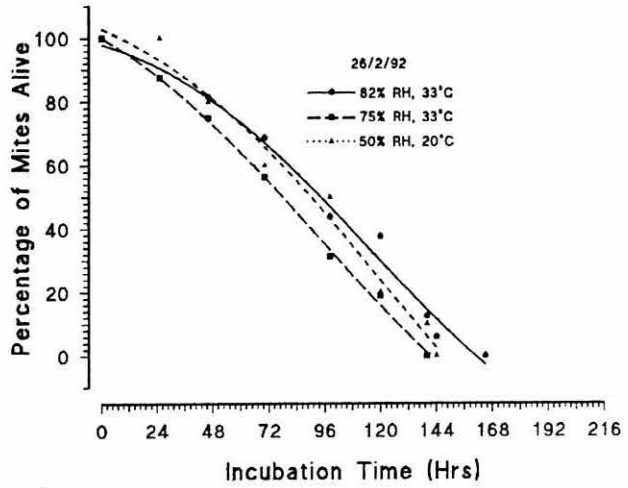


Figure 13.

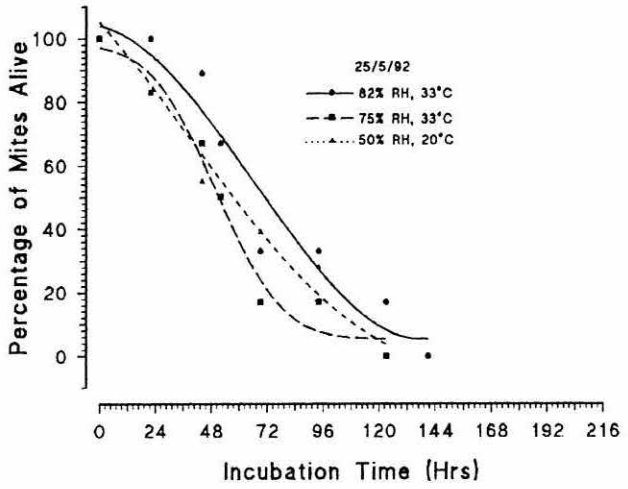


Figure 14.

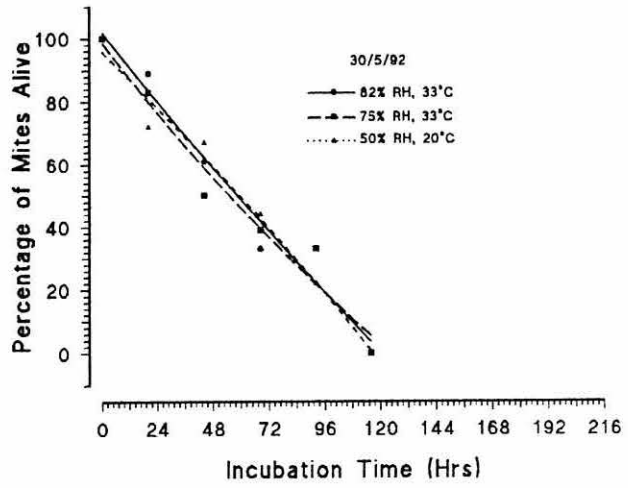
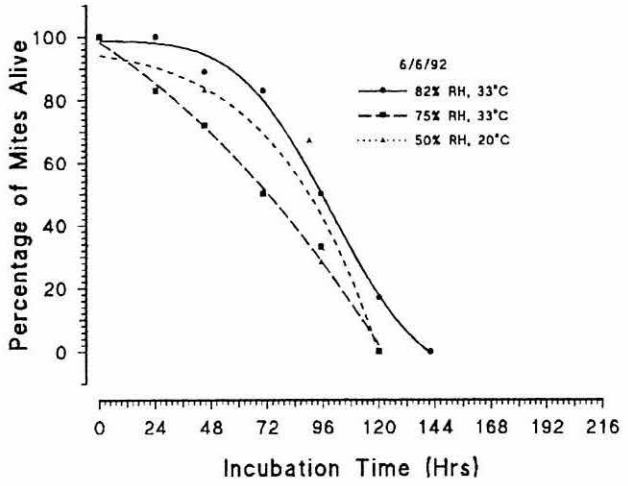


Figure 15.



Figures 16-19. Individual Mite Survival Off-Host profiles for the incubation conditions: 82% RH at 33°C; 100% RH at 33°C and 50% RH at 20°C.

Figure 16.

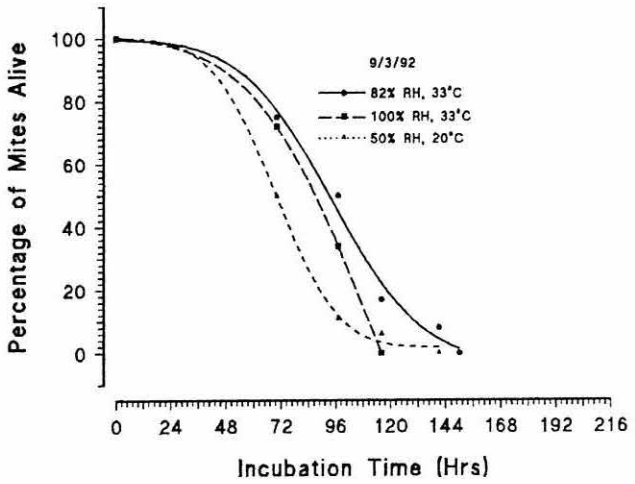


Figure 17.

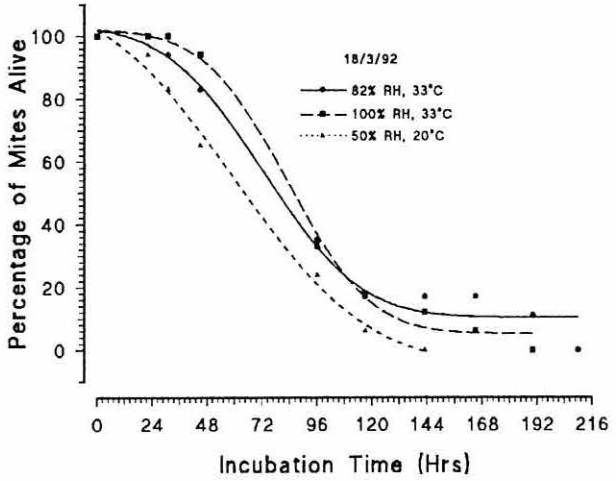


Figure 18.

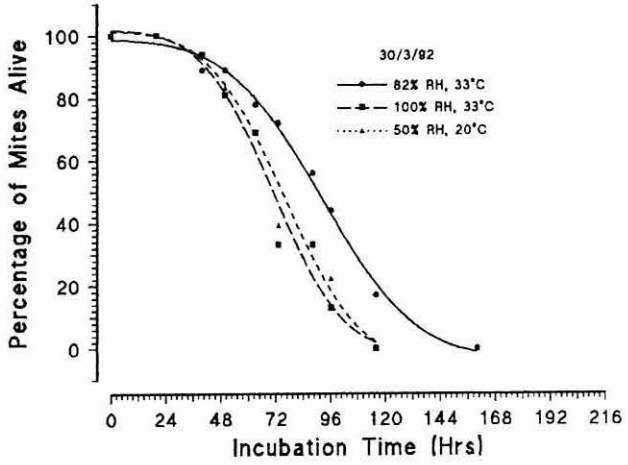
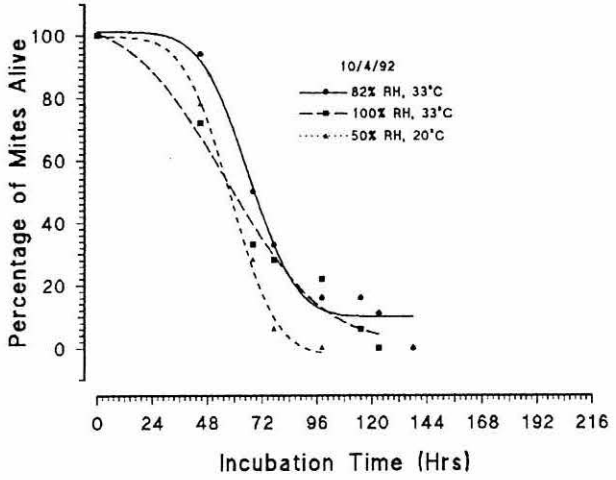


Figure 19.



Figures 20-23. Individual Mite Survival Off-Host profiles for the incubation conditions: 82% RH at 33°C; 100% RH at 33°C and 50% RH at 20°C

Figure 20.

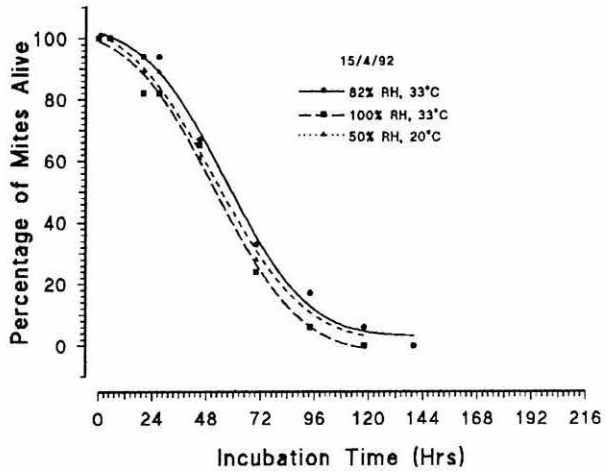


Figure 21.

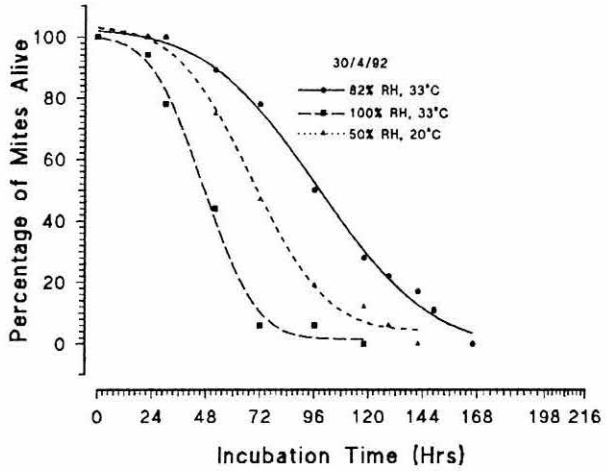


Figure 22.

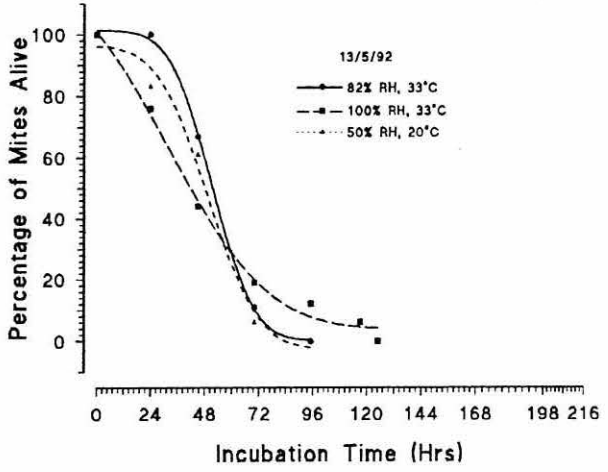


Figure 23.

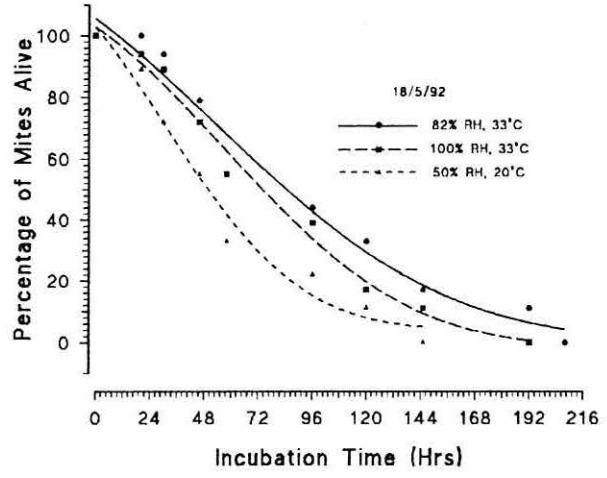
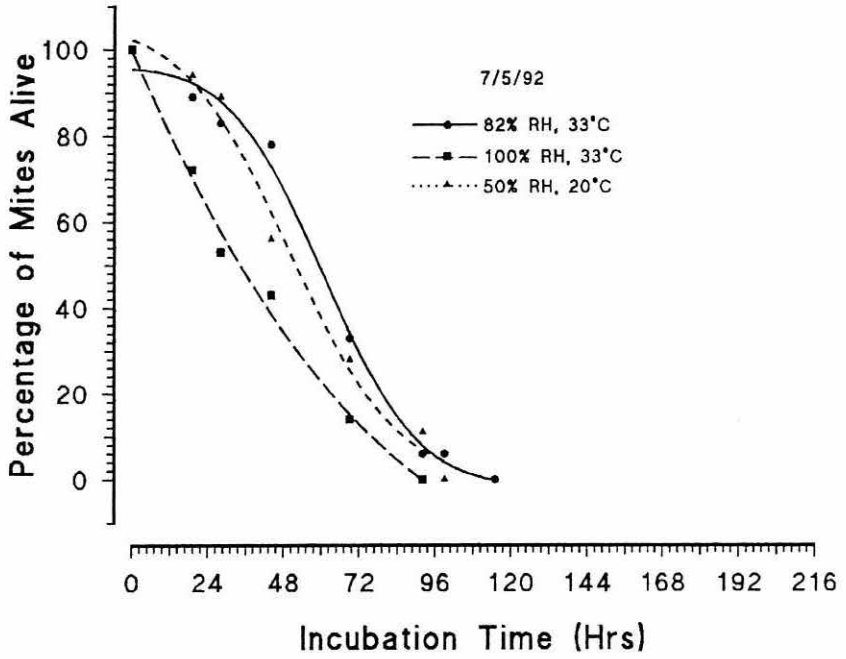


Figure 24. Individual Mite Survival Off-Host profile for the incubation conditions: 82% RH at 33°C; 100% RH at 33°C and 50% RH at 20°C.

Figure 24.



Discussion.

It is not sufficient to simply measure the temperature and relative humidity of the mite's natural environment and simulate them in an *in vitro* system. This would indeed give us an insight into the conditions that the parasites are exposed to in an *in vivo* situation, but those conditions are not necessarily the most benign to this infesting organism. It is important to remember that the mites cause an unnatural and irritating infestation that the sheep endeavours to control and combat, hence the responses of the sheep to the infestation are likely to be detrimental to the mite's survival.

As parasite and host co-evolve, common sense would suggest that a decrease in pathogenesis would be a selective advantage for the parasite, i.e. longer life for the host would maximise the reproductive potential of the parasite. Schmidt and Roberts (1989) suggest that the idea that "the least pathogenic parasite is the most successful" has become scientific dogma, not without its critics though (Anderson and May, 1982). With this philosophy, it could be argued that the "newer" the host-parasite interaction, the more likely there is to be a conflict between the two parties, and conversely, over the course of numerous generations, selective pressure would drive the system more towards commensalism. Of course, there is also selective pressure for the host to adapt to control the foreign organism outright and prevent subsequent re-infection or re-infestation. With this in mind, the situation we observe between *P. ovis* and it's ovine host could be argued to be in an "adolescent" stage of evolution. Some infestations, with particular strains of mites or with particular breeds of sheep, result in acute and serious disease, rapidly debilitating the host, often resulting in death if untreated. This suggests that there is a severe incompatibility between host and parasite. In other cases the disease takes on a more sedate, chronic character, causing the host little observable discomfort suggesting that the host-parasite relationship is tending towards a more symbiotic situation ("Symbiotic" is being used here, as originally proposed by de Bary in 1879, to describe any two organisms living in close association, commonly one living in or on the body of the other. Benefit to one or the other is not implied (Schmidt and

Roberts, 1989)). It can be seen then that the environment the mite colonises, which subsequently alters, may not be the best focus of our attention to determine the conditions for optimum survival of the mites.

It is well known that *P. ovis* mites on sheep migrate away from the initial site of infestation and disseminate radially to un-infested areas. It is not understood why the mites migrate in this manner. Kirkwood, (1986) proposes that the mites tend to be found at the lesion edge, after abandoning previously infested areas, because the exudate produced by the host quickly congeals making the environment unsuitable for the mites. It is possible that their food resources are becoming scarce as the infestation progresses and mite numbers increase. This may drive the mites to search for fresh and uninhabited pastures, perhaps from areas where the temperature and humidity are not ideal.

In my opinion it is improbable that this migration is stimulated by declining dietary resources, as a typical infestation results in the exudation of a multitude of potentially highly nutritious material; lipids, lymph, serum and so on that the mites are likely to feed on (Rafferty and Gray, 1986; Sinclair, 1988; Sinclair and Filan, 1989; Sinclair and Kirkwood, 1983). Also, far from being independent explorers, the mites are very gregarious animals and are often seen grouped together in large numbers both *in vivo* and *in vitro*, perhaps though, they move *en mass* once they have depleted the nutritional reserves from one occupied area. Never the less the mites do migrate away from areas they have previously occupied and are likely to be driven by a build up of their own excretory material in their environment stimulating the production of an immune response. Mites produce guanine-rich faecal material (Dinsdale, 1975; Vajropala, 1935) and *P. ovis* mites are no exception. It is quite possible that the guanine stimulates an immune response decreasing the suitability of the currently occupied area causing the mites to move. Indeed even dead *P. ovis* mites can stimulate the characteristic localised clinical signs of sheep scab indicating that simply the presence, or a component of mites, can cause disease rather than their feeding activities (P.G. Bates, personal communication). See also Chapter 5 for a discussion into the possibility of gut

derived bacteria playing a role in the pathogenicity of sheep scab. I would suggest that the mite's environment is constantly changing as they feed and excrete at the lesion edge. Their excretory products build up in these currently infested areas stimulating an immune response driving the mites onto un-infested areas.

A further point to mention is that there is a tremendously unpredictable variation in the way individual sheep respond immunologically and clinically to a *P. ovis* infestation (P.G. Bates, personal communication). This is due to a variety of variables including the strain of mite, immune status of the host, host disease history, host breed and so on. One infestation on one individual animal may produce an extremely elevated temperature, for example, while another animal may not experience this at all. This could obviously interfere with our interpretation of the picture as we cannot rely on the environment produced during an infestation to indicate the conditions that are most favourable for mite survival. If we were to depend on the measurements recorded from infested or even control animals alone, it would be an indirect method of determining the information we require. We must also investigate the mite's survival response to different environmental conditions without the numerous variables the host is likely to contribute.

Surprisingly, using our techniques, we could not demonstrate the expected elevated skin temperature associated with a *P. ovis* infestation. The significantly elevated rectal core temperature of infested sheep indicates the general fever that is characteristic of the disease, which would also be expected to raise the animals skin temperature. We would expect the elevated skin temperature to accompany the disease due to the irritation and inflammation which is concurrent with an infestation of this kind. Instead we found that there was no significant difference between the skin temperature of infested and naive control sheep. These animals were housed in similar enclosures under comparable environmental conditions and were similar breeds of sheep.

One feasible argument to explain these findings is based on the clinical nature of

sheep scab. As the infestation develops the lesion enlarges. The lesion area is characterised by a hyperkeratosis and the production of a moist, serous exudate. The exudate dries to a crust, several millimetres thick, and coupled with the thickening of the sheep epidermis, is likely to create an insulating layer where the lesion has developed. It is also conceivable that the evaporation of the moist exudate has a cooling effect on the skin surface. Both these occurrences are very likely to result in low temperatures being recorded. Areas not yet occupied by mites will experience minimal inflammation, none of the serous exudate and therefore the skin temperatures detected here are likely to be close to, if not normal.

With the electronic thermocouple we used to take our readings it was not possibly to penetrate through the thickened exudate to reach the epidermis. Instead we measured the temperature some millimetres above and areas outside the lesion edge. Since the mites migrate away from these previously infested areas and are seen to be feeding mainly at the lesion edge, on relatively healthy skin and not on the dried serous exudate, the data we have collected is likely to be a good indication of the temperatures the mites experience in an *in vivo* environment.

We attempted to measure the relative humidity at the level of the epidermis from our experimental and control animals using the Grant Meter and relative humidity probe. Unfortunately the membrane covering the probe (a nitro-cellulose type material) became easily saturated in the moist fleece, consequently giving us misleading readings. This incident did give us a good indication of the near saturated relative humidities the mites are likely to experience at this location which we have also shown, with this work, to be beneficial to mite survival off-host. Although statistical analysis of the data has perhaps not rewarded us with the clear-cut and unarguable evidence to suggest the best conditions for optimum mite survival, we have learned from our experience during these trials, features that cannot be corroborated with statistical analysis. At times it was foolish to continue experimenting with conditions that were obviously detrimental to mite survival, especially as the mites are a valuable resource, for the sole purpose of

producing sufficient material for statistical scrutiny. It could be seen quite clearly during the course of a few trials that 75% relative humidity and below caused the mites to desiccate quickly. The use of statistics though, only informs us that the mites die. Under other conditions, for example at 37°C, the mites tended to die relatively quickly also, but not necessarily due to desiccation. This illustrates the need to make observations on the whole situation as well as looking for significance using statistics.

The Survival Off-Host Trials that were conducted for this study were primarily concerned with the survival of *P. ovis* mites off-host and *un-fed*. There is, as yet, no functional *in vitro* culture system for *P. ovis* mites and the exact nutritional requirements of the mite are not understood. Hence it was thought unwise to introduce unnecessary variables, like experimental diets, into this study, which a) may have had a deleterious effect on mite survival and b) influenced the experimental relative humidity of the mite's micro-climate, both of which are likely to have given a false indication of the effects on survival of the temperature and relative humidity variables (see Chapter 8 for a study of mite survival off-host on a range of experimental diets). It must be considered though, that mites having access to feeding fluids, whether *in vivo* or in an *in vitro* culture system, which is the ultimate goal of this research, will be able to support their body-water balance which will have a beneficial effect on their survival, as Knülle (1967) demonstrated with the spiny rat mite, *Echinolaelaps echidninus*. The presence of a fluid diet directly beneath the mites will also contribute to the saturation of the mite's immediate environment. We have found that under starvation conditions the CEH of *P. ovis* lies between 75 and 82% RH and that the mite's *in vivo* environment is close to saturation, with the inclusion of feeding fluids in an artificial culture system the relative humidity threshold is likely to be reduced.

O'Brien *et al.* (1994) demonstrated that *P. ovis* mites and eggs will remain infective to other sheep when kept off-host in situations likely to happen in the field, i.e. within pieces of fleece snagged on fences, trailers and so on, for significantly longer than mite survival in this study. In their study, mites remained viable off-host for up to 16

days, in all seasons. In our study the mean maximal survival time for the mites was significantly less, approximately 150 hours. It must be pointed out than in O'Brien's study the ambient air temperatures were significantly lower than in our study, as they were investigating conditions off-host in a "field" situation. We know that mite's longevity can be enhanced at lower temperatures but egg production is correspondingly reduced. We investigated environmental conditions comparable to the on-host conditions, i.e. much higher temperatures which is likely to increase the mites metabolism and promote starvation. Wilson *et al.* (1977) conducted a similar study to O'Brien's work but it was conducted in southwest USA. They found mites failed to remain infective, on average, after 3 days off-host in contaminated pens and enclosures. There is a great inconsistency in the findings of many workers over the years on the survival of *P. ovis* off-host under various conditions (Stockman, 1912; Shilston, 1915; Bedford, 1915; Dill, 1920). One explanation must be the differing environmental conditions found where these studies were conducted as they were carried out at various locations around the world. Another is possibly due to different mite strains found in different locations around the globe as Roberts and Meleney (1971) demonstrated that certain mite strains survived adverse conditions more readily than others.

We investigated several other saturated salt solution briefly for our humidity generator. Solutions that produced relative humidities below 75% caused the mite to desiccate in a matter of hours and we did not pursue these trials further.

Compared to experiments which may use a significantly fewer number of mites, using tritiated labelled water vapour of measuring the weight loss of mite with extremely sensitive microbalances, for example, this trial has adequately demonstrated the environmental conditions that propagate optimum mite survival, without the use of expensive equipment or specific skills.

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Chapter 3.

An Histological Investigation and Computerised 3-Dimensional Reconstruction of the Digestive System of *Psoroptes ovis.*

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Summary.

The digestive system of *Psoroptes ovis* was investigated by light microscopy and serial sections were used to create a computerised 3-dimensional reconstruction of the system. The components of the digestive system of *P. ovis* resemble those described for other acarines, but the structural layout appears to be unique. The system consists of a cuticle-lined foregut and anal atrium separated by a microvilli-lined midgut. The foregut can be divided into a muscular pharynx and oesophagus. The main area of the midgut is a bi-lobed ventriculus composed of squamous and cuboidal epithelial cells. Cuboidal cells appear to detach from the midgut epithelia and enter the gut lumen where they degenerate. Ingested food material condenses into food balls, as it migrates antero-dorsally to the junction with the postventricular midgut. This organ is an extension of the midgut and can be further divided into the colon and post-colon. The colon is composed of similar cells to the midgut ventriculi and is assumed to have a similar function, the colon epithelial cells possess significantly longer microvilli and appears to perform an absorptive function. From the rectal tube food balls/faecal pellets are passed posteriorly into the cuticle lined anal atrium and voided. The entire digestive system contains a significant population of luminal bacteria.

Introduction.

The Acarine digestive system can be considered as a simple tube (Baker and Wharton, 1952) divided into three general, distinguishable regions: foregut, midgut and hindgut. The foregut and hindgut are of ectodermal origin and hence cuticle lined. The midgut develops from endodermal tissue and is specifically adapted for secretion and absorption (Evans, 1992). This arrangement is remarkably consistent throughout the Acari and Arthropoda (Barrington, 1967).

The anatomy of the digestive system of many groups of mites has been studied using conventional light microscopy (Rohnde and Oemick, 1967; Brody *et al.*, 1972; Woodring and Galbraith, 1976; Mothes and Seitz, 1981) and using electron microscopy techniques (Wright and Newell, 1964; Coons, 1978). These morphological studies are in general agreement about the location and structure of the pharynx, oesophagus and hindgut but there is great diversity in the structures that constitute the midgut.

No previous description of the digestive system of *Psoroptes ovis* has been published. Blake *et al.* (1978) extensively investigated the mouthparts of *P. ovis* but the remainder of the alimentary tract was left unexplored. An understanding of the structure and function of the digestive tract may help further studies on *P. ovis* in many ways. In particular it will help in the understanding of the dietary requirements of the mite, an area which is poorly understood. Accurate information on food requirements is essential for the development of an *in vitro* culture system for *P. ovis.*

It is the purpose of this study to investigate the digestive system of *P. ovis,* a mite of considerable and increasing veterinary importance, using conventional microscopy techniques, to compare this with the known structures of other mites and to create a computerised 3-dimensional (3-D) representation of the main components of the mite's digestive system.

Materials and Methods.

Mite Collection and Separation.

All mites were obtained from artificially infested sheep contained in isolated sheep-scab yards at the Central Veterinary Laboratory. Mites, usually CVL strain, were removed from infested sheep by careful scraping at the lesion edge, where they accumulate, with the broad side of a mounted needle and transferred into Durham tubes. Adult females were separated from males and larval stages by placing around 50 mites on a 4cm filter paper in the centre of a 4cm diameter petri-dish. This petri-dish was attached to the inside base of a 9cm petri-dish with a piece of Blu Tack ™ and the resulting "moat" filled with water. This produced a secure container in which to manipulate the mites. The petri-dish isolation unit was placed under a dissection microscope enabling the various stages to be easily identified. The adult females were removed and transferred into the Eppendorph incubation cages (see Chapter 2 for design) for fixation and subsequent processing. All experimental work described in this chapter was carried out on adult female mites as they are the largest life stage (approximately 600-750μm long) and consequently easier to process.

For the conventional histology investigated and presented in this study, two pre-treatment and fixation methods, out of several investigated, were found to produce acceptable results (see the Discussion section for more information):

1. Susa Fixation for Conventional Histology.

Usually six mites at a time were transferred into Eppendorph cages and agitated in 2% Tween 80 in distilled water (dH_2O) for 5 mins and rinsed in dH_2O for 5 mins. This washing stage removed any surface debris from their cuticle. They were then removed individually from the Eppendorph cages with a mounted needle and submerged under a drop of chilled Susa fixative (see Appendix A) in a solid watch glass with a layer

of dental wax covering the bottom. To facilitate the infiltration of fixatives, methanol dehydrating agents and resin, the mites were carefully punctured through their dorsal or ventral surface with an electrically sharpened steel or tungsten pin (Brady, 1965) avoiding internal areas of interest.

The mites were then transferred back into the Eppendorph cages and submerged in fixative. Trapped air bubbles were removed from the Eppendorph cages and mites by inverting the cages in the fixing medium, allowing the bubbles to gather on the inside of the gauze and sucking them out using a capillary pipette. The mites were fixed overnight at 4°C.

After fixation the fixative was poured off and the mites were rinsed in dH_2O for 3 x 10 minutes, dehydrated through an industrial methylated spirits (IMS) series; 50, 70, 90 and 3 x 100%, 10 minutes each, and infiltrated with 50% Historesin in absolute alcohol overnight at 4°C. During all washing, fixing, dehydrating and infiltrating procedures the mites were contained in Eppendorph cages and agitated using a rotary mixer at 4°C. The mites were then polymerised in fresh 100% Historesin overnight at room temperature. See Table 1. for a summary of the procedures used. Before the final resin became too viscous, the mites were manipulated with a mounted needle so they lay in a position that enabled them to be sectioned in a desired plane.

The blocks were cut at 4µm on an LKB 2218 Historange microtome and the sections expanded with dH_2O directly onto glycerine-albumin treated slides. After drying overnight at 50°C on a hot-plate the sections were treated with a variety of stains, the majority of which required the removal of residual mercuric chloride, deposited during Susa fixation. This was performed by washing in 0.5% iodine in 70% alcohol for one minute followed by 5% sodium thiosulphate for one minute. The slides were then washed for 3 minutes in running water.

Stains used were:

- Toluidine Blue.
- Ehrlich's Haematoxylin and Eosin.

(See Appendix A for staining procedures)

Following staining the sections were dehydrated through 50, 70, and 3x 100% IMS; 2mins each and mounted in DPX mountant.

Table 1. Summary of the procedures used for Susa fixation and Historesin embedding of *P. ovis* for conventional microscopy.

• Wash in 2% Tween 80 in dH_2O	5 mins.
• Rinse in dH_2O	5 mins.
• Mites pierced and Susa fixed at 4°C	Overnight.
• Rinse in dH_2O	3x10 minutes.
• Dehydration through 50, 70, 90 and 3x 100% IMS	10 minutes each.
• Infiltration with 50% Historesin in 100% IMS at 4°C	Overnight.
• Polymerised in 100% Historesin at room temperature	Overnight.
• (Post-sectioning) 0.5% Iodine in 70% methanol	1 minute.
• 5% Sodium Thiosulphate	1 minute.
• Rinse dH_2O	3 minutes.

2. Glutaraldehyde and Osmium Fixation for Conventional Histology.

The tissue blocks that were prepared for Transmission Electron Microscopy (TEM) (see Chapter 4.) were sectioned and examined at the light microscopy level also. The fixation procedure for these was as follows:

Mites were washed and rinsed as for Susa fixation in 2% Tween 80 in dH_2O. They were then submerged in chilled 4.2% glutaraldehyde (v/v) in 0.1 M phosphate buffer, pH 7.4 with 8% sucrose (w/v) added.

Mites were individually removed from the Eppendorph cages and submerged under a small drop of fresh fixative on a 9cm petri-dish. They were pierced as before with an electrically sharpened tungsten pin and again, to further aid infiltration of fixatives and resin, either the mouthparts or a portion of the mite's posterior was carefully but swiftly removed using a small scalpel blade. The legs were also often removed at the coxa. The samples were then transferred back into Eppendorph cages, usually six - ten per cage, air bubbles removed as before and fixed overnight at 4°C. The fixative was removed and the mites were rinsed for 3 x 10 mins in 0.1M phosphate buffer and secondarily fixed in 2% aqueous osmium tetroxide (OsO_4) for one hour. They were then rinsed for 3 x 10 mins in dH_2O, dehydrated through 50, 70, 90% and 3 x absolute ethanol, 10 minutes each and infiltrated with 50% Taab Transmit resin in absolute ethanol for 24hrs at 4°C, fresh 100% Transmit for 24hrs at 4°C before final embedding in fresh Transmit and polymerised at 60°C for 24hrs in individual moulds. During all washing, fixing, dehydrating and infiltrating procedures the mites were contained in Eppendorph cages and agitated on a rotary mixer at 4°C.

The blocks were trimmed on a LKB Pyramitome and 1 - 3µm sections were cut with fresh glass knives directly onto water with a standard LKB Ultramicrotome. Sections were transferred onto clean microscope slides using a small wire loop and baked dry on a hot plate at 60°C for a few minutes before being stained. Stains used were:

- Toluidine Blue.
- Ehrlich's Haematoxylin and Eosin.

(See Appendix A for procedures)

Following staining, sections were mounted in DPX mountant.

Table 2. Summary of the procedures used for Glutaraldehyde and Osmium fixation and Transmit embedding of *P. ovis* for conventional microscopy.

• Wash in 2% Tween 80 in dH_2O	5 mins.
• Rinse in dH_2O	5 mins.
• Mites pierced and Glutaraldehyde fixation at 4°C	Overnight.
• Rinsed in buffer	3x10 mins.
• 2% OsO_4 fixation in dH_2O	1 hour.
• Rinsed in dH_2O	3x10 mins.
• Dehydration through 50, 70, 90 and 3x 100% ethanol	10 minutes each.
• Infiltration in 50% Taab Transmit/ethanol at 4°C	24 hours.
• 100% Transmit at 4°C	24 hours.
• Polymerised in fresh Transmit at 60°C	24 hours.

Scanning Electron Microscopy.

The mite's external features were examined using scanning electron microscopy (SEM). Three fixation methods were employed.

1. Glutaraldehyde Fixation.

Whole adult female mites were placed in an Eppendorph cage and washed for a few minutes in acetone to remove superfluous surface material. They were then pierced in a posterior location to allow unobstructed movement of fixatives, dehydrating agents and so on. They were fixed in 4.2% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 overnight before being rinsed for 3 x 10 minutes in dH_2O and dehydrated as described above in a graded series of IMS. They were then taken through 50:50 IMS:acetone, 25:75 IMS:acetone, and 100% acetone, 3 x 10 minutes for each stage. The mites were then critical point dried in carbon dioxide over a 3 hour period, mounted on aluminium SEM stubs with double-sided adhesive tape before being gold coated in a Polaron Gold Sputter-Coater at 1.2kV, 10mA for 10 minutes and viewed with a Hitachi S520 Scanning Electron Microscope at 10kV.

2. Chloroform Fixation

Mites were washed as before in acetone, pierced and submerged in 100% chloroform. The chloroform was replaced every hour for three hours. The mites were then removed from the solvent and air-dried for three hours at room temperature, mounted on aluminium SEM stubs with double-sided adhesive tape before being gold coated and viewed as before.

3. Cryosystem Preparation.

Cryo preparation was performed using a Hitachi SEM and Emitec K1250 Cryogenic System. Mites were washed as before in acetone and individually mounted on brass cryostage blocks using a small amount of OCT medium (Tissue-Tec®) low temperature adhesive. They were then cryofixed in liquid nitrogen slush (approximately -210°C) withdrawn into the specimen chamber, evacuated and introduced into the pre-

cooled electron microscope, at approximately -150°C, to check for structural preservation and for the presence of surface ice crystals. The crystals, deposited during fixation, were sublimed off by raising the temperature of the SEM stage to approximately -80°C. The sublimation process could be visualised and monitored using the SEM conventionally. The stage temperature was reduced to approximately -150°C once the crystals had been removed. The specimens were withdrawn from the microscope into the specimen chamber, under vacuum, and transferred to the evacuated cryosystem preparation chamber chilled to approximately -150°C. Gold coating then followed for 1 minute with an argon plasma current of 20mA giving a coating thickness of approximately 20nm. The samples were then reintroduced into the chilled SEM and illuminated with a potential of 7-10kV.

4. Live Whole-Mounted Mites.

The pumping action of the mite's pharynx and oesophagus and the filling of the digestive system were examined by mounting several whole, live mites on microscope slides with coverslips, submerged under either phosphate buffered saline (PBS) or PBS stained lightly with acid fuchsin.

3-D Reconstruction of Digestive System.

Approximately 60 4μm Susa fixed, Historesin embedded serial sections were used to create a computerised 3-D reconstruction of the main components of the digestive system of *P. ovis*. The toluidine blue stained sections were enlarged graphically from microscope slides onto tracing paper using a microscope and scribing table. The conventional microscope used was modified so as to project a section image from the specimen stage, through the eye-piece and via a pair of small mirrors at right angles to each other to the underside of an electronic tracing table. Each section contained contours of the important features (cuticle, oesophagus, midgut, hindgut and so on) of the mite's digestive system which were initially traced onto translucent paper (approximately x500 magnification) then digitised and stored as co-ordinates in

individual files on computer. This information was then compiled into a graphical file consisting of the digitised constituents of the mite's digestive system, which was used to create the 3-D computer model.

Various computer programs (written or modified by Andrew Davies or Daffydd Roberts, Computer Department, at the University of Wales, Bangor) were used to manipulate the raw data to its final form in the database. Briefly:

- ROTATE.EXE rotates each digitised section, aligning the calibration points one above the other as they would appear in the intact organism. One calibration point becomes the origin (0,0) and the original co-ordinates created during the initial digitisation are modified in relation to this origin.

- MINMAX.EXE calculates the largest and smallest x and y co-ordinates the model contains, information necessary for the next programs.

- SHIFT.EXE moves all sections and aligns them so the largest is contained within the positive x and y axes.

- SCALE.EXE scales the sections to fit onto the specified presentation area (512 x 512).

- MODIFY.EXE names each section and contour specifically e.g. cuticle, oesophagus etc.

- CLOC04.EXE fixes any breaks or overlaps in the contours that might have occurred during the tracing and assembles the database of section co-ordinates, names and colours.

- ILLUS04.EXE is the program that produces the 3-D image from any of the numerous available viewpoints. The image can then be saved as a file and printed in a number of formats: stereo left and right, single image and so on.

In this case the desired images were displayed using ILLUS04.EXE and "captured" using a screen-capture program. The images, in PCX format, could then be imported into Corel DRAW!® (a graphics software package from the Corel Corporation) labelled and imported into a word processor and colour laser-printed.

Results.

Conventional Histology and Live-Mite Observations.

The Mouthparts

The articulated gnathosoma of *P. ovis* is situated anteriorly between the coxae of the first pair of legs where it maintains a curved profile, arching ventro-dorsally in a posterior direction [Figs. 1 and 2]. The gnathosoma steadily widens from approximately 15 to 60μm from its anterior extreme to the junction with the body or idiosoma.

The components of the mouthparts that contact the host's epidermis, consist of two fan-like, lateral projections arising from the termination of a pair of palps [Fig. 3], occasionally seen fused at their dorsal tips [Fig. 4]. The digestive system proper begins immediately posterior to these pseudorutellar prolongations with the pre-buccal or pre-oral cavity. Within this cavity lie a pair of mobile chelicerae possessing retro-facing teeth or barbs [Figs. 5 and 6]. From live whole-mite mounts, these have been observed to articulate in an antero-dorsal and lateral plane independently. The chelicerae are sealed within the pre-oral cavity dorsally by a membranous covering between the fused palps [Fig. 4] and ventrally by the base of the gnathosoma. A tube is therefore formed from the pseudorutellar prolongations to the junction with the pharynx.

The Foregut: Pharynx and Oesophagus.

Food material is actively sucked from the pre-oral cavity within the gnathosoma posteriorly into a short, narrow, cuticle-lined, muscular pharynx [Fig. 7]. The pharynx runs for approximately 70μm horizontally before reaching the junction with the oesophagus.

After being drawn through the pharynx, the ingested meal passes through a pharyngo-oesophageal valve and enters the cuticle-lined oesophagus, which runs postero-dorsally through the central nervous system for approximately 70μm [Figs. 7 and 8] to the junction of the midgut at its anterior extreme [Fig. 9]. The oesophagus has

a eight-pointed, star-shaped transverse profile. No specialised valve was observed at the junction between the oesophagus and the midgut. The pharynx and the oesophagus constitute the foregut.

The Midgut: Stomach or Ventriculus and Postventricular Midgut.

The midgut of *P. ovis* can be divided into two main structural areas from their appearances at the light microscopy level; the stomach or paired ventriculi and the postventricular midgut.

1. The Stomach or Ventriculus.

The largest component of the digestive system is the stomach or ventriculus which occupies the majority of the mite's body cavity when engorged and directly accepts ingested material from the oesophagus. No structural valve has been observed at this junction using conventional histological techniques.

Observations made while watching mites ingest blood or other coloured liquids, in a simple *in vitro* feeding system, suggest that the meal is not initially compartmentalised into a specific area of the stomach or ventriculi, but enters the stomach and distends both ventriculi equally, where it presumably begins to be processed. The structure of the stomach is a large, laterally displaced, bi-lobed central ventriculus [Figs. 10a-d and 11] which can be further divided into four pairs of smaller caeca [Fig. 12]; two are located antero-laterally at the junction with the oesophagus and the two other less well defined pairs are posteriorly located extensions of the bi-lobed ventriculus (see Fig. 10d for caeca with better definition). The caeca become more pronounced when the gut is distended with food, being bisected by dorso-ventrally running somatic muscles. From live mite observation these somatic muscles are seen to be actively contracting when the stomach is at least partially full, and are believed to play a role in gastric mixing and peristalsis, perhaps ultimately transferring processed food material into the post-ventricular midgut. They also seem to have a role to play in

oviposition, as they contract, compressing the body dorso-laterally and forcing the single or paired eggs through the ovipositor. Visceral muscles can also be seen through the translucent cuticle actively mixing the gut content while observing the mites feeding using a dissecting microscope. These muscles cannot be seen in sections at the light microscopy level. Figure 13 is a section demonstrating the plicated midgut epithelia seen when the gut is less distended by the ingested meal. Observing whole mounted mites ingesting stained PBS, suggests food material enters the midgut from the oesophagus and fills the whole stomach/ventriculi rather than being displaced into discrete areas. The food remains in the ventriculi, presumably being processed and mixed by peristalsis.

Epithelial cells lining the ventriculi are usually squamous or cuboidal. Cuboidal cells are often observed to be in various stages of "budding-off" from the epithelial cell layer [Figs. 14 and 15]. These cells elongate apically, detach from the epithelia and enter the gut lumen as spherical free-floating cells. Their staining characteristics change relative to the remaining epithelia, for example they stain intensely with toluidine blue. Ingested food material is often seen to condense into dense spheroids as it is processed in the ventriculi [Fig. 16]. Also frequently observed throughout the midgut are host derived eosinophilic lymphocytes [Fig. 17]. These can be differentiated from the free-floating epithelial cells as they have multiple lobed nuclei and are eosinophilic when stained with haematoxylin and eosin. This study has also demonstrated that a significant population of bacteria are frequently observed in the mite's digestive system, the majority of which are rods [Figs. 8 and 18]. The free-floating epithelial cells, the eosinophils and the bacteria are the only cellular materials that have been observed in the digestive system of *P. ovis*, suggesting that the majority of the gut content is of a liquid nature.

2. The Postventricular Midgut: The Colon and Post-Colon.

After storage and circulating around the midgut ventriculi, food material is transferred antero-dorsally to the postventricular midgut, leaving the stomach at a dorsal location [Figs. 19 and 20]. This organ can be divided into two distinguishable parts. The first, the colon, is a short, tubular organ lined with squamous and cuboidal, microvilli-lined epithelial cells, similar to those lining the stomach. This runs from the dorsal junction of the stomach, posteriorly for approximately 200μm. There follows a constriction in the epithelia and connective tissue and the post-colon follows [Fig. 21]. The epithelia in the post-colon is composed of similar squamous and cuboidal epithelial cells to the stomach, with possibly an increased prevalence of squamous cells, but the microvilli are significantly more elongated than those lining the stomach and colon [Fig. 21]. The post-colon descends ventrally between the two main lateral lobes of the stomach and passes posteriorly to join the anal atrium or hindgut [Fig. 22]. Faecal pellets, frequently seen containing bacteria, form during the transition along the colon to post-colon [Fig. 19] to hindgut.

The Anal Atrium.

The cuticle lined hindgut or anal atrium [Fig. 22] is a narrow, flexible tube approximately 200μm long connecting the post-colon with the anus and anal plates, through which the finally processed faecal pellets pass to the outside. Due to its narrow profile, the anal atrium is easier to detect in sections when distended during the transferral of faecal material along its length [Fig. 23], before passing through the internal and external anal plates to the outside [Fig. 24].

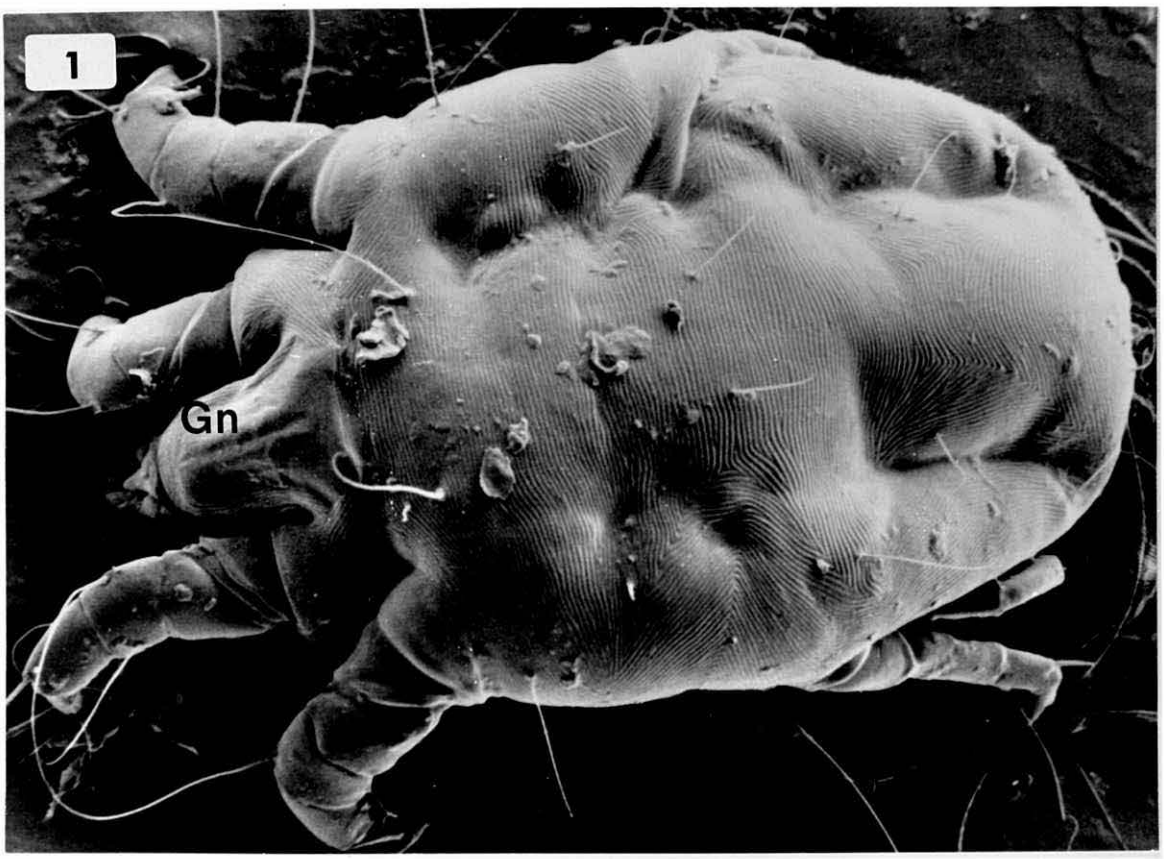
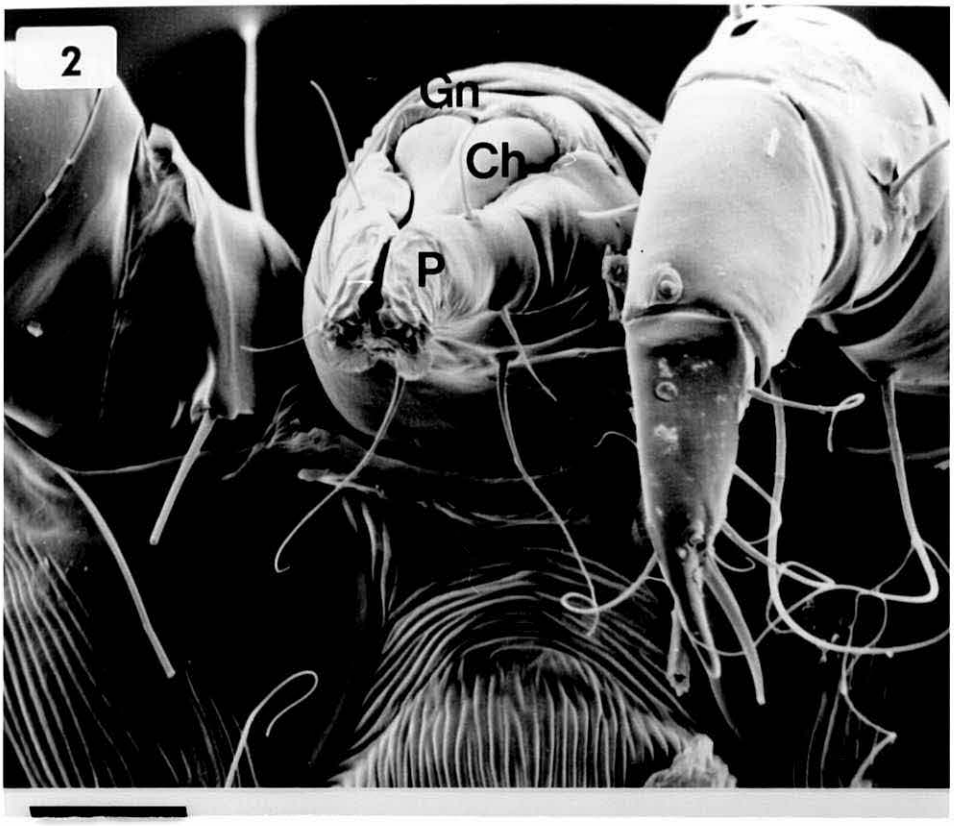


Figure 1. Dorsal view of an adult female *Psoroptes ovis* mite. The depressions in the cuticle reveal the internal attachment points of the dorso-ventral running somatic muscles. The typical arched profile of the gnathosoma (Gn) can be seen lying between the first pair of legs. Bar = 115μm.

Figure 2. Ventral view of the gnathosoma of *Psoroptes ovis* demonstrating the position of the gnathosoma (Gn) lying between the first pair of legs and arching ventrally from the attachment with the body. The palps (P) can be seen, although often seen fused together, their close association has been lost in this instance. The paired, mobile chelicerae (Ch) can be seen running through the channel formed by the close association of the two palps. Bar = 45μm.

Figure 3. *En face* view of the anterior extreme of the mite's mouthparts. These fan-like processes or pseudorutellar prolongations (PR) are the termination of a pair of palps. They are pressed against the host's epidermis and fluid material is presumably directed along the grooves, through the anterior opening (arrow) of the pre-buccal or pre-oral cavity. The palpal processes (PP) may serve to abrade the epidermal surface or anchor the mouthparts to the epidermis while fluids are ingested. Their precise function is not known. Bar = 8μm.


2
Gn
Ch
P

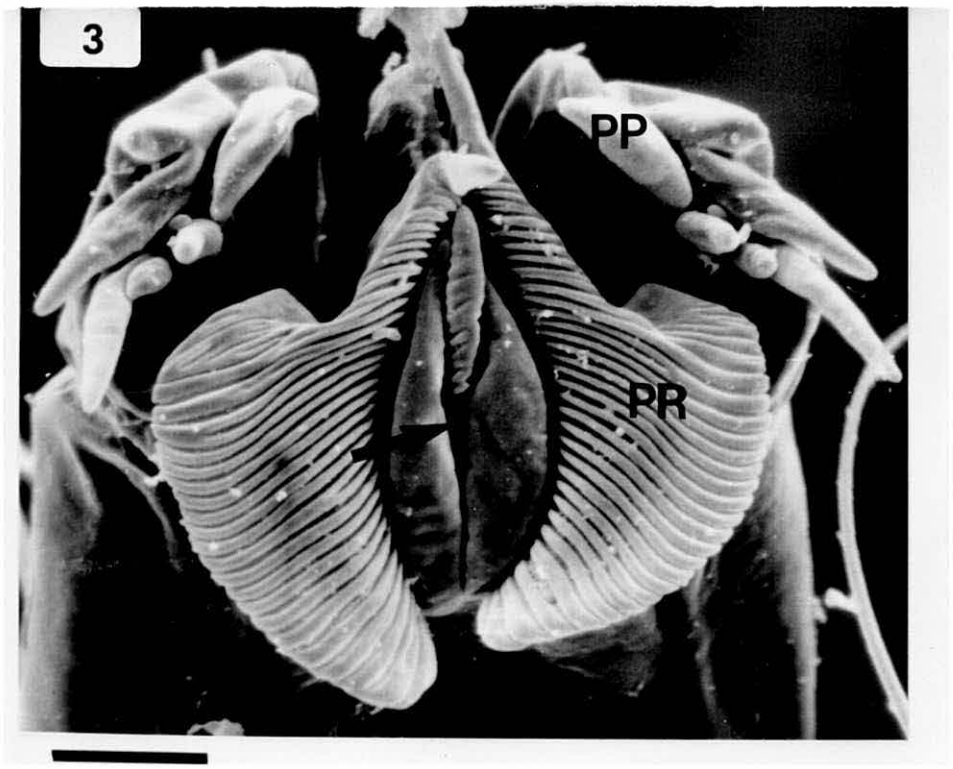

3
PP
PR

Figure 4. The gnathosoma viewed from a ventro-lateral position. The fused palps (P) forming the lateral margin of the gnathosoma can be seen as can the chelicerae (Ch) running through the central channel formed by the palps. Bar = 36μm.

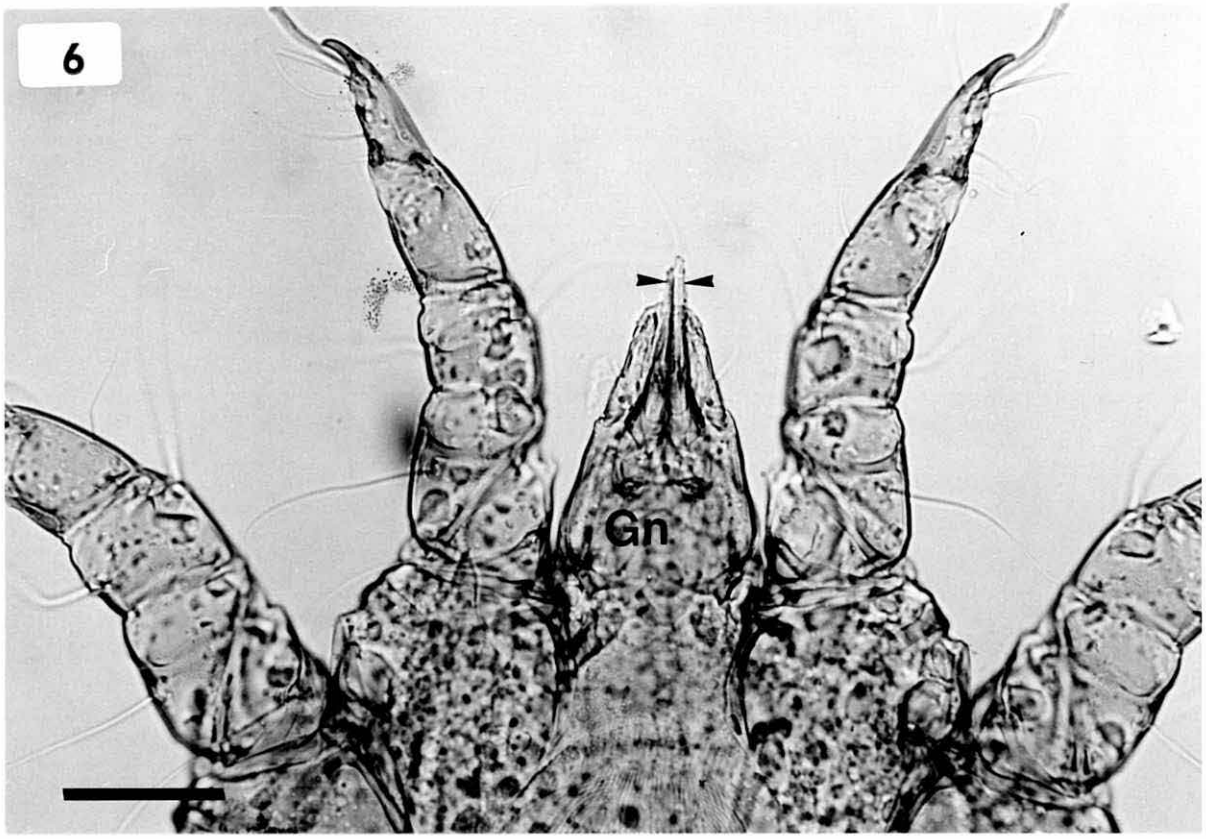
Figure 5. The anterior termination of the gnathosoma. The palps are not fused together in this example which allows the retractable, toothed chelicerae (Ch) to be observed. These possibly have an abrasive action on the host's epidermis but the feeding processes are not completely understood. The fan-like pseudorutellar prolongations (PR) and the anterior opening (arrow) of the mouthparts can be seen. Bar = 9μm.


4
Ch


5
Ch

Figure 6. Whole, live-mounted *P. ovis* (compressed dorso-ventrally) showing the retractable chelicerae (arrowheads) extended from the gnathosoma (Gn). They have been observed to extend and contract and articulate laterally, independently to each other with this method of preparation. Bar = 85μm.

Figure 7. Vertical-longitudinal section through an anterior portion of the mite. The gnathosoma is not included but visible is the narrow, sclerotized pharynx (Ph) and a group of pharyngeal dilator muscles (PDM) attached from the dorsal roof of the pharynx to the inside, dorsal surface of the gnathosoma. The pharyngo-oesophageal valve (POV) can be seen as can the oesophagus (Oe) running through the central nervous system (CNS) into the stomach (St) at an antero-ventral position. Bar = 54μm.


6
Gn

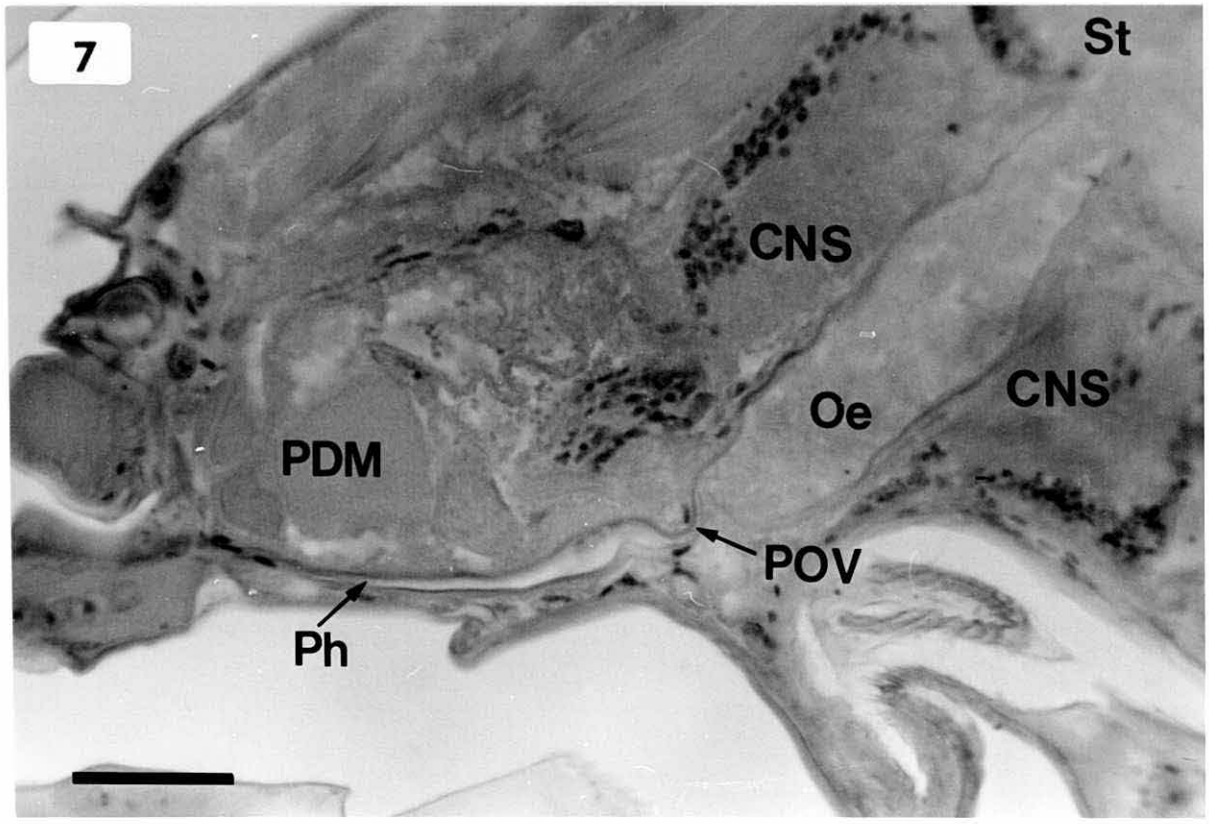
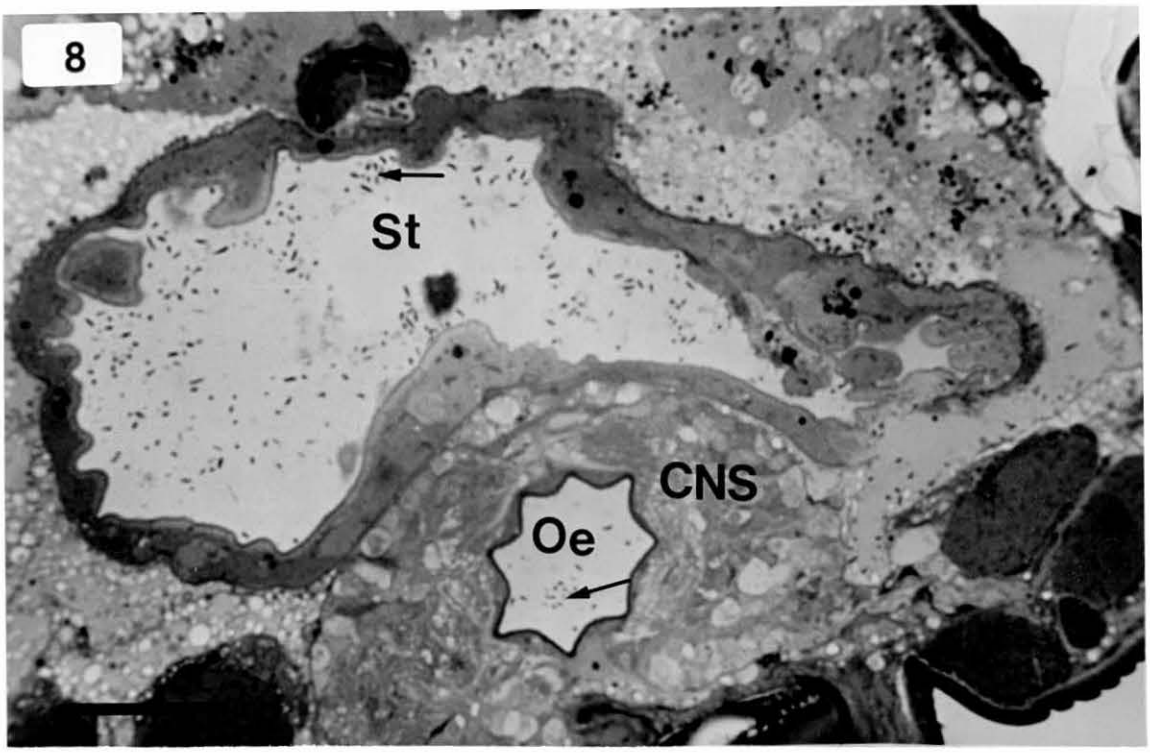
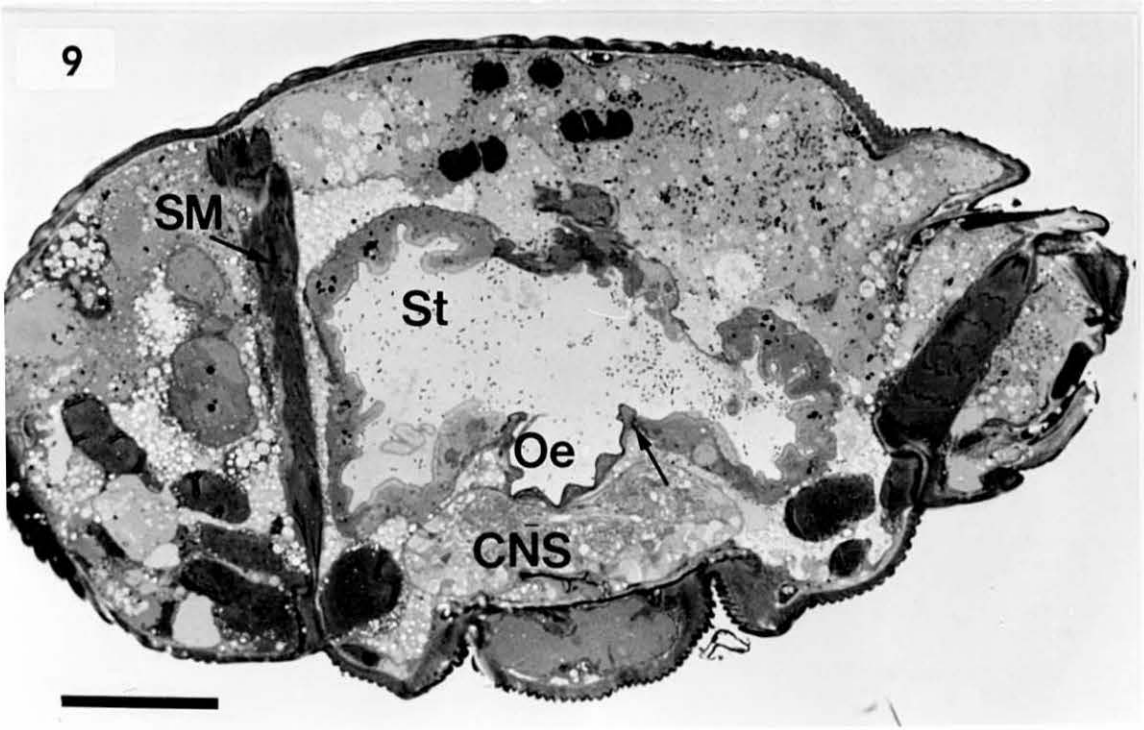

7
St
CNS
CNS
Oe
PDM
POV
Ph

Figure 8. Transverse section through the oesophagus (Oe), central nervous system (CNS) and an anterior portion of the stomach (St). The star-shaped transverse profile of the oesophagus can be clearly seen and the proximity of the surrounding CNS. Bacteria (arrows) can be seen in both the oesophagus and stomach. Bar = 46μm.

