"FACTORS INFLUENCING THE EPIDEMIOLOGY OF THE OVINE SARCOSPORIDIOSES; AND THE DEVELOPMENT OF *Sarcocystis tenella* IN SPECIFIC-PATHOGEN-FREE (SPF) SHEEP"

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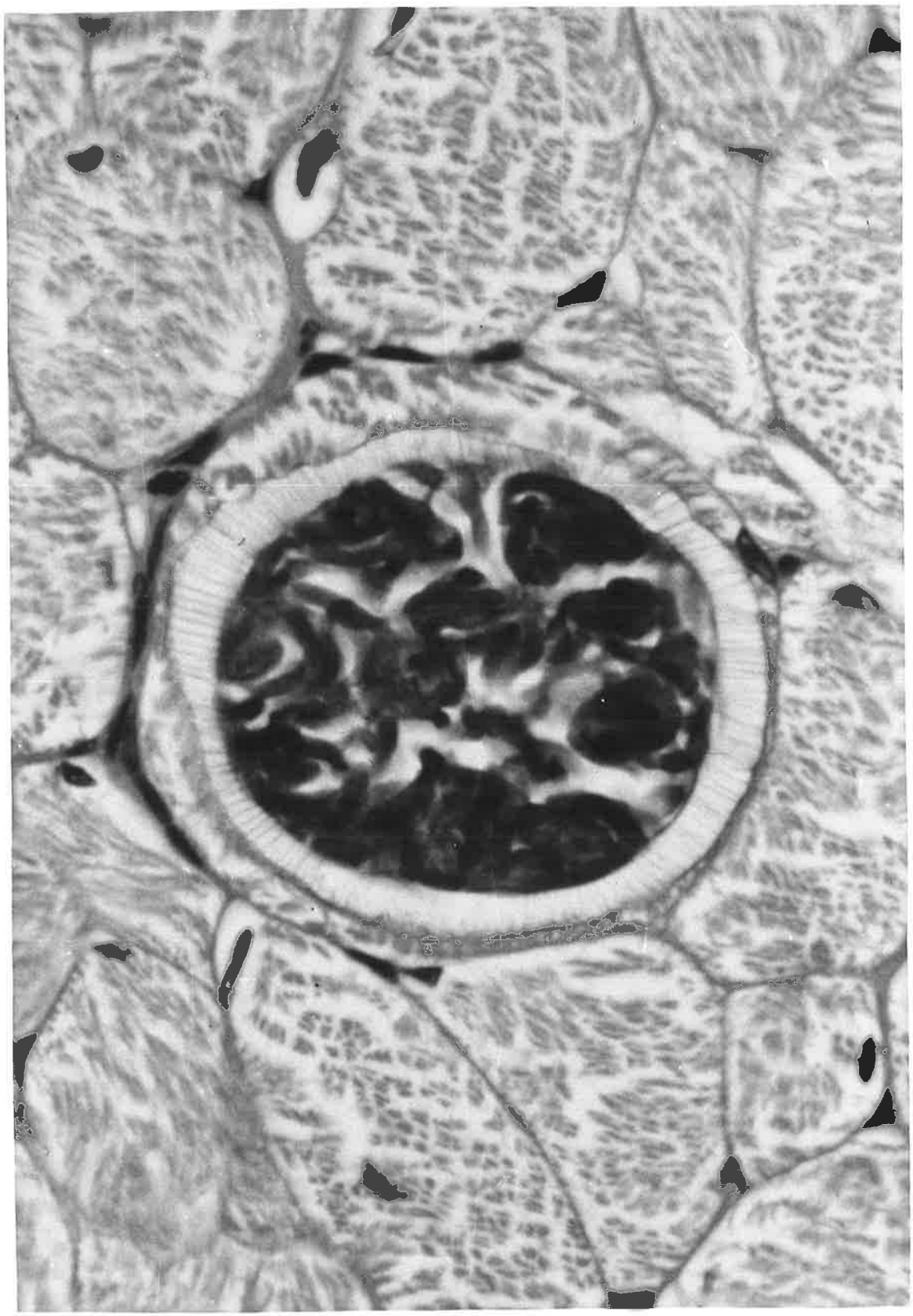
B.Sc. (Hons.)

A Thesis submitted to the University of Adelaide for the Degree of Doctor of Philosophy

July 1978



FRONTSPIECE. A microscopic sarcocyst of the sporozoan parasite *Sarcocystis tenella* (junior synonym *S. ovicanis)* within a skeletal muscle fibre of a sheep. (∿ 2,500 X mag.)

DEDICATED TO THE SUPPORT RECEIVED

FROM FAMILY, FRIENDS AND PET

TABLE OF CONTENTS

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cont..

...

cont..

...

cont..

...

cont..

APPENDICES

BIBLIOGRAPHY

COPY DISTRIBUTION

GENERAL SUMMARY

Selected factors were examined for their influence on the epidemiology of Sarcosporidial infections in sheep and the developmental stages of the predominant microscopic species of the parasite were determined.

Epidemiology in sheep

Sheep destined for slaughter were selected under a factorial design in which the age, sex, breed, location and season of sample were equally represented. They were examined for *Sarcocystis spp.* using gross, histological and immunoserological methods.

Macroscopic sarcocysts were found in 6.7% of the 864 sheep examined whereas microscopic sarcocysts were detected in 93.2%. Antibodies to *Sarcocystis spp.* were detected in 93.7% of the sheep with the Complement Fixation Test and in 96.9% with the Indirect Fluorescent-antibody Test. Significant differences in the prevalence of infections were observed within certain factors. More adults were infected than lambs and more animals were found infected in the dry season than during the wetter months of the year.

The intensity of infection by sarcocysts was determined for individual animals. Heavier infections were found during the dry season and also in locations with moist and temperate climates. It is postulated that the moist period preceding the dry season is that of maximum exposure to infection, which is consistent with the higher antibody titres found at that time.

The measurement of sarcocyst size indicated the existence of four size populations. The two microscopic polulations were found to be related to seasonal differences in infection. The two macroscopic populations may represent two separate species of the parasite and it was observed that the smaller population virtually disappeared during the wet season.

The properties of origin of the sheep were subject to investigations to determine ecological features related to the transmission of infections to

sheep. Managerial practices which provide the domestic carnivores with greater access to fresh meat were found to be more conducive to heavier infections.

Development in Sheep

Lambs were raised under specific-pathogen-free (i.e., Sporozoa-free) conditions and infected at weaning with *Sarcocystis* sporocysts harvested from the faeces of dogs. Twenty-nine lambs were sacrificed between 1.3 hours and 134 days post-inoculation (dpi) and all major organs and tissues were examined for developmental stages of the parasite.

The remnants of excysted sporocysts were found between 1.3 hours and 3 dpi within the gastro-intestinal tract. From 6-19 dpi, the first merogonous (or alternatively, schizogonous) stages of the parasite were found within arteriole endothelia of most organs and tissues, except those of the nervous, endocrine and reproductive systems. A second merogonous generation was detected from 21-34 dpi within the endothelial cells of capillaries throughout the body. An acute disease syndrome was associated with this prolific generation. Organisms morphologically different from the previous two stages were found at 36 dpi in hepatic sinusoids and lymph node capillaries. These may represent a third merogonous stage of the parasite.

Developing sarcocysts were detected within skeletal and cardiac musculature after 41 dpi and some were also found in the brain. Between 41-45 dpi, the sarcocysts appeared as small homogenous stages centrally located within the striated muscle fibres. These stages increased in size from 50-55 dpi and had undergone division forming numerous distinct metrocytes. The sarcocysts assumed their characteristic appearance after 60 dpi as they contained numerous cystozoites, peripheral metrocytes and were bounded by a radially-striated thick cyst wall.

On examination of the host antibody response to infection, it was found that complement-fixing antibodies appeared early in the course of

infection, peaked in intensity at 30 dpi and then gradually declined thereafter. Antibodies detected by the Indirect Fluorescent-antibody Test appeared later in infection, reached maximum intensity at 75 dpi and then persisted in titre to 134 dpi.

This thesis contains no material previously submitted by me for a degree in any university. All material presented was performed entirely by myself, except where specifically acknowledged.

(P.J. O'Donoghue)

ACKNOWLEDGMENTS

Research for this thesis was performed in the Veterinary Parasitology Unit at the Institute of Medical and Veterinary Science (I.M.V.S.) and the Zoology Department at the University of Adelaide. It was supported by a post-graduate scholarship and research grant from the Australian Meat Research Committee.

I am deeply indebted to my project supervisor, Dr. G.E. Ford, for the generous help and consultation given throughout the period of research, particularly with the development of the factorial-design survey and also for the critique of written drafts. I am also grateful to my university supervisor, Dr. R.I. Sommerville, for his professional and academic guidance and personal interest.

Excellent co-operation was received in field work from various personnel associated with the meat industry. Special thanks is given to :-

- the stock agents for assistance in establishing the trace system;
- the managerial staff of the various abattoirs for facilitating access;
- the meat inspection staff (Australian Bureau of Animal Health, Department of Primary Industry) for advice concerning sampling and providing the parasite for antigen preparation;
- the animal health advisers (Animal Health Branch, South Australian Department of Agriculture and Fisheries) for direction in the ecological studies;

and -

- the property owners involved in the survey for taking me into their confidence.

Numerous members of the I.M.V.S. technical staff provided assistance in several aspects of laboratory work for which I am extremely grateful. The animal attendant staff assisted with the production and maintenance of the specific-pathogen-free sheep used for experimentation. Dr. Ford's technicians provided the inoculum given to these sheep and also aided with the post-mortems and sample collection. Histopathology staff helped with section preparation and several other laboratories provided essential equipment.

I am also particularly indebted to several individuals from whom specific advice and assistance was gained :-

- Dr. L.L. Callow (Department of Primary Industries, Brisbane) for advice concerning immunofluorescence techniques;
- Miss R. Lewis (Computing Centre, University of Adelaide) for help with data processing and computer programming;

and -

- Mr. K. Burrows (Bureau of Meteorology, Adelaide) for providing access to climatic information.

Lastly, I wish to thank the I.M.V.S. photographer, Mr. M.R. Billett, and the medical illustrators, Miss A. Langen-Zeuffa and Mr.M.S. Hullan, for their advice and help with the preparation of material for presentation.

PREFACE

This thesis represents part of an applied research programme being carried out in Australia on Sarcosporidial infections in sheep. The research reported herein was orientated towards providing background information on the epidemiology and development of the parasite as a guide to future work. The studies are presented in three sections, as follows :-

The first section consists of a basic literature review of the parasite and presents general information on its appearance, distribution and mode of transmission. The nomenclature and terminology applicable to the parasite and its various stages of development are also reviewed and defined.

The second section deals with research on the epidemiology of infections by *Sarcocystis spp.* in slaughter sheep. A factorial-design survey was performed to determine the significance of pre-selected physiological and environmental factors on the prevalence and intensity of infections as measured through gross, histological and immunoserological studies. Numerous ecological features were also examined for each property involved in the survey to establish the existence of any relationships between the features and the intensity of infection.

The third section concerns research on the development of the microscopic species of the parasite, *Sarcocystis tenella* (junior synonym *S. ovicanis)*, in sheep raised under specific-pathogen-free conditions. The various developmental stages of the parasite are described from histological sections of sheep tissues taken at varying times after infection. The host antibody response to infection was examined by immunoserological methods and general observations were also made on other host responses to infection.

SECTION I:

GENERAL INTRODUCTION TO THE PARASITE

The fact that we are able to classify organisms at all in accordance with the structural characteristics which they present, is due to the fact of their being related by descent

Ray Lankester
(1847-1929)

I. GENERAL INTRODUCTION TO THE PARASITE

Prior knowledge of the Sarcosporidia is divided into two chronological categories for ease of presentation. The first category concerns the 'classical' knowledge about the parasite as obtained from the descriptive studies and reports on its occurrence made between the 1880's and the 1960's. The second category deals with recent knowledge of the parasite gained following the elucidation of its mode of transmission in the 1970's and the subsequent flood of research.

1.0 'CLASSICAL' KNOWLEDGE

In early literature, the organism was known only as a protozoan parasite typified by the occurrence of cysts within the muscles of various hosts. The resultant condition i s known as Sarcosporidiosis.

1.1 DESCRIPTION OF THE PARASITE

The Sarcosporidia are endoparasitic and are found as colonies predominantly within striated muscle fibres (i.e., skeletal and cardiac muscle). The morphology of these colonies, or sarcocysts, has been intensively studied under the light microscope (cf. Scott 1943, Ludvik 1959) and found to be very distinctive.

Sarcocysts are extremely variable in size and range from microscopic to macroscopic dimensions (from 10 µm up to 1 cm in length). They are very basophilic staining and contain many zoites, or Rainey's corpuscles, enclosed within a cyst wall. Larger sarcocysts are usually septate forming compartments which contain the zoites. The zoites are banana- to crescent-shaped and vary in size between species, ranging from 5-16 µm in length by 2-6 µm in width. They originate from near the cyst wall and in larger sarcocysts the older zoites, which are central within the cyst, appear to be degenerate. The cyst walls of the sarcocysts

vary in dimensions between species (ranging from 0.5-6.0 µm in width) and they also vary in appearance - usually being either smooth or radially-striated. Most sarcocyst walls have been described as double-layered with the inner primary layer being derived from the parasite and the outer secondary layer from the host.

Although characteristically cysts of muscle, some cysts reminiscent of *Sarcocystis* have occasionally been reported in other locations, such as the central nervous system (Brownlee 1936). However, in these extramuscular locations there may be confusion with other cyst-forming organisms, such as *Toxoplasma*, *Nosema (= Encephalitozoon)* and *Frenkelia (=* M organism) cysts in the nervous system and *Besnoitia (= Fibrocystis)* cysts in connective tissue (cf. Manwell & Drobeck 1953, Attwood & Sutton 1965, Frenkel 1973, Levine 1973). Only with experience can fine morphological differences between these various cysts be used for differential identification.

1.2 TAXONOMIC STATUS

Sarcocysts, or Miescher's sacs, were first described within the muscle fibres of the mouse by Miescher (1843) and they have since been reported in many other hosts. The generic names given to the parasite have been *Synchytrium* (Kühn 1865) and *Sarcocystis* (Lankester 1882) for colonies found in the domestic pig and *Miescheria* and *Balbiania* (Blanchard 1885) for those found in respectively, the mouse and the kangaroo. As the generic name used by Kühn was preoccupied and thus unavailable, the naming of the genus was attributed to Lankester and is known as *Sarcocystis* (Lankester 1882).

Railliet first named the parasite species found in sheep as *Miescheria tenella* (Railliet 1886a). He named them in the

discussion given after a paper presented by Moulè (1886) who was reporting the occurrence of cysts found microscopically in the muscular tissues of sheep. Shortly afterwards, Railliet also named the visible cysts found in the oesophagi of sheep as *Balbiania gigantea* (Railliet 1886b). Since then, the microscopic and macroscopic cysts found in sheep have been assumed to be the same parasite but of different ages; therefore, most infections in sheep have been reported as those of *Sarcocystis tenella* (Railliet 1886).

The exact taxonomic classification of the Sarcosporidia was the subject of much confusion and debate and they were usually reported in the literature as protozoan parasites of unknown taxonomic status (cf. Wenyon 1926). Once they had even been erroneously reported as being a fungus (Spindler & Zimmerman 1945). It was finally the result of electron microscopic studies on the zoite stages that established the Sarcosporidia as typical members of the class Sporozoa (Ludvik 1958, Sènaud & dePuytorac 1962). More specifically, this work established ultrastructural similarities between the Sarcosporidia and the better-known coccidial parasites (i.e., the Eimeriidae).

1.3 DISTRIBUTION OF THE PARASITE

Over 80 species of *Sarcocystis* have been named, usually in accordance with the different host species which have been found infected. Many more reports on its occurrence have been made in many localities without the naming of the parasite species. They have been found predominantly in herbivorous mammals, rodents, ground-feeding birds and lower vertebrates. There have also been occasional reports of sarcocysts found in man, carnivores and marine animals. The occurrence and distribution of *Sarcocystis spp.* has already been adequately reviewed for vertebrates

(Kalyakin & Zasukhin 1975) and for non-human primates (Karr & Wong 1975). Such reports and reviews reveal the Sarcosporidia to be world-wide in distribution and to occur in a great number of varied hosts.

Because the Sarcosporidia were so widespread, some authors suggested they may actually be a single zoonosological species and that species affiliation could not be derived from the sole characteristic of host occurrence (Alexeieff 1913, Panasyuk *et al.* 1971). This concept has since been shown to be erroneous with the recent determination of some of the life cycles of the Sarcosporidia.

1.4 LIFE CYCLE STUDIES

Early research into the transmission of Sarcosporidial infections was orientated along three basic avenues, that of direct host-to-host transmission, that via an insect vector and that involving a carnivorous host.

Initial research was performed on infections in laboratory rodents (Darling 1910, Nègre 1910) from which it was suggested that *Sarcocystis spp.* had a one-host life cycle where animals became infected via faecal contamination. This mode of transmission was also suggested for infections in sheep (Scott & O'Roke 1920) and pigs (Spindler *et al.*1946) and it was postulated that infective stages of the parasite were passed in the faeces and the urine. However, in retrospect it can be seen that seemingly successful infections found in this research may have resulted from extra-experimental contamination. Transplacental infections were also discounted in mice (Smith 1905) and sheep (Scott 1918) for the parasite was not detected in embryos or newly-born offspring taken from infected mothers.

The second avenue of research examined was that biting insects disseminated the infection between hosts. Numerous inoculation experiments failed to provide evidence for this mode of transmission (Smith 1901, Nègre 1907) as did research on sheep kept in insect-free conditions (Scott 1920). Sergent (1921) suggested this mode of transmission was highly possible for infections in cattle after he found *Sarcocystis* zoites in a blood sample taken from a calf, but it is now commonly thought that this finding was an artefact resulting from the rupture of a sarcocyst during sampling.

The third alternative suggested was that of a two-host life cycle where carnivores acted as definitive hosts and transmitted the infections to the herbivorous intermediate hosts (Minchin 1903, Crawley 1916). An early attempt to transmit *Sarcocystis sp.* from a sheep through a dog and back to sheep was unsuccessful (Scott 1915) but it is now known that this experiment failed because the time interval examined was insufficient for the infection in the dog to reach prepatency. Unfortunately, this avenue of research was discontinued at that time and was only recently resumed.

2.0 RECENT KNOWLEDGE

A major breakthrough in research on the Sarcosporidia occurred in the 1970's with the determination of an obligatory two-host life cycle for the parasite. There followed much research on the transmission and development of infections in various definitive and intermediate hosts. This led to several revisions of the nomenclature and terminology applicable to the Sarcosporidia.

2.1 TRANSMISSION STUDIES

Studies on the Sarcosporidia were re-orientated towards transmission via a definitive carnivorous host following some research which supported the earlier indications of the coccidial nature of the parasite's life cycle. It was found that zoites taken from sarcocysts in birds developed *in vitro* (Fayer 1970) forming sexual developmental stages similar to those found for other coccidia (Fayer 1972, Vetterling *et al*. 1973). Hutchison *et al*. (1970) then found that *Toxoplasma gondii*, another protozoan parasite apparently related to *Sarcocystis* (Soulsby 1968, Noble & Noble 1971), had a two-host life cycle where sexual development of the parasite occurred in the cat. Following these leads, Rommel *et al*. (1972) fed *Sarcocystis*-infected sheep meat to cats and found they excreted coccidian-like sporocysts in their faeces. This finally elucidated the mode of transmission for a species of *Sarcocystis*. Various other successful transmission studies have since been performed for other species of *Sarcocystis*, predominantly for those from meat animals to domestic carnivores. Sarcocysts from sheep have been transmitted to cats (Rommel *et al*. 1972, 1974) and to dogs (Ford 1974, Munday & Corbould 1974), but only the sheep/dog life cycle of the parasite has been definitely confirmed by several passages in animals raised specific-pathogen-free (Ford 1975).

Sexual developmental stages of the parasite were observed in the small intestines of the definitive hosts after they had been fed infected musculature. Macrogametocytes were firstly found in the lamina propia of the intestinal villi but microgametocytes have not been identified with certainty (Rommel *et al*. 1972, Munday *et al*. 1975). Oocysts were then found forming within the villi and after variable pre-patent periods (ranging from 7-15 days), free sporocysts were shed in the faeces of the definitive hosts. These

sporocysts were relatively uniform in size, measuring 12-16 µm in length by 8-10 µm in width, and were sporulated containing four sporozoites and a residuum (residual body). Observations made on these and other transmission studies resulted in several attempted revisions of the nomenclature.

2.2 REVISED TAXONOMY

It was noticed that successful transmission of ovine Sarcosporidia to carnivores was only achieved when macroscopically visible sarcocysts were fed to cats and when microscopic sarcocysts were fed to dogs (Munday & Rickard 1974, Rommel *et al.* 1974). It was therefore suggested that there were two species of *Sarcocystis* in sheep, a macroscopic species with the cat as the final host and a microscopic species with the dog as the final host. This led to some confusion as to the nomenclature applicable to each species.

Heydorn *et al.* (1975c) proposed a new nomenclature for the parasite species based upon a binomial combination of their two host species. The macroscopic species transmitted by cats was named *Sarcocystis ovifelis* and the microscopic species transmitted by dogs was named *Sarcocystis ovicanis*. However, this approach is not in accordance with the International Code of Zoological Nomenclature (1961) and is therefore not acceptable. As pointed out by Ashford (1977), the original naming of the ovine Sarcosporidia provides enough information for the correct historical nomenclature to be used. Macroscopic sarcocysts in sheep were named *Balbiania gigantea* (Railliet 1886b) after the microscopic sarcocysts had been named *Miescheria tenella* (Railliet 1886a). The macroscopic species transmissible to cats should therefore be named *Sarcocystis gigantea* and the microscopic species transmissible to dogs should be named *Sarcocystis tenella*. These species

names are different from those proposed in other recent reviews (cf. Levine 1977) but in my belief it is the correct and applicable nomenclature and is therefore used throughout this thesis.

Distinct ultrastructural differences between the cyst walls of macroscopic and microscopic sarcocysts from sheep have also been found (Bergman & Kinder 1975, Mehlhorn *et al.* 1976) thereby providing another criterion for the speciation of the ovine Sarcosporidia other than that of the transmission studies.

It is now obvious that the Sarcosporidia are coccidians and belong to the subphylum Apicomplexa (cf. Levine 1977) and it has been suggested that they be placed in the family Eimeriidae (Tadros & Laarman 1976) or separately in the family Sarcocystidae (Frenkel 1974). However, until much more is known about all the cyst-forming Sporozoa, the exact taxonomical classification of these organisms cannot be made.

2.3 TERMINOLOGY OF PARASITIC STAGES

Various infection studies have been carried out with *Sarcocystis* sporocysts from dogs being fed to calves (Fayer & Johnson 1973, 1974) and lambs (Gestrich *et al.* 1974, Munday *et al.* 1975). These studies revealed the existence of at least one asexual proliferative stage of the parasite prior to the appearance of the characteristic sarcocysts. These stages were similar to coccidial schizonts and were found within endothelial cells of arterioles and capillaries from 12-38 days after infection. However, using the terminology proposed by Levine (1971), these stages would be best known as meronts which contain merozoites. This terminology is used throughout this thesis to avoid any confusion with the established terminology applicable to the intestinal coccidians - e.g., the schizonts of *Eimeria spp.* (cf. Pellèrdy 1965).

The development of the characteristic sarcocysts has also been studied in calves (Mehlhorn *et al*. 1975c, Gestrich *et al*. 1975) and lambs (Mehlhorn *et al*. 1975d, Heydorn & Gestrich 1976). The sarcocysts have been described as forming zoites from spherical cells around the periphery of the cysts (Heydorn *et al*. 1975a). These cells have been termed metrocytes (Sènaud 1967, Mehlhorn & Scholtyseck 1973) and the zoites have been termed cystozoites (Hoare 1972) and bradyzoites (Frenkel 1973) by analogy with similar stages of *Toxoplasma*. Although the zoites have also recently been termed merozoites (Heydorn *et al*. 1975b), it was elected to use the term 'cystozoite' throughout this thesis to avoid any confusion with the previous zoite stages. This term is also more self explanatory as it relates better to the tissue 'cyst' stage of development.

SECTION II:

EPIDEMIOLOGY IN SHEEP

In Nature's infinite book of secrecy

A little can I read

William Shakespeare

(1564-1616)

II. EPIDEMIOLOGY IN SHEEP

Very little epidemiological research has been performed on Sarcosporidial infections in sheep apart from some abattoir surveys which have usually been carried out on unspecified animals of unknown origin. The following studies were undertaken to examine the influence of certain physiological and environmental factors on the epidemiology of infections in selected sheep available for slaughter within Australia.

INTRODUCTION

3.0 PROBLEM TO THE MEAT INDUSTRY

The infection of sheep by *Sarcocystis spp*. represents an economic problem to both the meat producers (the farmers) and the meat processors (the butchers). At this stage of our knowledge, the most observable effect on the meat industry is caused by macroscopic sarcocyst infections, and it is only recently that microscopic infections have been linked to reduced productivity.

3.1 MACROSCOPIC SARCOCYST INFECTIONS

Due to export regulations governing the hygiene of consumptible meat, the infection of sheep meat by macroscopic sarcocysts poses a particular problem for the various meat-exporting countries. In Australia, macroscopic infections are a common cause of carcase rejection and condemnation at meat inspection at the abattoirs. If more than five visible sarcocysts are detected in the musculature during meat inspection, present-day standards call for the rejection of the entire carcase from the meat export market. If the infection is 'light', in that the sarcocysts are few in number and are localized in specific muscles, the carcase may be trimmed free of such lesions and the meat may be sold on the local market. However, with 'heavy' infections, where the sarcocysts appear throughout the entire musculature, the carcase is

condemned and destroyed. This rejection and condemnation of meat is based more upon aesthetic factors than upon its known pathogenicity to humans, for the *Sarcocystis spp.* are not comsidered to be of any serious pathogenic significance (Soulsby 1968, Noble & Noble 1971).

The incidence of macroscopic sarcocyst infections in sheep is of such a degree that it constitutes a serious economic loss for the meat industry. Meat producers are offered lower prices for slaughter animals if they come from presumed heavily-infected areas, and meat processors suffer economically from loss of trade and increased handling costs. On examination of data collected by the Australian Bureau of Animal Health (Department of Primary Industry) on macroscopic sarcocyst infections detected in sheep at selected abattoirs during 1969-1973 (see Table 1), it was estimated that approximately $4 million was lost during this period due to this infection alone (estimate based on minimum carcase price for each half-year).

Year	1969	1970	1971	1972	1973
No. sheep inspected	12,871,469	26,000,134	31,010,697	29,307,145	17,398,947
No. sheep rejected	22,367	34,649	51,369	52,196	29,973
No. sheep condemned	50	2,504	4,565	12,597	16,341

TABLE 1 . Numbers of sheep rejected and condemned during 1969-1973 due to infections by macroscopic sarcocysts.

Research was undertaken to determine the present extent of macroscopic sarcocyst infections in slaughter sheep.

3.2 MICROSCOPIC SARCOCYST INFECTIONS

The rejection and condemnation of sheep meat due to Sarcosporidial infections only results from those by macroscopic sarcocysts. It does not take into account microscopic sarcocyst infections for they are not classified under any meat-export regulations as yet.

At the beginning of this project, no information was available on the epidemiology of microscopic infections in slaughter sheep apart from the occasional report on their occurrence. This was because most previous research had been orientated towards the descriptive histology of the organism.

It was only recently that the development of microscopic sarcocyst infections was observed to have a pathogenic effect in experimental lambs (Gestrich *et al*. 1974). Such being the case, these infections may be related to instances of reduced productivity in commercial animals, the economic effects of which are basically unseen. Studies were therefore performed to determine the extent and severity of microscopic sarcocyst infections in sheep available for slaughter within Australia.

Furthermore, the development of such infections was examined in experimental animals and is reported in Section III.

4.0 PREVIOUS EPIDEMIOLOGICAL OBSERVATIONS

Certain factors have long been supposed to influence the distribution and abundance of Sarcosporidial infections in sheep in South Australia. These factors have originated mainly from casual observations made by personnel associated with the meat industry (viz., from 'folklore') for almost no epidemiological information was available from historical scientific publications.

4.1 'FOLKLORE'

The exact geographic distribution and seasonal abundance of *Sarcocystis spp.* in sheep was unknown at the beginning of this project. However, casual observations made by various meat producers and processors indicated there may be regional and seasonal variations in the prevalence of macroscopic sarcocyst infections in sheep in South Australia (Ford 1974 pers. comm.).

Various observations relating to their distribution had also been made by personnel of the Australian Bureau of Animal Health (Department of Primary Industry) and the Animal Health Branch (South Australian Department of Agriculture and Fisheries). These observations suggested that the majority of macroscopically-infected sheep originated from two distinct locations - Kangaroo Island and Eyre Peninsula. Of a line of 1,510 sheep from Kangaroo Island slaughtered in 1970, 1,466 (97%) were rejected from export due to unacceptable levels of macroscopic sarcocysts. A survey of 7,479 sheep from Eyre Peninsula, slaughtered during 1970-1971, indicated that 1,496 (20%) of these sheep were infected with visible sarcocysts. These two locations, as well as others in the main sheep-producing areas of South Australia, were examined in this survey to determine whether any variations in both macroscopic and microscopic infections do exist. Regional differences

in both types of infection have been observed previously in other countries (Scott 1943, Meshkov 1973), but no geographic or climatic information was reported from which comparisons may have been made.

An examination of basic information collected by Mr. J.D. Habel (Sth. Aust. Dept. Agriculture & Fisheries) suggested the following relationships between the distribution of macroscopic sarcocysts in sheep from Eyre Peninsula and the following factors:-

(a) Environmental factors

. Rainfall

The distribution of macroscopic sarcocysts detected seemed to be loosely confined to a certain range of annual rainfall.

. Season

The prevalence of infection found at slaughter appeared to be slightly greater during the summer months.

(b) Physiological factors

. Age

Heavier infections appeared to occur in aged sheep (i.e., sound- to broken-mouth sheep).

. Sex

More ewes seemed to be infected than wethers.

Unfortunately, this data was insufficiently clear for detailed analysis; therefore, these factors were examined in this survey to determine their significance on both macroscopic and microscopic sarcocyst infections.

4.2 HISTORICAL OBSERVATIONS

Very little epidemiological information relevant to Sarcosporidial infections was available in the literature. Scott (1943), in a summary of his research, observed that the severity of microscopic sarcocyst infection depended upon the age of the sheep, its food, the restriction of its feeding habits and the climate, moisture and season. However, at the time of his research nothing was known of the coccidial nature of the parasite's life cycle, and it is now apparent that many of Scott's conclusions were erroneous. Fortunately, his descriptive style of reporting does provide some information that suggests seasonal differences in infection did occur. Heavier microscopic sarcocyst infections were recorded in sheep examined during the summer months in Wyoming (U.S.A.). As determined by information from the World Meteorological Organization (1970-75),a similar 'Mediterranean'-type climate prevails in South Australia. Some analogies may therefore be made between these two distant localities after the significance of season on infections has been determined for South Australia.

5.0 METHODS OF DETECTION

Sarcosporidial infections have generally been recognized by the detection of the parasite through gross and microscopic examinations. More recently, immunoserological studies have been employed to detect antibodies formed by the host against the parasite.

5.1 GROSS EXAMINATION

Most of the original reports on the Sarcosporidia have been made on macroscopic sarcocysts detected in the musculature of the host by gross (or visual) examination. Many sites of infection

have been described for various hosts and in sheep the striated muscle groups of the carcase, tongue and oesophagus are those usually found infected (Soulsby 1968). Various skeletal muscle groups were selected in the survey sheep for comprehensive visual examination in order to detect the maximum number of macroscopic infections as possible. The muscles selected included sites of infection which have been presumed to be predilection sites from casual observations made by meat inspection personnel. However, it is not known as yet whether they are predilection sites or even primary parasitized areas.

5.2 MICROSCOPIC EXAMINATION

The examination of host tissues under the light microscope to detect microscopic sarcocysts has been used for some time and has resulted in many reports on their occurrence (cf. Kalyakin & Zasukhin 1975). Numerous reports have also been made on the descriptive histology of these cysts using a variety of histochemical stains (Ludvik 1958, 1959). However, the use of histological section examination as an epidemiological tool has been limited to examinations of sheep in conjunction with transmission studies or immunoserological trials (Scott 1943, Awad & Lainson 1954), and has not yielded much epidemiological information. Prevalence surveys that have been performed have used alternative microscopic examination techniques, which included the examination of squash preparations of muscles (Moulé 1886, Meshkov 1973) and the examination of musculature subject to pepsin digest (Macchioni & Marconcini 1973). However, no critical studies have been performed to determine whether these techniques result in mechanical or chemical damage to the sarcocysts; therefore, it was elected to use histological sections stained with haematoxylin and eosin in this survey to detect microscopic sarcocyst infections.

Because the parasite is very basophilic staining, such an examination renders it readily observable within the basically eosinophilic musculature.

5.3 IMMUNOSEROLOGICAL EXAMINATION

The initial use of immunoserological tests for *Sarcocystis spp.* was largely to determine whether any antigenic cross-reactions occurred with other parasites. It was thought there was a cross-reaction between the Dye Test (DT) for *Toxoplasma* and that for *Sarcocystis* (Awad 1954), but this was not substantiated by further work (Cathie & Cecil 1957). No cross-reaction was found between these two parasites when the Complement Fixation Test (CFT) was used (Awad & Lainson 1954, Awad 1958), nor when the Indirect Fluorescent-antibody Test (IFAT) was employed (Markus 1973). However, the d'Ouchterlony gel diffusion technique used to detect antibodies to *Sarcocystis* was found to be very nonspecific (Petithory 1973).

More recently, immunoserological tests have been used to examine naturally-occurring and experimental infections by *Sarcocystis spp.* It has now been established that *Isospora hominis* infections in humans are actually *Sarcocystis sp.* infections (Mehlhorn *et al.* 1975b). Antibodies to such infections have been detected with the IFAT when using *Sarcocystis* antigen (Tadros *et al.* 1974) and no antigenic cross-reaction has been found between the IFAT's for *I. hominis* and *Toxoplasma gondii* infections in humans (Dymowska *et al.* 1974). It therefore appears that the IFAT for *Sarcocystis* infections is quite specific and it has been used with good success for infections in intermediate hosts; such as cattle and mice (Markus 1973, Wallace 1973), as well as in definitive hosts; such as cats, chimpanzees and humans

(Markus *et al.* 1974, Tadros *et al.* 1974). The CFT for *Sarcocystis spp.* has also been found to be specific and has achieved good success in the examination of infections in sheep (Munday & Corbould 1974). The CFT and the IFAT for *Sarcocystis spp.* were therefore selected for use in this survey to provide sero-epidemiological information on infections in slaughter sheep.

MATERIALS AND METHODS

6.0 SURVEY DESIGN

Rather than repeat an abattoir survey for sarcocyst infections in slaughter sheep, a more comprehensive survey was designed prior to sampling to provide indications of the factors influencing the epidemiology of such infections.

6.1 SELECTION OF FACTORS

Certain physiological and environmental factors, thought to be relevant to the distribution of the parasite from preliminary observations, were selected and incorporated into a factorial design. The host physiological factors chosen were the age, sex and breed of the sheep sampled and the environmental factors were the location and season in which they were sampled.

6.2 SUBDIVISION OF FACTORS

The subdivisions of each factor were primarily based on the availability of such sheep being slaughtered and are summarized in Table 2 :-

FACTOR	NUMBER OF SUBDIVISIONS	SUBDIVISION
Physiological		
Age	2	Young Aged
Sex	2	Ewe Wether
Breed	2	Merino Corriedale
Environmental		
Location	6	South-East Kangaroo Island Lower Eyre Peninsula Murray Mallee Mid Eyre Peninsula Pastoral Zone
Season	3	Wet Moist Dry
Replicates	6	

TABLE 2. The subdivisions of the selected factors incorporated in the survey.

Each subdivision was strictly specified to avoid any overlapping of samples which would only confound the interpretation of the results. The young sheep sampled consisted of animals from 9-15 months of age (i.e., lambs to 2-tooth) and aged sheep were those over 4 years of age (i.e., 8-tooth to broken-mouth). Very few rams are sold for slaughter; therefore, the two sexes sampled were ewes and wethers (castrate males). The breeds of sheep sampled were Merinos and Corriedales for there were not enough British breeds or other Comeback types (> 3/4 Merino) available for slaughter.

The selection of the sampling locations was based on geographic and climatic information, as shown in Figure 1. The locations represent the range of agricultural activities, natural vegetation types and soil types found throughout the main sheep-producing areas of South Australia. The order of presentation of the locations, which is kept consistent throughout this thesis, exhibits the gradations in the climatic parameters measured during the survey period. Because the gradation ranges are relatively universal for other sheep-producing areas, any differences observed in infection rates may be tentatively extrapolated to locations elsewhere.

The sampling seasons were selected on the basis of residual rainfall and represent the upper, middle and lower ranges observed prior to and during the sampling period (see Figure 2). The residual rainfall was calculated for each month by subtracting the estimated monthly evapotranspiration (approximately equal to 0.5 X the monthly evaporation, Weisner 1964) from the total monthly rainfall.

LOCATION	SE SOUTH - EAST	KI KANGAROO ISLAND	LE LOWER EYRE PENINSULA	MM MURRAY MALLEE	ME MID EYRE PENINSULA	PZ PASTORAL ZONE
AGRICULTURAL ZONE	GRAZING			CEREAL		PASTORAL
NATURAL VEGETATION	MALLEE HEATH			MALLEE		ARID GRASSLAND
SOIL TYPE	SOLONIZED PODZOLIC			CALCAREOUS SANDY		CALCAREOUS LOAMY
ANNUAL RAINFALL (mm)	700		400		100	
ANNUAL EVAPORATION (mm)	1400		1700		2000	
ANNUAL RESIDUAL RAINFALL (mm)	0		-300		-600	
MEAN MAXIMUM TEMPERATURE (°c)	22·5		27·5		32·5	
MEAN MINIMUM TEMPERATURE (°c)	7·5		5·0		2·5	

FIGURE 1. The ranges of geographic and climatic parameters applicable to the selected sampling locations.