Figure 9. Transverse section through the oesophagus (Oe), central nervous system (CNS) and the anterior portion of the stomach (St). This section demonstrates the junction between the oesophagus and the midgut and the close juxtaposition between the cuticle of the oesophagus and the epithelia of the midgut (arrow). A longitudinal section through a dorso-lateral running somatic muscle (SM) group can be seen. Bar = 70μm.


8
St
CNS
Oe


9
SM
St
Oe
CNS

Figures 10a-d. Four horizontal-longitudinal sections at approximately 100, 150, 200 and 250μm through the mite in a dorso-ventral direction (total mite thickness ≈300μm). The bi-lobed, laterally displaced left and right ventriculi (LV) and (RV) can be clearly seen as can the posterior extensions or caecae (arrows) of the ventriculi. The anterior extensions are not clear in these sections. Figures 10b-d show the oesophagus (Oe) and the central nervous system (CNS) which is especially clear in Figure 10d. Toluidine blue stained. Bar = 165μm.

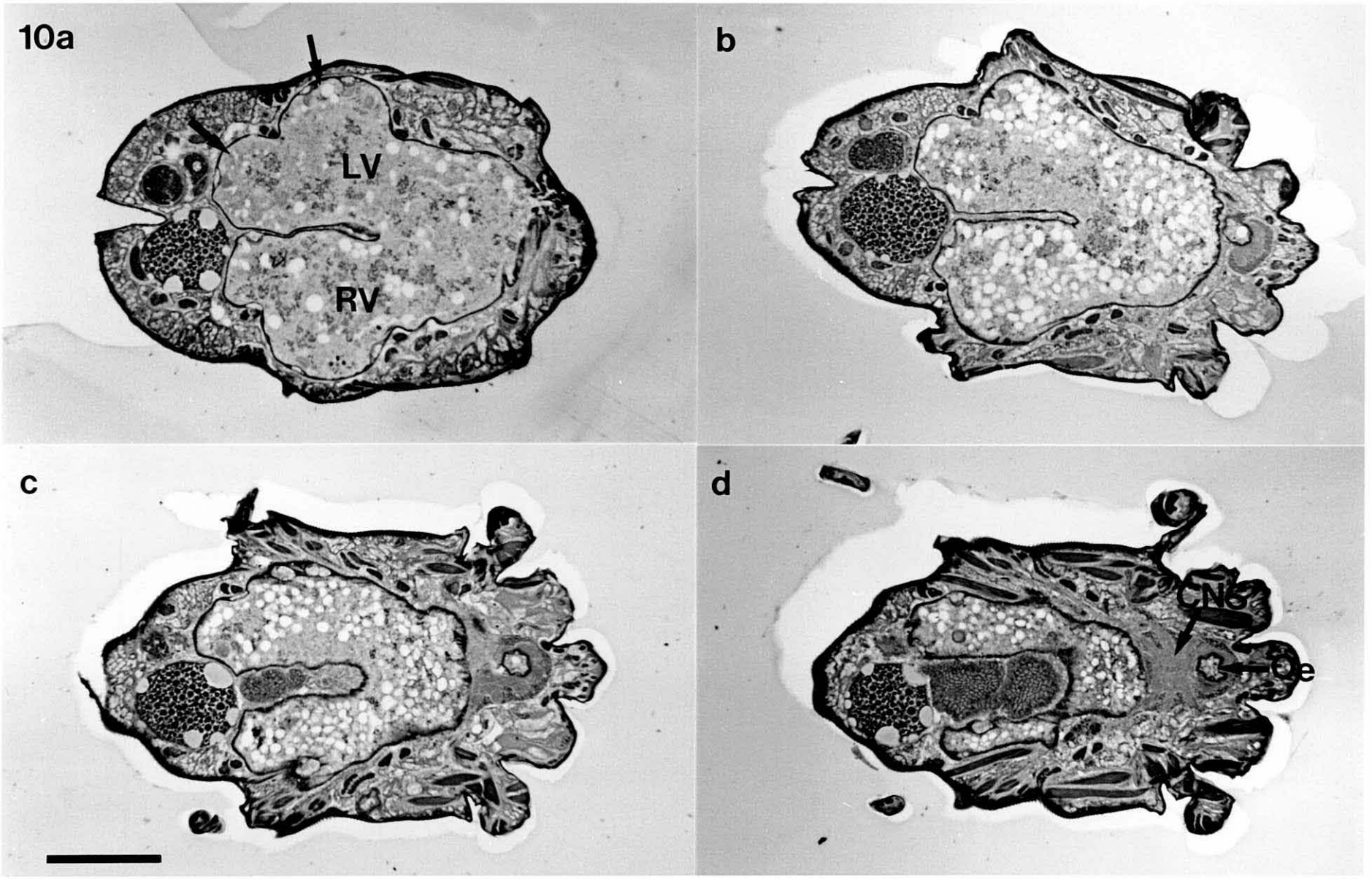
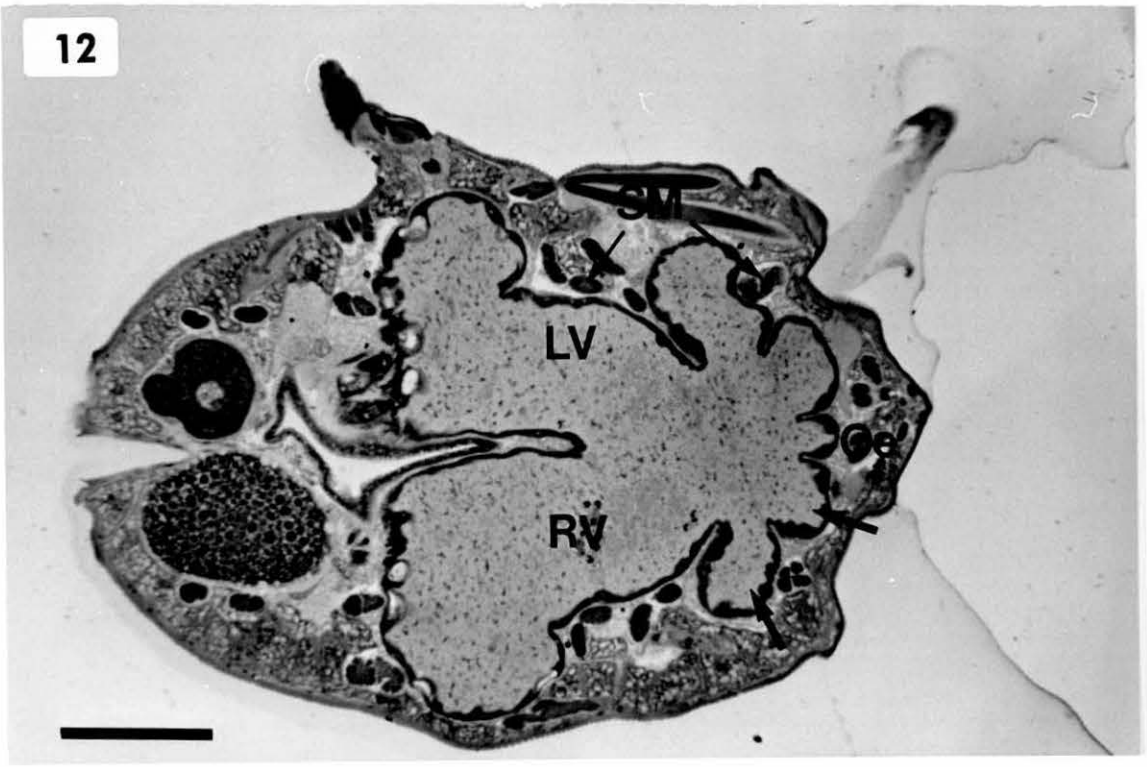

10a
LV
RV
b
c
d
CNG
e

Figure 11. A near central, vertical-longitudinal section through *P. ovis* showing part of a ventriculus (V). A membrane bound food ball (FB) can be seen just below the entrance into the postventricular midgut (thin arrow). The central nervous system also appears in this section (thick arrow). Bar = 135μm.

Figure 12. A central, horizontal-longitudinal section through *P. ovis* demonstrating the left and right ventriculi (LV) and (RV) respectively and the anterior caeca (arrows) protruding through the dorso-ventral running somatic muscles (SM). The junction of the oesophagus and midgut can also be seen (Oe). Bar = 125μm.

11

12



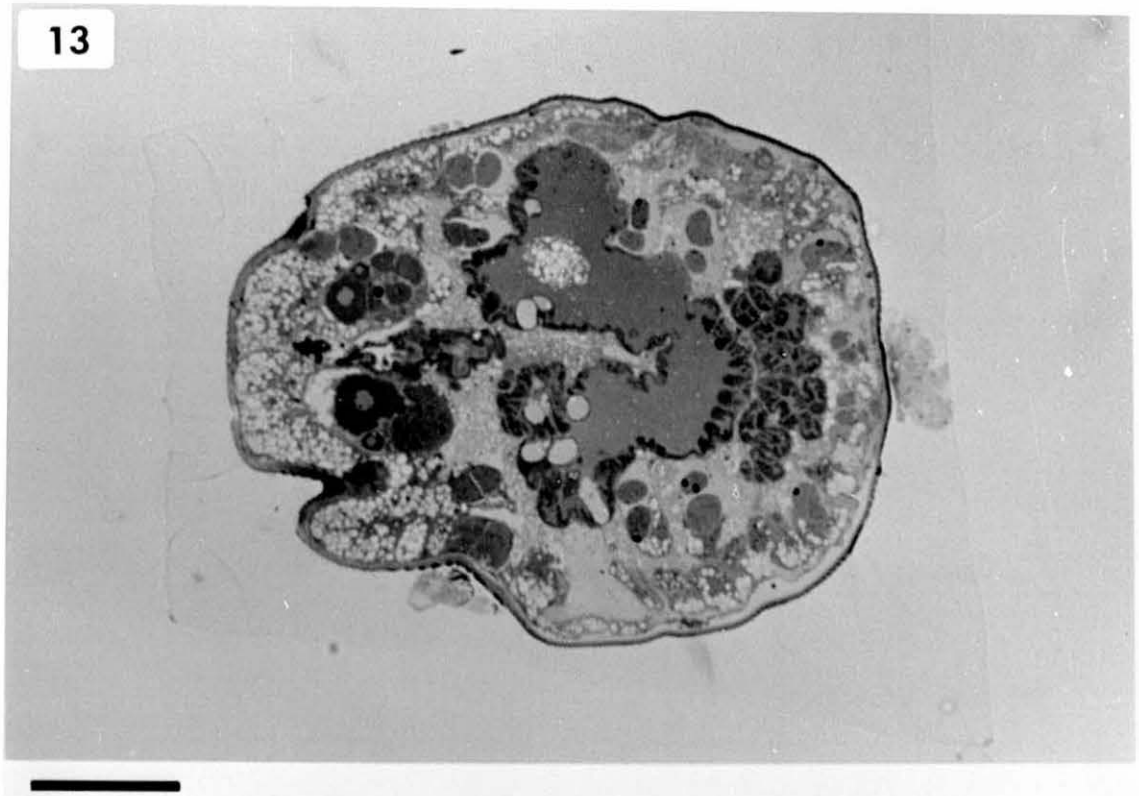
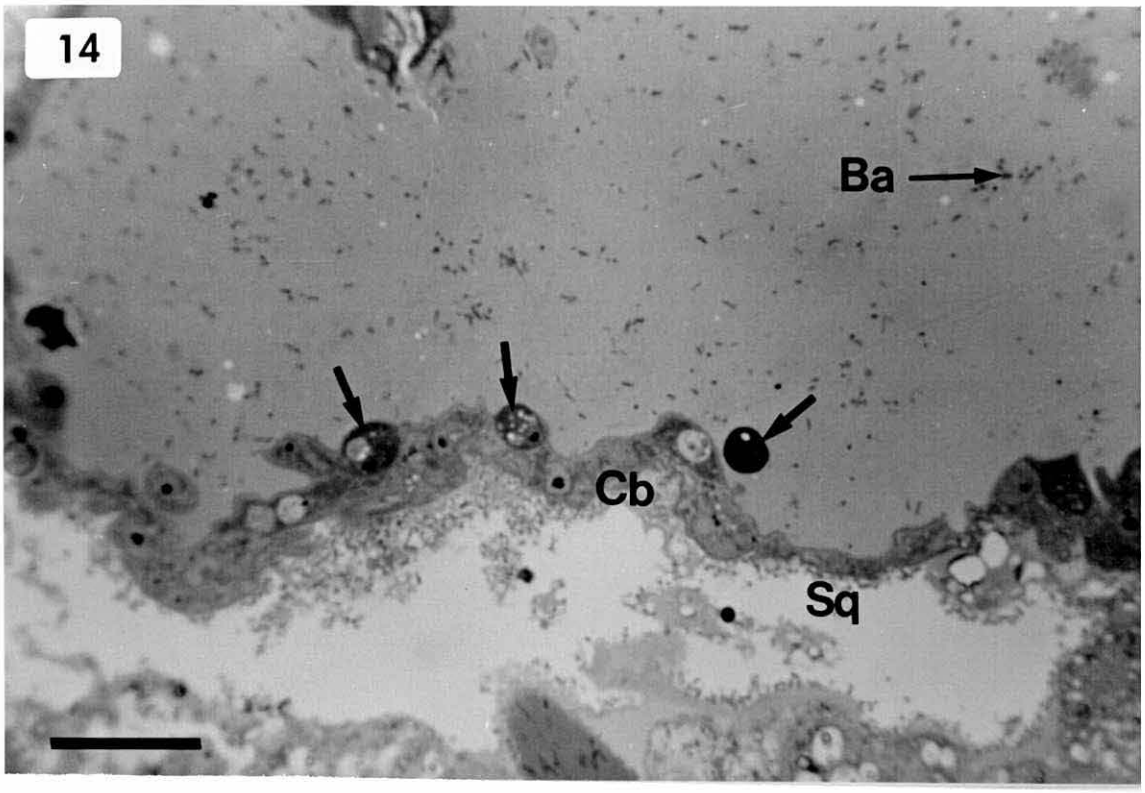
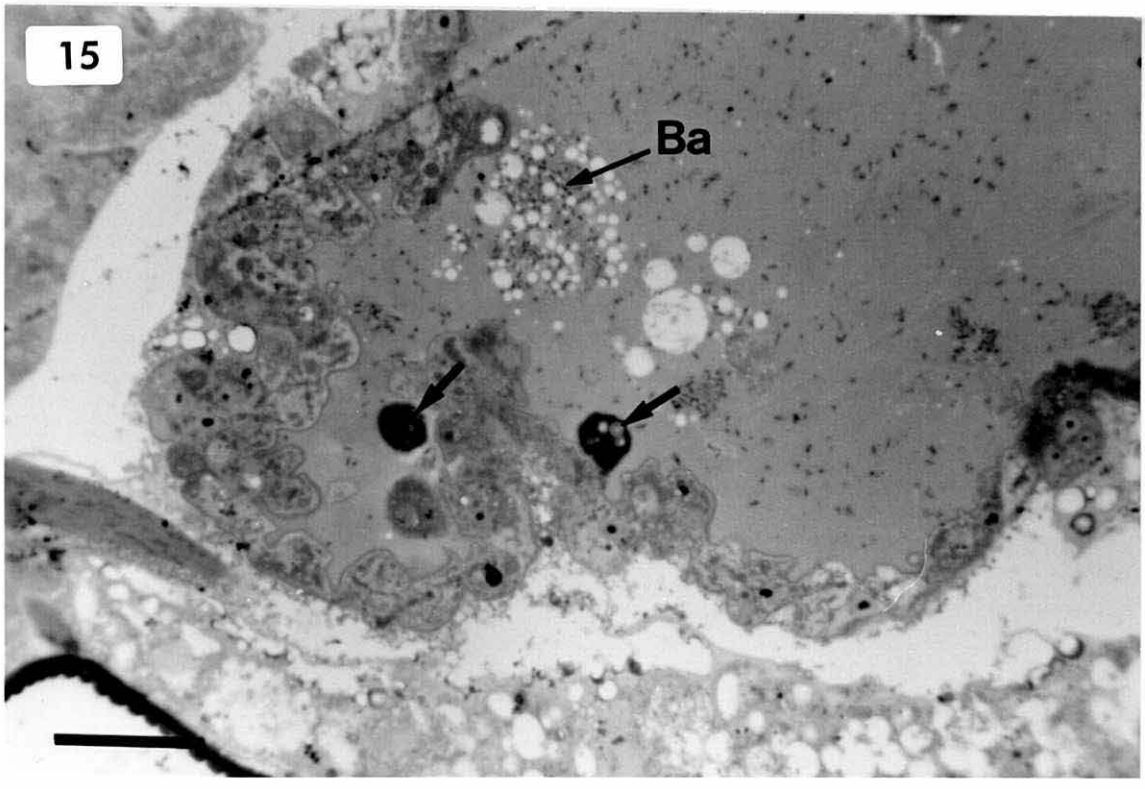


Figure 13. This approximately central, horizontal-longitudinal section through *P. ovis* demonstrates the plicated midgut epithelia observed when the gut is less distended with food material. Bar = 140μm.

Figure 14 and 15. These two sections showing two areas of the midgut ventriculi demonstrate the types of cells that constitute the midgut epithelium that are discernible at the light microscopy level. The cells range from squamous (Sq) to cuboidal (Cb) possibly due to localised distension of the gut. Cuboidal cells are often observed to be in various stages of enlarging and "budding-off" from the epithelium (arrows). These cells have different staining characteristics to the remaining cells, typically becoming more intensely stained as they develop and migrate into the lumen. Bacteria (Ba) can also be seen throughout the gut lumen of both these sections. Bar = 25μm.


14
Ba
Cb
Sq


15
Ba

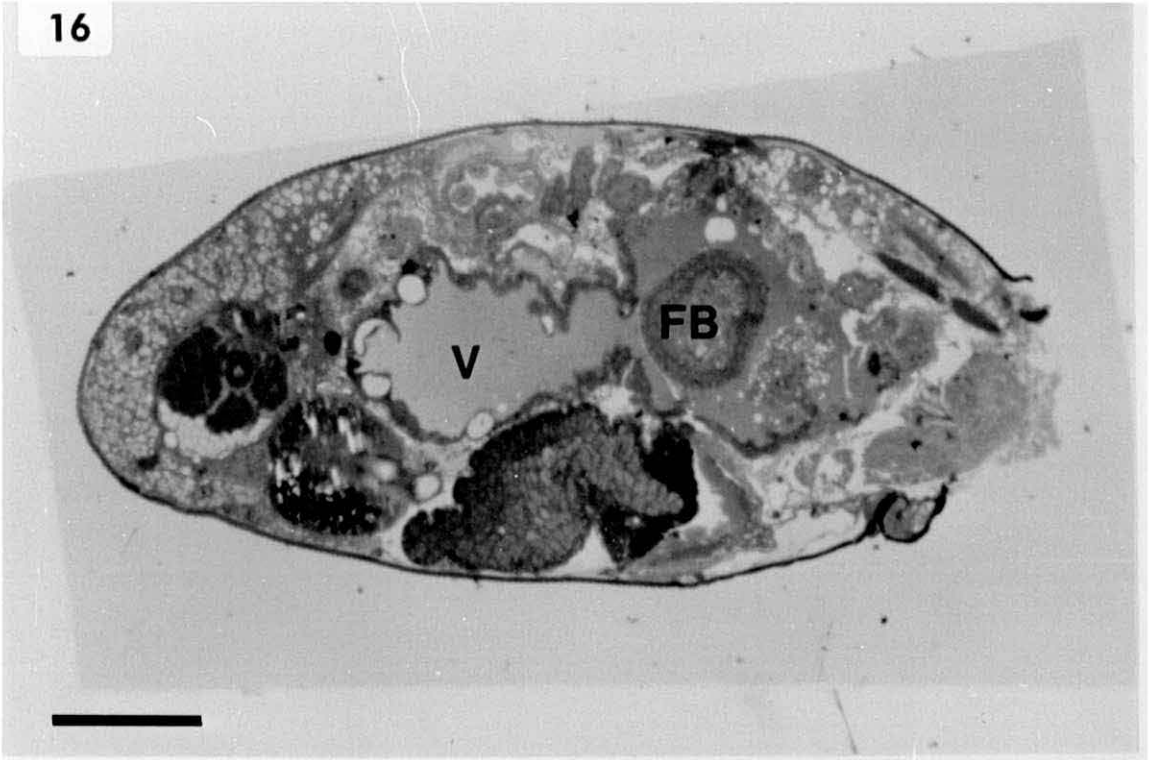
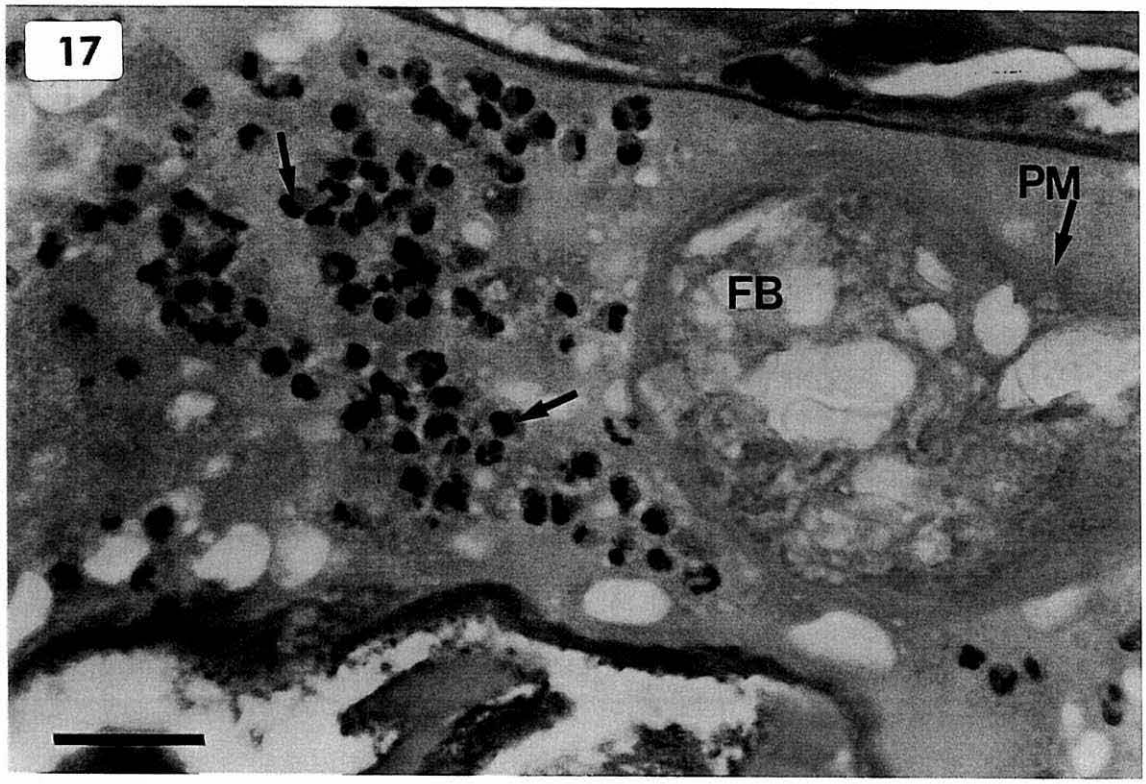


Figure 16. A near central, vertical-longitudinal section through *P. ovis* showing one of the midgut ventriculi (V) and food material condensing into a membrane bound food-ball (FB). Bar = 105μm.

Figure 17. This section through a midgut ventriculus demonstrates the frequently observed host-derived eosinophils (arrows). The clinical manifestation of sheep scab often results in the production of large numbers of eosinophils that migrate to the epidermis where they are often ingested by the mites feeding in this location. The section also shows a food-ball (FB) which seems to be enveloped in a type of peritrophic membrane (PM). Stain with haematoxylin and eosin. Bar = 30μm.

Figure 18. This horizontal-longitudinal section through the left ventriculus (LV) demonstrates the significant population of bacteria (Ba) that are frequently observed throughout the mite's digestive system and the dorso-ventrally running somatic muscles (SM). Also seen here are a group of host derived polymorphonuclear leukocytes, probably eosinophils (arrows) as described in Fig. 17. Toluidine blue stained.
Bar = 30μm.


17
PM
FB

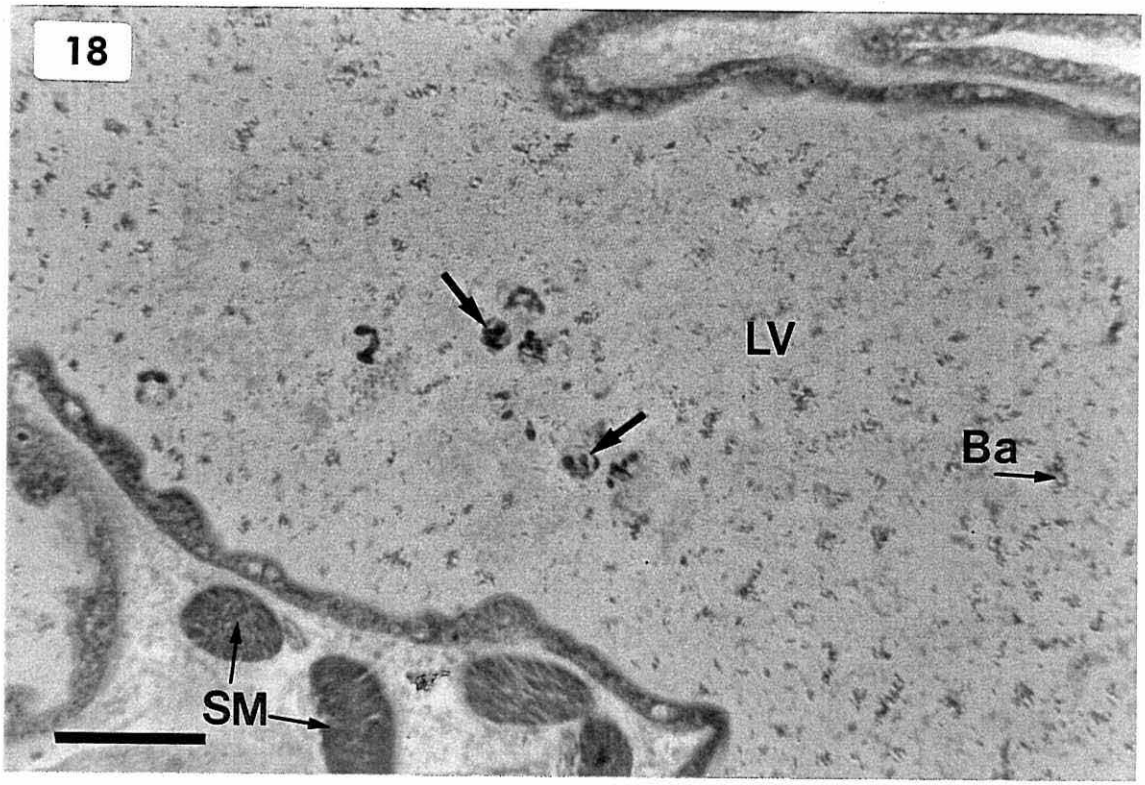
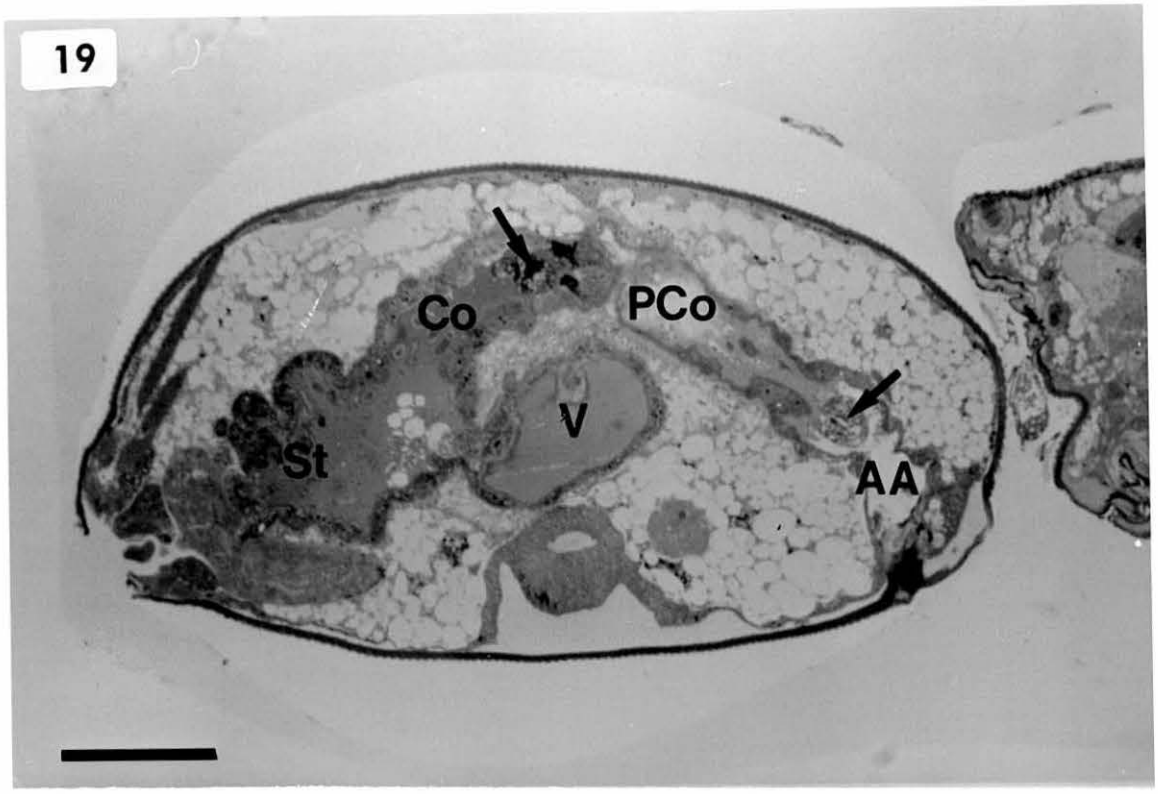

18
LV
Ba
SM

Figure 19. A central, vertical-longitudinal section through *P. ovis* demonstrating the position of the postventricular midgut (Co and PCo) in relation to the main body of the stomach (St). The postventricular midgut can be divided into two distinguishable regions. The first, the colon (Co) directly joins with the stomach and is a tubular organ composed of similar epithelial cells to the region that precedes it. There follows a constriction in the epithelium and the post-colon (PCo) begins. The post-colon descends between the two laterally displaced ventriculi (V) and is composed of epithelium that appears more squamous than the cells of the colon. This region is characterised by significantly longer microvilli than the colon or midgut ventriculi and presumably has an absorptive role. Two food-balls (arrows) can be seen being processed in the postventricular midgut. One is situated in the colon the other leaving the post-colon and entering the anal atrium (AA), the cuticle lined structure that ultimately transfers faecal material to the outside. Bar = 85μm.

Figure 20. This horizontal-longitudinal section shows the path of the postventricular midgut. A dorsal region of the right midgut ventriculus (RV) can be seen and also the junction between the stomach and the colon (St). The colon (Co), containing a food ball (arrow), and post-colon (PCo) are just distinguishable from each other. Bar = 105μm.


19
Co
PCo
V
St
AA

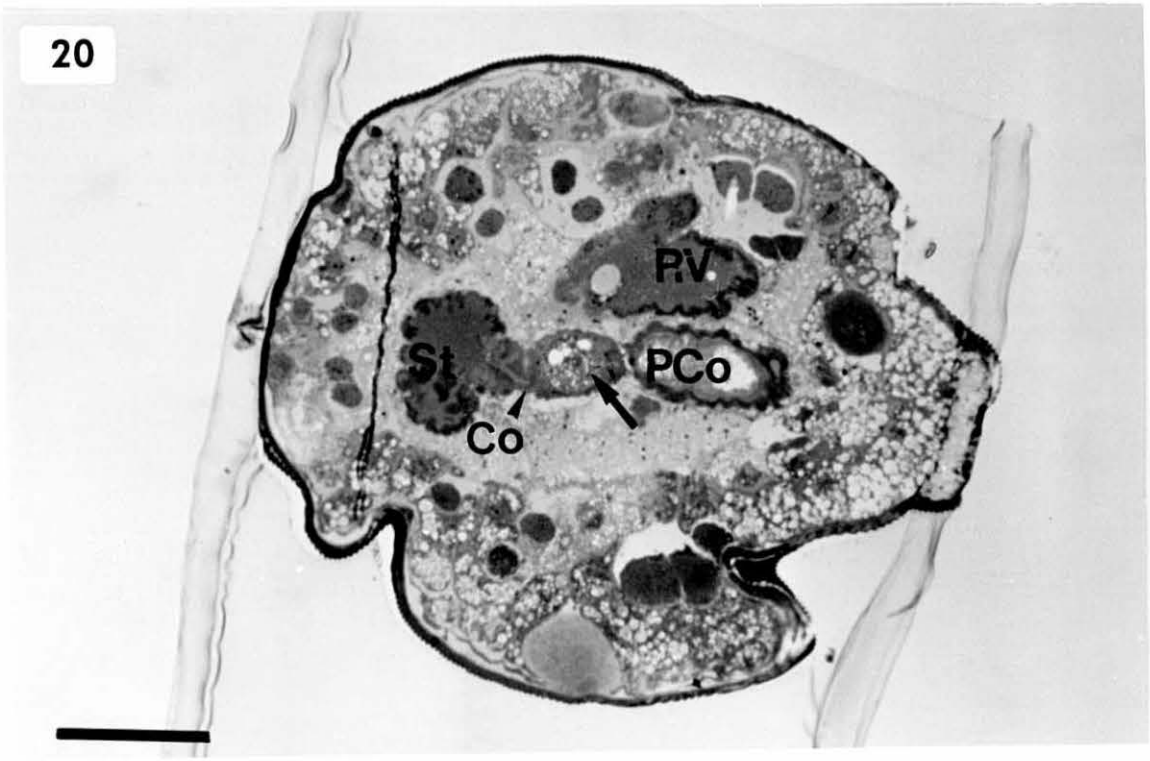
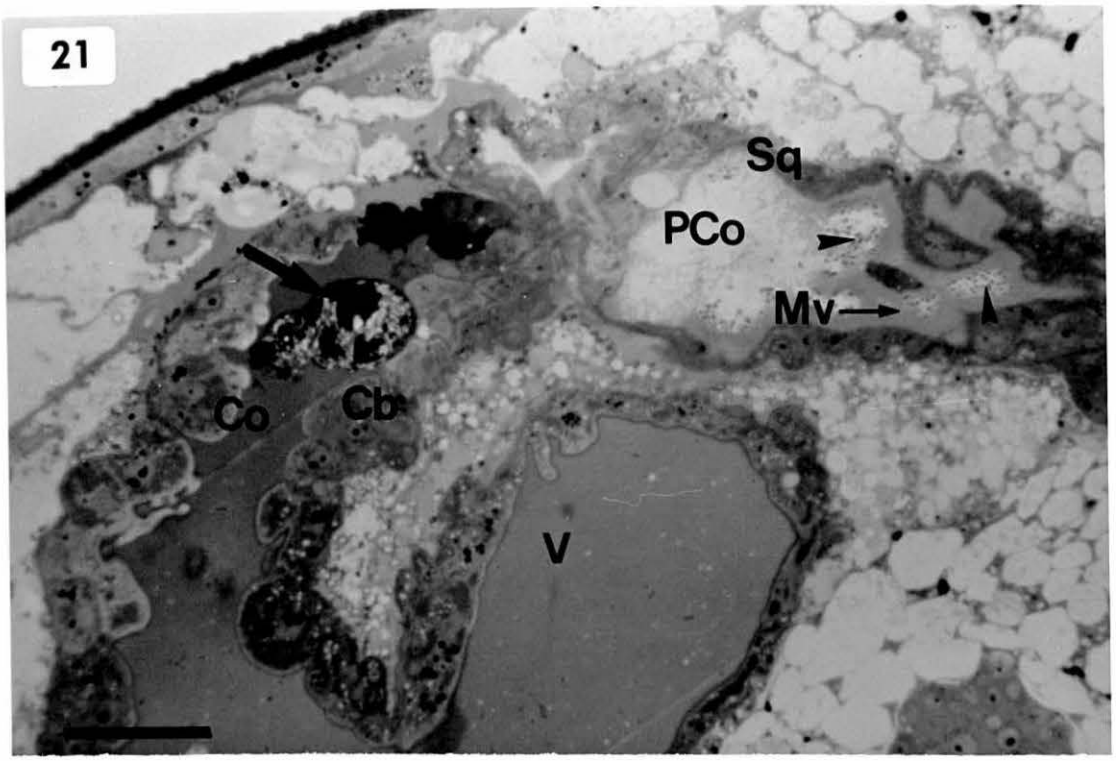

20
PV
St
PCo
Co

Figure 21. This enlargement from Figure 19 shows the region between the colon (Co) and the post-colon (PCo). The differences between the cuboidal epithelium (Cb) of the colon and the squamous (Sq) epithelium of the post-colon and ventriculi (V) are demonstrated in this figure. The extremely long microvilli (Mv) of the post-colon can also be seen in this enlargement. The food-ball (large arrow) can be seen closely associated with the epithelia of the colon and bacteria (arrowheads) are conspicuous in the post-colon. Bar = 25μm.

Figure 22. shows the colon (Co), post-colon (PCo) and portions of the left and right ventriculi (LV and RV) in a horizontal-longitudinal section. Also seen here is the cuticle lined hindgut or anal atrium (AA) leading from the post-colon to the outside. The anal atrium has a narrow profile when not distended by faecal material. Bar = 95μm.


21
Sq
PCo
Mv
Co
Cb
V

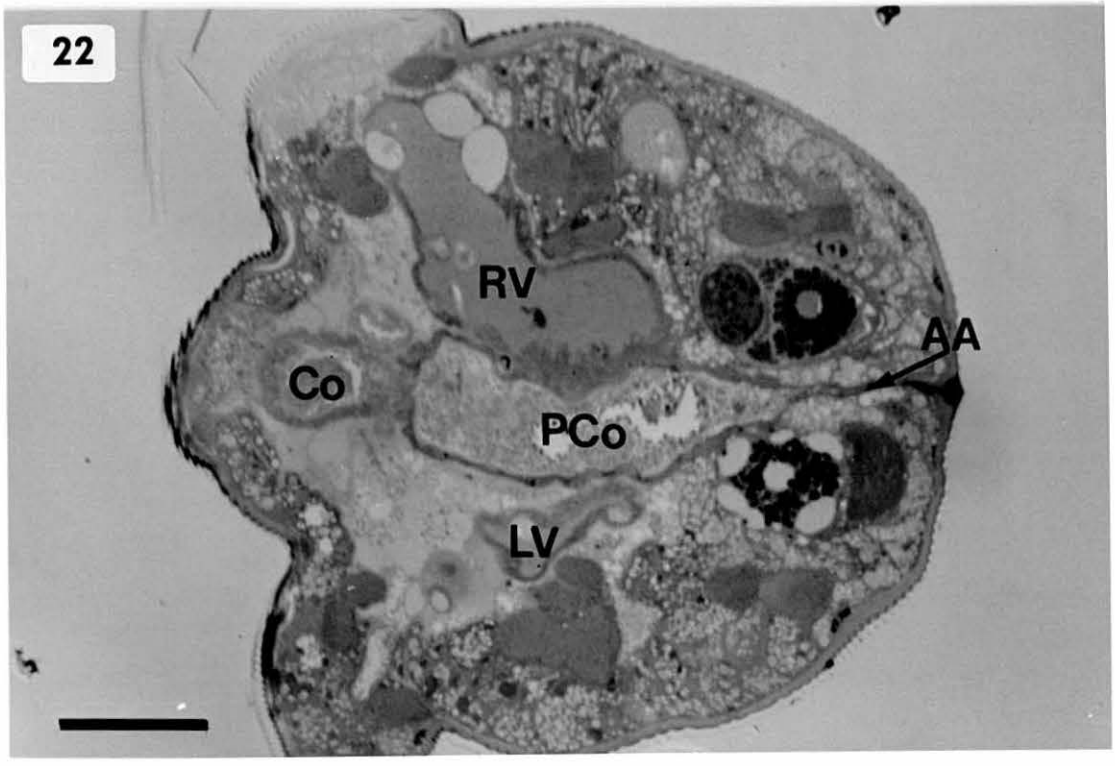
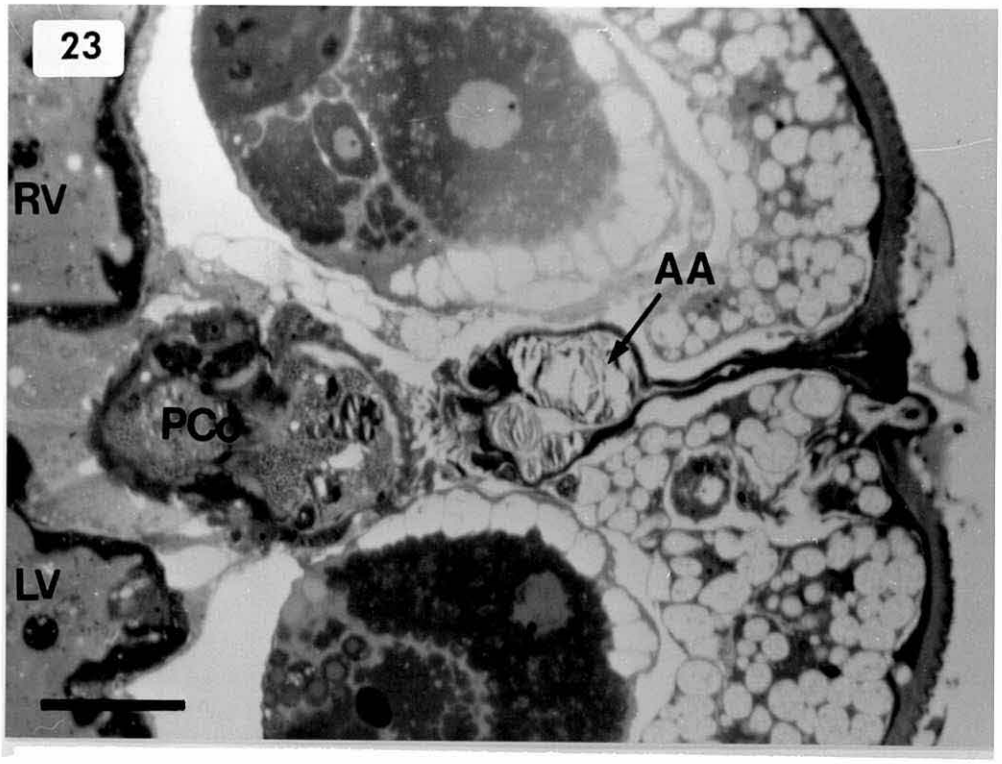
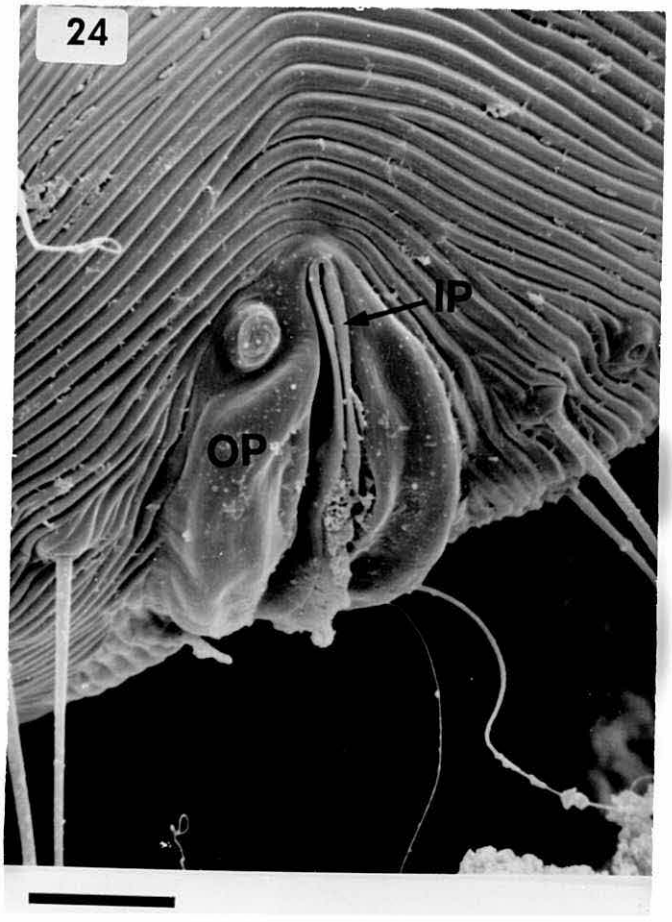

22
RV
AA
Co
PCo
LV

Figure 23. This horizontal-longitudinal section shows the posterior extreme of the mite. Visible at the left-hand edge of the figure are the left and right ventriculi (LV and RV). A portion of the post-colon (PCo) is visible as is the cuticle-lined anal atrium (AA) distended with faecal material. Bar = 30μm.

Figure 24. This scanning electron micrograph shows the mite's anus, composed of a pair of inner and outer anal plates (IP) and (OP). Faecal material is voided through the paired inner plates to the outside. Bar = 65μm.


23
RV
AA
PCo
LV


24
IP
OP

Computerised 3-Dimensional Reconstruction.

The computer software (ILLUS04) that creates the output for the (3D) model of the mite can present pictures of the mite from numerous viewpoints. Presented here are seven that will serve to help the reader to understand the physical structures that compose the mite's alimentary tract and the spatial relationship they have with each other [Figs. 25-31]. One further picture suggests the circulatory route of the ingested meal based on observations made while watching the mites feeding [Fig. 32].

Figure 25 demonstrates the whole adult female mite from a dorsal view. The cuticle sections have not been "filled" with colour and so appear transparent with coloured outlines, allowing the internal structures to be observed *in situ*. The pharynx is not included in any of these figures as it could not be easily observed in the histological sections. The oesophagus (blue) can be seen beginning at the posterior extreme of the gnathosoma and joining with the midgut (yellow) at an anterior position of this organ. The midgut or stomach, composed of two laterally displaced ventriculi, follows. This constitutes the largest organ in the digestive tract, extending deep into the posterior of the body cavity. This figure demonstrated the relatively huge volume the midgut occupies in relation to the body cavity. The remaining area behind the midgut ventriculi is occupied by oocytes and developing eggs. The postventricular midgut (PVM) (green) begins at a antero-dorsal position at the junction between the two ventriculi. The PVM is composed of two morphologically different organs. The first, the colon, begins at the junction between the stomach and the PVM and is a tubular organ following a posterior path dorsal to the ventriculi. The colon then descends ventrally between the two ventriculi to become the post-colon following a posterior path to the anal atrium or hindgut.

Figures 26 and 27 demonstrate the same features as the previous figure but from an antero-lateral and postero-lateral view respectively. Figure 26 shows the diagonal path the oesophagus takes from the gnathosoma, through the body to the midgut. Figure 27

demonstrates the path the post-colon takes in relation to the ventriculi and the pouch-like extensions or caeca the ventriculi posses.

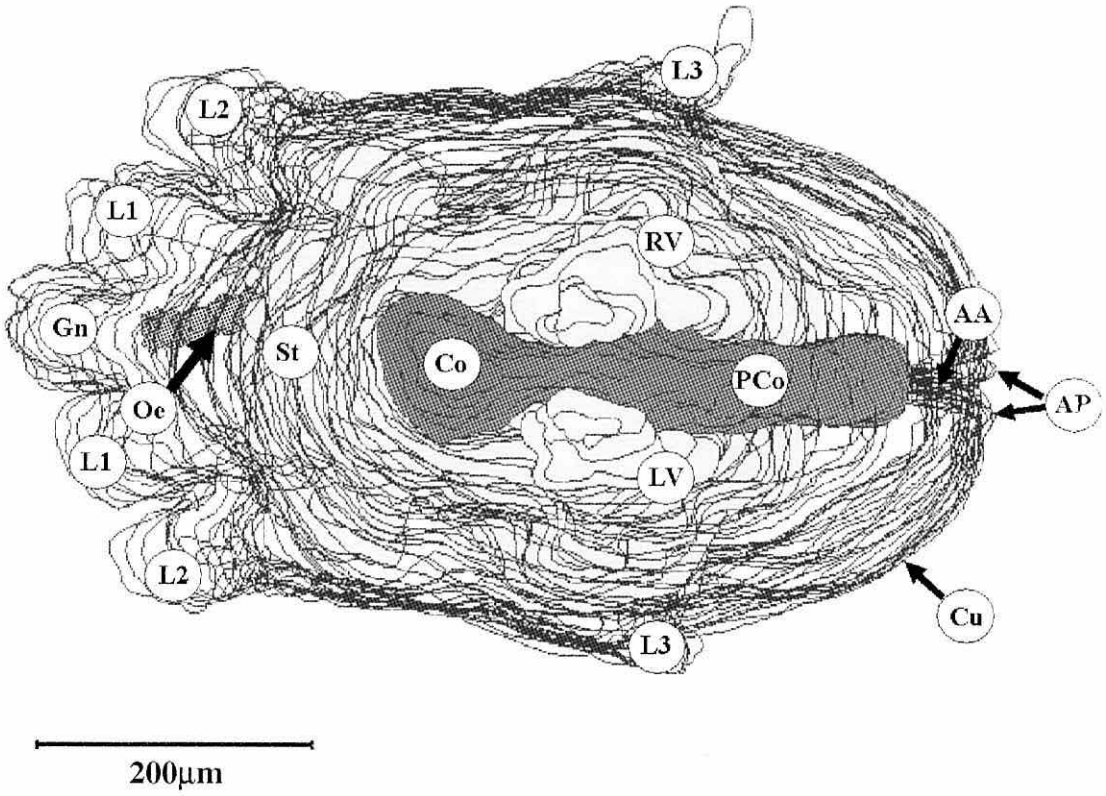
Figure 28 is an antero-lateral view of the digestive system with the body cuticle removed to reveal more clearly the internal organs. The path of the oesophagus is clear as is its insertion point into the midgut at an antero-ventral position, although it would be concealed by the CNS if this was included. The anterior caeca can be seen protruding from the stomach at an anterior position, either side of the oesophageal-midgut junction. The left and right ventriculi partially surround the post-colon as it descends to join the anal atrium.

Figure 29 is a postero-lateral view of the digestive tract with the body cuticle removed. The figure reveals similar features to the previous figure but also demonstrates the posterior caeca extending from the ventriculi during gastric distension.

Figure 30 is a dorsal view of the tract without the body cuticle and clearly demonstrates the overall structure of the system. The caeca can be more clearly visualised from this position. The postventricular midgut (the colon and post-colon) have been removed in Figure 31 to reveal that the ventriculi are isolated from each other towards the posterior, by the ventricular cleft and that the junction between the ventriculi and the colon is in an antero-dorsal location.

Figure 32 outlines a likely route ingested food material takes as it circulates around the digestive system. Material is drawn through the pharynx and oesophagus and enters the midgut, or stomach, at its anterior extreme. From here (having observed live mites feeding on blood or coloured PBS) the food material fills the two ventriculi. The material then must be transferred anteriorly and dorsally to the junction with the colon, after which it follows a postero-ventral path through the post-colon before being passed into the anal atrium or hindgut and voided as faeces through the anal plates.

Figure 25. Computerised 3-Dimensional Reconstruction of the Digestive System of *Psoroptes ovis* presenting a dorsal view, showing: the Body Cuticle (Cu); Gnathosoma (Gn); Legs 1-3 (L1), (L2) and (L3) (only the coxa were digitised and L4 leg sections were displaced during sectioning and were not digitised); Oesophagus (Oe); Stomach (St); Left and Right Ventriculi (LV) and (RV); Colon (Co); Post-Colon (PCo)(the differentiation between the colon and post-colon can not be easily from the figure); Anal Atrium (AA) and Anal Plates (AP). The Pharynx is not included.



Figures 26 and 27. 3-Dimensional Reconstruction of the Digestive System of *Psoroptes ovis* presenting an antero-lateral and postero-lateral view, showing: the Body Cuticle (Cu); Gnathosoma (Gn); Legs 1-3 (coxa only) (L1), (L2), (L3), (L4 not included); Stomach (St); Left and Right Ventriculi (LV) and (RV); Colon (Co); Post-Colon (PCo) Anal Atrium (AA) and Anal Plates (AP). The pharynx is not included.

Figure 26.

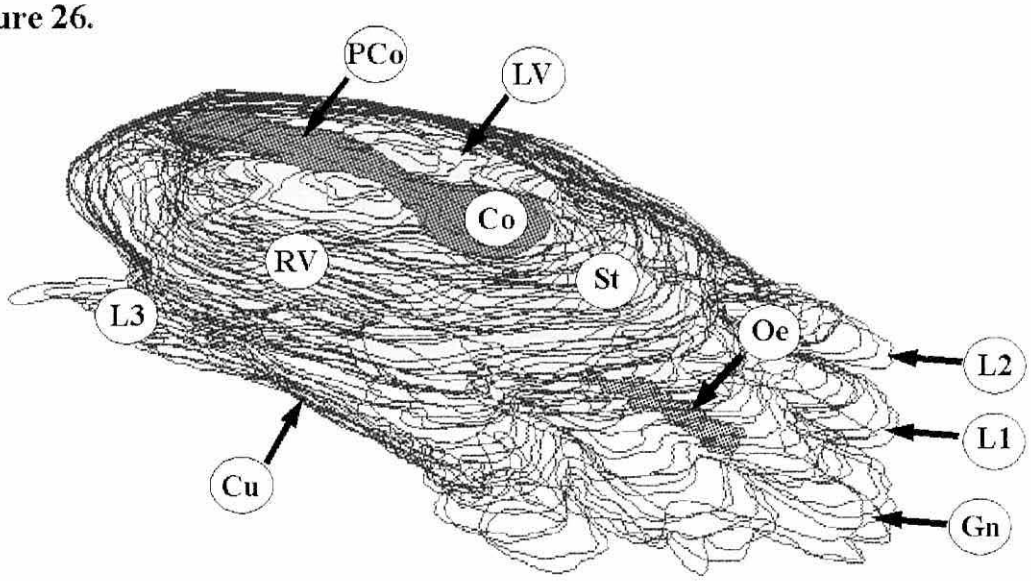
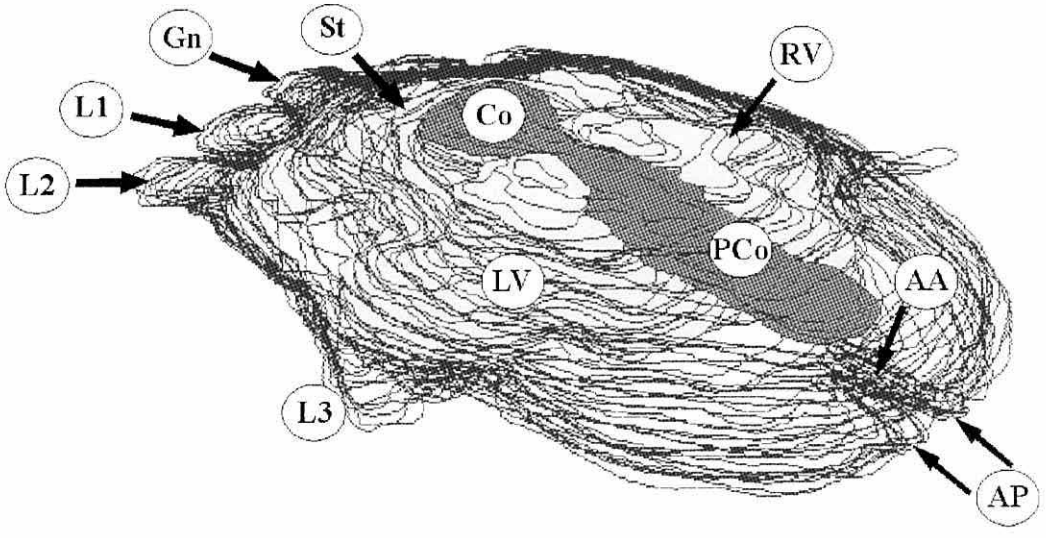


Figure 27.



200µm

Figure 28. Computerised 3-Dimensional Reconstruction of the Digestive System of *Psoroptes ovis* indicating the main components viewed from an antero-lateral viewpoint with the body cuticle removed. The postero-dorsal path of the Oesophagus (Oe) meeting the Stomach (St) at an antero-ventral area can be seen (the CNS is not included) as can the position of the Colon (Co) and Post-Colon (PCo) in relation to the laterally-displaced, bi-lobed ventriculi (LV) and (RV). The Anterior Caeca (ACa) can be seen protruding from the engorged stomach.

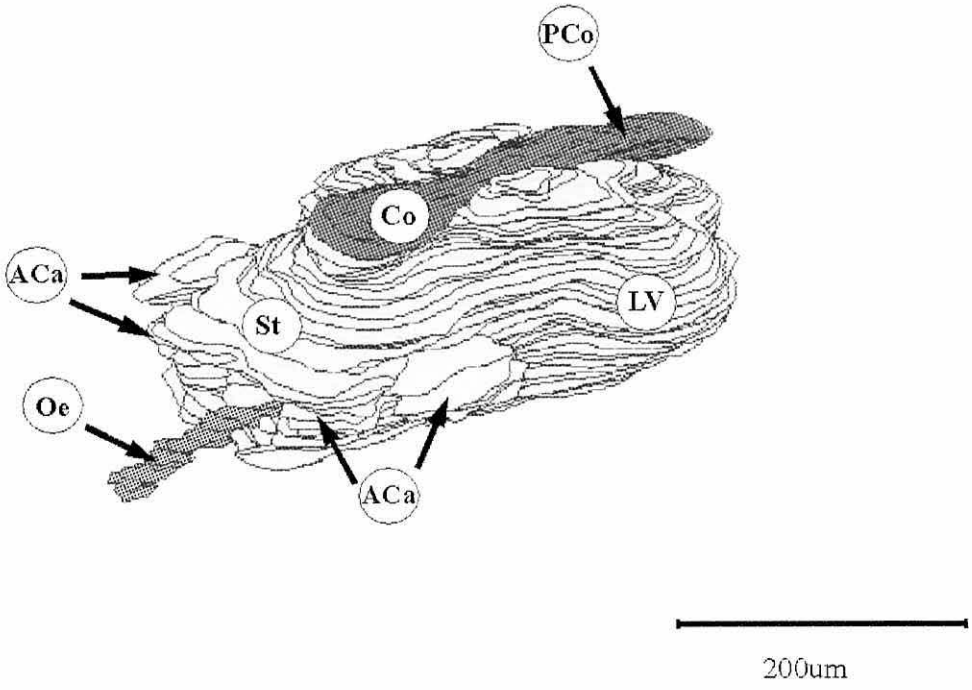
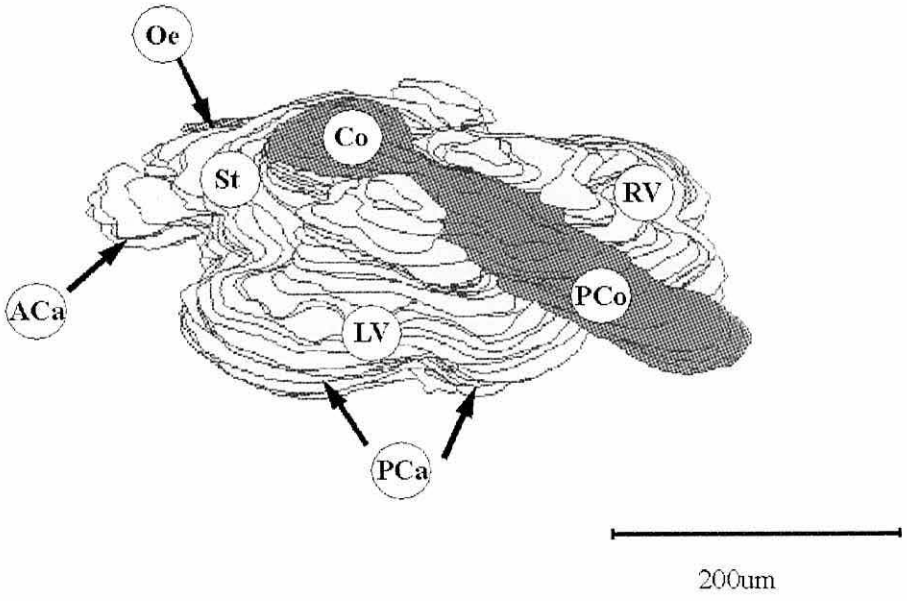


Figure 29. Computerised 3-Dimensional Reconstruction of the Digestive System of *Psoroptes ovis* indicating the main components viewed from a postero-lateral viewpoint. The Oesophagus (Oe) is only just visible, almost completely obscured by the Stomach (St). The Anterior Caeca (ACa) are clearly visible protruding from engorged stomach, the Posterior Caeca (PCa) are less well defined but can be seen here as lateral and posterior extensions of the Left and Right Ventriculi (LV) and (RV).



Figures 30 and 31. Computerised 3-Dimensional Reconstruction of the Digestive System of *Psoroptes ovis.* Figure 30 is a dorsal view of the mite's digestive system with the body cuticle removed. The anterior caeca (ACa) can be clearly seen, two at either side of the junction of the oesophagus (Oe) and the stomach (St). The posterior caeca (PCa) are less obvious extensions of the left and right ventriculi (LV) and (RV). The colon (Co) and post-colon (PCo) are included in Figure 30 but absent in Figure 31 to demonstrate the junction between the stomach and the colon (arrow) and to reveal more clearly the ventricular cleft dividing (VC) dividing the two ventriculi.

Figure 30.

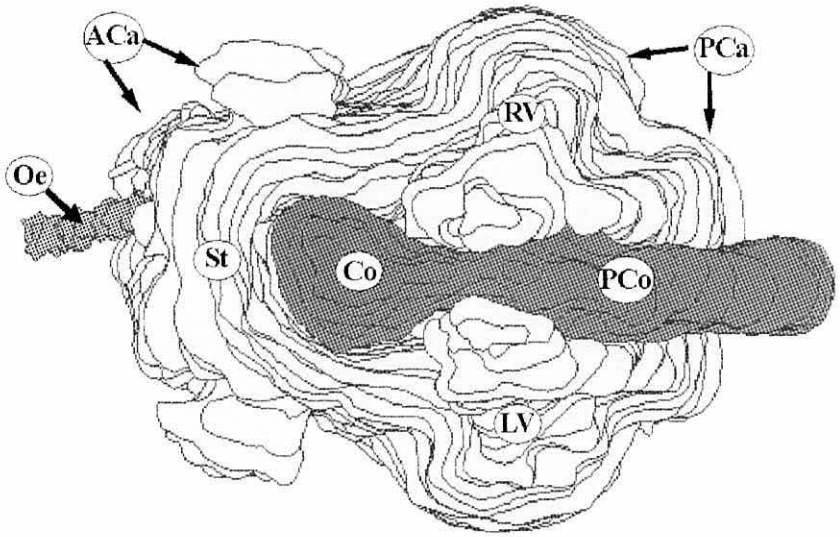


Figure 31.