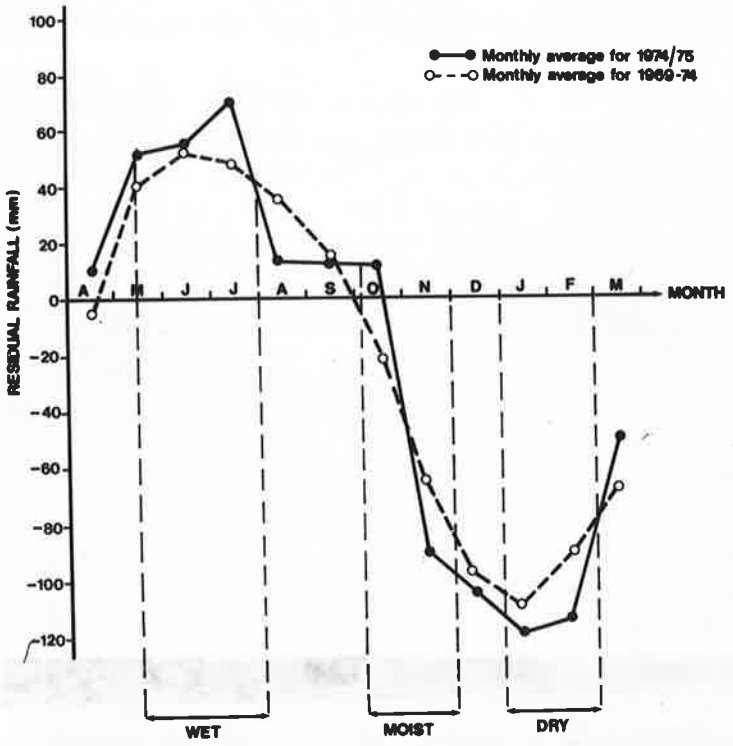


FIGURE 2. The ranges of residual rainfall applicable to the selected sampling seasons.

6.3 SPECIFICATION OF SHEEP

A total of 864 sheep from 144 properties was sampled under the factorial design. They were specified by every combination of each subdivision and each was replicated six times to achieve greater statistical significance. Therefore, for each season in each location, 48 sheep were sampled from 8 properties as specified in Table 3.

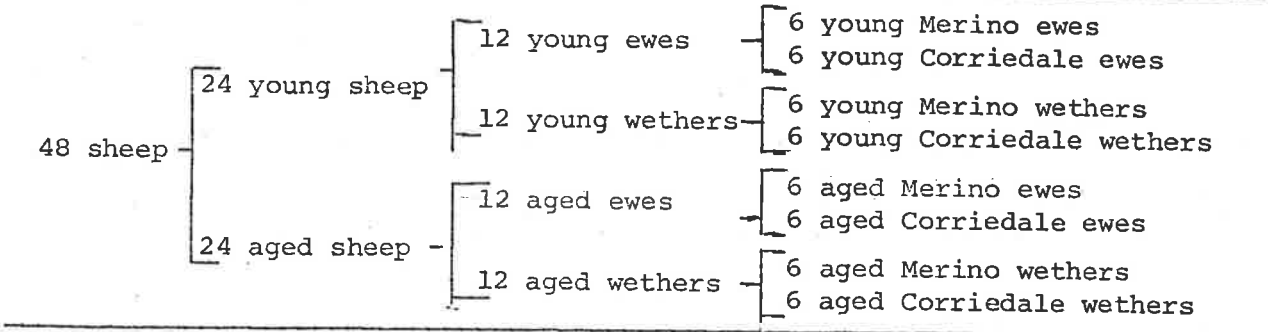


TABLE 3. Specified sheep sampled during each season from each location

7.0 SAMPLING SYSTEM

The specified sheep were selected from market sheep sold for slaughter during 1974 and 1975 and various samples were collected at the abattoirs. To facilitate the ecological studies, it was necessary to know the properties of origin of the sheep; therefore, a trace system had to be established.

7.1 TRACE SYSTEM

The basic format of the trace system employed is shown in Figure 3. The properties of origin of the sheep selected at the metropolitan and regional sale-yards were obtained from the records of the various stock agents. After their sale by auction, the sheep were marked on the fleece for identification purposes. The time and place of slaughter of the sheep were then obtained from the various meat buyers. Occasionally, a direct trace was available when some meat buyers bought stock directly from the properties. Each property of origin is shown for each sampling location in Figure 4.

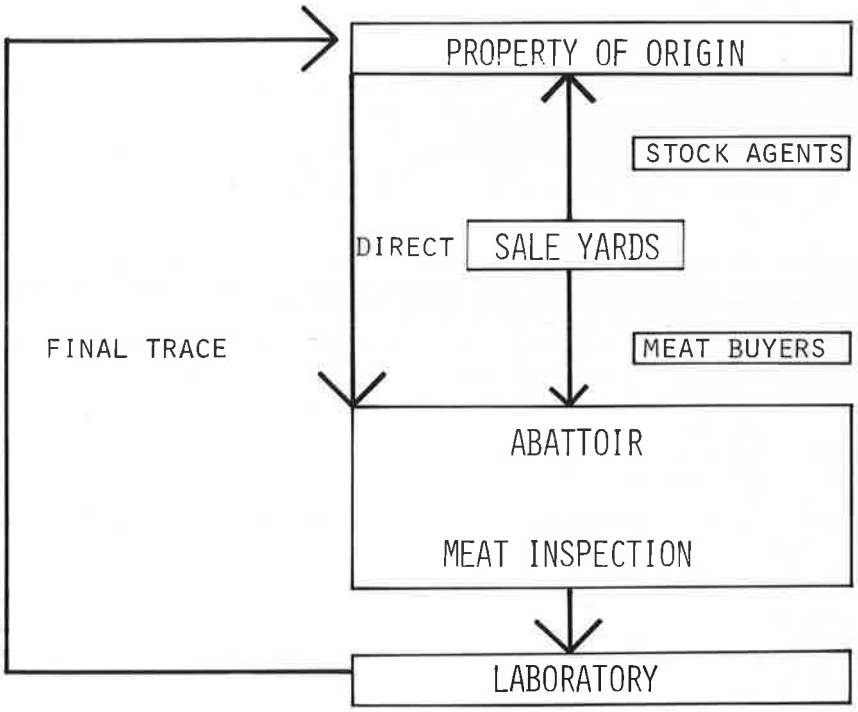


FIGURE 3. Format of the trace system employed to determine the properties of origin of the selected sheep.

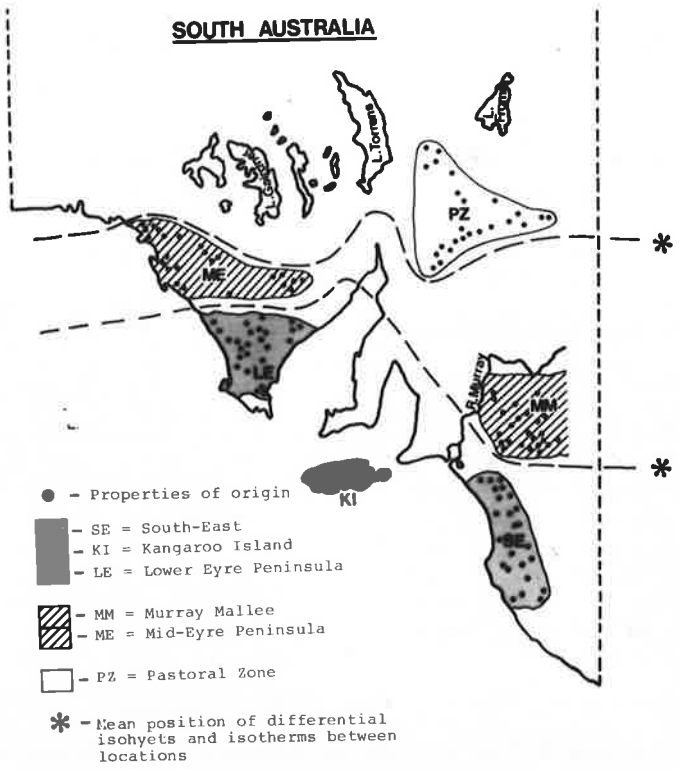


FIGURE 4. The properties of origin of the sheep for each location of sample.

7.2 ABATTOIR SAMPLING

At the metropolitan and regional abattoirs, the marked sheep were culled and then presented as a separate line for slaughter. At the moment of slaughter by exsanguination, 50 mls of blood was collected from each animal and an identification mark placed on each carcase.

Five tissue samples were then collected from the musculature of each sheep during the dressing procedure. The lesser diaphragm and the left oblique abdominal muscle were obtained from each dressed carcase. The proximal oesophagus and the apex of the heart were taken for each animal from the visceral inspection table and the posterior muscle groups of the tongue were excised from each severed head. These tissues were the most readily-accessible to obtain without interfering with slaughtering efficiency, and they were also presumed to be predilection sites of infection by the meat inspection personnel.

8.0 SPECIMEN STUDIES

Studies were then performed on the specimens collected to detect macroscopic sarcocysts, microscopic sarcocysts and antibodies to *Sarcocystis* using gross, histological and immunoserological examinations.

8.1 GROSS EXAMINATION

Every carcase is routinely inspected at the abattoir for a variety of parasitic and pathological lesions by the meat inspection personnel. A record was kept of their findings for the prevalence of macroscopic sarcocyst infections in the survey sheep.

A more detailed inspection of each carcase was then performed. The superficial aspect of the exterior and interior musculature of each carcase was closely examined, particularly the diaphragm and the thoracic andabdominal walls. Two longitudinal incisions were made either side of the tensor fascia-latae muscle in each flank and an internal exploration performed. The whole length of the oesophagus was examined visually and also tactilely to detect any gross subsurface sarcocysts.

Lastly, the posterior half of the tongue was closely examined through a series of cross-sectional incisions. The prevalence of sheep found infected with macroscopic sarcocysts by this examination was then recorded.

8.2 HISTOLOGICAL EXAMINATION

The tissue samples collected were formalin-fixed at the abattoirs and then cut into smaller segments in the laboratory after adequate fixation. These segments were processed routinely (i.e.,dehydrated, cleared and infiltrated) and then embedded in paraffin wax ensuring their random orientation within each block. Two histological sections were cut from each block at 5 µm thickness and stained with haematoxylin and eosin using standard staining procedures (cf.Thompson 1966). The stained sections were examined under a light microscope ensuring the minimum area of any section examined was 1.0 cm^2. The following information was then recorded:-

(a) Prevalence

The presence or absence of microscopic sarcocysts was noted for each of the 8,640 histological sections, thereby giving the prevalence of microscopic infection.

(b) Intensity

The number of microscopic sarcocysts encountered in each section was then measured using a planimetric count (Weibel 1972) on a 1 mm point grid magnified 3.25 X on to an overhead viewer. The intensity of microscopic infection for each tissue sample was then calculated stereologically (Elias *et al*.1971) using the formula :-

$$Nv = \frac{n}{A(\bar{D} + t - 2h')}$$

where

Nv = number of sarcocysts per unit volume

n = number of sarcocysts in each section

A = area of the section

$\bar{D}$ = mean diameter of the sarcocysts counted

t = section thickness

h' = height of optically or mechanically-lost sarcocysts

It was assumed that sarcocysts occupying less than half the section thickness could not be detected or fell out of the section; therefore, the optimum value of h' was taken as 2.5 μm.

(c) Cyst Size

The minimum diameter (width) of the first 10 sarcocysts encountered in each section was measured at 400 X magnification using an eye-piece graticule. Minimum diameter was measured in preference to that of maximum diameter (length) for it provides a much more stable indication of the size of the tubular sarcocysts when they are randomly sectioned. It is also better applied to stereological extrapolation and statistical analysis (Elias *et al*. 1971).

8.3 IMMUNOSEROLOGICAL EXAMINATION

The serum was removed by centrifugation from each blood sample collected and then stored frozen until examined. The sera were immunoserologically examined for antibodies to *Sarcocystis spp*. using the Complement Fixation Test and the Indirect Fluorescent-antibody Test.

8.31 Complement Fixation Test (CFT)

The CFT technique used was based on a microtest method (Anon. 1965) and was essentially similar to that described by Munday & Corbould (1974); therefore, it is only presented below in summary.

(a) Materials

The diluent used throughout the test was complement fixation saline (CFS) which was prepared from commercially-available sources (Oxoid diluent tablets).

The antigen was derived from visible sarcocysts obtained from slaughter sheep at the abattoirs. They were predominantly the long thin cysts found in the carcase musculature but did include some round fat cysts from the oesophagus. The cysts were excised intact from the musculature, washed twice in CFS and then lightly macerated to free the contained cystozoites. The larger cyst wall debris was sedimented out and the remaining solution diluted with CFS to give an approximate 10% suspension. This was frozen and thawed three times and then centrifuged at 2,000g for 60 minutes. The supernatant, which constitutes the antigen, was removed and preserved with 0.5% sodium azide. Each newly-prepared batch of antigen was standardized, by carrying out a chequer-board titration with a known positive serum, to give its highest working dilution.

Complement was obtained from guinea-pig serum and was preserved in Richardson's solution. Prior to use, the 50% haemolytic dose (C'H50) was determined for the

complement by titrating it against the indicator system and measuring the results optically on a photometer. The degree of haemolysis was plotted against the complement dosage on log/log paper and the working dilution of the complement to be used in the CFT was calculated as that equivalent to 5 times the C'H50 (e.g., see Figure 5).

The indicator system was prepared fresh before each test and consisted of a 3% suspension of sheep erythrocytes optimally sensitized in haemolysin. The appropriate haemolysin dilution was determined by titrating it against the complement working dilution and measuring the results optically on a photometer. The percentage haemolysis was plotted against the haemolysin dilution, thereby giving the dilution to be used in the CFT as that 25% stronger than the dilution at which the plateau of the graph began (e.g., see Figure 6).

The test sera were inactivated in a water bath at 58°C. for 30 minutes immediately prior to testing.

(b) Methods

The test sera were doubly diluted automatically in microtitre plates from 1:4 to 1:512. This left units of 0.025 mls of each serum dilution in each of the U-shaped wells. Equivalent volumes of antigen and complement were then added to each well and the reagents were fixed by incubation at 37°C. for 30 minutes. After fixation, 0.025 mls of the indicator system was added to each well and the reagents were thoroughly mixed during further incubation at 37°C. for 30 minutes. The test

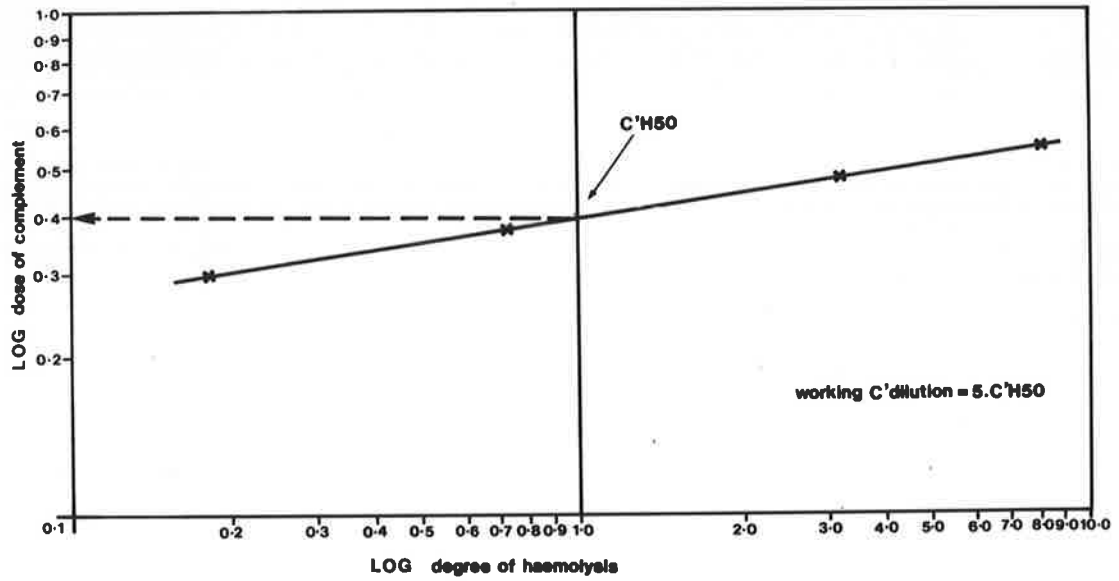


FIGURE 5. Example illustrating the calculation of the complement dosage to be used in the Complement Fixation Test.

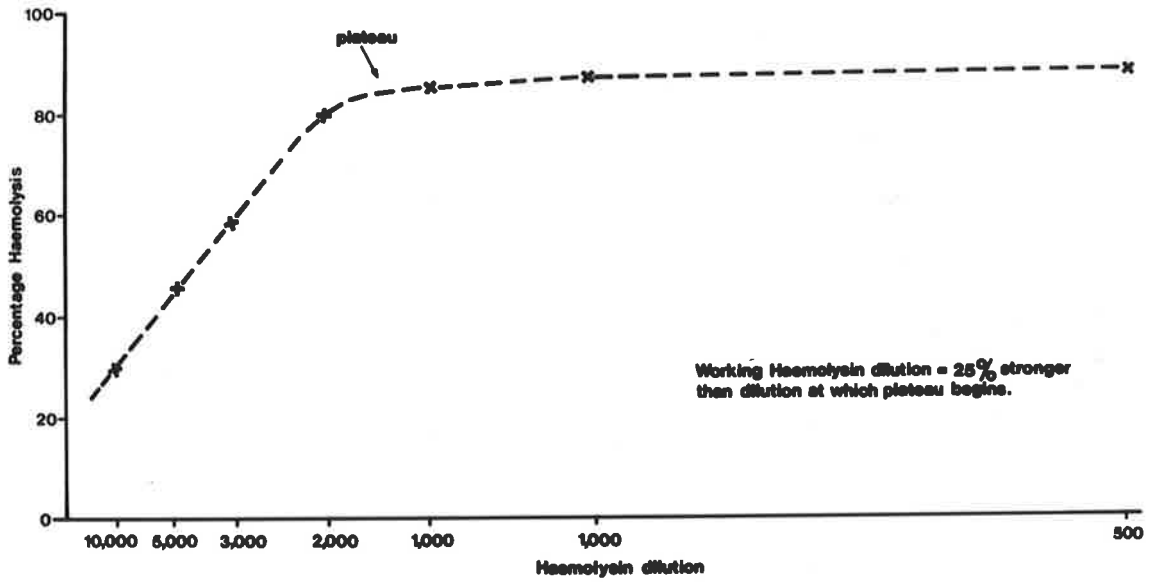


FIGURE 6. Example illustrating the calculation of the haemolysin dilution to be used in the Complement Fixation Test.

plates were then refrigerated for 2 hours before examination.

Known positive and negative sera were also included with the test sera to check the validity of the test results. An anticomplementary control was also run for each test serum and simply consisted of CFS being added to a duplicate 1:4 serum dilution instead of the antigen. Anticomplementary sera, which do not lyse the erythrocytes, were re-tested after de-anticomplementing by mixing with Kaolin in the ratio of 1:4 for 20 minutes and then centrifuging at 900g for 10 minutes.

(c) A negative result in the CFT was obtained when the erythrocytes were completely lysed and a positive result when no or only partial lysis occurred. The end-point of the CFT for each serum was taken as the last titration at which complete lysis had not yet occurred. For ease of analysis and presentation, these end-point titres were transformed on to a linear scale of 0 to 9, as shown in Table 4.

ACTUAL TITRE	TRANSFORMED TITRE
< 1:4	0
1:4	1
1:8	2
1:16	3
1:32	4
1:64	5
1:128	6
1:256	7
1:512	8
> 1:512	9

TABLE 4. Linear transformation of immunoserological titres

For sheep, an actual titre of 1:8 (or a transformed titre of 2) in the CFT was taken as indicative of infection by *Sarcocystis spp.*

8.32 Indirect Fluorescent-antibody Test (IFAT)

The IFAT technique used for *Sarcocystis* infections was developed from that being used for *Toxoplasma* infections in animals (Suzuki *et al.* 1965, Munday & Corbould 1971). The technique is described below with the steps found to be critical given in full and those which were just routine immunological procedures (cf. Weir 1973) summarized.

(a) Materials

The diluent used throughout the IFAT was standardized as phosphate-buffered saline (PBS) pH 7.2.

The antigen used was derived from visible sarcocysts obtained from slaughter sheep at the abattoirs. The sarcocysts used were confined specifically to the long thin cysts found in the carcase musculature, particularly the diaphragm, and it was found preferable to use those under 5 mm in length. The sarcocysts were obtained fresh at slaughter and were excised intact from the surrounding muscular and connective tissue. They were washed twice in PBS and then lightly macerated in 10 times their volume of PBS with added antibiotics (100 units of penicillin and 10 µg of streptomycin per ml). This suspension was allowed to stand for 30 minutes and then centrifuged at 900g for 5 minutes. The supernatant containing the released cystozoites was then washed vigorously 3 times in PBS in-between similar centrifugation. It was then diluted with PBS until there were approximately 30 cystozoites per high power field (400 X) under the light microscope. Drops of this antigen preparation were placed on clean glass slides by means of a platinum loop. The slides

were air-dried, fixed in 10% aqueous methanol for 1 minute and then stored frozen until use.

Anti-sheep immunoglobulins labelled with a fluorochrome were prepared in the laboratory (as summarized below) and were found to be more sensitive in the IFAT than the commercially-available reagents.

Sheep immunoglobulins were harvested from serum by salt fractionation using a 40% saturated ammonium sulphate solution (Heide & Schwick 1973). They were mixed with Freund's complete adjuvant and then inoculated into laboratory rabbits to produce anti-sheep immunoglobulins (Herbert 1973). These were harvested by salt fractionation from rabbit serum obtained 56 days post-inoculation and then tested for potency and specificity by respectively, a capillary-tube precipitin test and double diffusion in agar gel. Suitable anti-sheep immunoglobulins were then conjugated to fluorescein isothiocyanate in aqueous solution pH 9.0 at 4°C (Johnson & Holborow 1973), then further purified by passing through a Sephadex G-25 column. The labelled anti-sheep immunoglobulins were then stored frozen in aliquots until use.

The mounting medium used for the test slides was carbonate-buffered glycerol pH 9.0.

(b) Methods

The test sera were doubly diluted from 1:4 to 1:512 and one drop of each serum dilution was placed on the appropriate test area of antigen. The slides were incubated in a moist chamber at 37°C for 30 minutes

and then washed 3 times in PBS for 10 minutes each. Each test area was then overlaid with the labelled anti-sheep immunoglobulins and the slides incubated for a further 30 minutes. The slides were then washed 3 times in PBS for 10 minutes each, flushed in distilled water and mounted in the buffered glycerol before examination.

The prepared test slides were positioned under a microscope at 400 X magnification using transmitted dark-field illumination (tungsten lamp source). The tests were then read using transmitted ultra-violet illumination (200W mercury-vapour lamp) through a suitable light filtration system (excitor filter Diffus N, primary filter BG 12 and suppression filter K 530).

A negative control was run with each test serum and simply consisted of one antigen area being overlaid with PBS instead of serum. This control acted as a check for autofluorescence as well as indicating the degree of non-specific background staining. A positive serum of known titre was also included with each test batch to check the validity of the IFAT results

(c) Results

A positive result was obtained when the cystozoites exhibited uniform yellow-green fluorescence (e.g., see Figure 7). Partial fluorescence of the cystozoites was not regarded as significant (e.g., see Figure 8). The end-point of each IFAT was taken as the highest titre at which fluorescence last occurred. These titres were also transformed on to a linear scale of 0 to 9 (as shown in Table 4 for teh CFT) for ease of analysis and presentation. For sheep, a titre of 1:16 (or transformed titre of 3) in the IFAT was regarded as indicative of infection by *Sarcocystis spp*.

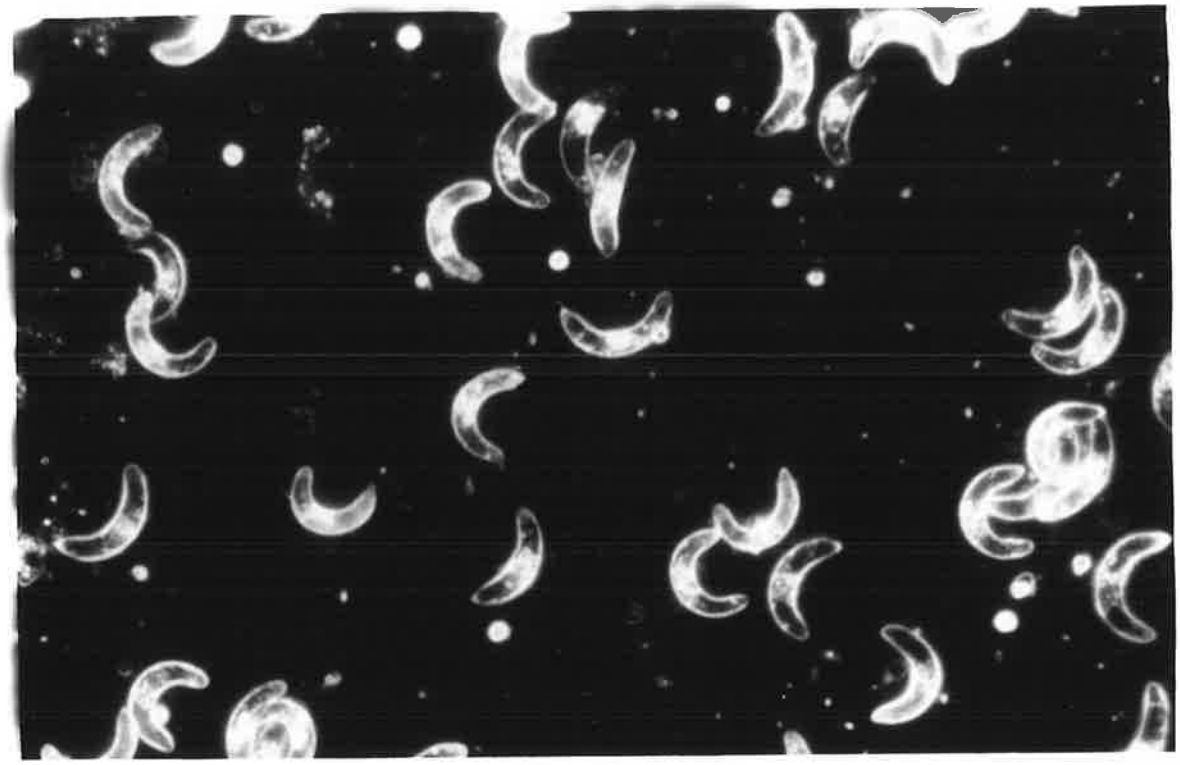


FIGURE 7. Cystozoites of *Sarcocystis sp.* exhibiting overall, circumferential fluorescence denoting a positive result to the Indirect Fluorescent-antibody Test. (~1,500 X mag.)

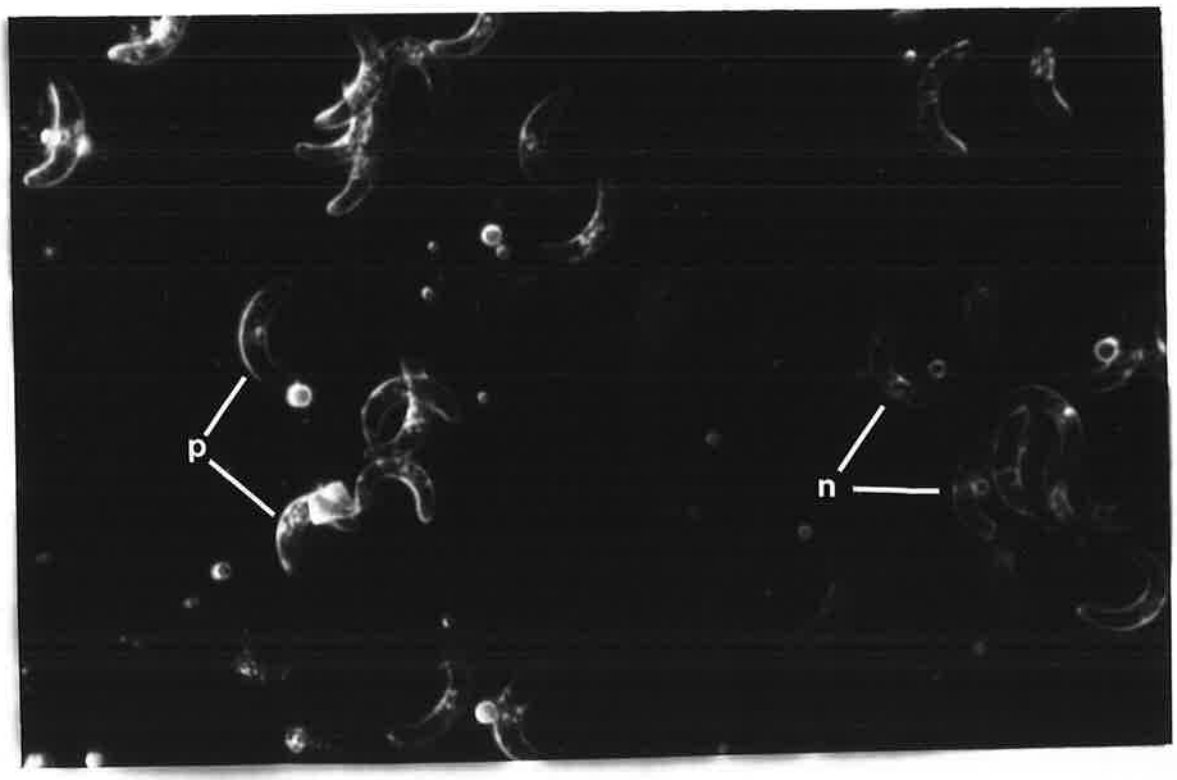


FIGURE 8. Cystozoites of *Sarcocystis sp.* exhibiting partial (p) and no (n) fluorescence denoting a negative result to the Indirect Fluorescent-antibody Test. (~1,500 X mag.)

9.0 ECOLOGICAL STUDIES

The 144 properties of origin of the survey sheep were visited during 1975 and the property owners were interviewed to gain any information which may be relevant to the distribution and abundance of the parasite.

9.1 SELECTION OF FEATURES

The selection of the ecological features included in the interviews assumed no pre-knowledge of the life cycle of the parasite or of any factors thought to influence its distribution. They were selected without bias to provide information about the history of the sheep slaughtered and their management - i.e.,

- origin and type of sheep
- grazing and yarding history
- history of known diseases and treatments
- contact with other animals

as well as general information about the property - i. e.,

- type of other domestic stock carried
- grazing and yarding facilities
- soil types, treatment and topography
- native and introduced flora
- native and feral fauna
- climatic conditions

A total of 83 ecological features was examined and was divided into those quantitive or qualitative in nature for statistical analysis. A complete list of the features examined is presented with the results in Appendix III under the following classifications

9.2 CLASSIFICATION OF FEATURES

For ease of presentation, the features examined were subsequently classified according to 3 phases of the life cycle of the parasite (see Figure 9).

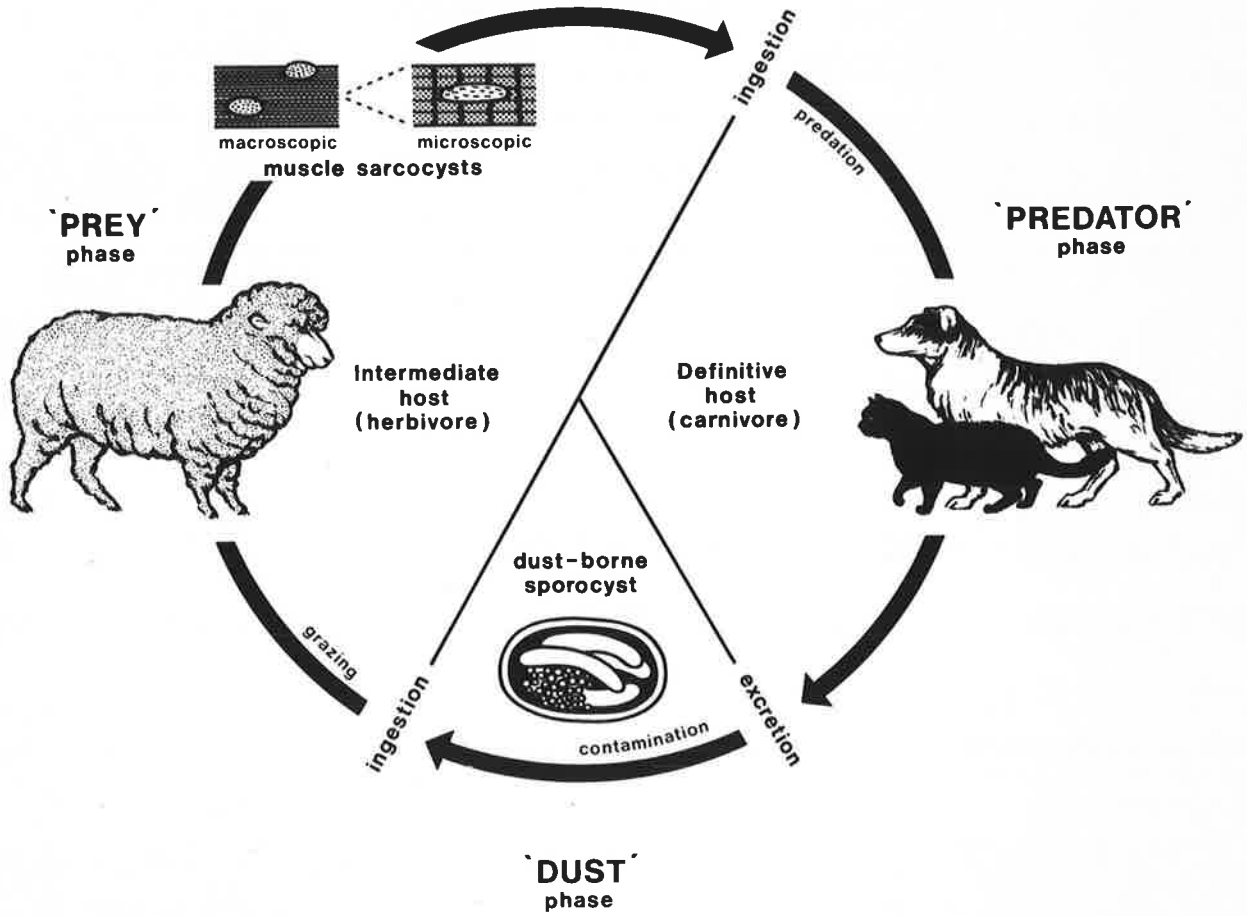


FIGURE 9. Diagrammatical life cycle of *Sarcocystis spp.* in sheep illustrating 3 basic phases of the life cycle.

Of the 83 ecological features examined, 31 were concerned with details of potential intermediate host animals and were classified under the 'prey' phase of the parasite's life cycle. A further 34 features were relevant to the various definitive host animals and were classified under the 'predator' phase of the life cycle. The remaining 18 features were related to the physical environment and were classified under the 'dust' phase of the life cycle. The importance of these classifications lies basically in determining the orientation of future research as will be seen in the discussion.

10.0 STATISTICAL ANALYSES

All results obtained were subject to statistical analysis to determine their level of significance upon the distribution and abundance of the parasite. The analyses were performed on geometric means derived from logarithmic transformations, thereby accounting for any possible skew distribution of the data.

10.1 ANALYSES OF VARIANCE

Each parameter measured in the factorial-design survey (as given in the results) was subject to an analysis of variance which was carried out by computer (programmed in Fortran language with 'subroutine AFACT' from the International Mathematical and Statistical Library). Most parameters were averaged for each property in the survey and weighted means were used for the data transformations to negate any possible diminution of data. These parameters were then subject to 5-factorial analyses of variance. The intensity of the antibody responses to infection was measured for each individual animal and was subject to 6-factorial analyses of variance. The levels of significance of the main effects and the 2 X interactions were determined for each analysis using the

F-test and the Student t-test.

10.2 CORRELATIONS

Correlations were sought between the various methods of detection employed in the survey by multiple regression analyses performed by computer (programmed in Fortran language with 'Statistical Subroutine Package 6400' from Control Data Library). Correlations between the quantitive ecological features and the intensity and cyst size of infection were sought using the same computerized multiple regression analyses. The qualitative ecological features were also examined for relationships with the intensity and cyst size of infection, but in these cases the Chi-squared test was used which tested the frequency distribution about the median.

RESULTS

11.0 PREVALENCE OF INFECTION

The prevalence of macroscopic sarcocysts, microscopic sarcocysts and antibodies to *Sarcocystis* were averaged for each property in the survey prior to analysis. The results of the analyses of variance and their statistical significances are summarized in Appendix I (Tables 1-4). The geometric means for each factor subdivision are presented in Table 5 :-

FACTORS AND SUBDIVISIONS		PREVALENCE (%)			
		SARCOCYSTS		ANTIBODIES	
		MACROSCOPIC	MICROSCOPIC	CFT	IFAT
TOTAL:		6.71	93.17	93.74	96.88
Age	Y	0	90.28	95.14	95.84
	A	13.43	96.07	91.44	97.92
Sex	♀	7.41	93.29	94.22	97.23
	♂	6.02	93.06	93.06	96.53
Breed	M	5.56	96.30	94.68	97.23
	C	7.87	90.05	92.60	96.53
Location	SE	3.48	90.98	90.98	97.23
	KI	10.42	98.62	94.45	97.92
	LE	9.73	93.06	95.84	95.84
	MM	2.78	93.75	91.67	96.53
	ME	12.50	90.28	94.45	95.84
	PZ	1.39	92.37	94.45	97.92
Season	w	3.82	80.56	91.32	93.75
	m	6.60	98.96	95.14	98.62
	d	9.73	100.00	94.45	98.27

TABLE 5: Summary of results of the prevalence of infection for each factor subdivision

11.1 PREVALENCE OF MACROSCOPIC SARCOCYSTS

At the abattoirs, macroscopic sarcocysts were detected in 5.79% of the survey sheep by the meat inspection staff. However, when using the more detailed gross examination they were found in 6.71% of the sheep. Upon analysis, significant differences in the prevalence results for macroscopic sarcocysts were observed for the factors of age, location and season (see Figure 10). Only aged sheep were found infected with macroscopic sarcocysts. More sheep were found infected from Kangaroo Island and both locations on Eyre Peninsula than from elsewhere. More of the sheep slaughtered during the dry months were infected than those slaughtered during the other sampling seasons.

11.2 PREVALENCE OF MICROSCOPIC SARCOCYSTS

Upon histological examination of the selected tissues, microscopic sarcocysts were detected in 93.17% of the survey sheep. Significant differences in these microscopic prevalence results were found for the factors of age, breed and season (see Figure 11). More aged sheep were infected with microscopic sarcocysts than young animals and more Merinos were infected than Corriedales. Less animals slaughtered during the wet months were found infected than those examined during the dry and moist months. No significant differences were observed between the sexes of sheep sampled or between the locations of sample.