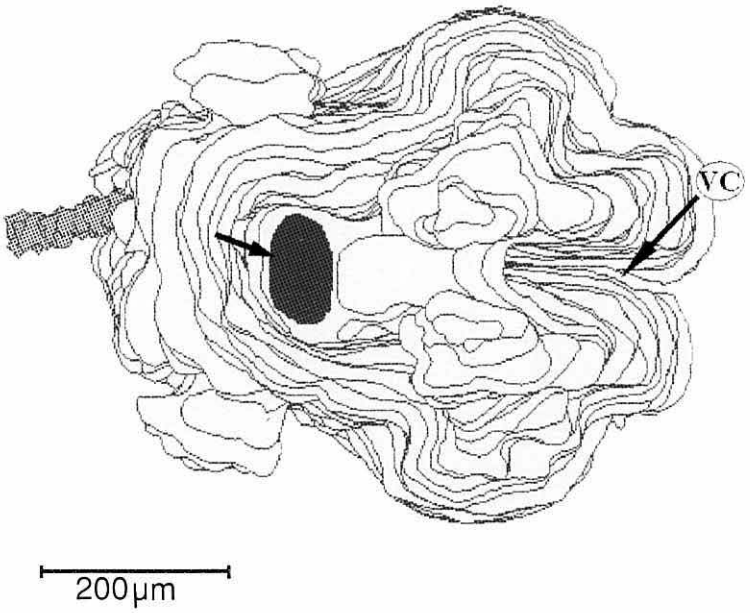
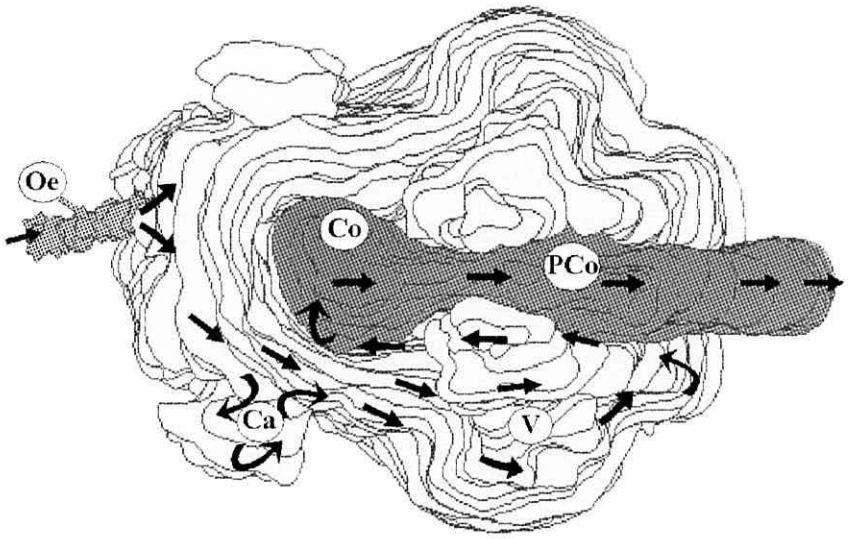


Figure 32. Computerised 3-Dimensional Reconstruction of the Digestive System of *Psoroptes ovis*. This dorsal view of the alimentary tract components demonstrates the proposed circulation of ingested food material around the digestive system. Food material passes through the oesophagus from the pharynx (not included here) and into the anterior region of the stomach. The ingested material fills both Ventriculi (V) from this position. After an undetermined period of time the semi-processed material is transferred anteriorly and dorsally to the Colon (Co) then posteriorly and ventrally through the Post-Colon (PCo) to the anal atrium or hindgut (not shown here).



200µm

Discussion.

P. ovis mites proved to be very difficult to investigate histologically. Due to their minute size (<750μm), it was practically impossible to dissect out the mite's digestive system without damaging it in the process. Instead it was found necessary to fix and embed the mites whole. This introduced several problems. The impervious nature of the mite's cuticle prevents the infiltration of fixatives through this resilient barrier. Instead the only initial route of entry for fixatives, without intervention, is through the digestive system via the pharynx and oesophagus or through the post-colon and colon via the anus and hindgut. This not only significantly hinders the rate of fixation but also impedes the exchange of dehydrating agents and so on which can result in shrinkage and distortion artefacts. These were the problems faced in our initial investigations with Bouin's fixative and JB4 (methacrylate) embedding. Subjecting the mites to a vacuum while fixing and infiltrating (Carranza *et al.*, 1987) did not alleviate the problems reported above. Attempts to avoid this infiltration problem using fixation by microwave irradiation (Carranza *et al.*, 1990; Leong *et al.*, 1985) was unsatisfactory. Instead it was found necessary to pierce the mites through the cuticle and remove legs or mouthparts to aid infiltration, dehydrating and embedding. This significantly reduced shrinkage, distortion and enhanced fixation.

Once it became clear that adequate structural preservation was being achieved, it was soon discovered that, again due to the nature of the mite's cuticle, the mites were often not adhering to the surrounding, polymerised resin, although it had successfully infiltrated into the mite's tissues. This resulted in many of the mite sections "falling out" of the surrounding resin while being cut on a conventional microtome. As many as 80% of the mite sections could be lost at a time.

There followed a significant effort to alleviate this further problem. It was attempted to dissolve the waxy epicuticle, which was likely to be responsible for this problem, with a great variety of solvents including acetone, chloroform,

trychloroethylene, acids, proteases and so on. None had any beneficial effect on section adhesion and some had a deleterious effect on the structural preservation of the mites. We investigated the effects of the solvents on the cuticle in an SEM study but no differences were observed between control mite cuticles and those treated with the solvents.

We reverted to embedding in paraffin wax but infiltration was poorer with this medium and due to the hardening effect heating had on the mite's tissues during treatment, they became poorly supported in the softer surrounding wax, introducing further cutting difficulties. Ester wax (a harder compound developed for histology) was investigated, as was pre-embedding our organisms in agar, both of which failed to give satisfactory results. Different resins were compared but neither Spurr nor Transmit resins relieved the difficulties.

Eventually through perseverance, we obtained approximately 80% serial sections from one Susa fixed and Historesin embedded mite. This provided a sufficient number of sections to produce the 3-D reconstruction models. It was also found that cutting individual 1 - 3μm sections from Spurr or Transmit resin embedded mites on an *ultra*microtome, although far more time consuming than cutting serial sections on a conventional microtome, resulted in significantly more mite sections remaining attached to the surrounding resin. This was probably due to the delicacy and reduced cutting speed which cutting in this manner requires. In summary, the recommended procedures for cutting mites of this nature would be to prepare the mites for TEM and cut individual sections using an ultramicrotome with glass or diamond knives, directly onto water. The sections can then be lifted from the water surface using a wire loop and transferred onto microscope slides. Surprisingly no references to this adhesion problem were found reported by other workers on similar mites. *P. ovis* mites possess sensory setae protruding from their cuticles but it seems they have fewer than many mite species that

have been successfully sectioned. These sensory hairs are likely to help the mites remain anchored to the embedding medium employed.

Only one of the three preparation methods used here for the SEM work produced constantly acceptable results. Both the glutaraldehyde fixation followed by critical point drying and the chloroform fixation and air drying procedures failed to produce replicable, acceptable structural preservation of the whole mite. The mouthparts, being less susceptible to distortion, tended to be well structurally preserved after all of the procedures, but only the cryosystem preparation procedures constantly gave good whole body preservation and was the quickest method of preparation.

In this investigation we found that the general plan of the digestive system of *P. ovis* is consistent with the arrangements described by Evans (1992) and Baker and Wharton (1952) for other members of the Acari. They describe the post-oral digestive tract being divisible into three basic regions characterised by their embryonic development. The foregut and hindgut are derived from ectodermal invaginations; stomodaeum and proctodaeum respectively (reviewed by Evans, 1992) and are connected by a midgut (mesentron) of endodermal origin. The foregut and hindgut, being invaginations of the body wall, are cuticle lined conveying-tubes whereas the midgut is lined with microvilli, and presumably specialised for secretion and absorption.

Throughout the literature on the Acari, the fore and hindguts remain most consistent in their structure and the nomenclature used to describe them. The foregut invariably consists of a cuticle-lined, sclerotized, muscular pharynx leading from the channels associated with the mouthparts to the oesophagus. This organ, generally lined with thinner cuticle, follows a posterior path to meet with the midgut or stomach at an anterior position. On the other hand, throughout the Acari the midgut and postventricular midgut demonstrate the greatest structural diversity. Unfortunately the terminology for the components of the digestive tract lying between the cuticle lined foregut and hindgut have not been standardised over the years, which has led to a certain

degree of confusion when authors use terms such as intestine, stomach, ventriculus and midgut to describe, essentially, the same organs. In this investigation we have followed the conventions of Evans (1992) by referring to the microvilli lined area that directly accepts food material from the oesophagus as the *stomach* (in the case of *P. ovis* this is composed of a pair of large laterally displaced ventriculi) which leads to the tubular microvilli lined *post-ventricular midgut*. This area can be subdivided into the colon and post-colon if, as Evans (1992) suggests, there are distinguishable differences between the two areas, as has been found in *P. ovis*. If the digestive system is homologous between the exit of the ventriculus and the cuticle-lined hindgut then the whole area is termed the *post-ventricular midgut*. This region is followed by the *anal atrium* which is analogous to the cuticle lined hindgut or rectum of Alberti and Crooker, (1985) and other workers.

Although the general structure of the digestive tract of *P. ovis* can be characterised by conventional nomenclature the appearance of the midgut is unique and does not resemble closely any system previously described or reviewed by Baker and Wharton (1952) or Evans (1992). An extensive literature search failed to find any information on any of the comparable mites like *Sarcoptes scabiei, Chorioptes* spp or *Demodex follicularum*. The greatest similarity is seen between the layout of *P. ovis* and the descriptions of *Dermatophagoides farinae* by Brody *et al.* (1972).

The mouthparts of *P. ovis* have previously been studied and extensively described by Blake *et al.* (1978) and to a lesser extent by Buxton, (1920); Sweatman, (1958); Griffiths, (1973); Rafferty and Gray, (1987). The light microscopy and SEM study by Blake *et al.*, (1978) more than adequately describes the structures and these authors hypothesise a probable, functional description for the mouthpart's operation, which we are in full agreement with. This present study is primarily concerned with the post-oral digestive system, hence no further description will be offered on the functional feeding apparatus of *P. ovis*, a brief description was included earlier in the Chapter for completeness. Instead we will discuss the components beginning with the pharynx.

The sclerotized pharynx of *P. ovis* is a muscular pump leading from the buccal cavity and begins the alimentary canal. Various authors have described this structure in other mites as being "Y" or "U" shaped in transverse section (Woodring and Cook, 1962; Woodring and Galbraith, 1976) and is controlled by a network of dilator and constrictor muscles sucking and pumping food material of a varied nature along its length. In this study we have viewed the pharynx in longitudinal section only and observed a large group of dilator muscles attached from the dorsal surface of the pharyngeal roof to the inside surface of the roof of the gnathosoma. No constrictor muscles were noted due to the specimen orientation during sectioning, but it is considered that the pharynx could regain its closed, relaxed state by the elasticity of the cuticular structures working antagonistically to the dilators, as has been reported by Alberti and Crooker (1985) in spider mites. Food material is drawn through the muscular pharynx, past the pharyngo-oesophageal valve, to the oesophagus which in the case of *P. ovis,* and in all literature viewed on other members of the Acari, is lined with a thinner layer of cuticle. A similar valve occurs in *Glycyphagus domesticus* Hughes and Hughes, (1939) and in *Caloglyphus mycophagus* (Kuo and Nesbitt, 1970). The oesophagus is star shaped in transverse section in *P. ovis* and food material is likely to be conveyed along the length of the oesophagus by peristaltic contractions of circular muscles and again the resilience of the cuticle acting antagonistically to the constrictors and the positive pressure developed from the action of the pharynx, could avoid the need for specific dilators. The oesophagus runs posteriorly and dorsally through the mite's central nervous system to meet with the midgut at its antero-ventral surface. The general structures and functions of the pharynx and oesophagus very closely resemble those described for a variety of mites (Hughes, 1954; Woodring and Galbraith, 1976; Mothes and Seitz, 1981) including *Dermatophagoides farinae* and *Tetranychus urticae* investigated by Brody *et al.* (1972) and Mothes and Seitz (1981) respectively.

Food material enters the midgut and initially fills both ventriculi of the stomach. The circulation of material around the main body of the midgut has not previously been

precisely determined, but we have proposed a likely route the ingested material takes [Fig. 30]. From observations made while watching mites ingesting blood or other coloured liquids, in a simple *in vitro* feeding system, it seems that the meal is not initially compartmentalised into a specific area of the stomach but enters the stomach, fills and distends both ventriculi equally, where it presumably begins to be processed. Baker and Wharton (1952) suggest that in the parasitic Acari the stomach is a small junction between the ventriculi or caeca. This is not the situation observed with *P. ovis*. They also suggest that in the Psoroptidae lateral ventriculi are absent. In our study we found that the laterally displaced ventriculi are the most prominent feature of the midgut, occupying the majority of the body cavity [Figs. 10, 11 and 23-29]. The oesophagus leads directly into the large cavity analogous to the stomach or ventriculi, again similar to the descriptions of Brody *et al.* (1972) for *D. farinae*. As the stomach fills both ventriculi distend laterally beyond the dorso-ventrally running somatic muscles, further dividing the ventriculi into smaller discrete pouches [Figs. 10 and 11]. These gut pouches or caeca have also been reported by Alberti and Crooker (1985) in the Actinotrichida. Whether these areas have a specific digestive role is unknown from this study but it is likely that they have a similar function to the main body of the stomach.

Considerable variation in the appearance of the midgut epithelia are to be expected depending on; state of engorgement, physiological activity of the animal, life-cycle stage and so on. This questions the validity of interpretations made from histological observations. This light microscopy study has effectively revealed the structural layout of the digestive tract of *P. ovis* but with the cellular resolution available to light microscopy there is a limit to the descriptions that can be made. However certain cell types can be observed which are consistent with descriptions of other mite systems by other authors. Many of these authors have described epithelial cells in the midgut of Acarines protruding into the gut lumen, detaching from the epithelial wall and eventually becoming free-floating. Coons (1978) describes midgut epithelial cells detaching from the gut wall and entering the lumen of *Macrocheles muscaedomesticae*

where they undergo cytoplasmic degeneration during intracellular digestion. Baker (1975), Mothes and Seitz (1981) and Alberti and Crooker (1985) all report similar findings in spider mites, *Histogaster carpio* and *Tetranychus urticae* respectively and agree on the digestive function of these free-floating cells. In the anterior portion of the stomach of *P. ovis* (and frequently in other locations) we have observed similar cells protruding from and then becoming free-floating in the gut lumen. It is very likely they have a similar function to the cells described by the previous workers. Their TEM studies reveal more cellular detail than this present study can provide and so no further comments can be made on the structure and possible functions of the cells found in *P. ovis.* It remains to be seen how the fine-structure of these cells compares to the descriptions of other authors (see Chapter 4).

From the stomach ventriculi food material is displaced in an antero-dorsal direction and passes through a junction on the dorsal surface of the stomach to the colon. Movement of food material into the colon is controlled by a valve (pyloric sphincter) (Evans, 1992). This valve is difficult to observe with the light microscopy techniques used in this study as the gut epithelia in the two regions is similar and in our sections the junction is dilated. The postventricular region of the digestive tract in Mesostigmata, Astigmata, Orbatida and Ixodida consists of two distinguishable regions; an anterior colon and a posterior post-colon. In the case of *P. ovis*, the colon begins after the pyloric sphincter and runs posteriorly for a short distance. The epithelium in this location is similar to the cells in the stomach being approximately cuboidal and microvilli lined. There follows a muscular constriction through which food is passed into the post-colon which follows a postero-ventral path, dropping between the two large laterally displaced ventriculi. The epithelium in the post-colon appears to be more squamous than in the colon, perhaps due to localised distension and the microvilli are significantly longer, an observation reported by Brody *et al.* (1972) for *D. farinae*. This area presumably has an absorptive role. Food material, which by this stage has condensed into faecal pellets, often containing a significant population of bacteria [Fig. 17], (investigated further in

Chapter 5), is transferred from the post-colon into the cuticle lined anal atrium or hindgut before being voided through the paired anal plates.

This conventional microscopy study has effectively demonstrated the histological structure and positions of the various components of the digestive system of *P. ovis* and that the gut content appears to consist of little host-derived cellular material, indicating the fluid nature of the mite's diet. The spatial relationship they have to each other and the overall layout of the alimentary tract is demonstrated by the 3-D reconstruction models. This gives us a far better understanding of the structures than conventional histology would alone. The components of the digestive system of *P. ovis* fall into the conventional, principal regions universally described for the Arthropoda, the precise structures and spatial relationship the components share with one another seems to be unique in the Acari but closely resembles the structure of *Dermatophagoides farinae* (Brody *et al.*, 1972). This study has effectively revealed the structural layout of the digestive tract of *P. ovis* but with the cellular resolution available to light microscopy there is a limit to the descriptions that can be made. It remains to be seen how the cellular composition of *P. ovis* appears and how this compares to other mite systems.

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Chapter 4.

Fine Structure of the Digestive System of *Psoroptes ovis.*

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Fine Structure of the Digestive System of *Psoroptes ovis.*

Summary.

The digestive system of *Psoroptes ovis* was studied using electron microscopy. The system consists of a cuticle-lined foregut and anal atrium separated by a microvilli-lined midgut. The foregut can be divided into a pre-oral cavity, muscular pharynx and an oesophagus constricted by an array of circular muscles. The microvilli-lined midgut consists of three morphologically discrete areas: a bi-lobed central ventriculus, a colon and a post-colon. The bi-lobed ventriculus is composed of two cell types; the most common are either cuboidal or squamous and possess short, apical microvilli, a single basally located nucleus, extensive rough endoplasmic reticulum and other components suggesting they are actively secreting. The other less common cells possess an extensive apical network of tubules and basally food vacuoles. They extend in to the gut lumen and become free-floating where they degenerate. These cells have a digestive function. The colon is composed of similar cells to the ventriculus. The post-colon posesses significantly longer microvilli than the preceeding areas and is assumed to have an absorptive role. Faecal pellets, often containing a significant amount of bacteria, leave the digestive system through the cuticle lined anal atrium.

Introduction.

In the previous chapter, the general structure and layout of the components of the digestive system of *Psoroptes ovis* was described and a computer software package was used to create various 3-dimentional models of the mite's alimentary canal. This has given precise information on the physical structure of the mite's digestive system which permitted a comparison of the morphology of the digestive tract of *P. ovis* with several other acarine systems.

That study also provided some information on the cellular components of the digestive system. This transmission electron microscopical study of the mite's gut has been performed to shed light on the intricate mechanisms and fine structure of the mite's digestive tract and give some indication of the nature of ingested meal, information vital for the development of an *in vitro* cultivation system. It is hoped this understanding will underpin a variety of further investigations. One interesting area would be to investigate the potential mite-borne agents responsible for the clinical symptoms of sheep scab. It is possible the disease is caused by an immune response initiated by a build up of antigenic mite products, not dissimilar to the processes involved in the related house-dust mite allergies. With the necessary tools, gold labelled antibodies for example, and the relevant TEM preparation procedures, investigated here, it will be possible to study the source of the antigens *P. ovis* produces. *P. ovis* mites contain a significant population of bacteria in their guts (see Chapter 5). These could play a role in the pathogenicity of the disease and the varying clinical symptoms that occur from different geographical outbreaks. Similar techniques to those described above could be employed to investigate this theory further.

The purpose of this investigation was to enhance the understanding of the digestive system of *P. ovis*, at the electron microscopy level, and to pave the way for many other highly relevant investigations.

Materials and Methods.

Mite collection and fixation.

Mites were obtained from infested sheep contained in secure isolation enclosures at the Central Veterinary Laboratory. They were removed from the lesion edge by careful scraping with the broad side of a mounted needle, transferred into Durham tubes, stoppered with cotton wool and placed inside larger diameter capped tubes for security and transportation.

The mites were removed from the tubes in the laboratory, separated into groups of adult females, washed, pierced, fixed in glutaraldehyde and osmium tetroxide and dehydrated by the methods previously described in Chapter 3 (Materials and Methods: *Mite Collection and Separation* and *Glutaraldehyde and Osmium fixation for Conventional Histology*). It was found essential to cut away a greater amount of the external mite-parts than was necessary for the light microscopical investigation. A large portion of the gnathosoma and the legs at the coxa were removed to aid infiltration, dehydration and so on. Two different embedding resins were investigated to determine which gave the best results for our purposes. These were Spurr (Spurr, 1969) and Taab Transmit resins. Table 1 summarises the general procedures for mite preparation and then outlines the specific procedures for the two resins examined.

***En Bloc* Staining During Specimen Dehydration.**

An *en bloc* staining procedure, with either aqueous or alcoholic uranyl acetate used during specimen dehydration, was investigated to improve staining characteristics of the sections. This was conducted as follows: The procedures summarised in Table 1 were followed until the dehydrating stage. For aqueous uranyl acetate *en bloc* staining, the specimens were incubated in 2% uranyl acetate in dH_2O for 30 mins after the post-OsO_4 rinses. The specimens were briefly rinsed of stain and dehydrated and embedded as described in the remainder of Table 1. For alcoholic uranyl acetate, the procedure involved incubating the mites for one hour in 2% uranyl acetate in 30% ethanol solution

between the 30 and 50% ethanol dehydrating stages. Stain was briefly rinsed off with filtered 30% ethanol and the remainder of the protocol was completed.

Table 1. Summary of the procedures used for Glutaraldehyde and Osmium fixation with Spurr or Transmit resin embedding of *P. ovis* mites for transmission electron microscopy.

• Wash in 2% Tween 80 in dH_2O at room temp	5 mins.
• Rinse in dH_2O at room temp	5 mins.
• Pierced and Glutaraldehyde fixed	Overnight.
• Rinsed in buffer	3x10 mins.
• 2% OsO_4 fixation in dH_2O at room temp	1 hour.
• Rinsed in dH_2O room temp	3x10 mins.
• Dehydration through 50, 70, 90 and 3x 100% ethanol	10 minutes each.

Spurr Embedding

• Infiltration in 50:50 Spurr:abs ethanol	12 hours.
• 100% Spurr	12 hours.
• Polymerised in fresh Spurr at 60°C	12 hours.

Taab Transmit Embedding

• Infiltration in 50:50 Transmit:Propylene oxide	12 hours.
• 100% Transmit	12 hours.
• Polymerised in fresh Transmit at 60°C	12 hours.

During all washing, rinsing, fixing, dehydrating and infiltrating stages the mites were contained within eppendorph cages and agitated on a rotary mixer. All stages were incubated at 4°C unless otherwise stated.

Specimen Block Sectioning.

For both resins investigated the polymerised blocks, each containing a single adult female mite, were orientated to be cut either transversely, horizontal-longitudinally or vertical-longitudinally. Trimming was performed with a LKB Pyramitome. Occasionally the block-face was trimmed to around half the length of the embedded mite (approximately $350\mu m^2$) to reduce block-face area to improve cutting. Usually though, the blocks were trimmed to allow a complete transverse or longitudinal section to be cut.

Trimmed blocks were transferred to a standard LKB Ultramicrotome where 1μm "thick" sections and mid-gold to silver sections were cut with fresh glass knives directly onto filtered dH_2O. Both thick and thin sections were carefully expanded with chloroform vapour for both Spurr and Transmit blocks. Thick sections from both blocks were transferred onto clean, de-greased microscope slides using a small wire loop and thin sections were carefully removed from the water surface into copper or nickel pyoloform treated 200 mesh grids, 2-4 sections per grid. The wire loop was frequently de-greased in chloroform to aid section transferral onto slides. Grids were transferred into a grid box and air-dried for at least an hour before being stained.

"Thick" Section Staining and Viewing.

The "thick" (1-3μm) sections once transferred onto slides were baked dry on a hot-plate at approximately 90^oC for a few minutes. Toluidine blue was applied to the slide to just cover the section and in the case of Spurr embedded mites, left for approximately 15 seconds on the hot-plate. Transmit resin takes up the stain more readily and so was stained for no more than 5 seconds, without using the hot-plate. Sections were viewed directly under a normal binocular microscope for orientation purposes but interesting sections were mounted in DPX under coverslips.

Ultra-Thin Section Staining and Viewing.

The general protocol for staining ultra-thin sections follows. Staining times varied from resin to resin and for the same resin depending on section thickness, stain freshness and so on.

The contrast enhancing property of Potassium Permanganate ($KMnO_4$), as a pre-uranyl acetate staining step (Soloff, 1973), was investigated. Grids were briefly wetted on the section side with dH_2O then floated, section side down, on a 0.9% solution of $KMnO_4$ in 0.1 M phosphate buffer at pH 7.4 (made fresh each week) for 5 minutes before being rinsed for 15 seconds in dH_2O. Grids were then floated on a drop of 2% aqueous solution of uranyl acetate, rinsed for 60 seconds by pipetting vigorously with dH_2O and floated on a drop of 0.2% aqueous lead citrate (Reynolds, 1963) rinsed again for 60 seconds and dried. Table 2 presents the procedures that produced, generally, the best staining results for each resin investigated.

Following staining, the grids were stored until dry in their boxes and viewed with either a Philips EM301 or an AEI Kratos Corinth transmission electron microscope.

Table 2. Summary of staining procedures and incubation times (minutes) for Spurr and Transmit embedded mites using $KMnO_4$, 2% uranyl acetate and lead citrate and including aqueous or alcoholic *en bloc* staining stages.

Resin Type	Aqueous *En bloc* Staining	Alcoholic *En bloc* Staining	$KMnO_4$	Uranyl Acetate	Lead Citrate
Spurr	-	-	5	10	5
Spurr	30	-	5	5	5
Spurr	-	60	5	-	5
Transmit	-	-	5	5	5
Transmit	30	-	5	5	5
Transmit	-	60	5	-	5

Results.

Chapter 3 describes the morphology and some cellular details of the digestive system of *P. ovis* using conventional microscopy. There will be features described in this present study that, to some extent, repeat the previous findings, but details will be added from this current study that are only observable using TEM techniques. A degree of repetition is used here to present the TEM information in an easily assimilable form for the reader.

Two different resins and several different staining regimes were investigated in order to find the procedures that produced best staining results for our organism. There was a significant difference between the staining characteristics of the two resins when either conventionally staining "thick" sections or the ultra-thin sections with uranyl acetate and lead citrate. Spurr sections took longer to stain to the desired intensity with toluidine blue, and with uranyl acetate and lead citrate, incubation times often had to be extended and repeated. The inclusion of either an aqueous or alcoholic *en bloc* stage dramatically reduced or eliminated the need of further staining, improved overall staining characteristics and is to be recommended (Table 2).

The Foregut: Pharynx and Oesophagus.

As described in Chapter 3, the foregut can be divided into the pharynx and the oesophagus. The fine-structure of these will be dealt with separately here.

The Pharynx.

The pharynx [Fig. 1] is a heavily sclerotized, cuticle-lined tube leading from the pre-oral cavity within the gnathosoma, to the junction with the oesophagus. Food material is actively sucked through the pharynx by the action of a group of three pharyngeal dilator muscles. These muscles are attached from the dorsal surface of the pharyngeal roof to the inside surface of the roof of the gnathosoma. Figure 1 also

demonstrates the pharyngeal teeth or ridges that overlie a thickened area of cuticle a short distance before the pharyngo-oesophageal valve (POV) separating the pharynx from the oesophagus. Food material must pass through this valve to enter the oesophagus.

The Oesophagus.

The oesophagus has an eight-pointed, star-shaped transverse profile [Fig. 2], begins immediately after the POV and runs dorso-caudally through the central nervous system to join with the midgut. The oesophagus is lined with a thinner cuticle than the pharynx and is encircled with an array of epithelial cells and circular muscles [Fig. 3] attached to each point of the star. Figure 4 is an enlargement of a portion of the oesophagus demonstrating the four components of the cuticle: waxy secretion layer, outer-epicuticle, inner-epicuticle, procuticle and the underlying epithelium and constrictor muscles. The oesophagus joins with the midgut or stomach, seems to extend slightly into the lumen and abruptly the cuticle gives way to the microvilli-lined epithelium of the midgut [Fig. 5].

The Midgut: Stomach or Ventriculi and Postventricular Midgut.

The midgut of *P. ovis* can be divided into the stomach or paired ventriculi, colon and post-colon. These will be discussed in turn.

The Stomach or Ventriculi.

Food material is pumped from the oesophagus to enter the midgut at its anterior extreme. The cells lining the midgut ventriculi appear to fall into two categories. The majority of the cells consist of a single layer of epithelial cells of variable thickness lined with short microvilli. The variable thickness appears to be dependant on the degree the gut is distended with ingested food. The cells vary from tall cuboidal to squamous [Fig. 6]. The majority of the stomach epithelium is low cuboidal to squamous after feeding [Figs. 7 and 8] and cells contain a single, large, usually basally located nucleus possessing a single nucleolus. Junctions between homologous epithelial cells are often very

convoluted giving the impression of a stratified epithelium and possess apical desmosomes [Figs. 7 and 8]. Extensive rough endoplasmic reticulum (RER) and small cristae type mitochondria are seen throughout these cells [Figs. 6, 7 and 8]. The RER has been seen stacked up inside apical regions of some stomach epithelial cells [Fig. 9] possibly suggesting the degeneration of the cell. Figure 9 shows two vesicles containing crystalline material, presumably nitrogenous waste. Figure 8 demonstrates the bacterial cells that are observed throughout the digestive tract. The microvilli of all the epithelial cells in the digestive system possess double plasma-membranes [Fig. 10], characteristic of many arthropods. These cells constitute the majority of epithelia of the stomach/ventriculi.

The second, less frequently observed, cell type occurs in many parts of the main body of the stomach ventriculi, possibly with greater frequency towards the anterior regions. These cells appear in various stages of transforming from their normal cuboidal state into detached, free-floating luminal cells. The cells are in the minority among the epithelial cells with only perhaps 8% of the epithelium at one time exibiting these dynamic characteristics. The cells become more spherical, often elongating apically, protrude from the surrounding epithelium and eventually break free and enter the gut lumen as spherical, free-floating cells [Fig. 11 and 12]. While still attached to the epithelium, the "budding" cells often contain several large vacuoles, frequently containing material of a similar nature to the lumen content. Apically they possess a dense network of tubules [Figs. 13 and 14] possibly indicating an active process of pinocytosis is occuring. Densely staining primary lysosomes are seen within these cells as are mitochondria and endoplasmic reticulum. The luminal surface of the cells is often devoid of microvilli while attached to the surrounding cells [Figs. 13-16] but the free-floating cells are frequently seen surrounded by microvilli [Fig. 12]. They contain a single nucleus similar to the surrounding cells [Fig. 16].

Another feature of these dynamic cells is that as they transform from being cuboidal cells resident in the gut epithelia to free-floating they degenerate, becoming intensively osmiophilic, frequently containing secondary lysosomes and small vesicles containing unidentifiable material [Figs. 11 and 12]. The ultimate fate of these cells is unclear; they have not been seen incorporated into faecal pellets.

Not to be confused with the free-floating epithelial cells are host-derived polymorphonuclear leukocytes [Fig. 17]. These, identified as eosinophils, are very frequently observed throughout the gut lumen and are distinguishable from the free-floating cells as they contain lobular, darkly staining nuclei and the cytoplasm is interspersed with large osmiophilic granules. Pseudopodia are seen as extensions of the cell membrane, microvilli are absent. It is not known if they have a deleterious effect on the mite. The eosinophils are the only host-derived cellular material observed in the mite's gut (it is not certain from where the bacteria originate) reinforcing the general belief that *P. ovis* feeds on a mainly liquid diet.

Throughout the midgut, the epithelial cells lie on a relatively thick basal lamina of homogenous composition. Attached to the exterior surface of this membrane at various positions are groups of visceral muscles [Fig. 7]. Directly adjacent to the basal lamina, again on the exterior surface, is an extremely lobular and amorphous intermediate tissue containing large stores of glycogen, indications of large lipid droplets and numerous mitochondria, often seen closely associated with the basal lamina [Figs. 6 and 7]. Finger-like protrusions of this tissue appear to intermittently puncture the basal lamina and penetrate some way into the basal portion of the epithelial cell above [Figs. 18-21]. Upon close examination at high magnification, it can be seen that the basal epithelial cell membrane invaginates with the intruding tissue, the basal lamina becomes an undetectably thin "sandwich" between the epithelial cell membrane and the intruding tissues cell membrane [Figs. 20 and 21] and a very close association between the two tissue types ensues.

Many of the cuboidal and squamous epithelial cells of the stomach/ventriculi often possess large vesicles containing concentric, osmiophilic, membranous structures and smaller vesicles [Figs. 22 and 23] These secondary lysosomes are found intermittently throughout the midgut epithelium.

The Postventricular Midgut: Colon and Post Colon.

The colon begins with the termination of the midgut ventriculi at an antero-dorsal position. The epithelial cells lining the colon are very similar to the cells lining the majority of the stomach ventriculi. The cells are mainly cuboidal epithelial cells containing the same distribution of organelles as before and are again surrounded basally by the amorphous, intermediate tissue described earlier, which also periodically punctuates the epithelial basal lamina of the colon epithelium.

Moving into the post-colon, the character of the cells changes significantly. The first observation to be made is the significantly longer microvilli extending apically from the post-colon cells into the gut lumen [Figs. 24-28]. Large nuclei are present in these cells [Fig. 24] as are numerous large vesicles containing an unknown material [Figs. 25 and 26]. Cell junctions are as convoluted as they are in the ventriculi and they also possess apical tight junctions [Fig. 27]. Secondary lysosomes are also present and numerous invaginations of the tissue underlying the basal lamina [Figs. 25 and 26]. The character of this tissue is similar to that previously described comprising of glycogen rosettes and mitochondria. Occasionally seen in this region at an apical region of the epithelial cells are small densely staining vesicles, possibly primary lysosomes [Fig. 28] suggesting these cells have a secretory role. These cells contain a significant number of small mitochondria.

Numerous bacterial cells are present free in the post-colon lumen [Figs. 24 and 25]. They do not seem to have a close association with the epithelial cells themselves. No budded cells are observed being produced from the epithelia or free in the post-colon.

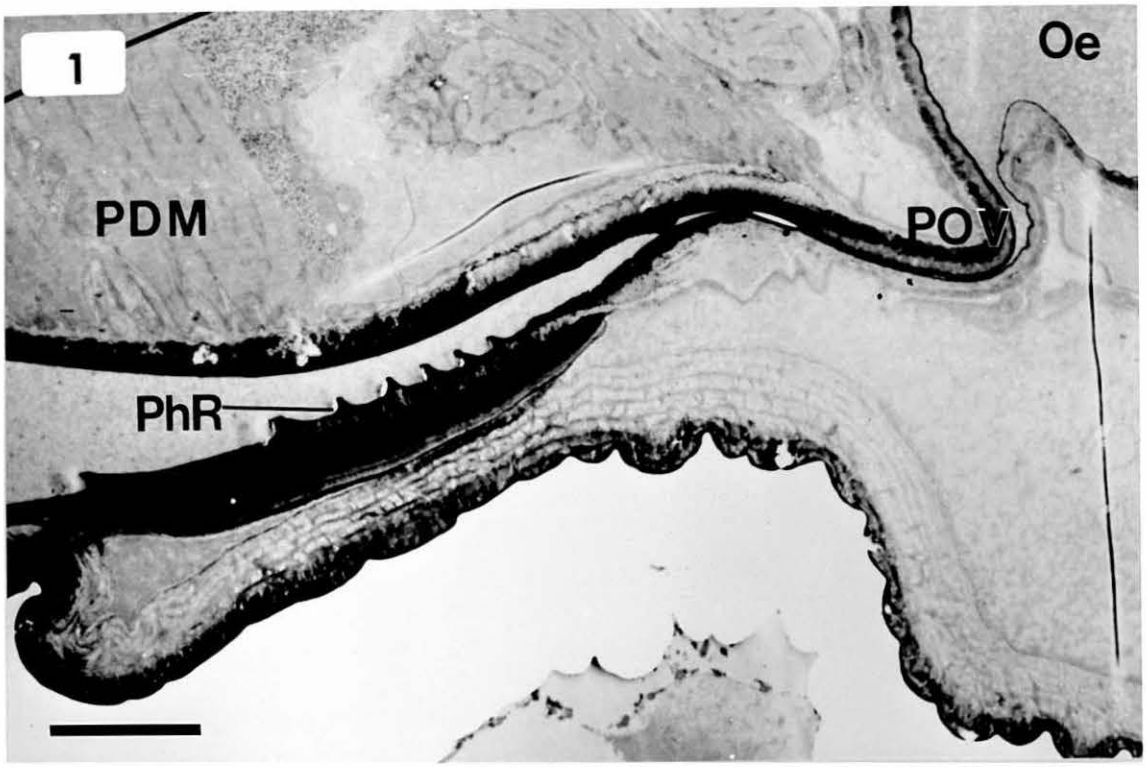
Towards the posterior end of the post-colon the epithelium becomes very squamous in character, still possessing relatively long microvilli and is more extensively invaginated basally with intermediate tissue invaginations than in the previously described areas [Figs. 25, 26 and 29]. Beneath the basal lamina extensive glycogen stores are observed as are numerous mitochondria. The microvilli-lined epithelium eventually gives way to cuticle-lined epithelium that constitutes the anal atrium. The basal lamina continues but is completely void of punctuating intermediate tissue. Food material by this stage has been processed and condensed into faecal pellets, containing a significant proportion of bacteria and appear to be enveloped in a peritrophic membrane of undetermined origin and is passed into the anal atrium or hindgut [Fig. 30].

The Anal Atrium or Hindgut.

The anal atrium is a cuticle-lined tube that conveys the processed food and waste material to be voided outside. The internal surface of the anal atrium often has a rough, irregular appearance [Figs. 30 and 31], the cuticle appears to be punctuated by pore canals [Fig. 32]. Two varieties of excreta are observed. The majority of this is material that remains once the ingested meal has been processed and frequently contains bacterial cells [Figs. 30, 32 and 33]. Less frequently observed is spherical crystalline material that is usually excreted without the presence of the more frequently produced conventional excreta. The processed faecal pellets are voided from the anal atrium to the outside presumably by the contraction of circular muscles. Faecal pellets measure approximately 15μm in diameter [Fig. 33] and contain a viable population of bacteria (see Chapter 5).

Figure 1. The heavily sclerotized pharynx in vertical-longitudinal section showing the-pharyngeal ridges (PhR), pharyngeal dilator muscles (PDM) attached to the dorsal surface of the roof of the pharynx, the pharyngo-oesophageal valve (POV) and the start of the oesophagus (Oe). Note the thickness of the pharyngeal cuticle in comparison to that of the oesophagus. Bar = 2μm

Figure 2. A transverse section through the oesophagus demonstrating the eight-pointed star shaped profile and its proximity to the central nervous system (CNS). Several bacterial cells (small arrowheads) can be seen in the oesophageal lumen (Lu) and the arrangement of the circular constrictor muscles (Mu) attached from each point of the star to the next. Several epithelial cells (large arrows) associated with the oesophageal cuticle can also be seen. Bar = 4μm.


1
Oe
PDM
POV
PhR

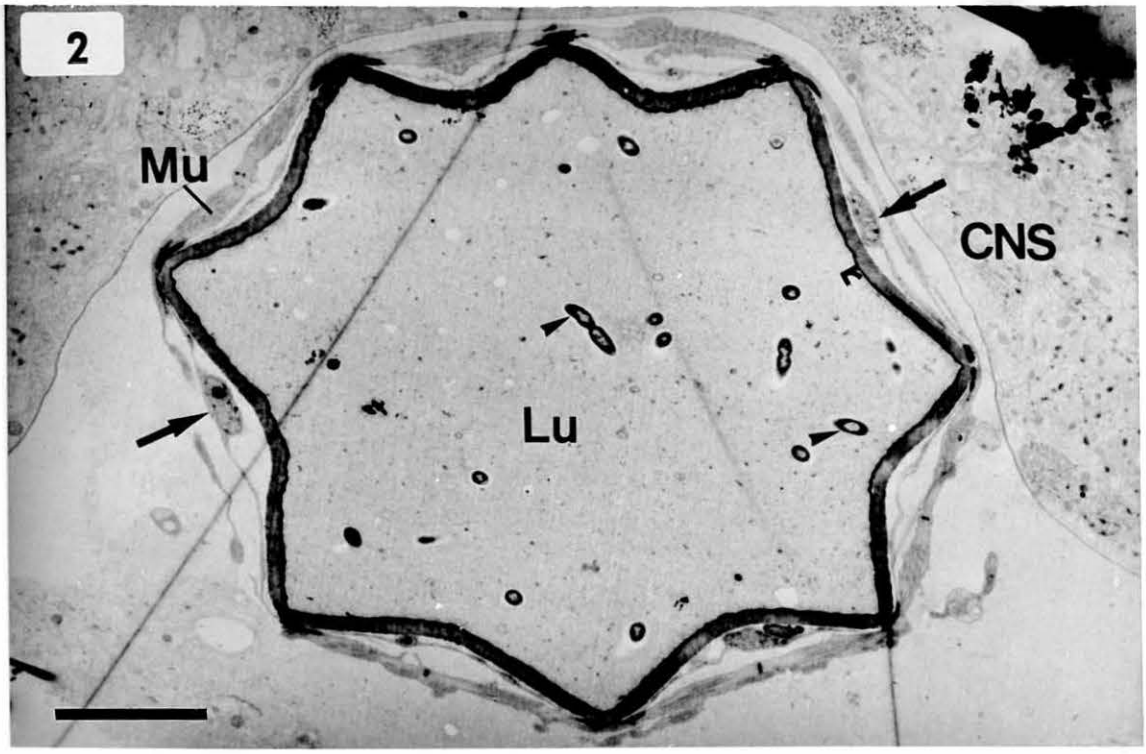
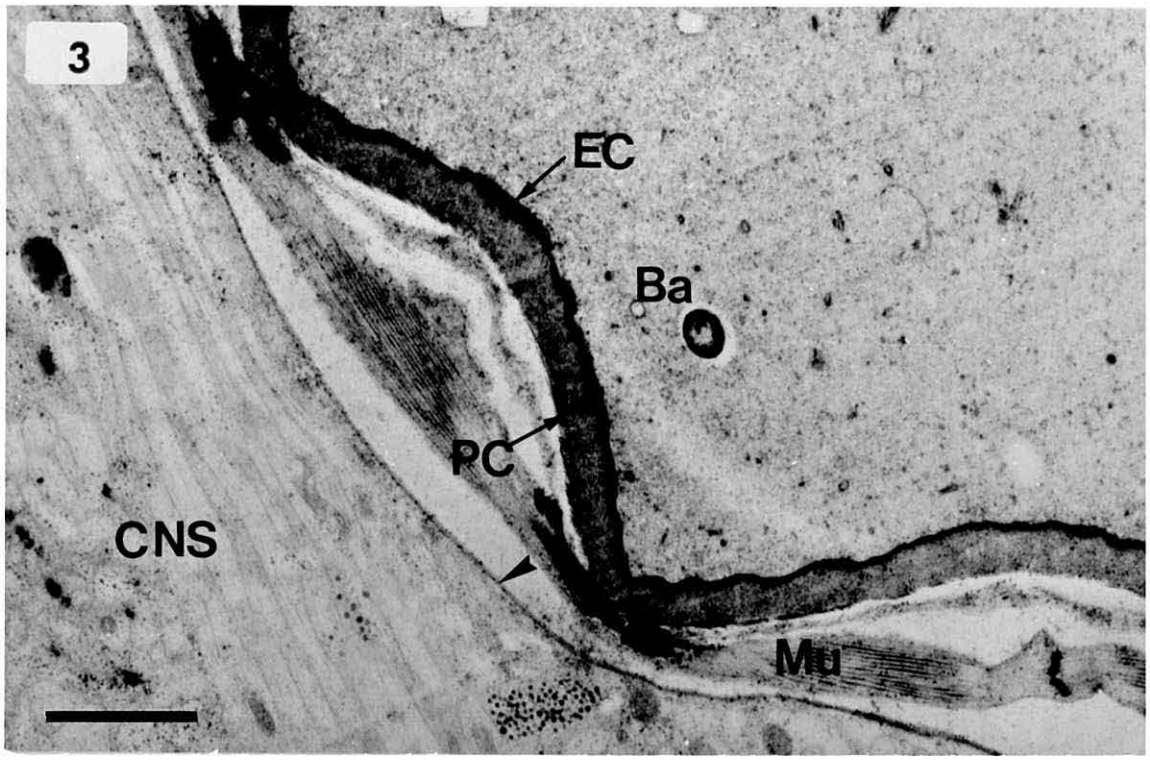

2
Mu
CNS
Lu

Figure 3. An enlargement of a portion of Figure 2 showing the oesophageal cuticle composed of a thin epicuticle (EC) and procuticle (PC). Circular muscle (Mu) attached to each oesophageal fold, the central nervous system (CNS) is also seen. The CNS is bounded by a capsule (arrow) isolating it from the oesophageal structures. Another bacterial cell can be seen in the lumen. Bar = 1.25μm.

Figure 4. Detail from a longitudinal section through the oesophagus and circular muscle (Mu). The lumen (Lu) is at the top of the picture. The figure also demonstrates the 4 cuticlar layers: waxy secretion layer (SL), outer-epicuticle (OE), inner-epicuticle (IE) and the procuticle (PC). An underlying circular muscle bundle (Mu) is visible. Bar = 0.3μm.


3
EC
Ba
PC
CNS
Mu

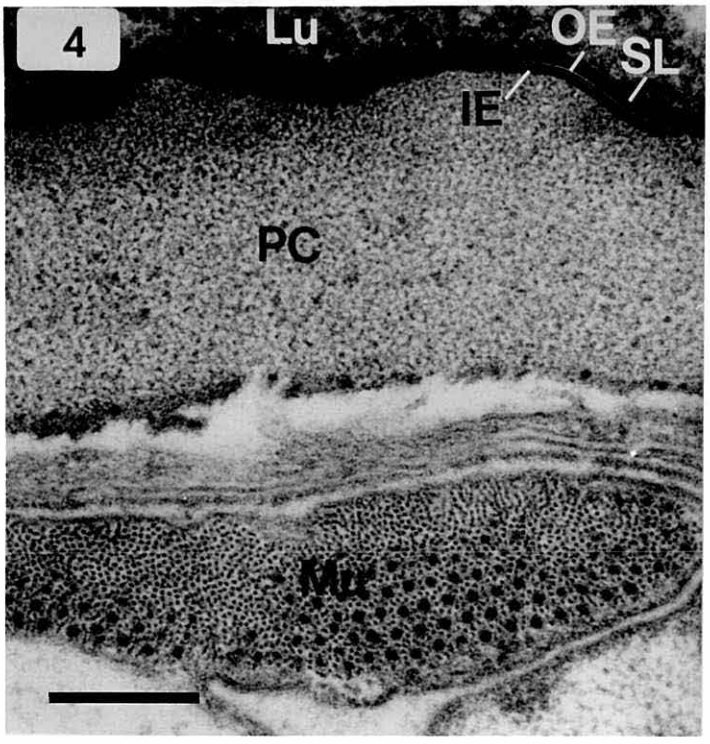
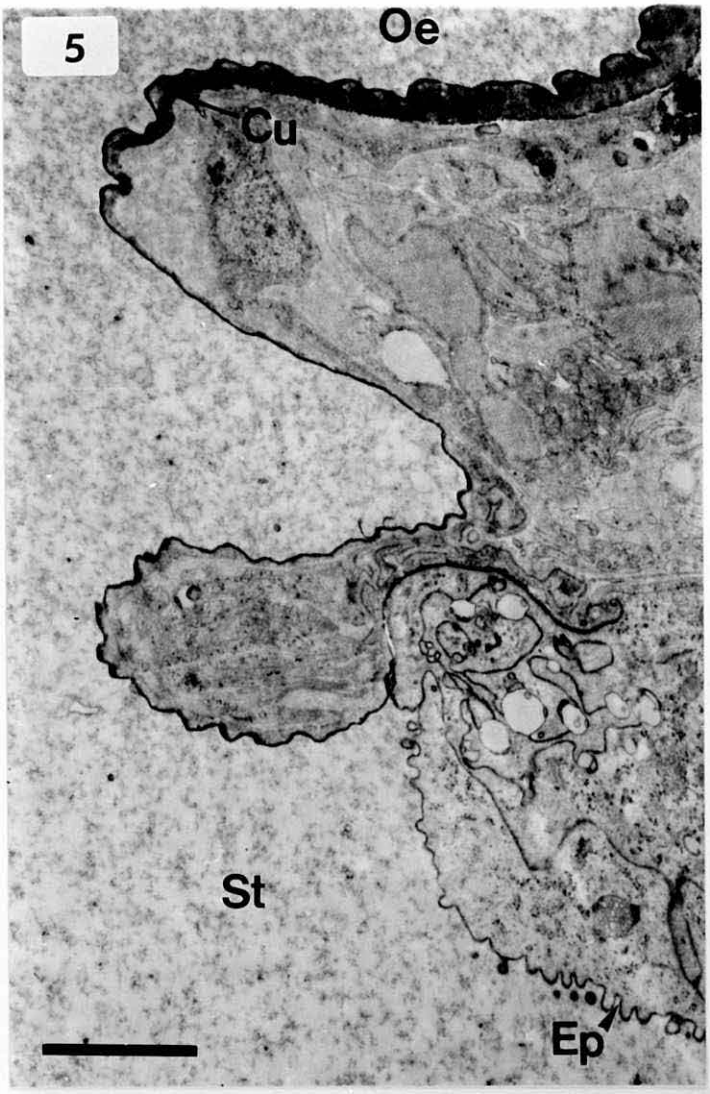

4
Lu
OE
SL
IE
PC
Mu

Figure 5. The junction of the cuticle-lined oesophagus (Oe) and the microvilli-lined midgut or stomach (St). The gradual thinning of the cuticle (Cu) giving way to the microvilli-lined epithelial cells (Ep) can be seen. Bar = 2.5μm.

Figure 6. This section demonstrates the variable thickness of typical stomach epithelial cells. The epithelium ranges from squamous (Sq) through cuboidal (Cb) to almost columnar (Cn). The location and distribution of the mitochondria (Mi) associated with the tissue lying outside the basal lamina (BL) can be seen. Large basally or medially located nuclei containing a single nucleolus (Nu) are characteristic of all the cells in the midgut epithelia. Lipid droplets (Ld) are present throughout the intermediate tissue (IT) lying beneath the epithelial basal lamina. This tissue also contains large reserves of stored glycogen (seen later in an enlargement). Bar = 5.5μm.


5
Oe
Cu
St
Ep

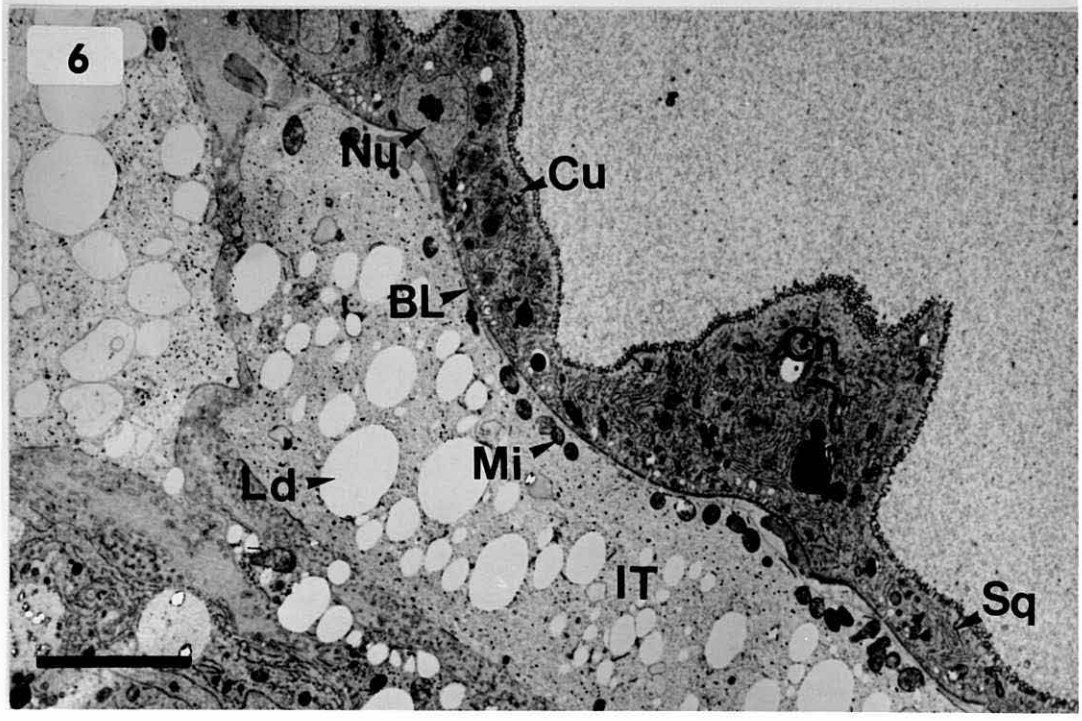

6
Nu
Cu
BL
Mi
Ld
IT
Sq

Figure 7. This figure demonstrates a portion of two typical squamous epithelial cells from the stomach. The short microvilli (Mv) adjacent to the gut lumen (Lu), large basally located nucleus (Nu) possessing a single nucleolus, small mitochondria (Mt) and extensive rough endoplasmic reticulum (RER) are all characteristic features of these cells. The convoluted lateral cell junction (CJ) between the two cells and apical desmosomes (D) are frequently seen throughout the midgut epithelia. The relatively thick, homogeneous basal lamina (BL) and the association the visceral muscle (Mu) has with it (*) is represented. The underlying lobular intermediate tissue (IT) and associated lipid (Ld) and glycogen (G) can be seen as can several intermediate tissue protrusions (arrowheads). Bar = 1.5μm.

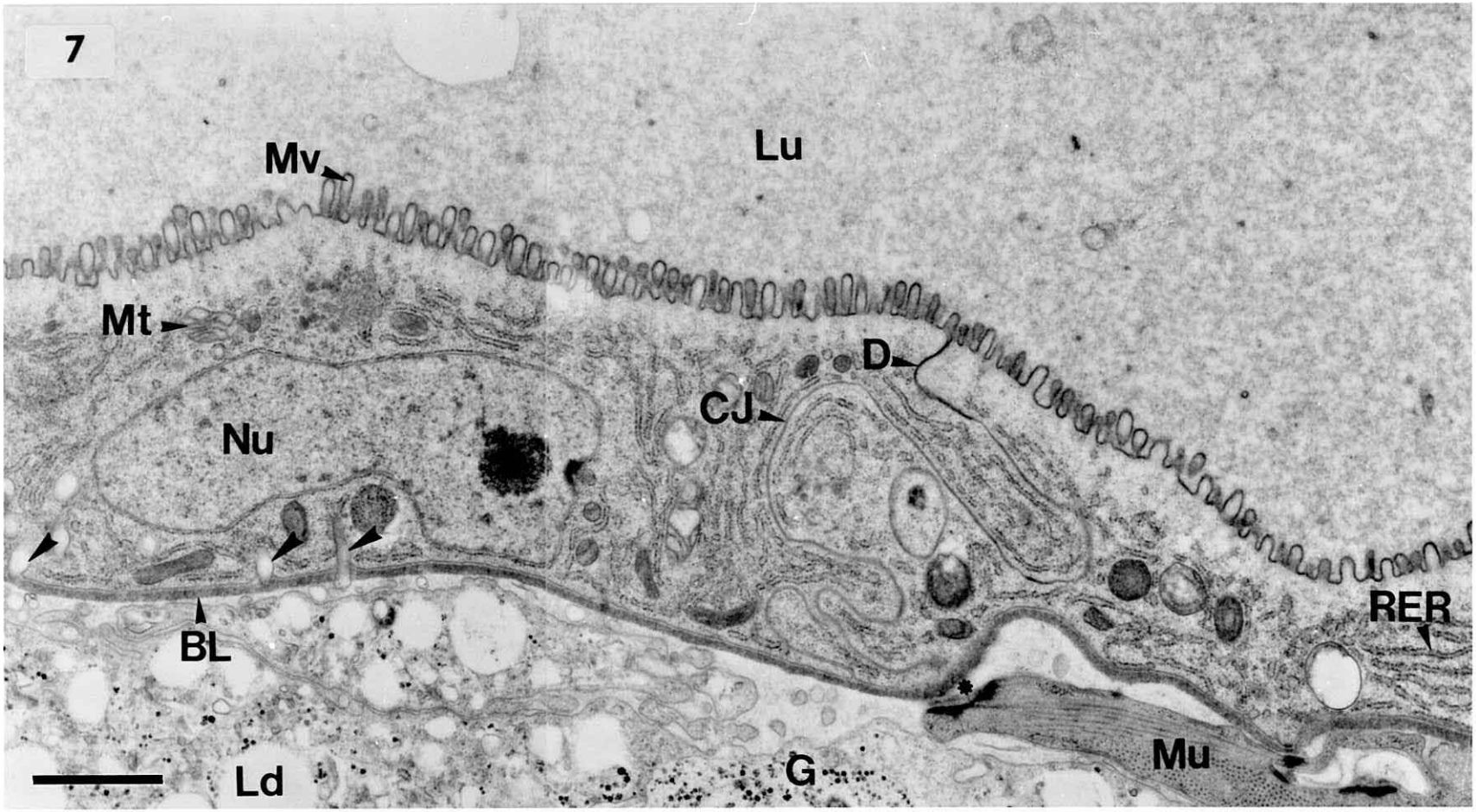
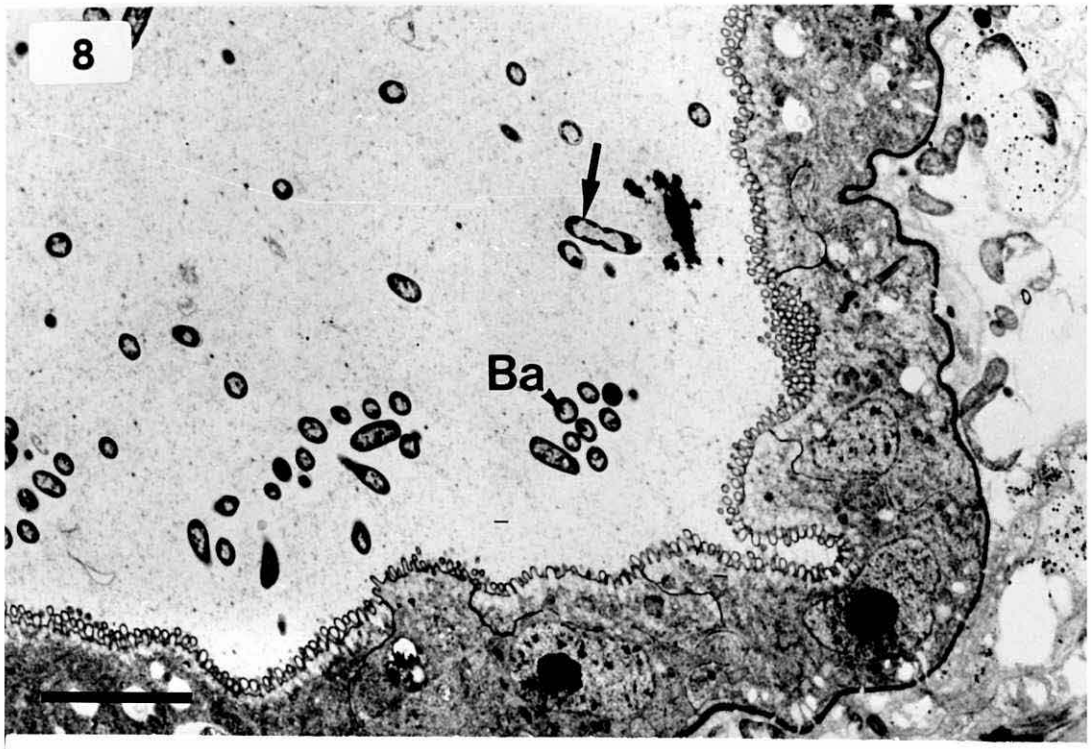
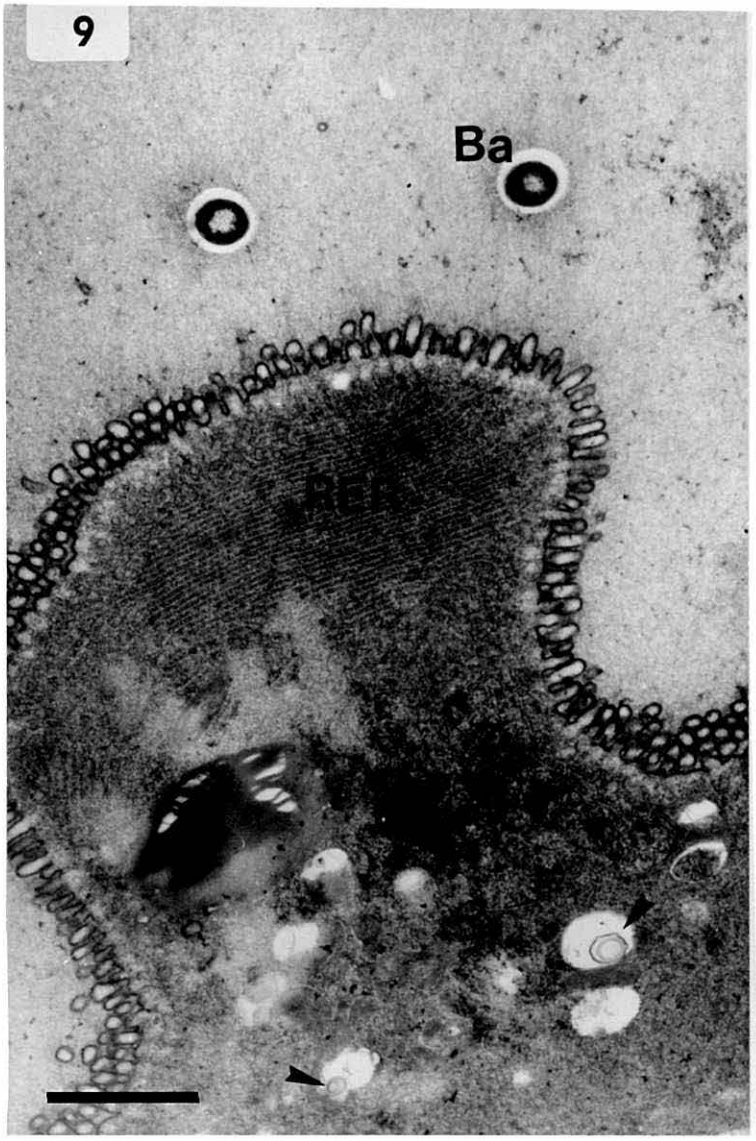

7
Mv
Lu
Mt
D
CJ
Nu
BL
RER
Mu
Ld
G

Figure 8. This figure of several cuboidal epithelia cells demonstrates the bacteria (Ba) frequently seen throughout the mite's digestive tract. These bacteria remain viable in the gut for some time as pairs of rods are often seen attached by their poles, indicating they were dividing during fixation (arrow). Bar = 3μm.

Figure 9. Epithelial cells are occasionally seen with large amounts of rough endoplasmic reticulum (RER) in uniform stacks or whorls. The cell contains several vesicles two of these appear to contain a crystalline material (arrowheads) possibly nitrogenous waste. This figure again demonstrated the bacteria (Ba) that are omnipresent in the gut. Bar = 1.75μm.


8
Ba


9
Ba

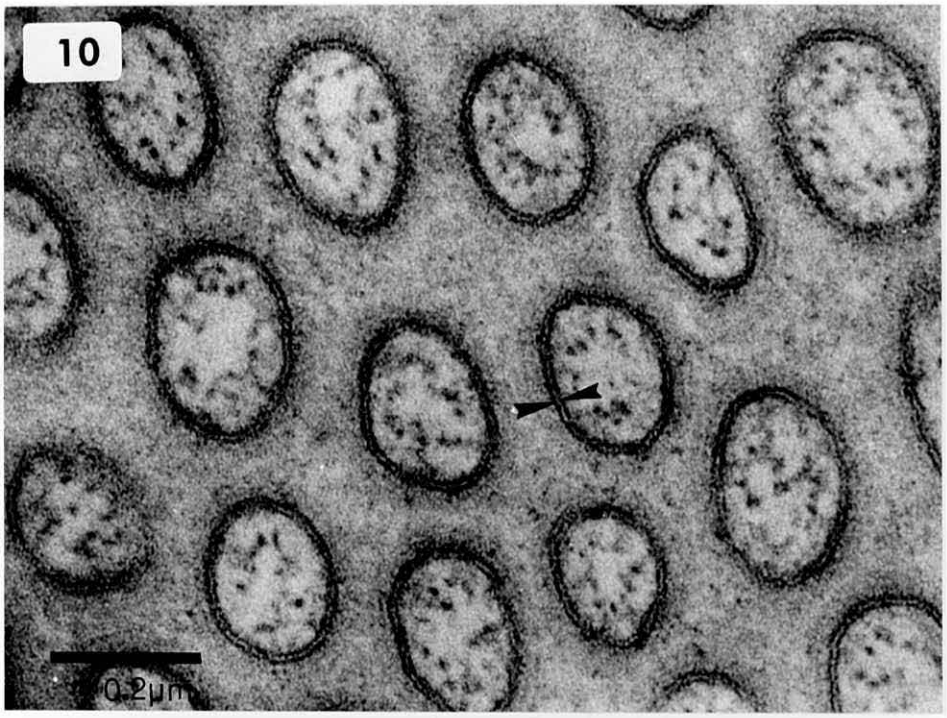
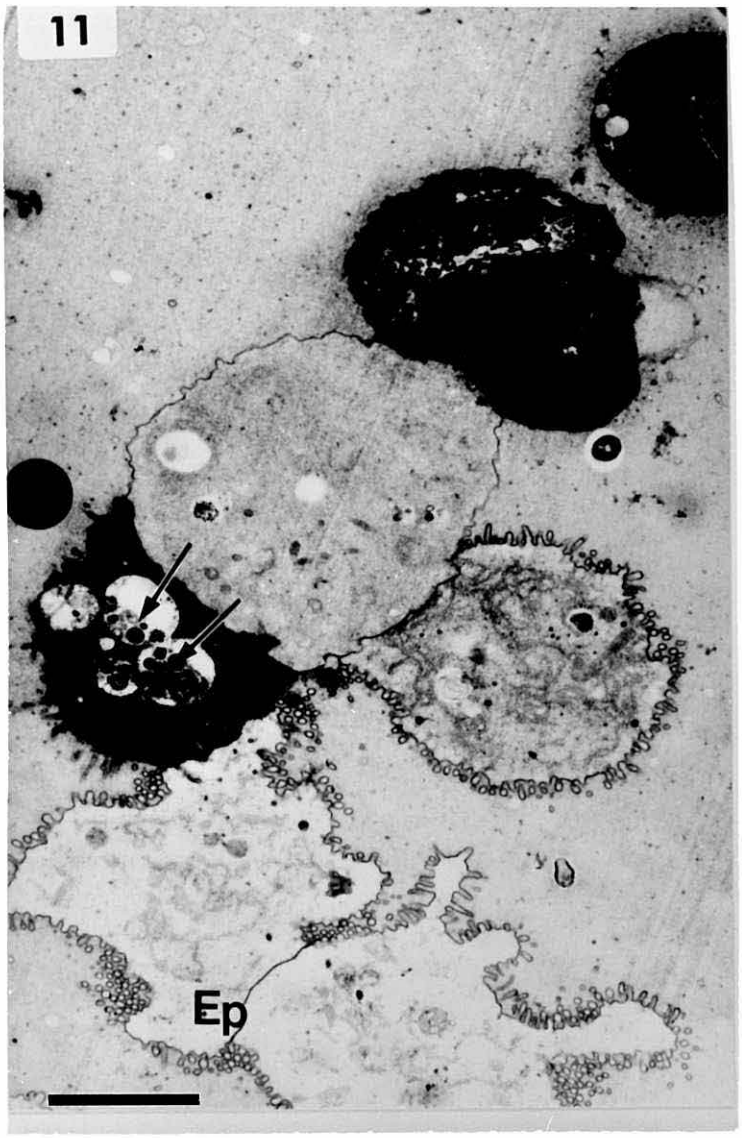


Figure 10. This transverse section through several epithelial microvilli demonstrates the double plasma membrane that appears to be characteristic of the digestive system microvilli.