11.3 PREVALENCE OF ANTIBODIES TO *Sarcocystis*

Antibodies to *Sarcocystis* detected by the CFT were found in 93.74% of the survey sheep. The only significant difference found for the prevalence of these antibodies occurred for the factor of age (see Figure 12). More young sheep were detected as having complement-fixing antibodies to *Sarcocystis* than for aged sheep.

Antibodies detected by the IFAT occurred in 96.88% of the sheep examined and their prevalence differed significantly for the factors of age and season (see Figure 13). More aged sheep were positive to the IFAT than young animals and less of those slaughtered during the wet months were positive than those slaughtered during the other sampling seasons.

In the following histograms, the levels of significance to the Student t-test are expressed for each of the relevant bar diagrams as follows:

$* = 0.05 > p > 0.01$

$** = 0.01 > p > 0.001$

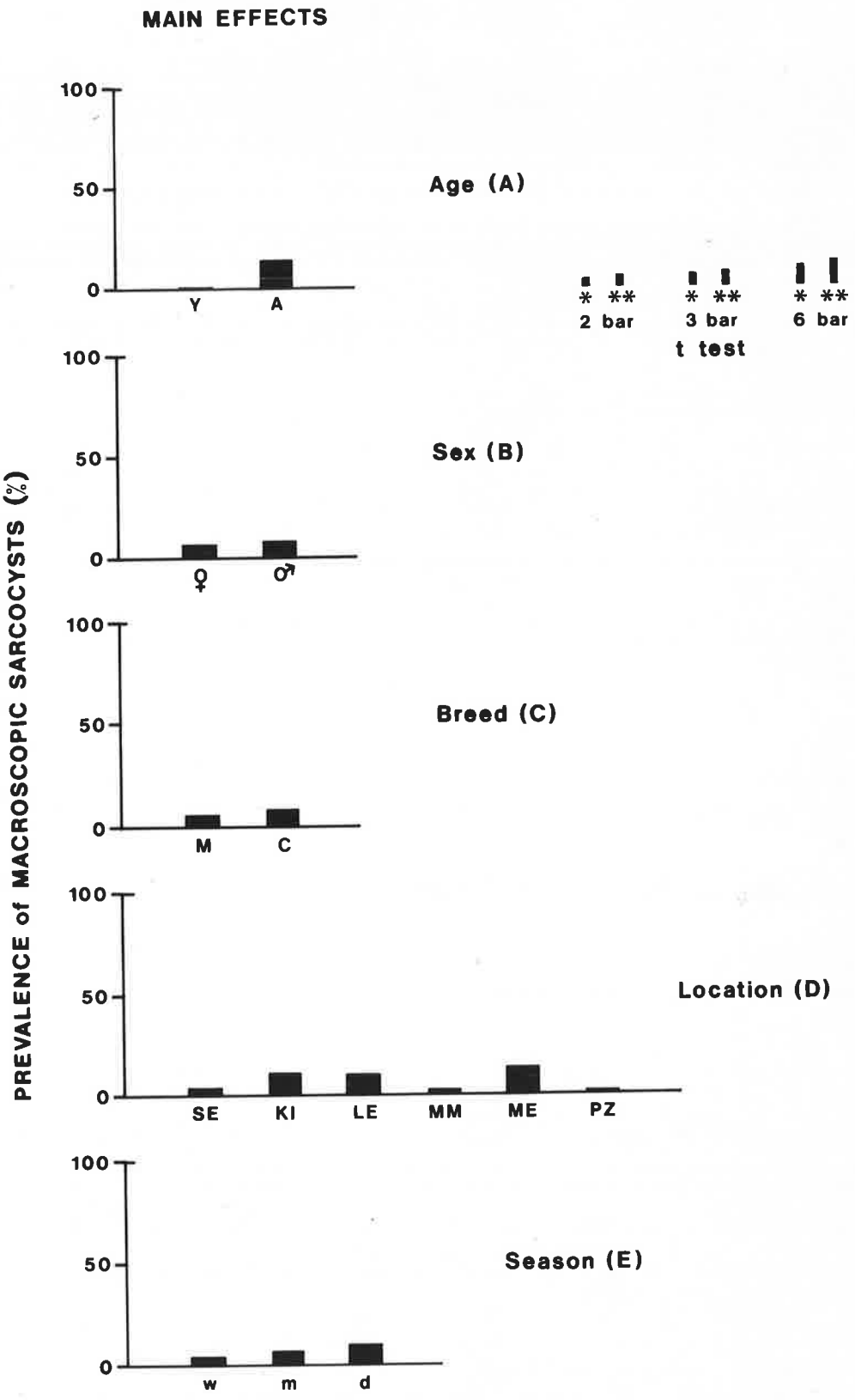


FIGURE 10. Prevalence of macroscopic sarcocysts in sheep for each factor examined in the survey - histograms of geometric means.

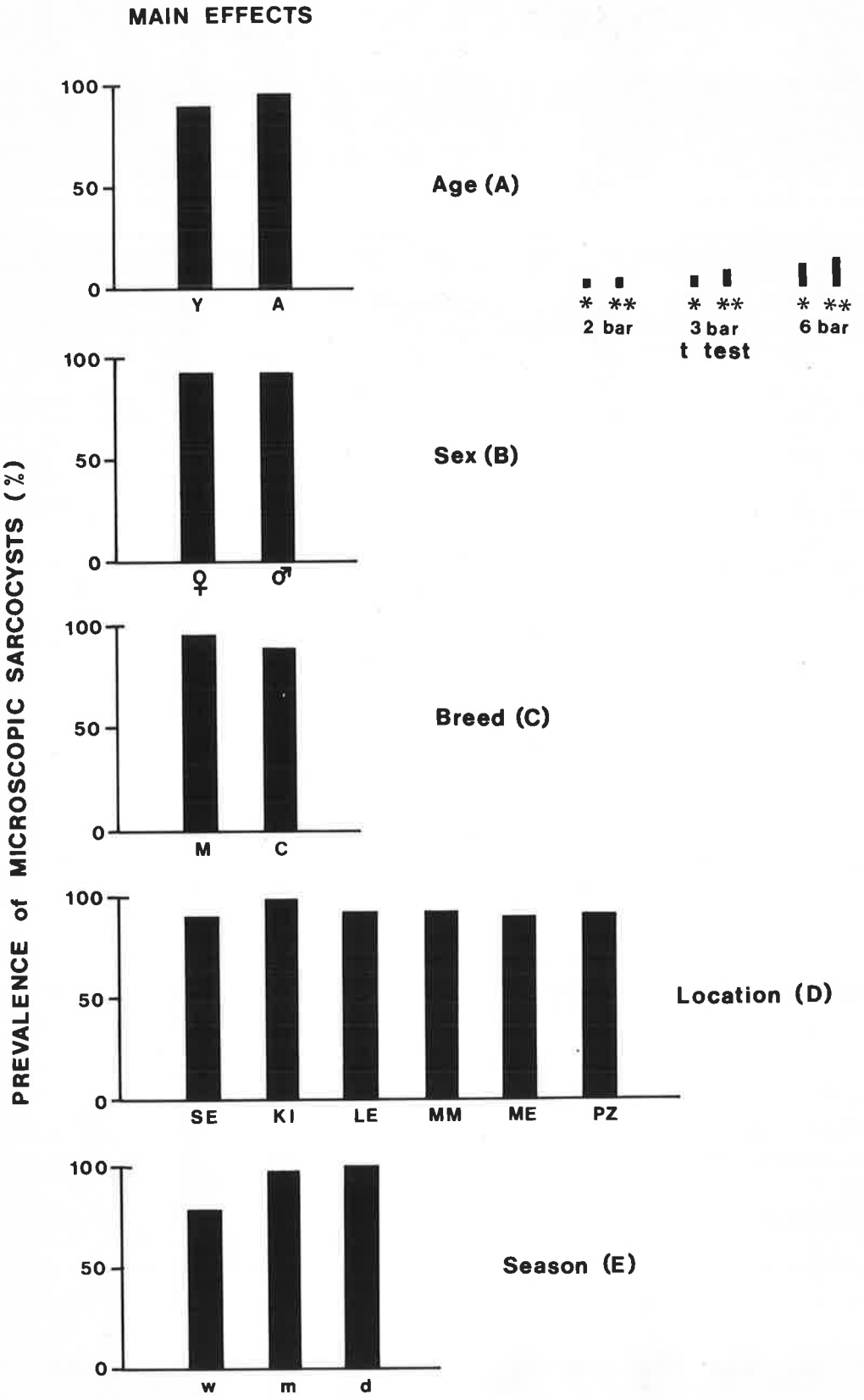


FIGURE 11. Prevalence of microscopic sarcocysts in sheep for each factor examined in the survey - histograms of geometric means.

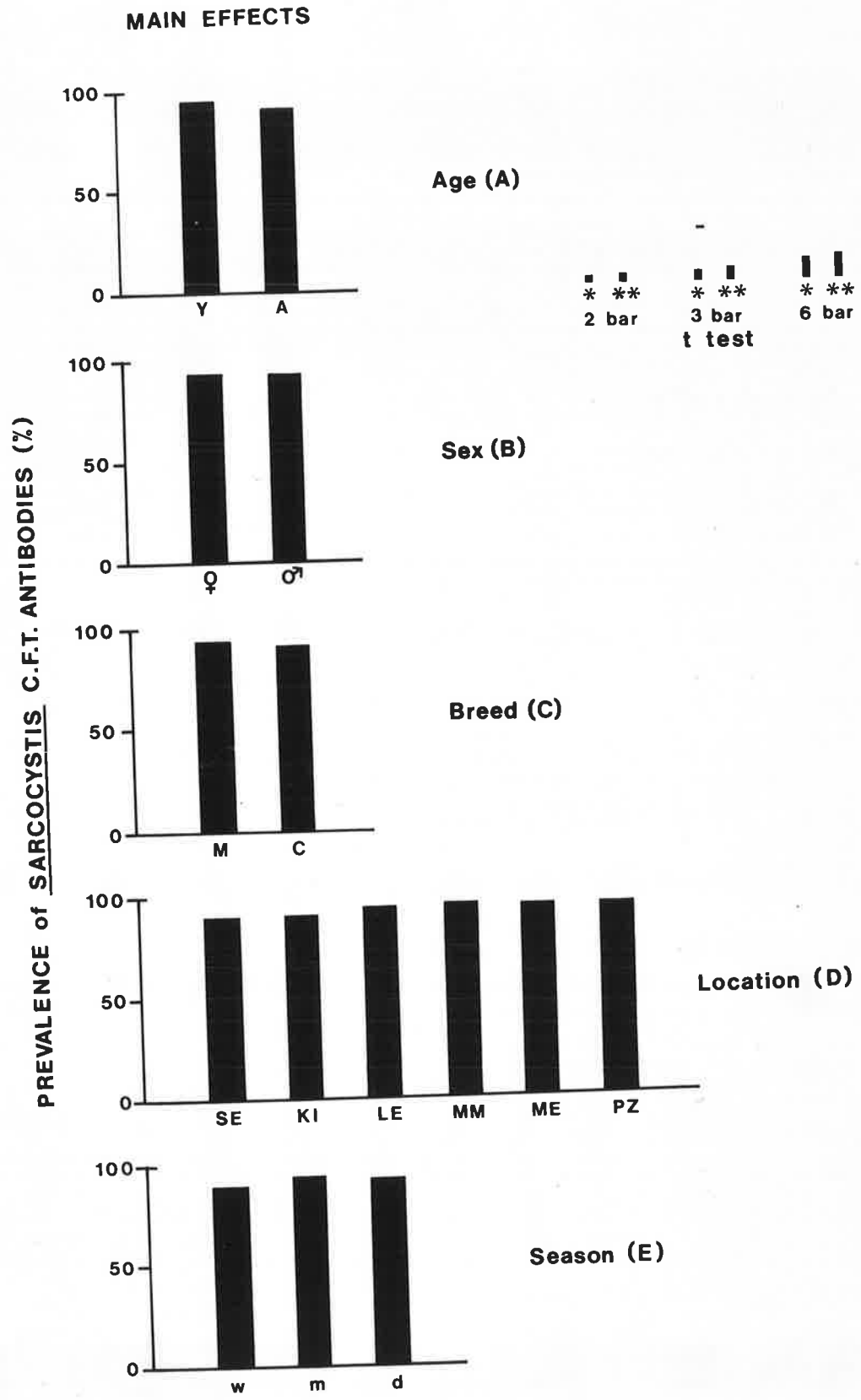


FIGURE 12. Prevalence of CFT antibodies to *Sarcocystis* in sheep for each factor examined in the survey - histograms of geometric means.

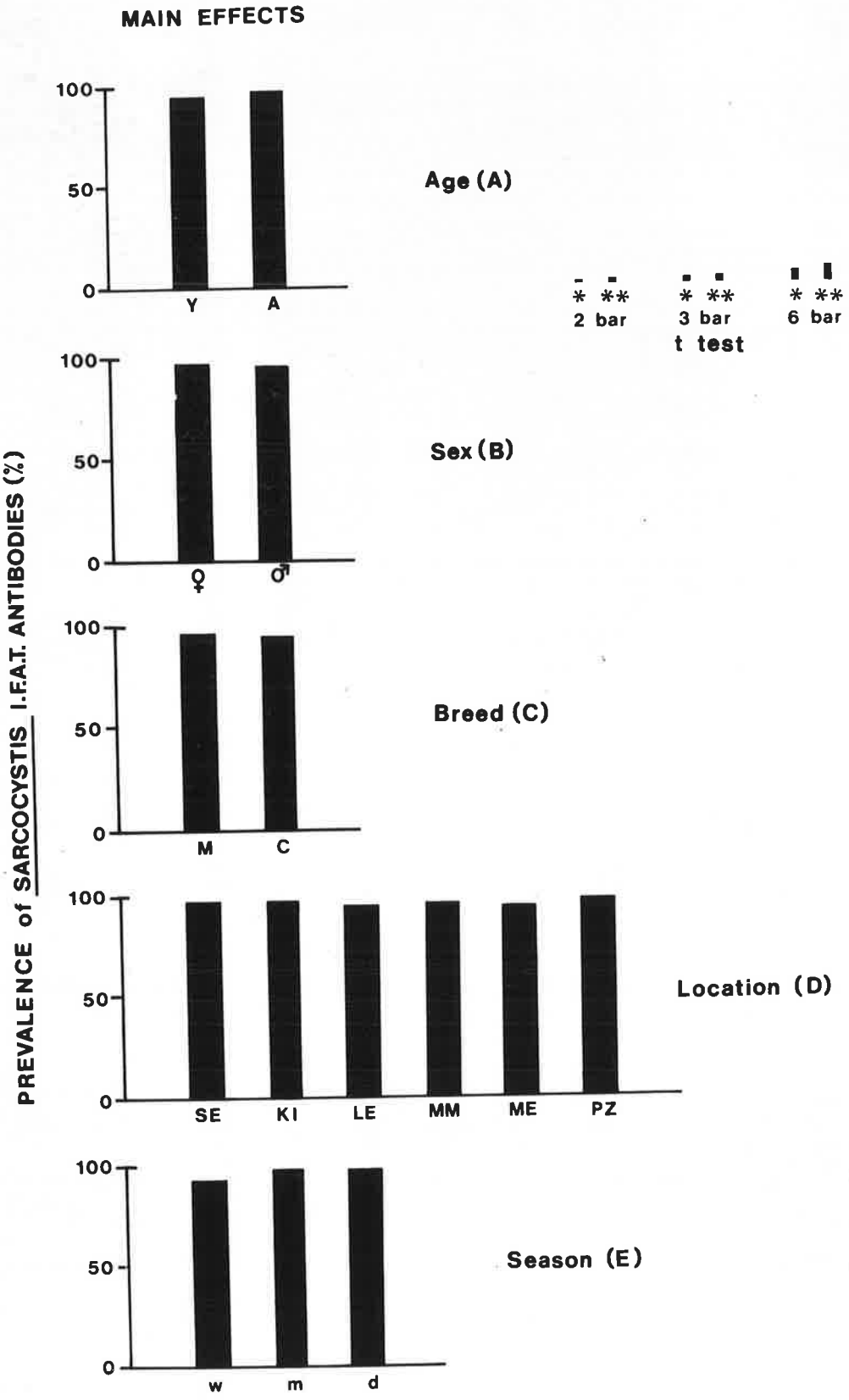


FIGURE 13. Prevalence of IFAT antibodies to *Sarcocystis* in sheep for each factor examined in the survey - histograms of geometric means.

12.0 INTENSITY OF INFECTION

The intensity of sarcocyst infection was averaged for each property in the survey prior to analysis whereas the intensities of the various antibody responses were analysed for individual animals. The results of these analyses of variance are summarized in Appendix I (Tables 5-7) and the levels of significance are also shown. The geometric means for each subdivision of each factor are given in Table 6 :-

FACTORS AND SUBDIVISIONS		INTENSITY		
		SARCOCYSTS	ANTIBODIES	
		Cysts per cc muscle ($\times 10^{-3}$)	CFT (Transformed titres)	IFAT
TOTAL:		2.41	3.60	4.47
Age	Y	2.25	4.09	3.86
	A	2.56	3.12	5.07
Sex	♀	2.36	3.61	4.42
	♂	2.45	3.59	4.51
Breed	M	2.43	3.56	4.50
	C	2.38	3.66	4.43
Location	SE	2.24	3.54	4.64
	KI	2.49	3.83	4.41
	LE	2.36	3.66	4.26
	MM	1.78	3.47	4.39
	ME	2.98	3.55	4.58
	PZ	2.58	3.58	4.52
Season	w	1.52	3.41	4.28
	m	2.19	3.85	4.59
	d	3.51	3.55	4.53

TABLE 6. Summary of results of the intensity of infection for each factor subdivision.

12.1 INTENSITY OF SARCOCYSTS

The intensity of sarcocyst infection (macroscopic plus microscopic) was quantitated as the number of sarcocysts per unit volume of tissue, and the grand mean for the survey sheep was 2.41×10^3 sarcocysts/cc muscle. Significant differences in the intensity of infection were found for the factors of location and season (see Figure 14). Lighter infections were found in sheep from the Murray Mallee than in those from the other sampling locations. Heavier infections were observed in the sheep slaughtered as the months became drier. No other factors were found to be significant.

12.2 INTENSITY OF ANTIBODIES TO *Sarcocystis*

The mean transformed titre for antibodies to *Sarcocystis* measured using the CFT was 3.60, which is approximately equal to an actual titre of 1:26. Significant differences in the intensity of the CFT antibody response occurred for the factors of age and season only (see Figure 15). Young sheep were found to have higher titres than aged sheep and animals sampled during the moist months exhibited higher titres than did those sampled during the other seasons.

For antibodies to *Sarcocystis* measured using the IFAT, the mean transformed titre was 4.47 which is approximately equal to an actual titre of 1:47. The intensity of these antibodies differed significantly for the factors of age, location and season (see Figure 16). Lower antibody titres were found in young sheep than in aged animals, in sheep from lower Eyre Peninsula than in those from the other locations and in sheep sampled during the wet months than in those sampled during the other two seasons.

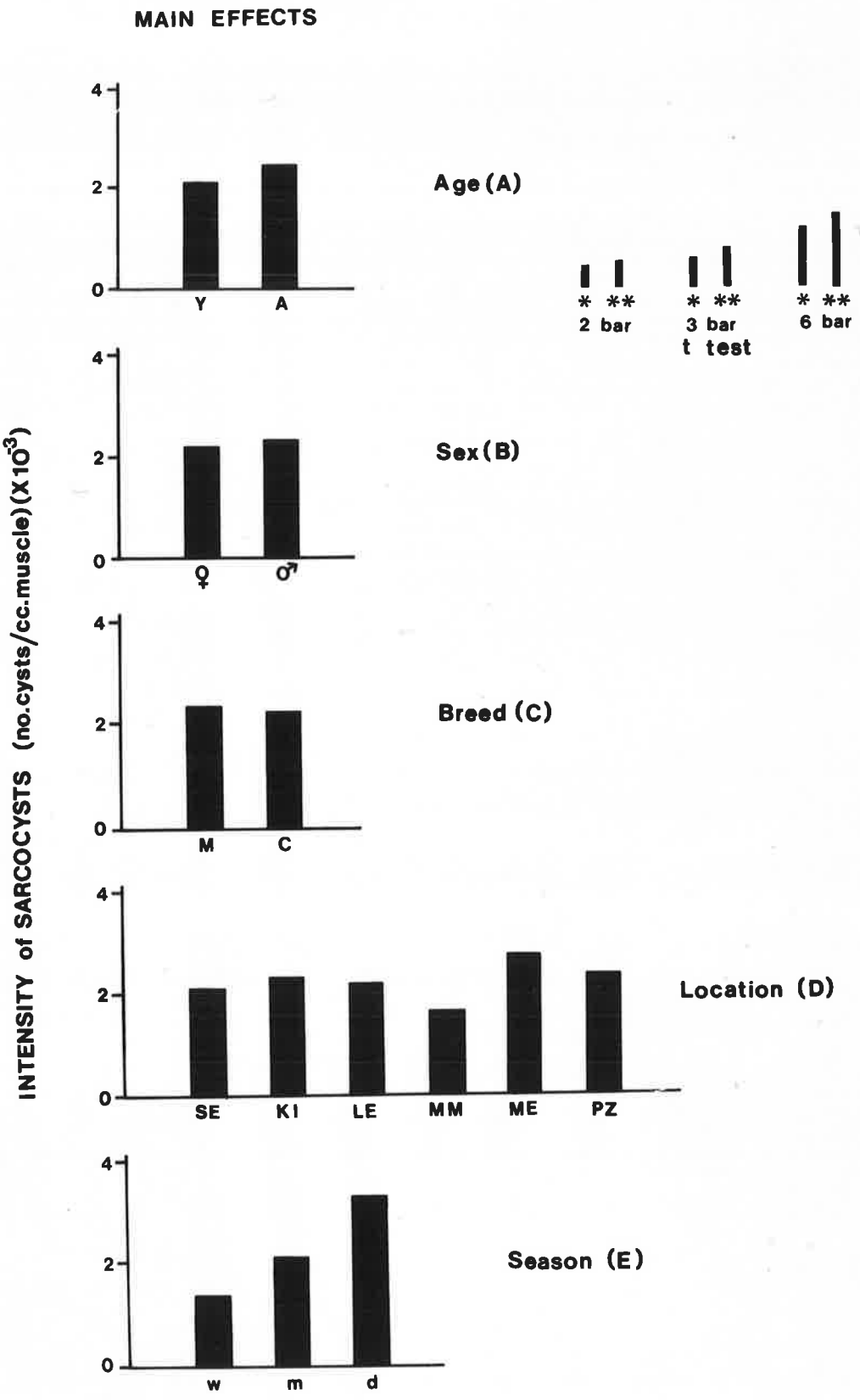


FIGURE 14. Intensity of sarcocyst infection in sheep for each factor examined in the survey - histograms of geometric means.

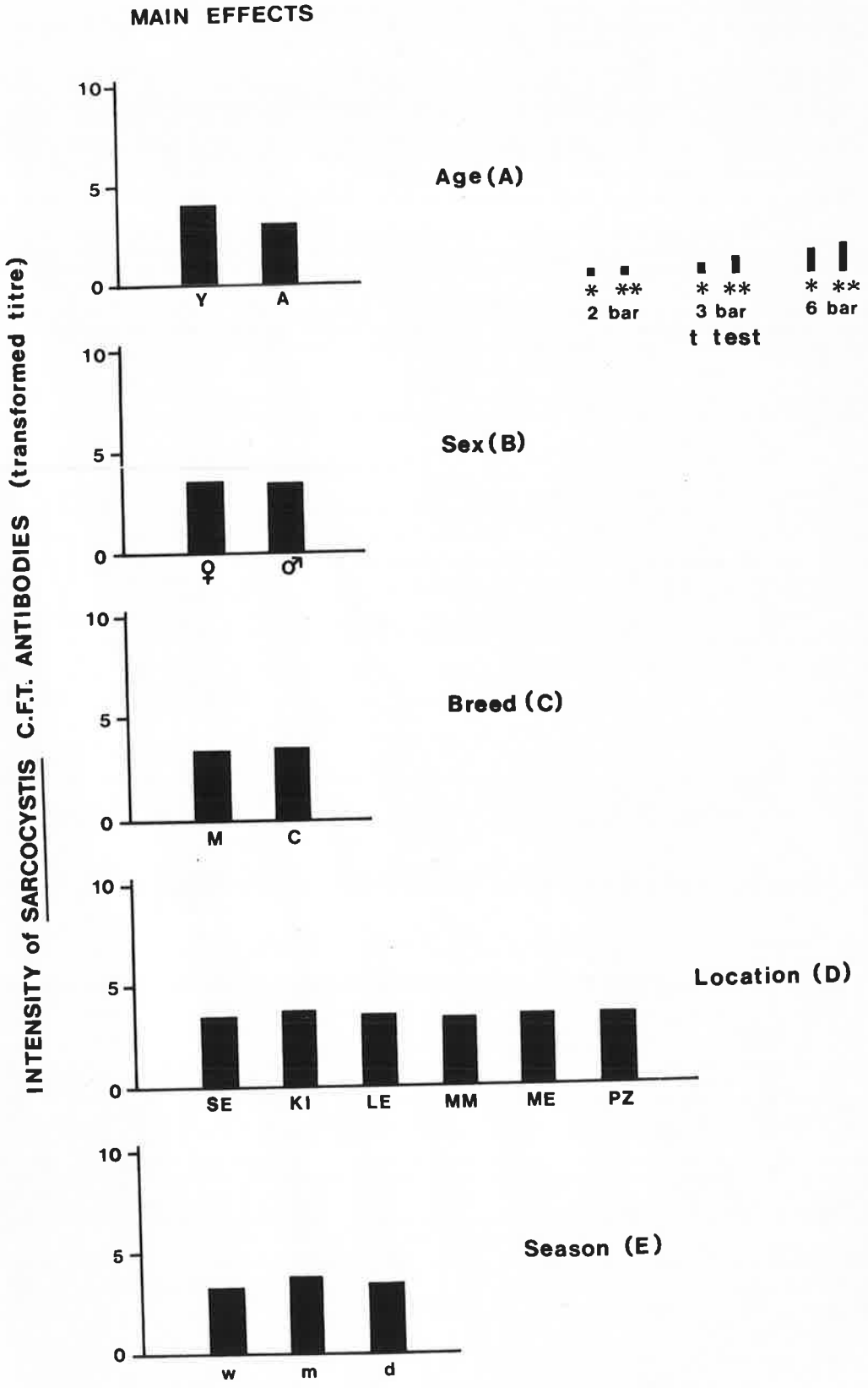


FIGURE 15. Intensity of CFT antibody response in sheep for each factor examined in the survey - histograms of geometric means.

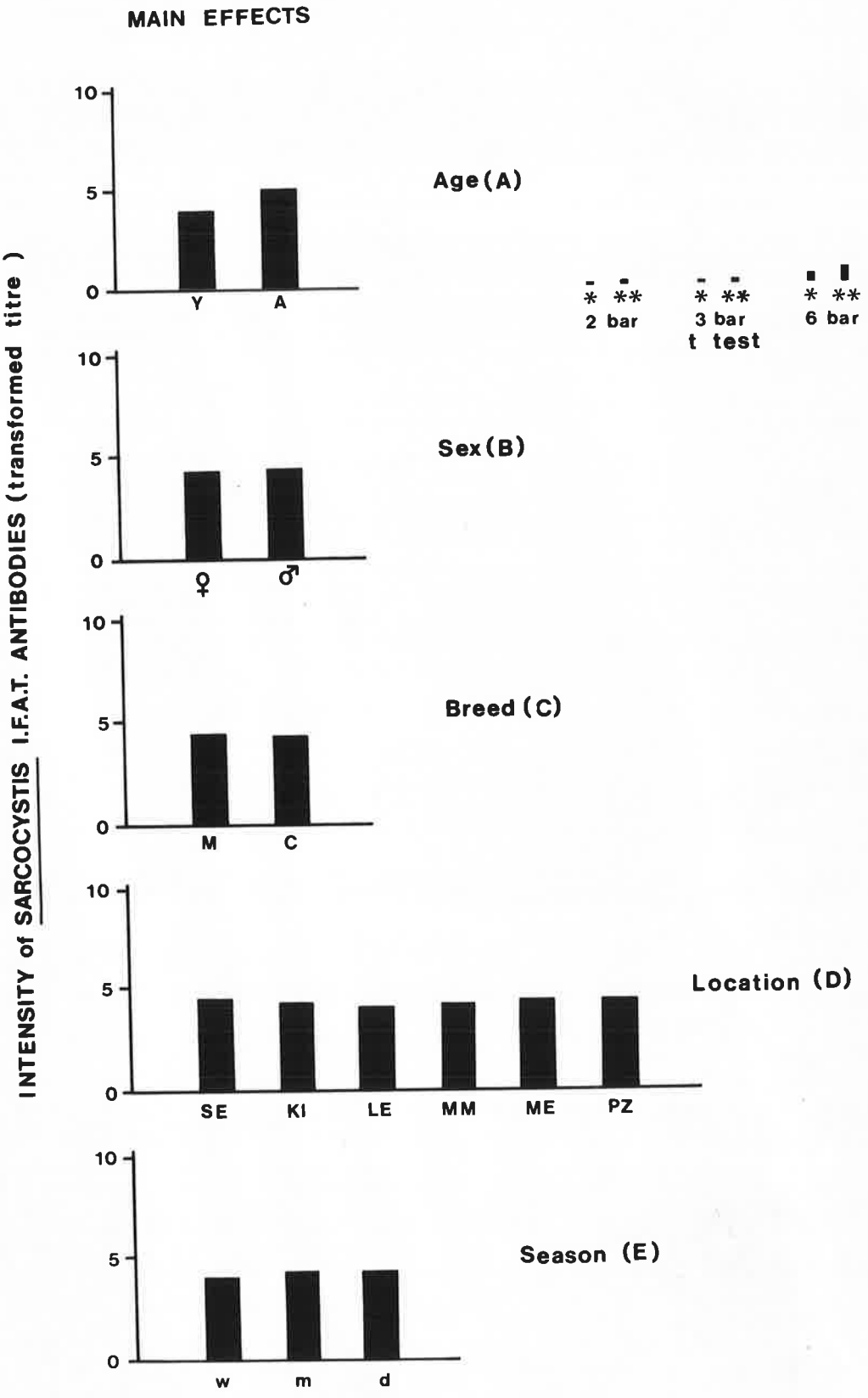


FIGURE 16. Intensity of IFAT antibody response in sheep for each factor examined in the survey - histograms of geometric means.

13.0 CYST SIZE OF INFECTION

The minimum diameters of the microscopic sarcocysts were averaged for each property involved in the survey before analysis. The results of such analysis, and the levels of significance, are summarized in Appendix I (Table 8). Because relatively few properties were infected with macroscopic sarcocysts, their minimum diameters were averaged for each infected sheep and analysed using the Chi-squared test which examined the size frequency distribution about the median. The results of this analysis are summarized in Appendix I (Table 9). The mean cyst sizes for both macroscopic and microscopic sarcocysts are given for each subdivision of each factor in Table 7 :-

FACTORS AND SUBDIVISIONS		CYST SIZE (μm) MACROSCOPIC SARCOCYSTS	CYST SIZE (μm) MICROSCOPIC SARCOCYSTS
TOTAL		956.74	34.77
Age	Y	0	30.83
	A	956.74	38.74
Sex	♀	920.47	34.30
	♂	1003.02	35.27
Breed	M	1100.26	35.13
	C	844.26	34.44
Location	SE	1225.00	35.41
	KI	833.09	35.59
	LE	813.39	34.71
	MM	962.50	33.38
	ME	1030.12	34.31
	PZ	1287.50	35.28
Season	w	1270.00	37.78
	m	1108.80	33.18
	d	687.05	33.39

TABLE 7. Summary of results of the cyst size of infection for each factor subdivision.

13.1 SIZE OF MACROSCOPIC SARCOCYSTS

The mean minimum diameter (i.e., mean width) of the macroscopic sarcocysts found in the survey sheep was 956.74 µm. Significant differences in the size occurrence of these cysts were found for the factors of age and season (see Figure 17). Only aged sheep were infected with macroscopic sarcocysts and there were more smaller cysts in sheep slaughtered during the dry months than in those sampled during the other seasons.

Two size populations of macroscopic sarcocysts were found when the grouped cyst sizes were plotted against their frequency (see Figure 18). These were arbitrarily named the 'fat' and 'thin' macrocyst populations and they had respective modes of 1250 µm and 350 µm.

13.2 SIZE OF MICROSCOPIC SARCOCYSTS

The mean minimum diameter of the microscopic sarcocysts detected in the survey sheep was 34.77 µm. The size of these microscopic sarcocysts differed significantly for the factors of age, sex and season (see Figure 19). Larger microscopic cysts were found in aged sheep than in young sheep, in wethers than in ewes and in sheep sampled during the wet months than in those sampled in the other seasons.

On plotting the grouped cyst sizes against their frequencies, two size populations were also found for microscopic sarcocysts (see Figure 20). The large microcyst population had a mode of 36 µm and the smaller a mode of 28 µm.

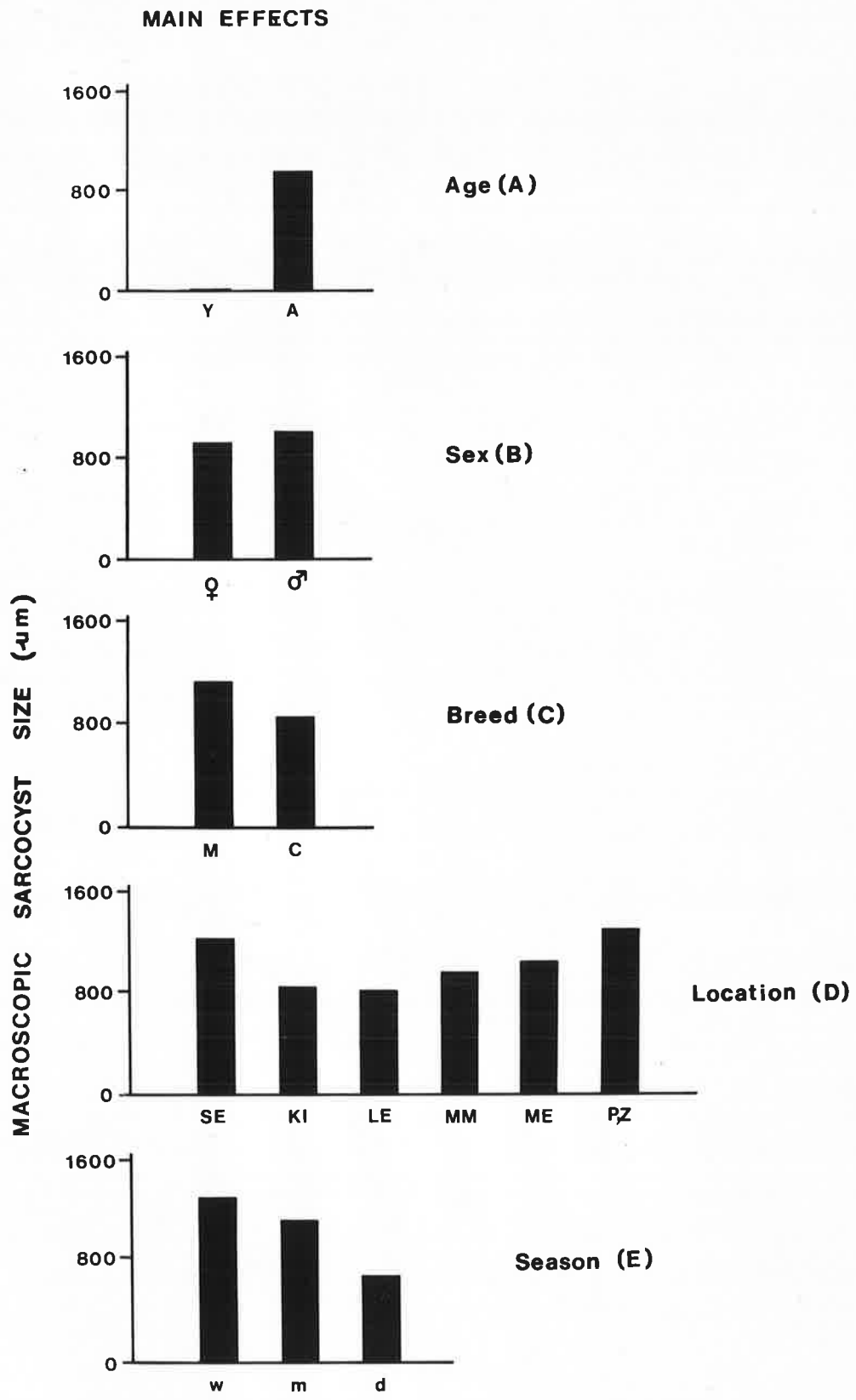


FIGURE 17. Sizes of macroscopic sarcocysts in sheep for each factor examined in the survey - histograms of means.