Figure 11 demonstrates several cells in various stages of "budding-off" from the stomach epithelia (Ep). The staining characteristics of the budding cells change to become intensively osmiophilic. Vacuoles (arrows) containing unidentifiable material are common inclusions in these cells. The cells are often void of microvilli but not exclusively it seems. Bar = 2.5μm.

Figure 12. This figure demonstrates two typical epithelial cell that have become free-floating in the gut lumen. The cells become characteristically intensively stained, often including various vacuoles (arrows). The cells contain what appears to be remnants of endoplasmic reticulum (not marked). Bar = 2μm.


11
Ep

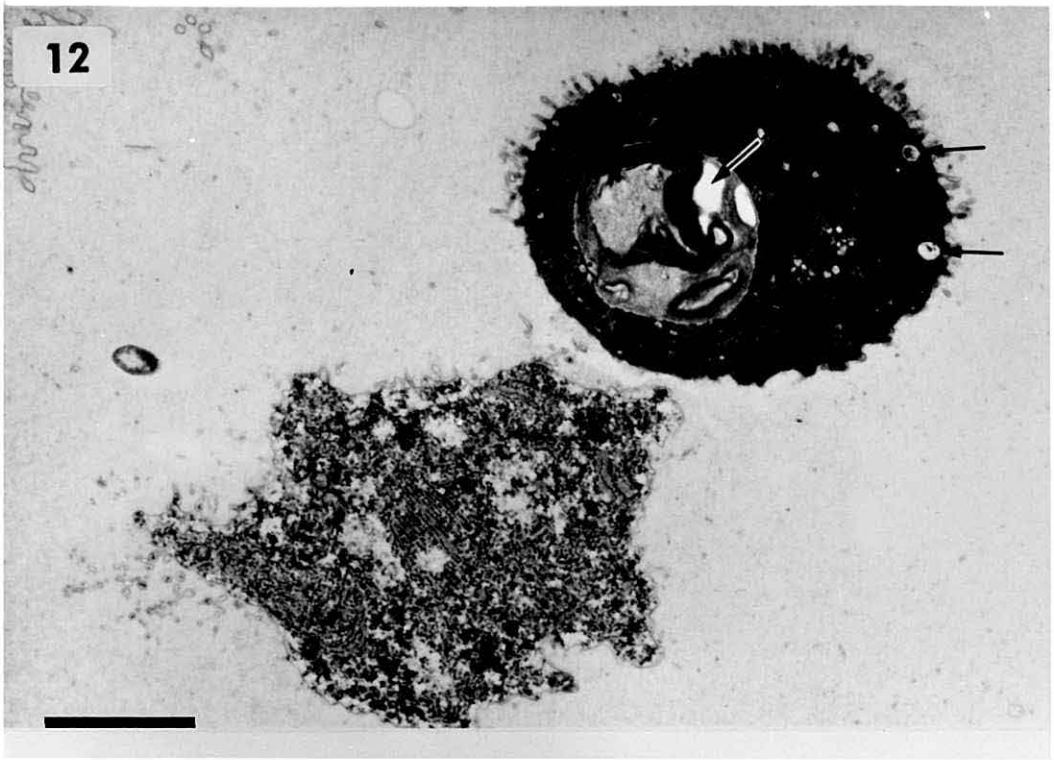
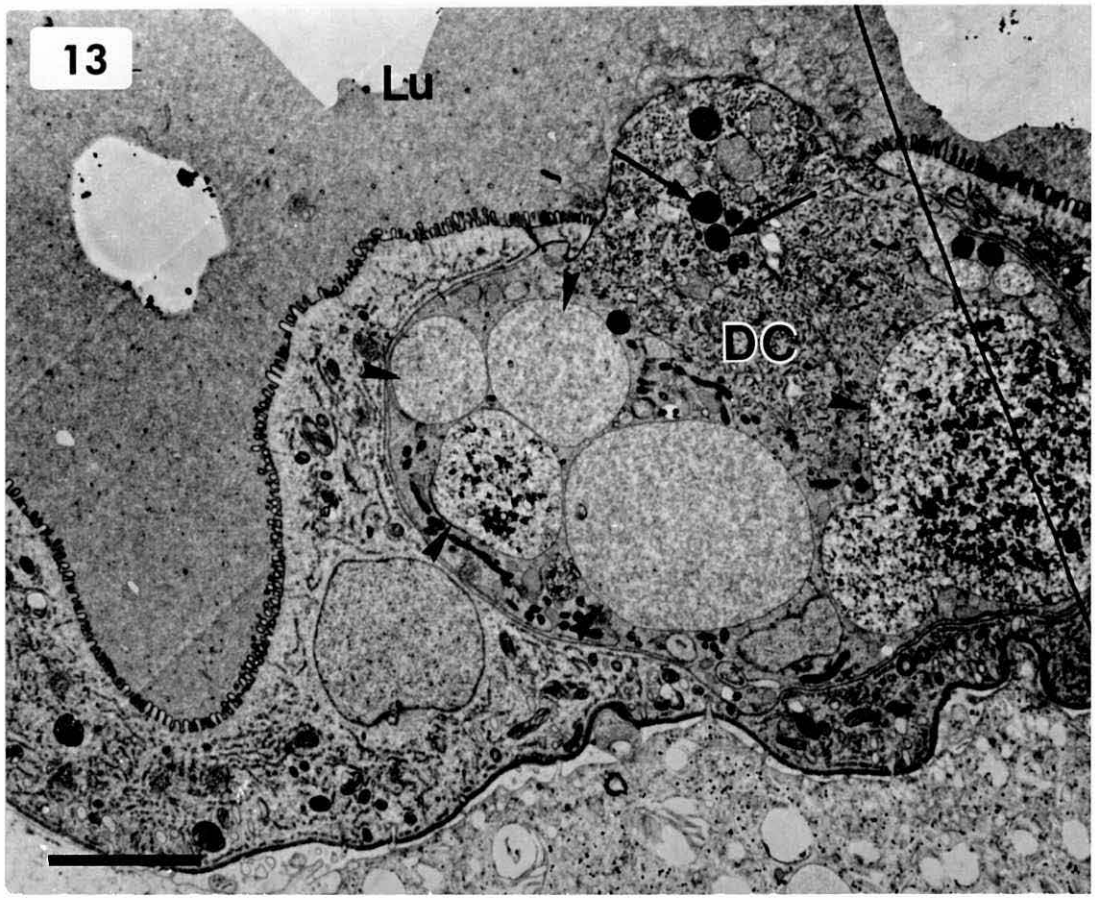
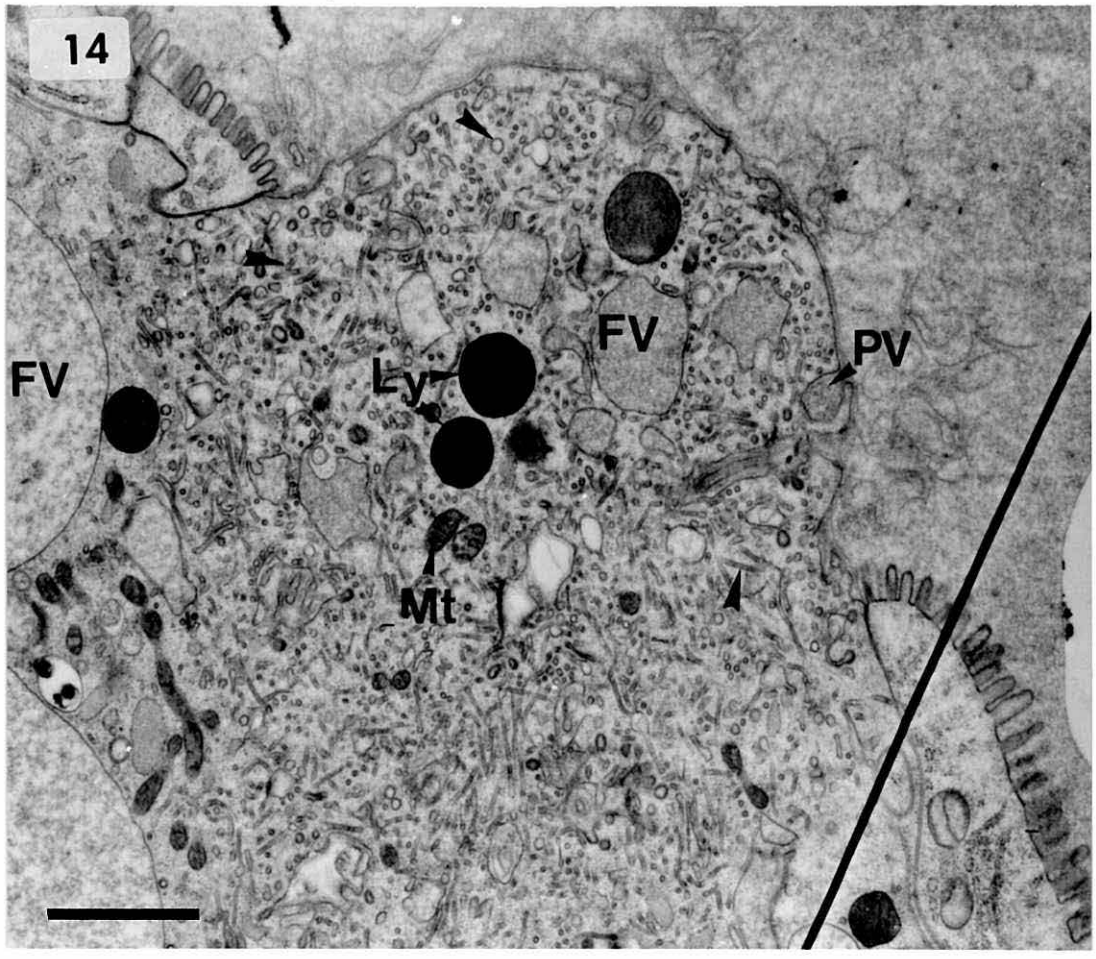

12

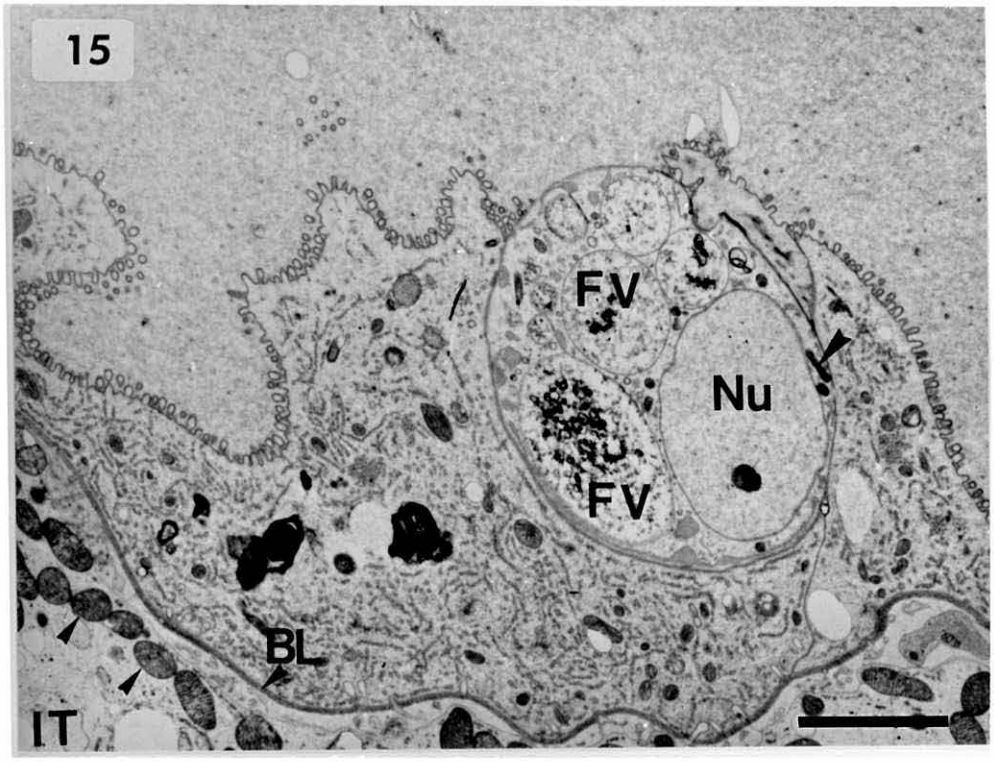
Figure 13 demonstrates a typical digestive cell (DC) in the process of migrating from the stomach epithelia into the gut lumen. The cell's apical surface, void of microvilli, can be seen to be extending into the gut lumen (Lu). Several lysosomes (arrows) can be seen and various food vacuoles are present in the base of the cell (arrowheads). Bar = 4μm.

Figure 14 is an enlargement of the apical region of Figure 13 demonstrating the extensive network of tubules (arrowheads) and food vacuoles (FV) characteristic of these digestive, budding cells. Small pinocytotic vesicles (PV) can be seen at the luminal border of the cell. Lysosomes (Ly) and small mitochondria (Mt) are also present. Bar = 1.6μm.


13
Lu
DC


14
FV
FV
Ly
PV
Mt

Figures 15 and 16 are two more examples of budding, digestive cells. Figure 15 contains several food vacuoles (FV) and appears to be in the process of leaving the epithelia. Numerous extra-epithelial mitochondria (small arrowheads) are seen to be in close association with the basal lamina (BL) within the intermediate tissue (IT). Figure 16 does not have any obvious vacuoles and is perhaps in an earlier stage of development. Small epithelial mitochondria (large arrowheads) are present and both cells are typically nucleated (Nu). Bars = 3μm and 2μm.


15
FV
Nu
FV
BL
IT

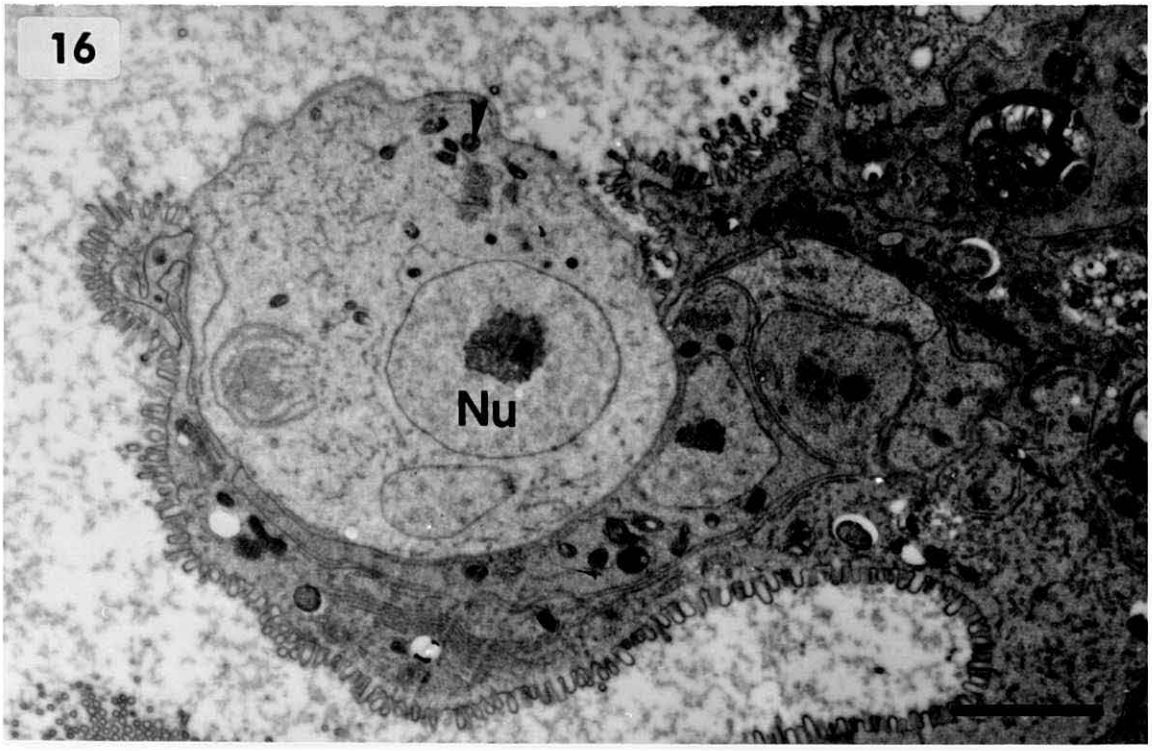
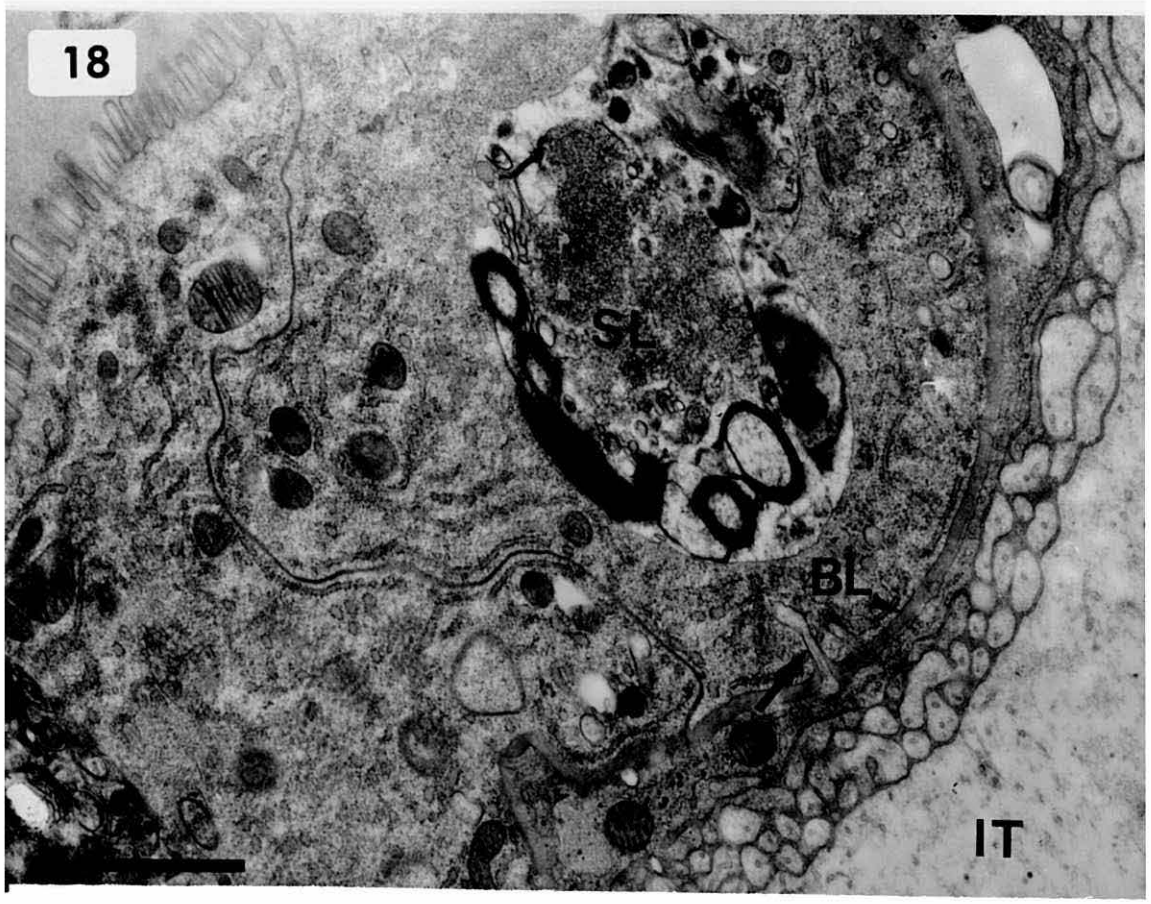
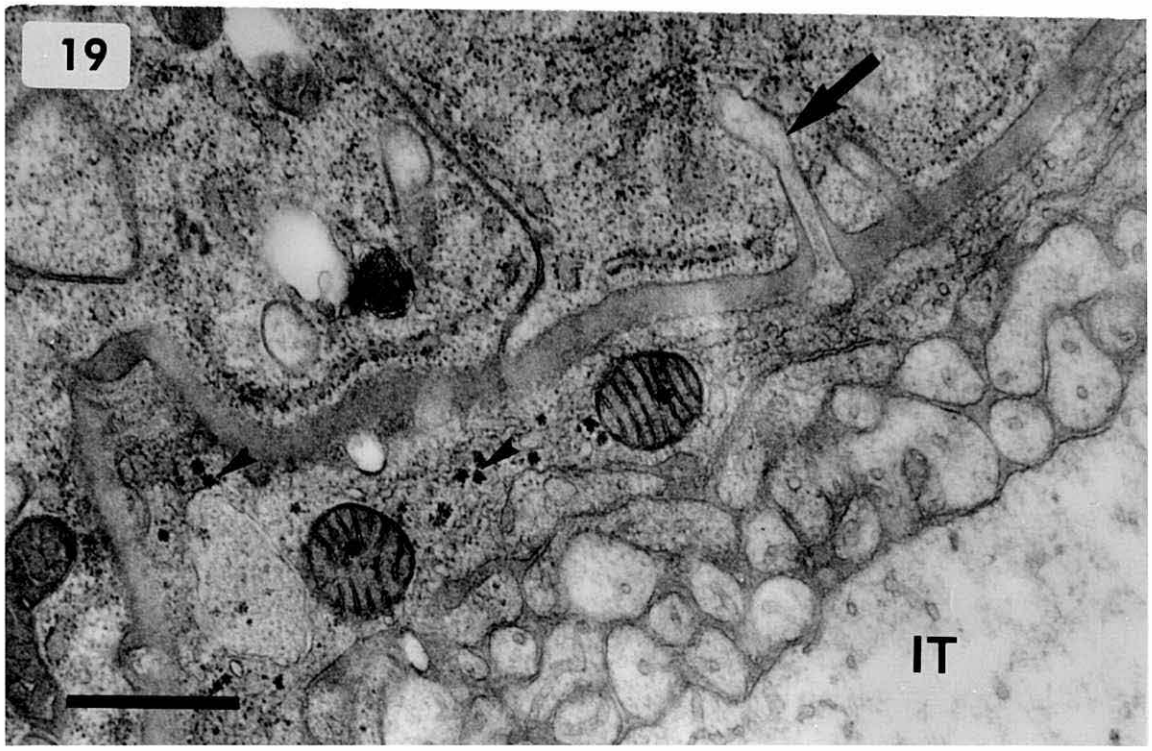

16
Nu

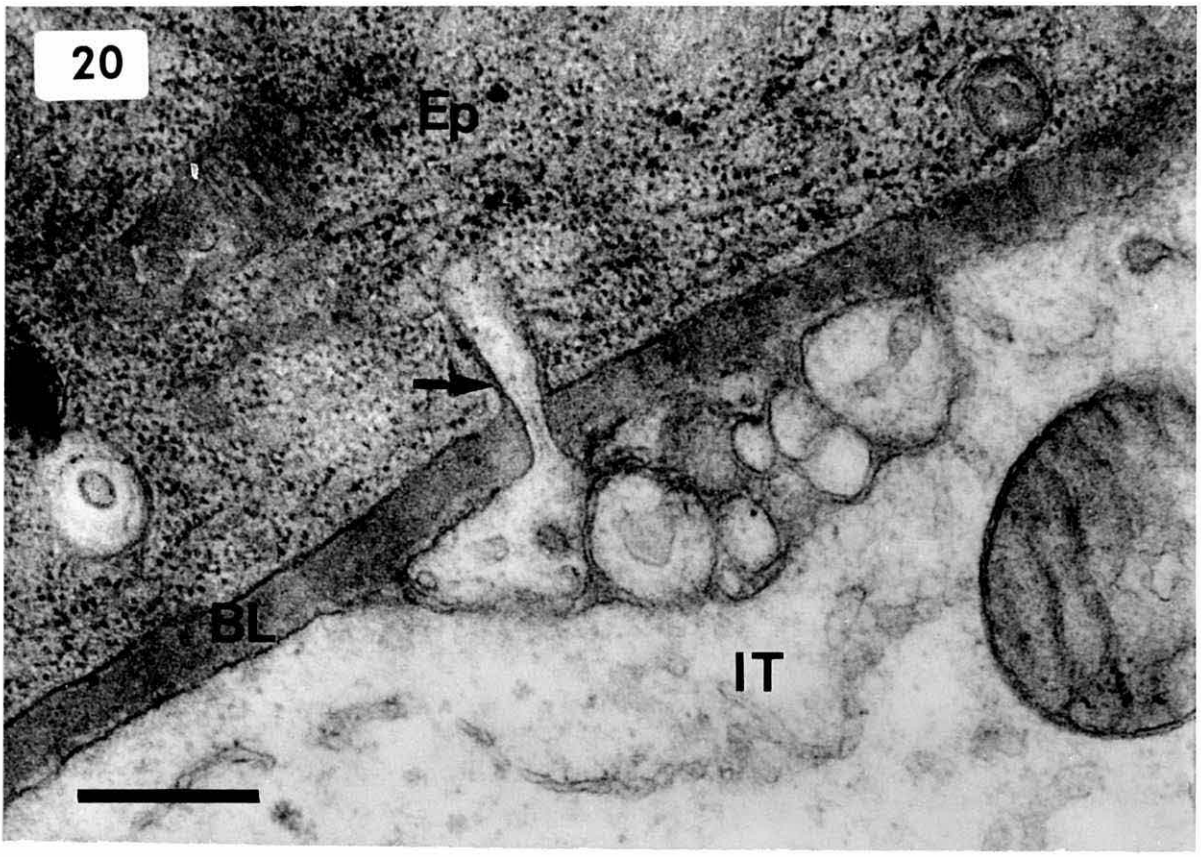
Figure 18. Section through portions of two typical midgut epithelial cells demonstrating the characteristic features of the cells and here, a large secondary lysosome (SL) containing osmiophilic material and other cell components. The basal lamina (BL) and intermediate tissue (IT) and the communication that appears between them is demonstrated here. Finger-like extensions (arrow) of the intermediate tissue are regularly seen to penetrate through the basal lamina and extend some distance into the base of the epithelial cells. Bar = 1.8μm.

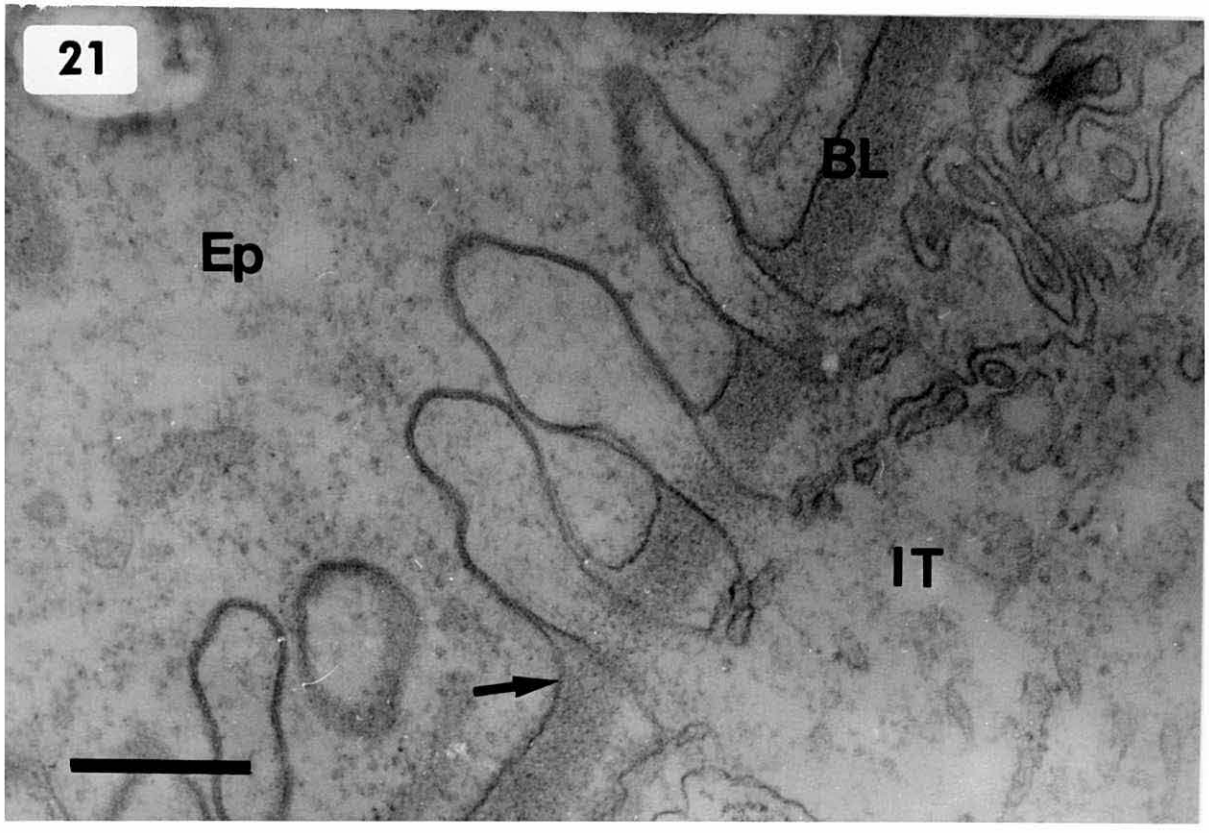
Figure 19. This enlargement of a basal portion of the previous figure magnifies the intermediate tissue (IT) extensions (large arrow). Two intermediate tissue mitochondria (∗) and glycogen rosettes (arrowheads) are labelled. Bar = 0.7μm.


18
SL
BL
IT

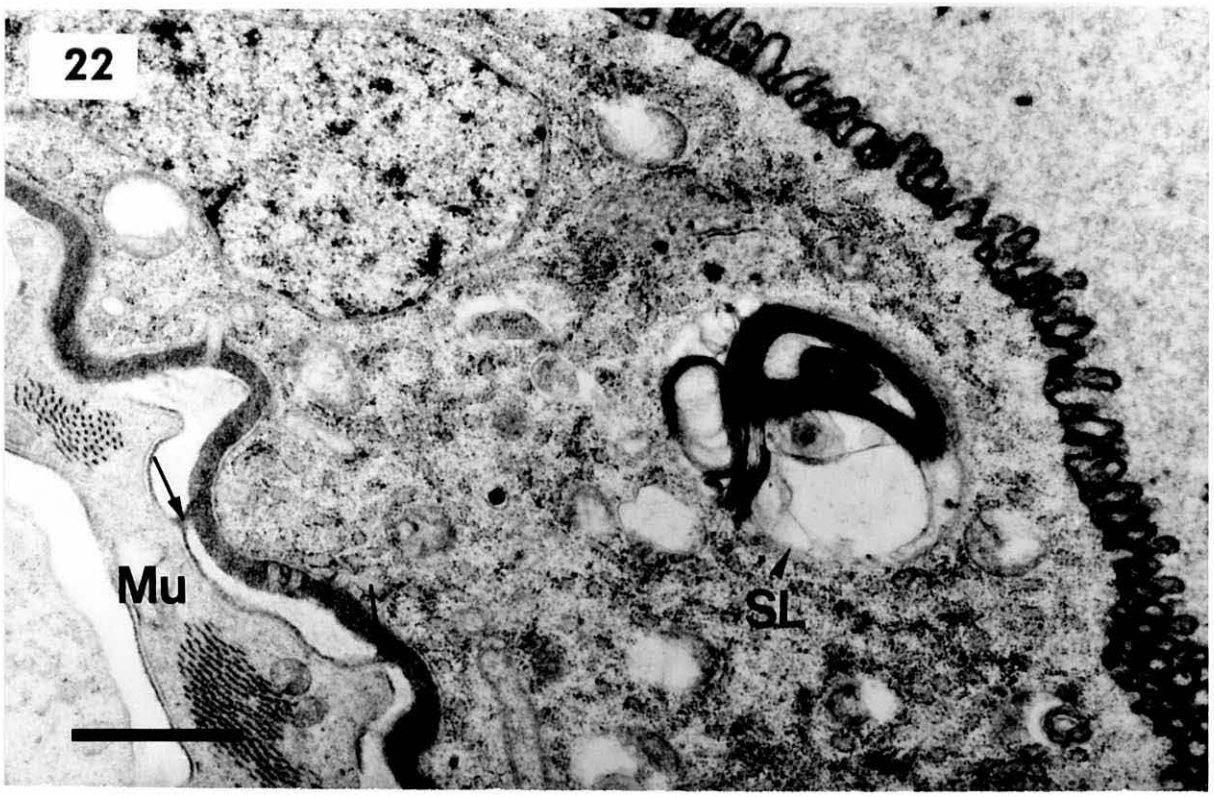

19
IT

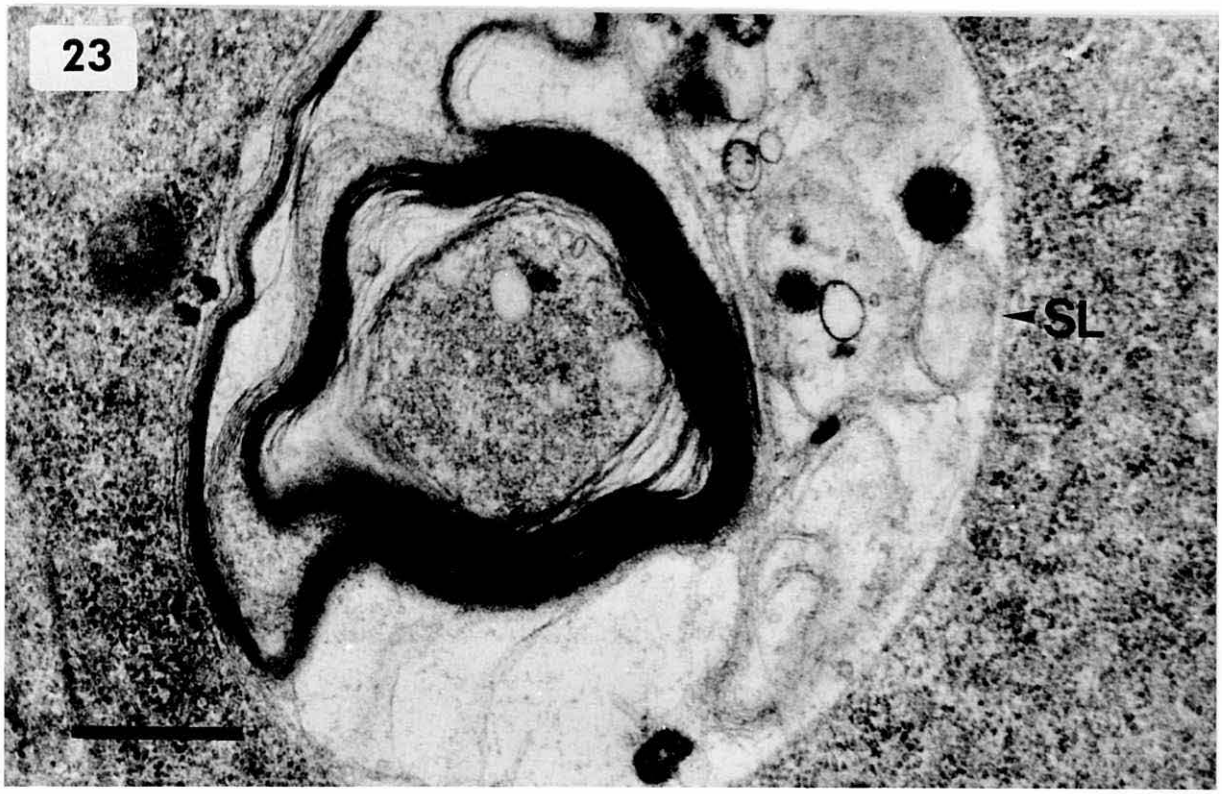
Figures 20 and 21 are magnifications of two other examples of the intermediate tissue (IT) protrusions. It can be seen that the epithelial cell (Ep) plasma membrane (arrow) invaginates basally into the cell and the cytoplasmic extensions of the intermediate tissue follows this invagination. The basal lamina (BL) follows the invagination, thinning between two plasma membranes. Bars = 0.44 and 0.28μm.


20
Ep
BL
IT


21
BL
Ep
IT

Figures 22 and 23 demonstrate two secondary lysosomes (SL) found in two typical stomach epithelial cells. They frequently contain concentric membranous structures as shown in the figures. Figure 22 also shows a section of visceral muscle (Mu) and its attachment to the epithelial cell basal lamina (arrows). Bars = 0.85 and 0.55μm.


22
Mu
SL


23
SL

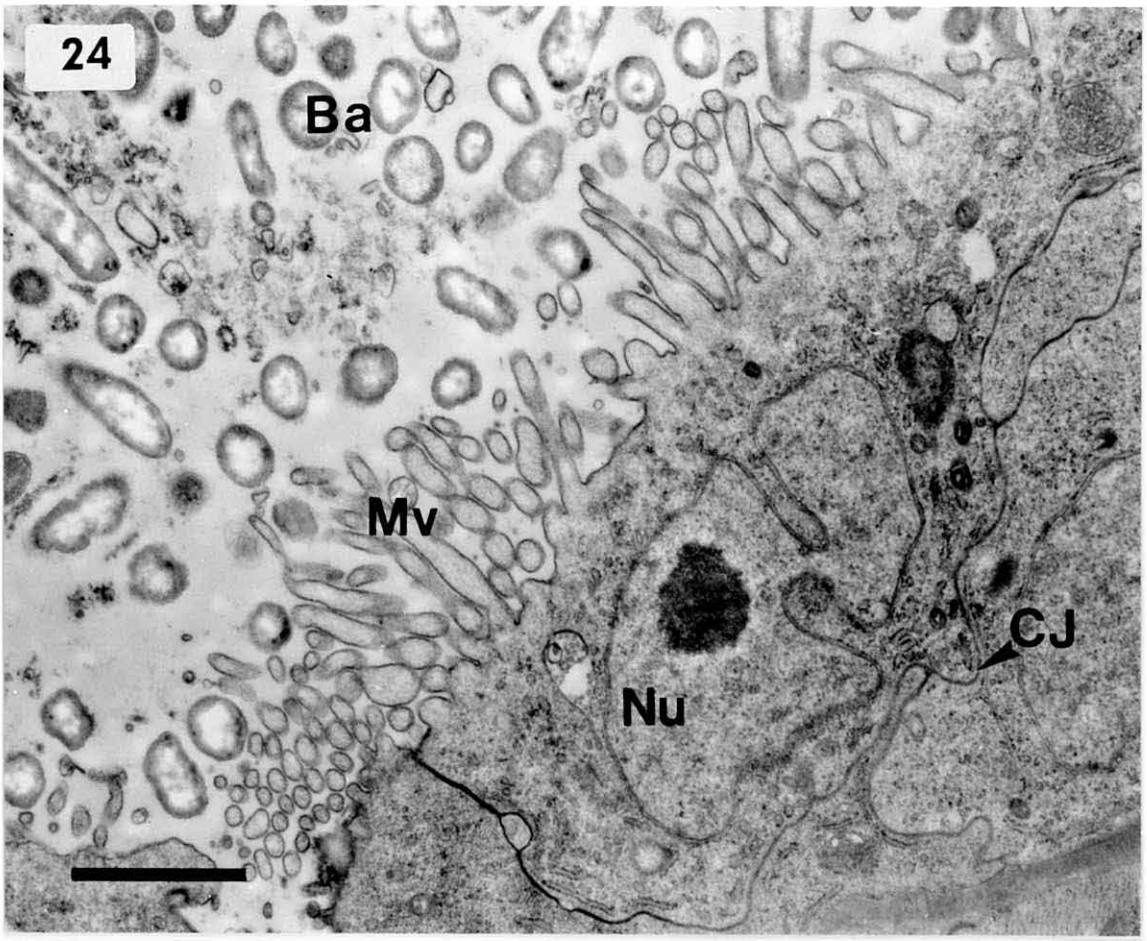
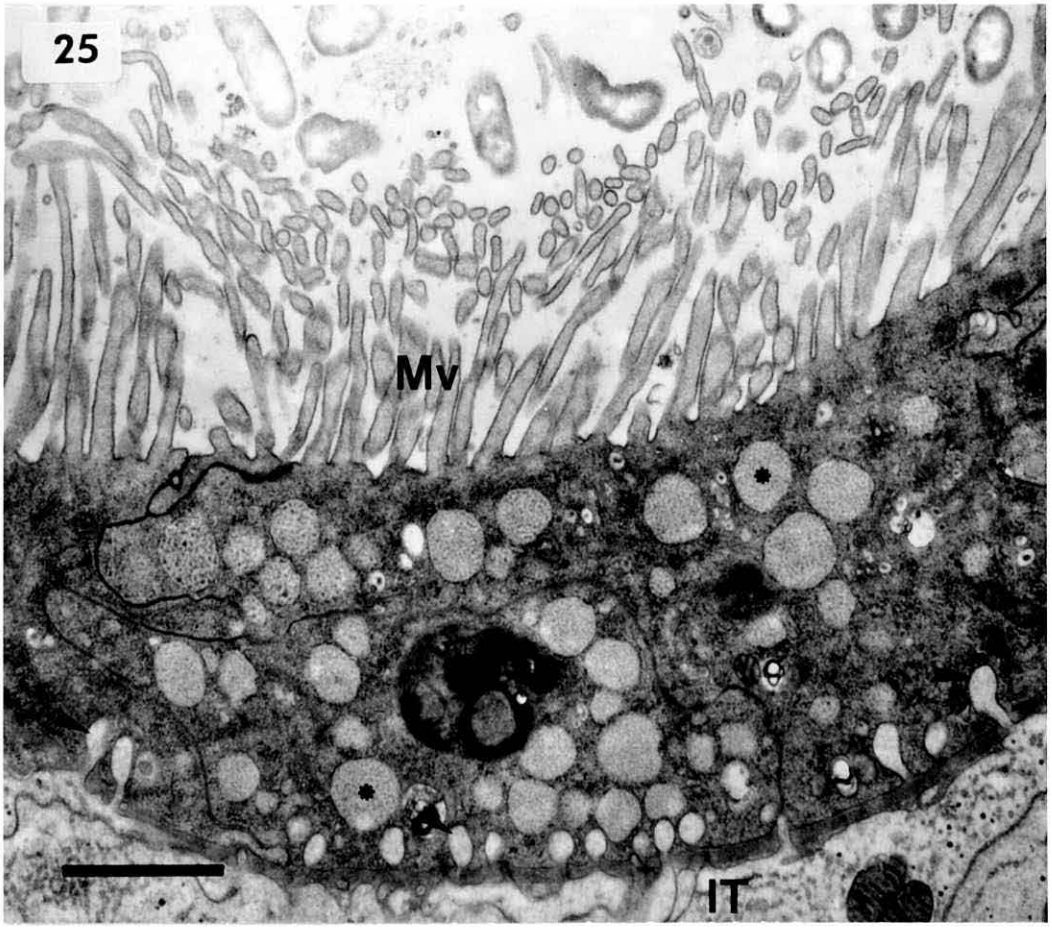


Figure 24. A characteristic cell of the post-colon, close to the junction with the colon, containing a large nucleus (Nu), single nucleolus and apically, long microvilli (Mv). Note the convoluted cell junctions (CJ) and the numerous bacteria (Ba) conspicuous in the lumen. Bar = 1.7μm

Figures 25 and 26. These two post-colon cells contain numerous vesicles (*) of unknown function. They are unlikely to be food vacuoles as seen in the digestive cells of the midgut ventriculi since the cells show no sign of tubules. A secretory role for the vesicles is possible but the long microvilli (Mv) and numerous intermediate tissue (IT) invaginations (arrowheads) also suggests an absorptive function for the cells. Figure 26 shows a section of a piece of visceral muscle (Mu). Bar = 1.6μm.


25
Mv
IT

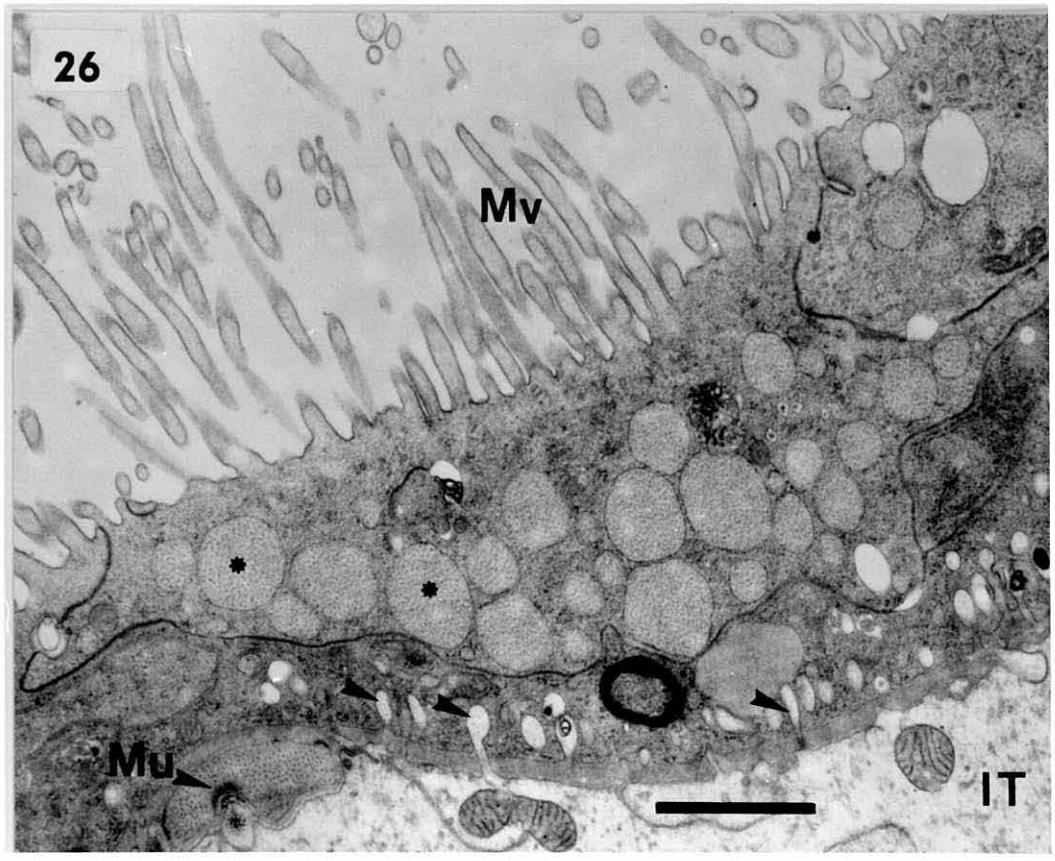
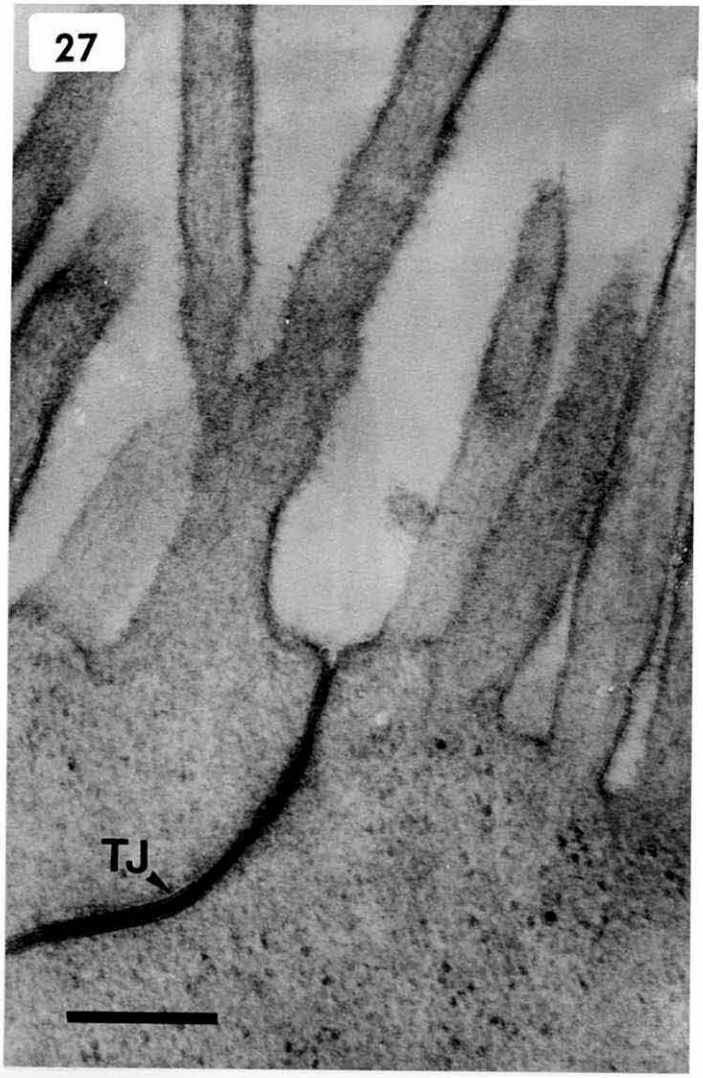
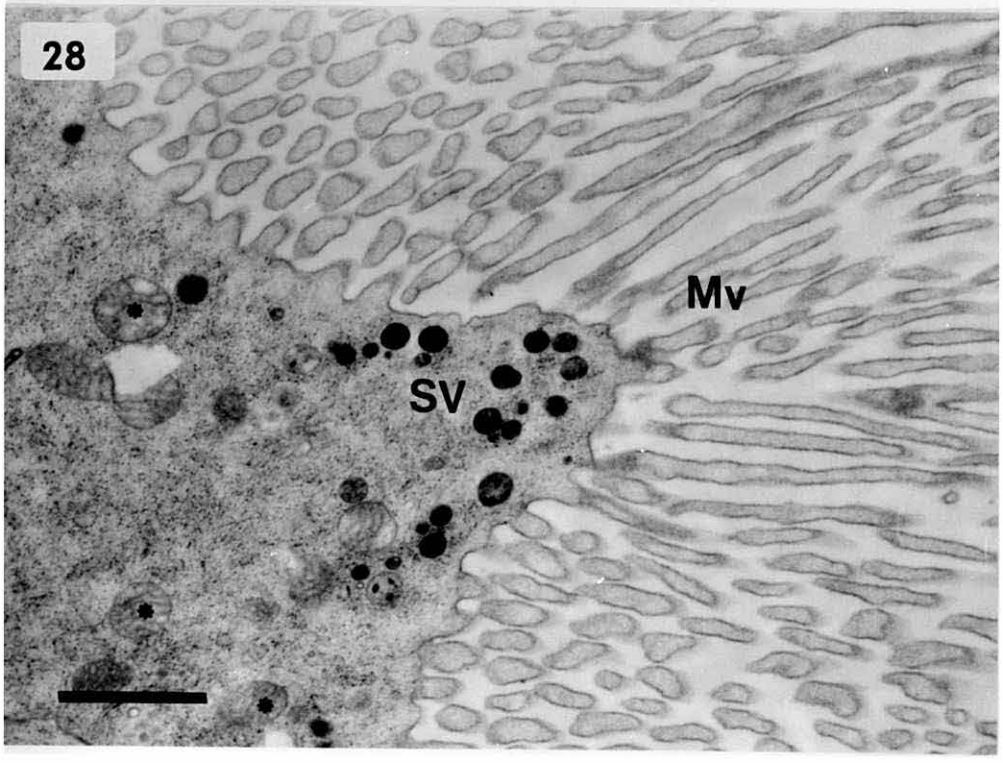

26
Mv
Mu
IT

Figure 27. The cells in the post-colon are joined apically by tight junctions (TJ), as are all the cells observed in the midgut. Bar = 0.2μm.

Belt desmasome

Figure 28. Apical secretory vesicles (SV) are occasionally observed in the post-colon epithelia, these, and the long microvilli (Mv), suggest a secretory role as well as an absorptive function of this area. Numerous mitochondria (*) are also present. Bar = 1.4μm.


27
TJ


28
Mv
SV

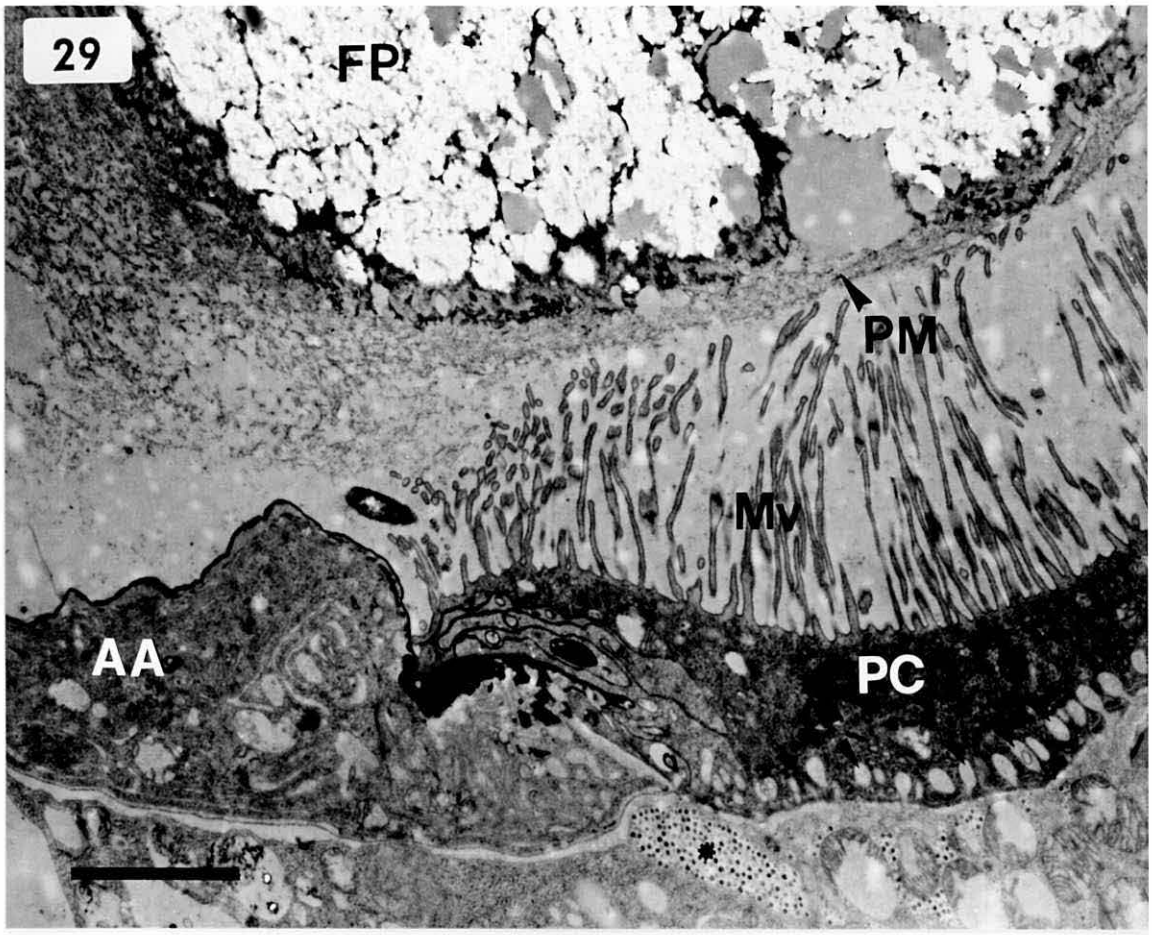
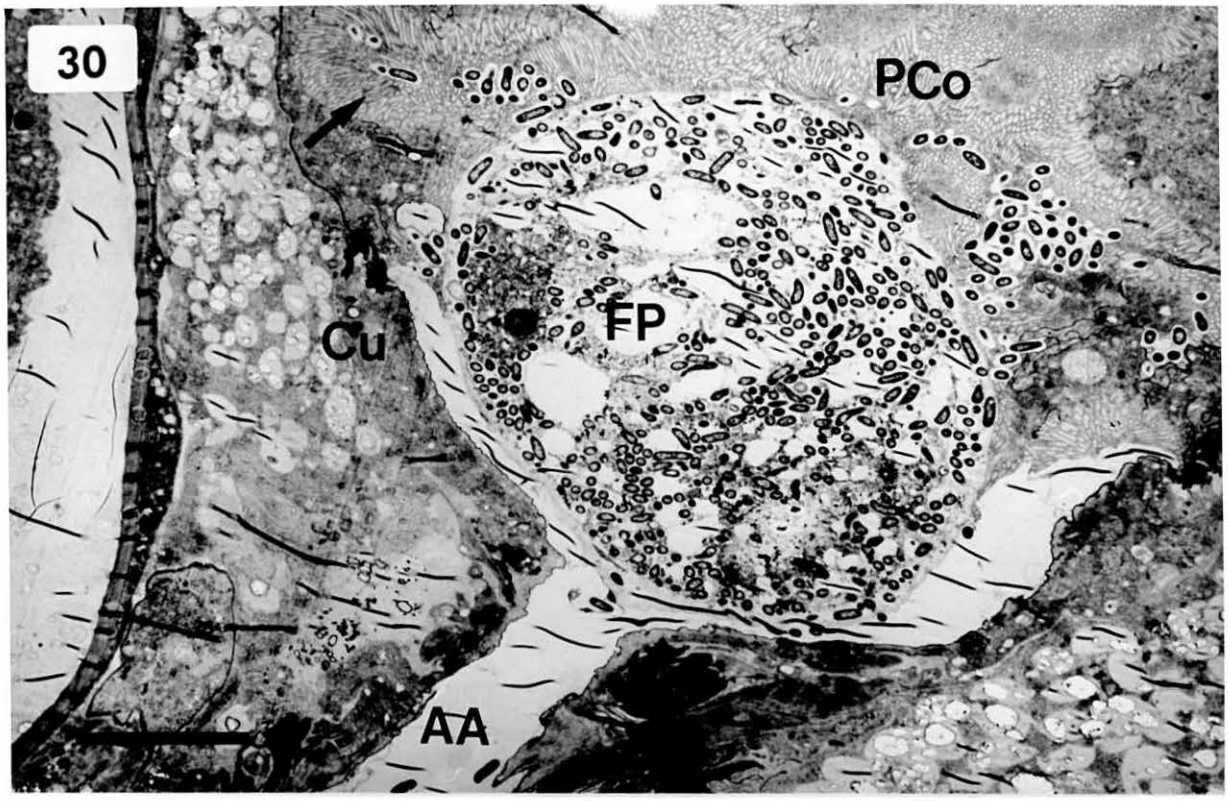


Figure 29. A section through the junction between the post-colon (PC), cuticle lined anal atrium (AA) and a faecal pellet (FP) surrounded by a peritrophic membrane (PM). The basal lamina of the post-colon epithelial cells is extensively punctuated by intermediate tissue invaginations (arrowheads), and together with the characteristically long microvilli (Mv) this again suggests solute absorption. A large store of glycogen (*) closely associated with (ruptured) mitochondria can be seen outside the epithelial cell basal lamina. Bar = 3μm.

Figures 30. Section showing the post-colon (PCo) and anal atrium (AA) junction, demonstrating the long microvilli (arrow) of the post-colon giving way to the cuticle (Cu) of the anal atrium. The figure demonstrates the typical excreta: a faecal pellet (FP) containing large amounts of bacteria. Bar = 7.8μm.

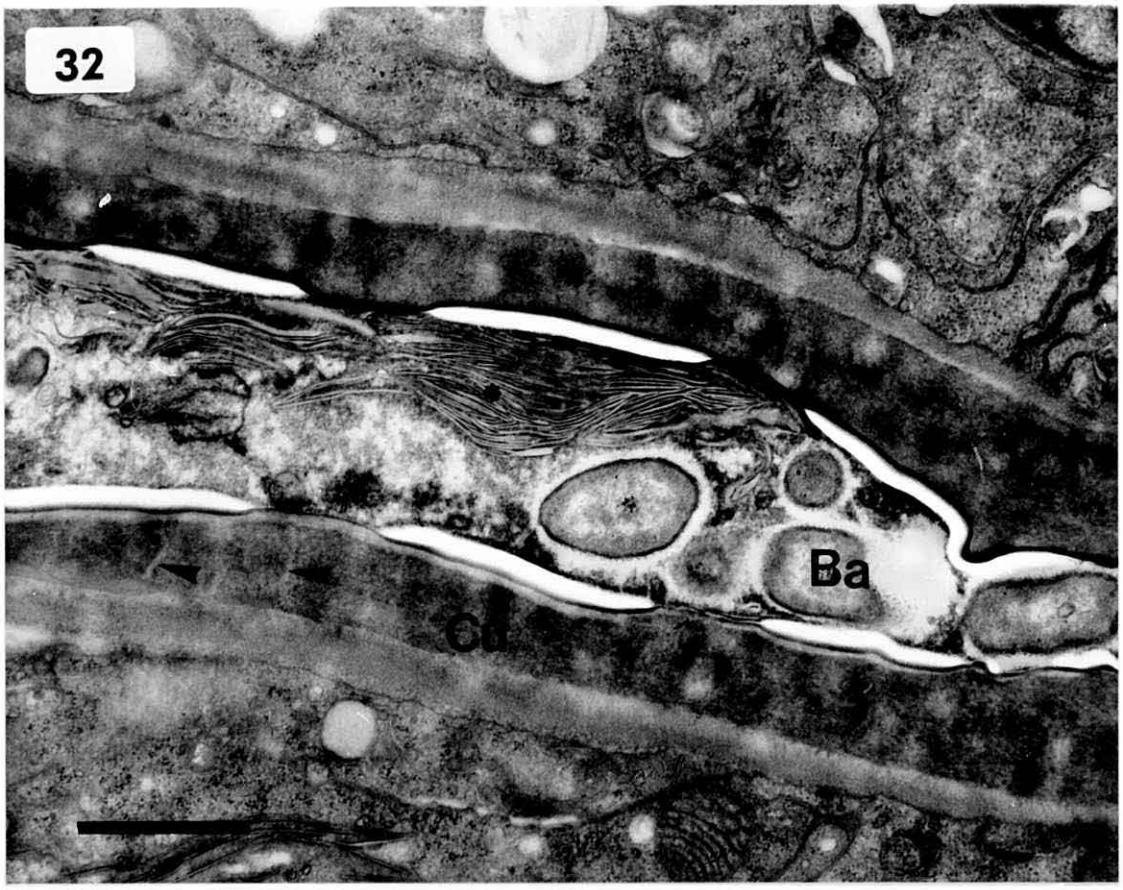
Figure 31 demonstrates a less frequently seen excreta; crystalline spheroids (arrows), distending the anal atrium (AA). Bar = 4.5μm.

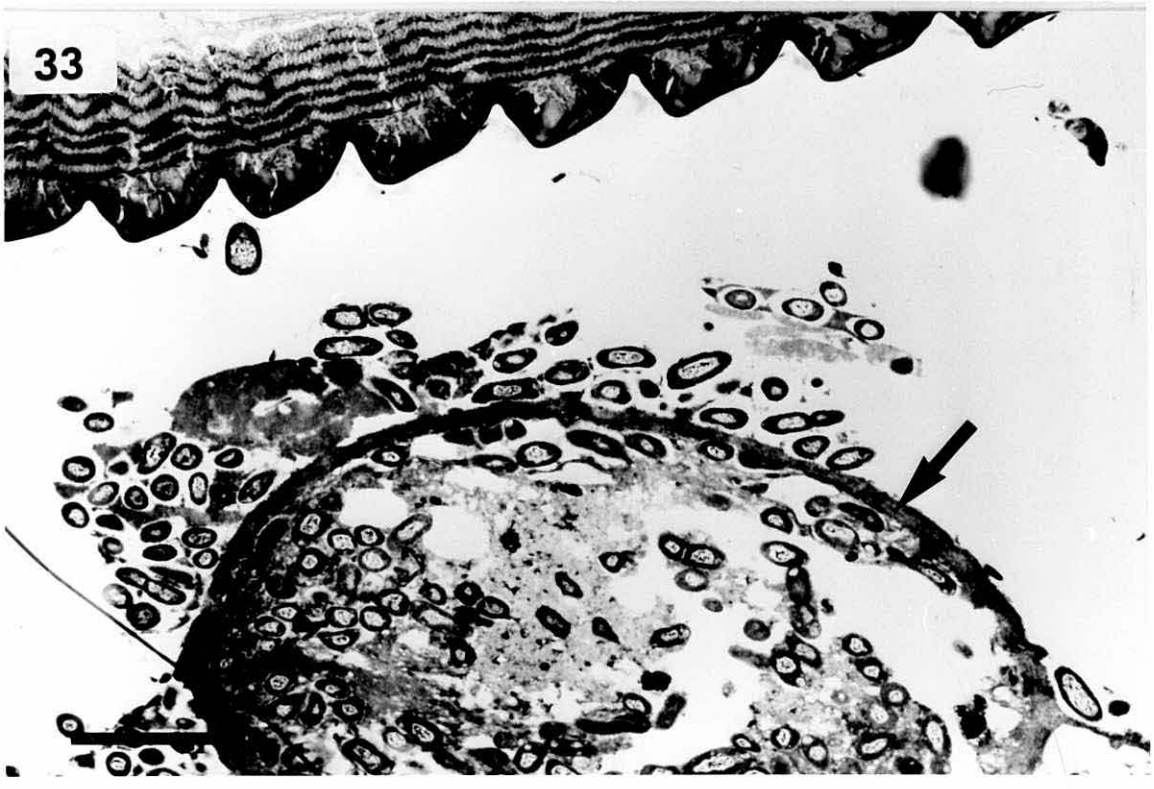

30
PCo
Cu
FP
AA


31
AA

Figure 32. A section through the anal atrium demonstrating the thick cuticle (Cu) and pore canals (arrows), punctuating the cuticle. The faecal material is uncharacteristically elongated here and contains bacterial cells (Ba) and an unidentified laminar substance (*), perhaps membranes. Bar = 1.4μm.

Figure 33. A section through a typical faecal pellet after being excreted. The peritrophic membrane (arrow) can be clearly seen enveloping the enclosed bacteria and excrement. The corrugated body cuticle appears at the top of the figure. Bar = 2.2μm.


32
Ba
cu


33

Discussion.

The foregut of *P. ovis* is composed of a muscular pharynx and an oesophagus surrounded by circular visceral muscles. Both these components are cuticle lined, the oesophagus possessing a thinner layer of cuticle than the pharynx. This arrangement is consistent with numerous mites and other arachnids (Evans, 1992; Baker and Wharton, 1952). The cuticle lining probably precludes any significant absorption of fluids and so the foregut is likely to serve as a tube, transferring ingested materials to the midgut. The pharyngo-oesophageal valve *P. ovis* possesses does not seem to be a universal feature of the Acari. Coons (1978) did not observe such a valve in the mesostigmatid mite *Macrocheles muscaedomesticae* but Brody *et al.* (1972) did in the more closely related astigmatid *Dermatophagoides farinae*.

The sclerotized ridges described on the floor of the pharynx could play a role in the physical maceration of ingested cellular material, although any cellular material observed in the digestive tract (eosinophils and bacteria) were intact, it seem that *P. ovis* feeds on an almost exclusively liquid diet. The function of the pharangeal ridges is unclear but may be a redundant, vestigial remnant indicating ancestrally different dietary components. A similar structure has been described for the house dust mite *D. farinae* (Brody *et al.*, 1972). This mite feeds on a more solid and particulate diet than *P. ovis* where the ridges may be involved in the breakdown of larger pieces of ingested material into more manageable sizes.

The arrangement of the muscle systems associated with the pharynx and oesophagus of several other mites have been studied to varying degrees (Brody *et al.*, 1972; Coons, 1978; Evans, 1992), all describe the arrangement of pharyngeal muscles into dilators and constrictors and indicate a pumping function for this area. Although only pharyngeal *dilator* muscles have been observed in *P. ovis*, due to the plane of sectioning, it should not be concluded that *constrictor* muscles are absent in *P. ovis* . In Chapter 3, the possibility that the resilience of the sclerotized cuticle could act

antagonistically to the dilating action of the pharyngeal dilator muscles, was discussed, as described in spider mites by Alberti and Crooker (1985). This is still possible but it is likely that *P. ovis* does possess constrictor muscles to complement the dilators. The circular muscle system of the oesophagus suggests an active food transfer action involving peristalsis. These features of the foregut are commonly seen throughout many of the Acarina (Brody *et al.*, 1972, Coons, 1978; Woodring and Cook, 1962).

The functional aspects of the digestive system of *P. ovis* are not directly comparable with any previously described system. There are some common ultrastructural features between this mite and other mites and arachnids studied by other workers, but there are differences between *P. ovis* and even the apparently closely related house dust mite *D. farinae*. Some of these differences must be due to variability in the distension of the midgut by ingested food and the corresponding effect this has on the character of the epithelial cells. Brody *et al.* (1972) describe certain characteristic areas of the midgut of *D. farinae*, proposing that the free-cells they found originated from an area of "active cells" in an antero-ventral position. From their descriptions, the budded cells closely resemble the free cells found in the midgut of *P. ovis*. They are generally round with numerous microvilli, contain large food vacuoles and smaller vacuoles containing material difficult to identify. Brody *et al.* (1972) also describe an area of the anterior midgut epithelia that appears, from their account, to synthesise a peritrophic membrane (PM). The presence of a PM has been reported and described by Wharton and Brody (1972) in *D. farinae* as well. We also found a membranous envelope around food balls in *P. ovis*. It was first noticed in conventional histological sections and appeared to surround food balls in the midgut ventriculi just ventral to the junction with the colon. In our later TEM study the structure was observed encapsulating processed food material seen passing through the post-colon into the anal atrium [Fig. 29] and surrounding excreted faecal pellets [Fig. 33]. Currently, peritrophic membranes are categorised into two types. Type I PMs are secreted over the entire surface of the midgut from microvilli or in restricted regions of it. Type II PMs tend to

be secreted from a band of cells towards the anterior of the midgut (Wigglesworth, 1972). Some peritrophic membranes do not conform to either of these situations (references in Eisemann and Binnington, 1994). The production site of the material in *P. ovis* is unknown at present, but from the little that is known about the membrane it appears to fall into the category of Type I.

There has been a significant amount of work carried out on some arachnid relatives of *P. ovis*, all demonstrating the dimorphic nature of the epithelia (*Cryptocellus boneti* (Arachnida: Ricinulei)(Ludwig *et al.*, 1994), Camel-spiders (Solifugae)(Ludwig and Alberti, 1992a), *Prokoenenia wheeleri* (Arachnida: Palpigradi)(Ludwig and Alberti, 1992b)). These investigations demonstrated that the midguts of these arachnids are composed of two cell types: *secretory* and *digestive* which are easily identifiable by their characteristic components. The *secretory cells* frequently contain large amounts of RER, cristae type mitochondria and conspicuous, apically located, electron dense secretory vacuoles. These vacuoles probably contain exoenzymes or zymogen which are stored in an inactive stage before being extruded into the gut lumen (Ludwig and Alberti, 1988). The *digestive cells* are larger than the secretory cells, contain more mitochondria than the secretory cells, less RER and contain characteristically, an extensive network of apical tubules.

The digestive process in this system seems to commence soon after food material fills the anterior midgut. The secretory cells extrude their secretory vacuoles into the lumen and extracellular digestion begins. The digestive cells take up the pre-digested food via pinocytosis. The apical network of tubules seems to act as a store of membrane for the pinocytotic vesicles, the small vesicles unify into larger nutritional or food vacuoles where intracellular digestion commences. These events have been described for all the arachnids cited in the previous paragraph.

The events that occur in the stomach ventriculi of *P. ovis* are less clear, the cells we have observed differ, at least in part, to this previously described system. We have

described two main types of cells in the stomach ventriculi of *P. ovis*, one type appears to be very similar to the digestive cells described previously thus, form now on, they will be referred to as *digestive cells*. It seems likely that they perform a similar role as they too possess an apical network of tubules, contain variously sized food vacuoles and there is strong evidence to suggest that they actively absorb fluids through pinocytosis [Figs. 13 and 14]. The digestive cells in *P. ovis* though, eventually leave the epithelia and become free floating in the gut lumen. At this stage they often contain small vesicles filled with an unknown degenerated material, possibly redundant cellular fractions or nitrogenous waste. Free-luminal cells have not been described as a function of the digestive cells in the previously cited work, (Ludwig *et al.*, 1994; Ludwig and Alberti, 1992a; Ludwig and Alberti, 1992b). The free-floating, digestive cells are not unique to *P. ovis* though. Coons (1978), describes similar cells occurring in *M. muscaedomesticae*, Baker and Krantz (1985) in *Rhizoglyphus robini*, Wright and Newell (1964) with *Anystis* sp. and Brody *et al.* (1972) in *D. farinae*. In all these cases the free cells are reported to begin degenerating once they become free-floating in the gut lumen. This also occurs with *P. ovis*. Only a small proportion of the ventriculi epithelial cells appear to have this digestive function in *P. ovis*, the remainder of the cells in this location do not obviously resemble the secretory cells previously described by other workers. They do contain a significant amount of RER suggesting they are possibly synthesising products for secretion, but no distinct evidence of secretory vesicles are seen. The digestive cells, though, often possess what appear to be secretory vesicles [Figs. 13 and 14] perhaps in *P. ovis* the "digestive" cells have a dual function. It has been reported that many arachnid digestive systems are equipped for both intracellular and extracellular digestion (Ludwig *et al.*, 1994; Ludwig and Alberti, 1992a; Ludwig and Alberti, 1992b, Mothes and Seitz, 1981) and that separate cells are responsible for either secreting materials for extracellular digestion while the digestive cells absorb the food material and digestive enzymes to perform intracellular digestion. In *P. ovis* a different situation may exist, the digestive processes could proceed through the following sequence of events: Food material is ingested and distends the stomach ventriculi. The digestive cells begin

absorbing the ingested fluids by pinocytosis and store the material in food vacuoles. The cells then secrete digestive enzymes from secretory vesicles into the food vacuoles and intracellular digestion begins. Processed nutrients may then be transferred from the epithelial cell into the intermediate tissue before the digestive cells migrates into the gut lumen where they degenerate. Alternatively, the digestive cells may migrate into the gut lumen with the semi-processed food material where it is released into the lumen and extracellular digestion would take place before being absorbed by further epithelial cells. Such theories could only be used as a guide to the extensive physiological and molecular investigations required before a clear understanding of the digestive processes in Acari will be available. Clearly *P. ovis* differs in several fundamental ways in its midgut ultrastructure from other Acari.

It has been suggested in at least one mite system that there exists only one general type of cell and that a constant process of cell enlargement, transformation and degeneration takes place (Wright and Newell, 1964). The picture we see of the fine structure of *P. ovis* could suggest this sequence of events. The cells other than the digestive cells we have labelled in *P. ovis* could be latent digestive cells, or as we have suggested, they could have this dual role of digestion and secretion.

Many workers have reported an "intermediate tissue" lying outside the basal lamina of the midgut epithelia containing glycogen stores and large lipid droplets (Coons, 1978; Ludwig and Alberti, 1992a; Reger, 1970). It has been concluded that this tissue has a storage function and suggested that it has a purpose similar to the fat-body of insects (Goyffon and Martoja, 1983). We have found an analogous tissue in *P. ovis* that contains significant reserves of glycogen, lipid droplets and mitochondria. A similar tissue was not reported by Brody *et al.* (1972) for *D. farinae.* All the workers who have reported this tissue, including ourselves, describe "finger-like" protrusions of the intermediate tissue penetrating through the epithelial cell basal lamina into the base of the epithelia where a very close association between the two tissue types results [Figs. 20 and 21]. The purpose of these invaginations is unresolved. Ludwig and Alberti (1992b)

suggest the transfer of ingested nutrients across the junctions is unlikely since they have found the invaginations in the base of secretory cells also. We have not identified any specific secretory cells but have observed the protrusions throughout the midgut basal lamina, especially in the post-colon region which appears to have a significant role in solute re-absorbtion [Figs. 25, 26 and 29]. We have not ruled out the nutrient transfer function of these structures. The similar structures found in the arachnid *Limulus polyphemus* were interpreted as neural tissue by Herman and Preus (1972).

The colon of *P. ovis* appears to be composed of similar cells to those lining the stomach ventriculi, consisting of a small number of cells in various stages of budding, but the majority lacking any distinct function except, obviously, to maintain the integrity of the digestive system. The epithelia of the post-colon of *P. ovis* possesses very characteristic significantly longer microvilli than in the midgut ventriculi and a PM is often seen surrounding food balls/faecal pellets closely associated with the microvilli. The source of the PM is unresolved. A similar description has also been reported for *D. farinae* (Brody *et al.*, 1972) except they suggest the PM originates from the shorter microvilli-lined cells in a more anterior position. The microvilli of much of the epithelial cells appears to have a double plasma membrane (DPM). Most insects guts have a conventional single plasma membrane with an extracellular glycocalyx adjacent to the gut lumen (Smith, 1968) but several studies have found microvilli with what appears to be a double plasma membrane (Baerwald and Delcarpio, 1983). It appears that *P. ovis* is one of the few arthropods possessing a double plasma membrane. The cells of the post-colon in *P. ovis* do not protrude into the gut lumen and seem not to be able to leave the epithelium, a feature reported by Coons (1978) also.

There is evidence to suggest not only an absorptive role for the post-colon (long, extensive microvilli and numerous intermediate tissue invaginations) but also a secretory function, again suggesting the duality of the gut epithelium. We have often observed large vacuoles filling the cytoplasm of squamous cells in this region [Figs. 25 and 26] which could be food vacuoles and apical, densely staining, possibly secretory vesicles

[Fig. 28]. RER and Golgi-bodies are not clearly present though. Brody *et al.* (1972) also suggest a secretory function of this area in the house dust mite. Large numbers of bacteria are frequently observed in this area, often very densely distributed, perhaps due to the concentrating effect of solutes being removed in this region. The bacteria will be investigated and discussed more extensively in the next chapter:

Food material, by the time it has passed through the colon and post-colon, has condensed into faecal pellets, surrounded by a PM and frequently contains a significant number of bacterial cells. The pellets are passed from the post-colon into the cuticle-lined anal atrium. A second, less frequently observed, material is seen being excreted through this region. The material has a crystalline appearance and is likely to be a nitrogenous, guanine waste, possible originating in the epithelial cells depicted in Figure 9. Pore canals can be seen punctuating the cuticle of the anal atrium [Fig. 32] as described by Brody *et al.* (1972) but not to the same degree as they have found. The function of these is unknown; the secretion of a cuticle-surface wax is a possibility, water absorption is perhaps unlikely as the post-colon is likely to perform this role proficiently. The faecal material is then passed along the length of the flexible anal atrium possibly by the contractions of circular muscles or by the pressure exerted by visceral muscles contracting in the colon and post-colon. Further details of the physical processes of excretion have not been investigated here.

We have established TEM techniques which will facilitate further studies into many aspects of the biology of *P. ovis.* Discussed briefly, earlier in this chapter, was the proposition to use techniques, determined in this study, to investigate the antigenicity of *P. ovis* in relation to its pathogenicity, possibly investigating the source of pathology-causing antigens and the immunological role of the bacterial fauna. This work can proceed more easily now we have a better understanding of the structures and cellular components that are likely to be involved. The presence of a PM-like structure surrounding food material in the gut has been revealed. This could also be investigated further as a vaccination target since it may have a vital role to play in digestion.

Much of this discussion has focused on the results of this study and their relevance to the *control* of *P. ovis* rather than their application in *sustaining* them. The long-term goal of this work though, is to produce an *in vitro* culture system to permit more extensive work to be carried out on *P. ovis*, to persue their ultimate control, so in effect the goal is common. Now we have a satisfactory understanding of the structure and ultrastructure of the mite's digestive system and that the mite, apparently, has a liquid diet, the foundations have been laid for further investigation to begin which will ultimately result in the control of this unpleasant disease.

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Chapter 5.

An Investigation into the Bacterial Fauna of the Digestive System of *Psoroptes ovis.*

Chapter 5.

An Investigation into the Bacterial Fauna of the Digestive System of *Psoroptes ovis.*

Summary.

Adult female *Psoroptes ovis* mites were removed from their ovine host, surface sterilised and the gut content liberated onto a variety of culture media to isolate the microorganisms associated with the mite's digestive system. Surface sterilised live mites were allowed to crawl freely on agar plates in order to investigate the viable bacteria excreted in faeces. Bacteria from all isolations appeared to be the same and were characterised as the enterobacter *Serratia marcescens*, known under certain circumstances as a pathogen of domesticated animals and arthropods.

Introduction.

The majority of work on arthropod gut microorganisms has concentrated on the insects, in particular pest insect species, and there is little literature on the microflora associated with the acari or arachnids.

Around 10% of known insect species harbour non-parasitic organisms, either intracellularly, or extracellularly, for example in the digestive system (Douglas, 1989). This is likely to be true for the acari and arachnids also. The bacteria found in the digestive system of insects have several functions. They can often be an important dietary component ingested while the organisms feed. *Damalinia ovis*, the biting louse of sheep, for example, ingests large quantities of bacilli, cocci and diplococci while they feed at the epidermis (Murray and Edwards, 1987). These are likely to be an important dietary constituent of the louse. Also, as termites cannot synthesise the necessary enzymes to digest cellulose, they possess areas of their digestive system modified to maintain a population of cellulose digesting microorganisms; a system crucial to the insect's survival (Bignell *et al.*, 1980). Derylo (1970) has demonstrated that the poultry lice *Eomenacanthus stramineus* and *Menopon gallinae* can harbour and transmit *Pasteurella multocida* causing severe cholera epidemics in fowl. There are many other examples of interactions between parasitic arthropods, their hosts and bacteria (Bignell, 1984).

While investigating the internal morphology of *Psoroptes ovis* using conventional histological methods, it became apparent that the mite's gut contained a significant population of bacteria [Fig. 1] also see Chapter 3. Little information could be gathered from the initial observations at the light microscope level except that from their appearance the majority of the microorganisms were rods. Further investigations of the mite's digestive system by transmission electron microscopy (TEM) (see Chapter 4), confirmed the bacteria to be mainly rods, with possibly a smaller population of cocci present [Fig. 2].

The origin and role of the bacteria was unknown and several possibilities were considered: The microorganisms could be abundant on the surface of their host and be ingested while the mites fed. This is very possible since the mites do not feed intercellularly like mosquitoes, but on the surface of their host (Blake *et al.*, 1978; Kirkwood, 1986; Rafferty and Gray, 1986; Sinclair and Kirkwood, 1983; Sinclair, 1988; Mathieson, unpublished), and sheep epidermis has an extensive flora (Burrell and MacDiarmid, 1983; Jansen and Hayes, 1987; Merritt and Watts, 1978; Murray and Edwards, 1987). Another possibility is that the microorganisms are symbionts playing a vital role in mite nutrition. Indeed it can be seen that the bacteria remain viable in the gut for some time as pairs of rods are often seen joined at their poles indicating that they had been dividing at the moment that they were fixed. It is not known though for how long the bacteria remain viable in the gut.

If the microorganisms are important for the mite's nutrition, then it is crucial for the development of an *in vitro* culture system that this area is investigated further. Additionally such studies could reveal a new target for the control of sheep scab using bacteriostats rather than potentially harmful acaricides. A third possibility for the role of the microorganisms is that they could be associated with the clinical development of the disease sheep scab. *P. ovis* mites excrete significant numbers of bacteria with their faecal pellets [Fig. 3] onto the surface of their host which could become established in this location and cause infection. The exact processes involved in the pathology of the disease are poorly understood but mite derived bacteria could play a very significant role.

It can hopefully be seen that it is of crucial importance to discover the role that these microorganisms play in their intra-luminal location in order for us to determine if they are an important nutritional requirement, an essential symbiont, a harmful transmittable pathogen and if they could be targeted in a novel control procedure.

This study was an attempt to culture and classify the types of bacteria present in the gut of *P. ovis*, thus laying the foundations for a more extensive, future study. These

results would enhance our understanding of the digestion of *P. ovis* and pave the way to a more successful *in vitro* culture system and perhaps ultimately to the development of alternative, less operator-harmful control measures against the disease.

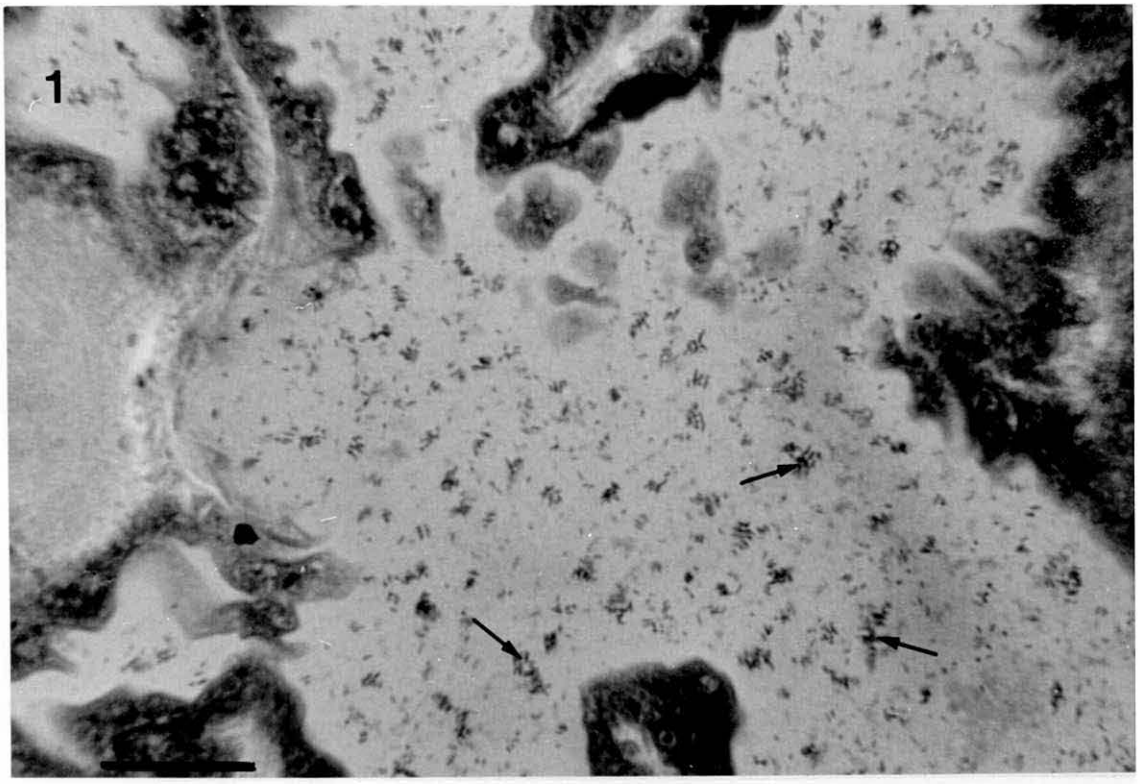
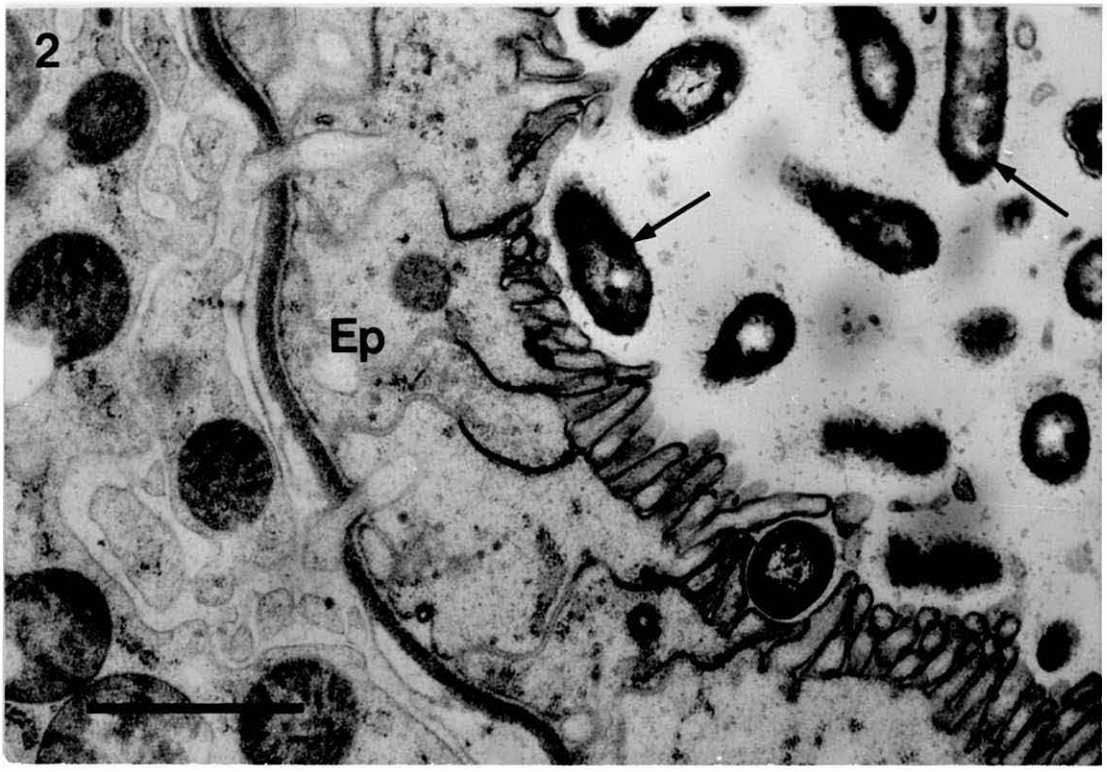


Figure 1. A light microscopy, horizontal-longitudinal section through an anterior region of the midgut ventriculus *Psoroptes ovis.* Numerous groups of bacteria (arrows) can be observed within the gut lumen throughout the section. Scale Bar = 20μm.

Figure 2. This transmission electron micrograph showing a portion of midgut lumen with associated epithelia (Ep) confirms the population of rods (arrows) present in the midgut of *Psoroptes ovis.* Scale Bar = 1.5μm

Figure 3. A transmission electron micrograph of a section through an excreted faecal pellet (FP) demonstrating the intact (and viable) bacteria constituting a significant proportion of its content. The mite's body cuticle (Cu) is visible at the top of the figure. Scale Bar = 3μm.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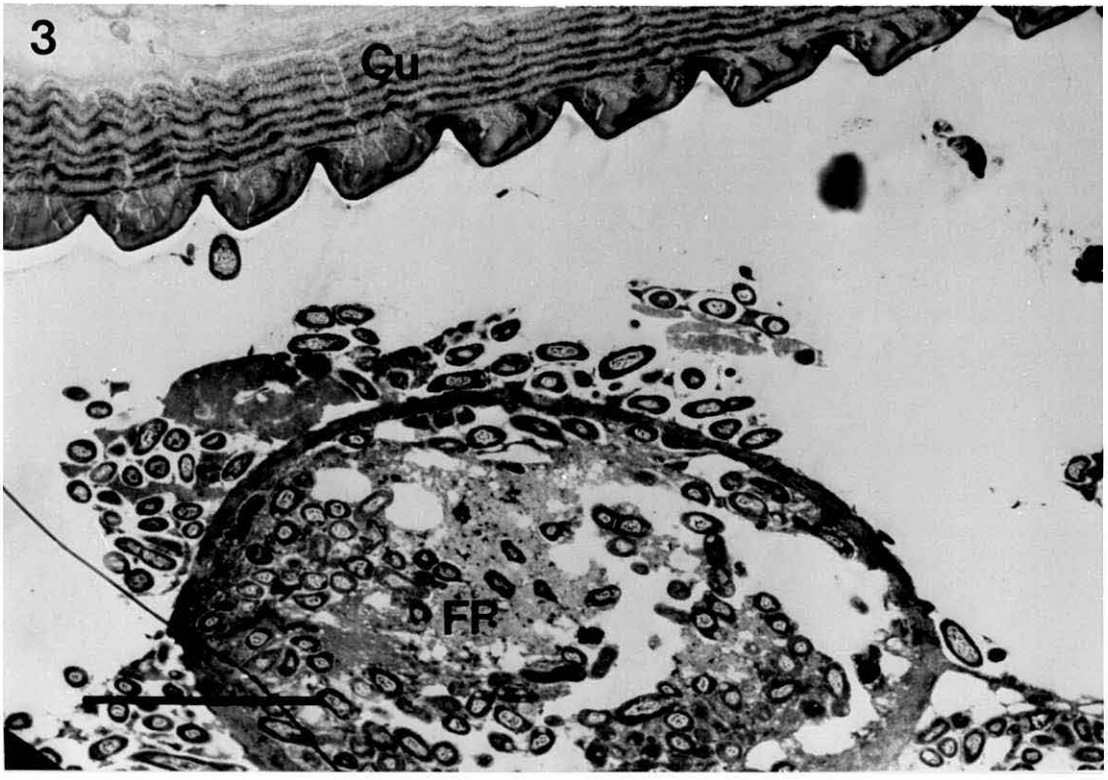
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Materials and Methods.

Surface Sterilisation of the Mite.

The normal habitat of this mite brings it into contact with a variety of microorganisms that become unavoidably attached to the cuticular surfaces. Before isolating the bacteria from the gut, the exterior surfaces of the mite must first be sterilised to establish that the bacteria were isolated from an internal location.

CVL strain mites were removed from their host, as previously described in Chapter 2, and divided into groups of six adult females, placed in individual Eppendorph cages (See Chapter 2. Materials and Methods) and exposed to a range of sterilising agents to determine the most efficient (Table 1.).

Table 1. Procedures for examining the surface sterilisation of *P. ovis.* Mites were immersed in each solution for the time (seconds) indicated.

Procedure No.	100% Acetone	70% Methanol	5% Milton's Fluid
1.	-	-	30
2.	-	30	-
3.	-	30	30
4.	-	-	60
5.	60	-	60
6.	-	60	-
7.	-	60	60
8.	30	60	-
9. (control)	-	-	-

The mites were air-dried in their Eppendorph cages and, with a pair of sterilised watchmaker's forceps, removed individually, dragged carefully for a few seconds over a marked area on a nutrient agar (NA) and 10% sheep's blood in nutrient agar (SBA) plates. The mites were then discarded and the labelled petri-dishes were incubated at 33°C overnight to be examined the following morning for signs of bacterial growth.

Isolation of Bacteria from the Gut of *P. ovis.*

As reported below Sterilisation Procedure 8. was the most convenient and effective. Once the mites had been through the sterilisation procedures they could remain in the 70% methanol for in excess of 60 seconds before the next procedures were carried out. Three different methods were employed to isolate the bacterial fauna from the digestive system of *P. ovis*. These were;

- Whole-mite squashes.
- Live mite-trail cultures.
- Pierced dead-mite cultures.

Whole Mite Squashes.

Following sterilisation procedure 8 each mite was carefully drawn over a marked area on the surface of a blood agar petri-dish. They were then transferred to a sterilised strip of glass coverslip (approximately 10 x 5mm) and macerated quickly with a pair of sterile needles before dropping the coverslip into a few mls of labelled, sterile nutrient broth (NB). This was repeated with six adult female mites. Both NB and associated agar plates were incubated at 33^{o}C overnight. The agar plates were inspected the following day and if there was any sign of microbial growth the broths were discarded as they could not be considered to consist of the internal bacteria alone.

Live Mite Trail Cultures.

Some of the mites remained alive after the sterilisation procedures and so, rather than squashing the mites and liberating the gut content into NB, they were placed on NA or SBA plates with sterile forceps, and allowed to crawl freely around on the plates so any viable bacteria excreted with the faeces could be investigated. These plates were incubated at 33^{o}C overnight. Six adult female mites were used; three for each plate type.

Pierced Dead-Mite Cultures.

Some of the mites killed during sterilisation, were again drawn across a marked area on a SBA plate to test surface sterility before being placed onto a fresh NA or SBA plate and pierced through the midgut with a sterile, electrically sharpened tungsten needle (Brady 1965) to liberate the gut content. These plates were incubated with the others at 33°C overnight. Again six adult female mites were used.

Examining the Bacterial Growth.

After overnight incubation at 33°C the broths and agar plates were examined for microbial growth. Several individual mite-trail colonies and pierced-mite colonies were removed by wire loop from the original plates and inoculated into NB, where they were allowed to grow for 48hrs before being stored at 4°C with the mite squash cultures.

Isolating Bacterial Colonies

All the new NB cultures were diluted in sterile phosphate buffered saline to 10^{-2}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-8} of the original culture. 0.1ml of each culture was smeared with a glass rod onto NA and SBA plates. After 48hr incubation at 33°C, representative colonies were removed from the appropriate dilutions to investigate further. These colonies, numbering 24 in all, were removed from the dilution plates by wire loop and streaked onto new NA and SBA to produce single colonies. After 24hrs, when the colonies had grown sufficiently, small fractions of each colony were removed and examined for motility, Gram staining, morphology, catalase activity and the ability to grow anaerobically. The growth of isolates on McConkey's agar, King Ward and Rayney 'A' (KWA) and KWB plates, as well as SBA and NA were also investigated. Colonies of interest were then plated from the single colony plates onto NA slopes in sterile universal tubes and sent to the National Collections of Industrial and Marine Bacteria (NCIMB) in Aberdeen for further biochemical tests and classification.

Results.

Two of the six squashed-mite cultures showed no sign of bacterial growth. This suggests that the sterilisation procedure was bordering on the severe rather than the benign or that these mite's guts were axenic. All the live mites from the mite trail experiment and the pierced mites produced bacterial colonies as did the whole-mite homogenates.

The results from the tests carried out on all the discrete colonies isolated, suggested that they were all Gram negative, catalase positive, motile, facultative anaerobic rods with similar dimensions of approximately 1.3 x 0.35μm. There were too many isolates to practically and economically send all for further characterisation, but we could distinguish two physically definable biotypes. These two different colony morphologies were repeatedly observed from our single plate isolates. From the first and subsequent subculturing, the colonies had the following appearances: The majority of the colonies growing on the NA, SBA, KWA and KWB after 48hrs were circular-entire, smooth, opaque, cream coloured, low convex colonies around 4mm in diameter. The second biotype were very similar but were slightly more translucent than the other more widespread opaque colonies.

On McConkey's agar the colonies all grew at a similar rate, shape and size and the difference previously described was still discernible. Both biotypes decolourised the red pigment from the agar, taking up the colour themselves in the process and to some extent the more translucent colony appeared more pink than the opaque colony.

On KWA both colonies grew and were still recognisable as they were originally described but the overall growth was not as rapid as with either the NA, SBA or McConkey's agar plates. The KWA plate was rather dehydrated which could explain the observation. The colonies were again discrete on KWB plates but the overall growth rate was reduced again. No fluorescence was observed with either the KWA or KWB.

Overall, the different bacteria grew with the same characteristic morphologies on all the different types of media we used, leading to the initial conclusion that two different bacterial types had been isolated from the digestive tract of *P. ovis*. These were sent to the NCIMB on nutrient agar slopes in universal tubes.

The NCIMB have a two tiered characterisation procedure. The primary report, based on repeats of the tests we carried out and combined with observations on spore formation, growth temperature limit, oxidase test and fermentation in glucose, suggested both the isolated monocultures were from the family Enterobacteriaceae. The secondary report, based on a more extensive biochemical analysis using API test strips (API 20E, Bio-Mérieux, UK, Ltd) and further biochemical tests, the NCIMB concluded they were both strains of *Serratia marcescens*. The slightly different plate appearances we observed were explained by strain differences.

Discussion.

An extensive literature search has revealed that *Serratia marcescens* has drawn a significant amount of interest from an entomological, medical and veterinary perspective, for an in-depth review see Grimont and Grimont, (1978). The genus *Serratia* is composed of several species, some strains being chromogenic, producing a red, blood-like pigment, prodigiosin (Williams and Hearn, 1967). *S. marcescens* was wrongly thought to be saprophytic for many decades, obtaining nutrients from dead and decaying organic materials.

Throughout historic literature there are accounts of bread and especially consecrated wafers (Hosts) bearing the tell-tale "blood stains" of *S. marcescens* (Gaughran, 1969). Religious intolerance and superstitious paranoia have led to much actual bloodshed directly associated with the bacteria (see Gaughran (1969) and Yu (1979) for a historical viewpoint on *S. marcescens).*

In the entomological world, *S. marcescens* and other species of the genus have been isolated from the digestive tract of healthy, diseased and dead insects like crickets (Bucher, 1963) and moths, termites, wasps and bees (Grimont and Grimont, 1978). It seems though, that the bacterium has a largely pathogenic role rather than being a commensal or symbiont (Steinhaus, 1959).

Douglas (1989) suggests that the intracellular microflora of insects can be divided into two groups: *the guest microorganisms* associated with a variety of cell types and *the mycetocyte symbionts* which are restricted to a single specific host cell type- the mycetocyte. The guest microflora are generally prokaryotic and have been isolated from the majority of insect Orders studied. Douglas reports that experiments eliminating guest microorganisms from hosts have shown no observable effects on survival, growth or fecundity of the insects concerned but there does seem to be a relationship between incompatible cross-matings of different insect "strains" and their associated specific

microflora (Yen and Barr, 1973). In contrast, the mycetocyte symbionts that have been studied show clear advantages to the insects that harbour them. Disrupting the mycetocytes from insects causes restricted growth and premature death (Douglas, 1989). Douglas explains that these symbionts are not a general feature of insects but seem to be confined to relatively few families studied; sucking lice, cockroaches, beetles, and aphids for example. Although mycetocytes have been described in gut epithelial cells of some acari (Coons, 1978), there is little information to be found in the literature describing the role the bacteria play in these mites. This present investigation appears to be the first to link *S. marcescens* with the Acari and indeed the Arachnids.

It has been suggested that the insects (and most probably other orders of arthropods) living on a nutritionally restricted diet have adapted to utilise the metabolic activities of symbionts, in particular mycetocytes, to synthesise components their diets lack (Buchner, 1965). Obligate haematophagus insects, e.g. anopluran lice, have mycetocyte symbionts, whereas fleas, who feed on blood in the adult stage also, but who's larvae feed on a more varied diet of detritus, lack them. This theory proposes that earlier life-cycle stages store the necessary factors found in an alternative diet for later in the organism's development, or utilise the activities of mycetocytes (Buchner, 1965). The food materials available to *P. ovis* at the epidermis; lipids, bacteria, serum, lymph and occasionally blood, are likely to be sufficiently nutritionally diverse to satisfy the mite's metabolic requirements, without the need for mycetocyte symbionts. It is possible that the bacteria we isolated from *P. ovis* play a digestive role similar to the function of mycetocytes, although the microorganisms we observed were exclusively extracellular in the gut lumen. Experiments depleting the gut of it's natural fauna using bacteriostats would give us a good indication of the importance of these organisms.

The bacteria we observed in the gut were so numerous that it is feasible that they may contribute significantly to the mite's nutritional intake in a similar fashion to the bacteria ingested by *Damalinia ovis* (Murray and Edwards, 1987). We cannot say for

certain whether this is the case or not but, again, experiments depriving the mites of bacteria in their diet would enlighten us on the subject.

The question of whether the bacteria could play a role in the pathogenicity of the disease is both an interesting and exciting proposition. We have observed that the mite's faecal pellets often contain a significant concentration of intact viable bacteria and so it is possible that any pathogenic agent in the mite's digestive system could reach the outside environment and establish itself in this location. Surface sterilised mites allowed to crawl freely on blood agar plates leave a trail of bacterial colonies that presumably have originated from the gut. The mite's do not seem to possess salivary glands but they may well regurgitate gut material and digestive enzymes through reverse peristalsis, onto the surface of the sheep and this is another way potentially pathogenic microorganisms may reach the skin. Whatever the route, any pathogenic microorganism contained within the digestive system is very likely to be excreted onto the host's epidermis. Once the bacteria become established on the skin it is very possible that they contribute directly to the irritation that accompanies the infestation. Vines (1995) reported that skin bacteria can readily break down the triglycerides, that are present in sebum, into free fatty acids, these can be irritating to the skin by breaking down membranes leading to inflammation. These activities may be advantageous to the mite, in a digestive role while the bacteria and sebum are contained within the mite's digestive system, but if the bacteria establish themselves on the skin surface, severe irritation could result. This is a possible explanation for some of the clinical manifestations of sheep scab.

The nature of the disease sheep scab is such that infested animals often scratch themselves so severely they cause small skin haemorrhages. This would enable the bacteria to gain a suitable entry into the host's blood system which could result in the development of septicaemia. Blood feeding Hemiptera, from the family Reduviidae, transmit South American trypanosomiasis in a similar manner. They deposit the protozoan *Trypanosoma cruzi* with their faeces, around the small puncture wounds they cause while

feeding. The Protozoa are rubbed into the irritating wound where they can then establish themselves. It has already been mentioned that Derylo (1970) demonstrated the role poultry lice play in the transmission of pathogenic bacteria causing cholera in poultry, so it can be seen that this transmission route is very possible.

The clinical pathology of *P. ovis* infestations is little understood, but bacterial involvement, although of unknown origin and genus, is known to cause complications like subcutaneous abscesses in infested sheep (Mathieson, personal observation). *S. marcescens* has been documented to cause disease in vertebrates including septicaemia in various domesticated animals such as foals, pigs and fatally so in goats (Wijewanta and Fernando, 1970). The interaction that occurs between infested sheep and the microflora of *P. ovis* must be investigated further to determine if there is a relationship between the bacteria and the disease.

The clinical development of sheep scab can be, among other factors, dependent on geographical location of the outbreak (P.G. Bates, personal communication). Some outbreaks are characterised by severe, acute pathology while some cause very few clinical signs at all. Also in the *same* geographical location there is a huge variation in the disease incubation period (from 3 weeks to very many months) in similar sheep breeds under the same conditions and the course of infestation can again vary from moderate to intense (Dermot O'Brien, personal communication). The cause of these geographical and localised differences in the pathogenicity of sheep scab infestations is unclear but, there are aspects of the mite's biology that are likely to remain constant wherever the outbreaks occur, which could perhaps be ruled out. For example, it is unlikely for the mite's physical feeding mechanisms alter from place to place or for the antigenic character of the mite's faecal material (which is believed to contribute to the clinical response) to change. Perhaps a more likely explanation is variations in the microflora of mites, of sheep or an interaction between the two. Work carried out at the Central Veterinary Laboratory in 1983 on the bacteriology of *P. ovis* infestations was concerned with the isolation of

particular bacteria that could be associated with sheep scab infestations. They investigated the bacteria found on seven *P. ovis* naive control-sheep and 12 infested sheep. *Serratia marcescens* was isolated from all 12 infested sheep and with nine of the 12 animals, this was the only bacteria isolated. *S. marcescens* was not isolated from any of the control animals (unpublished data). It is very interesting that this bacteria is likely to have originated from the gut of the infesting parasite and deserves immediate further investigation.

This work has established that there is a viable population of *Serratia marcescens* in the digestive system of *P. ovis* which can be transmitted alive in mite faeces. The TEM study shows that bacteria are present throughout the microvilli-lined digestive tract (between the foregut and the hindgut) and have not been observed anywhere else outside the mite's digestive system. A small number of bacteria have been observed in the oesophagus also, whether they are a component of the mite's last meal or have been displaced from the midgut during fixation cannot be determined from these observations. It is possible that other bacterial species are present and that more sophisticated isolation and culturing methods will show this. Further studies are required to obtain a more complete account of the species present and perhaps to establish a more precise description of the location of these bacteria, although the minute size of the mite's digestive system may preclude it's dissection and manipulation.

The fact that at least one bacterial species is capable of at least surviving in the gut of the mite is exciting, and now it remains to discover the role, if any, that these bacteria play in the life of the mite and the aetiology of sheep scab.

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Chapter 6.

An Investigation into the Diet of *Psoroptes ovis* using SDS PAGE and Western Blotting Techniques.

Chapter 6.

An Investigation into the Diet of *Psoroptes ovis* using SDS PAGE and Western Blotting Techniques.

Summary.

"Fed" and "Starved" *Psoroptes ovis* extracts were investigated by SDS-PAGE and western blotting techniques. The extracts were separated by electrophoresis, stained with Coomassie Blue or silver stained and the banding patterns compared with each other and with extracts of material the mites are likely to ingest of ovine origin. Two bands were missing from the starved banding profile that were present in the fed extract. These corresponded to proteins of approximately 27 and 50 kDa which were dominant in profiles of separated whole sheep serum and "scab exudate". The separated extracts were electroblotted onto nitrocellulose paper and probed with antisera to sheep erythrocyte stroma, sheep whole serum and sheep serum albumin. All antisera demonstrated affinity to components in the fed extract and to a lesser extent in the starved extract. It was concluded that *P. ovis* ingest serum components which are likely to be present in the surface exudate associated with clinical sheep scab.

Introduction.

The development of a practical, functional *in vitro* culture system for *Psoroptes ovis* mites would be tremendously advantageous to *Psoroptes* research. Unfortunately, due to the physical requirements necessary to house infested animals in isolation, and the financial demands such an operation would require, only a few exclusive establishments are able to conduct research into *P. ovis*. With the development of an artificial cultivation system many more institutions would be able to investigate the numerous aspects of the mite's biology, ecology and disease aetiology which would dramatically and rapidly increase our knowledge of *P. ovis* and ultimately increase the likelihood of bringing sheep-scab under control.

As discussed in Chapter 2, if we are to have any success in producing an artificial cultivation system, it is imperative that we understand at least the key components of the mite's natural environment. Chapter 2 presented an investigation of the environmental conditions (temperature and relative humidity) conducive to optimal mite survival off-host. This and the following chapter investigate the dietary composition of *P. ovis* using biochemical and immunological techniques. This information is imperative to the successful production of an *in vitro* culture system.

It has been attempted, with limited success, to maintain colonies of *P. ovis* mites on several different liquid diets that they are likely to encounter in their ectoparasitic environment (see Chapter 8). Although the mites *feed* on the experimental diets, the colony fails to maintain itself suggesting the diet may be sub-optimal. So a more direct approach to determine the dietary requirements was embarked upon.

Presented here is an investigation attempting to identify certain components of the mite's ingested meal by using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), modifying the methods of Laemmli (1970) and western blotting techniques, based on the methods of Towbin *et al.* (1979). The principles of the work was to separate the components of whole "fed" and "starved" mite homogenates by

molecular size using SDS-PAGE in order to identify the components ingested by the mites. The banding patterns of the fed and starved mite extracts can then be compared to patterns produced when known materials are electrophoretically separated, e.g. serum, serum albumin and so on. A similar experimental rationale has been successfully used as the basis of an investigation into the prey preferance of predatory mite *Typhlodromus pyri* (Murray and Solomon, 1978; Dicke and de Jong, 1988).

SDS-PAGE bands of interest can then be transferrered electrically onto nitrocellulose paper (NCP) (western blotting) and immunoblotted with antisera specific to known materials that are suspected to have been ingested from the epidermis (e.g. sheep serum). By comparing fed mite homogenate with a starved mite homogenate and the reactions with the known antisera, it may be possible to determine the components in the fed mite's gut and those missing from the starved mite's gut. The information gained on the materials present in the extracts is limited only by the number and specificity of test antisera available.

Before the western blotting was attempted, a pilot Dot Immunobinding or "Dot-Blot" assay (Hawkes, 1986) was conducted to determine, using simpler and less laborious procedures, if the more informative western blotting procedures would be likely to be successful. This simpler technique involves adsorbing the mite extracts directly onto the nitrocellulose paper where they are probed by the known antisera. The extracts are not separated by SDS-PAGE and so the information gained is less specific.

This investigation was undertaken to try to determine specifically, some of the components of the diet of *P. ovis*, information which is crucial for the development of a functional *in vitro* culture system.

Materials and Methods.

Mite Collection and Preparation.

A number of mites, from various stock colonies of different strains, were removed from their ovine host by the methods described in Chapter 2. Within minutes of being removed the mites were fixed in liquid nitrogen (LN_2) and stored at -20°C until needed. Mites were stockpiled until several hundred mites were collected, stored in separate Durham tubes with approximately 50-100 mites per tube (=Fed group). A second sample of mites were collected, separated into groups of adult females and washed by the methods described in Chapter 2. They were then incubated at 82% relative humidity and 33°C on filter paper moistened with phosphate buffered saline (PBS) to stimulate "feeding" and clearing of the mite's gut for approximately 96 hours. This allowed any food material to pass from the gut and leave the mites in a "starved" condition. After the 96 hour starvation period, dead mites were removed and the remainder were fixed in LN_2 and stored in Eppendorph tubes at -20°C. This was also continued until sufficient numbers of mites were collected (=Starved). These procedures produced a Fed group and a Starved group of mites with around 600-700 mites in each group.

When sufficient numbers of mites had been collected they were, in turn, removed from storage, thawed and sheep derived surface contaminants were thoroughly washed off. This was especially necessary for the fed mites. The largest pieces of fleece, exudate and larval stages could be removed by forceps and mounted needles but the remaining debris was removed by washing the mites in Eppendorph cages (see Chapter 2 for design) using the following procedures:

Approximately 50 mites were placed in an Eppendorph cage. The cage was inverted (gauze uppermost) and placed in the base of a tube, slightly wider than the Eppendorph. The tube was filled with chilled (approx. 0°C) 2% Tween 80 in (PBS). Air bubbles, that attached to the mites and collect against the gauze, were removed with

a capillary pipette. The mites were vigorously agitated by pumping the detergent through the gauze, around the cage with the pipette (20 times). The tube was sealed and placed inside a slightly larger tube containing ice. The whole assembly was then agitated on a rotating mixer for 20 minutes. The contaminated detergent was removed and replaced with fresh washing solution and the whole procedure was repeated (with particular attention to the pipette agitation) a further three times. After the last wash the detergent was rinsed away with chilled distilled water (dH_2O) and the mites were finally, briefly rinsed with 100% acetone. The mites were then removed from the Eppendorph cage onto clean filter paper, previously dipped in liquid paraffin wax to prevent paper fibres contaminating the mites. This was placed in a petri-dish placed inside a larger container of dry ice. Mites were counted and any remaining larval stages removed. The washed mites were counted into groups of 100 adult females, transferred into Eppendorph tubes and then re-frozen in LN_2 and stored at -20°C.

These procedures were repeated with both the fed and starved mites. Fed mites generally needed a more extensive washing procedure as they had not been previously washed as the starved mites had. By this stage approximately 700 mites from each group were stored at -20°C.

Homogenate Preparation.

The purpose of this exercise was to rupture the mite's cuticle and gut to release the materials the mites had ingested. Consequently it was not necessary to thoroughly homogenise the samples and these procedures were employed: One group of 100 washed mites were removed from -20°C storage at a time. The mites were carefully removed from the Eppendorph tube with a clean mounted needle and transferred to the bottom of an ice-cold 0.1ml Griffiths tube and 750µl of chilled dH_2O added. The plunger was inserted and received 10 firm, half-twists followed by 10 half-plunges (from the surface of the homogenate to the bottom of the tube). The plunger was carefully removed to the neck of the tube and a further 250µl of dH_2O was used to rinse the

remaining homogenate from the plunger. During these procedures, the tube was intermittently placed on ice to keep warming and protein degradation to a minimum.

The Griffith tube and homogenate were then floated, using a polystyrene support, in 200mls of 2% concentrated household detergent (Fairy Liquid) in water, chilled to approximately 2°C and sonicated for 10 minutes. The homogenate was transferred to a 0.5ml Eppendorph tube and centrifuged at approximately 1000g for 5 minutes (4000 rpm using an microcentrifuge). The supernatant was carefully removed and transferred to a 1000µl Eppendorph tube and kept on ice while the remainder of the mites were processed. The remaining mites from the group were processed, as above and added to the previously prepared homogenate. A Sigma (modified Lowry (1951)) protein assay was performed on the two preparations and the pooled homogenate was stored at -20°C in 30µl aliquots. The fed and starved mites were processed on different days to reduce the chance of contamination between the groups.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Throughout this investigation 12% resolving and 4% stacking gels were most commonly used. Occasionally a 15% resolving gel was used to limit the migration of the smaller protein molecules. The gel reagents and proportions required to produce these acrylamide percentages are presented in Table 1. 10ml of each gel is sufficient to cast two mini gels 1mm thick.

Table 1. Gel reagents and quantities used to produce 12% and 15% resolving and 4% stacking gels.

	12%	15%	4%
Distilled water	3.5ml	2.35ml	6.1ml
1.5M Tris-HCL pH 8.8	2.5ml	2.5ml	-
0.5M Tris-HCL pH 6.8	-	-	2.5ml
10% (w/v) SDS stock	100µl	100µl	100µl
Acrylamide/Bis (30% stock) (degas for ≥15mins)	4.0ml	5ml	1.3ml
10% ammonium persulphate (APS) (fresh daily)	50µl	50µl	50µl
TEMED	5µl	5µl	10µl
Total Monomer (approx)	10ml	10ml	10ml

Electrophoretic profiles were generated for the two mite homogenates by SDS-PAGE using Bio-Rad's Mini-PROTEAN II equipment and generally following the manufacturers instructions. Briefly, paired glass plates (8.2 x 10.2 x 0.1cm and 7.2 x 10.2 x 0.1cm) separated by 1.0mm teflon spacers were clamped in a cassette and mounted on a gel casting stand. This provides a receptacle to contain the resolving gel, in this case 12% acrylamide, made by combining the reagents, excluding the APS and TEMED, described in Table 1. To initiate polymerisation the mixture was warmed under tap water at approximately 40 - 50°C, the APS and TEMED added and swirled gently to mix. This mixture was pipetted carefully between the glass plates to the desired level and overlaid with 1ml of butanol to prevent dehydration of the setting gel. The gel was left to polymerise either at room temperature or at 37°C to increase the rate of polymerisation. Once set (after approximately 30mins) the butanol was removed from the top of the gel and distilled water used to thoroughly rinse its surface. The stacking gel mixture, consisting of the components described in Table 1, (again omitting the APS and TEMED) was warmed, polymerising agents added and pipetted carefully into the space above the resolving gel. This space was also partially occupied by a teflon comb

cut and inserted to give the required well pattern. The comb remained inserted into the polymerising stacking gel until polymerisation, carried out as before, was complete (approx. 30 mins), at which point it was carefully removed and the wells rinsed 5 times with dH_2O. Two gels were usually cast together at one time. Cassette pairs were attached together with the electrode core and placed inside the electrophoretic tank. The base of the tank was filled with the running (electrode) buffer (see Appendix B) making contact with the anode and the upper reservoir of the electrode core filled with buffer making contact with the cathode.

The amount of mite extract loaded per gel well was dependant on the procedure to be followed, i.e. whether the gels were to be Coomassie Blue or silver stained, or western blotted. The proceeding methods describe the procedures that followed and the quantities of mite extracts required, Table 2 summarises the quantities of mite extracts used and the corresponding protein concentrations loaded for the various post-electrophoresing procedures carried out.

Mite Extract Concentrations for Coomassie Blue Staining Gels and Western Blotting.

If the gels were to be Coomassie Blue stained or western blotted, 10µl aliquots of both mite extracts were diluted with reducing sample buffer (see Appendix B), at a ratio of 1:2, heated for 5 minutes at 95°C and 20µl loaded per 5mm well. A 10mm well was used for western blotting in which case 30µl of sample/buffer were loaded at the same dilution as before. Fed and starved mite homogenate protein concentrations were different, as expected (fed: 2.25 µg/µl and starved: 1.5 µg/µl) but both extracts were diluted identically to directly compare banding intensities for both conventionally and immunologically stained proteins. This gave approximately 15µg of fed mite extract and a corresponding 10µg of starved extract loaded per 5mm well or 22.5µg and 15µg of extracts for a 10mm well. They were separated under denaturing conditions on 4% stacking and 12% resolving gels, each gel was run until the bromophenol blue in the sample buffer had reached the bottom of the gels (approx. 1 hour at 200 volts). The

apparent molecular weights of the separated proteins were determined by comparing them with molecular weight markers diluted to be Coomassie Blue stained and run on the same gel. After electrophoresis the unit was dismantled and the glass-plate/gel assemblies were removed from the cassettes.

Proteins separated by SDS-PAGE can be visualised by staining in Coomassie Blue stain. For this procedure the gels were carefully removed from the glass plates and immersed in 0.1% Coomassie Blue R-250 in dH_2O:ethanol:glacial acetic acid (50:40:10 by volume) filtered to remove unwanted particles. They were then incubated overnight at room temperature and de-stained in gel fixative solution; dH_2O:methanol:glacial acetic acid (50:40:10). The fixative was removed intermittently as it removed stain from the gels and fresh replaced until the desired band intensity was achieved. The gels were then dried onto blotting paper on a gel slab-drier for 2 hours, dried, photographed and stored as above.

Mite Extract Concentrations for Silver Staining gels.

Silver staining is far more sensitive in detecting proteins (10 to 50 times) and so a smaller concentration of mite extracts were required. The above SDS-PAGE procedures were followed but both mite extracts were diluted 1:5 with reducing buffer, giving approximately 7µg of fed and 5µg of starved mite extract loaded per 5mm well. The gels were run for approximately 1 hour at 200 volts again. The apparent molecular weights of the separated proteins were determined by comparing them with molecular weight markers diluted to be silver stained and run on the same gel. The gels were silver stained according to the Bio-Rad Silver Stain Kit instructions, dried, photographed and stored as above.

Table 2. Dilutions, total protein content (brackets) and volume of fed and starved mite extracts loaded for the various post-electrophoresing procedures carried out. All dilutions are with reducing sample buffer.

SAMPLE	Coomassie Blue Staining 20µl loaded/5mm well	Western Blotting 30µl loaded/10mm well	Silver Staining 15µl loaded/5mm well
Fed Extract	1:2 (15µg)	1:2 (22.5µg)	1:5 (7µg)
Starved Extract	1:2 (10µg)	1:2 (15µg)	1:5 (5µg)

The Preliminary Dot Immunobinding Assay.

For this assay supported NCP is washed in dH_2O for a few minutes and air dried. Using a soft pencil, rectangular strips (approximately 5 x 30mm) were ruled onto the NCP and labelled. 0.5µl of fed mite extract (giving a protein concentration of approximately 1.0µg) was pipetted onto the centre of each labelled, marked area on the NCP and allowed to air dry at room temperature for 20 minutes. The NCP was then washed in TTBS (0.05% Tween 20 in transblotting solution, see Appendix B) for 10 minutes and cut into strips (5 x 30mm) containing the adsorbed fed mite extract. Non-specific binding of the antiserum was blocked by incubating the strips in 2% milk powder in TTBS for 15 minutes. This procedure allows an irrelevant and undetectable protein to bind to sites on the NCP which could otherwise bind free proteins (antibodies in this case) non-specifically leading to high background staining. The blocking solution was removed and the strips incubated in three dilutions of the three commercial acquired antisera and Normal rabbit serum (i.e. 12 strips were required) for 4 hours. The three dilutions used were: 1:100, 1:200 and 1:400 of the antisera in 2% milk powder in TTBS. The primary antisera used in the investigation were:

- Rabbit anti-sheep Whole Serum
- Rabbit anti-sheep Serum Albumin and
- Rabbit anti-sheep Erythrocyte Stroma
- Normal rabbit serum

All primary antisera were purchased from Nordic Immunochemicals.

After the primary incubation the strips were washed in TTBS for 3 x 10 minutes to remove the primary antiserum and then reblocked in the usual blocking solution for 15 minutes. The strips were then incubated for 2 hours 30 minutes in the secondary antisera. Throughout this study goat anti-rabbit IgG conjugated with horseradish peroxidase (IgGAM/PO)(Sigma Immunochemicals) was used, diluted to 1:750 in 2% milk powder in TTBS. The secondary antiserum was then washed off in TTBS for 3 x 10 minutes and the binding revealed in 4-chloro-1-naphthol. (see Appendix B). Table 3

summarises the procedures used in the dot blot assay and Table 4 shows the primary and secondary antisera and dilutions used in this assay.

Table 3. General procedures for the Dot Immunobinding assay used to screen mite extracts for antisera specificity.

1. Wash in dH_2O (then air dry)	5 mins
2. Dot between 0.1 and 2µl of extract onto NCP and air dry (protein concentration 0.1-1.0 µg)	20 mins
3. Cut NCP into strips and mark area for dotting with pencil if required	
4. Wash in TTBS	10 mins
5. Block non-specific binding with 2% milk powder in TTBS	15 mins
6. Wash in TTBS	10 mins
7. Incubate in Primary antiserum	2 hours-overnight
8. Wash in TTBS	3 x10 mins
9. Repeat 5	
10. Incubate in Secondary antiserum	2 hours -overnight
11. Wash in TTBS	3 x10 mins
12. Reveal with 4-chloro-1-naphthol	few minutes

Table 4. Primary and secondary antisera and dilutions used in the Dot-Immunobinding assay. Both primary and secondary antisera were diluted in 2% milk powder in TTBS.

Primary Antisera	Primary Antisera Dilutions	Secondary Antisera	Secondary Antisera Dilutions
Rabbit anti-sheep whole serum	1:100, 1:200, 1:400	Goat anti-rabbit IgGAM/PO	1:750
Rabbit anti-sheep serum albumin	1:100, 1:200, 1:400	Goat anti-rabbit IgGAM/PO	1:750
Rabbit anti-sheep erythrocyte stroma	1:100, 1:200, 1:400	Goat anti-rabbit IgGAM/PO	1:750
Normal rabbit serum	1:100, 1:200, 1:400	Goat anti-rabbit IgGAM/PO	1:750

Lane Visualisation with Pyronine-Y Staining and Electroblotting.

Before proteins are electroblotted onto NCP a dye (Pyronine-Y) was introduced into the gel which was electrophoresed behind the separating proteins and transferred to the NCP during electroblotting. The dye indicates the positions of the lanes in which the proteins will be found making identifying the relevant lanes easier. The stain appears as a small coloured band on the NCP facilitating the localisation of the relevant lanes. The procedures are as follows:

Stain composition

3mg Pyronine-Y
3ml dH_2O
1ml Glycerol

Load ≤8µl of the stain per 5mm well approximately 5 minutes before the bromophenol blue front reaches the end of the gel (the exact time depends on the voltage used and the acrylamide concentration of the gels. A degree of trial and error is required). The stain will be electrophoretically transfered into the stacking gel. If the gel is run for too long after the stain is loaded, it may be transferred into the resolving gel which may be undesirable when it comes to immunoblotting the NCP. The pink stain from the Pyronine-Y does fade with time though.

Western Blotting: Electro-transfer of Proteins onto Nitrocellulose Paper.

The previous methods of Boyce and Brown (1991) and Boyce *et al.* (1991) were followed but modified for this study in the following ways: For western blotting mite extract samples were prepared as for Coomassie Blue staining, i.e. 10µl of each sample was diluted with 20µl of sample buffer and heated at 95°C for 5 minutes. 30µl of each mite extract/buffer preparation was loaded into a 10mm well. This corresponded to 15µg of fed extract and 10µg of starved extract loaded. Each extract was run on a separate gel and molecular weight standards were also run on each gel. Proteins were separated for approximately 1 hour at 200V, until the bromophenol blue front had reached the bottom edge of the gel, the Pyronine-Y was loaded approximately 5 minutes before the run was complete. Electroblotting was carried out using a Bio-Rad

Transblotting Kit in accordance with the manufacturers instructions. Briefly, the glass plates containing the gels were unclamped from the cassettes and one of the plates carefully removed from the gel surface. The remaining plate and gel were immersed in electroblotting buffer (see Appendix B), the gel carefully separated from the glass and left to equilibrate for 30 minutes in the buffer. If two gels were being processed they were kept in separate labelled containers. Supported nitrocellulose paper was cut to rectangles 1cm larger than the gels, rinsed in dH_2O and then immersed in electroblotting buffer. Two sheets of Whatman filter paper, grade 1, were cut slightly larger than the NCP and soaked in buffer for 30 minutes also. Soaking the materials in this manner allowed them to expand, absorbing the buffer and equilibrating. Two pieces of absorbant pad (supplied with the kit) were also immersed with the other components. The materials were assembled together after soaking, held between the two hinged plates of the perforated perspex "blotting sandwich", supplied with the kit. The order of the components were as follows: Perspex support, adsorbant pad, filter paper, SDS-PAGE gel, NCP, filter paper, adsorbant pad and finally the other perforated perspex plate. Care has to be taken to ensure the components are assembled in the correct order in relation to the orientation of the perspex plates. One side represents the cathode the other, the anode. It is imperative that the NCP is on the anode side of the gel and the gel is closest to the cathode. Two gels can be electroblotted in each transfer unit.

The transfer tank is filled with chilled electroblotting buffer (approx. 4°C) and the above described assemblies inserted. A cooling device, in this case an ice filled insert bath, is inserted also. This acts as a heat sink, absorbing the heat energy produced during the transfer process. The unit was connected to the constant voltage power supply unit (Bio-Rad) and electroblotted for 1 hour at 100 volts (≈200-300mA). After this time the power was disconnected, the assembly removed from the transferring tank and the gels and NCP carefully separated. Gloves were worn on all occasions the gels or NCP were being handled. The NCP was marked with a soft pencil to label and indicate the lane orientation. The gels were silver stained to indicate how much of the separated proteins

had been transferred. The NCP was rinsed briefly in transblotting solution (TBS) (see Appendix B) to remove any acrylamide contaminating the NCP surface, air-dried, labelled and stored at 4°C between two pieces of filter paper, usually for no longer than 24 hours.

Positive and Negative Controls for Immunoblotting.

To positively control the test antisera and to determine rough dilutions of test antisera to immunoblot the mite extracts with, diluted whole sheep blood (1:200 in PBS then 1:3 in sample buffer, 20µl loaded/5mm well) was electrophoretically separated under reducing conditions and electro-transferred onto NCP using the methods described in the previous section. The NCP was cut into strips containing the molecular weight standards and the diluted sample, using the Pyronine-Y bands as guides. The protein standards were stained in Coomassie Blue overnight (as described for gel staining) to reveal their positions. A faster method of staining was occasionally used. This involved staining the protein standard strip in a 0.1% solution of Amido Black in dH_2O for 3 hours. It is then destained in 20% methanol, 7% acetic acid until the desired band intensity is achieved (few seconds). The strips containing the sample proteins were incubated in 2% milk powder in TTBS for 1 hour at room temperature while being agitated to block non-specific binding. After blocking the strips were washed 3 times for 5 minutes in TTBS and then incubated in an appropriate dilution of primary antiserum:

- Rabbit anti-sheep Whole Serum
- Rabbit anti-sheep Serum Albumin and
- Rabbit anti-sheep Erythrocyte Stroma.

Primary antisera dilutions used were 1:100, 1:200, 1:400 and 1:800 in 2% milk powder in TTBS. The NCP strips were washed 3 times for 5 minutes in TTBS and incubated in the secondary antisera, Goat anti-Rabbit IgG/PO diluted to 1:200, 1:400 and 1:800 with 2% milk powder in TTBS. 12 x 3mm strips were needed in all.