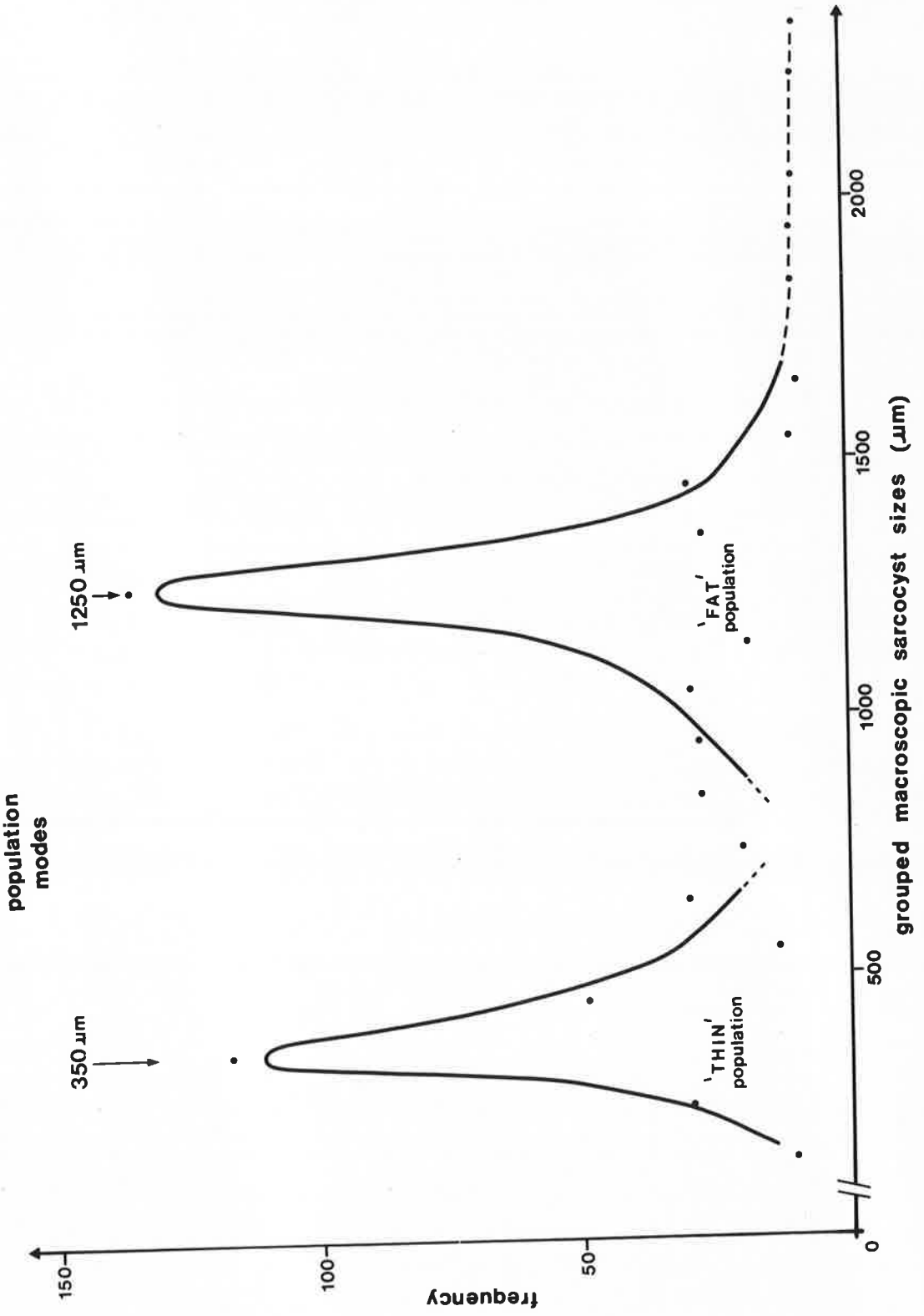


FIGURE 18. Frequency distributions for the two macroscopic sarcocyst size populations.

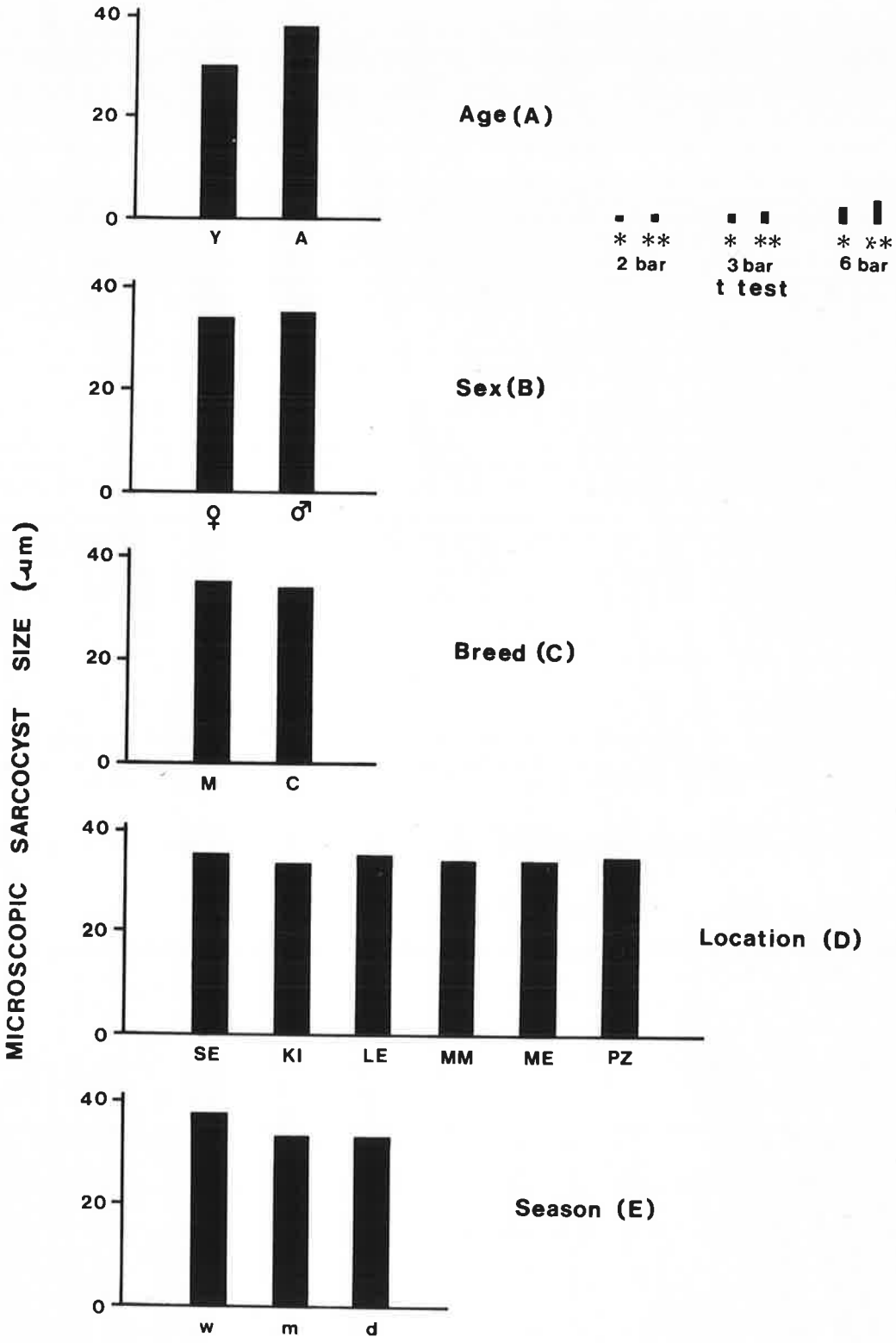


FIGURE 19. Sizes of microscopic sarcocysts in sheep for each factor examined in the survey - histograms of geometric means.

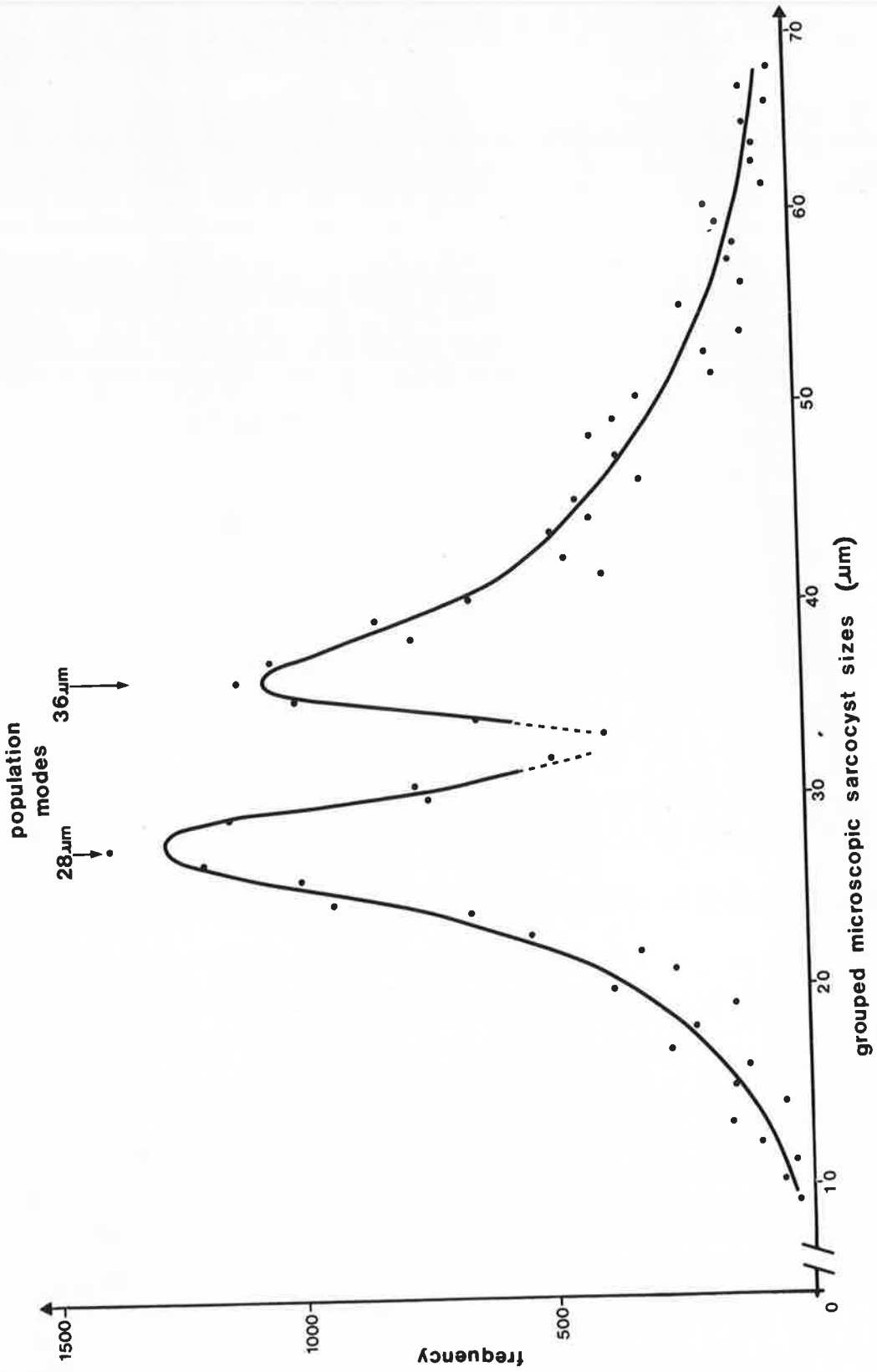


FIGURE 20. Frequency distributions for the two microscopic sarcocyst size populations.

14.0 INTERACTIONS OF FACTORS

The levels of significance of the interactions occurring between the factors are given for each parameter measured in the survey in Appendix I (Tables 1-8). The geometric means of all the 2 X interactions are listed for each parameter in Appendix II (Tables 1-8). The 2 X interactions found to be statistically significant were predominantly those involving the factors of location and season, or both. However, only those considered to have biological implications are presented in the results below, together with brief descriptions of the source of interaction.

14.1 INTERACTIONS INVOLVING LOCATION

The factors age and location (A x D) exhibited significant 2 X interactions for the following parameters:-

(a) Prevalence of microscopic sarcocysts (see Figure 21a). More lambs than aged sheep were infected with microscopic sarcocysts from lower Eyre Peninsula and Murray Mallee whereas less lambs were infected than aged sheep in the other sampling locations.

(b) Intensity of CFT-antibody response (see Figure 21b). Higher CFT-antibody titres were found in lambs than in aged sheep in almost all locations, especially in those from Kangaroo Island.

(c) Size of microscopic sarcocysts (see Figure 21c). Larger microscopic sarcocysts were found in aged sheep than in lambs in all the locations sampled, particularly in those from Kangaroo Island and mid-Eyre Peninsula.

The factors sex and location (B X D) interacted significantly for the following parameter:-

(d) Prevalence of macroscopic sarcoycsts (see Figure 21d). More ewes than wethers were found infected with macroscopic sarcocysts from mid-Eyre Peninsula, whereas more wethers were infected than ewes from lower Eyre Peninsula.

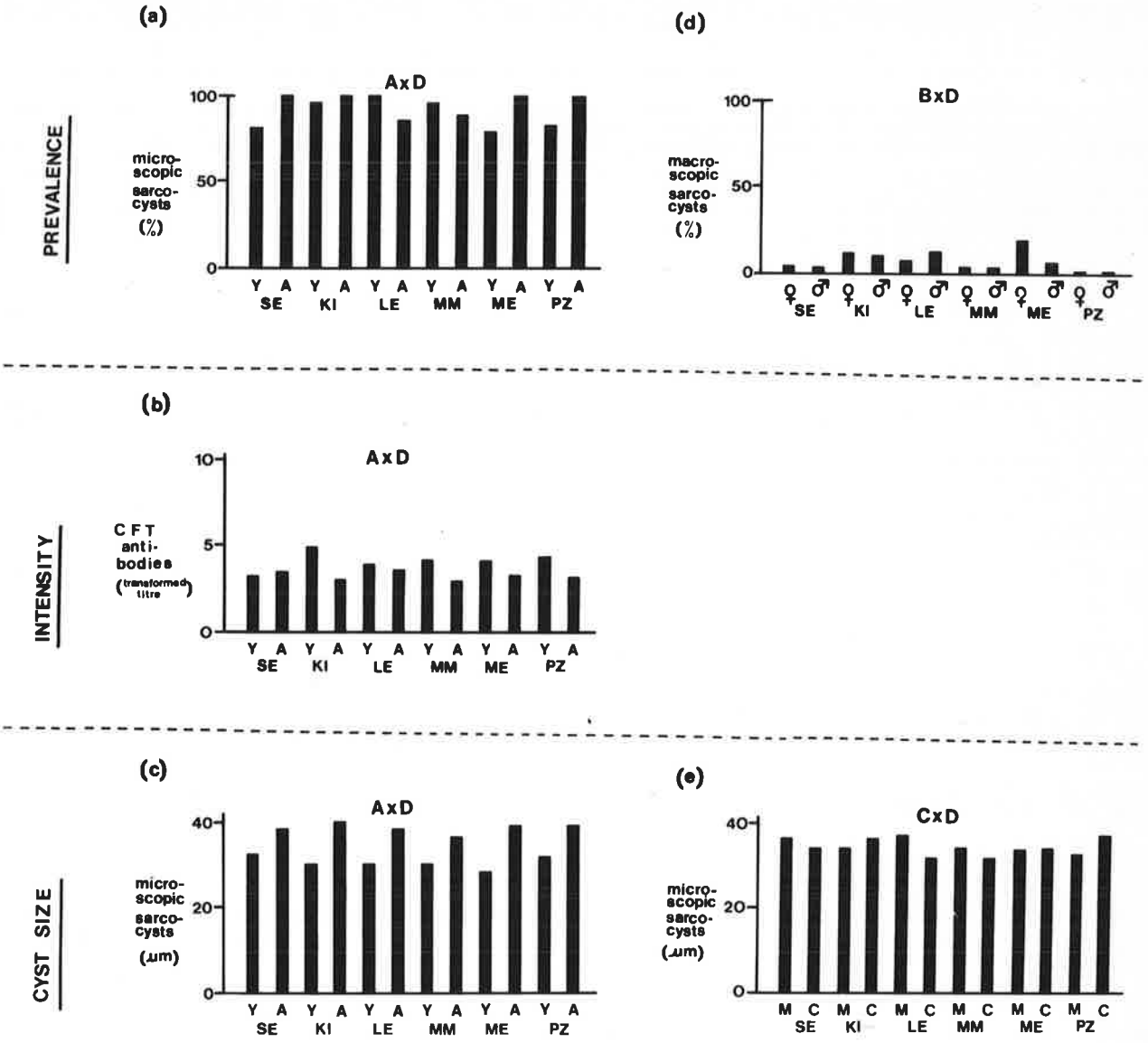


FIGURE 21. Significant 2 X interactions between factors which involve that of location - histograms of geometric means.

Breed and location (C x D) interacted significantly for one parameter, as follows :-

(e) Size of microscopic sarcocysts (see Figure 21e).

Larger microscopic sarcocysts were found in Merinos than in Corriedales in all sampling locations except Kangaroo Island and the Pastoral Zone.

14.2 INTERACTIONS INVOLVING SEASON

The factors age and season (A x E) exhibited significant 2 X interactions for the following parameters :-

(a) Prevalence of microscopic sarcocysts (see Figure 22a).

Less lambs were infected with microscopic sarcocysts than aged sheep during the wet season of sample.

(b) Size of microscopic sarcocysts (see Figure 22b).

Smaller microscopic sarcocysts were found in lambs than in aged sheep during all seasons sampled, but the cyst size difference between the two age groups was the greatest during the wet and dry seasons.

Sex and season (B x E) were found to significantly interact for two parameters, as follows :-

(c) Prevalence of IFAT-antibodies to *Sarcocystis* (see Figure 22c).

Less ewes were found with IFAT antibodies than wethers during the wet season than during the other seasons of sample.

(d) Intensity of IFAT-antibody response (see Figure 22d).

Higher antibody titres were detected in ewes than in wethers during the dry season whereas wethers had higher titres than ewes in the other seasons.

The factors of breed and season (C x E) interacted significantly for the following two parameters :-

(e) Prevalence of microscopic sarcocysts (see Figure 22e).

Fewer Corriedales than Merinos were found infected with microscopic sarcocysts during the wet season.

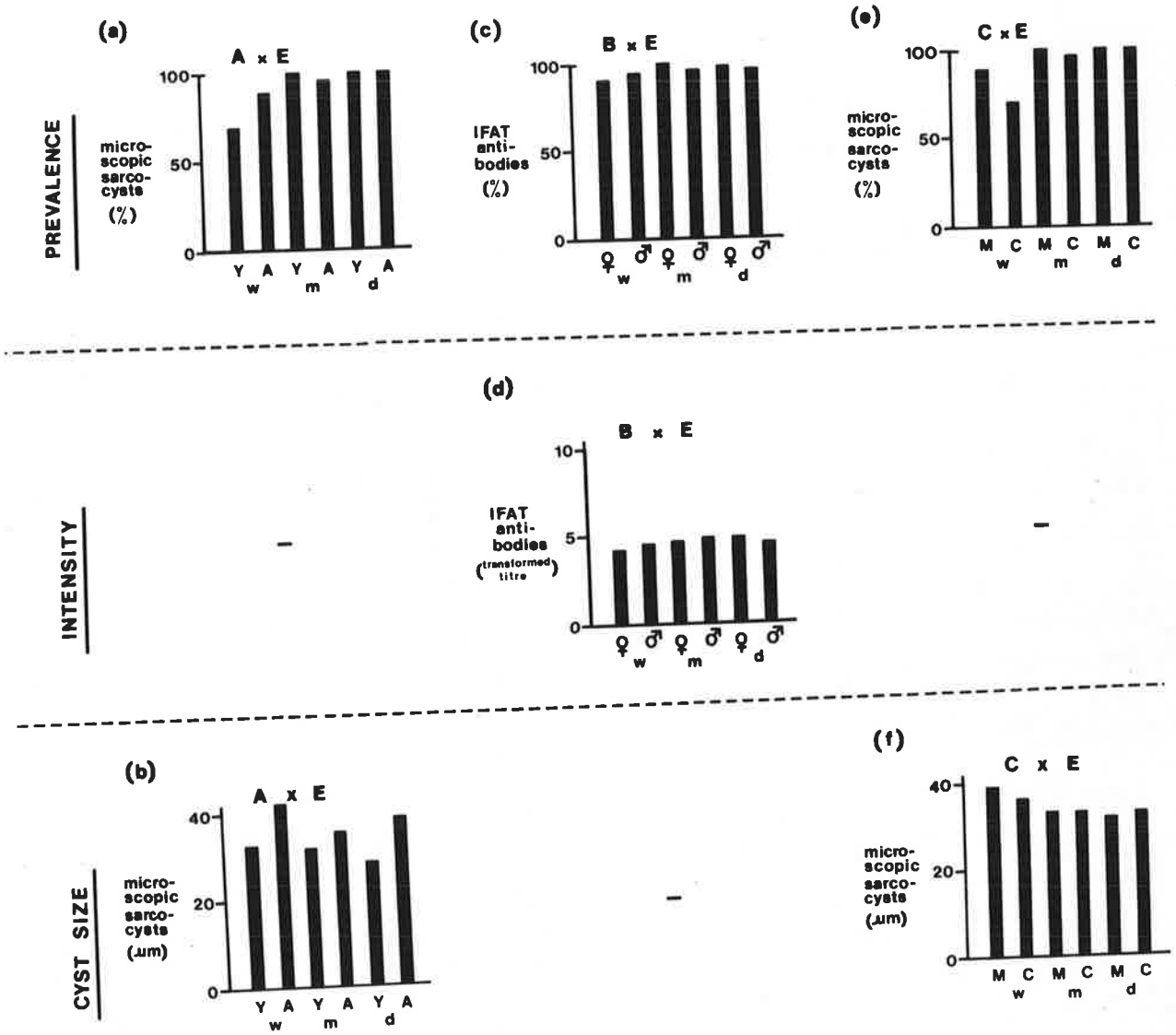


FIGURE 22. Significant 2 X interactions between factors which involve that of season - histograms of geometric means.

(f) Size of microscopic sarcosysts (see Figure 22f).

Larger microscopic sarcocysts were found during the wet season, particularly in the Merinos sampled rather than in the Corriedales.

14.3 INTERACTIONS BETWEEN LOCATION AND SEASON

Significant 2 X interactions between the factors of location and season (D x E) were found for the following parameters:-

(a) Prevalence of microscopic sarcocysts (see Figure 23a).

Less animals were found infected with microscopic sarcocysts during the wet season in all sampling locations, particularly in the South-East and mid-Eyre Peninsula.

(b) Prevalence of CFT-antibodies to *Sarcocystis* (see Figure 23b).

Less sheep were positive to the CFT during the wet season of sample in all locations, especially in those from the South-East.

(c) Intensity of sarcocyst infection (see Figure 23c).

Heavier sarcocyst infections were detected in sheep from all locations as the seasons became brier, particularly during the dry season in mid-Eyre Peninsula and the Pastoral Zone.

(d) Intensity of CFT-antibody response (see Figure 23d).

Lower CFT-antibody titres were detected during the wet season in the South-East than during the other seasons in the other locations.

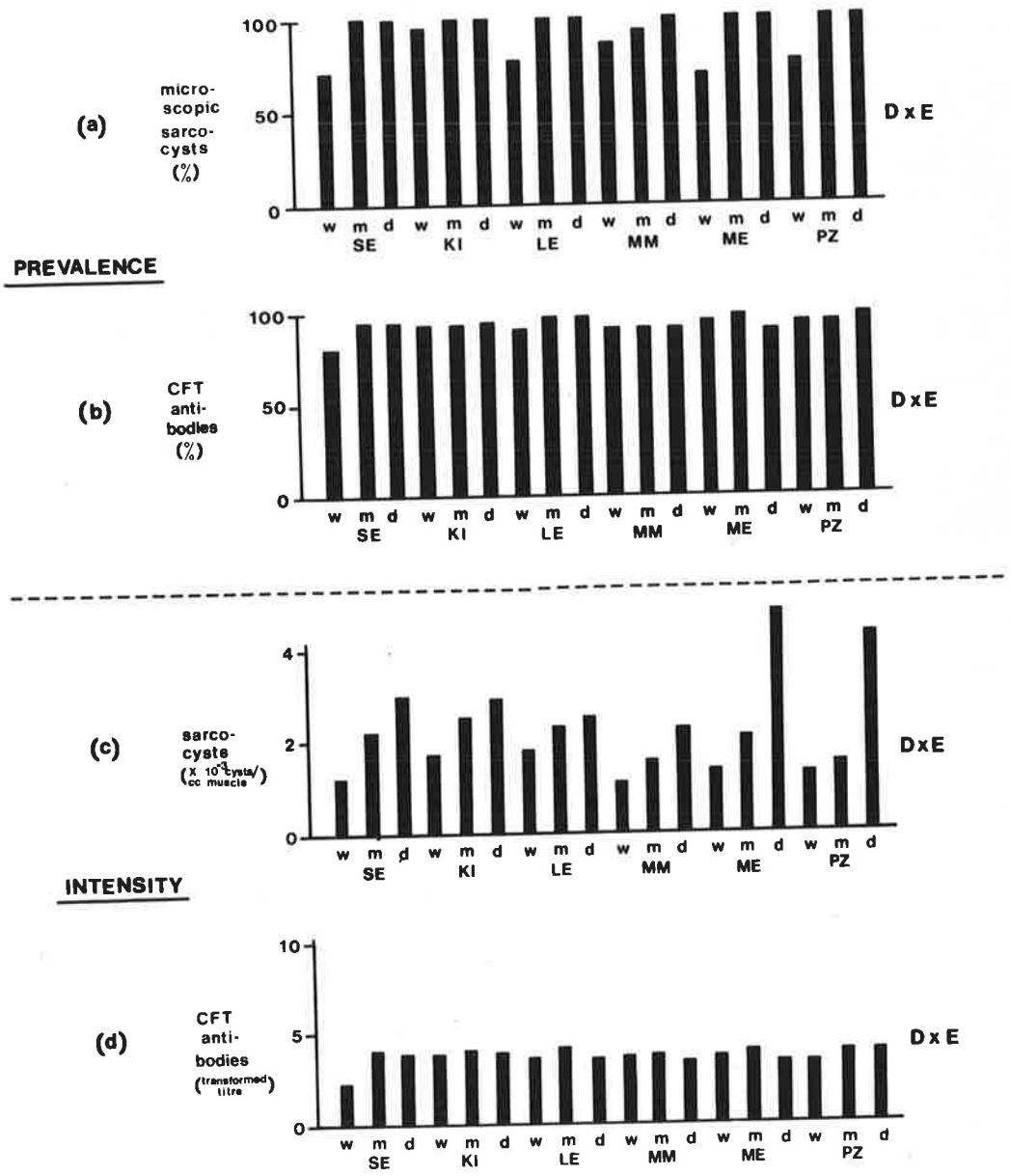


FIGURE 23. Significant 2 X interactions between the factors of location and season - histograms of geometric means.

15.0 CORRELATIONS OF PARAMETERS

Various combinations of the parameters measured in the survey were analysed by multiple regression to determine whether any correlations existed between them. These analyses dealt with correlations between the various methods of detection employed to determine the prevalence and intensity of infection.

15.1 CORRELATIONS BETWEEN METHODS

Relationships were sought between the results of the gross, histological and immunoserological examinations. The results of these analyses are given with their multiple correlation coefficients (r) and levels of significance in Table 8.

x		y	r	Sig.
PREVALENCE RESULTS				
Gross examination	X	CFT	0.052	ns
Gross examination	X	IFAT	0.121	ns
Histological examination	X	CFT	0.123	ns
Histological examination	X	IFAT	0.595	**
CFT	X	IFAT	0.144	ns
INTENSITY RESULTS				
Histological examination	X	CFT	0.214	ns
Histological examination	X	IFAT	0.283	ns
CFT	X	IFAT	0.049	ns

(ns = not significant; ** = 0.01>p>0.001)

TABLE 8. Summary of multiple regression analyses between the various methods of detection employed.

A significant positive correlation was found between two of the methods employed to determine the prevalence of infection. This correlation occurred between the results of the histological examination (used to detect microscopic sarcocysts) and the results of the IFAT (used to detect antibodies to *Sarcocystis)* (see Figure 24). The number of sheep reacting to the IFAT increased with the number of sheep found to be infected upon histological examination.

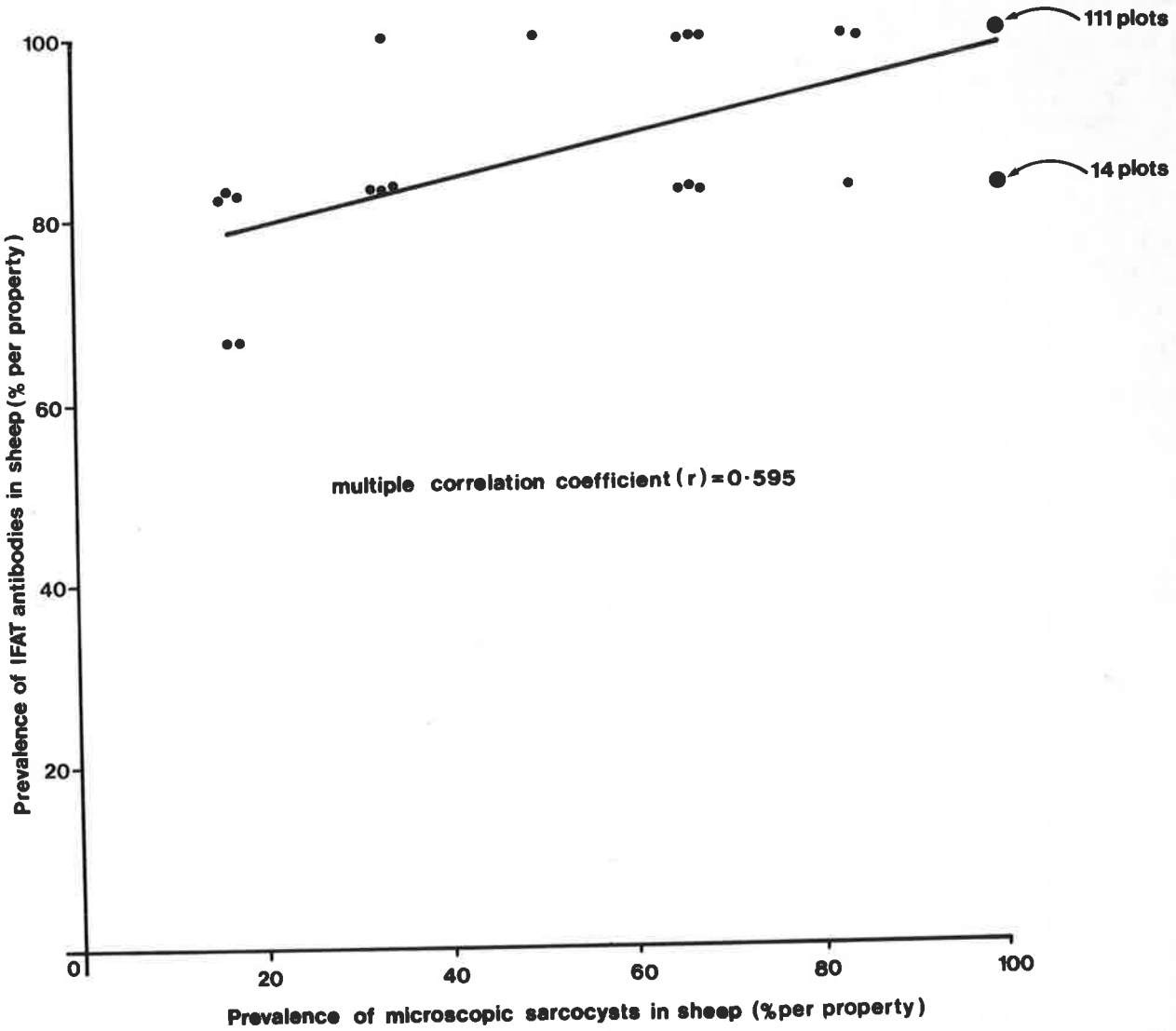


FIGURE 24. Significant positive correlation between the prevalence of infection as determined by histological examination and the Indirect Fluorescent-antibody Test.

16.0 CORRELATIONS OF ECOLOGICAL FEATURES

Relationships were sought between the ecological features determined for each property in the survey and the intensity of sarcocyst infection on those properties. They were also sought between the ecological features and the mean size of the sarcocysts (macroscopic plus microscopic) found in the sheep on the properties.

16.1 QUANTITATIVE ECOLOGICAL FEATURES

The results of the multiple regression analyses between the quantitative ecological features and the intensity of infection, and also the mean sarcocyst size, are summarized in Appendix III (Table 1). No significant correlations were found.

16.2 QUALITATIVE ECOLOGICAL FEATURES

The results of the Chi-squared analyses between the qualitative ecological features and the intensity of infection, as well as the mean sarcocyst size, are given in Appendix III (Tables 2-4). The geometric means of the intensity of infection are shown in Table 9 for each ecological feature found to be statistically significant.

CLASSIFICATION	FEATURE	SUBDIVISIONS	INTENSITY ($\times 10^{-3}$ cysts per cc muscle)
'PREDATOR'	DOGS' FOOD SUPPLY	- fed fresh meat	2.49
		- fed refrigerated meat	1.34
	RATION SHEEP (disposal of trimmings)	- food for carnivores	2.34
		- burnt or buried	1.77
'DUST'	TEMPORARY SHEEP YARDS (degree of repair)	- poor	2.66
		- good	1.99
	WATER SOURCE	- mixed	4.05
		- from pipeline	2.39
		- rain catchment	2.09
		- bore water	1.73

TABLE 9. Mean intensity of sarcocysts per property for each significant ecological feature.

Heavier sarcocyst infections were found in sheep from properties where the owners :-

(a) fed their domestic dogs fresh meat (i.e., mutton or beef) rather than meat which had been refrigerated for at least a week prior to feeding;

(b) used the carcase trimmings (i.e., flaps, hocks and heads) from their ration sheep as food for the domestic carnivores instead of disposing of it by burning or burying;

(c) allowed their temporary sheep yards in the pastures to decline to a poor state of repair (such as becoming overgrown with vegetation), rather than being maintained in a good state; and -

(d) obtained the water for their stock from multi-derived sources (predominantly rain catchment in dams plus river water from pipelines), rather than from single sources (such as bore water).

No significant differences in the mean size of the sarcocysts were found for any of the qualitative ecological features examined.

DISCUSSION

This survey on Sarcosporidial infections in slaughter sheep provides the best indication to date of the extent of the infections within an area of Australia. However, it must be noted that the prevalence of infections within this survey does not represent the prevalence of infections throughout the area of Australia examined, for only certain classes of sheep were sampled from the total sheep population available. This was because the survey was designed to provide much more information on the epidemiology of the infections other than their abundance. It incorporated several physiological and environmental factors which could be statistically tested to determine their influence on such infections. Before discussing the significance of these factors, basic information is presented on the epidemiology of the sarcocyst infections detected in the survey sheep.

17.0 MACROSCOPIC SARCOCYST INFECTIONS

Observations made in the course of this project on the prevalence, intensity and size of the macroscopic sarcocysts found in the slaughter sheep are discussed below.

17.1 PREVALENCE

The prevalence of macroscopic sarcocyst infections has previously been gauged from figures compiled by the meat inspection staff at the various abattoirs. However, not all macroscopic infections are detected at the abattoirs. Within the survey, meat inspection personnel found macroscopic infections in 5.8% of the sheep, whereas 6.7% were detected as infected when a more detailed examination was performed. The increase in the number of animals found infected does not necessarily reflect an inadequate examination of the sheep carcases by the meat inspectors. They examine specific muscles from each carcase which are presumed to be predilection sites of infection, but as yet no definitive work has

been performed to confirm this casual observation. As the meat inspectors also examine each carcase for other parasitic and pathological lesions, it is simply impractical for them to employ more stringent examination procedures for macroscopic sarcocysts alone due to the rapidity of the slaughter procedure. However, to gain prevalence results which are much closer to actual infection rates, a comprehensive gross examination must be performed, as has been advocated in the U.S.S.R. (Meshkov & Kotomindev 1976).

The results of detailed gross examinations performed on slaughter sheep in other surveys carried out during this period of research are summarized in Table 10.

COUNTRY	MACROSCOPIC PREVALENCE (%)	NO. SHEEP EXAMINED	AUTHORS
Italy	17.0	127	Macchioni & Marconcini 1973
Iran	11.6	6120	Afshar *et al*. 1974
Australia (Tas.)	23.0	304	Munday 1975
U.S.S.R.	95.7	1291 carcases	Meshkov & Kotomindev 1976
	63.1	1783 heads	

TABLE 10. Prevalence of macroscopic sarcocysts in sheep detected by detailed gross examinations.

Unfortunately, direct comparisons between these results and those of this survey cannot be made because the sheep examined consisted of random abattoir samples and were not strictly specified into distinct classes. However, the results do illustrate that macroscopic sarcocyst infections are found in many sheep-producing areas of the world. The economic impact of these infections in other countries is not known, but in Australia (which is a major meat-exporting country) they do represent quite a severe problem due to the rejection and condemnation of infected meat.

17.2 INTENSITY

The present-day criteria used by Australian meat inspectors for the rejection and condemnation of sheep meat infected with macroscopic sarcocysts are based upon the intensity of such infections. These criteria were applied to the 58 survey sheep found with macroscopic sarcocyst infections, as shown in Table 11.

CLASSIFICATION	NO. MACROSCOPIC CYSTS PER CARCASE	NO. SHEEP INFECTED	MEAN CYST SIZE (µm)
'Light'	less than 5	27	1096.5
'Medium'	between 5 & 15	5	975.0
'Heavy'	greater than 15	26	818.6

TABLE 11. Intensity criteria for macroscopic sarcocyst infections applied to the survey sheep.

Of the 27 sheep found to have 'light' macroscopic infections upon detailed examination, only 19 were detected as infected by the meat inspectors. The remainder were not detected because the sarcocysts were either overlooked or were not apparently visible as they were within the musculature and not on the superficial aspects of the carcase. The 5 sheep found with 'medium' infections were rejected from the meat-export market and were only available for local markets after they had been trimmed free of any visible sarcocysts. This removal of cysts is cosmetic in nature for it is based more upon aesthetic factors than on the parasite's known pathogenicity to humans (cf. Soulsby 1968, Noble & Noble 1971). The 26 sheep detected with 'heavy' infections were condemned from human consumption and were either destroyed or processed for pet food after sterilization. On information gained from personnel associated with the pet food industry and from pilot trials carried out in this laboratory (Ford 1975 pers. comm.), such sterilization procedures were found to be sufficient to render

the parasite inviable; as determined by its non-transmissibility to cats.

Although most of the sheep carcases were utilized for production in some form, the loss of trade and increased handling costs associated with the latter two categories of infection constituted a directly-observable economic problem for the meat industry. Future research on macroscopic sarcocyst infections should therefore be orientated towards gaining information which may eventually be utilized for the control of such infections.

17.3 CYST SIZE

Certain indications were gained from the examination of the mean sizes of the macroscopic sarcocysts which suggested that they may actually be infections by two separate species of the parasite. The differences found between macroscopic sarcocysts which led to this postulate are outlined as follows.