The non-specific binding of the secondary antiserum to the separated proteins was also investigated. This was carried out by blocking NCP strips of separated and electro-blotted fed mite extract, and immunoblotting them with various dilutions of secondary antiserum. Dilutions used were 1:50, 1:100, 1:200 and 1:400 in the usual dilutant. The procedures were performed as before, excluding the primary antisera incubations.

Normal rabbit serum was immunoblotted against electro-blotted fed mite extract as a negative control. Primary and secondary antibody dilutions, determined previously from this section (Primary: 1:200, Secondary: 1:500) were immunoblotted following the procedures described above. Again goat anti-rabbit IgG/PO was the secondary antiserum which was revealed by incubating the NCP strips in enzyme substrate solution (4-chloro-1-naphthol in methanol/H_2O_2). Specific binding was revealed by the development of deep purple bands.

Immunoblotting Mite Extracts.

The electro-blotted NCP was removed from storage and cut into strips containing the molecular weight standards and the mite extracts, using the Pyronine-Y bands as a guide. The protein standards were either stained in Coomassie Blue or Amido Black to reveal their positions. The 10mm sample lanes were cut into 3 equal strips each containing approximately 5μg and 3μg of protein for the fed and starved mite extracts respectively. The strips were incubated in 2% milk powder in TTBS for 1 hour at room temperature while being agitated. After blocking the strips are washed 3 times for 5 minutes in TTBS and then incubated in an appropriate dilution and volume of primary antiserum (suggested by the results from the previous section) in 2% milk powder in TTBS for 3 hours at room temperature, with agitation. Normal rabbit serum was blotted at the dilutions used for the test antisera as a negative control (see Table 5 for antisera dilutions used in this investigation). Strips were then washed in TTBS, 3 times for 5 minutes and incubated in goat anti-rabbit IgG/PO for 2 hours at room temperature, with agitation. Finally the NCP was washed as before and incubated in 4-chloro-1-naphthol in

methanol/H_2O_2 as before to reveal the specific binding. In all the above described procedures (blocking, incubation, washing and so on) the NCP strips were contained within Universal tubes. They provided a useful receptacle which allowed a convenient volume of solutions and antisera to be used. The processed NCP strips were photographed to make a permanent record as the colour fades with time.

Comparison of Sheep Derived Materials with Mite Extracts using SDS-PAGE.

The electrophoretic profile of whole sheep serum and "scab" exudate were compared with the profiles of the mite extracts. The two samples and mite extracts were run as before under reducing conditions on a 4% stacking and 12% resolving gel. Several dilutions of the samples were used in reducing sample buffer. The "scab" exudate, being a viscous, semi-liquid, was prepared for electrophoresis in the following way: An equal volume of scab exudate and PBS were vigorously agitated in a 1.5ml Eppendorph tube for 3 minutes by hand. The preparation was centrifuged at 1000g for 5 minutes (4000 rpm using an microcentrifuge) The soluble fraction was pipetted off and dituted 1:3 with reducing sample buffer for silver staining or 1:2 for Coomassie Blue staining to give final dilutions of 1:6 and 1:4 for silver and Coomassie staining respectively. The sheep serum was prepared by diluting the undiluted serum sample to 1:250 in PBS and then 1:2, 1:4 or 1:8 with sample buffer for silver staining, giving final ditutions of 1:500, 1:1000 and 1:2000. For Coomassie Blue staining the undiluted sheep serum was diluted in PBS to 1:50 and then with sample buffer 1:4 and 1:6 to give the final dilutions of 1:200 and 1:300. All samples, and molecular weight markers, were heated at 95^{o}C for five minutes and 15µl of each sample loaded per 5mm well. Table 6 summarises the sample dilutions of scab exudate and serum used for either Coomassie Blue or silver staining that were investigated.

Table 5. Primary and secondary antisera dilutions and compositions used in the Immunoblotting investigation. All antisera dilutions are in 2% milk powder in TTBS.

Primary Antisera	Primary Antisera Dilutions	Secondary Antisera	Secondary Antisera Dilutions
Rabbit anti-sheep whole serum	1:200 (50µl of antiserum + 10ml of 2% milk powder in TTBS)	Goat anti-rabbit IgG/PO	1:500 (20µl of antisera + 10ml of 2% milk powder in TTBS)
Rabbit anti-sheep serum albumin.	1:200 (diluted as above)	Goat anti-rabbit IgG/PO	1:500 (diluted as above)
Rabbit anti-sheep erythrocyte stroma	1:200 (diluted as above)	Goat anti-rabbit IgG/PO	1:500 (diluted as above)
Normal rabbit serum	1:200 (diluted as above)	Goat anti-rabbit IgG/PO	1:500 (diluted as above)

Table 6. Final dilutions of "scab" exudate and whole serum samples for SDS-PAGE and Coomassie Blue or silver staining.

	Final dilutions of samples loaded for either silver or Coomassie staining.	
SAMPLE	Silver Staining	Coomassie Blue Staining
"Scab" Exudate Preparation	1:6	1:4
Whole Sheep Serum	1:500, 1:1000, 1:2000	1:200, 1:300

Results.

The Preliminary Dot Blot Immunoassay.

All the test antisera (anti-sheep whole serum, anti-serum albumin and anti-erythrocyte stroma) demonstrated a positive affinity to the fed mite extract at dilutions from 1:100, 1:200 and 1:400. The normal rabbit serum showed no affinity to the mite extract at any of the dilutions investigated.

Comparison of Various Extracts Separated by SDS-PAGE.

Figure 1 compares the banding pattern of fed and starved mite extracts (Coomassie Blue stained) separated under reducing conditions with a 15% resolving gel. Limited information on the differences between the two extracts can be gleaned from this due to the limited resolution of the gel. There are though, possibly two stained areas discernible from the fed extract that are fainter or absent in the starved extract. These correspond to molecules of approximately 50 and 27 kilodaltons (kDa) respectively. Figure 2 shows the comparable banding patterns between a fed mite extract and both a "scab" sample and a sheep serum sample separated on a 12% resolving gel. Three distinct bands can be seen in all three samples corresponding to molecular weights of approximately 66, 50 and 27 kDa. The bands representing the smaller two proteins correspond to the two bands demonstrated in Figure 1. Figure 3 shows a gel run to separate a fed and starved extract, similar to Figure 1 but prepared for silver staining. This gel also demonstrates the banding profiles of "scab" exudate and sheep whole serum. The fed extract bands generally all stain more intensively than the starved bands, but the band resolution is not clear enough to destinguish any particular bands apparent in the fed extract but absent in the starved. There are definite bands at approximately 50 kDa in both the fed and starved extracts (again visibly fainter in the starved case) and a corresponding band with both the "scab" and serum samples.

Figure 1. Fed and Starved mite extract separated under reducing conditions. (15% resolving gel Coomassie Blue stained)

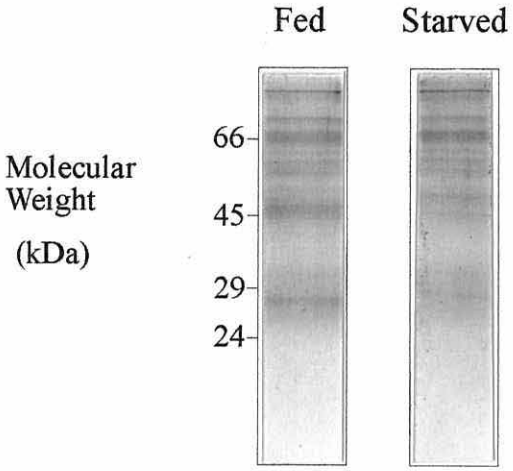


Figure 2. Fed mite extract, scab extract and sheep serum separated under reducing conditions. (12% resolving gel, Coomassie Blue stained)

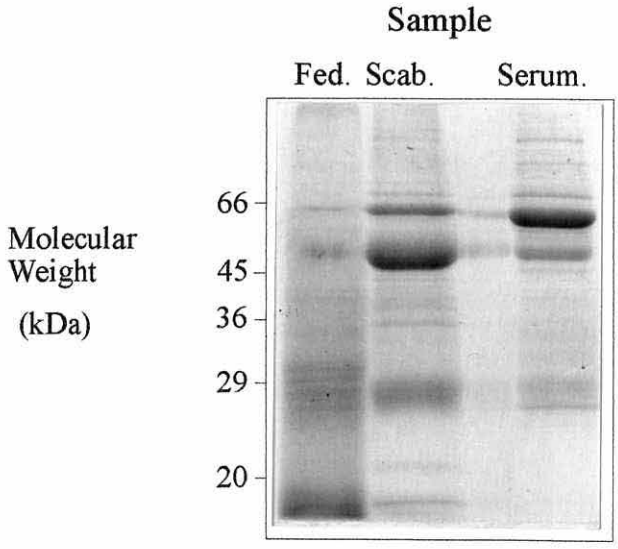


Figure 3. Fed and Starved mite extracts separated under reducing conditions with scab extract and three dilutions of sheep serum. (12% resolving gel, silver stained)

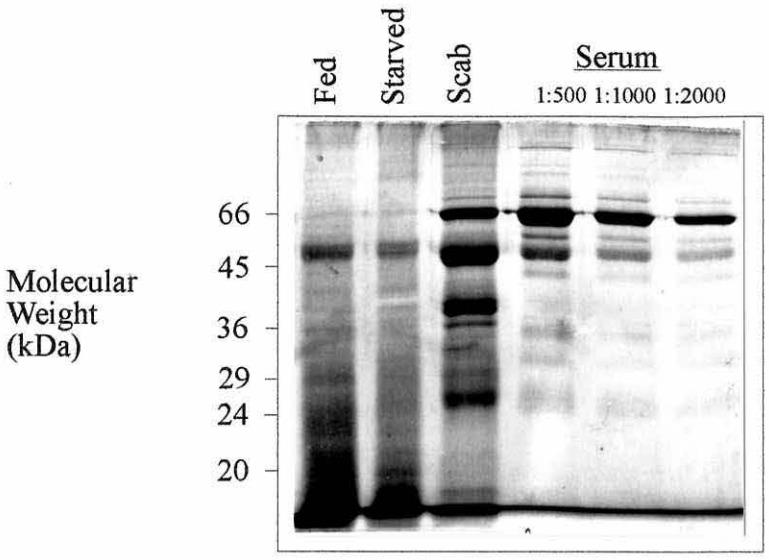
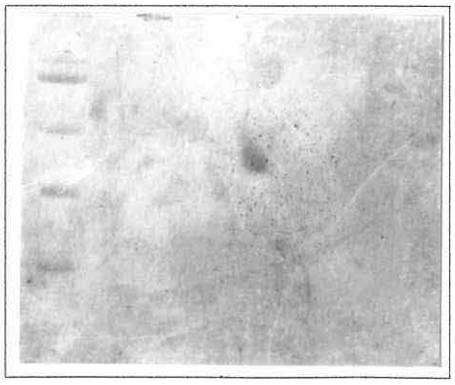


Figure 4. Silver stained gel of various samples after 1 hour electroblotting at 100 volts demonstrating complete transferral of samples.



Positive and Negative Controls for Western Blotting.

In all of the positive control procedures (immunoblotting whole sheep blood with the three anti-sheep test antisera) there were positive reactions of varying degrees, depending on the dilutions of the primary and secondary antisera. A secondary purpose of this positive control investigation was to determine at what dilutions to use the antisera to probe the mite extracts. From these results it was decided to use 1:200 and 1:500 dilutions of primary and secondary antisera respectively.

The non-specific binding of the secondary antiserum (Goat anti-Rabbit IgG/PO) to the mite extracts was investigated but no binding was detected at any of the dilutions used. The normal rabbit serum negative control investigation also failed to produce any detectable binding at the dilutions used for the mite extract investigation.

Comparison of Fed and Starved Immunoblot Banding Profiles.

Silver staining the gels after electroblotting for 1 hour at 100 volts suggested that an insignificant amount of the protein samples, or standards, remained in the gel, at the detection resolution of the silver stain [Fig. 4]. This electroblotting procedure was repeated throughout the investigations.

Figure 5 shows the results from immunoblotting separated fed and starved mite extracts with rabbit anti-sheep whole serum. One major and approximately 10 minor bands are present in the fed extract but only one is discernible in the starved extrtact, which is significantly fainter than the corresponding band in the fed extract. The major protein that has been detected in the fed extract is approximately 30kDa. Rabbit anti-sheep serum albumin [Fig. 6] was immunoblotted against the two extracts also. The fed extract differs from the starved extract by possessing four bands that the starved extract either is missing or is significantly fainter than fed extract. The bands correspond to molecules of approximately 66, 50, 37 and 30kDa. The final antiserum immunoblotted was rabbit anti-sheep erythrocyte stroma (cytosol) [Fig. 7]. In this case the differences between the extracts is less definite. Both extracts appear to contain a component of

Figure 5. Fed and Starved mite extract immunoblotted with Rabbit anti-Whole Sheep Serum.

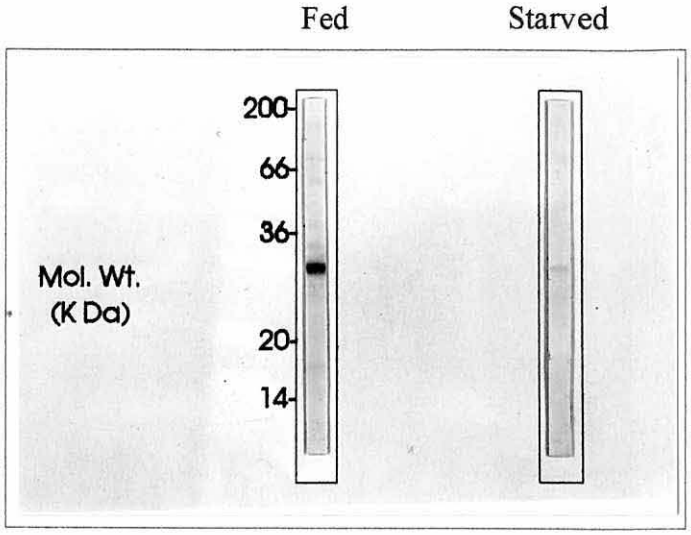


Figure 6. Fed and Starved mite extract immunoblotted with Rabbit anti-Sheep Serum Albumin.

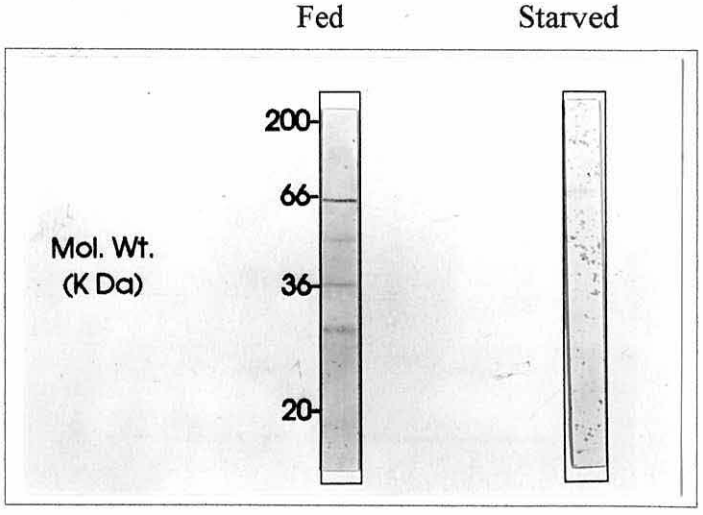
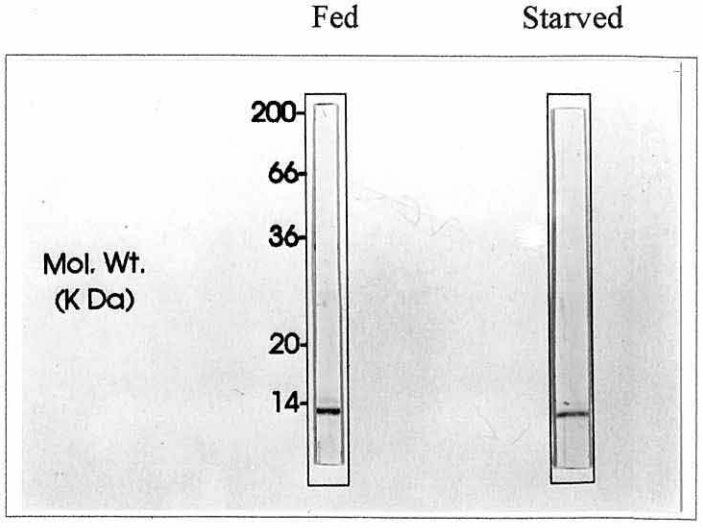


Figure 7. Fed and Starved mite extract immunoblotted with Rabbit anti-Erythrocyte Stroma



erythrocyte stroma of approximately 14 kDa molecular weight. There are also faint bands indicating two proteins; just smaller and larger than 14 kDa. All three bands are perceivably fainter in the starved extract.

Discussion.

P. ovis mites are generally difficult to work with immunologically, due to their minute size, exacerbated in this case by the experimental design. It was necessary to acquire two very comparable sets of mite extracts, from essentially exactly the same number of mites. Adult female *P. ovis* mites are no larger than 750μm in length, but although their gut takes up the majority of the body cavity, a large number of individual organisms were needed to provide sufficient protein for the assays. The unpredictability of mite availability subsequently leads to difficulties in obtaining sufficient numbers of mites. If the mites were larger then obviously fewer animals would be required for each assay, and it would also be more likely to be able to dissect out the digestive system, further simplifying the situation as purer mite extracts would be obtainable, containing fewer mite body proteins which undoubtedly complicates the electrophoretic profile. Weighing the mites was not practical for the experimental design used in this study as it would not have been an accurate enough way to obtain two groups of directly comparable mites. The starved mites would weigh less than the fed mites and so the starved extract would contain more mites which is likely to have an effect on the composition of the extract. Therefore the mites had to be individually counted and then extensively but carefully washed. This made the mite extracts a very precious material to produce and in addition to the sporadic availability of the mites, this limited the number and types of assays that could be performed with the extracts. Nevertheless this investigation has given at least some indication of the components that are present in the mite's gut, are likely to be digested there and hence indicate some of the materials likely to be important dietary requirements for the mites.

It was not possible, from the SDS-PAGE investigation to accurately identify as many bands, as envisaged, present in the fed but absent in the starved extracts. We have shown though, that the fed extract contains two protein bands (representing molecules of 27 and 50 kDa respectively), absent from the starved extract, which correspond to two of three major bands seen in both the sheep serum and the scab exudate profiles. Both

extracts contained a large number of bands, many of which are undoubtedly derived directly from the mite, rather than being host-derived materials. This is likely to obscure the banding pattern of the host-derived materials. As suggested earlier, if it was possible to dissect out the mite's gut then significantly purer extracts could be obtained which would help to clarify the electrophoretic profiles.

Despite the lack of clarity the stained gels have demonstrated, we have acquired some relevant information from this study. It does appear that the mites ingest a significant amount of the exuded materials characteristically found at the epidermis during a sheep scab infestation, and that this appears to have a similar electrophoretic profile to serum. It may be argued that it is not surprising that the mites seem to ingest (perhaps among other substances) a material of this nature considering the immediate environment of the mite and the likely nutritional components of the exudate. It has previously been discussed though that attempting to feed the mites on a serum diet has met with little success and so further investigations were required to try and more precisely identify the dietary components of *P. ovis.* It seems from information generated from the stained gels that *P. ovis* mites do at least ingest components with a similar electrophoretic profile to scab exudate and whole serum. Do the mites contain the "scab" components or does the scab contain components of the mite? On the face of it, it is not possible to say. The scab material undoubtedly contains mite excrement and fractions of dead mites, but the sheep whole serum sample separated electrophoretically demonstrates a profile similar to the scab profile. This sample would not have any *P. ovis* components contaminating it. We have to conclude then, that from the three bands that appear to be common to the mite extract, the sheep whole serum sample and the scab sample are components that are likely to be exuded onto the epidermis during an infestation and which the mites ingest.

The immunoblots revealed some interesting and specific information concerning the nature of the ingested meal. The anti-whole serum and the anti-serum albumin probes both again suggest that the mites ingest and indeed digest at least some

components of serum. In both these cases the banding pattern of the fed mite extract is perceivably stronger than the corresponding starved profile. The "quality" of the starved extract is discussed in more depth later, but it appears that the starvation procedure effectively resulted in the gut content diminishing, why else would we repeatedly see less definite banding patterns in the starved extract. Indeed the respective protein concentrations of the two extracts (fed: 2.25 μg/μl and starved: 1.5 μg/μl) suggested this earlier on in the investigation.

The strong positive reaction to the sheep erythrocyte stroma is puzzling because it seems *P. ovis* mites feeding on sheep rarely ingest erythrocytes. This was concluded by the failure to detect haemoglobin in fed mite homogenates (Rafferty and Gray, 1987). Mites are occasionally seen to be stained slightly red with ingested blood through their translucent cuticle, rather than being their usual "pearly white" colour, but the vast majority of the mites do not have the opportunity to ingest erythrocytes. Given the opportunity though, mites will ingest erythrocytes in an *in vitro* feeding system, for example (Deloach, 1984; Mathieson, unpublished) or from small haemorrhages that can result when the sheep scratch themselves severely enough when relieving the disease-associated pruritis. The fact that there appears to be a strong reaction in both fed and starved extracts could be because there is a strong affinity and avidity between the molecules adsorbed to the NCP and the antiserum, not necessarily because there is a relatively large amount of the specific molecule present. By diluting the primary antisera further, it may be possible to demonstrate visually the differences between the fed and starved extracts and their affinity to the anti-erythrocyte stroma. If there was a significant amount of erythrocyte stroma in the gut, then one would expect the mites to appear more red than they were but any "reddened" mites were removed during preparation at the counting stage as they do not represent the majority of the population and it was thought would perhaps interfere with the results. It is possible that the mites somehow ingest an amount of erythrocyte stroma but do not possess the necessary proteases to digest the molecules hence the starved mite extract appears to contain a

comparable amount of the material. The anti-sheep erythrocyte stroma antiserum may conceivably be cross reacting with another host (or even mite) derived molecule which is detected in both the fed and starved extracts.

With the benefit of hindsight, although it has been possible to demonstrate some differences in the gut content between fed and starved *P. ovis* mites, the work was possibly a little adventurous and a greater volume of work would perhaps have been possible if fed mites alone were investigated. There would be no need to laboriously count the mites, simply washing, weighing and homogenising them would suffice. With a greater volume of fed mite extract it would be practical to run full size gels which would give us a better band resolution and a more precise indication of the proteins present in the homogenate and following electroblotting. It would be interesting to compare the banding pattern of fed *P. ovis* mites separated by SDS-PAGE with that of another distantly related mite, a plant sap feeder for example. The body proteins of the two mites are likely to be similar which could be identified and disregarded allowing the remaining components (likely to be food material) to be investigated. The experiment would require the use of full size gels and a larger concentration of mite extracts to increase the resolution of the gel.

The quality of the "starved" mite extract may be questioned, due to the difficulty of keeping mites alive off-host long enough to starve them. We only kept the mites off-host under unfed conditions for 96 hours as after this time significant numbers of mites began to die. Other workers have reported mites remaining viable, and therefore healthy and possibly "fed", for up to 15 days under field conditions (O'Brien *et al.*, 1994). A consequence of this is that our "starved" mites' guts possibly contained more stored food material than was anticipated which were detected as bands on the stained SDS-PAGE gels. The mites that survived off-host for longer periods than the incubation period used here may have cleared their guts of food material and may be utilising stored food metabolites though, in which case our mites may well have cleared their guts adequately. Another occurrence which may have complicated the electrophoretic profiles is the effect

of large molecules, ingested as dietary requirements, being catabolised during digestion. This is very likely to happen as large molecules are cleaved by digestive enzymes resulting in more numerous smaller molecules. This could possibly confuse the electrophoretic profiles of the two extracts. Rather than the fed extract showing bands that the starved extract lacks, the starved extract banding pattern is likely to show a number of smaller molecules that the fed extract lacks!

A further consideration to be made concerning the starved extract is that since the mites are being starved, it is possible that physiological changes are occurring within the mite which may disrupt normal processes of digestion. Even simply removing the mites from their natural environment may disrupt them sufficiently to alter these normal digestive processes. This could well interfere with the nature of the "starved" mite gut content. The protein concentrations of the two extracts did suggest that the starved extract contained less total protein than the fed extract though, and the immunoblots demonstrated a visible difference between the contents of fed and starved animals, indicating some of the components ingested and possibly digested by the mites. The reason why the immunoblots revealed visual differences between the fed and starved extracts and the stained gels failed to clearly show differences, is probably due to the poor resolution that accompanies staining the gels with Coomassie Blue or the silver staining techniques. Immunological staining methods are far more specific and sensitive than other more conventional methods of staining. As discussed earlier, producing purer mite extracts and running the samples on full sized gels would be likely to produce clearer banding patterns.

It is likely that *P. ovis* mites utilise nutritionally the abundant lipids that are found around the sheep epidermis and there are various studies that have attempted to demonstrate the presence of lipid in the digestive tract of *P. ovis* mites (Sinclair, 1988; Sinclair and Filan, 1989). Whether lipids could be identified from mite extracts using the immunoblotting techniques employed in this study is uncertain due to the difficulty in raising antisera to lipids. Work of this kind would help to clarify the suggestions that *P.*

ovis mites ingest and digest a significant amount of surface lipid from the epidermis of infested sheep.

The SDS-PAGE techniques used here have been used successfully in the past to determine the dietary composition of a variety of other arachnids. Murray and Solomon (1978) developed a technique to detect some species of prey consumed by mites and insects. Their methods detected prey enzymes within the gut of single predacious mites (*Typhlodromus pyri*) using SDS-PAGE techniques and subsequent staining the gels for specific, prey esterase activity. Dicke and de Jong (1988) extended these techniques to examined the prey preference of the same mite by using similar techniques. It seems that the materials ingested by *P. ovis* are far more numerous and perhaps less able to be labelled than the diets of the predatory mites discussed above.

Although this study has not been able to (and was not expected to) identify the specific nature and composition of the diet of *P. ovis*, it has certainly demonstrated that it is possible to identify certain materials that are present in extracts of fed mites and absent or less abundant in starved mite extracts. There is now strong evidence that *P. ovis* mites appear to ingest and digest certain serum components which have a similar electrophoretic profile to the exudate produced during a *P. ovis* infestation. The mites do not regularly feed on whole blood and they may or may not feed on other surface components- that remains to be proved by further work. This work has layed a solid foundation for both *in vitro* culture investigations and work on vaccine development.

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Chapter 7.

Further Investigations into the Diet of *Psoroptes ovis* using Conventional and Immuno-histological Techniques.

Chapter 7.

Further Investigations into the Diet of *Psoroptes ovis* using Conventional and Immuno-histological Techniques.

Summary.

The location and nature of the diet of *Psoroptes ovis* was investigated by probing frozen naive sheep skin sections with antisera to whole mite extract and identifying the regions that showed affinity to the antiserum with conventional staining. In addition, comparable frozen skin sections were stained with commercial antisera with specificity to materials potentially ingested by the mites to compare the staining characteristics of the two types of antisera. The study has shown that the *P. ovis* mites ingest material with an antigenic profile similar to sheep whole serum, sheep serum albumin and sheep erythrocyte stroma and it is very likely that the mites ingest materials with this composition.

Introduction.

In Chapter 6, the dietary components of *Psoroptes ovis* were investigated using SDS-PAGE and Western Blotting techniques. This approach has shed some light on the little understood and important subject of the diet of *P. ovis*. An understanding is important in order to produce an *in vitro* feeding system capable of the rapid and inexpensive screening of putative antigens if a vaccine-based control approach is to be attempted. As discussed in the previous chapter due to the limited success of feeding and maintaining colonies of *P. ovis* off-host, a more direct approach was necessary to determine the dietary constituents the mites required. In this chapter the nature of the diet of *P. ovis* is investigated further, in particular the source of the mite's food is sought using immunohistological and conventional histological techniques.

During the course of an infestation a wide range of potentially nutritious materials are available to the mites. Wright and Deloach (1980) demonstrated that *Psoroptes cuniculi* (now believed to be a strain of *P. ovis* (P. G. Bates, personal communication)) reared on rabbits ingest erythrocytes and suggested this was because the mites pierced the rabbits capillaries, this will be discussed later. They then went on to demonstrate that *P. ovis* mites from a variety of hosts (sheep, cattle and again rabbits) ingest significant amounts of erythrocytes in artificial infestations of rabbit's ears. They suggest that *P. ovis* mites from whatever source animal are capable of piercing the rabbit's ear capillaries to gain access to the erythrocytes. Mites feeding on sheep though, do not accumulate significant amounts of haemoglobin during feeding (Rafferty and Gray, 1987). The erythrocytes they do occasionally ingest probably originate from host induced epithelial trauma as a result of the intense itching associated with the disease. The clear difference in the depth of capillary beds on sheep trunks and rabbit's ears are probably the determining factor.

It is generally agreed that the mites do not pierce the epidermis, as suggested by Shilston (1915), but are perhaps able to abrade the loose stratum corneum to access

material below (Rafferty and Gray, 1987; Mathieson, unpublished) or that they simply suck fluids without first causing any surface trauma (Kirkwood, 1986). The deposition of antigenically active faecal material which is believed to stimulate an immune response, resulting in the production of a moist, serous exudate, which is also possibly a significant dietary factor, is recognised as contributing significantly to the disease (Rafferty and Gray, 1987; Sinclair and Filan, 1989). Bacteria originating in the digestive system of the mite (investigated in Chapter 5), could play a role in stimulating an immune response and the characteristic exudate that accompanies an infestation.

In this study antisera to whole "fed" mite extract and also whole "starved" mite extract have been raised and used to immunoblot sheep skin sections, naive for *P. ovis* infestations. The fed mite antiserum should bind to proteins in the skin sections to demonstrate the location of the mite's ingested meal and the starved antiserum should not. Conventional light microscopy, combined with staining characteristics of comparable skin sections probed with commercial antisera to materials the mites potentially ingest, would then be used to identify the specific areas and nature of the materials the mites ingest.

Materials and Methods.

Fed and Starved Mite Extract Production.

The same fed and starved mite extracts prepared for Chapter 6 were used for the work carried out in this investigation. See Chapter 6 Materials and Methods section for details. Briefly for clarity, mites were regularly collected from infested sheep maintained at the Central Veterinary Laboratory, immediately fixed in liquid nitrogen (LN_2) and stored at -20°C until sufficient mites had been collected. A second group of mites were incubated off-host at 33°C, 82% relative humidity, unfed for approximately 96 hours before being fixed in LN_2 and stored at -20°C. When sufficient numbers of mites had been collected the two groups were extensively washed, homogenised, protein concentration determined, aliquoted into 30µl fractions and stored at -20°C.

Antisera Production and Screening.

Seven CBA/CA male mice were used to raise the experimental antisera. They were weighed and randomly assigned to receive either the fed or the starved mite homogenate. Four mice were used to raise the anti-*P. ovis* "fed" antiserum (α-POF) and three for the anti-*P. ovis* "starved" antiserum (α-POS). Each mouse received three intra-peritoneal inoculations in total, one injection on each inoculation day; the first at day zero, the second 14 days post primary inoculation (PPI) and the final inoculation at 28 days PPI. For the primary inoculations the antigen was injected with an equal volume of Freund's Complete Adjuvant (FCA), each mouse received approximately 75µg or 56µg of protein in <100µl of inoculant for the fed and starved groups respectively and approximately half the respective protein concentration in Freund's Incomplete Adjuvant (FIA) for the secondary and tertiary inoculations, again each mouse received less than 100µl of inoculant at each inoculation [Table 1].

Table 1 A summary of the amount of protein (in μg) inoculated into each mouse from the two groups on each inoculation day. Values are approximate, based on the original Sigma modified Lowry protein assay performed.

Total protein inoculated per mouse (μg) for each inoculation.			
	Primary: t=0	Secondary: t= 14 (days)	Tertiary: t= 29 (days)
Fed Extract	75 in FCA	35 in FIA	35 in FIA
Starved Extract	56 in FCA	25 in FIA	25 in FIA

The mice were test bled by withdrawing approximately 750μl of blood by retro-orbital sinus puncture from each mouse at 45 days PPI. The blood from each experimental group was pooled and the serum extracted by incubating the blood in the collection tubes for 2 hours at 37°C then overnight at 4°C. The blood clot was removed and the remaining serum centrifuged at 3000 rpm for 10 mins with a bench centrifuge. The serum was aliquoted into 20μl fractions and stored at -20°C until required. The mice were exsanguinated by cardiac puncture two days later once the antisera had been screened for specificity to the antigens by the methods previously described. The sera were initially tested using a Dot Immunobinding assay based on the methods of Hawkes (1986) and then later by Western Blotting (see below). Normal mouse serum (NMS) was prepared from a group of three CBA/CA mice to be used as a negative control in the various assays.

The Dot Immunobinding Assay.

This "dot-blot" assay was performed 45 days PPI to quickly determine whether the mice had produced specific antisera to the two mite extracts, before the mice were exsanguinated.

For this assay supported NCP is washed in dH_2O for a few minutes and air dried. Using a soft pencil, rectangular strips (5 x 30mm) were ruled onto the NCP and labelled.

0.5µl of either fed or starved mite extracts (giving protein concentrations of approximately 1.0µg and 0.75µg of fed and starved extracts respectively) were pipetted onto the centre of each labelled, marked area on the NCP. The NCP was then washed in TTBS for 10 minutes and cut into strips (5 x 30mm) containing either the adsorbed fed or starved mite extract. Non-specific binding of the antiserum was blocked by incubating the strips in 2% milk powder in TTBS (see the Appendix C) for 15 minutes. The blocking solution was removed and the strips incubated in three dilutions (1:100, 1:500 and 1:1000 in 2% milk power in TTBS) of either the α-POF or the α-POS for 4 hours. The strips were then washed in TTBS for 3 x 10 minutes to remove the primary antiserum and re-blocked in the usual blocking solution for 15 minutes. Following this the strips were incubated for 2 hours 30 minutes in the secondary antisera (goat anti-mouse IgGAM conjugated with horseradish peroxidase(/PO)) (Sigma Immunochemicals) diluted to 1:750 in the usual dilutant. The secondary antiserum was then washed off in TTBS for 3 x10 minutes and the binding revealed in 4-chloro-1-naphthol as usual (Appendix B). See Table 3 Chapter 6 for a summary of the procedures used in the dot blot assay and Table 2 below, for the combinations of antigens and antisera used in this assay.

Table 2. Combinations of antigen (mite extract), adsorbed to the NCP and mouse sera used to probe the antigens used in the dot immunoblot assay.

Antigen (Mite Extract)	Serum Immunoblotted
Fed *P. ovis*	Mouse anti-Fed *P. ovis* extract
Starved *P. ovis*	Mouse anti-Starved *P. ovis* extract
Fed *P. ovis*	Normal mouse serum
Starved *P. ovis*	Normal mouse serum

Western Blotting.

The Western blotting procedures conducted here have been previously described in Chapter 6 but are briefly described here for clarity and completeness.

For Western blotting, approximately 11μg and 7μg of fed and starved homogenate respectively, were loaded per 5mm mini gel lane. Sigma wide molecular weight markers were run to determine approximate molecular weights of the samples. The homogenate was separated on a 4% resolving and 12% separating gel for 60 minutes at 200 volts using a Bio-Rad Mini PROTEAN II electrophoresis kit. The separated proteins were transferred electrophoretically using a Bio-Rad Electroblotting kit at 100 volts for one hour onto NCP following the manufacturer's instructions.

The nitrocellulose sheets were dried and cut into strips containing the sample proteins and markers using the Pyronine-Y bands as guides. The markers were stained either using Amido Black or Coomassie Blue. Each 5mm sample strip was cut into two longitudinally and stored at -20°C for no longer that one week.

Immunoblots were carried out with the antisera generally following the methods of Boyce *et al.* (1991) with the following alterations: Non-specific binding of antisera was blocked by incubating the NCP strips in 2% milk powder in TTBS for 1 hour and rinsing in TTBS for 2 x 5 minutes. Strips were incubated for 4 hours in a 1:200 dilution of either α-POF, α-POS or NMS in the normal dilutant. Following a 2 x 5 min wash in TTBS the strips were incubated in a 1:1000 dilution of goat anti-mouse IgGAM/PO for 2hrs 30mins. Specific binding was revealed using 4-chloro-1- naphthol in the usual manner.

Frozen Section Preparation for Immuno and Conventional Staining.

The sheep skin-sections were prepared at a nearby abattoir where ewes and lambs were routinely being slaughtered. A whole lamb skin and fleece, with no known history or clinical signs of sheep scab, was purchased and processed moments after it had been mechanically removed from the sheep. The fleece was clipped to within a few

millimetres of the epidermis at several locations on the dorsal region of the skin. Using a scalpel blade, $20mm^2$ skin sample areas, including all the dermal layers and the sub-dermal connective tissue, were removed from the skin and cut into smaller approximately 5-$8mm^3$ tissue blocks. The blocks were mounted on cryo-stubs with either moistened filter paper or OCT medium (Tissue Tek) and plunged into LN_2. The stubs remained under LN_2 until they were returned to the laboratory where they were covered in Parafilm® to reduce the effects of desiccation, and stored at -70°C. All processes were carried out as quickly as was practical to help preserve the antigenicity of the tissue.

6-8μm sections were cut at between -10 to -25°C on a mechanical Slee cryostat and picked up on clean untreated glass coverslips. They were stored at this temperature until further processing was carried out, no more than 2 hours later.

In order to maximise the staining characteristics of the sections by stabilising the position of molecules and reducing the possibility of them leaching from the tissue, the effects of various post-sectioning fixation processes were investigated.

Three different procedures were investigated:

- No post-section fixation
- 100% Acetone for 20mins
- 4% Paraformaldehyde in phosphate buffered saline (PBS) pH 7.4 for 5 minutes.

The sections were removed from -25°C storage and immersed in fixative for the specified length of time. They were rinsed in dH_2O for 2 x 5 minutes and then either immunologically or conventionally stained.

Immunohistological Staining.

Two immunohistological techniques were investigated; a *three-tier* peroxidase anti-peroxidase (PAP) system and a *two-tiered* immunoperoxidase (IP) system.

Peroxidase Anti-Peroxidase (PAP) system.

Sections were air dried following the procedures of the previous section and mounted face upwards and level on glass microscope slides using small pieces of Blue Tak to keep the coverslips raised above the microscope slide. The slides were labelled according to the procedures that were to follow. To block endogenous peroxidase interfering with the specific immunological staining, incubating some sections in 1% phenylhydrazine hydrochloride (PHH) in PBS for 30 minutes was investigated. Sections were incubated in the solution, made fresh daily, and rinsed for 15 minutes in PBS (1 x 5 mins in PBS then 2 x 5 mins in PBS at 37°C). There followed an incubation in 0.1% Trypsin in 0.1 $CaCl_2$ (aqueous) for 20 minutes. The trypsin was rinsed off in ice cold dH_2O and a non-specific binding of the secondary antiserum was blocked by incubation in 2% goat serum in PBS for 45 minutes at room temperature. Incubation in primary antisera then followed. Numerous different dilutions of primary, secondary and tertiary antisera were investigated for the three antisera stages in this procedure, but to avoid unnecessary confusion, described here will be the dilutions that were found to be most efficient. Approximately 250µl of the primary antisera (see Table 4), diluted in PBS was pipetted onto the surface of each section and incubated at either 37°C for 2 hours or at 4°C overnight. The primary antisera were washed off for 2 x 5 minutes in PBS and the secondary antisera applied (see Table 4), diluted as before in PBS and incubated under the same conditions as above for 2 hours. The secondary antisera were washed in 2 x 5 minutes in PBS and the final, tertiary antisera applied. In this case, a mouse or rabbit PAP diluted in PBS to 1:400. This final antisera was incubated as before for either 2 hours at 37°C or overnight at 4°C. Under both conditions the sections were incubated inside closed containers with moistened cotton wool balls to minimise the evaporation of the antisera. The tertiary antisera was washed off as before and the specific binding revealed using diaminobenzidine/H_2O_2 (DAB/H_2O_2)(Sigma) following the manufacturers instructions. When the desired stain intensity was reached the reaction was stopped by the addition of dH_2O or PBS. Excess water was removed from the surface of the sections and they were mounted in Aquamount (BDH). Table 3

peroxidase anti-peroxidase procedures described above and Table 4 shows the various antisera and dilutions used in this investigation.

Table 3. A summary of the PAP procedures for staining frozen sections.

1.	Block endogenous peroxidase activity with 1% PHH in PBS (optional)	30 mins
2.	Wash in PBS 1 x 5 mins, and 2 x 5 mins at 37°C	15 mins
3.	0.1% Trypsin in 0.1% $CaCl_2$ (aqueous)	20 mins
4.	Wash in ice cold distilled water	10 mins
5.	Block in 2% goat serum in PBS at room temperature	45 mins
6.	Primary antiserum, see Table 4	2 hours at 37°C or overnight at 4°C
7.	Wash in PBS	10 mins
8.	Secondary antiserum, see Table 4	2 hours at 37°C or overnight at 4°C
9.	Wash in PBS	10 mins
10.	Mouse PAP	2 hours at 37°C or overnight at 4°C
11.	Wash in PBS	10 mins
12.	Wash in Tris-HCL pH 7.6	10 mins
13.	DAB/H_2O_2 (Sigma)	approx. 5 mins
14.	Wash in dH_2O or PBS	1 min
15.	Mount under glass coverslip with Aquamount (BDH)	

Table 4. Antisera dilutions for the three tiered PAP procedures performed in this investigation, all dilutions are in PBS. Dilutions of the positive controls (rabbit anti-sheep erythrocyte stroma and rabbit anti-sheep whole serum) and negative control (normal mouse serum and normal rabbit serum) are included.

Primary Antisera	1° Antisera dilution	2° Antisera (dilution)	3° Antisera (dilution)
Mouse α-POF	1:100	Goat anti-mouse IgG (1:400)	Mouse PAP (1:400)
Normal mouse serum	1:100	As above	As above
Rabbit anti-sheep erythrocyte stroma.	1:400	Goat anti-rabbit IgG (1:400)	Rabbit PAP (1:400)
Rabbit anti-sheep serum albumin.	1:1000	As above	As above
Rabbit anti-sheep whole serum	1:2500	As above	As above
Normal rabbit serum	1:400	As above	As above

The non-specific binding of the secondary and tertiary antisera was investigated, as was the non-specific staining of the DAB/H_2O_2. This was carried out by incubating sections following the protocol described in Table 3, but omitting the unnecessary steps. The following combinations of antisera and reagents were investigated:

- Secondary and tertiary antisera, and DAB/H_2O_2
- Tertiary antiserum and DAB/H_2O_2 and
- DAB/H_2O_2 alone.

Immunoperoxidase (IP) System.

The two tier system using a peroxidase conjugated secondary antiserum is a similar system to the PAP method but involves only two antisera instead of the three used in the PAP system. The procedures were conducted exactly as for the PAP system (see pervious section) but the secondary antiserum was Goat anti-mouse IgGAM/PO or Goat anti-rabbit IgGAM/PO. Table 5 summarises the procedures involved and Table 6 shows the various antisera and dilutions used in this investigation.

Table 5. Summary of the procedures used in the two-tier immunoperoxidase system.

1. Block endogenous peroxidase activity with 1% PHH in PBS (optional)	30 mins
2. Wash in PBS 1 x 5 mins, and 2 x 5 mins at 37°C	15 mins
3. 0.1% Trypsin in 0.1% $CaCl_2$ (aqueous)	20 mins
4. Wash in ice cold distilled water	10 mins
5. Block in 2% goat serum in PBS at room temperature	45 mins
6. Primary antiserum, see Table 6	2 hours at 37°C or overnight at 4°C
7. Wash in PBS	10 mins
8. Secondary antiserum, see Table 6	2 hours at 37°C or overnight at 4°C
9. Wash in PBS	10 mins
10. Wash in Tris-HCL pH 7.6	10 mins
11. DAB/H_2O_2 (Sigma)	approx. 5 mins
12. Wash in dH_2O or PBS	1 min
15. Mount under glass coverslip with Aquamount (BDH)	

Table 6. Antisera dilutions for the two tiered antibody procedures performed in this investigation, all dilutions are in PBS. Dilutions of the positive controls (rabbit anti-sheep erythrocyte stroma, rabbit anti-sheep whole serum and rabbit anti-sheep serum albumin) and negative control (normal mouse serum and normal rabbit serum) are included.

Primary Antisera	1° Antisera dilution	2° Antisera (dilution)
Mouse α-POF	1:80, 1:100	Goat anti-mouse IgGAM/PO (1:250)
Normal mouse serum	1:80	As above
Rabbit anti-sheep erythrocyte stroma.	1:400	Goat anti-rabbit IgGAM/PO (1:250)
Rabbit anti-sheep serum albumin.	1:1000	As above
Rabbit anti-sheep whole serum	1:2500	As above
Normal rabbit serum	1:400	As above

As with the PAP investigation, the non-specific binding of the secondary antisera was investigated, as was the non-specific staining of the DAB/H_2O_2. This was carried out by incubating sections following the protocol described in Table 5, but omitting the unnecessary steps. The following combinations of antisera and reagents were investigated:

- Secondary antisera, and DAB/H_2O_2 and
- DAB/H_2O_2 alone.

Conventional Staining.

Frozen sections cut and fixed as described above were conventionally stained to establish the structural layout of the sheep skin used in this investigation. The stains used were:

- Ehrlich's Haematoxylin and Eosin (H&E)
- Periodic Acid Schiff
- Sudan Black
- Toluidine Blue

The sections were stained according to the specific procedures for each stain (see Appendix C). Several sections were stained in both H&E and Sudan Black. Following the final rinses in H_2O, the sections were mounted under coverslips in Aquamount (BDH), removing the need to be dehydrated.

Results.

The Dot Immunobinding Assay.

The dot blot assay effectively demonstrated that antisera, with specific affinity to the two experimental mite extracts (fed and starved), had been raised in the inoculated mice 45 days PPI [Figs. 1a and 1c] and was detectable, with only a minimal staining reduction throughout the range of antisera dilutions investigated. Normal mouse serum was immunoblotted against the two extracts also but no affinity was detectable between the antiserum and the extracts at any of the dilutions investigated [Figs. 1b and 1d].

Western Blotting and Immunoblotting.

The Western blots were performed to determine if the α-POF antiserum and the α-POS antiserum recognised a different set of proteins when used to probe the fed and starved extracts separated by SDS-PAGE and Western Blotting. If the starved extract did contain only mite body proteins then the antisera should only contain antibodies to these body proteins. The α-POF antisera should recognise *all* the mite body and ingested proteins indicating that the α-POF and α-POS antisera could be used to probe sheep skin sections in the manner described above in the Introduction.

The Western Blots of the mite extracts probed with the two antisera produced a more complicated picture than anticipated. Figure 2a shows the banding pattern seen when the fed extract is probed with the α-POF antisera. There are at least 16 bands recognised by the α-POF antisera between 29 and 205 kDa with 4 major bands of approximately 66, 80, 116 and 205 kDa. The pattern seen with starved extract immunoblotted with α-POS antiserum [Fig. 2b] also demonstrates these major proteins but also a uniform group of 12 evenly spaced bands between 38 and 64 kDa. The blot for fed extract blotted with α-POS antiserum [Fig. 2c] shows a similar pattern to Figure 2a but there are two bands missing, representing proteins of approximately 44 and 49 kDa respectively and the 12 evenly spaced bands appear fainter than in Figure 2b or the following blot. The last experimental blot investigated; starved extract blotted with α-

POF antiserum [Fig 2d] is very similar to Figure 2b, again showing the 4 major bands and the 12 bands between 38 and 64 kDa. Normal mouse serum demonstrated no detectable affinity to either extracts when used to blot at the dilutions used in this assay.

The results of this immunoblotting investigation suggest that the two antisera did not demonstrate sufficient differences in the proteins that they recognise to enable a direct meaningful identification of specific dietary components (see later for a more in-depth discussion of these results). The α-POF antiserum alone was used for the remaining immunohistology.

Figures 1a-d. Dot blots of Fed and Starved mite extracts immunoblotted with three dilutions of mouse α-POF, mouse α-POS and normal mouse serum (NMS).

Figure 1a. Fed mite extract immunoblotted with three dilutions of mouse α-POF.

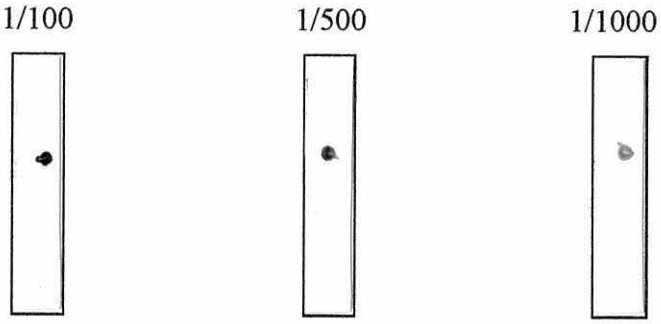


Figure 1b. Fed mite extract immunoblotted with three dilutions of NMS.

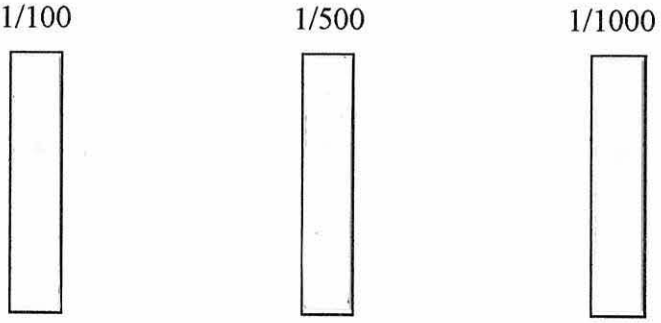


Figure 1c. Starved mite extract immunoblotted with three dilutions of mouse α-POS.

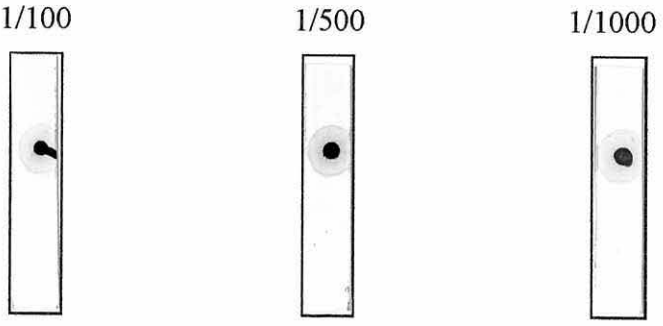
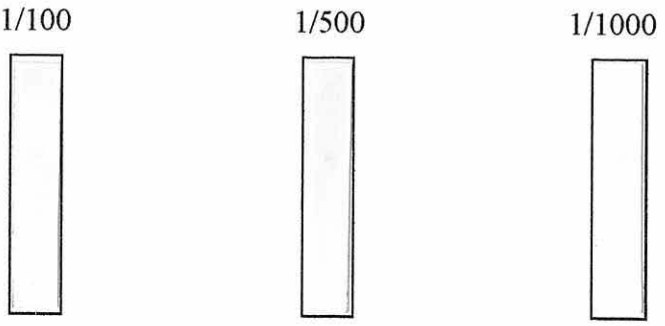
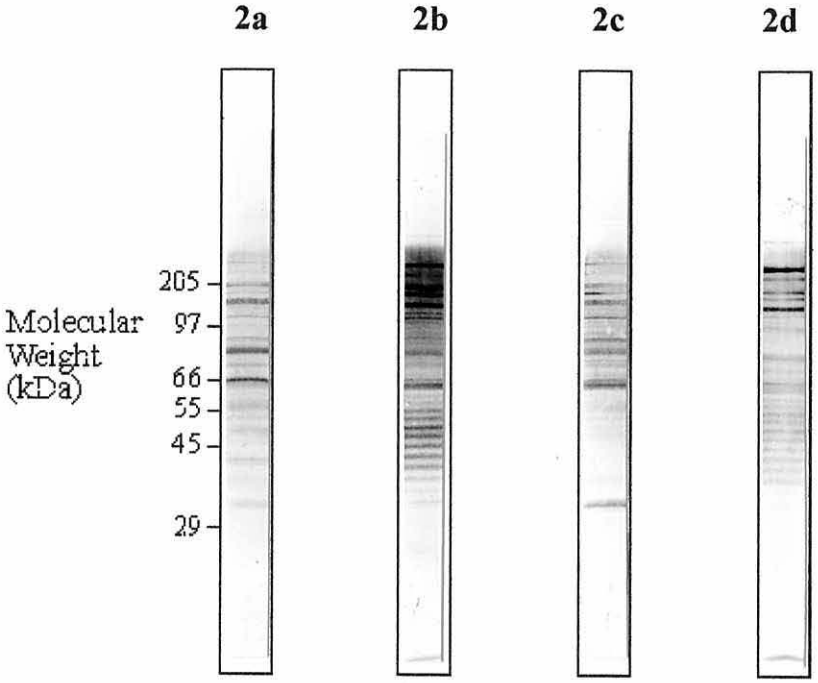


Figure 1d. Starved mite extract immunoblotted with three dilutions of NMS.



Figures 2a-2d. Electrophoretically separated fed and starved mite extracts immunoblotted with mouse α-POF and mouse α-POS antisera. **2a:** Fed mite extract immunoblotted with mouse α-POF; **2b:** Starved mite extract blotted with mouse α-POS; **2c:** Fed mite extract blotted with α-POS; **2d:** Starved mite extract blotted with α -POF.



Conventional Histology and Immunohistology.

The first few figures presented here are for orientation purposes and to demonstrate the location of the major structures found in the dermal layers and some of the secretions associated with the structures. Figure 3 is a vertical section through the dermal layers of lamb skin stained with H&E. The section demonstrates the relatively thin epidermis, the dermis and the epithelial lined wool follicles and a wool root in the upper half of the figure. The lobular sebaceous glands associated with the wool follicles are also clearly seen. Deeper in the dermal tissue the apocrine sweat glands are visible. The wool arrector muscle can also be seen and darkly stained material is conspicuous adjacent to or interspersed with the stratum corneum. Figures 4 and 5 are further enlargements of several of the typical holocrine sebaceous glands found throughout the dermal tissue, stained in this example with H&E. The nature of the sebum can be seen to change from its original state at the base of the gland to being eosinophilic as the cells break down to release their contents into the wool follicle. The base of the wool root also demonstrates its intensely eosinophilic character. Figures 6 and 7 are stained with both H&E and Sudan Black to demonstrate the location and source of the lipids associated with the stratum corneum. The lipids are produced by the sebaceous glands where they progress along the wool roots to reach the surface of the epidermis.

The sections immunologically stained with α-POF antiserum have identified some locations from where the mites possible gain their nutrition. Figure 8 is a vertical section through the skin demonstrating all the typical structures described for Figure 3. The α-POF antiserum has bound to all the epithelial surfaces, clarifying the positions of the dermal structures and has stained the stratum corneum relatively intensely. The cells of the sebaceous glands show some affinity to the antiserum but mainly in the basal portions of these glands where the cells are intact before breaking down to release their contents [Figs. 8 and 9]. The epithelia of the wool follicles also appear to have stained positively for α-POF [Figs. 8 and 10] as have components of the sweat glands. Figure 11, also stained with α-POF, again demonstrates the material found adjacent to the stratum

corneum that stains relatively intensely with this antiserum and very intensely stained cells just beneath the dermis. These are likely to be polymorphonuclear leukocytes. Normal mouse serum was used to immunoblot skin sections but no affinity to any specific area was detected at any of the dilutions investigated [Fig. 12]. The non-specific staining of DAB was investigated but produced no discernible staining. The various post-sectioning fixation procedures investigated indicated no benefit to carrying out these extra procedures in this investigation nor did attempting to eliminate the effects of endogenous peroxidase activity. The possibility that the secondary antiserum (goat anti-mouse IgG/PO) binding non-specifically was investigated but no activity was detected [Fig. 13].

Figures 14 shows a vertical skin section stained with PAS to demonstrate the location of carbohydrates that the mites may have access to and utilise. The figure shows several primary and secondary wool follicles and the abundant carbohydrate that appears to be closely associated with these structures. Towards the bottom of the figure several sections of a coiled sweat gland are visible, in this case showing only mild staining characteristics with PAS. Figure 15 on the other hand, also stained with PAS, shows an enlargement of a sweat gland demonstrating an area adjacent to the gland lumen that stains intensively for carbohydrate. Several sections of wool roots can be observed in this figure, areas of which also stain intensely with PAS.

The sections immunoblotted with commercial antisera demonstrate the location in the sheep skin of some of the antigens these antisera have affinity to. Both rabbit anti-sheep serum albumin and anti-sheep erythrocyte stroma stain similar areas to each other in the sheep skin investigated here [Figs. 16 and 17]. In both cases the epidermis and the epithelial lined structures appear to stain positively for the immunoblotted antisera and the antisera both stain material lying above the stratum corneum to varying degrees, as seen in previous sections. The staining characteristics of rabbit anti-sheep whole serum was investigated at several dilutions. Figure 18 shows a skin section immunoblotted with a 1:2500 dilution of the antiserum and the typical uniform staining observed

throughout the skin layers, with possibly a greater degree of activity associated with the epidermis. Normal rabbit serum was used at comparable dilutions to the experimental antisera to immunoblot skin sections but no significant binding was detected [Fig. 19].