(a) Differences in intensity of infection

The mean sizes of the macroscopic sarcocysts were found to differ between the various intensity criteria of infection (see right-hand column of Table 11). It appeared that the size of the sarcocysts became smaller as the intensity of infection increased. This observation initially suggested a 'crowding-effect' probably resulting from intra-species competition for physiological or nutritional requirements; as has been observed for some helminths (cf. Ford 1967 p.324). However, upon closer examination of the data, the size differences were found to be due to variations in the distributions of the two size populations of macroscopic cysts found in the survey sheep. The size populations occurred with almost equal frequency in the sheep population sampled, and they were arbitrarily named the 'fat' and 'thin' populations. The

larger 'fat' population had a mode of 1250 µm in width and the smaller 'thin' population one of 350 µm. A division between the two populations was initially made at the mid-point of their modes (i.e., at 900 µm). Lightly-infected sheep were usually infected with cysts from the 'fat' population, whereas heavily-infected sheep were infected with those from the 'thin' population. However, there were several 'heavy' infections which were comprised solely of cysts from the 'fat' population, which suggests the mean size differences observed were not related to a 'crowding-effect'. They simply resulted from variations in the occurrence of the two macroscopic size populations.

(b) Differences in sites of infection

Mean size differences in the macroscopic sarcocysts were also found between the various sites of infection examined in the sheep (as shown in the left-hand column of Table 12).

SITE OF INFECTION	TOTAL POPULATION OF MACROSCOPIC SARCOCYSTS		'FAT' POPULATION (>900 µm)		'THIN' POPULATION (< 900 µm)	
	Mean Size (µm)	No. Cysts	Mean Size (µm)	No. Cysts	Mean Size (µm)	No. Cysts
Oesophagus	1031.3	439	1360.1	264	535.6	175
Tongue	810.7	142	1520.0	51	416.7	91
Skeletal muscle	982.5	58	1448.1	11	567.3	47
Diaphragm	262.5	22	-	0	262.5	22

TABLE 12. Mean sizes of macroscopic sarcocysts for each site of infection found.

On average, larger macroscopic sarcocysts were found in the oesophagus and smaller cysts in the diaphragm.

Although these mean size differences initially suggested different 'growth potentials' of cysts in the different infection sites, they were found to be related to variations in the distributions of the two size populations (see right-hand columns of Table 12). The majority of cysts from the 'fat' size population were found in the oesophagus and the remainder in the tongue and skeletal musculature. Although cysts from the 'thin' population were detected in all of the sites examined, approximately half of them were found in the oesophagus. Only cysts from the 'thin' population were found in the diaphragm and they were slightly smaller than their counterparts elsewhere. These variable distributions of the two size populations do account for the mean size differences found between the sites of infection when the two populations are combined.

(c) Differences in morphology

Macroscopic sarcocysts representative of both size populations were examined much more closely under the light microscope. All cysts were found to contain septae and their cystozoites were of uniform size (mean dimensions of 12.3 µm in length by 4.1 µm in width). However, the inner (or primary) cyst walls of sarcocysts from the 'fat' population were always found to be thicker than those of the 'thin' population cysts; measuring 1-2 µm in width compared with 0.5-1.0 µm (see Figures 25 & 26). The outer capsules (or secondary cyst walls) were also much larger and more clearly defined in cysts from the 'fat' population. The initial arbitrary division of cysts into either population at the mid-point of their modes (at 900 µm) was found to be still quite valid for morphologically different

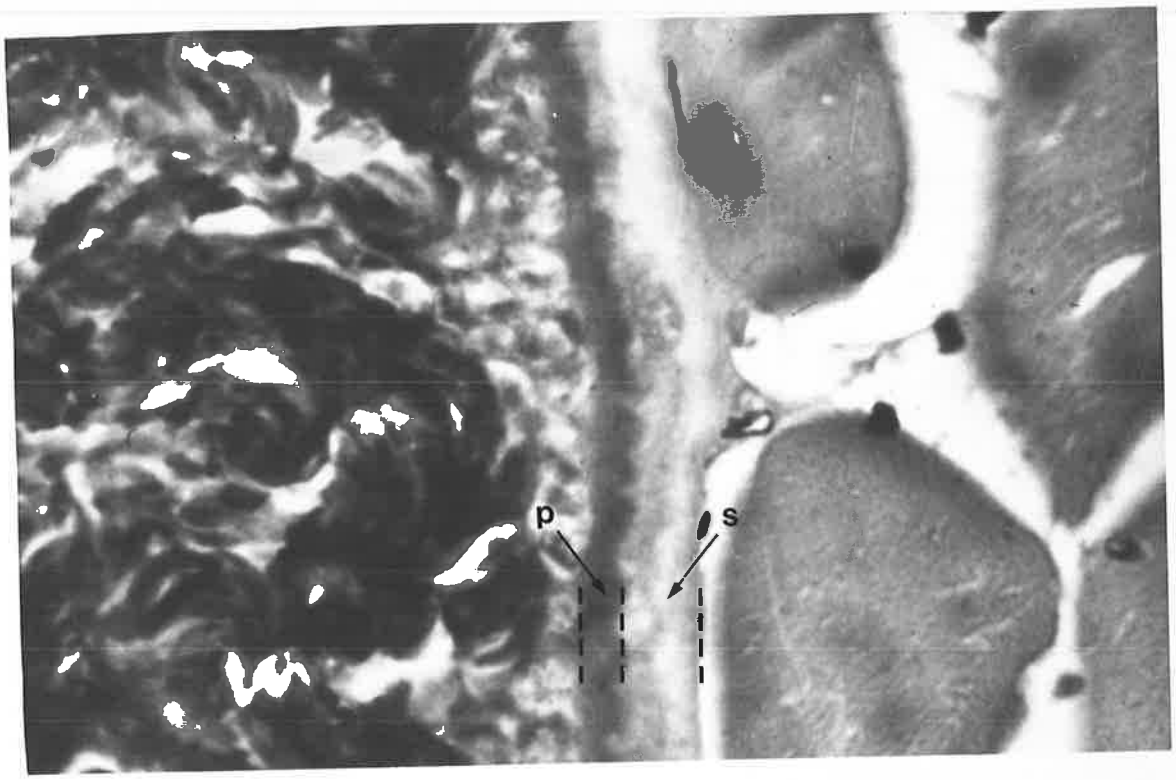


FIGURE 25. Macroscopic sarcocyst from the 'fat' size population illustrating the thick primary cyst wall (p) and the clearly-defined secondary cyst wall (s). (~1,500 X mag.)

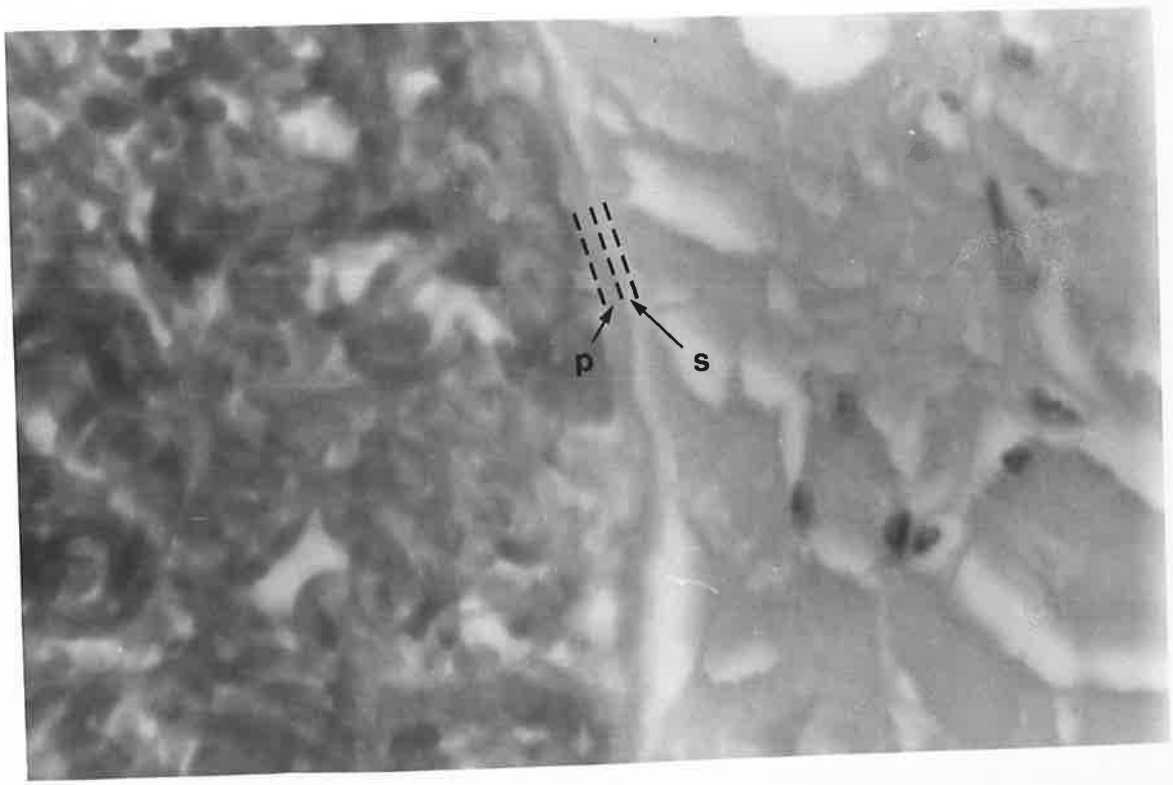


FIGURE 26. Macroscopic sarocyst from the 'thin' size population illustrating the thin primary cyst wall (p) and the virtual absence of a secondary cyst wall(s).(~1,500 X mag.)

cysts only overlapped this point to a very small degree. Because of the differences observed, it is hereby postulated that the 'fat' and 'thin' size populations of macroscopic sarcocysts found in sheep may actually represent two different species of *Sarcocystis*. These postulated species appear to be different in size, in cyst wall morphology and also in their distribution between the sites of infection within the sheep hosts.

Recent research carried out in New Zealand does provide partial support for this postulate. Two distinct (but overlapping) size populations were found for carcase cysts in sheep (i.e., macroscopic sarcocysts obtained from the diaphragm and flank muscles only), and the walls of cysts from these two populations were also reported to be structurally different (Collins *et al.* 1976). On information gained from these authors, the size populations reported were comparable with those detected in this project, although the respective modes were slightly larger. The structural differences mentioned between the cyst walls of these two populations were also similar to those found in this survey (Collins 1977 pers. comm.).

(d) Differences in ultrastructure

Ultrastructural differences between the cyst walls of 'fat' and 'thin' carcase cysts have subsequently been detected (Collins & Hartley 1977 pers. comm.). The primary cyst walls of 'thin' carcase cysts were found to have 'hair-like' protrusions containing almost no microtubules or vesicles. Those of 'fat' carcase cysts had 'cauliflower-like' protrusions containing numerous microtubules and

vesicles, which is identical with that described for some oesophageal cysts (cf. Mehlhorn & Scholtyseck 1973, Mehlhorn *et al.* 1976). The measurements given for these oesophageal cysts (i.e., 1-15 mm in size) are equivalent to those found for 'fat' population cysts; therefore, it appears that the 'fat' macroscopic cysts in the oesophagus and the carcase musculature are one and the same species. Although the cyst walls of 'thin' macroscopic cysts from the oesophagus and the carcase muscles appear identical under the light microscope, it is not known whether they are ultrastructurally similar. At this stage of our knowledge, electron microscopy has provided partial evidence in favour of the postulated speciation of macroscopic sarcocysts, but much more research must be carried out to further delineate between these species.

(e) Note on nomenclature

As most of the previous research on macroscopic sarcocyst infections in sheep has been performed on oesophageal sarcocysts, some pertinent observations on taxonomy may be made, as follows. The original naming of macroscopic sarcocysts in sheep as *Sarcocystis gigantea* was made on visible cysts detected in oesophagi (Railliet 1886b). Because the 'fat' population cysts appear to be identical in size with these cysts, it follows that this species is *S. gigantea*. Furthermore, the taxonomical revision of macroscopic infections in sheep was based on the transmission of large oesophageal cysts to cats (Heydorn *et al.* 1975c); therefore, it is most probable that the proposed new name of *S. ovifelis* also refers to 'fat' macroscopic infections. In this case, the 'fat'

macroscopic cysts should take taxonomic precedence and be referred to as those of *S. gigantea* (junior synonym *S. ovifelis*), whereas the 'thin' macroscopic cysts are a hitherto un-named species. However, initial transmission studies with 'fat' and 'thin' cysts failed to distinguish between them for both were infective to cats only (Collins *et al.* 1976). Therefore, although they have been postulated as two separate species, both must still be referred to as *S. gigantea* (junior synonym *S. ovifelis*) infections until some definitive work is performed to obtain their type species.

18.0 MICROSCOPIC SARCOCYST INFECTIONS

Various observations made on the prevalence, intensity and size of the microscopic sarcocysts found in the survey sheep are discussed below.

18.1 PREVALENCE

In contrast to the low levels of macroscopic infections found in the slaughter sheep, the prevalence of microscopic sarcocyst infections was extremely high. Upon histological examination, 93.2% of the sheep were detected as being infected with microscopic sarcocysts. Similar prevalence results have been found in sheep in other countries when researchers have used specialized techniques to detect microscopic infections; as summarized in Table 13.

These results do give a general indication of the extent of microscopic sarcocyst infections in slaughter sheep throughout the world. Unfortunately, direct comparisons between these results and those of this survey cannot be made because the slaughter sheep examined were not specified into distinct classes as were those of this survey, but consisted of random abattoir samples.

COUNTRY	TECHNIQUE EMPLOYED	MICROSCOPIC PREVALENCE (%)	NO. SHEEP EXAMINED	AUTHORS
United Kingdom	Histological Sections	54.7	245	Awad 1958
Iran	"	99.1	5412	Afshar *et al.* 1974
Netherlands	"	18.0	193	Kruijf & Bibo 1976
U.S.S.R.	"	50.0	1872	Vel'yaminov 1976
U.S.S.R.	Squash Preparations	97.5	480	Meshkov 1973
Italy	Pepsin Digests	95.0	127	Macchioni & Marconcini 1973
Sardinia	"	86.3	1383	Arru & Cosseddu 1976
U.S.A.	HCI Digests	59.4	1095	Seneviratna *et al.* 1975

TABLE 13. Prevalence of microscopic sarcocysts in sheep detected by specialized microscopic examination techniques.

However, although there may be variations between the levels of detection of the various techniques employed, it does appear that the prevalence of microscopic infections in Australia is as high as that found in other sheep-producing countries. At present, these infections pass through the abattoirs undetected as they are not classified under any meat-hygiene regulations; therefore, they do not represent any directly-observable economic problem for the meat industry.

However, rather than dismiss microscopic sarcocyst infections in sheep as unimportant, greater emphasis must be placed on them as a problem because of the recently-observed effects that the establishment of these infections has on meat production. Experimental infections in lambs have been shown to be pathogenic and even lethal (Ford 1974 pers. comm., Gestrich *et al.* 1974, Munday *et al.* 1975). Such infections may therefore be related to

instances of decreased productivity in commercial animals, the extent of which is as yet unknown. Much more research has to be carried out to determine the significance of such infections on meat production, wool production and also on the reproductive performance of sheep. Until this occurs, the economic impact of microscopic sarcocyst infections remains relatively unrecognized.

18.2 INTENSITY

Microscopic sarcocysts were found in sheep from each of the 144 properties involved in the survey. Because of their widespread occurrence, more information was gained from examining the intensity of microscopic infection on each of the properties. The average intensity of microscopic infection was 2,410 sarcocysts per cc of muscle. Although this seemed extraordinarily high, the intensity values were derived stereologically from randomly-orientated sections and were not biased towards higher values. They were normally distributed and had upper and lower limits of 98,210 and 70 cysts per cc of muscle. The only differences observed for the intensity of infection were between the various sites of infection examined, as shown in Table 14.

SITE OF INFECTION	INTENSITY OF INFECTION ($\times 10^{-3}$ cysts/cc muscle)	NO. SHEEP INFECTED	MEAN CYST SIZE (μm)
Heart	4.23	719	35.45
Tongue	2.95	707	35.84
Oesophagus	1.86	510	35.21
Diaphragm	1.67	554	33.16
Skeletal muscle	1.30	471	34.46

TABLE 14. Means of intensity and size of microscopic sarcocysts for each site of infection examined.

Cardiac muscle was the most heavily-infected tissue found in the survey sheep, whereas the striated muscle groups of the various other tissues examined were less infected. As microscopic sarcocyst infections appear to initially become established via the host circulatory system (see Section III and cf. Gestrich *et al.* 1975), the heavier intensities of infection found in the heart may have resulted from its higher vascularity. Such being the case, it could be argued that highly-vascularized striated muscles become more heavily infected than those which are poorly vascularized. However, without critically sampling muscles of differing physiological activities, nothing conclusive can be ascertained as to the validity of this postulate. Research must be performed to determine whether such predilection sites of infection do exist.

Although cardiac muscle was found to be the most heavily-infected tissue for microscopic sarcocyst infections, no macroscopic sarcocysts have previously been observed in the heart muscles of sheep (O'Donoghue 1975 pers. observation, Meshkov 1973, Meshkov & Kotomindev 1976). It appears that cardiac muscle is the only striated muscle which is not conducive to the development of macroscopic sarcocysts.

18.3 CYST SIZE

Microscopic sarcocysts in sheep result from infections by at least two known species of *Sarcocystis:* those of *S. tenella* and *S. gigantea*. Certain features which may be used to distinguish between these species are outlined for each as follows.

(a) *S. tenella*

The microscopic cysts of *S. tenella* (junior synonym *S. ovicanis*) require the dog as the final host (Rommel *et al.* 1974, Ford 1975), and it is generally accepted that these cysts remain microscopic in form for the life of the intermediate host. Ultrastructural studies have revealed that these cysts apparently do not have secondary cyst walls and that their primary cyst walls have regularly-folded 'palisade-like' protrusions which do not contain any fibrillar or tubular elements (Mehlhorn *et al.* 1975d, Mehlhorn *et al.* 1976). Under the light microscope, these protrusions give the appearance of a radially-striated thick cyst wall when they are projecting perpendicularly from the surface of the cyst (see Figure 27). However, in some instances, the protrusions lie flat against the surface of the cyst (Hartley 1977 pers. comm.); therefore giving the appearance of a non-striated thin cyst wall (see Figure 28).

(b) *S. gigantea*

The macroscopic cysts of *S. gigantea* (junior synonym *S. ovifelis*), which require the cat as the final host (Rommel *et al.* 1974), must have developed from microscopic cysts of the same species. Although this has not yet been confirmed by infection studies, some microscopic cysts have been described as being ultrastructurally similar to macroscopic cysts from the oesophagi of sheep (Bergman & Kinder 1975). The walls of these microscopic cysts were composed of a double-layered secondary cyst wall and a primary cyst wall which had 'cauliflower-like' protrusions containing numerous microtubules. This description is identical with that of the large macro-

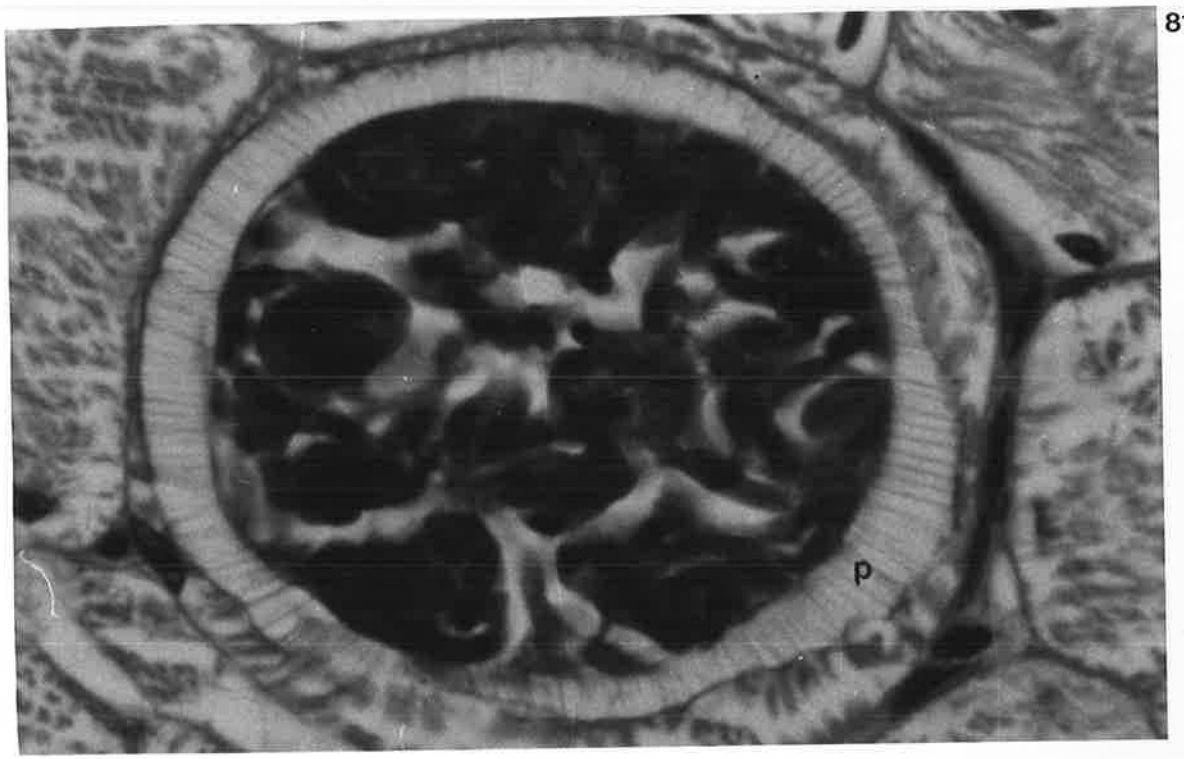


FIGURE 27. Microscopic sarcocyst belonging to the species *S. tenella* exhibiting the characteristic thick primary cyst wall (p) with its radial striations. (~1,300 X mag.)

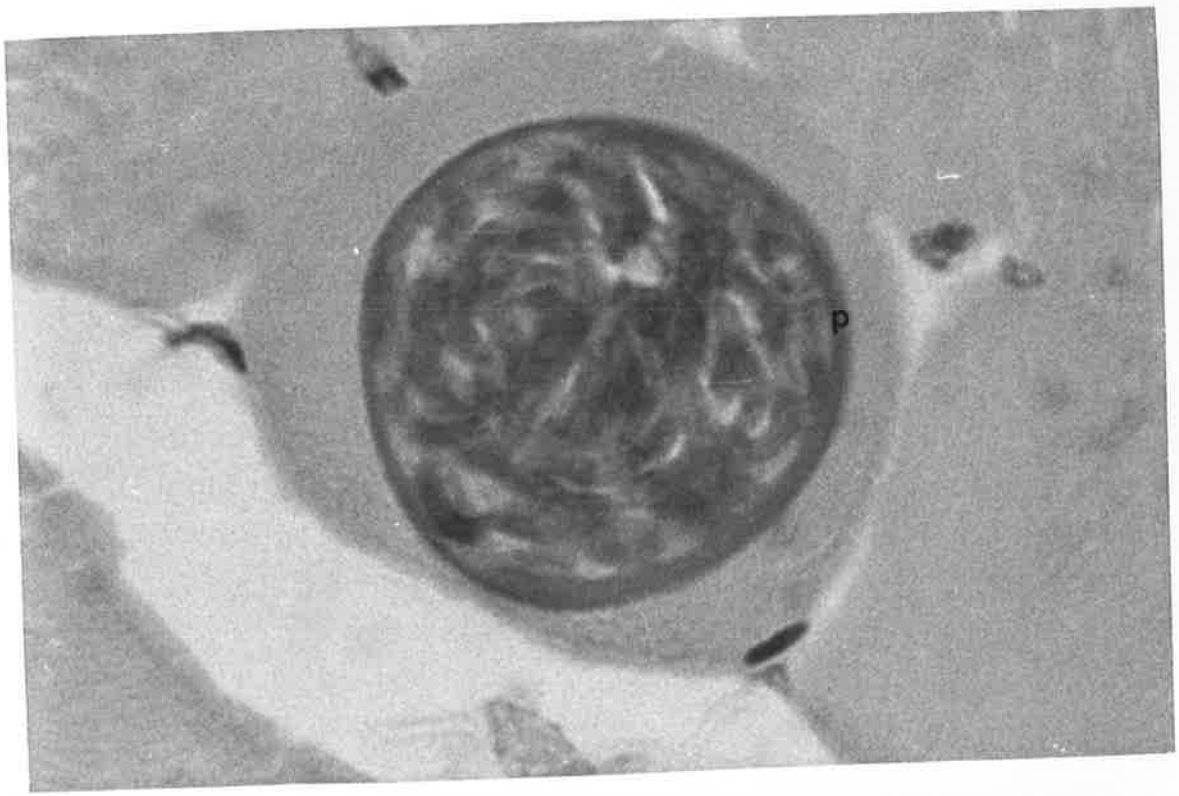


FIGURE 28. Microscopic sarcocyst belonging to either the species *S. tenella* or *S. gigantea* owing to the non-definitive appearance of its thin primary cyst wall (p). (~1,300 X mag.)

scopic cysts from the oesophagi of sheep (Mehlhorn & Scholtyseck 1973, Mehlhorn *et al*. 1976) which could be allocated to the 'fat' macroscopic population formerly described. It therefore appears that these microscopic cysts are the precursors of the 'fat' macroscopic cysts of *S. gigantea*. No evidence of a separate microscopic form of the 'thin' macroscopic cysts of *S. gigantea* has been found as yet. Under the light microscope, the walls of these microscopic cysts appear as non-striated thin cyst walls (see Figure 28), which is similar to the appearance of the *S. tenella* cysts whose protrusions lie flat against the cyst surface.

Morphological differences at the light microscope level can therefore only be used to allocate a portion of the microscopic sarcocysts found in the survey sheep to those of the species *S. tenella*. The remainder of the cysts may belong to either species due to the similar appearance of their cyst walls. Despite this problem in identification, it was determined that the two size populations of microscopic sarcocysts found in the survey sheep were not related to infections by the different species. Approximately 15% of the microscopic sarcocysts found had radially-striated thick cyst walls, measuring from 1.0-2.5µm in width. These cysts could be allocated to those of the species *S. tenella* and it was found they were normally distributed over the two size populations and were not restricted to either one. The remainder of the microscopic sarcocysts found had non-striated thin cyst walls (measuring 0.5-

1.0μm in width) and these could not be allocated to either the species *S. tenella* or *S. gigantea* due to their identical light microscopic appearance.

The two microscopic size populations were also not related to size differences between the various sites of infection examined, as the mean cyst sizes for the different sites were relatively uniform (refer back to right-hand column of Table 14). It appears that the two microscopic size populations of sarcocysts found in this survey were either just fortuitous to sampling or resulted from differences between the factors sampled. The latter alternative is further explored in Section 20.5 for the factor of 'season of sample'.

19.0 ANTIBODIES AGAINST INFECTION

During the development of the two immunoserological tests used in this survey to detect host antibodies to *Sarcocystis spp.*, various observations were made on the antigenicity of the parasite; and these are discussed below. The prevalence of antibodies to *Sarcocystis spp.*, as detected by the Complement Fixation Test (CFT) and the Indirect Fluorescent-antibody Test (IFAT), are also discussed for the survey sheep sampled.

19.1 GENERAL OBSERVATIONS

The prevalence and intensity of host antibodies against infection were more closely related to the prevalence and intensity of infection by microscopic sarcocysts than they were to that by macroscopic sarcocysts. As the antigens used in the immunoserological tests were derived from macroscopic sarcocysts, the tests must be assumed to be genera-specific rather than species-specific. This would result from common antigenic sites existing between the various *Sarcocystis* species, which is similar to that found for many other infections caused by parasites; such as hydatid disease, schistosomiasis, filariasis and amoebiasis (cf. Ambroise-Thomas 1976). The results of the immunoserological tests must therefore be discussed in relation to all the species of *Sarcocystis* found in the sheep rather than for separate species.

However, during the development of the IFAT, differences in antigenicity were observed between the two size populations of macroscopic sarcocysts. Cystozoites derived from cysts of the 'thin' population provided the only consistently-effective antigen tested, whereas those obtained from 'fat' population cysts did not exhibit very strong or specific antigenic properties. Although this antigenic difference could be taken as further evidence for the postulated speciation of the two populations, it should first be examined by more exacting immunological methods. It was found that the washing procedure of the cystozoites to be used as antigen was very critical. If they were not washed effectively, they only exhibited partial fluorescence over their surface when reacted against positive serum. This suggested that the washing procedure was removing some substrate which was blocking the antigenic sites on the

cystozoites. To remove this substrate from cystozoites derived from 'fat' macroscopic cysts, they usually had to be washed so vigorously that mechanical damage resulted, thereby rendering them unsuitable for use as antigen. The antigenic difference observed between the two populations may therefore have been the result of differential blocking of antigenic sites between the populations by this hypothetical substrate. This substrate may have been antibodies which had penetrated the cyst walls and coated the cystozoites. This is quite feasible because large molecules and even micro-organisms, such as enzymes and bacteria, have been shown to penetrate into sarcocysts (Mehlhorn 1975, Mehlhorn & Sénaud 1975). Furthermore, the differential permeabilities of the cysts to antibodies could simply reflect their different ultrastructures. The cyst walls of 'fat' cysts certainly appear to be transporting more material across them for their protrusions do contain large numbers of vesicles and invaginations whereas those of 'thin' cysts do not (cf. Mehlhorn *et al.* 1976, Collins & Hartley 1977 pers. comm.). However, such discussion must remain strictly speculative until much more research has been carried out on the immunology of the Sarcosporidia.

19.2 CFT ANTIBODIES

Complement-fixing antibodies to *Sarcocystis spp.* were very prevalent within the survey sheep, being found in 93.7% of them. The few surveys carried out in the past which have used the CFT as a sero-epidemiological tool have revealed similar antibody levels, as shown in Table 15.

COUNTRY	PREVALENCE OF CFT ANTIBODIES (%)	NO. SHEEP EXAMINED	AUTHORS
United Kingdom	80.0	45	Awad & Lainson 1954
United Kingdom	69.8	245	Awad 1958
Australia (Tas.)	98.4	304	Munday 1975

TABLE 15. Prevalence of CFT antibodies to *Sarcocystis spp.* in slaughter sheep.

Within this survey, 88% of the sheep positive to the CFT were found to be infected with sarcocysts. This level of detection was not high enough to achieve a significant correlation between the prevalence of CFT antibodies and the prevalence of sarcocysts, for the CFT does have some short-comings. 'False negative' reactions to the CFT were observed in 52 sheep which did have detected sarcocyst infections. This lack of antibodies in the presence of the parasite was found to be related to the age of the host, and is discussed further in Section 20.1. 'False positive' reactions to the CFT were found for 50 sheep in which sarcocyst infections were not detected. These reactions may be explained if the level of histological detection was not adequate enough to demonstrate the existence of all sarcocyst infections. This was found to be the situation for 10 of these animals which were re-examined.

19.3 IFAT ANTIBODIES

The IFAT for the detection of antibodies against *Sarcocystis spp.* has been used primarily in conjunction with experimental infections of various host species and not as a sero-epidemiological tool. The use of the IFAT in this survey on slaughter sheep has provided evidence that it may be used as an effective

test in screening programmes on livestock rather than the alternative of examining post-mortem samples. A significant positive correlation was found between the prevalence of infection as determined by the IFAT and by the histological examination. Antibodies to *Sarcocystis spp.* were found by the IFAT in 96.9% of the survey sheep and sarcocysts (macroscopic plus microscopic) were detected in 93.3%. Only 13 'false negative' reactions to the IFAT were found in animals infected with sarcocysts. As for the CFT, these reactions were shown to be related to the age of the host and are discussed in Section 20.1. Of the 837 sheep positive to the IFAT, only 30 of these were found not to be infected with sarcocysts. These 'false positive' reactions were probably due to the inadequate level of detection used in the histological examination, resulting in not all infections being detected. As these apparently 'false' reactions to the IFAT for *Sarcocystis spp.* occur very infrequently, they do not detract from the test's usefulness in providing general sero-epidemiological information about such infections in sheep. The one possible disadvantage of the IFAT is that it cannot be used to differentiate between infections by individual species of the parasite.

20.0 SIGNIFICANCE OF SELECTED FACTORS ON INFECTIONS

The survey was designed to examine the significance of selected physiological and environmental factors upon the Sarcosporidial infections found in the slaughter sheep sampled. The factors found to be statistically significant in their influence on such infections are discussed below and their biological ramifications are indicated.

20.1 AGE OF HOST

The age of the sheep sampled was found to be significant for both macroscopic and microscopic sarcocyst infections, as well as for the antibodies formed by the host against infection.

(a) Macroscopic sarcocyst infections

Infections with macroscopic sarcocysts were only detected in the aged sheep sampled (i.e., those over 4 years of age) and none were found in any of the young sheep examined (i.e., lambs between 9 and 15 months of age). Most other research workers have also failed to detect macroscopic infections in lambs (Meshkov 1973, Seneviratna *et al.* 1975, Meshkov & Kotomindev 1976) apart from two exceptions; the 83 lambs from South Australia reported to be infected with visible sarcocysts (Whiting 1972) and that of a single lamb from Tasmania in which macroscopic carcase cysts were found (Munday 1975). Unfortunately, the exact ages of these lambs were not determined; therefore, it is not known whether they are comparable with those sampled in this survey. They may have belonged to the interim age group (i.e., those between 15 months and 4 years of age) which was not examined in this survey due to its design in avoiding overlapping samples. Sheep of this interim age group have been found infected with macroscopic sarcocysts in South Australia (O'Donoghue 1975 pers. observation) as well as in other locations, such as Tasmania (Munday 1975).

The virtual absence of macroscopic sarcocysts in lambs is thought to result from the time interval required for such infections to become visible. Experimental infections of sheep with sporocysts of *S. gigantea* (junior synonym

S. ovifelis) from cats have so far failed to determine the actual time interval necessary for macroscopic development, but no visible sarcocysts were detected even after 15 & 18 months of infection (Heydorn 1976 pers. comm. to Ford, Munday 1976 pers. comm.). Further research must be carried out to firstly, confirm the life cycle of this species of *Sarcocystis* and secondly, to determine the chronological sequence of macroscopic development.

(b) Microscopic sarcocyst infections

More aged sheep sampled were infected with microscopic sarcocysts than were lambs. This result appeared to reflect the longer period of exposure to infection by aged sheep and their greater likelihood of becoming infected. If this was the situation, it would also be expected that aged sheep become more heavily infected than lambs. However, no significant differences in the intensity of infection were observed between young and aged sheep. More intense infections have even been found in lambs than aged sheep in other countries (Meshkov 1973). Sheep therefore do not become more heavily-infected with age. Whether this is related to the maintenance of a biological equilibrium or the result of transient infections is unknown as yet. Experim ental infections have previously been performed successfully on sheep which were already infected (Munday & Rickard 1974, Heydorn 1976 pers. comm. to Ford); thereby illustrating that secondary infections can occur. However, until more is known about challenge infections and the host immune responses to infection, little can be deduced about the actual effect the age of the host has on microscopic sarcocyst infections.

The mean size of the microscopic sarcocysts was greater in aged sheep than in lambs; thereby suggesting the continued development of the parasite with age. However, on examination of the sizes of microscopic sarcocysts found in young and aged sheep respectively, it was found that the larger microscopic size population occurred more frequently in lambs than in aged sheep. The sizes of the microscopic cysts in aged sheep were more evenly distributed over the two size populations. The occurrence of this large size population in lambs could be indicative of the microscopic sarcocysts which are developing into macroscopic cysts. However, no morphological differences were observed between the two size populations to substantiate this postulate (as has been discussed in Section 18.3). The larger size population was found to occur predominantly in animals examined during the 'wet' sampling season, and is therefore further discussed in Section 20.5.

(c) Antibodies against infection

The differences observed between the two age groups for the prevalence and intensity of CFT antibodies to *Sarcocystis spp.* were found to be related to the duration and titre of the antibody response following infection. As determined from the experimental infections reported in Section 30.1, complement-fixing antibodies appear early in the course of infection, reach maximum titre approximately 1.0-1.5 months after infection and then steadily decline thereafter. In animals which have been infected for quite some time, these antibodies may have declined below the detectable level, if not disappeared altogether. In the survey, 44 of the 52 apparently 'false negative' reactions to the CFT did occur in very aged sheep in which sarcocysts were detected. These

reactions resulted in more lambs being CFT positive than aged sheep. Furthermore, as most of the infected lambs found in the survey would have only become infected quite recently to sampling, their antibody titres would be higher than those in aged sheep where infections would be more established. This would account for the observation in the survey where CFT antibody titres were greater in lambs than in aged sheep.

The differences found between young and aged sheep for antibodies detected by the IFAT were also related to the duration and titre of the host antibody response following infection. The studies on the experimental infections reported in Section 30.2 demonstrated that IFAT antibodies appear later in the course of infection; usually at the same time that the sarcocysts become established in the musculature. Some lambs may even not yet have developed antibodies detectable by the IFAT even though they may have demonstratable sarcocyst infections. This was found to be the case in 12 of the 13 apparently 'false negative' reactions to the IFAT occurring in the survey for they were found in very young lambs which did have low levels of sarcocysts. These reactions reduced the number of lambs reacting to the IFAT and resulted in more aged sheep being positive to the test. The IFAT antibodies detected in the experimentally-infected sheep achieved maximum titre approximately 2.5-4.0 months after infection and these persisted in intensity for much longer. Aged animals with established infections would therefore have higher antibody titres to infection than the recently-infected young animals. In the survey, this was found to be the case as aged sheep did have greater antibody titres to the IFAT for *Sarcocystis spp.* than did lambs.

The variations observed in the antibodies formed against infection by young and aged sheep do partially reflect the status of infection within them. However, very little is known about their antibody responses to multiple or challenge infections, although initial observations indicate that antibodies are formed by the host against subsequent infections (O'Donoghue 1976 unpublished data, Munday 1976 pers. comm.). This secondary antibody response confounds any attempt to extrapolate back to the animal's age of initial infection from the immunoserological results, but it does account for the few aged sheep which had relatively high antibody titres to infection. The usefulness of these tests in indicating the status of infection within individual animals appears to be in discriminating between acute and chronic infections by the Sarcosporidia. This is further discussed for the experimental infections reported in Section III.

20.2 SEX OF HOST

The only significant difference found in Sarcosporidial infections between the two sexes of sheep sampled occurred for the size of the microscopic sarcocysts.

(a) Microscopic sarcocyst infections

Wethers were found to have larger microscopic sarcocysts than ewes. This difference was not related to wethers having more microscopic sarcocysts which could be allocated to the larger size population than did ewes. It may have resulted from actual physiological differences between the two sexes or from different managerial procedures relating to them. Although there is not enough basic information in this survey

to resolve the cause of this cyst size difference between sexes, attention should firstly be given to the effects of the breeding season of the sheep on the cyst size of infection. At this time the physiological differences between the sexes would be the greatest for the ewes would either be in oestrous, gestation or lactation. Differences in the management of the sheep would also be more diverse during this period for most of the attention paid to the sheep would be on the breeding ewes. Further research must be performed to determine the exact significance of the sex of the host on these sarcocyst infections, particularly during the breeding season of the hosts. It is also imperative to determine whether there is any detrimental involvement by the parasite on the reproductivity of the host, for this is of economic concern.

20.3 BREED OF HOST

The breed of the sheep sampled in the survey was significant for the prevalence of the microscopic sarcocyst infections only.

(a) Microscopic sarcocyst infections

More Merinos were found infected with microscopic sarcocysts than were Corriedales. This difference in the prevalence of infection could reflect a breed characteristic where Corriedales are less susceptible to microscopic infection than Merinos due to some physiological trait. Alternatively, the difference may have resulted from variation in the managerial procedures applicable to the different breeds of sheep. Corriedales are usually kept as general utility animals, being used for meat production (lamb and mutton) as well as for wool production; whereas Merinos are kept mainly for wool production, and only secondarily for

meat (predominantly in the form of mutton). Because these two breeds of sheep are utilized for different produce at different times of their lives, their management would be expected to vary accordingly. Unfortunately, the significance of such physiological and managerial differences between breeds on the microscopic infections cannot be determined from this survey. Research should be undertaken to examine these features because of their relative importance to primary production. If microscopic sarcocyst infections are found to depress meat and wool production, the exact significance of any breed differences in infection would be of prime concern due to the different uses of these breeds for such production.

20.4 LOCATION OF SAMPLE

The sampling locations examined in this survey were selected on the basis of geographic and climatic information and they also represent the ranges of natural vegetation types, soil types and agricultural activities found throughout the main sheep-producing areas of South Australia. Significant differences were observed between these locations for both macroscopic and microscopic sarcocyst infections, as well as for the IFAT antibodies formed against infection.

(a) Macroscopic sarcocyst infections

On a local basis, it was found macroscopic sarcocyst infections were more prevalent in sheep sampled from Kangaroo Island and both locations on Eyre Peninsula than in sheep from the other locations examined. This finding supports the casual observations made by various personnel associated with the local meat industry in that these locations were thought to be those predominantly plagued by macroscopic

infections. However, contrary to other observations made by the same personnel, macroscopic infections were found in the other sampling locations; such as the South-East and the Pastoral Zone. Macroscopic infections are not restricted to specific locations as previously thought, but are more widespread across the state of South Australia. As such, the discrimination employed by the meat buyers against property owners in locations presumed to be those infected (resulting in lower prices offered for stock) cannot be wholly justified because macroscopic infections are only slightly more prevalent in these locations than elsewhere.

When examining the finer details of the locations which initially led to their selection, it was observed that macroscopic infections occurred predominantly in sheep from locations which had mid-range climates (i.e., those which may be colloquially referred to as having 'moist and temperate' climates). Fewer macroscopic infections were found in sheep from locations with more extreme climates (i.e., 'wet and cold' or 'dry and hot' climates). This observation initially suggested that climatic parameters limited the prevalence of macroscopic infections. However, the Murray Mallee was a location also examined in the survey which had a 'moist and temperate' climate but in which only a few macroscopic infections were found. Furthermore, macroscopic infections were found to be very prevalent in sheep from Tasmania; averaging approximately 66% (Munday 1975); which has a 'wet and cold' climate by analogy with the ranges examined in this survey. Because of these anomalous findings, it appears that the climates of the locations cannot be directly related to the differences in the prevalence of macroscopic sarcocyst infections.

Such differences in prevalence may be related to the different geographical features of the various locations. Macroscopic infections were more prevalent in locations where the natural vegetation consisted mainly of mallee (i.e., small *Eucalyptus* trees which produce stems from large underground lignotubers). Such vegetation is capable of sustaining quite large populations of feral and native animals which may contribute to the greater transmission of infections in these locations. However, the majority of the land in these locations has been cleared of natural vegetation because the main agricultural activity is cereal crop production. The grazing of sheep is usually only secondary to this production; therefore, the management of the sheep would vary from that employed in basically sheep-producing areas so as to be more compatible with the alternate agricultural use of the land. Whether such differences in sheep management would be more conducive for macroscopic sarcocyst infections to occur is unknown. It is also unknown whether the different soil types of these locations (being predominantly podzolic or sandy) influence the extent of pasture contamination by the parasite due to their different characteristics; such as drainage, texture and topography. All of the above-mentioned geographical features must be examined to determine why location differences in macroscopic infections occur. This is especially important if future research is to be orientated towards the control of infections and the consequent production of so-called 'clean' areas.

(b) Microscopic sarcocyst infections

The intensity of infection of the survey sheep by microscopic sarcocysts differed significantly between the sampling locations. Heavier infections were found in sheep from the mid-Eyre Peninsula

and lighter infections occurred in sheep from the Murray Mallee. These differences in the intensity of infection must reflect the levels of pasture contamination by the parasite within the locations and/or the longevity of the infectious stages of the parasite on the pastures. It is unlikely that the latter differs significantly due to the influence of climate for the heavily- and lightly-infected locations had similar climates. Different levels of pasture contamination may have resulted from variations in the managerial procedures employed between these geographically distinct locations. The topography of the locations may also be important in limiting infections, as indicated by previous research in the U.S.S.R. Heavier microscopic infections were found in sheep from the mountainous regions than in sheep from the low-lands or plains (Meshkov 1973). However, such differences in intensity may also have resulted from variable managerial procedures between the locations. Research must be orientated towards providing information on which managerial procedures are more conducive for the existence and maintenance of microscopic sarcocyst infections. This is further explored in the ecological studies discussed in Section 22.0.

(c) Antibodies against infection

Lower antibody titres to the IFAT for *Sarcocystis spp.* were found in sheep from the locations which had mid-range 'moist and temperate' climates. These locations had relatively high levels of infection by both macroscopic and microscopic sarcocysts; therefore, the lower IFAT antibody levels in these sheep may simply reflect the status of infection in them. The sheep may have become infected at an earlier age

due to the comparatively high level of pasture contamination in these locations. If so, the antibody titres to the IFAT may have declined lower in the aged sheep from these locations than in their counterparts elsewhere. Although this appeared to be the situation on examination of the titres in aged sheep from these locations, it cannot be substantiated from the survey results. This is because of the unknown nature of the host's antibody response to naturally-occurring infections as the sheep must be exposed to continuous or repeated potential infection. The effect of the host's immunity to infections subsequent to initial infection requires further investigation before the exact immunological status of the naturally-infected host can be determined.

20.5 SEASON OF SAMPLE

The season in which the sheep were sampled was found to be very significant in its influence on the Sarcosporidial infections detected in the sheep. It must be noted that these sampling periods do not represent the empirical seasons of the Southern Hemisphere, but rather represent the upper, middle and lower ranges of residual rainfall found in South Australia. The parameter of residual rainfall was used to select the sampling periods because it provides a crude index of the 'potential dessication' applicable to the local environment; particularly during the dry season when negative values are obtained as the potential for evaporation is limited by the lack of rainfall. Significant variations were found between these 'seasons' for both macroscopic and microscopic sarcocyst infections, and also for the various antibodies detected against infection.

(a) Macroscopic sarcocyst infections

More macroscopic infections were found in sheep sampled

during the dry season than in those sampled during the moist and wet seasons. Seasonal variations in the prevalence of such infections have previously been reported in the U.S.A. More macroscopic infections were observed in sheep examined during the dry summer months in Wyoming (Scott 1943) and Michigan (Seneviratna *et al*. 1975). Although the average monthly rainfalls of these locations were within the ranges examined in this survey (cf. World Meteorological Organization 1970-75), it is not known whether their residual rainfalls were likewise compatible because no monthly evaporation data was available for the calculation of this parameter. Direct comparisons with these results could not be made in any case, because the sheep examined consisted of random abattoir samples and were not specified into distinct classes. However, from all of the results it does appear that the high prevalence of macroscopic sarcocyst infections in sheep during the dry summer months may be an epizootiological feature of this parasite. Unfortunately, the influence of residual rainfall (or 'potential dessication') on the prevalence of these infections cannot be directly determined for the parasite must have been within the sheep hosts for quite some time to have become visibly detectable. Certain indications as to the cause of the seasonal difference in infections were gained from the examination of the sizes of the macroscopic sarcocysts, as follows.

The mean size of the macroscopic sarcocysts found in the survey sheep became greater as the sampling seasons became wetter. This result was found to be due to a seasonal variation in the frequencies of the two macroscopic size populations, as shown in Table 16.

SEASON	'FAT' POPULATION (> 900 µm)		'THIN' POPULATION (< 900 µm)	
	No. cysts	Mean Size (µm)	No. cysts	Mean Size (µm)
Wet	105	1321.0	7	468.4
Moist	164	1497.3	103	510.2
Dry	88	1303.1	194	420.6

TABLE 16. The frequency and mean size of the two macroscopic size populations for each season of sample.

Sarcocysts which could be allocated to the 'fat' macroscopic size population were found throughout the sampling seasons whereas very few cysts of the 'thin' population were detected during the wet season. The low frequency of 'thin' macroscopic sarcocysts in sheep during the wet season in South Australia has been confirmed subsequent to this survey (Ford 1977 pers. comm.). It therefore seems unlikely that the virtual absence of these cysts in sheep examined during the wet seasons was just fortuitous to sampling. To account for such large differences in their frequencies between seasons, the 'thin' macroscopic sarcocysts must regress or disappear in infected sheep in the period immediately prior to the wet season. The lower prevalence of macroscopic sarcocysts observed in sheep during the wet season in Michigan, U.S.A., was also attributed to their disappearance in the preceding season (Seneviratna *et al.* 1975), but unfortunately no information was provided to determine which size population these cysts may have belonged to. These authors postulated that the cysts disappeared most probably by rupture but no

supportive evidence was given. The disappearance of the macroscopic cysts could also be related to their degeneration, for calcified sarcocysts are occasionally found in the musculature of sheep (Jubb & Kennedy 1963). However, no evidence of ruptured or calcified sarcocysts was found in any of the survey sheep upon histological examination. The depletion of macroscopic sarcocysts in sheep prior to the wet season and their mode of disappearance still poses an enigma which remains to be resolved. More stringent examinations of the parasitological and immunological state of sheep relative to infection during future moist and wet seasons (i.e., autumn and winter) may help to elucidate an answer to this problem.

(b) Microscopic sarcocyst infections

More sheep were found infected with microscopic sarcocysts during the drier sampling seasons than during the wet season. Heavier microscopic infections were also found in sheep examined during the drier seasons. These results are partially supported by those of previous studies carried out in the U.S.A. More microscopic infections were found in sheep during the summer months in Michigan (Seneviratna *et al.* 1975) and more intense infections were reported in sheep examined during the dry season in Wyoming (Scott 1943). As previously mentioned, direct comparisons between these results and those of this survey cannot be made due to the different survey designs, even though the seasonal climatic parameters of these various locations appear to be similar (cf. World Meteorological Organization 1970-75). However, certain extrapolations may be drawn from the results regarding the differing level

of microscopic infections between seasons, as follows.

The experimental infections reported in Section III ascertained that characteristic microscopic sarcocysts became detectable in the musculature of sheep approximately 2-3 months after infection. Furthermore, the sizes of these cysts were within the size ranges observed for the microscopic sarcocysts detected in the survey sheep during the dry season. It is therefore quite feasible that some sheep found infected during the dry season may actually have become infected in the preceding moist season. As more prevalent and heavier microscopic infections were found in the dry season, it follows that the preceding moist period may be that of maximum exposure to infection. Such being the case, the level of pasture contamination by the infectious stages of the parasite would be greater during this moist period than at other times of the year. This observation initially suggests a seasonal variation in the survival of the infectious stages of the parasite; where the moderate moist period exerts less influence on survival than the more extreme wet and dry periods. The actual climate of these periods (of which residual rainfall is only one parameter) may act to limit the survival of such stages, but at present, little is known about the resistance of these stages to any climatic extremes. Seasonal differences in pasture contamination may also be influenced by the various managerial procedures utilized throughout the year. Those employed during the moist season may be more conducive to the spread of the organism over the pastures than those employed during the other seasons. As can be seen, much more research must be per-

formed before anything conclusive can be determined as to the contributory factors resulting in seasonal differences in infection. At this stage, it can only be postulated that the moist period preceding the dry season (i.e., spring) is the time of maximum exposure to microscopic infections.

The mean size of the microscopic sarcocysts detected in sheep during the wet season was greater than that of cysts found in sheep during the moist and dry seasons. This size difference was observed to be due to the higher prevalence of sarcocysts which could be allocated to the larger microscopic size population during the wet season of sample. The reason for the predominance of this size population during the wet season is not known, but it may be indicative of a period of maximum growth of the parasite. Such growth during the wet season may be related to the physiological, nutritional or immunological state of the infected host at this time. Until each of these factors has been comprehensively examined for its influence on microscopic sarcocyst infections, little can be determined about the biological significance of this large microscopic size population found during the wet season.

(c) Antibodies against infection

Higher antibody titres, as measured by the CFT and the IFAT for *Sarcocystis spp.*, were found in sheep sampled during the moist season than in those examined during the wet and dry seasons. This result indicated that the moist sampling season was the period of greatest immunoserological activity for the survey sheep. The elevated humoral responses of the sheep at this time are suggestive of

relatively recent infection, and possibly even re-infection. From the experimental infections reported in Section III, it was observed that antibodies detectable by the CFT and the IFAT can achieve similar titres to those found in the survey sheep during the moist season after only 20 & 40 days of infection. It is therefore consistent that the higher antibody titres to infection found during the moist season reflect the period of maximum exposure to infection (i.e., spring).

21.0 SIGNIFICANCE OF FACTOR INTERACTIONS ON INFECTIONS

The interpretation of the significant 2 X interactions found between the factors examined in this survey is very difficult due to their complex nature. Many other features may have acted to determine the actual biological interactions which were found to be statistically significant in this survey. Until much more is known about Sarcosporidial infections in sheep and the many features relevant to their distribution and abundance, the biological implications of these interactions can only be discussed speculatively, as follows.

21.1 INTERACTIONS INVOLVING LOCATION

Significant 2 X interactions involving the factor of location were found for both macroscopic and microscopic sarcocyst infections as well as for the intensity of the CFT antibody response.

(a) Macroscopic sarcocyst infections

The prevalence of macroscopic infections was greater in ewes than in wethers from the mid-Eyre Peninsula whereas the situation was the reverse in the lower Eyre Peninsula. When the prevalence results were pooled for both locations, more ewes were observed to be infected than wethers; which was similar to that previously found upon analysis of preliminary observations collected on Eyre Peninsula (by Mr. J.D. Habel, Sth. Aust. Dept. Agric. Fish.). The reasons for the sex

differences in macroscopic infections between adjacent locations are obscure as yet, but they may be related to differences in the management of the two sexes between locations.

(b) Microscopic sarcocyst infections

More lambs than aged sheep were infected with microscopic sarcocysts from lower Eyre Peninsula and Murray Mallee whereas more aged sheep were infected than lambs in the other locations. The higher prevalence of infection in lambs in these two locations suggests their exposure to infection at an earlier age resulting in more infections becoming histologically detectable. Whether this could be related to the seasonal pasture contamination differing between locations must be examined in the future.

The microscopic sarcocysts found in aged sheep were larger than those in lambs in all sampling locations, particularly in Kangaroo Island and mid-Eyre Peninsula. It is not known whether this result reflects a greater 'growth potential' of the sarcocysts with age in particular locations or whether it was simply fortuitous to sampling.

Larger microscopic sarcocysts were detected in Merinos than in Corriedales in all locations except those of Kangaroo Island and the Pastoral Zone. Whether this resulted from a breed characteristic for infection which was dependant on location or resulted from different managerial practises applicable to the breeds in the various locations is also not known as yet.

(c) Antibodies against infection

The intensity of CFT antibodies was greater in lambs than in aged sheep in all locations, especially within Kangaroo Island. This result initially suggested these lambs were younger than those sampled elsewhere in that their antibody titres to infection were still quite high. However, the lambs were similar in age to their counterparts in other locations. It may have been that the lambs were exposed to greater levels of infection resulting in more intense antibody responses for Kangaroo Island was found to have very prevalent and relatively intense infections.

21.2 INTERACTIONS INVOLVING SEASON

Significant 2 X interactions involving season were found for microscopic sarcocyst infections and IFAT antibodies to infection.

(a) Microscopic sarcocyst infections

Fewer lambs than aged sheep were infected with microscopic sarcocysts during the wet season than during the other seasons. This seasonal difference reflects the lower pasture contamination postulated for the wet season. It is more obvious in lambs as they have not been exposed to infection in previous years.

The microscopic sarcocysts detected in aged sheep were larger in size than those in lambs (greatest size differences occurred during the wet and dry seasons). Although more microscopic cysts could be allocated to the larger size population during these two seasons than in the moist season, the exact biological significance of the differing distributions of the populations is unknown.

More Merinos than Corriedales were found infected with microscopic sarcocysts during the wet season than during the other seasons; and the sarcocysts were also larger in Merinos at this time. Such results suggest a breed characteristic for infection which is most pronounced during the wet season (i.e. Merinos may be more susceptible to the establishment and development of infections than Corriedales, especially during the wet months). This could be related to seasonal differences in the physiologies of the two sheep breeds; as possibly occurring during their breeding seasons. However, such speculation requires substantiation which must be gained from future studies.

(b) Antibodies against infection

More wethers than ewes were positive to the IFAT during the wet season whereas during the other seasons the reverse situation was observed. The source of interaction lay in the variable numbers of ewes (not wethers) found to react to the IFAT (fewer in wet season). The intensity of the IFAT antibody response also varied and was greater in ewes than wethers during the dry season. Although these differences in antibodies to infection were restricted mainly to ewes, it is not known whether they were related to the immune status of the females during reproduction.

21.3 INTERACTIONS BETWEEN LOCATION AND SEASON

Significant 2 X interactions between location and season were found for the prevalence and intensity of microscopic sarcocysts and the CFT antibodies to infection.

(a) Microscopic sarcocyst infections

The seasonal variation found for the prevalence of microscopic infections (i.e. lower during wet season) was more evident in the South-East and mid-Eyre Peninsula than elsewhere. These two locations were geographically and climatically different, therefore their lower prevalences of infection appear not to have resulted from common environmental causes. Rather, similar managerial procedures less conducive to the transmission of infections may have been employed at this time in both locations.

The seasonal variation found in the intensity of infections (i.e. heavier as seasons became drier) was very pronounced in the mid-Eyre Peninsula and Pastoral Zone. Because these locations had relatively warmer and drier climates than the others, the heavier infections found may be indicative of greater pasture contamination or greater longevity of the infectious stages of the parasite under such climatic conditions. Alternatively, they may have resulted from managerial procedures being employed at this time which were more conducive to the transmission and dissemination of infections.

(b) Antibodies against infection

The South-East presented the major source of interaction with season for the prevalence and intensity of CFT antibodies. Both parameters were lower in sheep sampled during the wet season from the South-East than from elsewhere. Such a result may reflect a lower immune status to infection in sheep from this location although it does not appear that infections were less prevalent or severe within them.

As can be seen, much more research must be performed upon Sarcosporidial infections in sheep to determine the complex biological interactions occurring between all the features which act to limit the distribution and abundance of the parasite.

22.0 SIGNIFICANCE OF ECOLOGICAL FEATURES ON INFECTIONS

Relationships were found between several of the 83 ecological features examined on each property involved in the survey and the intensity of sarcocyst infections in the sheep from those properties. The intensity of infection was examined rather than the prevalence of infection for sarcocysts were found in sheep from each of the 144 properties. It must be noted that the relationships found were gained from statistical analyses between each individual feature and the intensity of infection, and not from multi-factorial analyses on various combinations of features. Furthermore, the numbers of observations in the subdivisions of the features were not orthogonal as were those for the survey factors but were obtained fortuitously. This means that only tentative interpretations can be made on the relevance of the features found to be statistically significant on the intensity of infection. However, this initial research is nonetheless important as no previous ecological studies have been performed on Sarcosporidia. The analyses were performed to determine whether any single ecological feature played a major role in limiting the extent of sarcocyst infections in sheep. In this manner, certain indications may be gained for the direction of future ecological studies. The following discussion on the significance of the ecological features is given for each of the 3 phases allocated to the life cycle of the parasite; the 'prey', 'predator' and 'dust' phases.

22.1 'PREY' PHASE OF LIFE CYCLE

No significant relationships were found between the intensity of sarcocyst infection in the sheep and any of the 31 ecological features examined which were pertinent to the various 'prey' animals (i.e., potential intermediate hosts). This result does

not reflect the unimportance of the management of the various types of stock carried on the properties in influencing the intensity of sarcocyst infections. It also does not indicate a relative uniformity in the type and number of stock kept on the properties nor in the managerial procedures applicable to such stock. Rather, it indicates that no single feature examined exerted a great influence on the intensity of infection. Collectively, several features may influence the intensity of infection on the properties, but they are not apparent when analysed individually. However, the lack of significant relationships found for this phase of the life cycle may be used to draw a tentative general conclusion. Future research may best be orientated not on the 'prey' animals which are found to be infected, but on the 'predator' and 'dust' phases of the life cycle which serve to produce and disseminate the infections. In other words, research into the potential control of infections may best be orientated towards 'prevention' rather than 'cure'.

22.2 'PREDATOR' PHASE OF LIFE CYCLE

Two of the 34 ecological features pertaining to the number, types and management of the various 'predator' animals on the properties examined (i.e., potential definitive hosts) were found to have significant differences in the intensity of sarcocyst infection in the sheep sampled from those properties. Lighter sarcocyst infections were found on properties where the management procedure was to feed the domestic dogs on meat which had been refrigerated for at least a week prior to feeding rather than on fresh meat. Lighter infections were also found on properties where the carcase trimmings of the ration sheep butchered on the properties were disposed of rather than used as food for the domestic dogs and cats. Although these two managerial procedures

were found to be statistically significant on infections, it must be noted that the analyses were performed on relatively low numbers of observations. Only approximately 15% of the property owners fed their dogs refrigerated meat and only 8% did not feed the carcase trimmings to their dogs and cats. However, even with the increased elements of doubt which must be assigned to these results, they do appear to have a biological significance for both do limit the access of the domestic carnivores to potentially-infected meat. Because dogs and cats have been shown to act as definitive hosts for the ovine Sarcosporidia (Rommel *et al*. 1972, Ford 1974), the restrictions in the feeding of such meat to them would reduce their likelihood of becoming infected and thereby transmitting the infections. It may be that these features are very significant in reducing the levels of infection and could be recommended to property owners as good strategems to facilitate partial control of infections. The situation is not quite so simple, however, for many other features may also contribute towards limiting the successful transmission of the parasite; such as the carnivores' accessibility to the pastures, their toiletry habits and the longevity of the infectious stages of the parasite on the pastures. Until more comprehensive ecological studies are performed on a multi-factorial basis, the exact biological significance of these features on sarcocyst infections remains largely unknown.

Previous research has been performed on the effects that the processing of beef infected with *Sarcocystis spp*. has on the infectivity of the parasites to cats (Gestrich 1974, Gestrich & Heydorn 1974) and dogs (Fayer 1975). Observations made on the critical values of cooking, refrigeration and freezing of the meat indicated that the bovine sarcocysts were rendered unviable after

heating to a temperature of 65-70°C or freezing at -20°C for 3 days; whereas they were still viable after refrigeration at 2°C for up to 18 days. The effects that the processing of sheep meat infected with *Sarcocystis spp.* has on their viability is not known, but from the results of the ecological studies it appears that refrigeration for at least a week prior to feeding may be significant. However, preliminary research in this laboratory has indicated that mutton infected with microscopic sarcocysts is still infective to dogs after 7 days refrigeration at 4°C, and in one instance even after freezing at -18°C for 7 days (Ford 1976 pers. comm.). The effects on the viability of the parasites due to the other forms of processing infected meat are not known, but no significant differences were observed in the intensities of infections on properties where the domestic carnivores were fed meat which had been cooked or frozen. Whether such processing was not of sufficient time intervals or at critical enough temperatures to affect the parasites is indeterminate as yet. Further research must be undertaken to determine the exact critical values of the various forms of meat handling and processing on the viability of the Sarcosporidia if their significance in limiting the successful transmission of infections is to be assessed.

22.3 'DUST' PHASE OF LIFE CYCLE

Only 2 of the 18 ecological features examined on each property which were relevant to the 'dust' phase of the life cycle (i.e., the physical environment) were found to be statistically significant for differences observed in the intensity of sarcocyst infections in sheep from those properties.

Firstly, heavier sarcocyst infections were found on the 52 properties which had temporary sheep-yards in the pastures which had been allowed to decline to a poor state of repair rather than

being maintained in good condition. These temporary yards are used predominantly for herding to cull stock and for lamb marking (i.e., castration, tail-docking and mulesing). The yards in poor condition may have constituted localized areas of infection for the sheep thereby resulting in the heavier sarcocyst infections observed. This may have resulted from such yards allowing freer access to potentially-infected carnivores and also because these yards usually contained vegetation upon which any enclosed sheep may graze. However, until more work has been carried out to enable the delineation of focal areas of infection, little can be determined about the actual biological significance this feature appears to have on sarcocyst infections in sheep.

Secondly, significant differences in infections were observed between properties which utilized different sources of water for their stock. Heavier sarcocyst infections were found on properties using multi-derived water sources (usually water from pipelines plus that from rain catchment) than on the properties relying on water from single sources (such as bore-water). This result may simply reflect a greater 'surface area for contamination' phenomenon. In other words, river water transported to the properties in pipelines and the water collected into dams by natural rain catchment present a greater surface area for potential contamination by the dust-borne infectious stages of the parasite than does the water from bores. However, as the water sources utilized on the properties are very dependent upon the geographical and climatic location of the properties, the differences observed in infections may have resulted from locational differences in infections due to some other feature or features. To determine those features and their relative importance in limiting the extent of sarcocyst infections requires very critical studies between locations where

each feature is strictly specified.

In conclusion, it appears that future ecological studies must be based upon multi-factorial analyses and these should be orientated towards the feeding and defaecating habits of the domestic and feral carnivores as well as the physical environment the parasite encounters.

SECTION III:

DEVELOPMENT IN SHEEP

A scientific observation is always a committed observation; ...; it shows by demonstration; ...; it reconstructs the real after having reconstructed its representation

Gaston Bachelard
(1884-1962)

III. DEVELOPMENT IN SHEEP

Greater significance must be assigned to microscopic sarcocyst infections in sheep beacuse of their extremely high prevalence and their recently-observed pathogenic effects on the host during the establishment of infection. At the commencement of this project, very little was known about such infections; therefore, a series of experiments was designed to determine the various stages of development of the microscopic species, *Sarcocystis tenella* (junior synonym *S. ovicanis*), in sheep.

INTRODUCTION

23.0 DEVELOPMENTAL STAGES OF THE PARASITE

In 1972 an obligatory two-host life cycle was determined for a species of *Sarcocystis* in the course of 'in vivo' transmission studies (Rommel *et al.* 1972). Since then, several two-host life cycles have been determined for various species of *Sarcocystis*. However, most of the recent research has been confined to the transmission of infections and ultrastructural studies on the sarcocyst stages of the parasite. Only a few laboratories have detected and described some morphological stages, presumed to be the parasite, within the tissues of various intermediate hosts following infection and before the appearance of the characteristic sarcocysts.

23.1 PRELIMINARY OBSERVATIONS

Fayer & Johnson (1973, 1974) infected calves with what they referred to as *S. fusiformis* sporocysts from dogs' faeces. They found stages morphologically similar to coccidial schizonts within, or in close proximity to, the vascular endothelia in various tissues of calves examined 23-33 days post-inoculation (dpi). The infected tissues included those of the lymphatic, respiratory,

urinary, digestive and nervous systems and the musculature. Developing sarcocysts were also found within the skeletal musculature of calves after 54 dpi. Wallace (1973) fed specific-pathogen-free mice with oocysts taken from the faeces of a stray cat. Although he described these infective oocysts as *Toxoplasma*-like, they were most probably a species of *Sarcocystis* for they seemed to be responsible for the formation of sarcocysts in the musculature of the mice between 42 and 70 dpi. Wallace also found cystic stages in the muscles of the mice between 14 and 40 dpi and he hypothesized these to be schizonts of *Sarcocystis* which presumably led to the formation of the sarcocysts. Rzepczyk (1974) fed *Sarcocystis sp.* oocysts and sporocysts from the snake *Morelia spilotes variegata* to a rat *(Rattus fuscipes)* and found cyst-like bodies associated with the endothelial nuclei of capillaries in skeletal muscle at 11 dpi. Gestrich *et al.* (1974) infected 2 lambs with *Sarcocystis* sporocysts from dogs and found what they described as schizogonous stages containing merozoites in several organs of the lambs at 24 and 25 dpi.

These reports seem to implicate the presence of at least one previously-unknown stage of the parasite in the intermediate hosts following infection and before the appearance of the sarcocysts. These stages were found predominantly in association with the vascular endothelia and appeared between 11 and 40 dpi. Although most of the above authors have referred to these stages as 'schizonts', it was elected to use the term 'meront' throughout this thesis to avoid any confusion with the established terminology applicable to the intestinal coccidians (cf. General Introduction).

23.2 DESCRIPTIVE HISTOLOGY

The stages found in the experimentally-infected calves (Fayer & Johnson 1973, 1974) were described as mostly immature, measuring 7.6-26.6 µm in length by 3.8-13.3 µm in width and containing 3-50 nuclei. A few were also described as being nearly mature in that they contained prominent nuclei with some associated cytoplasm, thereby indicating zoite formation. The cysts found in the mice (Wallace 1973) were spherical to elliptical in shape and measured from 15-35 µm in diameter. They were thin-walled and contained homogeneous contents with numerous basophilic granules which could be taken to be the nuclei of the parasites. The cyst-like bodies found in the rat (Rzepczyk 1974) measured 13-34 µm in length by 7-16 µm in width. Some were also described as being mature in appearance as they contained distinguishable zoites which measured 5 µm in length by 2 µm in width. Unfortunately, no definitive description was given for the morphological stages found in the experimentally-infected lambs (Gestrich *et al.* 1974). However, from a photograph accompanying the report in the literature and its corresponding magnification, a cyst-like stage was measured at 6 µm in diameter and the contained zoites at 1.5 µm in length by 0.5 µm in width.

These descriptions of morphological stages presumed to be those of *Sarcocystis spp.* are suggestive of proliferative stages of the parasite due to the occurrence of zoite formation. Such zoites are referred to as 'merozoites' throughout this thesis (cf. General Introduction) and their reported dimensions were quite variable. Unfortunately, none of this research has rigidly precluded the possibility of previous host infection by *Sarcocystis spp.* nor concomitant infections by other sporozoan parasites. All of the results must therefore be subject to a certain element of doubt.

In fact, some of the stages described may be subject to variable interpretation as those occurring for other parasites in their normal or aberrant hosts; e.g., *Eimeria spp.* schizonts in the intestinal tract, early *Klossiella spp.* colonies in the kidney and *Toxoplasma sp.* or *Nosema cunniculi* cysts in the nervous system. Research was undertaken to clarify this portion of the life cycle for the species *Sarcocystis tenella* by carrying out experimental infections in sheep raised under specific-pathogen-free conditions. In this manner, any developmental stages found could be definitely ascribed to those of *S. tenella*, and the sequence of development of the parasite from the infective sporocyst to the characteristic sarcocyst could be established.

24.0 HOST RESPONSES TO INFECTION

Various observations have previously been made on several aspects of the intermediate host response to Sarcosporidial infection; usually incidental to preliminary transmission studies. These observations dealt mainly with the seroconversion of the host following infection and with any general pathological changes in the host which could possibly be attributed to the infection.

24.1 ANTIBODY RESPONSES

Munday & Corbould (1974) infected a 10-day-old, colostrum-deprived lamb with *Sarcocystis sp.* sporocysts obtained from dog faeces and found the lamb seroconverted to a positive titre in the *Sarcocystis* Complement Fixation Test (CFT) at 31 dpi. Munday & Rickard (1974) repeated this observation in two more similarly-infected sheep, and also in a further three sheep which had been infected with *Sarcocystis sp.* sporocysts obtained from cat faeces. All five sheep were reported to have no antibodies to *Sarcocystis* before challenge, and to have developed complement-fixing antibodie

to infection between 15 and 47 dpi. However, the possibility of concomitant or extraneous infection was not eliminated. Wallace (1973) found that the mice which developed sarcocysts in their musculature following infection with unspecified oocysts (most probably a species of *Sarcocystis)* from a cat, developed antibodies to infection at 30 dpi as detected by the Indirect Fluorescent-antibody Test (IFAT).

To determine the sequence of the host antibody response to the experimental infections carried out in this study, the CFT and the IFAT were employed to trace the development of such antibodies to infection. In this manner, the sequence of development of the parasite could be compared with the sequence of development of the host antibodies which may possibly affect such parasitic stages. Furthermore, by determining the chronological sequence of development of these various antibodies to infection, certain indications may be gained as to the significance of the antibody titres found to infection in the sheep examined in the epidemiological survey (cf. Section II).

24.2 OTHER HOST RESPONSES

Fayer & Johnson (1973, 1974) found that most of their experimentally-infected calves became ill between 23 and 33 dpi and some even died during this period. The calves had been infected with 0.25-1.0 million sporocysts and the developing disease syndrome was described as acute and severe and characterized by anorexia, cachexia, weight loss and anaemia; as determined by the general good health of the un-infected controls. Gestrich *et al.* (1974) also found that the lambs infected with 1.6-2.0 million sporocysts exhibited poor health, loss of appetite and anaemia prior to their death at 24 and 25 dpi. It appears that Sarcosporidial infections of such magnitudes can lead to the

onset of a disease syndrome in the intermediate hosts which can be lethal in certain cases. Certain records were therefore kept within this project on the general health of the experimentally-infected lambs even though general pathology and patho-physiology were not incorporated as parts of this thesis.

MATERIALS AND METHODS

25.0 EXPERIMENTAL ANIMALS

Sheep available from the various commercial markets were unsuitable for use as experimental animals because of the extremely high prevalence of natural infection by *Sarcocystis spp.* (as shown in Section II). Experimental animals were therefore specially bred for hand-rearing under specific-pathogen-free (SPF) conditions. The pathogens excluded from these animals were sporozoan parasites, so in the context of this document, the term SPF is analogous to 'Sporozoa-free'. The production and maintenance of the SPF animals was essentially similar to that described by Ford (1975) but was more rigidly controlled due to the availability of better facilities.

25.1 PRODUCTION OF SPF LAMBS

Fifty big-framed Merino ewes were mated with a Border Leicester ram and consequently marked on the rump by a dye-in-oil mixture which had been placed on the brisket of the ram. In this manner, the approximate date of lambing was obtained. Cross-breeding was favoured as it produces a hardy offspring more suitable for hand-rearing (Belschner 1962, Spedding 1970) as well as for early weaning (Langlands & Donald 1975). During gestation, the ewes were housed under cover in clean surroundings and fed on a pelletted diet. They were also treated with a coccidiostat (Embazin) to reduce the risk of transplacental or post-natal parent-to-progeny infection. The lambs were immediately removed from their mothers after normal parturition and transferred to the dust-proof SPF shed. Only 3 of the 41 lambs born were allowed to suckle from their mothers to obtain natural colostrum; but only after the ewe's teats had been sterilized with absolute alcohol.

After having suckled once only, these lambs were also removed to the SPF shed (see Figure 29 for design of SPF shed).

In the outer receiving-room of the shed, all lambs were scrubbed thoroughly clean in warm soapy water to remove any fortuitous sporozoan cysts. The lambs were then passed into the middle shower-room, washed again and then swabbed with a bacteriocidal solution to combat any fortuitous bacterial infections. The lambs were then taken into the inner SPF holding-rooms, towelled dry and penned separately on wire-mesh grids above floor level. For the first 3 days, the lambs were bottle-fed on an artificial colostrum mixture which consisted of warm reconstituted powdered-milk fortified with lactose, cod liver oil, egg and mineral and vitamin supplements. Neat sheep serum, shown to be free of antibodies to *Sarcocystis spp.* by the CFT and the IFAT, was also added to the artificial colostrum. This was given in an attempt to replace natural colostral antibodies for temporary protection against natural infections (cf. Pout *et al.* 1973). After the first 3 days, the lambs were fed on reconstituted powdered-milk from a lamb-bar (see Figure 30) and then gradually weaned on to water and a pelletted diet over the next 67 days. Lucerne chaff was made available to the lambs ad lib. at 25 days of age, and at this time each lamb was inoculated with ruminal bacteria administered via a stomach tube. The bacteria were obtained through a ruminal fistula in a coccidia-free sheep which was housed indoors. They were given to the lambs at biological dose rates (cf. Moir 1951) at 25 days of age for the anatomy of the digestive tract slowly changes from that accommodating milk digestion to one conducive to rumination at this time (Swenson 1970).