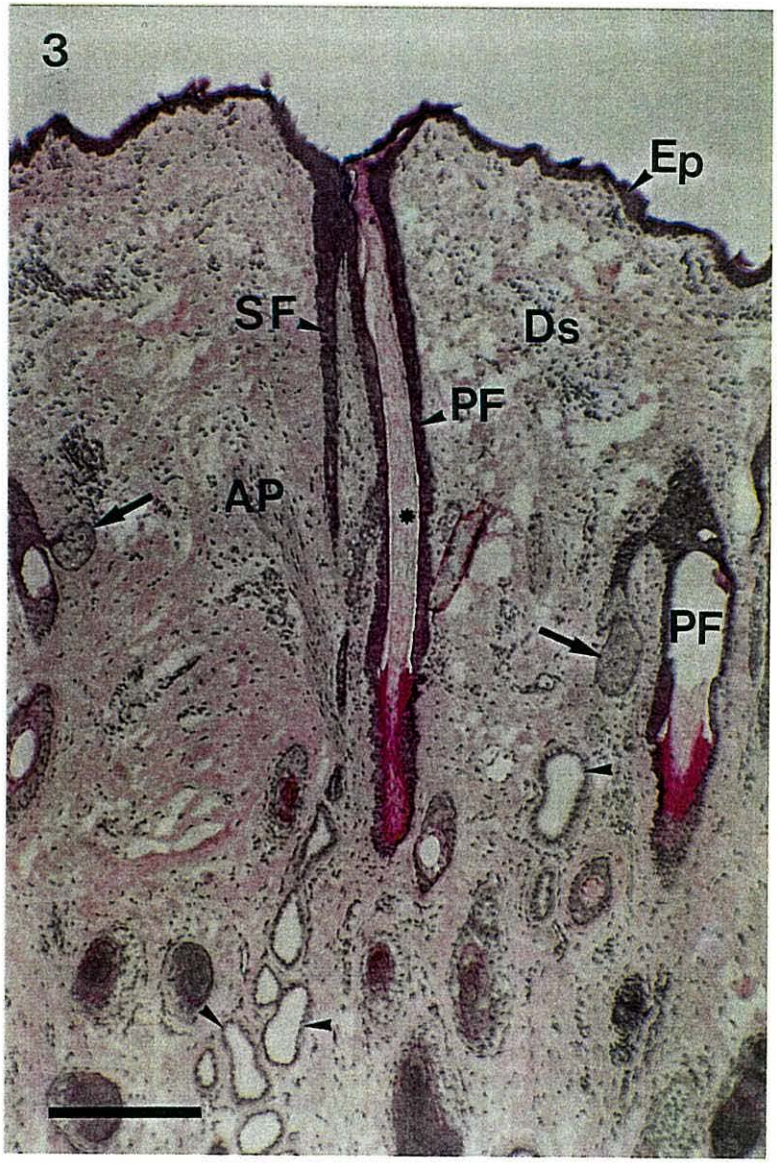
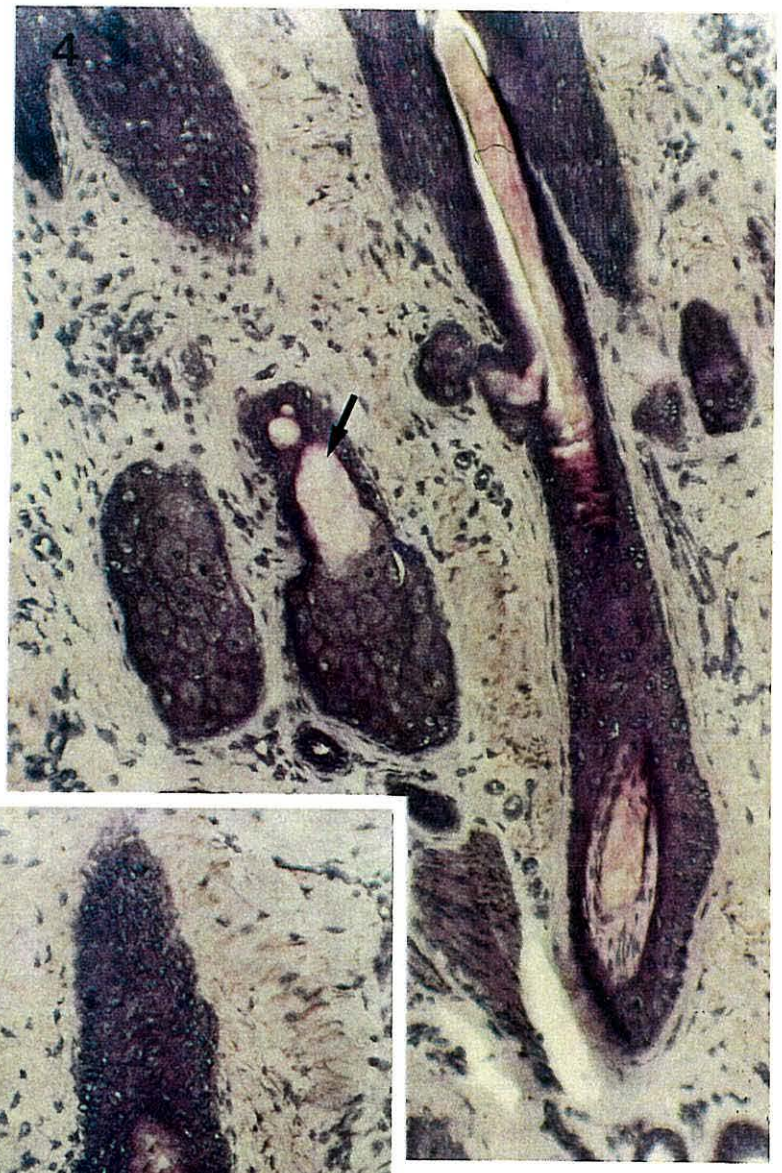


Figure 3. The skin layers and structures seen in a vertical longitudinal section of sheep skin from a mid-lateral location. Epidermis (Ep); dermis (Ds); primary and secondary wool follicles (PF) and (SF); wool root (*); sebaceous glands (arrows); coiled sweat gland (arrowheads); arrector pili muscle (AP). H&E. Bar = 500μm

Figures 4 and 5. Two wool follicles with associated sebaceous glands. Note the staining characteristics of the sebum changes to being eosinophilic as these holocrine cells break down releasing their contents into the sebaceous gland duct (arrows) and wool follicle. H&E. Bar = 200μm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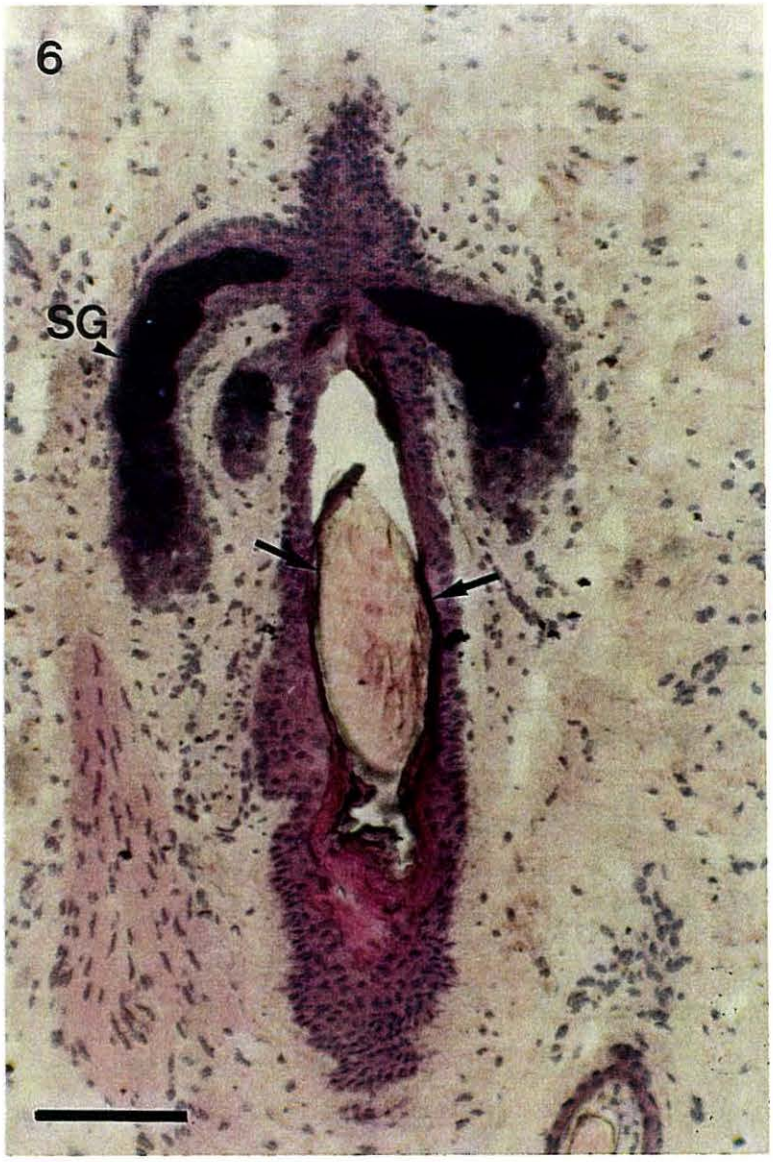
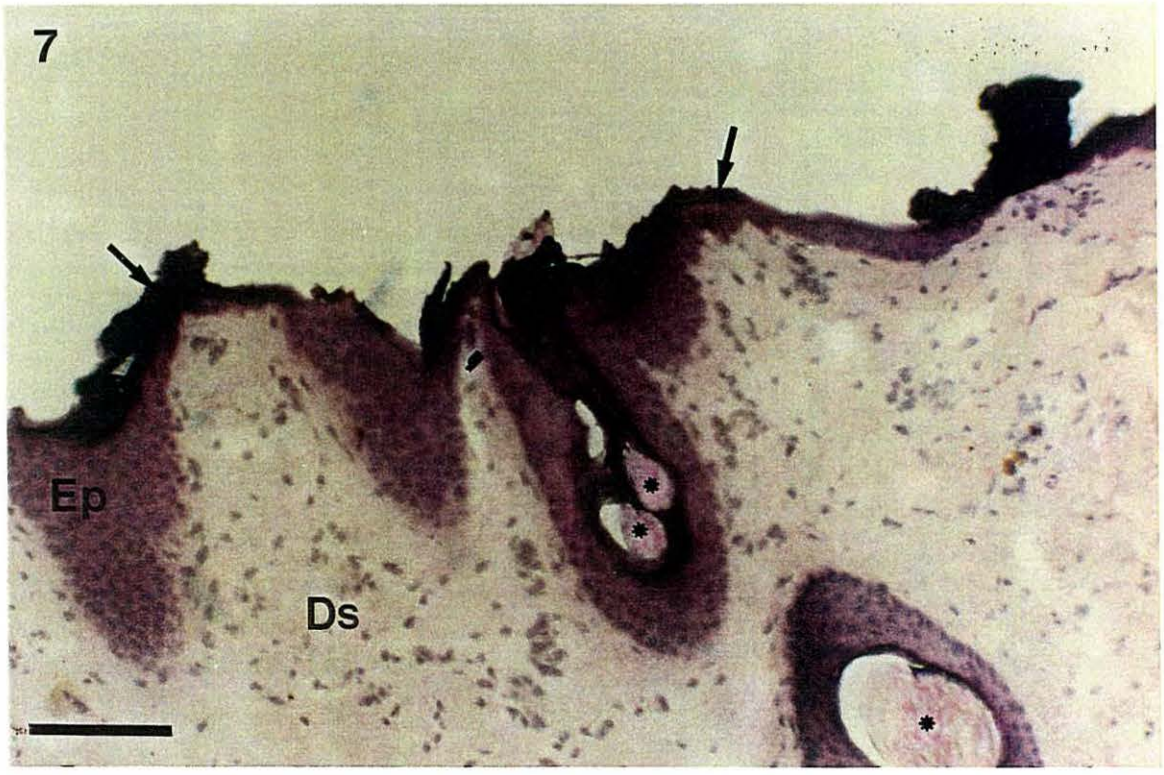
5

Figures 6. A section through a portion of a wool follicle and sebaceous glands (SG). The lipid content of the whole gland is demonstrated as is the sebum coating the wool root (arrows). H&E and Sudan Black. Bar = 200μm

Figure 7. A section through the epidermis (Ep), upper dermis (Ds) and several wool follicles (∗). The surface of the epidermis is extensively covered with lipid deposits (arrows) which can also be detected in surrounding the wool roots. H&E and Sudan Black. Bar = 200μm


6
SG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Ep
Ds

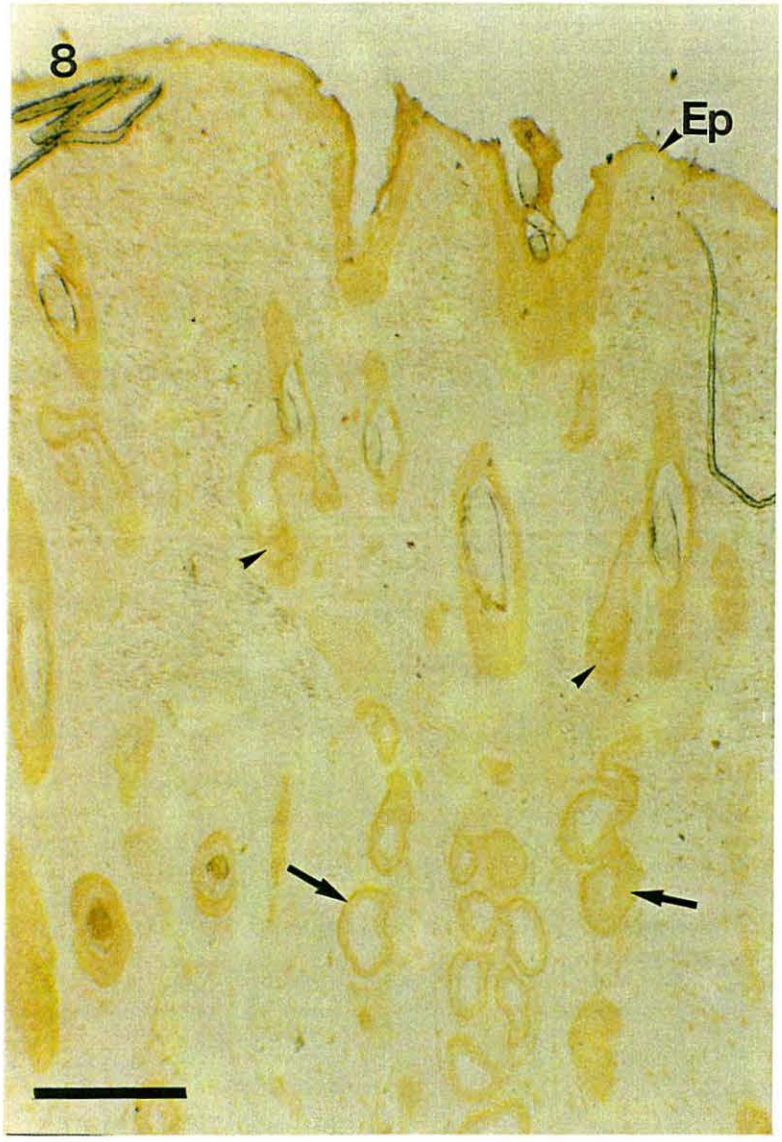
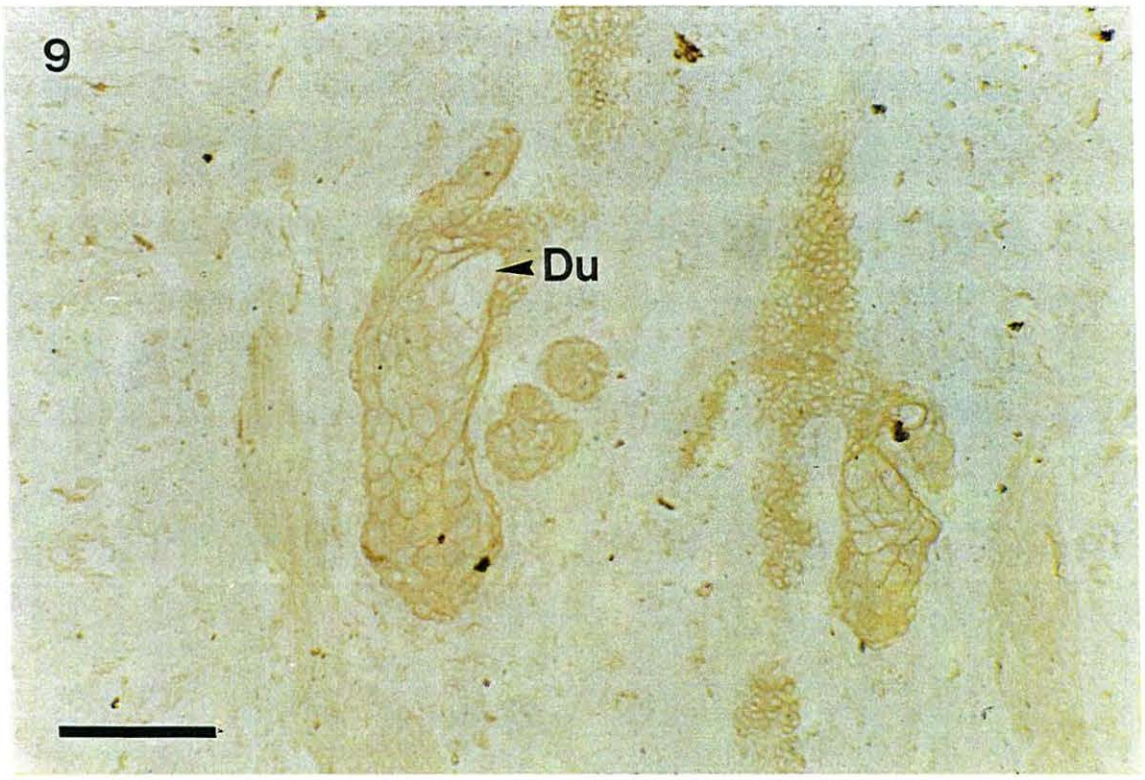


Figure 8. A vertical section through the skin layers taken from a mid-lateral position stained with mouse anti-fed mite extract (α-POF) in a 3 tiered PAP system. The epidermis (Ep) and the epidermal cells lining the sweat glands (arrow) and the cells of the sebaceous glands (arrowheads) stain positively. Superficial material on the epidermis also stains positively for α-POF antiserum. Bar = 500μm

Figure 9. An enlargement of two sebaceous glands stained with α-POF in a 2 tiered IP system. The stain demonstrates a gradual loss of affinity from the base of the gland to the gland duct (Du) where the cells have broken down to release their contents.
Bar = 200μm

Figure 10. A transverse section through several wool follicles demonstrating the specific binding of the follicle epithelial cells when stained with α-POF antiserum. PAP system. Bar = 200μm


9
Du

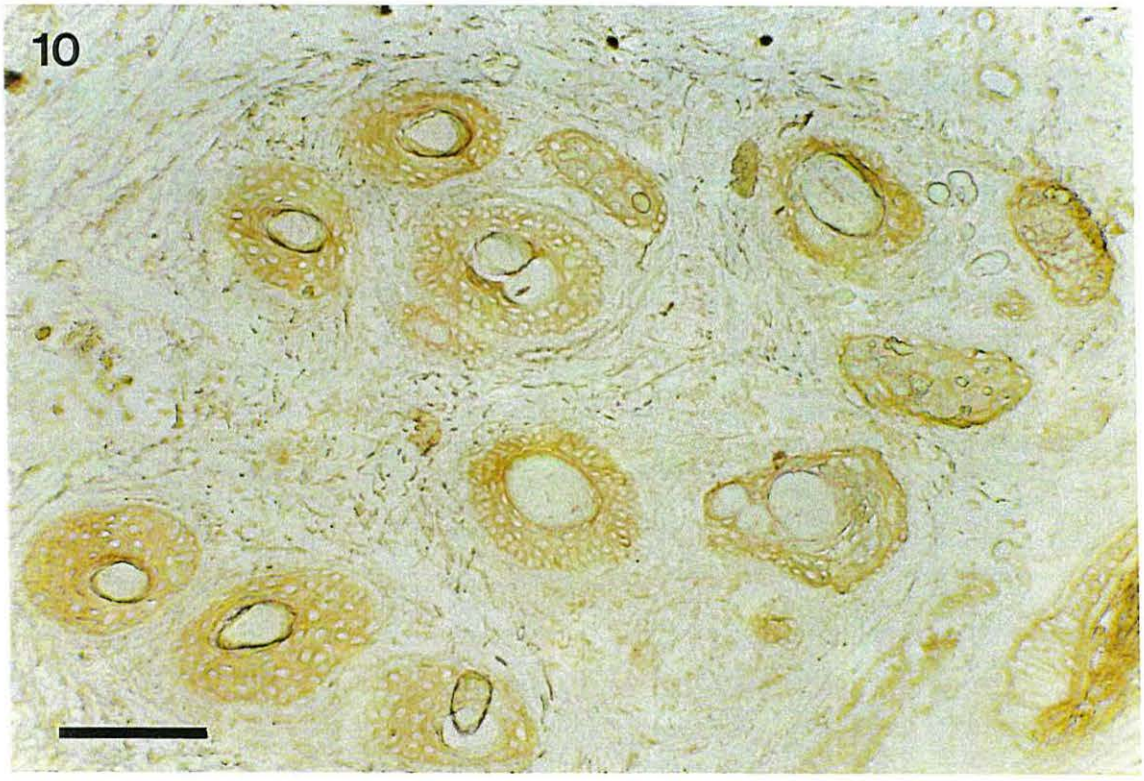
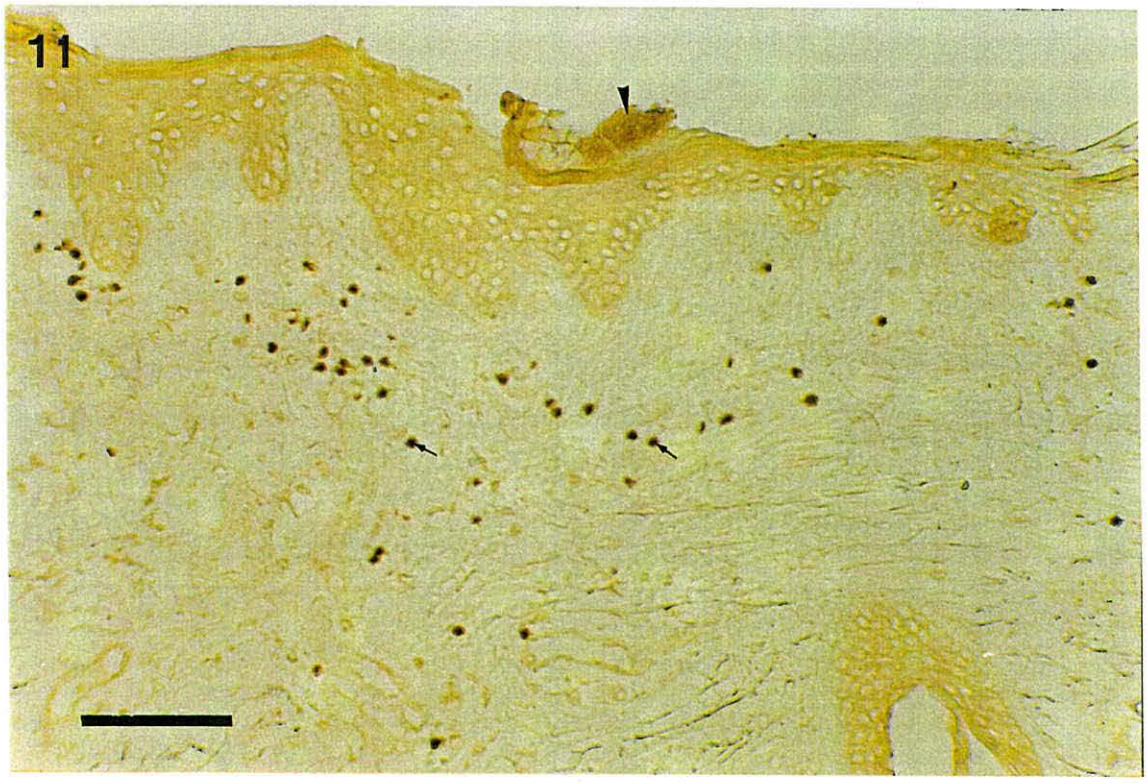

10

Figure 11. A vertical section through the skin layers stained with α-POF in an IP system demonstrating the superficial material found adjacent to the epidermis (arrowhead), the stained epidermis and intensively stained polymorphonuclear leukocytes in the dermis (small arrows). Bar = 200μm

high peroxidase in white blood cells.

Figure 12. A control vertical skin section probed with normal mouse serum at an equivalent dilution to the IP system used for the α-POF investigations. Bar = 200μm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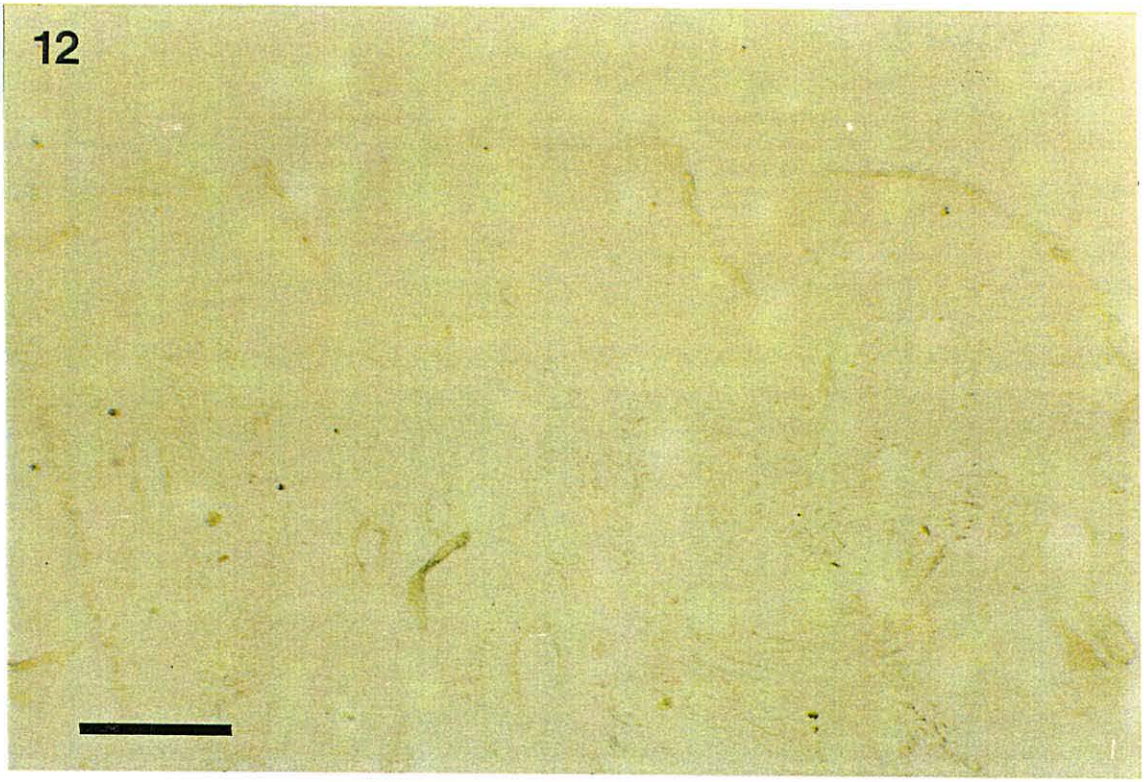
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Figure 13. A control vertical skin section probed with the secondary antiserum (goat anti-mouse IgG/PO) used in the IP system. Bar = 500μm

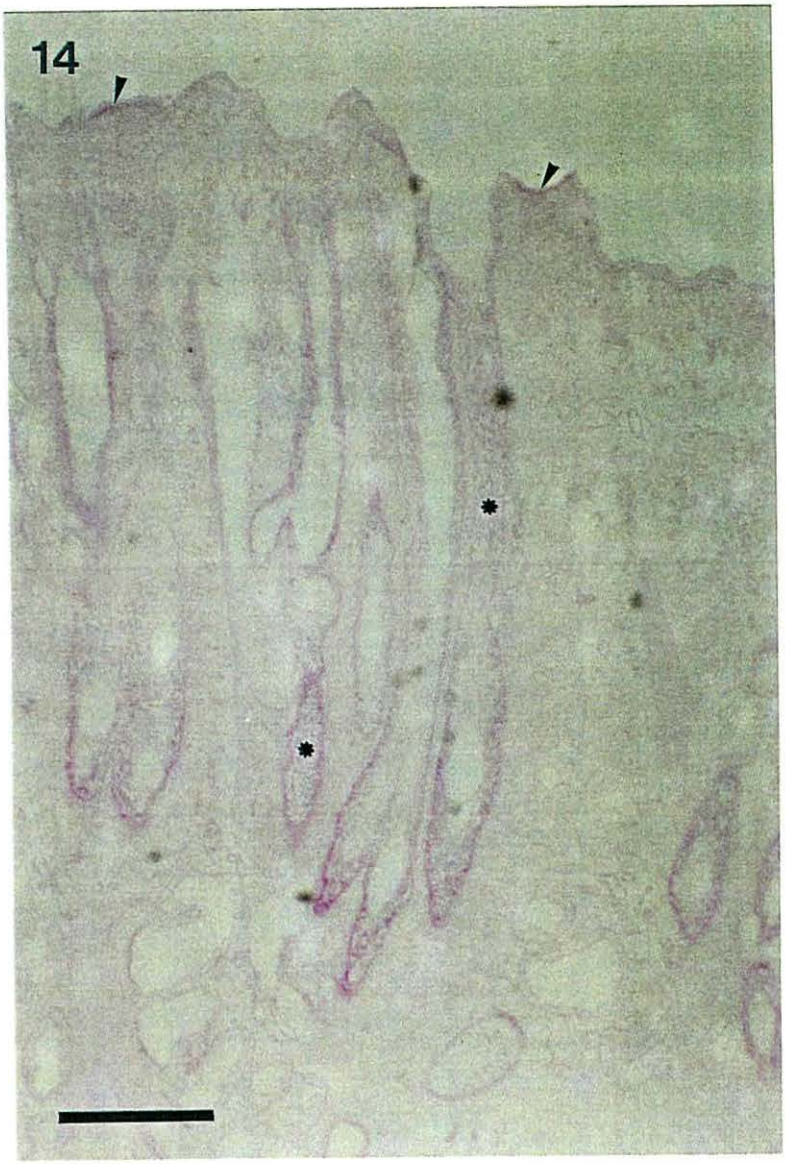

Figure 14. A vertical section through the skin layers demonstrating the location of carbohydrates associated with a group of wool follicles (*). Some surface material also stains positively (arrowheads). PAS. Bar = 500μm

Stain may be lipid in frozen sections.

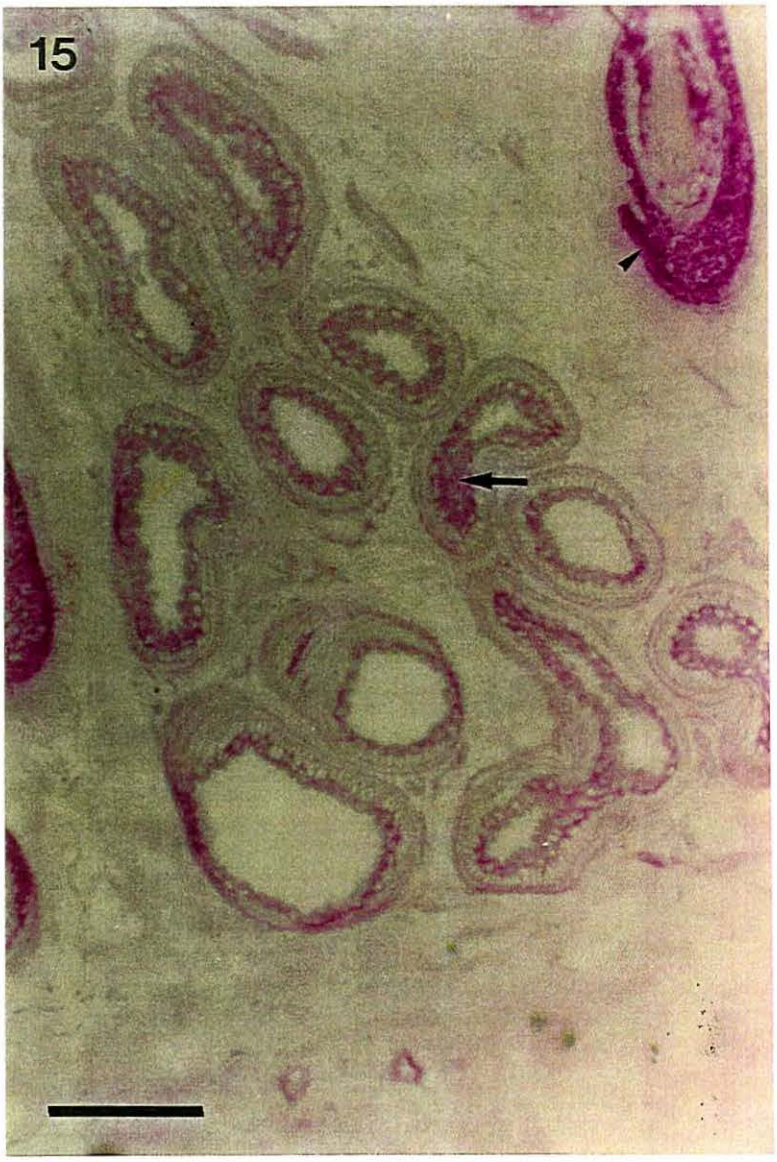

Figure 15. This section through a coiled apocrine sweat gland demonstrates the carbohydrate rich material in the lumen of the gland. A wool follicle (arrowhead) is also present showing the extensive carbohydrate associated with the follicle. PAS.
Bar = 200μm

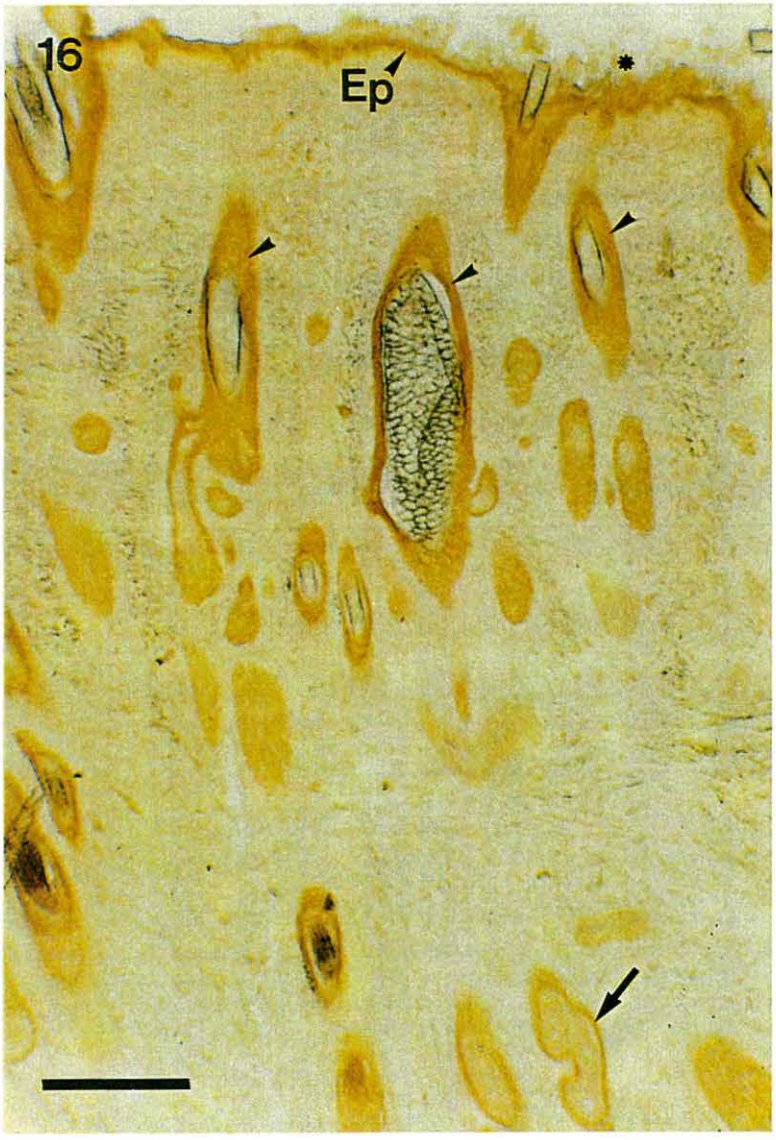

Figure 16. This vertical section of lateral skin illustrates the specific binding that results when skin is probed with rabbit anti-sheep serum albumin. The epidermis (Ep), wool follicles (arrowheads) and regions of the sweat glands (arrow) specifically stain with this antiserum. Material adjacent to the epidermis shows some affinity to the antiserum also (*). IP. Bar = 500μm

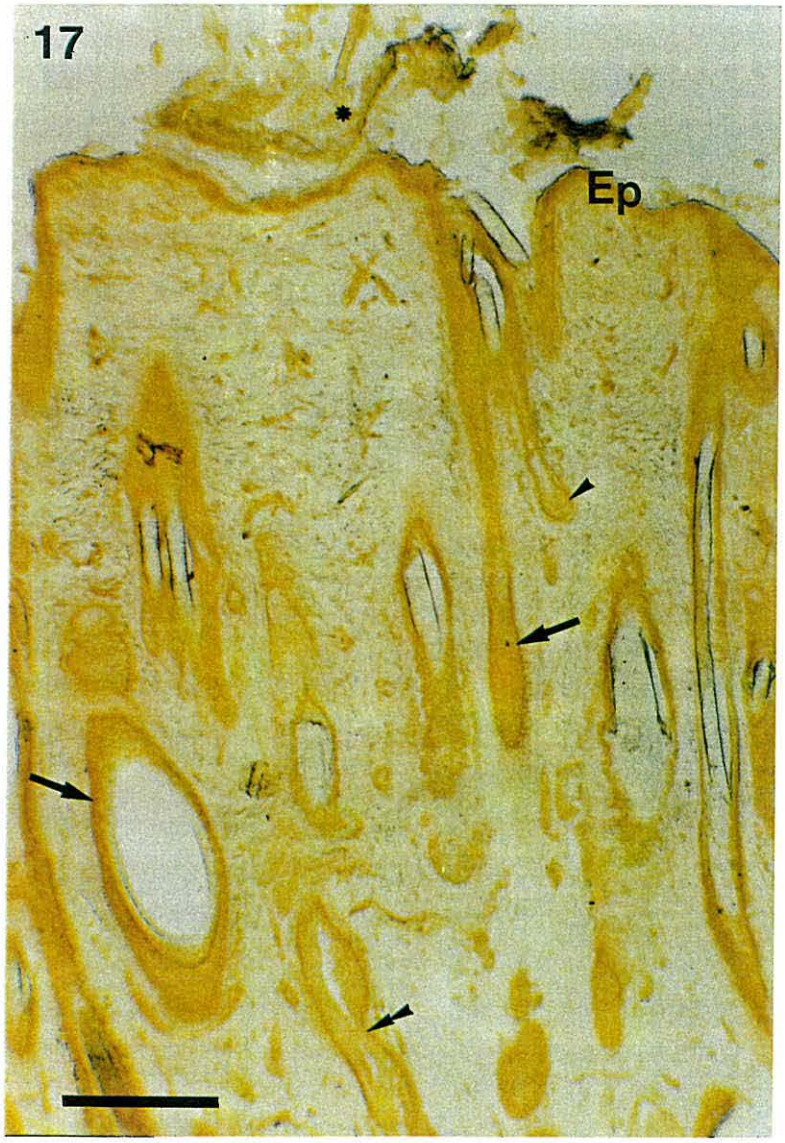

Figure 17. A vertical section of mid-lateral skin illustrating the staining pattern seen using rabbit anti-sheep erythrocyte stroma. Again there is a reaction to the superficial material on the epidermis (*), the epidermis (Ep), wool follicles (arrows), portions of the sebaceous glands (arrowhead) and sweat glands (double arrowhead). IP. Bar = 500μm

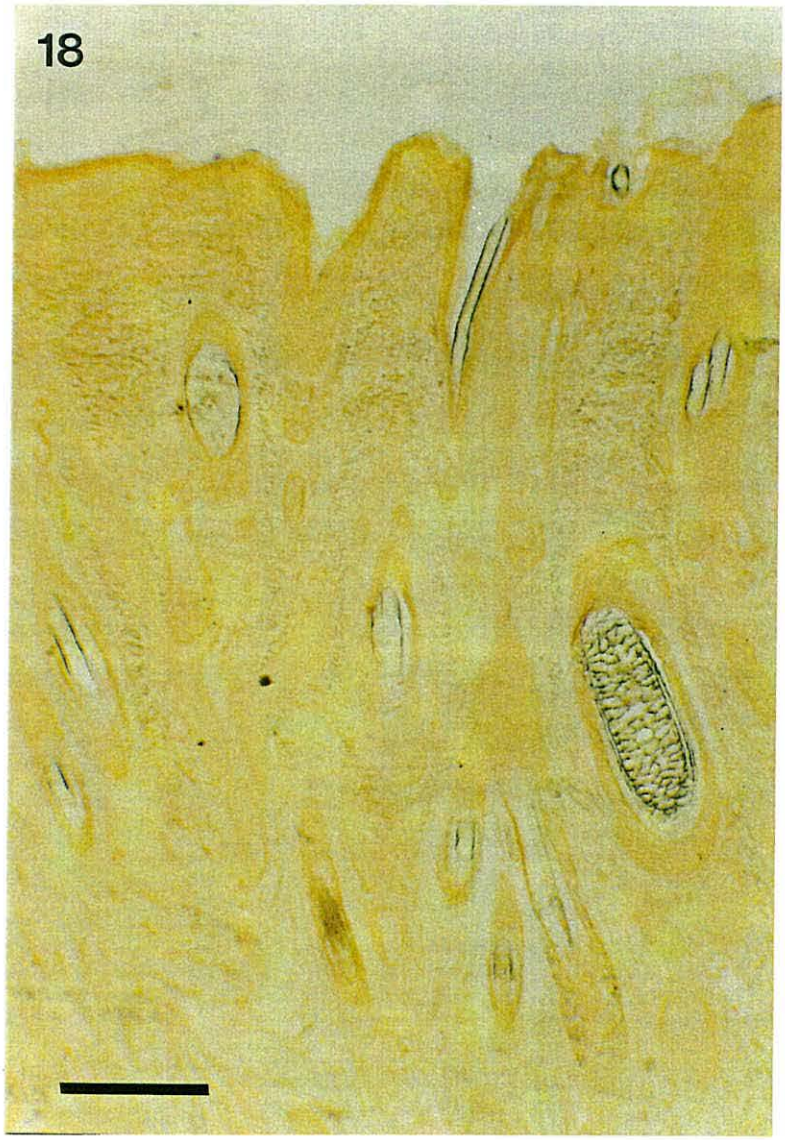

Figure 18. This vertical section shows the typical staining characteristics of a section stained with rabbit anti-sheep whole serum. The stain has reacted mildly throughout the dermis, more intensely to the epidermis and to a greater degree on the apical surface of the epidermis. IP. Bar = 500μm

Figure 19. This control section has been blotted with normal rabbit serum at the lowest dilution used for the other rabbit antisera investigated (1:400). IP. Bar = 500μm

Discussion.

The preliminary dot-blot investigation of the experimental mouse antisera demonstrated that antisera with specific affinity to the two mite extracts had been successfully raised. There was a limit to the amount of time that could be spent on this study, therefore the intervals between the three inoculations had to be kept to a minimum, and the period after the final inoculation until exsanguination, ideally should have been longer than the 47 days allowed in this study. The bloods were pooled from the experimental animals for the test bleeds and for producing the final antisera. With more time and with more antigen with which to screen the antisera, the bloods from the individual animals could have been tested separately to determine how each of the animals had responded to the inoculations and to investigate the antibody titres of each individually. With the limited time and resources we had for this study, it was necessary to carry out our investigation with some concession to the situation, nevertheless, the dot blot assay did demonstrate that we were successful in producing useful antisera specific to at least some of the materials we were interested in and worthy of further investigation.

The Western Blots of the two mite extracts blotted with either mouse α-POF or the mouse α-POS antisera revealed a more complicated picture than was previously anticipated. It was envisaged that the fed mite extract would contain both mite body-proteins and food materials ingested by the mites and that the resulting antiserum produced (α-POF) would have affinity to the mite's food and body proteins (this *is* probably the case with the α-POF). It was hoped the starved extract would contain only mite body proteins with only an insignificant amount of food proteins. We would then be able to compare the banding patterns of various extract/antisera permutations to determine which components of the extract were mite derived proteins and which were sheep derived. With this rationalism, the immunoblot of fed extract blotted with α-POF antiserum should demonstrate more bands than the starved extract blotted with α-POS antiserum, which would represent some proteins derived from the food materials

ingested by the feeding mites. Figures 2a and 2b fail to show this. In fact the opposite is seen, i.e. the starved extract blotted with α-POS antiserum has identified more bands than the fed extract blotted with α-POF. There are many bands common to both profiles, four of these stain intensely throughout the antisera-extract permutations investigated and are likely to be mite-body proteins. These bands correspond to proteins of approximately 66, 50, 37 and 30kDa respectively. Those bands obviously missing from Figure 2a and present in Figure 2b are relatively small molecules (38-64 kDa) and this could represent fragments of larger molecules ingested and, at least, partially digested by the mites, this could explain why they appear in immunoblots of starved extract blotted with α-POS antiserum and fed extract blotted with α-POS antiserum. The protein assays from this and the previous chapter demonstrated that the fed extract had a higher protein content than the starved extract, which would be expected, which again suggests that the additional bands seen in Figure 2b (and to a lesser extent in 2c and 2d) are products of digestion.

Figure 2c demonstrates a similar picture to the previously described figure. The blot presented here is starved extract blotted with α-POF antiserum. If the above described situation was occurring, then we would expect the immunoblotted antiserum (specific to mite body proteins and the ingested food proteins) to recognise the body proteins, showing a similar pattern to Figure 2a and the other blots, but also to recognise the food fragments that were demonstrated in Figure 2b. This is indeed the picture that is seen. The 12 "food" bands are less definitely stained in this blot though, possibly because the α-POF antiserum used to blot the starved extract contained fewer antibodies to the semi-digested food components since this extract would contain a greater proportion of newly ingested materials, resulting in a less distinct reaction between antiserum and extract. These assays have been used more as a qualitative assay than a quantitative assay but the reproducibility of the results must justify tentative conclusions on the reaction *quantity*.

The final permutation of extract and antiserum [Fig. 2d], fed extract blotted with α-POS antiserum, should demonstrate a similar banding pattern to 2b or 2c by showing the mite body proteins and fewer of the whole food molecules with possibly a number of the smaller molecules representing the breakdown products of the ingested meal, Figure 2d does show this although the 12 bands representing molecules of 38-64 kDa are fainter than in Figures 2b and 2c possibly because the fed extract contained fewer of the broken down molecules and the α-POS antiserum contained fewer antibodies to these components, as described for the situation seen in Figure 2c.

Lamb skin was used in preference to ewe skin as the lambs were less likely to have come in contact with sheep scab mites and therefore less likely to have immunological experience of the mites and their products which would undoubtedly interfere with staining characteristics of the α-POF antisera. The lamb skins were carefully examined for any clinical signs of sheep scab before one was chosen to be further processed. None of the skins examined showed any visible indication of sheep scab exposure nor did any of the flock before they were killed.

The α-POF antiserum (and the α-POS) stained material in the region of the stratum corneum, the epidermis and epithelial lined structures in the skin sections [Figs. 8 and 11]. The basal portions of the sebaceous glands also showed affinity to the antiserum [Figs. 8 and 9]. An infestation of *P. ovis* results in an increase in the production of surface skin lipids (sebum) (Sinclair, 1988) which the mites are likely to ingest from the epidermis (Sinclair and Filan, 1989), this is probably a component of the superficial material that stains positively for α-POF in the region of the stratum corneum. The sebaceous gland duct lumen, which transfers sebum from the sebaceous glands to the wool follicle, does not appear to stain positively with the α-POF, though. This is likely to be due to the antiserum not containing antibodies with affinity to the lipids that the sebum is composed of. It can be seen from the H&E stained sections that the staining characteristics of the sebaceous glands changes from the base of the gland to the junction with the wool follicle. Where the sebaceous gland cells break down to release

their contents the cellular material becomes obviously eosinophilic, whereas the majority if the gland stains normally with haematoxylin [Figs. 4 and 5]. It is possible then that the cells, or cell contents, may also have antigenic epitopes that change as the cellular contents are released from the cells into the gland lumen. Sebaceous glands stained with Sudan Black though, reveal that the entire structure stains positively for lipid [Fig. 6.]. It is possible that although the Sudan Black recognises the whole sebaceous gland structure as being composed of lipids, the α-POF antisera only contains antibodies to the intact gland cells, before they break down and release their content. It is difficult to draw firm conclusions from these results but they do go at least some way to corroborate the findings of Sinclair and Filan (1989) that *P. ovis* mites ingest skin lipids that probably originate from the sebaceous glands, although the lipids in the apical portion of the sebaceous glands do not appear to react with the α-POF antiserum, the cells of the sebaceous glands themselves do. This situation may be a question of kinetics. Sebum accumulates on the skin and in the gland but passes rapidly through the duct.

It is known that the mites do not ingest observable amounts of host-derived cellular material of epidermal origin (sloughed skin cells for example)(see Chapters 3 and 4) so the staining of the epidermis, epithelia and stratum corneum is not likely to indicate that the antiserum is recognising the epithelial cells directly, rather a material that is closely associated spatially with the epithelial cells. It has been demonstrated (Chapters 3 and 4) that the mite's digestive tract contains significant numbers of bacterial cells, some of which are likely to have originated from the extensive population of bacteria that are the normal fauna of sheep skin (Murray and Edwards, 1987). If the α-POF antiserum was detecting these bacteria (it is likely that the fed mite extract contains antibodies to the bacteria found in the digestive system) then the stain would appear to be seen superficially on the epidermis and not follow so intensely the wool follicles, this is in fact what was observed. We have discussed that the surface staining could also be due to some components of the sebum staining which is still a possibility, but the α-POF antiserum cannot differentiate between these components in this type of assay as the

antiserum undoubtedly consists of a multitude of antibodies to different skin components, it can only demonstrate the locations the antiserum has specific affinity to. If the antiserum was binding to the sebaceous lipids (or a material associated with the lipid) then we would expect that the stain would be detected along the length of the wool follicle, between the wool root and the epithelial cells. This is not seen. The epithelial cells of the wool follicle are stained but it is likely that the antiserum does not recognise the sebaceous lipids as such, which obviously does not mean that they are not being ingested.

Aside from the superficial staining, the α-POF antiserum also demonstrates an affinity for the epidermis [Figs. 8 and 11]. As briefly discussed earlier it seems improbable that the staining is due directly to a reaction between cellular material from the superficial skin layers and the antiserum, because of the absence of epithelial cells found in the mite's digestive system (see Chapters 3 and 4), but more likely to be associated with inter or intracellular fluids that become accessible to the mites during an infestation and which are more probably ingested from this location. By comparing the staining characteristics of the commercial antisera to the pattern seen with α-POF, this may help to reveal why the epidermis and epidermis lined structures stain with α-POF. Both the rabbit anti-sheep erythrocyte stroma and the anti-sheep serum albumin antisera demonstrated similar staining characteristics to those seen for the α-POF antiserum [Figs. 16 and 17]. Does this mean that the mites are ingesting significant amounts of these components in their normal diet? The serum albumin antiserum should stain areas where this protein is found and should be a specific indication of where *whole* serum is located also. The figures demonstrate that the serum albumin protein is detected specifically throughout the epidermis and regions of both the sebaceous glands and the sweat glands as are the erythrocyte stroma proteins. It was postulated earlier that the staining (with α-POF) observed with the sebaceous glands was possibly specifically due to the antiserum recognising sebum components. This is still a possibility, but the anti-sheep erythrocyte stroma and anti-sheep serum albumin both intensively stain similar

areas of the sebaceous glands [Figs 16 and 17]. This suggests that the recognised material is an erythrocyte component or serum albumin and not necessarily a sebum component. The staining that is observed with sebaceous glands and the sweat glands, detected by these two antisera is likely to indicate the presence of blood components in the vicinity of the two glands, they are after all liable to have a significant vascular system which may explain why the two commercial antisera and the α-POF antiserum reacts to these areas. The lumen of the sweat gland and the underlying cells demonstrate some affinity to the anti-erythrocyte, the anti-serum albumin and the α-POF antisera. It is unclear exactly what component of the sweat gland the mites may be utilising from the staining picture seen here, but the secretions of this gland have been shown to be rich in carbohydrate [Fig. 15]. It would be expedient for the mites to utilise this potentially nourishing resource which is very likely to be produced profusely during an infestation as the mites attempt to reduce the elevated temperature that accompanies an infestation (as discussed in Chapter 2).

The anti-sheep erythrocyte stroma reacted relatively intensely to areas of the skin, similar areas to those highlighted by the α-POF antiserum. This would suggest that the mites could be ingesting these blood components or that the α-POF antiserum is reacting or cross-reacting with some other antigen found at these locations. *P. ovis* mites will ingest erythrocytes from *in vitro* feeding systems (Deloach, 1984; Mathieson, unpublished), their feeding behavior on host is likely to be more complicated though. Wright and Deloach (1980) and Deloach and Wright (1981) investigated the feeding activities of *P. ovis* mites infesting rabbit's ears and since they demonstrated that the mites ingest a significant quantity of haemoglobin, concluded that the mites can pierce into the ear capillaries and ingest erythrocytes from there. This may not be the case though. The capillaries are likely to be damaged by the scratching activities of the rabbits or the inflammatory response that accompanies a *P. ovis* infestation, whether in rabbits or sheep, which would eliminate the mite's need to pierce the capillaries. The capillaries in rabbits ears are closer to the epithelial surface than in sheep skin so it is less

likely that even if the mites can feed invasively that they can access material from inside capillaries when feeding on sheep. *P. ovis* mites reared on sheep are occasionally seen to be stained red to varying degrees with what is presumed to be components of erythrocytes or intact erythrocytes (we believe that the mites are not capable of ingesting whole erythrocytes as the mite's pharynx is not sufficiently wide to accommodate intact red blood corpuscles and we have not observed erythrocytes in the digestive system). These reddened mites only represent a small proportion of the population of mites on each infested animal and are believed to ingest blood components opportunistically from small haemorrhages caused by the scratching activity of sheep (P. G. Bates, personal communication). These mites may represent a sub-group that do actively abrade the epidermal surface to cause small feeding lesions into capillaries, especially in rabbits, where the blood vessels are closer to the skin surface. Indeed the mite's mouthparts appear to be capable of at least abrading the epithelia (Blake *et al.*, 1978) but it is not clear how *P. ovis* mites use their feeding apparatus, the vast majority of mites collected at any time do not appear to have ingested a significant quantity of intact erythrocytes though. This corroborates the work by Rafferty and Gray (1987) who found that *P. ovis* mites feeding on sheep do not contain detectable levels of haemoglobin but do when infesting rabbit's ears. In Chapter 4 we described the mite's pharyngeal teeth which could be responsible for mechanically lysing erythrocyte membranes. So it is possible that the mites may ingest a small quantity of erythrocytes or erythrocyte components opportunistically from small skin lesions caused by the sheep scratching or from the inflammation that accompanies a *P. ovis* infestation, but which is not sufficient to be detected by the methods used by Rafferty and Gray (1987) but sufficient to be detected by the immunological methods employed here. In the previous chapter it was demonstrated using SDS-PAGE and western blotting techniques that the mite extracts do contain detectable amounts of erythrocyte stroma, even though the mites were discarded from the extract if they appeared in any way reddened.

The anti-sheep whole serum stains the skin sections to varying degrees [Fig. 18]. The epidermis stained more strongly than the dermis and presents a picture similar to the staining seen with α-POF. If the mites were ingesting a significant amount of serum then we would expect the α-POF to stain similar areas to the anti-sheep whole serum, this does appear to be what we see. The mites undoubtedly ingest other materials found associated with the epidermis which makes the overall picture slightly difficult to interpret.

This study did not proceeded as smoothly nor has it been as straightforward to interpret as was previously desired, but at least some relevant information has been acquired. Firstly we have shown that it is possible to raise antiserum to mite components using mice as experimental animals, antiserum which could be used in a variety of immunological assays. In this study the antiserum was used immunohistologically to demonstrate the location of sheep-derived materials (from *P. ovis* naive animals) that the mites ingest while feeding. The specific staining picture observed has been difficult to interpret but it has been demonstrated that the mites ingest fluid materials closely related to the epidermis which are likely to consist of serum and various blood components. Staining with commercial antisera has shown that the mites ingest material that are present in the skin from the same location in epithelial cells where erythrocyte and other serum components are found. From this work we cannot say whether the mites ingest these materials but this information coupled with the results of the previous chapter indicate that the mites are ingesting detectable amounts of erythrocyte components and at least certain components of serum. Further studies investigating the reaction between various blood components (lymph, plasma, serum, erythrocytes and so on), immunoblotted with the α-POF antiserum would produce more information on what materials the mites are actually utilising. This was not undertaken due to time constraints and insufficient α-POF antiserum following the immunohistological investigations conducted and presented here. The previous chapter investigated the antithesis to this,

immunoblotting fed and starved mite extracts with commercial antisera to various potential food materials derived from sheep.

The conventionally stained skin sections have shown that there appears to be a significant amount of lipid found on the skin surface, which originates from the sebaceous glands and which the mites are likely to ingest. We have demonstrated that there is material present in a similar location on the sheep's skin surface that stains positively with α-POF. It is unlikely that the sebaceous lipids are directly being detected at this location since the α-POF antiserum does not appear to recognise the lipids in the sebaceous gland ducts or surrounding the wool roots, it is possible though that the antiserum is detecting material very closely related to the lipids. Bacteria found on the skin surface are capable of breaking down the skin lipids which are rich in tryglycerides into free fatty acids and other metabolites (Vines, 1995) which are likely to be present in the mite extracts and thus the α-POF antiserum may recognise indirectly the immunological character of the lipid combined with the surface bacteria.

In this study we could not use our intended control antiserum (α-POS) since the mite sample population used to raise the antiserum were not sufficiently starved and so the antiserum contained antibodies with specificity to the mite's food materials. In future, with a more efficient "starving" procedure or using *P. ovis* raised exclusively on rabbits, it would be possible to produce this alternative antiserum which would be a useful tool to investigate this important subject further. Alternatively another mite species, with sufficiently different dietary requirements to *P. ovis*, perhaps *Dermatophagoides farinae* or a detritivorous soil mite extract could be used.

The work here has only investigated the staining characteristics of antisera used to probe naive sheep skin. Future work should also investigate the changing staining pattern seen when skin from infested animals is investigated. This would indicate whether the mites are utilising materials that arise from the skin during an infestation or if

they are driven away by the reaction they stimulate. It would also give us much needed information on the histopathology of sheep scab infestations.

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Chapter 8.

The Development of an *in vitro* Feeding Device for *Psoroptes ovis*.

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The Development of an *in vitro* Feeding Device for *Psoroptes ovis*.

Summary.

Four devices are described for feeding *Psoroptes ovis* mites off-host. The devices are described in the chronological order in which they were investigated -each new device attempts to overcome difficulties experienced with the previous system. The first device described used sheep blood agar from which the mites fed directly. The latter three systems used nylon gauze, filter-paper or a combination of the two through which to feed the mites on a variety of fluid diets. Each device is described in detail, as are the benefits and limitations of each. Data from preliminary investigations into the maximum survival attained on two experimental diets suggest that sheep plasma is a more appropriate diet than defibrinated sheep blood.

Introduction.

The long term goal of this thesis is to investigate the feeding biology of *Psoroptes ovis* which is little understood and to direct the information gathered towards producing an *in vitro* feeding system for the mites. The advantages of the existence of such a system are obvious. There would be far less need to depend on artificially infesting sheep, or rabbits, to study the mites and their reaction to candidate chemotherapeutic compounds or alternative control treatments. The system would provide a means of studying the mite's feeding activities, nutritional requirements as well as physiological, behavioural and developmental events. Novel control compounds, putative vaccination agents and so on could be more easily screened in an artificial feeding device and the responses of the mites monitored more closely than if they were free on sheep. Trials on sheep would not be completely eliminated but the dependence on them could be significantly reduced. Ultimately it may be possibly to maintain self sustaining populations of mites off-host, further reducing the reliance on infesting experimental animals. All these benefits would alleviate the immense costs that accompany keeping large, infested experimental animals in secure isolation and the unfortunate suffering that is inevitable with *P. ovis* infestations.

Artificial feeding systems have been developed to varying degrees of success for a variety of parasitic and haematophagus arthropods. Deloach (1984) produced a simple gauze feeding system for *P. ovis* to investigate the stimulatory effects of various fluids on feeding. Limited success was achieved by Wirtz *et al.* (1987) in feeding *Simulium damnosum*, the dipteran vector of River Blindness, through either silicon or baudruche membrane. Tarshis (1958) described a membrane feeding system for mosquitoes and fleas. Soft ticks have been successfully fed through a variety of membranes (Campbell and Oliver, 1984) and Bauer and Aigner (1978) developed an artificial device used to feed tsetse flies. Haematophagus mites have been successfully fed artificially by a number of workers. Durden and Linthicum (1992) have produced a system that

maintains a colony of mesostigmatid mites which feed through a gauze from suckling mice. Although this system does not replace the need for experimental animals, mice are perhaps a little more expendable than other larger hosts. The parasitic mites of honey bees, *Varroa jacobsoni* and *Acarapis woodi* have been successfully fed through a synthetic membrane system (Bruce *et al.*, 1991) and various workers have produced feeding devices for poultry mites using either gauzes or membranes (Crystal, 1986; Fletcher and Lancaster, 1984; Kirkwood, 1971). *P. ovis* mites do not penetrate into capillaries when reared on sheep, unlike the haematophagus insects and mites, so existing membrane feeding systems are unlikely to be modifiable to conform to the needs of *P. ovis*. The house dust mites, *Dermatophagoides* spp., can be maintained off-host on a variety of solid diets such as mixtures of yeast and human hair (Arlian, 1975) but their habitat, feeding mechanisms and dietary composition all differ to the parasitic and fluid feeding existence of *P. ovis* and so again, these previously developed *in vitro* feeding systems are not suitable for feeding *P. ovis* mites.

This work describes and discusses the advantages and limitations of various artificial feeding devices in a preliminary study to investigate the practicalities of producing a functional *in vitro* feeding device for *P. ovis*.

Materials and Methods.

Mite collection and preparation.

All mites were collected from artificially infested sheep at the Central Veterinary Laboratory by the methods previously described in Chapter 2. If necessary the mites were removed from exudate collected with the mites using mounted needles and carefully freeing the mites from the hardening material with the aid of a dissection microscope. The cleaned mites were usually separated into groups of adult females and introduced into the feeding device being investigated. Before each feeding trial commenced the mites were kept off-host, unfed for 1 hour at 82% relative humidity and 33°C in eppendorph cages (described in Chapter 2 Materials and Methods).

Experimental Diets.

Three experimental diets were investigated:

- Sheep blood agar (Device 1)
- Whole defibrinated sheep blood (Device 2, 3 and 4)
- Sheep plasma (Device 2, 3 and 4)

The blood and blood components came from sheep maintained for blood collection and had no previous history of sheep scab nor had they been treated with systemic or topical pesticides. Blood or blood components for all feeding trials came from these animals.

The procedures for introducing the experimental diets into each feeding device were particular to the system being investigated and will be described individually in the following sections. For all systems the number of mites remaining alive were recorded twice daily. Mites were considered "alive" if they demonstrated active mobility of at least four of their eight legs. Dead mites were removed during each routine inspection of the devices.

Feeding Devices.

Four feeding devices will be described here in the chronological order in which they were developed.

1. Mites on Sheep Blood Agar Plates.

10 adult female mites or 20 mites of mixed stage and sex were placed onto the surface of sheep blood agar bacteriological plates (nutrient agar with 10% sheep's blood). The agar plates were placed inside a large shallow container of water to impede any mites that escaped. The number of mites that fed could be easily determined by the colour change caused by the ingested erythrocytes or erythrocyte components. The mites were observed for a short period of time before the feeding devices were incubated overnight at 33°C. No attempt was made to manipulate the relative humidity the mites were exposed to as they were enclosed beneath a petri-dish and therefore the atmosphere was assumed to be near saturated.

2. Petri-Dish Gauze Feeder.

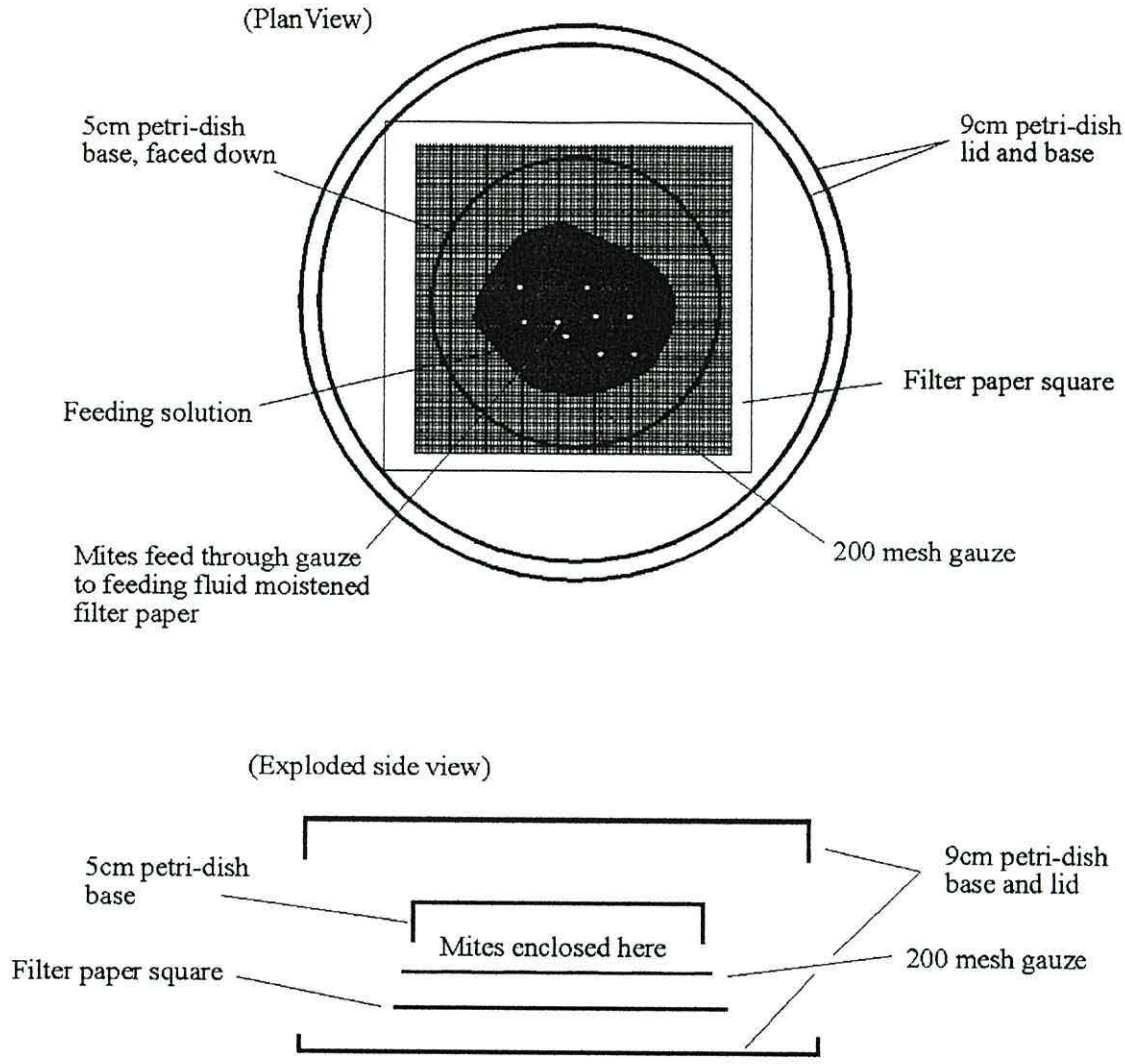
This system uses the permeability of nylon gauze and the absorbency of filter-paper to feed the mites. The mites (usually 10 adult females per trial) were placed on top of 200 mesh gauze (approx. $6cm^2$) lying over the filter-paper cut to similar dimensions and contained within the base of a 9cm plastic petri-dish. The mites are secured beneath the upturned base of a 5cm petri-dish held against the gauze beneath the lid of a 9cm petri-dish. The whole assembly was held together with rubber bands stretched around the larger petri-dish, this holds the smaller upturned petri-dish in place and prevents the mites from escaping [Fig. 1]. Sufficient feeding solution was applied to the filter-paper to moisten the lower surface of the gauze, but not allowing the fluid to be drawn through to the upper surface, before the larger petri-dish lid was secured on top. The whole assembly was placed on a hot-plate at approximately 35°C to create a temperature gradient to attract the mites to the base of the device. The feeding solution was re-applied when necessary once it had been dispersed and absorbed by the filter-paper by

removing the upper petri-dish and pipetting a small amount of fresh blood at the edge of the gauze and filter-paper exposed at the edge of the smaller petri-dish. Every 2-3 days the soiled filter-paper was replaced by dismantling the device, carefully separating the gauze and filter-paper and replacing it with a clean piece. New feeding solution applied and the device re-assembled. These are the general procedures used for investigating the potential of this device.

Two feeding trials were conducted from which data was recorded. Two groups of 10 adult female mites were incubated in separate, identical devices and fed on defibrinated sheep blood and incubated at 33°C. The feeding solution was introduced by the methods described above, the number of mites remaining alive was recorded twice daily, dead mites were removed when the feeding solution was re-applied. The mites were not transferred to clean feeding devices, one set of gauze and filter-paper was used throughout.

In these preliminary investigations the relative humidity within the device was not manipulated since the mites were enclosed beneath a petri-dish. The relative humidity was therefore assumed to be near saturated. The activity of the mites was easily observable through the clear plastic petri-dishes using a dissection microscope.

Figure 1.



Plan and exploded side-view of the Petri-dish Gauze Feeder. The mites feed through a 200 mesh gauze to filter-paper moistened with the feeding solution in the base of a 9cm plastic petri-dish. A small petri-dish base is up-turned and placed over the mites. The lid of the larger petri-dish is placed over the whole assembly and secured with rubber bands, thus holding the smaller petri-dish in place and preventing the mites from escaping.

3. Perspex Feeding Cylinder.

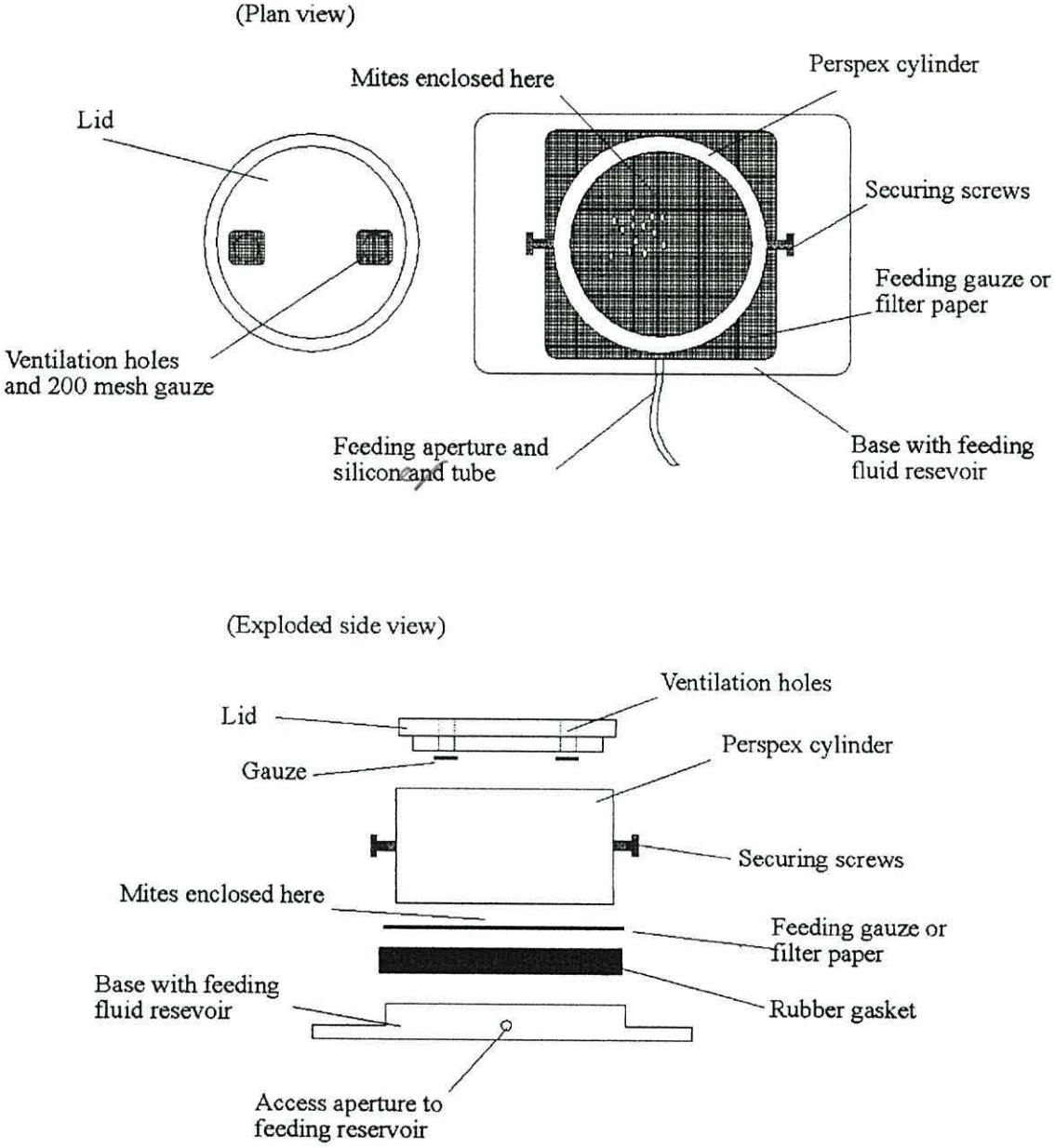
This device consists of a 2cm tall x 2.5cm diameter perspex cylinder with a removable perspex lid. Two small screws are secured into two tapped holes opposite each other on the outside of the cylinder. The lid has two 5mm hole covered on the inside surface with 200 mesh gauze. This is designed to help the circulation of air and to equilibrate the relative humidity inside the device with that outside. The mites feed through either 90, 100 or 200 mesh nylon gauze or Whatman grade 4 filter-paper, placed on top of a rubber gasket, to the feeding solution contained within the well of a separate plastic base. The cylinder is secured to the base by rubber bands stretched around the feeding base and securing screws on the perspex cylinder. The cylinder is therefore pressed against the gauze and the rubber gasket preventing the feeding solution from escaping from the feeding reservoir, securing the mites inside the device and making the whole apparatus easier to manipulate, thus keeping disruption of the mites to a minimum. The base has a small aperture, laterally placed, through which the feeding solution is injected to the fluid reservoir using a syringe and needle via a narrow silicone tube inserted into the aperture [Fig. 2a and b]. Sufficient feeding-fluid is injected until the fluid moistens the under-surface of the gauze only, or moistens the filter-paper if used. The narrow gauge of the tube prevents the fluid from leaking back out of the feeding chamber. The fluid is replenished daily as it evaporates and congeals. After 24 hours incubation the soiled gauze was washed by injecting a few millilitres of warmed phosphate buffered saline (PBS) at approximately 30°C through the feeding solution inlet tube and withdrawing it. This is repeated several times until the majority of the congealed feeding solution had been cleaned from the gauze. When filter-paper was being used, the device was taken apart, the mites were carefully removed from the soiled material and placed on fresh filter-paper in another device. Routinely, 10 adult female mites were investigated per trial but occasionally 20-30 mites of mixed development and sex were investigated.