Thirty-four (83%) of the lambs born were successfully hand-

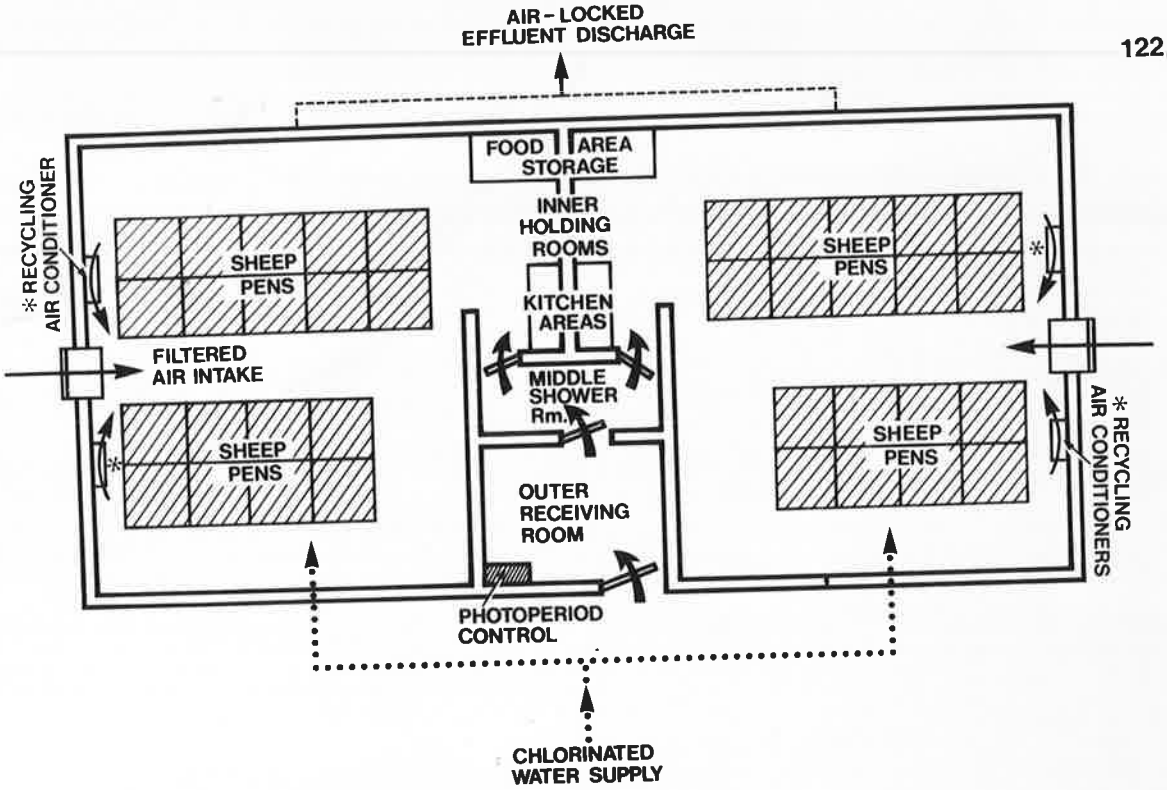


FIGURE 29. Floor plan of the dust-proof SPF shed depicting aspects of its design and controlling mechanisms.

FIGURE 30. Cross-bred experimental lambs being hand-reared within the dust-proof SPF shed.

reared and subsequently used for experimentation. The remaining lambs died during rearing, usually within 3 days of birth.

25.2 MAINTENANCE OF SPF LAMBS

The SPF shed was basically designed to be 'dust-proof', in that dust- or air-borne sporozoan cysts were excluded from its confines. To this end, all incoming air was passed through 5 µm filters. The initial sterilization of the shed was carried out by steam-cleaning and formaldehyde gas saturation, thereby applying heat and toxic chemicals to any enclosed contaminants. All equipment and clothing used within the shed was autoclaved prior to its introduction and all food entering the shed was sterilized either by autoclaving or with ethylene oxide under pressure. All food was aired within the shed for at least 10 days before use to avoid any toxic effects due to residual sterilizing agents (Hann 1975 pers. comm.). Personnel entering the shed disrobed, showered thoroughly and dressed in sterile clothing and head-dress once inside the holding rooms. The internal air temperature was maintained between 18°C & 25°C by the use of re-cycling air-conditioners and the photo-period was kept constant at 13 hours per day.

Each lamb was regularly examined from birth during weaning to ensure they were 'Sporozoa-free' and remained so. These examinations were performed on blood and faecal samples. Serum from each lamb was tested for antibodies to *Sarcocystis spp.* using the CFT and the IFAT, and each was also tested for antibodies to *Toxoplasma gondii* by the use of the IFAT. Faeces were prepared by alternative sedimentation in water and floatation in a magnesium sulphate solution (modification of McMaster technique described by Whitlock 1948), then examined at 400 X magnification under a light microscope for coccidial oocysts or sporocysts.

26.0 EXPERIMENTAL INFECTIONS

The SPF lambs were infected with varying doses of *Sarcocystis tenella* (junior synonym *S. ovicanis)* sporocysts which were obtained from experimentally-infected laboratory dogs. The lambs were sacrificed at varying times after infection and numerous specimens collected for comprehensive examination.

26.1 SOURCE OF INOCULUM

Beagle dogs were raised without access to fresh meat in a 'Sporozoa-free' colony and used as the definitive hosts to provide the inoculum to be given to the SPF lambs. The dogs were fed fresh sheep meat (i.e., hearts and diaphragms) which had been obtained from various metropolitan abattoirs and was shown to be infected with microscopic sarcocysts by histological examination. The dogs' faeces were then collected daily for up to 5 weeks after feeding and aliquots of each collection were examined for coccidial oocysts or sporocysts using the modified floatation technique formerly mentioned (cf. Whitlock 1948). Only dogs which yielded pure infections of *Sarcocystis tenella* sporocysts were used as sources of inoculum. This ensured that only one organism was being introduced into the SPF lambs, thereby allowing the study of that infection alone. The sporocysts were harvested from suitable faeces by alternative sedimentation in water and floatation in a sucrose solution (modification of technique described by Whitlock 1959). The sporocysts were then stored at $4^{o}C$ in distilled water not more than 1 cm deep. Immediately prior to inoculation, the sporocysts were quantitated by counting them in a Neubauer Haemocytometer chamber (modification of eosinophil count described in Dacie & Lewis 1968). The sporocysts were then inoculated into the SPF lambs at the required dose rates.

26.2 INOCULATIONS

Seven experimental series of infections, and one series of un-infected controls were performed as shown in Table 17.

The SPF lambs were selected for the series of infections on the basis of similar age and body weight but irrespective of their sex. All animals were inoculated per os. at morning feeding time with the required number of sporocysts. The un-infected controls received inoculations consisting of normal saline only. Larger doses of sporocysts were given to lambs in the initial series (i.e., those destined for sacrifice early after infection) to increase the chances of detecting the infecting organisms. Smaller doses were given in the series thereafter to minimize the risk of the death of the host due to any pathogenic effects resulting from infection.

All lambs were infected after they had been weaned except for the 5 animals in series V. This series was performed to examine whether lambs were susceptible to infection immediately after birth, as detected by the immunoserological tests employed. This may give an indication as to the efficacy of any maternal immunity transferred to the offspring via colostral antibodies. These lambs were then able to be sacrificed at varying times after infection, if they did become infected, to accommodate gaps in the other experimental series.

The un-infected control animals were regularly tested by immunoserological and faecal examinations for any contaminants which may have entered the shed. At post-mortem, they were also critically examined for natural and extraneous infections by other sporozoan parasites.

SERIES	LAMB NO.	SEX	AGE AT INFECTION (days)	INOCULUM DOSAGE (no. sporocysts)	TIME OF SACRIFICE (days post-innoculation)
I	42	♀	163	2 million	1 dpi
	51	♂	155		
	40	♀	164		
	52	♀	153		
II	50	♀	155	1 million	3-15 dpi
	53	♂	145		
	49	♀	155		
	55	♀	145		
III	07	♀	92	0.5 million	15-25 dpi
	35	♀	125		
	26	♀	138		
	38	♀	123		
IV	29	♂	129		25-35 dpi
	02	♂	92		
	04	♀	92		
	00	♀	103		
V	11	♀	1		Variable
	09*	♂	1		
	14	♂	3		
	10*	♀	3		
	12*	♂	7		
VI	36	♂	125	0.25 million	35-50 dpi
	30	♂	129		
	17	♂	99		
	28	♂	131		
VII	22	♂	99		55-130 dpi
	13	♂	109		
	23	♀	85		
	56	♀	174		
CONTROLS	43	♂	-	-	75-150 days of age
	25	♀			
	41	♀			
	08	♀			
	21	♂			

(* denotes those lambs which received natural colostrum from their mothers)

TABLE 17. Series of experimental infections of SPF lambs with *S. tenella*.

26.3 SPECIMEN COLLECTION

All lambs were sacrificed at the required post-inoculation time by exsanguination and duplicate specimens were collected as quickly as possible (usually less than 10 minutes). Tissues prone to severe post-mortem autolysis, such as gut and brain, were collected first to minimize the amount of any degeneration occurring. To this end, the series I lambs were anaesthetised with sodium pentobarbitone and the gut samples were perfused with fixative before sacrifice (similar to technique described by Cawthorne *et al.* 1973). The specimens collected from each lamb represented all the major organs and tissue systems and are shown in Table 18.

Every five days during the course of infection, blood was collected from each lamb via the jugular vein. The serum was removed by centrifugation and then stored frozen until examination. Various observations were also made every 5 days on the general health of the experimental and control lambs. Clinical pathology was also maintained for other studies not reported within this thesis.

27.0 SPECIMEN STUDIES

All tissue specimens collected were examined under the light microscope after histological processing using routine and specialized techniques to detect any developmental stages of the parasite. All sera were subject to immunoserological examination to detect any host antibodies formed against the parasite.

27.1 HISTOLOGICAL EXAMINATION

The first of each duplicate tissue sample was immediately fixed in 4% glutaraldehyde in Millonig's buffer (Millonig 1961); except for the samples from the gastro-intestinal tract which were fixed by perfusion with Serra's fluid. The fixed tissues were

DIGESTIVE SYSTEM

(1) Tongue

(2) Oesophagus

(3) Rumen

(4) Reticulum

(5) Abomasum

(6) Duodenum

(7) Jejenum

(8) Ileum

(9) Caecum

(10) Colon

(11) Liver

(12) Pancreas

CARDIO-VASCULAR SYSTEM

(13) Heart (apex)

(14) Heart (interventricular septum)

(15) Aorta

(16) Peripheral blood (from jugular vein)

(17) Bone marrow (from tibia)

LYMPHOID SYSTEM

(18) Spleen

(19) Thymus

(20) Axillary lymph node (L.N)

(21) Bronchial L.N.

(22) Prefemoral L.N.

(23) Mesenteric L.N. (duodenal)

(24) Mesenteric L.N. (ileal)

RESPIRATORY SYSTEM

(25) Bronchus

(26) Lung

URINARY SYSTEM

(27) Kidney

(28) Ureter

(29) Bladder

NERVOUS SYSTEM

(30) Cerebrum

(31) Cerebellum

(32) Thoracic spinal cord

(33) Optic nerve & retina

ENDOCRINE SYSTEM

(34) Pituitary gland

(35) Thyroid

(36) Adrenal

REPRODUCTIVE SYSTEM

(37) Ovary/Testis

(38) Mammary gland

MUSCULATURE

(39) Lesser diaphragm (skirt)

(40) Masseter (cheek)

(41) Brachio-cephalic (neck)

(42) Infra-spinatus (shoulder)

(43) Posterior pectoral (axilla)

(44) Oblique abdominal (abdomen)

(45) Tensor fascia-latae (flank)

(46) Anterior semimembranous (rump)

(47) Posterior semimembranous (tail)

TABLE 18. Tissue specimens collected from each lamb at post-mortem

processed routinely into paraffin wax blocks and 2 histological sections were cut from each block ar 5 µm thickness on a microtome. The sections were then stained with haematoxylin and eosin using standard staining procedures (cf. Thompson 1966). Better contrast of basophilic parasitic elements was obtained by over-staining with haematoxylin and then differentiating for slightly longer. Thin smears of the peripheral blood and bone marrow specimens were made, fixed in 70% methanol for 5 minutes and then stained with Wright's stain (Romanovsky base).

Each of the 3,296 stained sections was closely examined under a light microscope at 400 & 600 X magnification. Any parasitic stages found were measured under oil immersion at 1000 X magnification using an eye-piece graticule. Each stage found was photographed at these various magnifications; as was a 0.01 mm micrometer scale. The dimensions of fine morphological elements within the parasitic stages were then measured from uniformly-enlarged photographs. The intensity of infection by the developmental stages of the parasite was also stereologically calculated for each lamb as the number of stages per cc of tissue examined (determined as formerly described in Section II).

Several other histological stains were also employed to examine aspects of the histochemistry of the developmental stages of the parasite; and also to check for any bacterial contaminants within the tissues resulting from fortuitous infections. Additional histological sections were cut from various tissues and stained with the following :-

Periodic acid Schiff's reagent

[for PAS positive elements]

neutral Giemsa stain

[for metachromatic cytoplasmic granules]

van Gieson's stain

[for collagen fibres]

Krajian's stain

[for reticulin fibres]

Gram's stain

[for Gram positive bacilli]

Zil Nielson's stain

[for acid-fast bacilli]

(cf. Thompson 1966)

and alkaline Giemsa-colophonium stain

[recommended for sporozoans]

(Garnham 1975 pers. comm. to Ford)

27.2 FLUORESCENT EXAMINATION

The second of each duplicate tissue sample was wrapped in aluminium foil and snap-frozen by immersion in a 1:1 mixture of dry-ice and ethanol. The frozen tissues were then sectioned at 5-8 µm thickness on a frozen microtome and the sections air-dried. The fluorescent examination performed consisted of subjecting each section to a fluorescent-labelled antibody stain by the indirect method. This technique is essentially similar to the Indirect Fluorescent-antibody Test except it tests for the presence of the antigen (i.e., the parasite) rather than the antibody formed against the parasite. It was adapted from that described for examining *Toxoplasma* infections by Calderon *et al.* (1973) and is outlined as follows :-

(a) Materials

Sheep serum shown to contain antobodies against *Sarcocystis spp.*, as detected by the CFT and the IFAT, was obtained from a known infected animal. The immunoglobulins were harvested by salt fractionation and these provided the initial reagent; the anti-*Sarcocystis*

immunoglobulins.

Anti-sheep immunoglobulins (raised in the rabbit) were conjugated to fluorescein isothiocyanate (as previously described in Section II) and these provided the secondary reagent.

(b) Methods

Each section was covered with the anti-*Sarcocystis* immunoglobulins and incubated in a moist chamber at 37°C for 30 minutes. They were then washed 4 times with phosphate-buffered saline (PBS) pH 7.2 for 10 minutes each. The sections were then overlaid with the conjugated anti-sheep immunoglobulins and incubated for a further 30 minutes in the moist chamber at 37°C. Each section was then washed 4 times in PBS for 10 minutes each, flushed with distilled water, counter-stained with 2% methylene blue and then mounted in carbonate-buffered glycerol pH 9.0. It is important that the sections are not allowed to dry out at any stage of the staining procedure.

A negative control was also prepared which simply consisted of a section being covered with PBS instead of the anti-*Sarcocystis* immunoglobulins. This control acted as a check for autofluorescence and also gave an indication of the amount of nonspecific background fluorescence occurring in the sections.

The stained sections were examined under a light microscope at 600 X magnification using alternatively, transmitted dark-field illumination (tungsten lamp source) and incident ultra-violet illumination (200-W mercury-vapour lamp) through an adequate light filtra-

tion system (excitor filter Diffus N, primary filter BG 12 and suppression filter K 530).

(c) Results

Fluorescent reactive structures were yellow-green in appearance and each was examined minutely and measured using an eye-piece graticule. Unfortunately, the large degree of nonspecific background fluorescence found in the sections rendered this technique ineffectual in many instances. However, the location of the reactive structures within the tissues was recorded to determine whether such sites of reactivity were analogous to known sites of infection as determined by other studies.

27.3 IMMUNOSEROLOGICAL EXAMINATION

All serum samples were tested for host antibodies to *Sarcocystis spp.* using the Complement Fixation Test and the Indirect Fluorescent-antibody Test (as described beforehand in Section II).

RESULTS

28.0 HISTOLOGICAL EXAMINATION

On microscopic examination of the stained histological sections, various developmental stages of *Sarcocystis tenella* (junior synonym *S. ovicanis*) were found in the tissues of all the experimentally-infected lambs. The sites of detection, descriptive histology, intensity of infection and aspects of the histochemistry of these developmental stages are presented below.

It is also important to note that no sporozoan parasites were detected in any of the 5 negative control lambs upon histological examination; nor in any of the other lambs prior to their experimental infection as determined by examinations of faeces and blood.

28.1 DETECTION OF DEVELOPMENTAL STAGES

Three basically different stages in the life cycle of the parasite were detected within various organs and tissues of the experimental lambs at varying times after infection. In chronological order from the time of infection, these stages were;

- the remnants of the infective sporocysts found in the gastro-intestinal (G-I) tract,
- several merogonous stages found in various organs and tissues throughout the body and finally,
- the developing sarcocysts found within the musculature.

(a) Sporocysts

Intact sporocysts were recovered from samples of gastro-intestinal content from the 4 lambs sacrificed 1.3-24.0 hours after infection. The remnants of sporocysts were found within gastric pits, near gastric epithelial cells, within the crypts of Lieberkühn in the small intestine and between intestinal villi in the 5 lambs examined from 1.3-72.0 hours post-inoculation (see Table 19).

TISSUE	HOURS POST-INOCULATION				
	1.3	4	12	24	72
(4) Reticulum	+	-	-	-	-
(5) Abomasum	+	-	-	+	+
(6) Duodenum	-	+	+	+	-
(7) Jejenum	-	-	+	-	-
(8) Ileum	-	-	-	-	+
	42	51	40	52	50
	LAMB NO.				

(+) sporocyst remnants detected
(-) sporocyst remnants not detected

TABLE 19. Sites of detection of remnants of infective sprorocysts.

The epithelial and cryptic cells in close contact to the sporocyst remnants were usually intact and only in a few instances did they appear to be damaged or degenerate. No sporozoites from the sarcocysts were found free within the lumen of the G-I tract or in the surrounding tissues; nor were any found within the cytoplasm of cells in the epithelium or lamina propria. However, mitotic figures in the epithelial cells and micro-organisms within the lumen did add to the difficulties in identification which were overcome during microscopic examination.

(b) Merogonous stages

Numerous merogonous stages of the parasite were found in various organs and tissues throughout the bodies of the 16 lambs sacrificed from 6-36 days post-inoculation (dpi). The infected tissues included elements of the digestive, cardiovascular, lymphoid, respiratory, urinary, nervous and endocrine systems and the skeletal musculature (see Table 20). However, no merogonous stages were detected in the rumen, jejunem, ileum, aorta, peripheral blood, bone marrow, bronchi, ureter, ovary/testis, mammary gland and the masseter or oblique abdominal muscles of any of the infected lambs.

TISSUE	DAYS POST-INOCULATION															
	6	9	12	15	17	19	21	23	25	27	28	31	32	34	36	36
(1) Tongue	-	-	-	-	-	-	+		+	-	-	+	+	-	-	-
(2) Oesophagus	-	-	-	-	-	-	-	-	+		-	-	-	+	-	-
(3) Rumen	-	-	-	-	-	+	-	-	+			-	-	-	-	-
(4) Reticulum	-	-	-	-	-		+	-	+			-	-	-	-	-
(5) Abomasum	-	-	-	-	-	-	-	-	+			-	-	-	-	-
(6) Duodenum	-	-	-	-	-	-	-	-	+		-	-	-	-	-	-
(9) Caecum	-	+	-	-	-	-	-		+			+	-		-	-
(10) Colon	-	-	-	-	-	-	-	-	+		-	-	-	-	-	-
(11) Liver	-	-	-	-	+	+	-	-	+	-	+	+	+	+	+	+
(12) Pancreas	-	-	-		-	-	-	+	-			+	+	-	-	-
(13) Heart (apex)	-	-	+	-	-	-	-	+	+	-	-	-	+	-	-	-
(14) Heart (1-V)	-	+	-	+	-	+	-	-	+	+	+	-	-	+	-	-
(18) Spleen	-	-	-	-	-	-	-	-	+	+	-		-	-	-	-
(19) Thymus	-	+	-	+	-	-	-		+		-	+	-	-	-	-
(20) Axillary L.N.	+	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-
(21) Bronchial L.N.	-	-	-	+	-	-	-	-	+	-	+	-	+	+	-	+
(22) Prefemoral L.N.	-	-	+	-	+	-	+	+	+	+	-	-	-	-	+	-
(23) Mes. L.N.(duo.)	-	-	+	-	-	-	-	-	+	+	-	-	-	-	+	-
(24) Mes. L.N.(il.)	+	-		-	-	-	-	-	+	+	+	+	-	-	-	-
(26) Lung	-	-	-	-	+	-	+	-	+	+	-	-	-	-	-	-
(26) Kidney	+	-	+	-	+	-	-	+	+	-	+	+	-	-	-	-
(29) Bladder	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
(30) Cerebrum	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-
(31) Cerebellum	-	-	-	-	-	-	-	+	+	+	+	-	+	-	-	-
(32) Thor. spinal cd.	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
(33) Optic Nerve		-		-	-		+			+	+	-			-	-
(34) Pituitary gland	-		-	-		-	-	-	-			-	+	-	-	
(35) Thyroid	-	-	-			-	+	-	-	+		-	-	-	-	-
(36) Adrenal	-	-	-	-	-	-	-	-	-	-		+	-	-	-	-
(39) Lesser diaphragm	-	-	-	-	+	-	-	-	+	-	+	-	-	-	-	-
(41) Brachio-cephalic	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
(42) Infra-spinatus	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(43) Post.pectoral	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
(45) Tensor fascia-latae	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-
(46) Ant.semimembr.	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
(47) Post.semimembr.	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
+) Meronts detected	53	49	55	07	35	26	38	14	29	36	02	04	10	12	00	09
-) Meronts not detected	LAMB NO.															
) Tissue not sampled																

TABLE 20. Sites of infection by the merogonous stages of *S. tenella*.

The majority of the merogonous stages were found to be definitely within the endothelial cells of the vascular system and the remainder were in very close association to endothelial cells even though it was not obvious they were within them from the plane of section. The exact sites of infection by the merogonous stages was found to be relatively definitive with regard to the time after infection, as follows.

- From 6-19 dpi, the merogonous stages were found predominantly within the endothelial cells of arterioles. These arterioles were those supplying the G-I tract, the lymphoid tissues and the muscalature as well as the hepatic arterioles in the liver, those of the vasa vasorum in the heart and the alveolar arterioles in the lung. Merogonous stages were also found at this time in the capillary network in renal glomeruli between the afferent and efferent arterioles. No developmental stages of the parasite were found in the nervous, endocrine or reproductive systems during this period of infection.

- From 21-34 dpi, merogonous stages were only detected within endothelial cells of capillaries, and such stages were more widespread throughout the body. The sites of infection included the subepithelial capillaries in the G-I tract, hepatic sinusoids, interlobular pancreatic capillaries, those of the vasa vasorum in the heart, throughout the lymphoid tissues, in alveolar walls of the lung, in the renal medulla and glomeruli, throughout the white and grey matter of nervous tissue, in the pars nervosa of the pituitary gland, the interfollicular capillaries of the thyroid, those in the adrenal cortex and lastly, the capillaries supplying the skeletal musculature. No stages were found in the reproductive system.

- In the 2 lambs examined at 36 dpi, very few merogonous stages were found and they were confined to the hepatic sinusoids in the liver and the cortical capillaries around the germinal centres in the lymph nodes.

(c) Sarcocysts

Developing sarcocysts were found within the musculature of the 8 lambs sacrificed from 41-134 dpi (see Table 21). Only striated muscle fibres were found to contain such stages and these included the muscle groups of the tongue and oesophagus as well as the predominant cardiac and skeletal muscles. Very occasionally, developing sarcocysts were found in association with nervous tissue elements; such as the Perkinjie fibres in the heart and the white matter of the cerebrum and cerebellum. These stages were usually located very close to either large capillaries or even venules.

TISSUE	DAYS POST-INOCULATION							
	41	45	50	55	65	75	111	134
(1) Tongue	+	+	+	+	+	+	+	-
(2) Oesophagus	+	+	+	+	+	+	+	+
(13) Heart (apex)	+	-	+	+	+	+	+	+
(14) Heart (I-V)	-	-	+	+	+	+	+	+
(30) Cerebrum	-	-	-	-	+	+	+	-
(31) Cerebellum	-	-	-	+	+	-	-	-
(39) Lesser diaphragm	+	+	+	+	+	+	+	-
(40) Masseter	+	+	+	+	+	+	+	-
(41) Brachio-cephalic	-	+	+	+	+	+	+	+
(42) Infra-spinatus	+	+	+	+	+	+	+	+
(43) Post.pectoral	+	-	+	+	+	+	+	+
(44) Oblique abdominal	+	+	+	+	+	+	+	+
(45) Tensor fascia-latae	+	+	+	+	+	+	+	+
(46) Ant. semimembranous	+	+	+	+	+	+	+	+
(47) Post. semimembranous	+	+	+	+	+	+	+	+
	30	17	28	22	13	23	11	56
(+) sarcocysts detected (-) not detected	LAMB NO.							

TABLE 21. Sites of detection of sarcocysts of *S. tenella*.

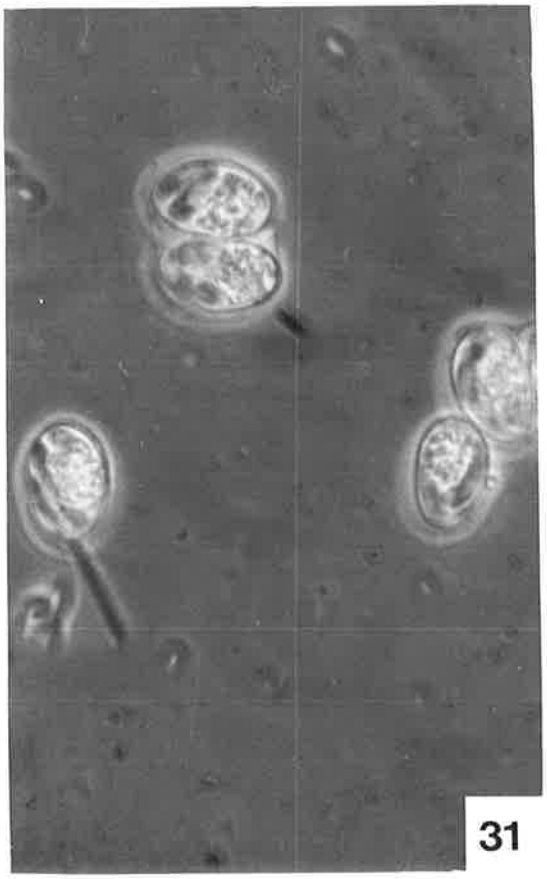
28.2 DESCRIPTIVE HISTOLOGY

The various times after infection (i.e., dpi) at which different sites of infection were observed corresponded exactly to those times at which different morphological stages of the parasite were found. The descriptive histology of these stages is therefore presented under a similar format; that for the sporocyst remnants, the merogonous stages and lastly, the developing sarcocysts.

(a) Sporocysts

The intact sporocysts recovered by the floatation technique from samples of the G-I content of lambs 1.3-24.0 hours post-inoculation were relatively characteristic in form. They measured from 14.6-16.1µm in length by 9.1-11.0µm in width (average dimensions 15.3 x 10.5µm) and all contained 4 sporozoites and a residuum (see Figure 31).

The sporocyst remnants found within the G-I tract from 1.3-72.0 hours post-inoculation usually consisted of the ruptured sporocyst walls trapped or wedged within the lumens of the gastric pits or intestinal crypts (see Figure 32). Most remnants were double-layered and they were irregular in shape as though they had collapsed. Some remnants were observed to have ruptured at one end only. All such stages measured from 6.0-15.0µm in length by 4.5-7.5µm in width. No sporocyst remnants contained any sporozoites and only a few enclosed what appeared to be a circular or oval residuum (measuring 1.5-3.5µm in diameter). Occasionally, the epithelial cells surrounding the remnants appeared to be degenerate, in that their cytoplasmic content projecting towards the lumen was extensively damaged and only their basal nuclei were *in situ* (see Figure 33).



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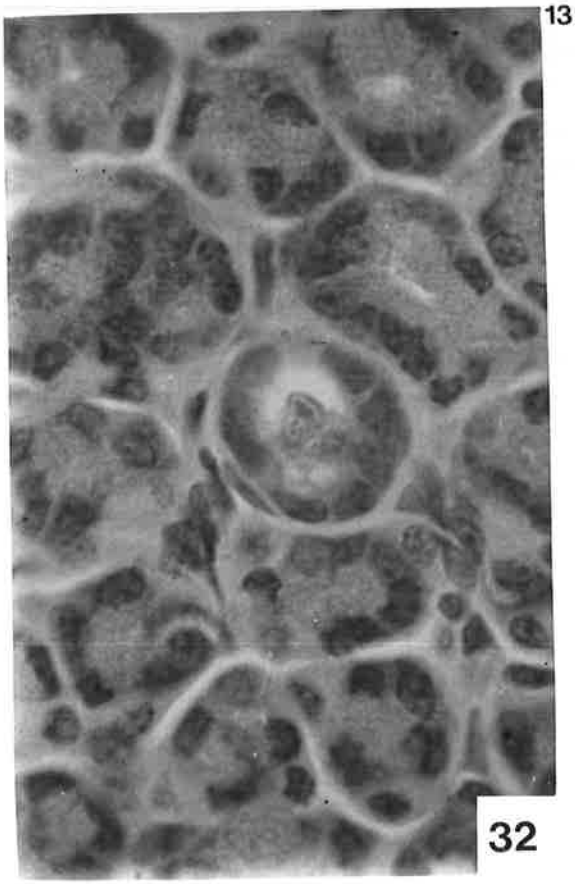

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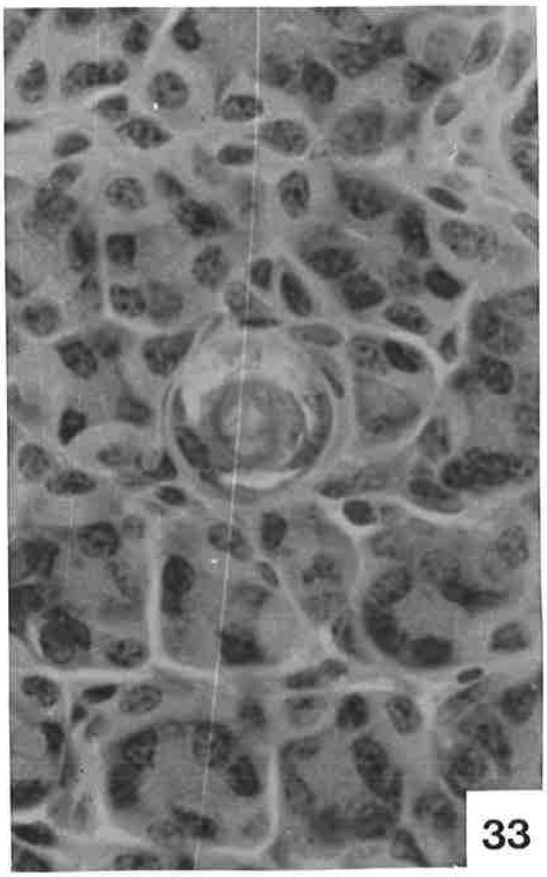


33

Infective sporocysts of *S. tenella*

FIGURE 31. Intact sporocysts recovered from lumen of small intestine at 12 hours post-inoculation (pi).

FIGURE 32. Remnants of excysted sporocyst within crypt of Lieberkühn of duodenum at 12 hrs. pi.

FIGURE 33. Remnants of excysted sporocyst within degenerate crypt of Lieberkühn of jejenum at 24 hrs. pi.

(all ~1,000 X mag.)

(b) Merogonous stages

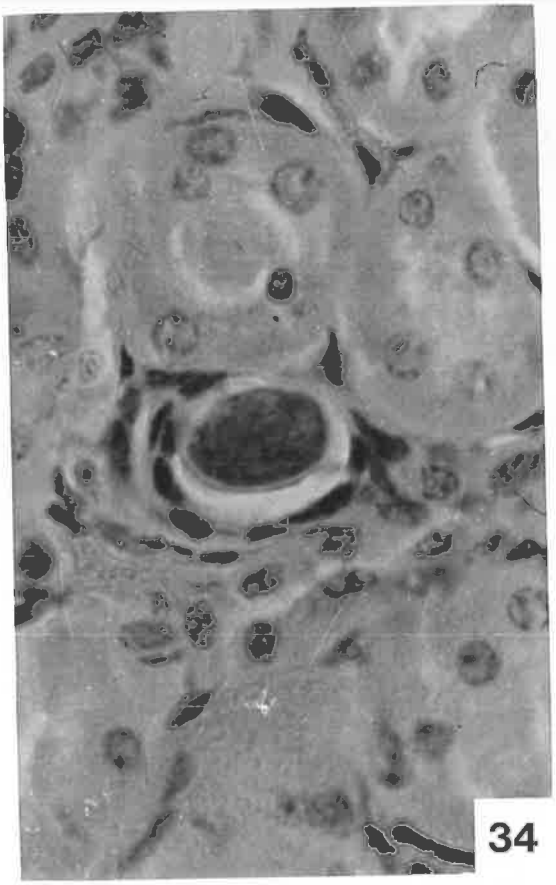
Three morphologically different merogonous stages of the parasite were detected in the lambs from 6-36 dpi. These stages occurred in a definite chronological sequence which was analogous to the times after infection at which the sites of infection varied. The dimensions of these 3 merogonous stages are given in Table 22.

DAYS POST-INOCULATION	6-19	21-34	36
SITE OF INFECTION	arterioles	capillaries	sinusoids & capillaries
	6-9dpi	21-23dpi	
UNDIFFERENTIATED MERONT	11.5-15.2 x 6.0-8.5 (14.0 x 7.4)	11.5-15.9 x 5.3-9.7 (14.5 x 8.4)	-
divides into			
	12-19dpi	25-34dpi	36dpi
DIFFERENTIATED MERONT	13.4-35.5 x 12.2-15.1 (22.3 x 13.4)	12.0-27.4 x 7.5-15.3 (15.2 x 10.6)	6.9-8.2 x 4.3-5.5 (7.4 x 5.1)
containing			
	18-28 zoites (24)	18-38 zoites (30)	6-9 zoites (8)
MEROZOITES	1.4-1.9 x 0.5-1.1 (1.5 x 0.6)	1.1-2.1 x 0.3-0.8 (1.6 x 0.5)	1.8-2.3 x 0.4-0.8 (2.1 x 0.6)

(means given in round brackets)

TABLE 22. Size ranges and mean dimensions of the 3 merogonous stages and their merozoites (in μm).

- From 6-19 dpi, the first merogonous stages of the parasite were observed within the endothelial cells of arterioles. Undifferentiated ovoid bodies first appeared within the cytoplasm of the endothelial cells from 6-9 dpi (see Figure 34). These meronts were relatively homogeneous in appearance, deeply basophilic staining and averaged 14.0µm in length by 7.4µm in width. They usually obscured the host cell nuclei and partially distended the endothelial cell into the lumen of the arteriole. From 12-19 dpi, the stages detected had undergone division and increased in size, now averaging 22.3µm in length by 13.4µm in width. These differentiated meronts contained numerous merozoites (see Figure 35) with an average number of 24 per meront. The merozoites exhibited prominent basophilic nuclei and slightly eosinophilic anterior ends. They were relatively elongate in shape with mean dimensions of 1.5µm in length by 0.6µm in width. The merozoites were contained within the cytoplasm of the host cell but it was not possible at the light microscopic level to determine whether they were bound within a separate membrane (as distinct from the cellular membrane). In most cases, the parasitized cell was extremely distended to the extent that it virtually occluded the lumen of the arteriole (see Figure 36).



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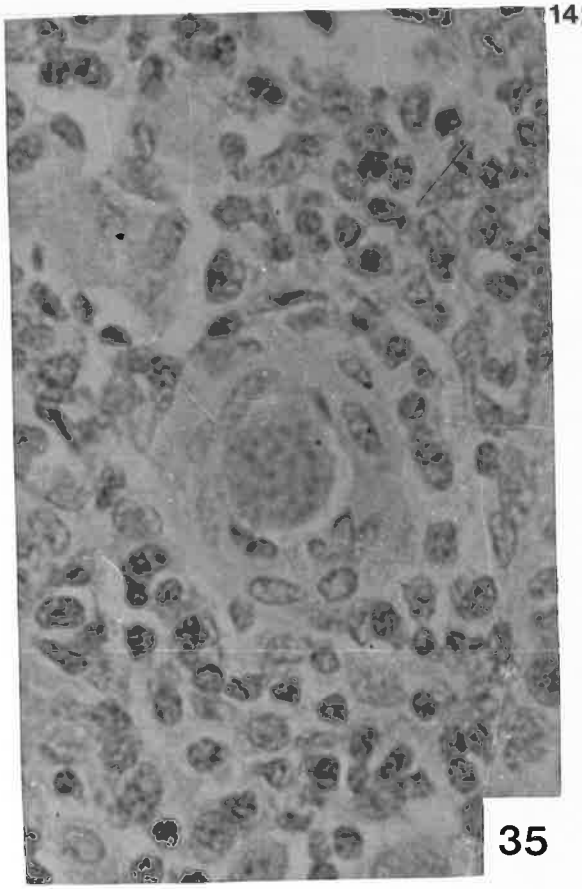

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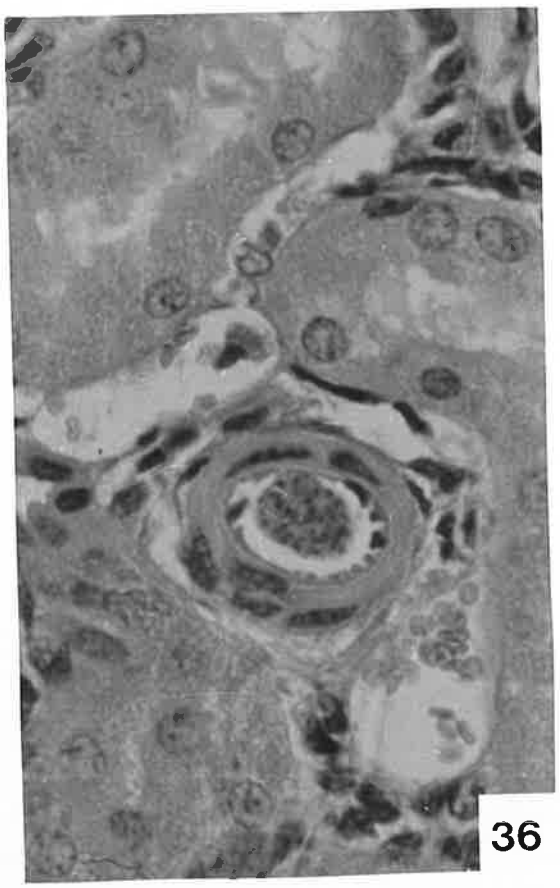

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First generation meronts of *S. tenella*

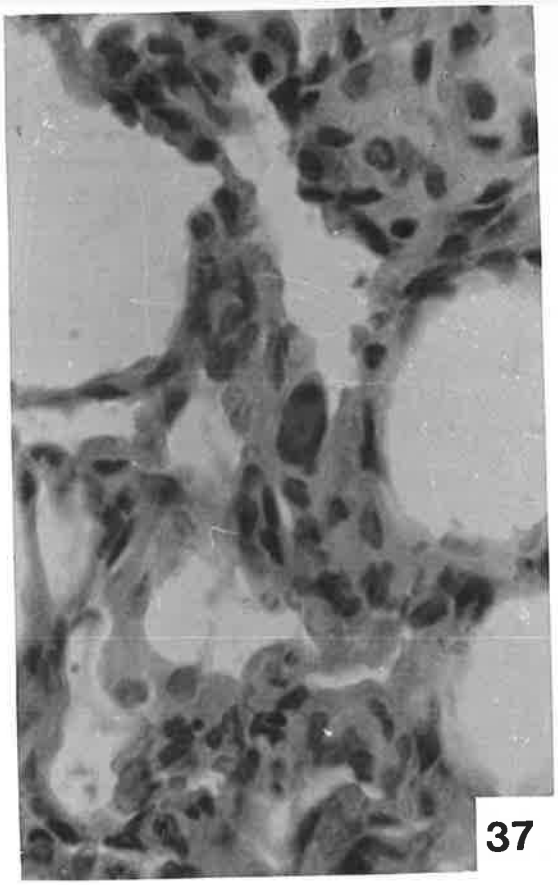
FIGURE 34. Large basophilic meront (undifferentiated) within endothelium of arteriole in renal medulla at 6 dpi.