Mite survival was compared for two feeding fluids: sheep plasma- prepared by rapidly spinning down fresh whole sheep blood, and defibrinated sheep blood from the same stock animals. The two feeding fluids were investigated in parallel in identical feeding devices. 10 adult female mites were introduced into each device and fed on equal volumes of feeding fluid. They were fed once daily and before each second and subsequent feed the gauzes were washed by the previously described methods. The number of mites remaining alive in each device was recorded twice daily, dead mites were removed as they arose. 3 replicates were conducted for each feeding medium. These trials used 200 mesh gauze throughout, filter-paper or the finer meshed gauzes were not as strictly investigated.

Behaviour and feeding activities of the mites were easily observed through the perspex lid of the feeding chamber. Temperature and relative humidity were kept constant by incubating the device in an incubator at 33°C, 82% relative humidity. The desired relative humidity was achieved by suspending the feeding devices above a saturated solution (aqueous) of potassium chloride (Wiston and Bates, 1960) in 17 x 11.5 x 6cm sandwich boxes (Stewart Plastics, crystal boxes, type 145) [Fig. 2b]. It was not possible to heat the base of the device therefore this system did not use a temperature gradient to stimulate feeding.

Treating the gauzes to reduce its permeability to the feeding medium was briefly investigated. The 200 mesh gauze was dipped in paraffin wax and suspended vertically in an oven at 55°C with tissue paper clamped to the lower end to absorb excess wax. This precedure rendered the gauze more hydrophobic by coating the individual nylon filaments with a thin film of wax. The finer mesh material was sprayed on both surfaces with PTFE waterproofer to achieve the same effect. These modifications were not investigated in a controlled fashion but comments on their function will be made in the Results and Discussion section.

Figure 2a.



Plan and side-view of the Perspex Feeding Cylinder. The mites are enclosed within the perspex cylinder by the lid above and by the gauze or filter-paper below. Rubber bands secure the cylinder and feeding gauze against the rubber gasket and prevent loss of feeding solution from the fluid reservoir. The feeding solution is introduced into the base of the device through a small aperture via a silicon tube inserted into the aperture. The fluid fills the feeding reservoir and moistens the gauze or filter-paper from where it can be ingested. The mites can be observed feeding and counted through the clear lid of the device.

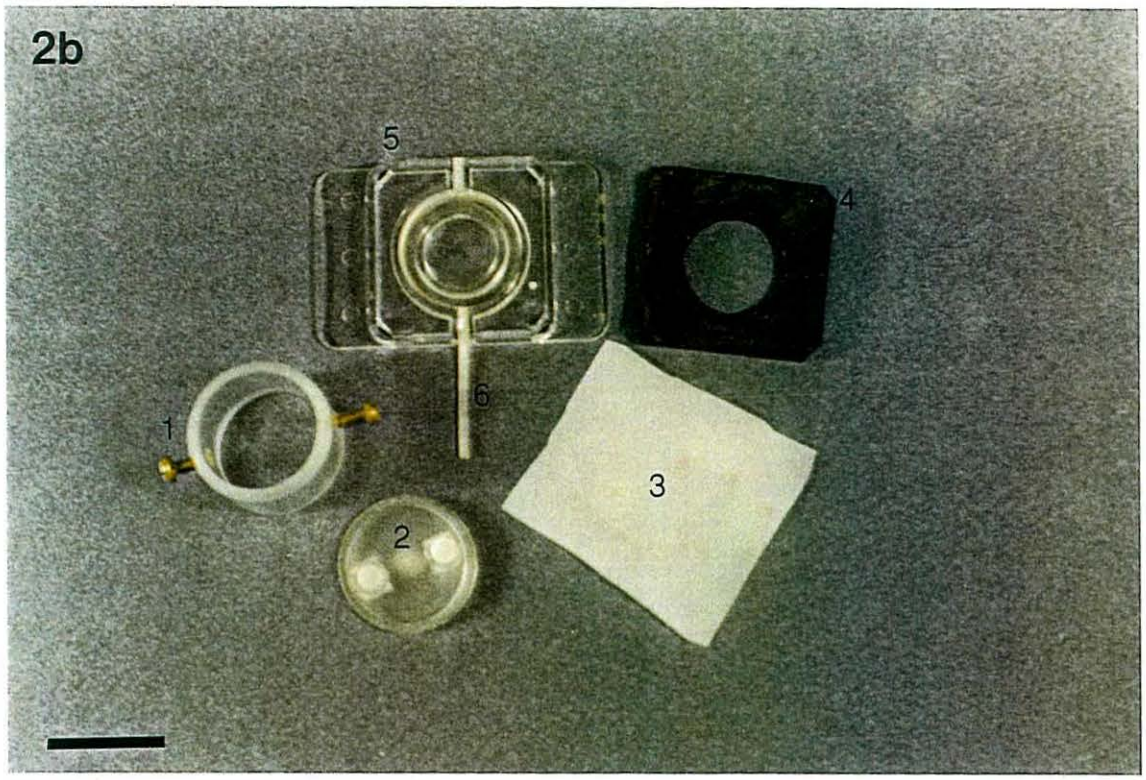


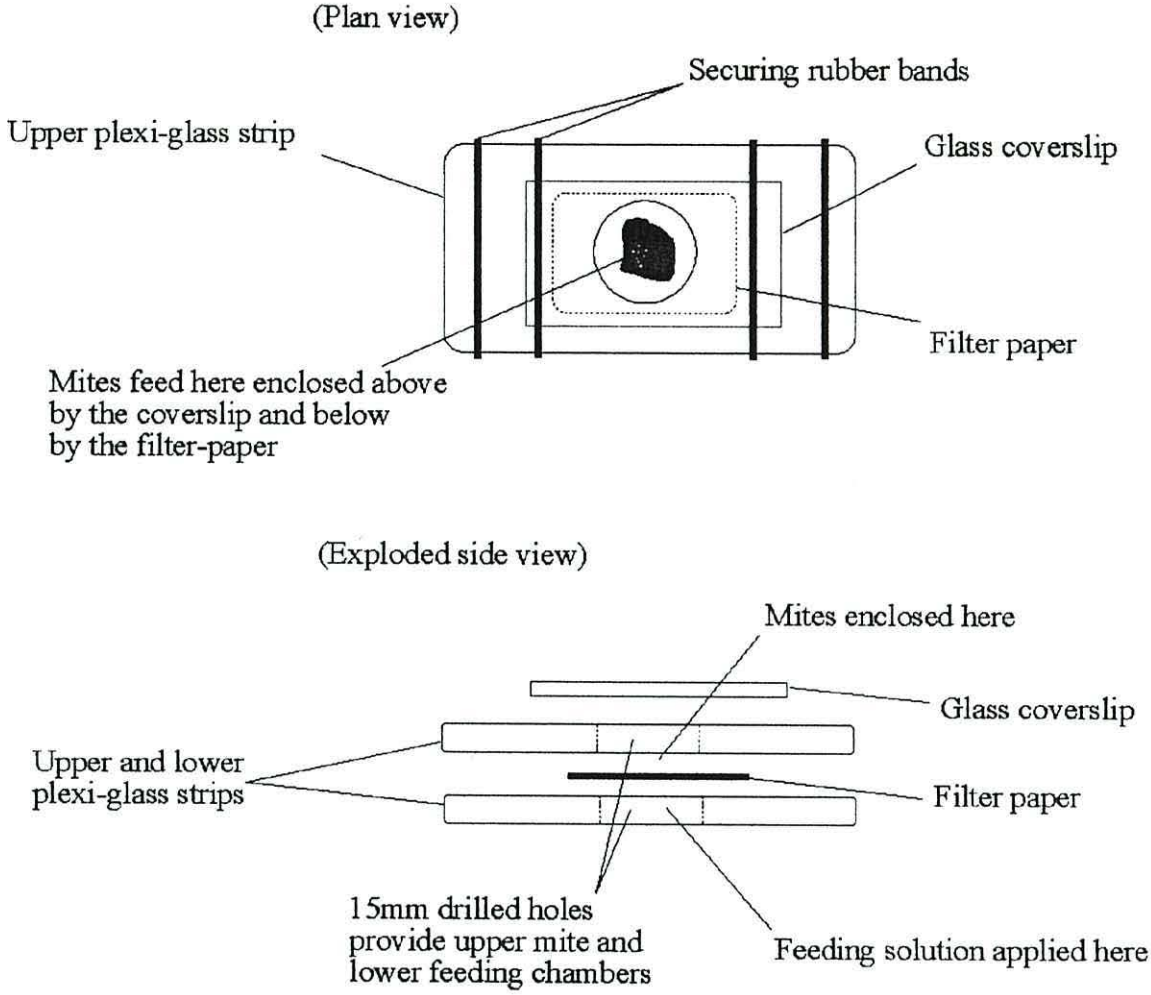
Figure 2b. The components used to construct the Perspex Feeding Cylinder. The mites are enclosed within the perspex cylinder (1) by the lid above (2) and below by the gauze or filter-paper (3). Rubber bands secure the cylinder and feeding gauze against a rubber gasket (4) which prevents loss of feeding solution from the fluid reservoir (5). The feeding solution is introduced into the base of the device through a small aperture via a silicone tube (6) inserted into the aperture. The fluid fills the feeding reservoir and moistens the gauze or filter-paper from where it can be ingested. Bar =25mm.

4. Plexi-Glass Feeding Device.

This device consists of two 60 x 30 x 3mm plexi-glass strips with a 15mm hole drilled through the centre of each. The two strips are placed one on top of the other, the holes adjacent, with Whatman grade 4 filter-paper cut to 20 x 20mm placed between the two. A pair of rubber bands stretched around the two ends of the strips secures the filter-paper in place. The mites are placed onto the filter-paper and enclosed within the cylindrical feeding area (3 x 15mm) by a glass coverslip cut down in length to approximately 25 x 20mm and secured with another pair of rubber bands [Fig. 3a and b]. The feeding solution is applied to the under surface of the filter-paper through the lower hole by inverting the device and replenishing twice daily.

A preliminary survival trial was conducted to investigate the survival of mites in this device fed on either sheep plasma or defibrinated sheep blood. The two feeding fluids were investigated in parallel in identical feeding devices. 10 adult female mites were introduced into each device and fed on equal volumes of feeding fluid, applied by the methods described above and replenished twice daily. 3 replicates were conducted in close succession for each feeding medium. The number of mites remaining alive was recorded twice daily, dead mites were discarded by removing the rubber bands securing the coverslip to access the feeding area. The temperature was controlled by incubating the devices at 33°C, the relative humidity could not be manipulated as the unit was sealed and therefore was assumed to be near saturated. After approximately 3 days the feeding solution had usually congealed reducing the amount of fresh solution that could be absorbed. At this stage the mites were removed from the soiled feeding device and transferred into a clean one. This was carried out for both groups whether it was necessary for both or not.

Figure 3a.



Plan and side-view of the Flat Plexi-Glass Feeding Device. The mites are placed inside the aperture of the upper plexi-glass strip on the filter-paper and enclosed beneath the glass coverslip placed on top and secured with rubber bands. The feeding solution is introduced by inverting the device and applying it through the aperture of the lower strip.

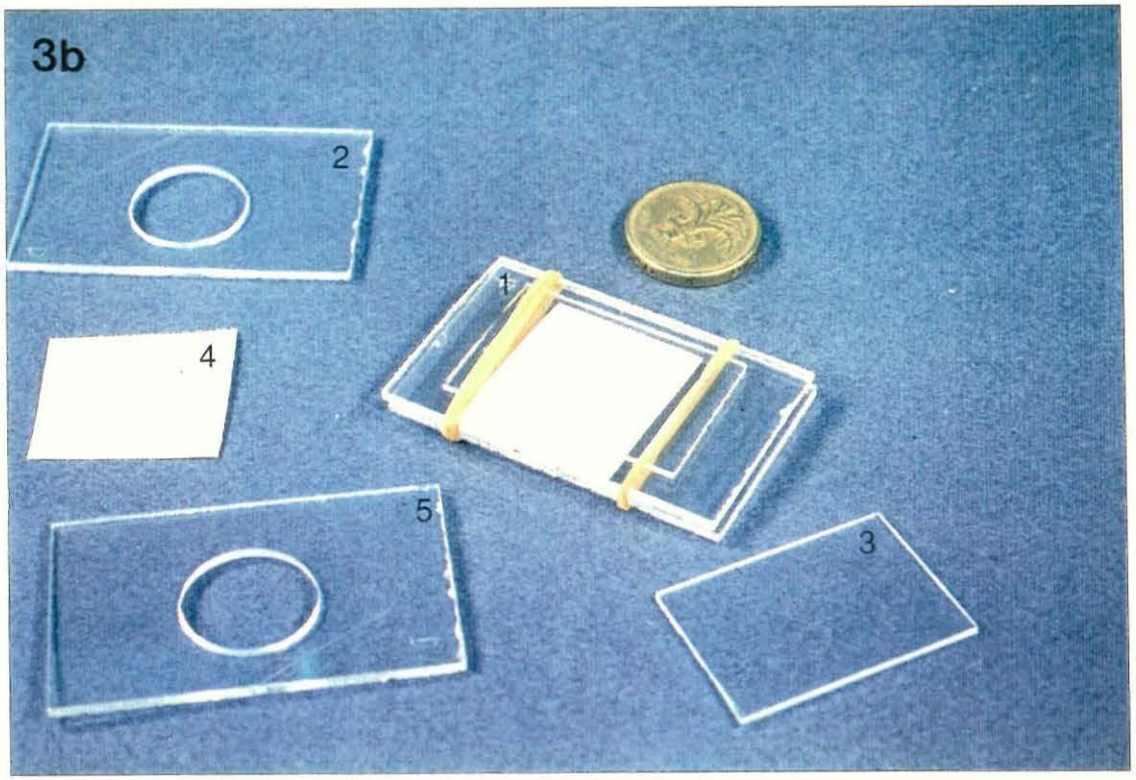


Figure 3b. The components used to construct the Plex-Glass Feeding Device (1). The mites are enclosed within the aperture of the upper plexi-glass strip (2) enclosed by a glass cover slip above (3) and below by the filter-paper (4). The device is held together by elastic bands. The feeding solution is introduced by inverting the device and applying it through the aperture of the lower strip (5).

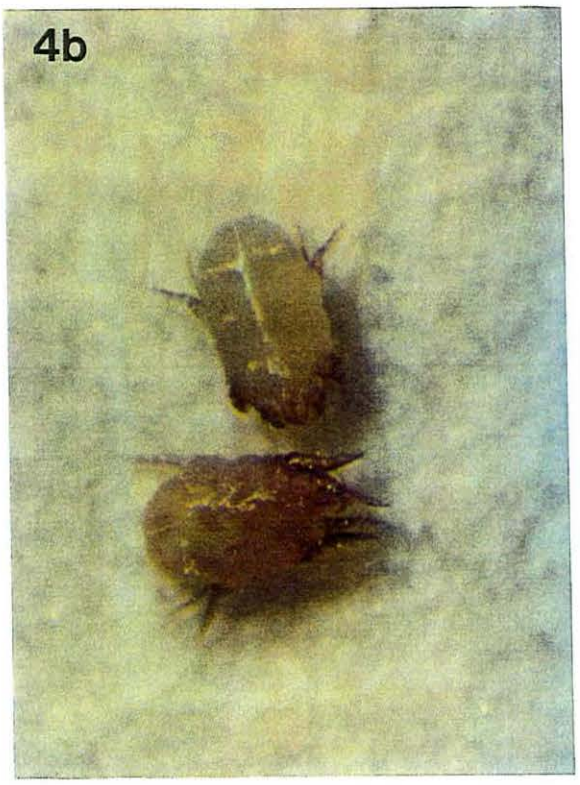
Results and Discussion.

The data for the survival of mites in the second two feeding devices are not directly comparable with each other as the trials were not run in parallel. With some initial investigations comparing mite survival on different experimental diets and with the observations made during the investigations, significant conclusions can be made about the merits and limitations of each device, which will enhance future development of a fully functional *in vitro* **feeding** system and pave the way the production of perhaps an *in vitro* **culture** system in the future.

Device 1: Mites on Sheep Blood Agar Plates.

With this device the mites wandered briefly on the surface of the agar, impeded to varying degrees depending on the size of the individual mites, by the viscous nature of the material. After a few seconds they paused and appeared to be feeding from the plate surface as their mouth-parts could be seen ventrally curved towards the substrate surface, characteristic of feeding *P. ovis* mites. It was soon noticed by the changing colouration of the mites' bodies that they were ingesting material from the feeding substrate, as they became reddened internally with erythrocyte components [Figs. 4a and b].

Several other mites from earlier life stages and several adult males were placed onto the agar surface and incubated overnight at 33°C. The following day a proportion of the mites remained alive, generally the larger stages, as it appeared that the surface moisture and viscosity of the medium overcame the earlier life stages and engulfed them, preventing them from moving and presumably affecting their normal respiration. Also, as they tried to move over the surface of the agar the clawing action of the mites' legs, during normal locomotion, often caused them to dig themselves under the agar surface becoming trapped. A further difficulty the mites faced was from the bacteria they undoubtedly carry on their body surface and others that they excrete with their faeces (see Chapters 3, 4 and 5) which become established on the SBA and very quickly over-



Figures 4a and 4b. Mites that have ingested blood components (erythrocytes or haemoglobin) become reddened as the material within the digestive system is visible through their translucent cuticle. These figures both compare a blood-fed and a plasma fed mite. Bar =1000μm.

came them, especially since they were incubated at an elevated temperature. In retrospect, the introduction of a bacteriostat into the feeding medium (paraformaldehyde or an antibiotic, for example) may alleviate this bacterial problem, although the effect this may have on the mite's normal gut fauna, the role of which is unknown, may be deleterious to the mite's survival.

This device demonstrated that it is possible to feed the mites using very simple and readily available materials. It is not possible to state how much longer than overnight the mites may have survived without the problems caused by the bacteria, as this system was not pursued any further, but all the mites observed fed to a certain extent before they were engulfed by surface moisture or bacteria. The following device was developed in an attempt to alleviate the various difficulties experienced with this simple initial device.

Device 2: Petri-Dish Gauze Feeder.

The second feeding device, using nylon gauze placed over feeding-fluid moistened filter-paper, was developed directly after the previous system. The gauze was introduced to raise the mites above the feeding substrate and thus prevent the "drowning" observed in device 1. SBA was abandoned because of the difficulties with bacterial invasion. An immediate advantage of this new system was that by using fluid moistened filter-paper, rather than SBA, several different feeding solution can be easily investigated and compared, although this was not done here. The mites were also far more mobile than they had been when they were on the SBA. For a short time after being introduced into the device they actively crawled over the gauze surface, unhindered by surface moisture. They soon became stationary and appeared to be feeding from the fluid below and after several minutes their bodies became reddened by the blood components they were ingesting. No record was taken of the numbers that had fed, but a significant number (perhaps 50%) did successfully feed, whether to repletion or not could not be determined.

The mites generally remained alive for a mean maximum of approximately 114 hours. By this time the gauze and filter-paper had again become saturated with congealed blood. The mites could have been transferred into a clean device but a new device had been developed which was designed to alleviate the difficulties experienced with congealing feeding solution and was therefore likely to produce superior survival. Although device 2 was not investigated in a strictly scientific manner, it has demonstrated that it is possible to stimulate the mites to feed in a relatively simple system. Whether the temperature gradient enhanced the feeding stimulation is unknown but feeding trials without this feature (i.e. with the previous and subsequent devices) has demonstrated that it is perhaps not necessary.

The survival results were not as promising as one would expect, especially if compared with the survival of mites off-host and unfed (see Chapter 2). One would expect their survival to be enhanced if the mites were allowed to feed. The mean maximum survival time of **unfed** mites incubated under the same conditions was 147.7 hours ±9.2 (SEM) whereas in this preliminary investigation the **fed** mites survived for only 114 hours ±18.0. Adult female *P. ovis* mites are believed to survive for an average of 40 days (960 hours) on-host (Bates, 1991) and up to 15 days under field conditons off-host (O'Brien *et al.*, 1994). The conditions in the device or the feeding fluid appears therefore to be far from optimal. The results from this study with device 2 are not conclusive though as only a very small number of trials were conducted and the poor survival results could be attributed to several factors. One is undoubtedly due to the physical problems the mites face when the feeding solution congeals, blocking the gauze and also solidifying to the mites appendages and disrupting their normal functioning. It is also possible that the relative humidity in the device is too high and causes deleterious effects. In consequence, another device was developed taking into consideration both the ventilation and solidifying feeding medium problems. It is unlikely that the introduction of defibrinated sheep blood, components of which the mites naturally feed upon (see Chapters 6 and 7), would reduce the longevity of the mites in comparison to a

starved group. The effects of two different feeding solutions are investigated more thoroughly in the next device.

Device 3: Perspex Feeding Cylinder.

This device was developed to alleviate the limitations and problems experienced with the previous system. The gauze or filter-paper again raised the mites above the feeding solution but there were two further alterations. The first was the introduction of ventilation holes into the lid of the device, which it was assumed would allow the internal environment to equilibrate with that outside. Secondly, the problems experienced earlier with the feeding fluid congealing to the mites and gauze were alleviated to some extent by introducing a design feature which allowed the gauze to be washed intermittently. This did not appear to disturb the mites to any great extent although it was occasionally necessary to turn the mites back onto their legs as they would become overturned during the washing procedures and become trapped by the surface tension forces of a thin film of the cleaning fluid that remained after cleaning. The advantages of cleaning the mites this way appeared to outweigh the disadvantages.

The mites readily fed through the gauzes or on the filter-paper. When defibrinated sheep blood was under investigation the mites became reddened by the ingested blood components within an hour. Sheep plasma was also investigated. Data from preliminary investigations suggest that plasma produces better survival times than the defibrinated blood. In two of the three paired trials the mites survived longer when fed on plasma [Fig. 5]. Overall, the mean maximum survival times on defibrinated blood and plasma were 152 hours ±22.3 (SEM) and 220 hours ±32.7 respectively (related t-test, $p=0.16$). The survival times for mites fed on plasma compares favourably with those mites incubated under similar conditions but unfed (data from Chapter 2)(unrelated t-test, $p=0.16$). Mites fed on blood did not survive longer than the unfed group ($p=0.9$) [Fig. 6] . These preliminary findings reflect to some extent the observations made by Deloach (1984). Although in that study the *number* of mites stimulated to feed on a

variety of diets was under investigation rather than the *survival* times of mites. Deloach (1984) demonstrated that the mites feeding response to blood was reduced compared to plasma, 50% plasma, serum and even water. In our study only a small number of replicates were conducted to investigate the two feeding solutions, if more replicates were conducted perhaps significant differences would be demonstrable.

Figure 5. Maximum Survival Times (hours) for the three paired replicates investigating mites fed on either Sheep Plasma or Defibrinated Sheep Blood, using the Perspex Feeding Device. The devices were incubated at 33°C at 82% relative humidity.

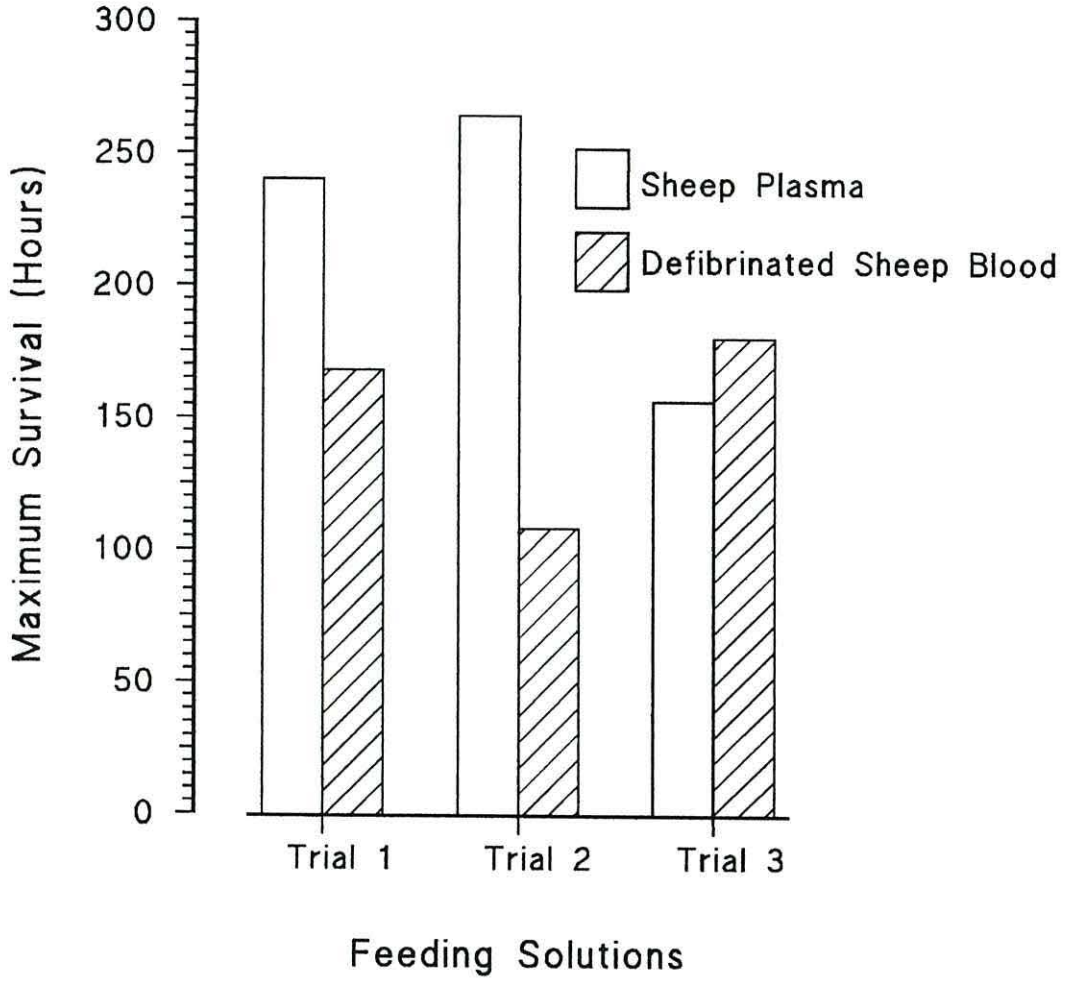
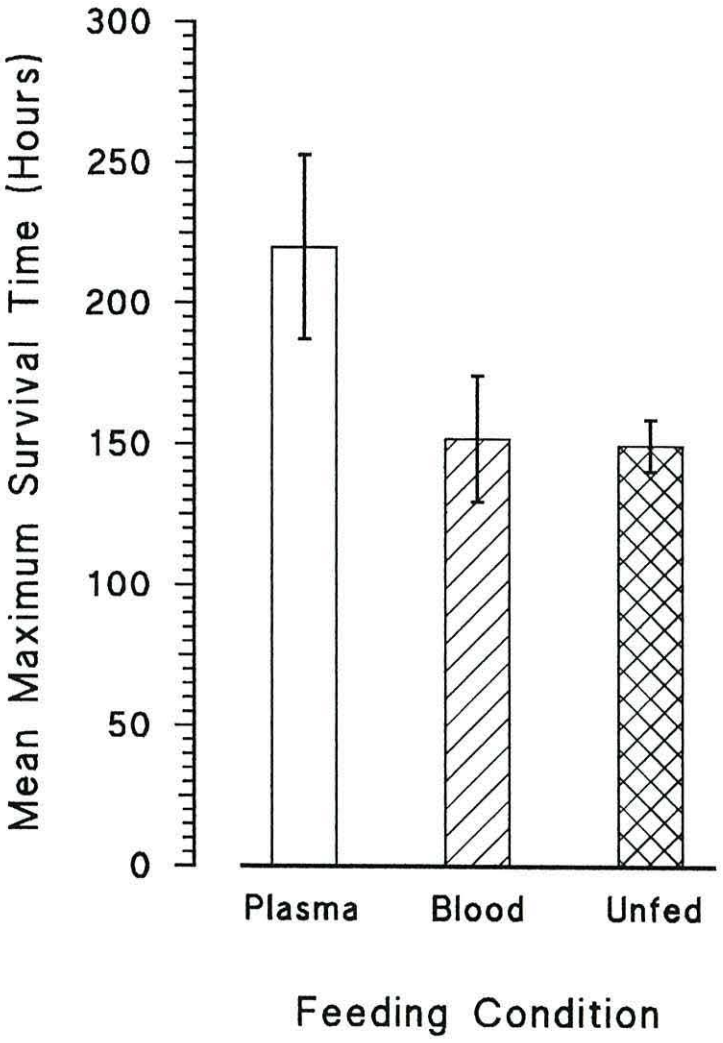


Figure 6. Mean Maximum Survival Times (hours) of three paired replicates investigating mites fed on either Sheep Plasma or Defibrinated Sheep Blood in the Perspex Feeding Device. Data for unfed mites is included, all mites were incubated at 33°C at 82% relative humidity.



Related t-tests were carried out on the mean maximum survival times attained for mites fed on plasma and blood. Unrelated t-tests were carried out on the data from the two fed groups of mites and the data previously collected for unfed mites. There was no significant difference found between the mean maximum survival times of mites fed on plasma or blood ($p=0.16$) or between plasma fed mites and unfed mites ($p=0.16$) nor blood fed and unfed mites ($p=0.9$). Standard error of the means (±SEM) are shown.

This feeding system has demonstrated that it is possible to maintain a sample of mites off-host, fed under conditions that reflect the conditions the mites experience on-host for up to 12 days. This still falls some way short of the 40 days Bates (1991) believes to be an average life-span of *P. ovis* females. Although from this preliminary study it was not possible to demonstrate a significant difference between the survival of the mites on the different diets or unfed, the initial results propose that sheep plasma is a more suitable feeding material than sheep blood. The reason why blood appears to produce inferior results is unclear. Various workers have been able to maintain mites off-host for longer periods (Stockman, 1912; Shilston, 1915; Bedford, 1915; Dill, 1920; O'Brien *et al.*, 1994) but in those cases the mites were exposed to lower temperatures as they were investigating the survival of mites off-host under conditions they are likely to find themselves naturally i.e. sheep enclosures, scratching posts and so on. Although these conditions may enhance survival, the mites' normal physiological functions are likely to be reduced which would undoubtedly affect feeding, ovipositing and so on.

A great advantage of having a functional *in vitro* feeding system would be its use in screening novel control compounds, whether systemic or topical acaricides or candidate antisera for vaccine control. A requirement of such a feeding system would be that it would have to be able to stimulate the mites to feed and maintain them for a sufficient time for the deleterious effects of the novel agent to be compared with the mites' normal death rate. Whether this device, in its current state, is capable of such trials has not yet been studied but could open the doors to some very worthy investigations. It may be possible to enhance the longevity of mites off-host combining off-host and on-host trials by shuttling the mites between the feeding device and experimental sheep. The mites could be removed from sheep and exposed to the test compound (a new chemical acaricide for example) in a feeding device and allowed to feed for a time before being replaced back on sheep, perhaps enclosed to prevent them migrating too far. The effects of the compound could then be observed more precisely

on-host with the deleterious effects of the *in vitro* device reduced. The incubation time in the device and the exposure to the control agent could be varied to determine the optimum feeding time for maximum mortality and so on.

This perspex feeding device initially stimulated the mites to produce eggs but after a few days no further eggs were deposited. Their subsequent development was observed but only a small proportion of them hatched. Whether a proportion of the females had not been inseminated or whether the conditions were inappropriate for further development is unknown. The few 1st stage larvae that emerged did not survive more than 24 hours or so, they succumbed to the same surface tension difficulties described previously. The main purpose of these studies was to develop a *feeding* system rather than a *culture* system so egg deposition and development and the difficulties experienced by the earlier life-stages was not an immediate concern and therefore not investigated further.

The surface tension problems, first experienced when the gauze was being washed, manifested itself more severely later in the trials. The problem was created by an interaction between the feeding fluid, the gauze and the mites close association with the two. Occasionally the capillary action of the gauze drew the feeding fluid through to where the mites were contained and engulfed them in the fluid. Due to the size of the mites they became immobilised in the solution and quickly perished [Fig. 7]. This undoubtedly affected the survival of the group. To alleviate this problem the gauzes were coated with either paraffin wax of a PFTE waterproofing spray. The wax treatment alleviated the problem to some extent; the feeding fluid tended to remain below the gauze for longer, minimising the mites' disruption [Fig. 8]. The waterproofing spray did not improve the situation to any observable degree. Further trials were not conducted with these modifications due to time restraints but it merits further investigation.

It is doubtful whether membranes would be an appropriate material through which to feed *P. ovis* because the mites are unlikely to be able to abrade a synthetic

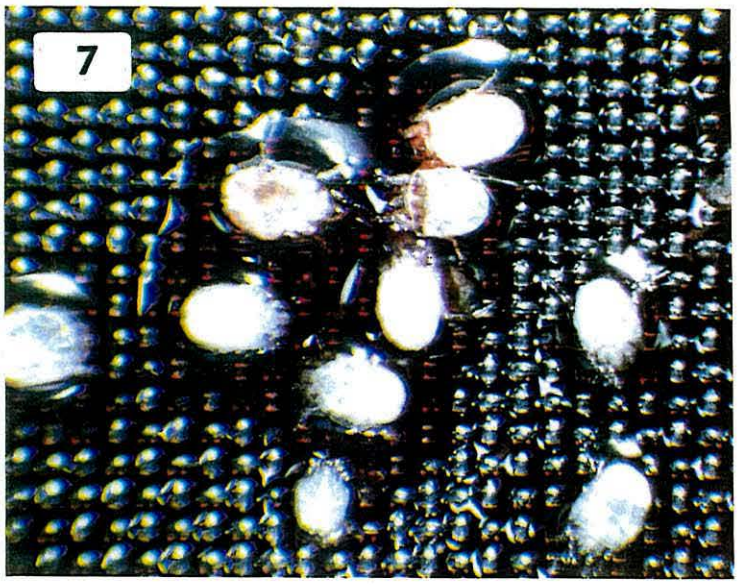


Figure 7. Several adult female *Psoroptes ovis* mites feeding on sheep blood through untreated 200 mesh nylon gauze. Note the blood is drawn through the gauze surrounding and engulfing the mites.

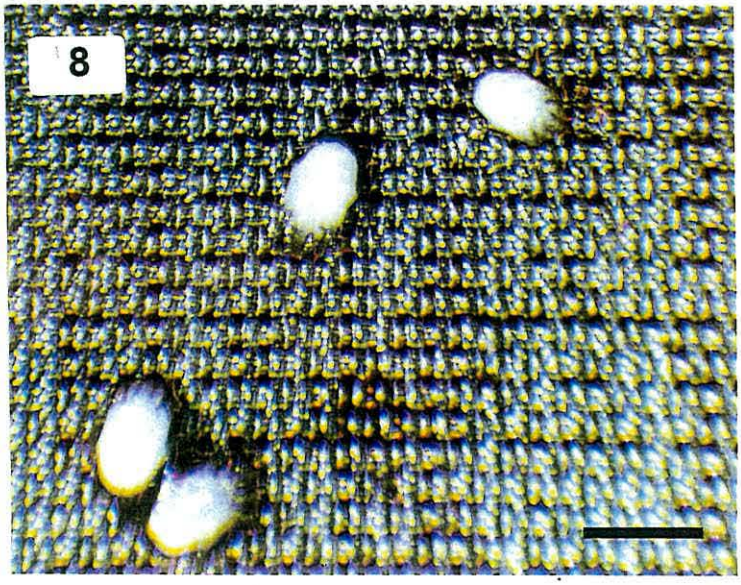


Figure 8. These adult female mites are seen feeding through 200 mesh nylon gauze treated with paraffin wax. It can be seen that the amount of fluid drawn through the gauze has been reduced. Bar =1000μm.

membrane. Mosquitoes can be successfully fed through natural lambskin condoms. Unfortunately we were unable to find a supplier for the material but briefly investigated the suitability of sausage skins (rolled sheep small intestine) through which to feed the mites. Scanning electron microscopy was then used to examine the skin for evidence of piercing or abrasion. The sausage skin was permeable to the feeding solution removing the mites' need to disrupt the membrane. The results were inconclusive.

A final problem experienced with device 3 was perhaps since the system did not incorporate a temperature gradient to attract the mites to the feeding substrate, the mites often failed to remain in contact with the gauze or filter-paper and instead wandered around the inside of the device, often gathering between the lid and the side of the perspex cylinder. They would remain there, not attempting to feed unless they were manually moved down to the feeding solution. This problem coupled with the difficulties with the feeding fluid being drawn into the feeding area stimulated the necessity to develop a device which would alleviate these obstacles.

Device 4: Plexi-Glass Feeding Device.

Although the previous device overcame certain problems experienced with the previous two systems other unforeseen difficulties arose. The plexi-glass feeding device was developed to confront the difficulties experienced with the mites migrating away from the feeding fluid and the problems associated with the feeding solution saturating the nylon gauze and disrupting the mites' normal behaviour. This was achieved by significantly reducing the height of the feeding chamber from 20mm to 3mm and exchanging the feeding gauze for filter-paper. It was not possible to wash the filter-paper (as it had been when gauze was used) but this did not seem to be too much of a sacrifice.

The data generated from the survival trial comparing the longevity of mites on blood and plasma was comparable to the data produced by device 3. In all of the paired trials, mites fed on plasma survived longer than mites feeding on blood [Fig. 9]. When

the results are combined the mean maximum survival time on plasma was 200 hours ± 25.0 (SEM) and 164 hours ±18.3 for blood [Fig. 10]. Related t-tests were carried out on the mean maximum survival times attained for mites fed on plasma and blood and unrelated t-tests were carried out on the data from the two fed groups of mites and the data previously collected in Chapter 2 for unfed mites. There was no significant difference found between the mean maximum survival times of mites fed on plasma or blood ($p=0.11$) or between blood fed mites and unfed mites ($p=0.49$) but mites fed on plasma did survive for significantly longer on plasma compared to unfed mites ($p=0.02$).

Figure 9. Maximum Survival Times (hours) for the three paired replicates investigating mites fed on either Sheep Plasma or Defibrinated Sheep Blood in the Plexi-glass Feeding Device.

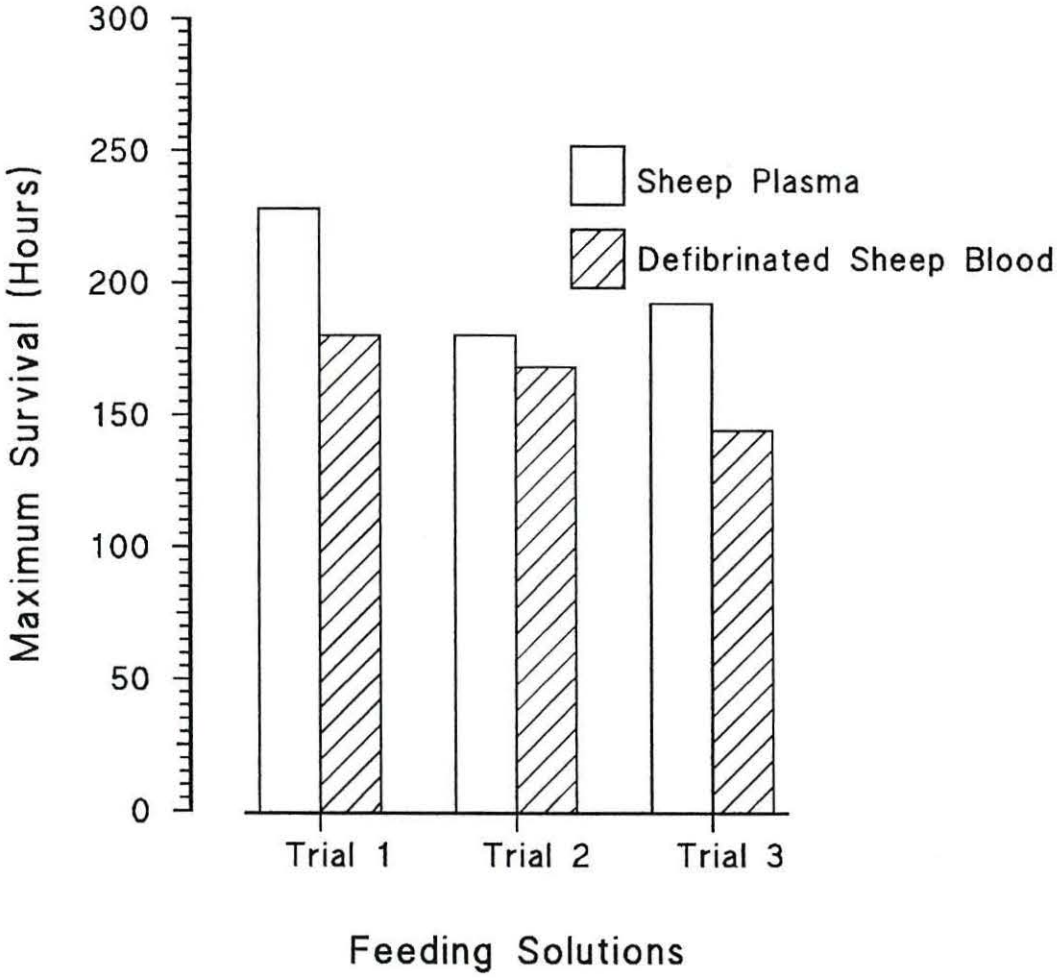
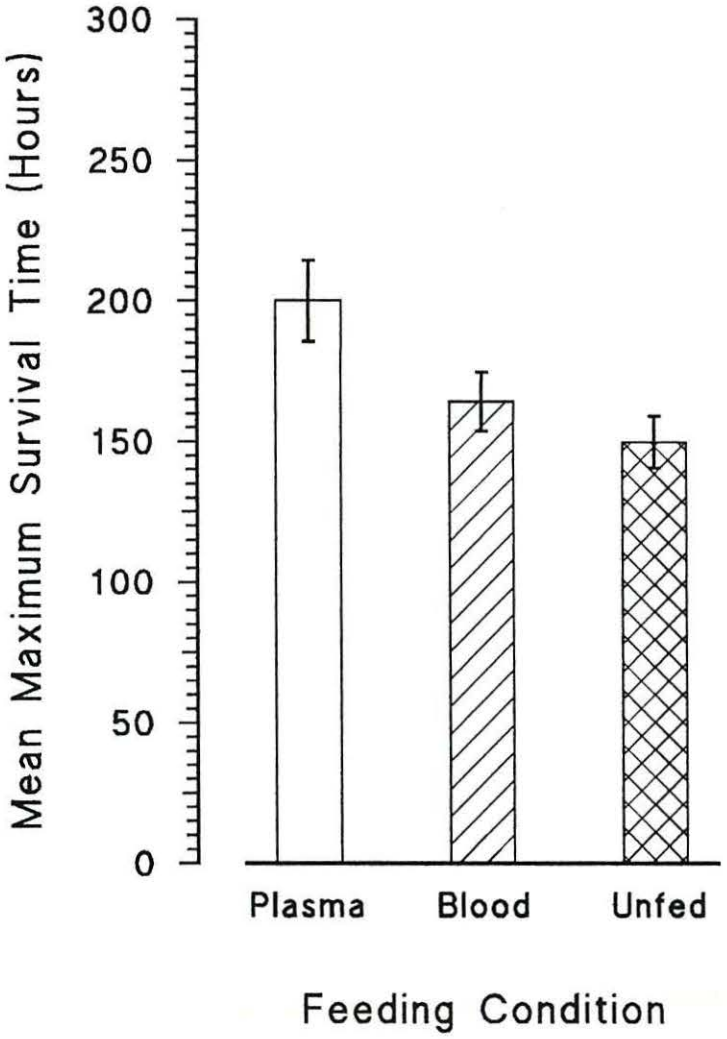


Figure 10. Mean Maximum Survival Times (hours) of three paired replicates investigating mites fed on either Sheep Plasma or Defibrinated Sheep Blood in the Plexiglass Feeding Device, including data for unfed mites.



Related t-tests were carried out on the mean maximum survival times attained for mites fed on plasma and blood. Unrelated t-tests were carried out on the data from the two fed groups of mites and the data previously collected for unfed mites. There was no significant difference found between the mean maximum survival times of mites fed on plasma or blood ($p=0.11$) or between blood fed mites and unfed mites ($p=0.49$). Mites fed on plasma survived for significantly longer on plasma compared to unfed mites ($p=0.02$). Standard error of the means (±SEM) are shown.

Comparing the results from the survival trials of the two devices, the Standard Error of the Means were smaller with the Plexi-Glass Feeder than they were with the Perspex Feeder, possibly due to the reduced disruption caused by feeding fluid leaking into the feeding area and a greater proportion of the mites feeding due to the reduced height of the feeding area. The mean maximum survival times are not significantly different to each other (Table 1). Only when the results are compared with the previously performed survival off-host unfed trials, does there appear to be a significant difference between the survival conditions. Due to the small number of replicates conducted only tentative conclusions can be made about the implications of these results.

Table 1. Mean maximum survival times (hours) and Standard Error of the Means (SEM) for two feeding devices and two feeding solutions. Number of trials in each case was three.

Feeding Solution	Perspex Feeding Device		Plexi-Glass Feeding Device	
	Mean Max. Survival	SEM	Mean Max. Survival	SEM
Plasma	220	32.7	200	14.4
Blood	152	22.3	164	10.5

As with the previous device, there were several eggs laid under both feeding conditions (blood or plasma), within the first few hours of incubation. Their development was observed but, as before, very few matured even to first stage larvae. The number of eggs produced in any of the feeding devices was far fewer than the 2 per day reported to be laid on host (Kirkwood, 1986). It appears that the conditions in the devices are not suitable for egg development, but as described earlier, the main purpose of these studies was to develop a *feeding* system rather than a *culture* system so egg deposition and development and the difficulties experienced by the earlier life-stages was not an immediate concern and therefore not investigated further. Nevertheless, poor egg production is a clear indication that nutrition or other conditions are sub-optimal.

The design of this device made it difficult to replace the filter-paper once it had become soiled. This was also experienced with the previous device when filter-paper was used. Unfortunately the next best alternative was gauze which had other problems associated with it. It was possible to remove the mites to a clean feeding system, but this practice was not convenient, especially if large numbers of devices and mites were under investigation. It would be beneficial if these systems, or a derivative of them, could feed the mites and keep them viable for an extended period of time, it would be more likely that the devices could be used successfully to screen candidate antisera for vaccination control programs and other new control agents. There are also a host of questions to be answered on many aspects of the mites' feeding behaviour which could be more easily studied in a device that could maintain mites for a longer time.

All of the devices investigated and described above have demonstrated that it is very possible to feed *P. ovis* mites off-host, whether simply on blood agar or a more complicated filter-paper or gauze based device. Of the two feeding fluids investigated it could be tentatively suggested that plasma appears to stimulate better survival than defibrinated blood, more trials are needed before any firm conclusions are drawn. The mites do feed on both fluids but whether they feed to repletion or whether the diets are sufficiently nutritious to fulfil the mites' metabolic activities, is not known. The feeding media should be tested with a variety of supplementary compounds vitamin B12, for example. The low numbers of eggs produced and the poor development that followed is likely to be attributable to several factors. The conditions inside the devices may be sub-optimal or even deleterious to mite survival, especially the earlier stages. The problems the adults and earlier developmental stages experience with feeding fluid saturating the gauze or filter-paper may also be harmful to the developing eggs. Further work is needed to develop a feeding substrate which allows the mites access to the feeding material but which minimises the disruption to the feeding mites. One of the many commercial breathable fabrics, Goretex ™ for example, may hold some of the key features necessary for an effective material.

It appears that *P. ovis* mites will feed from a variety of materials *in vitro* (Deloach, 1984), whether they are nutritious or not. It is important then that we do not depend on the mites to inform us what they naturally feed upon or what constitutes a diet that is nutritionally complete. The limited survival experienced with the latter two devices, as well as the poor egg development, could be a result of the experimental diets not being sufficiently nutritious. This would explain why initially the mites deposit eggs but subsequently very few, if any, appear. It is possible that the mites use the nutritional resources from their last meal taken on-host to fuel the production of the eggs, subsequently deposited in the *in vitro* feeding devices, and that the succeeding meals from the devices are deficient in factors essential for egg production. These problems may be reduced if a basic experimental diet, plasma for instance, were to be enriched with other nutritious materials; serum albumin, lanolin, erythrocyte components and vitamins for example, and the devices were modified to alleviate the problems associated with fluid and gauze capillarity. As they stand though the perspex feeding device and the plexi-glass device have both demonstrated that they are sufficient to allow the mites to feed and survive off-host. In this study we have only demonstrated that the mites survive significantly longer than control unfed mites when incubated in the plexi-glass feeding device and fed on sheep plasma ($p=0.02$). It is likely that further survival trial replicates would demonstrate additional significant differences between feeding solutions and the devices. It is important to remember that it is not necessarily imperative to maintain the mites off-host for an extended length of time for the purposes of screening novel control materials. The only requirement is to be able to demonstrate that the candidate control compound produces significantly higher mortality rates than the control environment. Both of these two devices potentially already have these attributes, only conducting screening trials will demonstrate this and should be investigated immediately.

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Chapter 9.

General Discussion.

Chapter 9.

General Discussion.

This project comprises a wide range of investigations all related to the eventual production of an *in vitro* culture system for *P. ovis*. It has been noted that this long-term goal needs to be approached in stages and here investigations have centred on producing an *in vitro* feeding system.

The development of an artificial feeding device for *P. ovis* could have been targeted directly by aiming a significant effort towards producing an *in vitro* feeding device and investigating the mites' survival on a variety of fluids and enriched feeding media. With time and a constant supply of mites an optimum feeding fluid would eventually have been developed. At the same time the mites' natural environment could have been investigated and the feeding device modified accordingly. This *modus operandi* may have succeeded in producing a more successful device with greatly enhanced mite survival and perhaps more significant egg production and development. Alternatively, the outcome may have been disappointing leaving us with a great deal of data and various devices falling short of our intended goal. The approach we chose to take has resulted in the investigation of many more interesting and fruitful subjects delving into numerous previously un-investigated areas crucial for the overall comprehension of this extremely important ectoparasite.

The many subject areas that were investigated, and the techniques that were developed and used in the realisation of our long term goal began with determining the environmental conditions that would produce optimum mite survival off-host. The study demonstrated that the mites are rather more sensitive creatures than previously appreciated and do not appear to be robust when removed from their natural environment. The results of our study suggested that the mites' natural environment is likely to be characterized by a near saturated atmosphere and indeed longevity was enhanced at the higher relative humidities investigated. These findings must be put in

perspective and considered when incorporated into an artificial feeding device. The perspex feeding device described in Chapter 8 was designed to include ventilation holes to allow the manipulation of the environment inside the device and the equilibration with that outside. The subsequent difficulties that arose with this device made it necessary to incorporate further design features in a new system (the plexi-glass device) which at the time abandoned the use of ventilation holes. Whether this caused deleterious effects on survival is not known. It may be that the near saturated atmosphere within the plexi-glass device was similar to the mites' natural environment and did not interfere with the mites' normal activities. Although from our studies we have shown that the mites survived for a significantly longer time at 82% relative humidity and 33°C compared to the other conditions investigated, it is possible that even these conditions are sub-optimal for mite survival or perhaps for egg production and development. Sheep scab is a disease that manifests itself in a range of guises, the clinical signs vary and are likely to create a range of environments the mites must cope with. Whether the environment selects for mites that can withstand the conditions or whether the mites can survive under a range of conditions is unknown but the situation could obviously be rather complicated. Further investigations into finding the optimum conditions for survival and development of all stages may prove very fruitful.

The structure and ultrastructure studies demonstrated that the digestive system of *P. ovis* follows the general constructional characteristics of a typical arthropod (cuticle-lined fore and hindgut with a microvilli-lined midgut) but the precise layout of the constituents appears to be unique. Of the other acarine digestive systems that were compared to *P. ovis*, the alimentary canal of the house dust mite *Dermatophagoides farinae* does show some similarities in both the general structure and the cellular activities. Both systems, and many other arachnids, have characteristic cells in the midgut that absorb luminal material by pinocytosis, leave the gut epithelia before degenerating- demonstrating intracellular digestion. Sheep scab mites also possess an intermittent tissue described for many other acarines, found adjacent to the epithelial

basement membrane and anchored to these cells by finger-like protrusions. The lipid and glycogen inclusions and the close relationship the tissue has with the epithelia, likens it to the fat-body of insects and suggests it has a secondary metabolic function. If we are to assume that *P. ovis* evolved from a free-living or semi-parasitic life-style, perhaps inhabiting the bedding of domestic animals, before exploiting its chosen host further and becoming an obligate ectoparasite, it is perhaps not surprising that *D. farinae* with its close association with the human habitat possesses a similar digestive system to a parasitic mite such as *P. ovis*. Unfortunately there was little structural or ultrastructural information on other medically or veterinarily important parasitic mites to be found in the literature with which to compare *P. ovis*. It would be interesting to compare and contrast the digestive systems of other parasitic mites with *P. ovis* and their respective diets.

These microscopy studies revealed that the mites' diet appeared to be almost exclusively liquid. There was no evidence to indicate that the mites were ingesting a significant quantity of erythrocytes or other cellular material from the epidermis. The study demonstrated that they do ingest a significant number of host-derived eosinophils. These are characteristically found at the epidermis during a *P. ovis* infestation where they are ingested by the mites along with other fluid materials. Whether they have a deleterious effect on the mite is unknown but it is plausible that the eosinophils' lysosomal enzymes could disrupt digestion in some way, either by active release or passive release during digestion of the cells. Alternatively, they may be an important dietary component. Further studies may be able to demonstrate any significant role these cells play.

The ultrastructure study demonstrated that the mites possess a peritrophic membrane which appears to be formed in the post-ventricular midgut. Faecal pellets are voided from the mites wrapped in the membrane. It has been suggested that the antigenicity of this material could contribute to the pathology of sheep scab and that it could be used as an antigen in a vaccination program. Additional investigations are

needed to clarify the role that this structure plays in the mite's digestion and its role in disease pathology.

The structural and ultrastructural studies also showed there is a significant population of bacteria resident in the mites' digestive system and these are liberated in the mites' faeces. Further investigations demonstrated that bacteria from the faeces and digestive system are viable and these proved to be the enterobacter *Serratia marcescens* which from previous studies has been closely linked with the disease sheep scab and life-threatening infections in other animals. This reveals, potentially, a very interesting situation in which the bacterial fauna of *P. ovis* could explain the variation in disease pathology that occurs in different geographical locations, and indeed in different animals in the same outbreak, and expose a new target area for the control of the disease. The bacteria could also be an important source of nutrition for the mites or perhaps have an important digestive role. Whatever the significance the bacteria have to the disease or the mites' physiological functioning, the subject deserves immediate and thorough attention.

The histological investigation of the mite's digestive system was undertaken as a prelude to an immunohistological study envisaged to determine the mite's nutritional requirements by immunoblotting mite sections with antisera to materials the mites were likely to ingest. This study did not in fact proceed due to the difficulties experienced sectioning the mites and preserving antigenicity following fixation and embedding. It is likely though, with perseverance, the relevant techniques could be determined and further information on the mites' nutritional requirements attained. The techniques could also be used to prove the antigenic properties of the peritrophic membrane or other mite-derived materials.

These initial investigations into the structure and ultrastructure of *P. ovis* revealed many interesting features of this mite's digestive system and the techniques developed have paved the way for further studies investigating the function and cellular events that were previously unknown.

Two chapters were dedicated to determining the dietary requirements of *P. ovis* to conclusively demonstrate the material that the mites normally ingest and digest. This information is not only necessary for the production of an *in vitro* feeding or culture system but also much needed for the correct approach for the production of novel control procedures. It is absolutely necessary to have at least some understanding of the host-derived materials the mites are exposed to so the control agent can be presented in the correct manner. The first investigation examined electrophoretic profiles of fed and starved mites and demonstrated that the mites ingest material with a very similar electrophoretic profile to sheep serum. The assumption the material is sheep serum was confirmed in the following investigation which used conventional and immuno-histological techniques to determine the location and nature of the materials the mites ingest. It is possible that sheep scab control may be achieved through vaccination. This would only be possible if the mites ingest significant quantities of host-derived materials containing antibodies raised to a vital component of the mites. These two studies have demonstrated that the approach is viable. It remains to be seen if sheep can be immunologically stimulated to control a *P. ovis* infestation, perhaps using an *in vitro* feeding device in place of live sheep trials to screen candidate antisera.

Appendix A.

A. Composition of Susa's Fixative and Procedures for Conventional Histological Staining for all resins investigated in Chapter 3. (Following the removal of excess mercuric chloride, if applicable)

Susa's Fixative:

1. Mercuric Chloride 45g
2. Distilled H_2O 800mls
3. Trichloroacetic acid 20g
4. Glacial acetic acid 40mls
5. Formaldehyde (40%) 200mls

Toluidine Blue:

1. Toluidine blue — 30 secs on hot-plate at 50°C.
2. Drain off excess stain
3. Wash in tap water — until stain no longer dissolves (few secs).
4. Dry on hot-plate and fix coverslip with DPX.

Ehrlich's Haematoxylin and Eosin:

1. Ehrlich's Haematoxylin — 30-40 mins.
2. Wash in tap water — 1 min.
3. 1% acid alcohol — few secs.
4. Wash in tap water — few secs.
5. Few drops of lithium carbonate — to blue.
6. Wash in running water — 1 min.
7. 1% Eosin — 10 mins.
8. Wash in tap water — few secs.
9. Dry on hot-plate and fix coverslip with DPX.

Appendix B.

1. Reagents and Gel Preparation for Bio-Rad "Mini-PROTEAN II" SDS-PAGE Slab Gel used in Chapter 6.

A. Acrylamide/bis (30% T, 2.67% C)
(Available commercially from Bio-Rad)

B. 1.5 M Tris-HCL, pH 8.8
27.23g Tris base (18.15g/100ml)
≈80ml dH_2O
Adjust to pH 6.8 with 1N HCL. Make to 100ml with dH_2O and store at 4°C.

C. 0.5 M Tris-HCL, pH6.8
6g Tris base
≈60ml dH_2O
Adjust to pH 6.8 with 1N HCL. Make to 100ml with dH_2O and store at 4°C.

D. 10% SDS
Dissolve 10g SDS in dH_2O with gental swirling and bring to 100ml with dH_2O.

E. Sample Buffer (SDS denaturing buffer)(store at room temperature)

Distilled water	4.0ml
0.5 M Tris-HCL, pH 6.8	1.0ml
Glycerol	0.8ml
10% (w/v) SDS	1.6ml
2-β-mercaptoethanol	0.4ml
0.05% (w/v) bromophenol blue	0.2ml
TOTAL VOLUME	8.0ml

F. Running (electrode) Buffer (5X concentration)

Tris base	9g
Glycine	43.2g
SDS	3g

to 600ml with dH_2O
Store at 4°C, dilute 60ml 5X stock with 240ml dH_2O for one run.

Appendix B.

1. Reagents and Gel Preparation for Bio-Rad "Mini-PROTEAN II" SDS-PAGE Slab Gel used in Chapter 6.

A. Acrylamide/bis (30% T, 2.67% C)
(Available commercially from Bio-Rad)

B. 1.5 M Tris-HCL, pH 8.8
27.23g Tris base (18.15g/100ml)
≈80ml dH_2O
Adjust to pH 6.8 with 1N HCL. Make to 100ml with dH_2O and store at 4°C.

C. 0.5 M Tris-HCL, pH6.8
6g Tris base
≈60ml dH_2O
Adjust to pH 6.8 with 1N HCL. Make to 100ml with dH_2O and store at 4°C.

D. 10% SDS
Dissolve 10g SDS in dH_2O with gental swirling and bring to 100ml with dH_2O.

E. Sample Buffer (SDS denaturing buffer)(store at room temperature)

Distilled water	4.0ml
0.5 M Tris-HCL, pH 6.8	1.0ml
Glycerol	0.8ml
10% (w/v) SDS	1.6ml
2-β-mercaptoethanol	0.4ml
0.05% (w/v) bromophenol blue	0.2ml
TOTAL VOLUME	8.0ml

F. Running (electrode) Buffer (5X concentration)

Tris base	9g
Glycine	43.2g
SDS	3g

to 600ml with dH_2O
Store at 4°C, dilute 60ml 5X stock with 240ml dH_2O for one run.

Appendix B.

1. Reagents and Gel Preparation for Bio-Rad "Mini-PROTEAN II" SDS-PAGE Slab Gel used in Chapter 6.

A. Acrylamide/bis (30% T, 2.67% C)
(Available commercially from Bio-Rad)

B. 1.5 M Tris-HCL, pH 8.8
27.23g Tris base (18.15g/100ml)
≈80ml dH_2O
Adjust to pH 6.8 with 1N HCL. Make to 100ml with dH_2O and store at 4°C.

C. 0.5 M Tris-HCL, pH6.8
6g Tris base
≈60ml dH_2O
Adjust to pH 6.8 with 1N HCL. Make to 100ml with dH_2O and store at 4°C.

D. 10% SDS
Dissolve 10g SDS in dH_2O with gental swirling and bring to 100ml with dH_2O.

E. Sample Buffer (SDS denaturing buffer)(store at room temperature)

Distilled water	4.0ml
0.5 M Tris-HCL, pH 6.8	1.0ml
Glycerol	0.8ml
10% (w/v) SDS	1.6ml
2-β-mercaptoethanol	0.4ml
0.05% (w/v) bromophenol blue	0.2ml
TOTAL VOLUME	8.0ml

F. Running (electrode) Buffer (5X concentration)

Tris base	9g
Glycine	43.2g
SDS	3g

to 600ml with dH_2O
Store at 4°C, dilute 60ml 5X stock with 240ml dH_2O for one run.

2. Buffers and Reagents for Electroblotting and Immunoblotting used in Chapter 6.

A. Electroblotting Buffer (1 litre):
3.03g Tris base
14.4g Glycine
200ml ethanol
Add dH_2O to make 1 litre, adjust to pH 8.3.

B. Transblotting Solution (TBS)
20mM Tris base
500mM NaCl
Adjust pH to 7.5

C. Tween Transblotting Solution (TTBS)
0.05% Tween-20 in TBS

D. Non-Specific Blocking Solution
2% Marvel (milk powder) in TTBS

E. Enzyme Substrate Solution
a. 20mg 4-chloro-1-naphthol in 4ml methanol
b. 10ml TBS warmed to 37°C in a Universal tube
Add a. to b. plus 10µl of H_2O_2

Appendix C.

A. Procedures for Conventional Histological Staining used in Chapter 7.

Frozen sections were removed from -25°C storage and submerged in the first staining reagent for all the following stains.

Ehrlich's Haematoxylin and Eosin. (for general structure)

1. Ehrlich's Haematoxylin	30-40 mins.
2. Wash in running tap water	1 min.
3. 1% acid alcohol	few secs.
4. Wash in running tap water	few secs.
5. Few drops of lithium carbonate	to blue.
6. Wash in running water	1 min.
7. 1% Eosin	10 mins.
8. Wash in running tap water	few secs.

9. Remove excess water and mount under coverslip in Aquamount.

Periodic Acid Schiff. (for carbohydrates)

1. 0.5% aqueous Periodic acid	10 mins.
2. Wash in running tap water	5 mins.
3. Rinse in dH_2O	few secs.
4. Schiff's reagent	10 mins.
5. Wash in 0.05M sodium bisulphate	3 x 2 mins.
6. Wash in running tap water	5 mins.

7. Remove excess water and mount under coverslip in Aquamount.

Sudan Black B. (for lipids)

1. Rinse frozen section very rapidly in 70% methanol solution.
2. Stain in Sudan Black B (saturated in 70% methanol) 5 mins- 1 hour.
3. Rinse in 70% methanol very rapidly.
4. Wash in running tap water 10 mins.
5. Remove excess water and mount under coverslip in Aquamount.

- Section that were stained in both H&E and Sudan Black B were first stained in Sudan Black to procedure 4 and then stained as normal in H&E.

Toluidine Blue. (for general structure)

1. Toluidine blue	2 mins.
2. Wash in running tap water	few secs.

3. Remove excess water and mount under coverslip in Aquamount.