FIGURE 35. Differentiated meront containing numerous merozoites in arteriole endothelium in prefemoral lymph node at 12 dpi.

FIGURE 36. Mature meront containing merozoites within distended endothelium of arteriole in renal medulla at 17 dpi.

(all ~1,000 X mag.)

- From 21-34 dpi, the second merogonous stages of the parasite were detected within the endothelial cells of capillaries. These stages were more numerous and far more widespread throughout the tissues than the former stages. Intracytoplasmic ovoid bodies were firstly detected within the capillary endothelial cells from 21-23 dpi (see Figure 37). These undifferentiated meronts were very homogeneous in nature and deeply basophilic staining. They averaged 14.5µm in length by 8.4µm in width and generally occupied most of the cellular cytoplasm thereby forcing the nuclei to one side (usually away from the capillary lumen). From 25-34 dpi, the stages detected had undergone differentiation and now contained numerous merozoites. These merozoites were similar in appearance to those of the first merogous stages; measuring on average 1.6µm long by 0.5µm wide and possessing prominent nuclei. In some instances, the merozoites were arranged in a circular or rosette formation within the meront (see Figure 38). The meronts themselves were larger in size than the undifferentiated stages detected beforehand, having the mean dimensions of 15.2µm in length by 10.6µm in width; but they were smaller in size than the first merogonous stages. They also occupied most of the host cell cytoplasm and usually obscured the structure of the capillaries (see Figure 39). The actual position of the meronts within the endothelial cells was best seen in longitudinal or transverse sections through capillaries, as was the distention caused to the parasitized cell (see Figure 40). From 27-34 dpi, free red blood cells were occasionally observed within the tissues around sites of infection by the meronts thereby indicating that local damage was occurring to the capillaries resulting in petechial haemorrhages (see Figure 41). From 31-34 dpi, the merozoites appeared to be slightly larger in size as though they were almost mature (see Figure 42).



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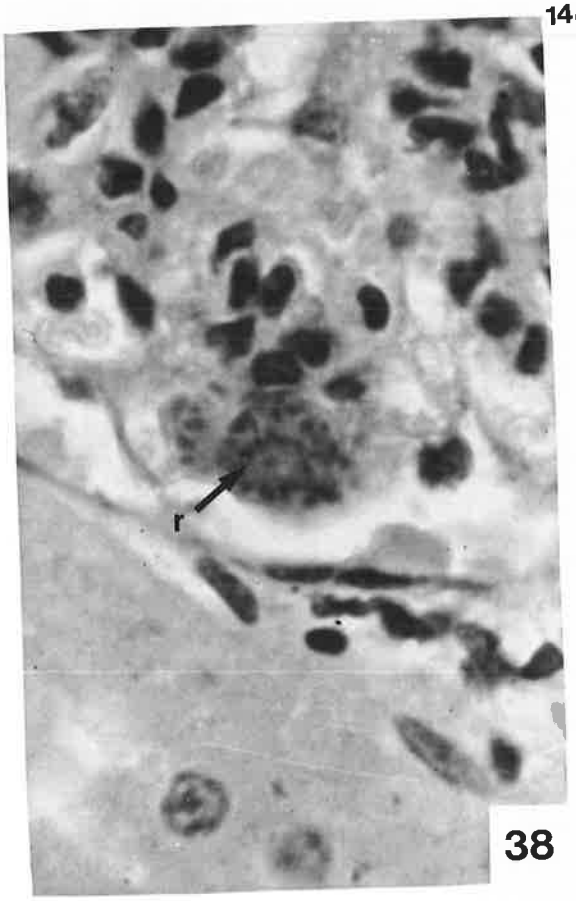

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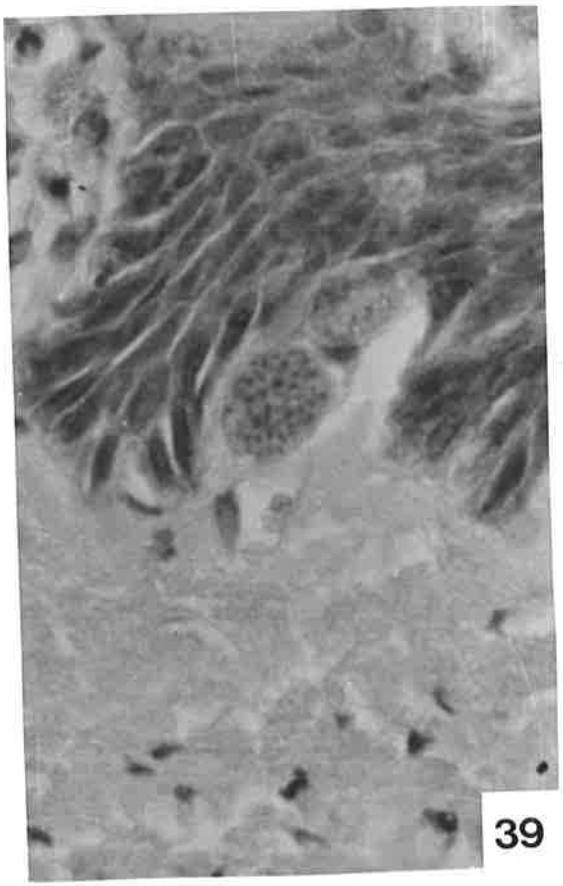

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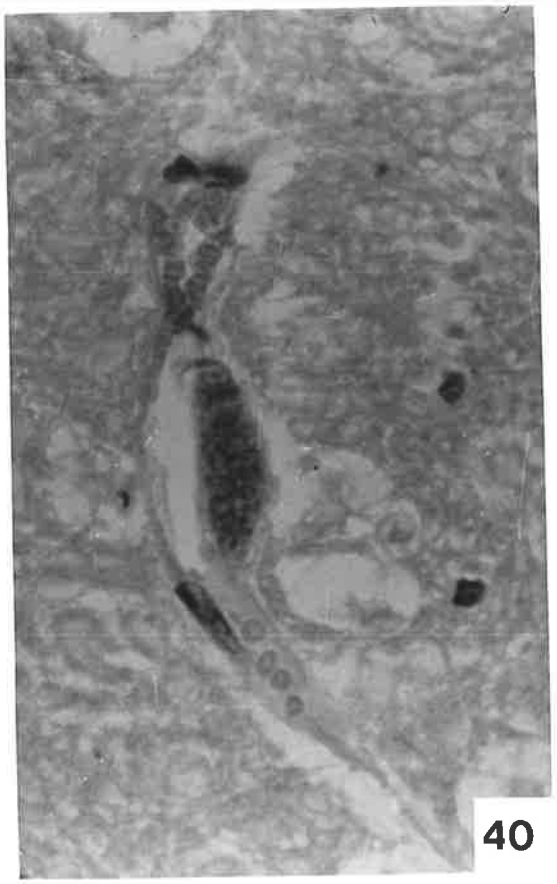
Second generation meronts of *S. tenella*

FIGURE 37. Small basophilic meront (undifferentiated) within endothelium of capillary in lung at 21 dpi.

FIGURE 38. Differentiating meront containing merozoites within renal glomerulus at 25 dpi. Note rosette formation (r) of some of the zoites.

FIGURE 39. Differentiated meront containing numerous merozoites associated with subepithelial capillary in oesophagus at 25 dpi.

(all ~1,000 X mag.)



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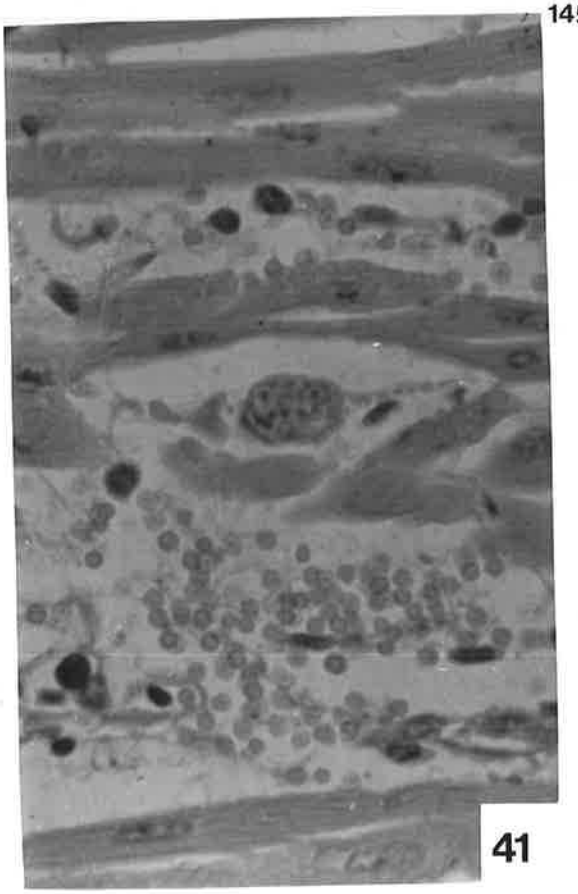

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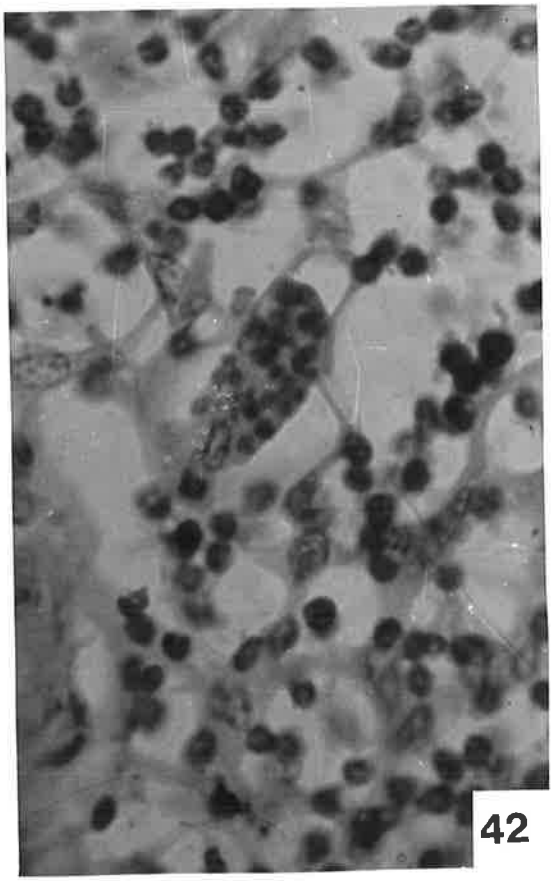

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Second generation meronts of *S. tenella*

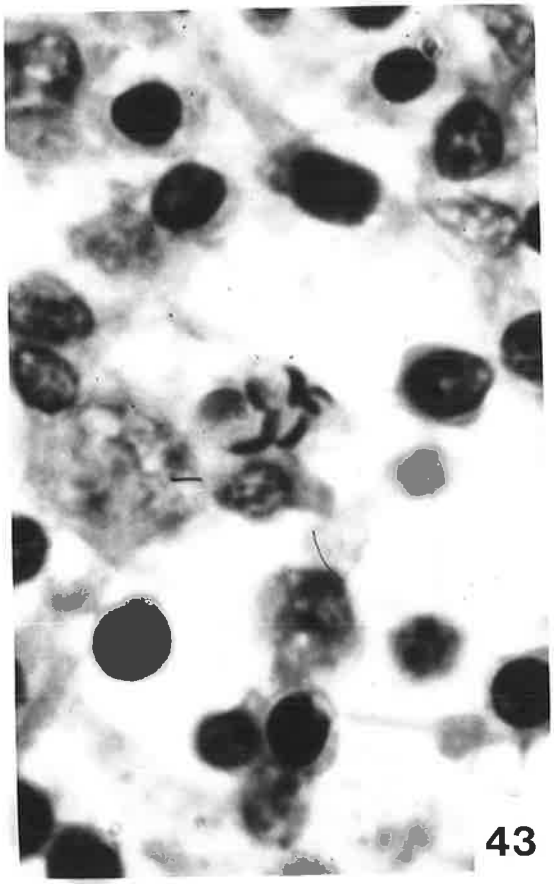
FIGURE 40. Meront containing numerous merozoites within capillary endothelium of cerebral white matter at 27 dpi.

FIGURE 41. Differentiated meront located in skeletal muscle (posterior pectoral) at 31 dpi. Note localized haemorrhagic syndrome.

FIGURE 42. Meront containing large mature merozoites in axillary lymph node at 34 dpi.

(all ~1,000 X mag.)

- At 36 dpi, the third merogonous stages of the parasite were found within the endothelial cells of sinusoids and capillaries. No undifferentiated stages (or ovoid bodies) were observed prior to 36 dpi. Very few merogonous stages were detected and they were much smaller than the preceding two merogonous stages; measuring 7.4µm in length by 5.1µm in width. They were always differentiated and contained fewer merozoites (average number of 8 per meront) which were quite different in size and shape from those of the previous two stages (see Figure 43). The merozoites were very basophilic staining, homogeneous in appearance and their nuclei were not obvious. They were large and crescent-shaped with mean dimensions of 2.1µm in length by 0.6µm in width. Occasionally the merozoites were clumped within the cytoplasm of the host cell and only isolated zoites were apparent (see Figure 44). The merogonous stages also distended the host endothelial cells into the lumen of the blood vessels and shrinkage artefacts were quite often found around them in the sections (see Figure 45).



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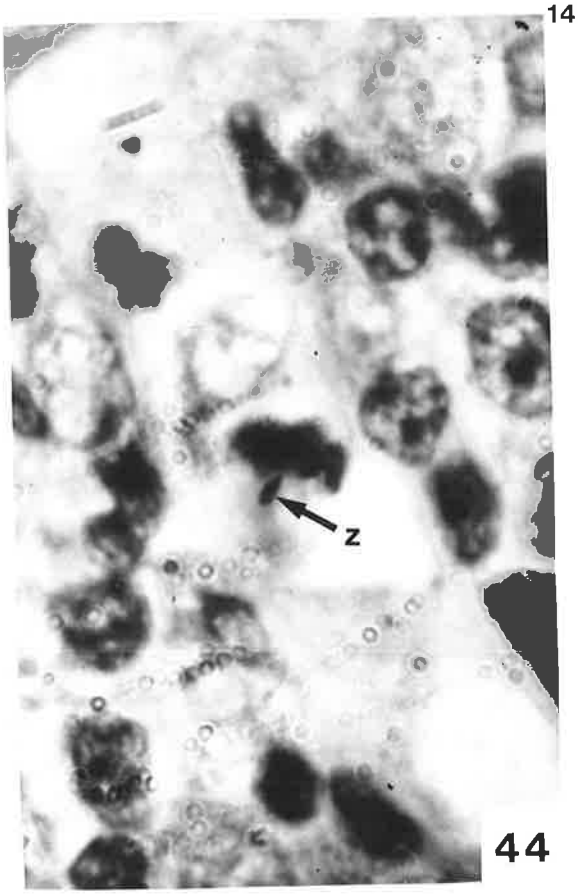

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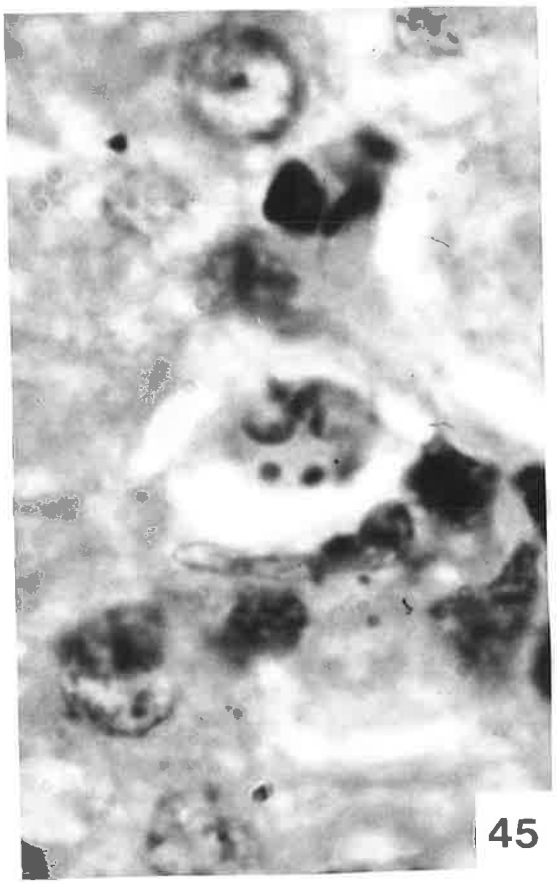

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Third generation (?) meronts of *S. tenella*

FIGURE 43. Small meront containing several crescent-shaped merozoites within bronchial lymph node at 36 dpi.

FIGURE 44. Basophilic meront within mesenteric lymph node at 36 dpi. Note single isolated zoite (z) to one side of the meront.

FIGURE 45. Meront containing several merozoites within hepatic sinusoid at 36 dpi.

(all ~2,000 X mag.)

(c) Sarcocysts

Three distinct stages were observed in the development of the intracellular muscle sarcocysts between 41 and 134 dpi. These stages occurred at definite times after infection and their dimensions, and those of their internal elements, are given in Table 23.

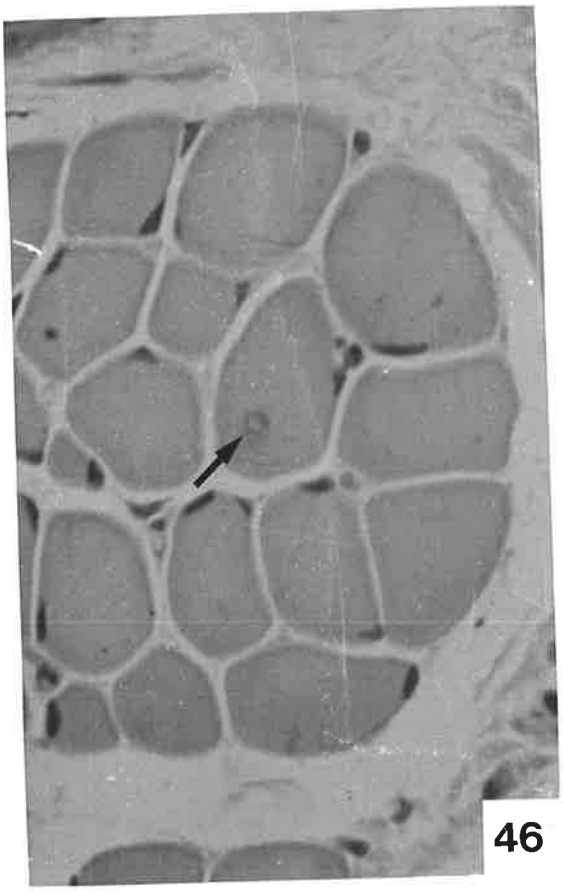
DAYS POST-INOCULATION	41-45	50-55	65-134
STAGE OF INFECTION	invasion	division	growth
INTRACELLULAR MUSCLE CYST	14-33 x 3-6 (24.5 x 4.5)	38-110 x 7-15 (81 x 9)	28-184 x 15-36 (94 x 22.5)
bounded by a			
CYST WALL	-	thin & smooth 0.2-0.8 (0.5)	thin & smooth 0.3-0.7 (0.6)
			thick & radially-striated 1.5-2.4 (1.8)
and containing		-	
		14-85 cells (35)	0-40 cells (15)
METROCYTES	-	3.1-4.4 x 1.5-2.3 (4.0 x 2.1)	2.1-4.6 x 1.9-3.4 (3.7 x 2.8)
and			
			8-140 zoites (48)
CYSTOZOITES	-	-	4.5-10.5 x 1.5-4.5 (9.5 x 4.0)

(means given in round brackets)

TABLE 23. Size ranges and mean dimensions of the developing sarcocyst stages and their internal elements (in µm).

- At 41 & 45 dpi, invading stages of the parasite were found within the striated muscle fibres. These stages were circular to elongate in shape, homogeneous in appearance and lightly basophilic staining (see Figure 46). They were variable in size averaging 24.5µm in length by 4.5µm in width. Although these stages were very discrete and always centrally located within the cytoplasm of the muscle fibres, they did not appear to have a definite cyst wall (see Figure 47). The stages also did not contain discernible internal elements; such as metrocytes and cystozoites.

- At 50 & 55 dpi, dividing stages of the parasite were detected within the musculature. Such stages were much larger than their predecessors, with mean dimensions of 81µm in length by 9µm in width, and they were very elongate in shape and located within the centre of the muscle fibres parallel to the direction of the fibre (see Figure 48). These intracellular stages were bounded by a distinct cyst wall which was thin and smooth in appearance and measured 0.5µm in diameter. Numerous basophilic cellular bodies were found throughout the cysts and the average number was 35 per cyst (see Figure 49). These cells were dividing metrocytes and they were spherical to ovoid in shape and measured 4.0µm long by 2.1µm wide. No cystozoites were detected within these cyst stages at this time of infection.



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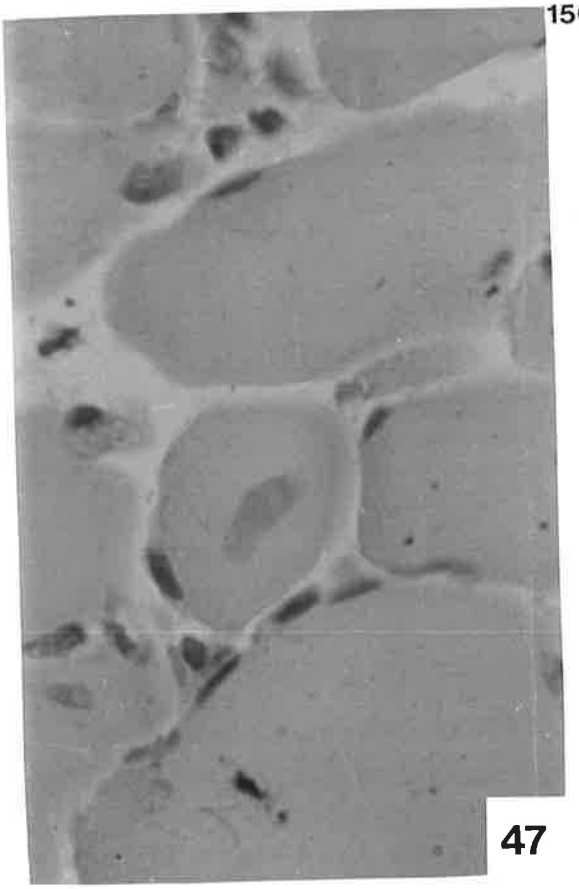

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48

Intracellular muscle stages of *S. tenella*

FIGURE 46. Small basophilic stage within single fibre of infra-spinatus skeletal muscle at 41 dpi.

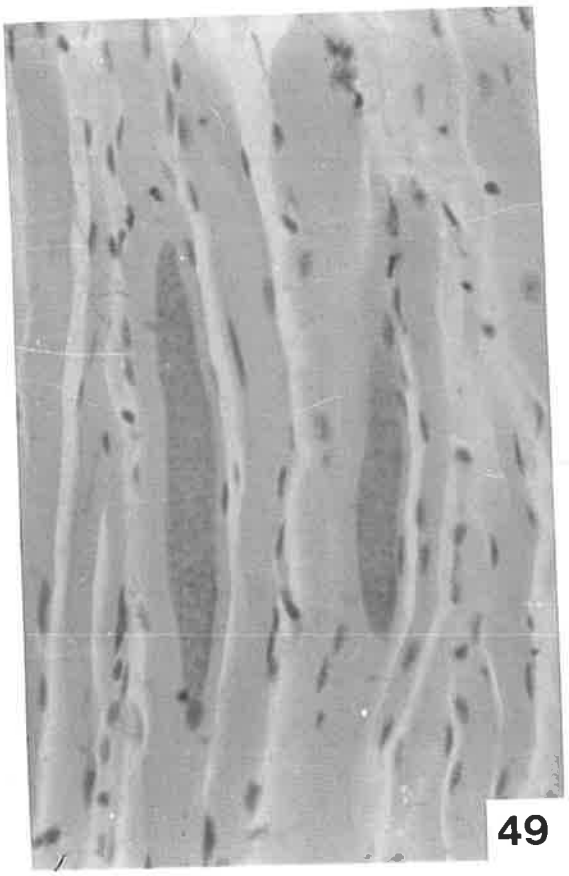
FIGURE 47. Elongate stage containing no discernible elements within tensor fascia-latae muscle fibre at 45 dpi.

(both ~1,000 X mag.)

FIGURE 48. Longitudinal section of dividing stage within lesser diaphragm muscle fibre at 50 dpi.

(~500 X mag.)

- From 65-134 dpi, characteristic sarcocysts were observed developing within the cardiac and skeletal musculature. The sarcocysts were extremely variable in size and had the mean dimensions of 94µm in length by 22.5µm in width. They occupied the majority of the sarcoplasm of the muscle cells and were aligned within them in the direction of the fibres. The cysts were always surrounded by a cyst wall and at the light microscopic level it invariably appeared as a single layer. However, two different types of cyst walls were detected. The majority of the sarcocysts (82%) were bounded by a thick cyst wall which was radially-striated in appearance and measured 1.8µm in diameter (see Figure 50). The remainder of the sarcocysts had thin, smooth cyst walls measuring 0.6µm in diameter. The different cyst wall morphologies were not dependent upon the sites of infection for both types of cysts were found throughout the various muscular tissues examined. All of the sarcocysts contained basophilic cystozoites which were crescent-to- banana-shaped and averaged 9.5µm in length by 4.0µm in width. The number of cystozoites within each cyst varied according to its size and their average number per cyst was 48. The zoites were situated within compartments formed by septae inside the sarcocysts and shrinkage artefacts frequently exaggerated their grouped appearance (see Figure 51). Ovoid metrocytes were detected around the internal periphery of most sarcocysts and they were similar in appearance to those of the dividing stages detected earlier; measuring 3.7µm in length by 2.8µm in width. The sarcocysts found in the cerebrum and cerebellum between 55 & 111 dpi were smaller than their counterparts elsewhere (average of 31µm long by 16µm wide) and were relatively characteristic in appearance except that they lacked a distinct cyst wall. Only rarely was any cellular reaction to infection observed with the histological sections and in those rare cases, it usually took the form of a lymphocytic aggregation around the parasitized muscle fibre.



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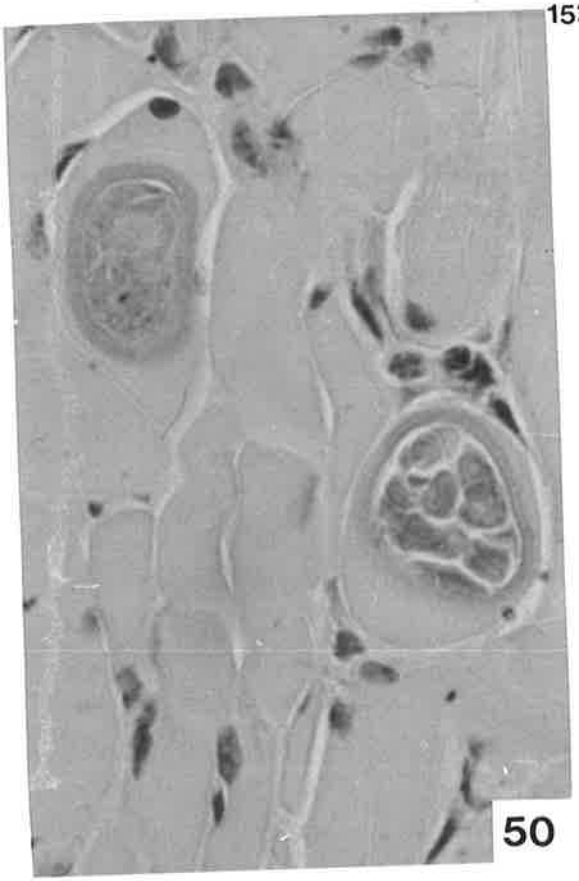

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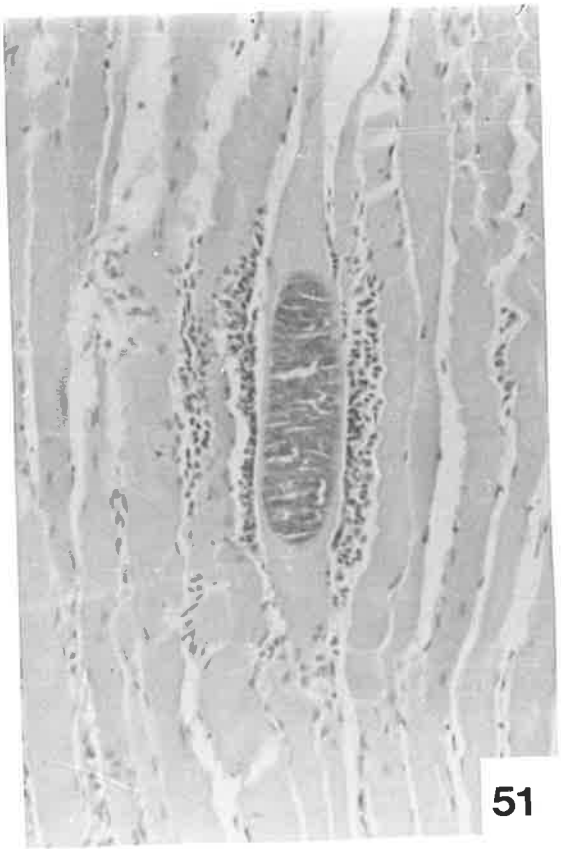


51

Intracellular muscle stages of *S. tenella*

FIGURE 49. Dividing stages containing numerous ovoid metrocytes within oblique abdominal muscle at 55 dpi. (~500 X mag.)

FIGURE 50. Cross section of sarcocysts containing cystozoites within masseter muscle fibres at 75 dpi. Note radially-striated thick cyst walls. (~1,000 X mag.)

FIGURE 51. Characteristic sarcocyst within semimembranous muscle fibre at 111 dpi. Note shrinkage artefacts between cystozoites and also nuclear infiltration around fibre. (~300 X mag.)

28.3 INTENSITY OF DEVELOPMENTAL STAGES

The intensity of infection by the various developmental stages of the parasite was stereologically calculated for each infected lamb and arbitarily expressed as the number of stages per cc of tissue. The stereological calculations included all tissues examined which were not infected thereby providing averages which were not biased towards higher values. Because the lambs were infected with varying doses of sporocysts, the intensities of infection were adjusted to a uniform infective dosage of 0.5 million sporocysts; as given in Table 24. These adjustments thereby allowed tentative comparisons to be made between the intensities of infection in the individual lambs.

The adjusted intensities of infection by the merogonous stages of the parasite were plotted against the post-inoculation times (see Figure 52) and two major peaks in intensity were observed. The first merogonous stages of the parasite steadily increased in intensity from 6 dpi, peaked at 15 dpi and then declined thereafter until 19 dpi. The second merogonous stages achieved a much greater intensity of infection at 27 dpi after a dramatic increase from 21 dpi and followed by a considerable decrease until 34 dpi. The third merogonous stages of the parasite were only detected at 36 dpi and they occurred with a very low intensity of infection, as did the sporocyst remnants detected from 1.3-72.0 hours post-inoculation.

The adjusted intensities of infection by the developing sarcocyst stages of the parasite were also plotted against the time after infection (see Figure 53). The intensity of sarcocyst infection increased dramatically from 41 dpi, peaked in intensity at 65 dpi and then steadily declined thereafter until 134 dpi. The increase in intensity was observed for the invading and dividing stages of the intramuscular cysts whereas the peak in intensity and the subsequent decrease occurred for the characteristic sarcocysts.

LAMB NO.	INOCULUM DOSE RATE (no. sporocysts)	POST-INOCULATION TIME	INTENSITY OF INFECTION (X 10^{-3}stages/ cc of tissue)	INTENSITY ADJUSTED TO UNIFORM DOSE RATE (X 10^{-3}stages/cc tissue)
42		1.3 hours pi.	0.015 sporocysts	0.004 sporocysts
51		4 "	0.017 "	0.004 "
40	2 million	12 "	0.021 "	0.005 "
52		24 "	0.020 "	0.005
50		3 days pi.	0.008 "	0.004 "
53		6 "	0.011 meronts I	0.005 meronts I
49	1 million	9 "	0.021 "	0.011 "
55		12 "	0.032 "	0.016 "
07		15 "	0.021 "	0.021 "
35		17 "	0.014 "	0.014 "
26		19 "	0.009 "	0.009 "
38		21 "	0.015 meronts II	0.015 meronts II
14		23 "	0.039 "	0.039 "
29		25 "	0.062 "	0.062 "
36*	0.5 million	27 "	0.073 "	0.146 "
02		28 "	0.066 "	0.066 "
04		31 "	0.031 "	0.031 "
10		32 "	0.022 "	0.022 "
12		34 "	0.009 "	0.009 "
00		36 "	0.014 meronts III	0.014 meronts III
09		36 "	0.007 "	0.007 "
30		41 "	0.020 sarcocysts	0.040 sarcocysts
17		45 "	0.143 "	0.286 "
28		50 "	0.380 "	0.760 "
22		55 "	1.392 "	2.782 "
13	0.25 million	65 "	4.092 "	8.184 "
23		75 "	2.183 "	4.366 "
11**		111 "	1.742 "	1.742 "
56		134 "	0.280 "	0.560 "

* Lamb 36 received 0.25 million sporocysts
** Lamb 11 received 0.5 million sporocysts

TABLE 24. The intensity of infection by the various developmental stages of *S. tenella* as derived from stereological calculations and also adjusted to a uniform infective dose of 0.5 million sporocysts.

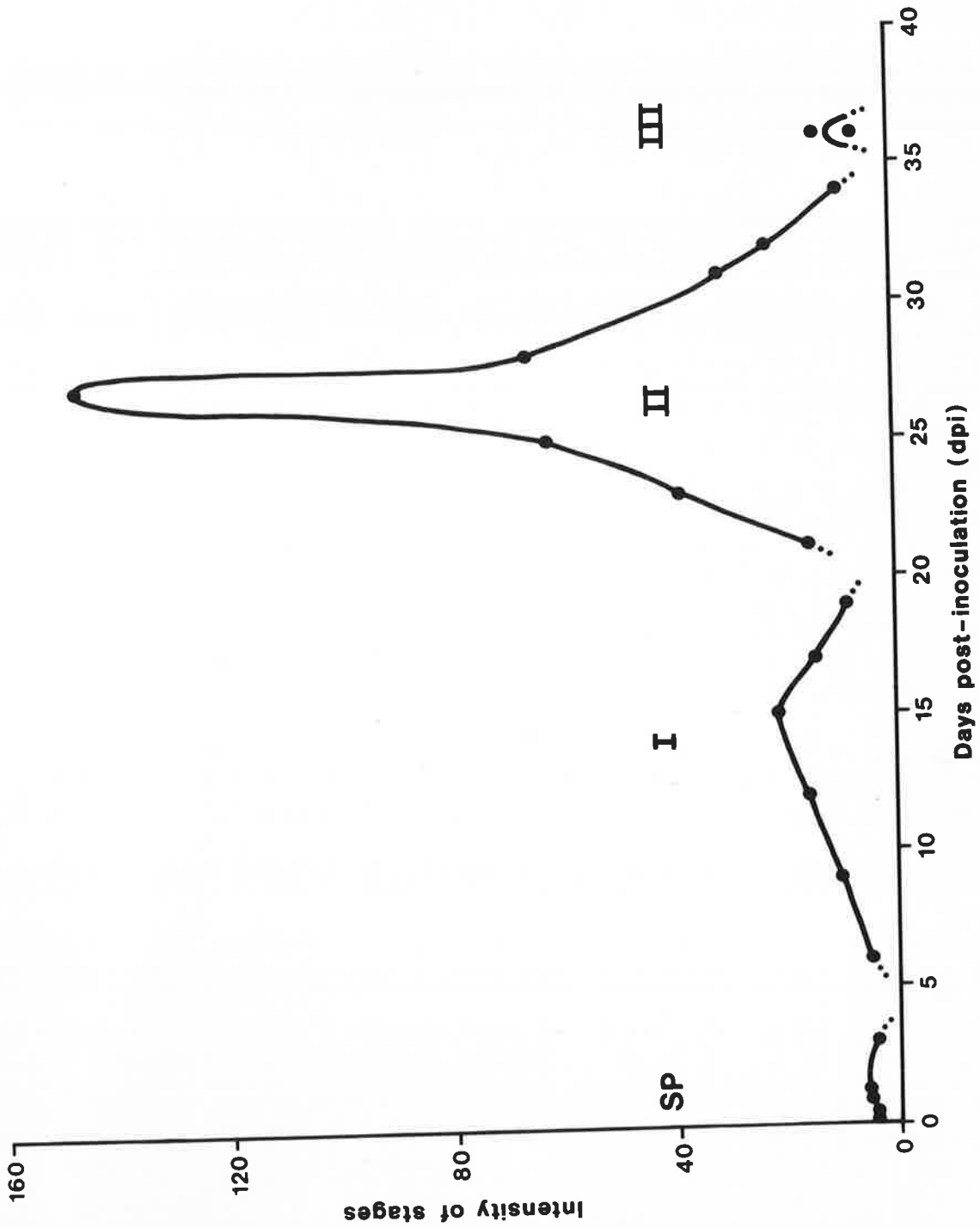


FIGURE 52. Intensity of infection of the lambs by the merogonous stages of development of *S. tenella* (derived from stereological calculations and corrected to a standard infective dose).

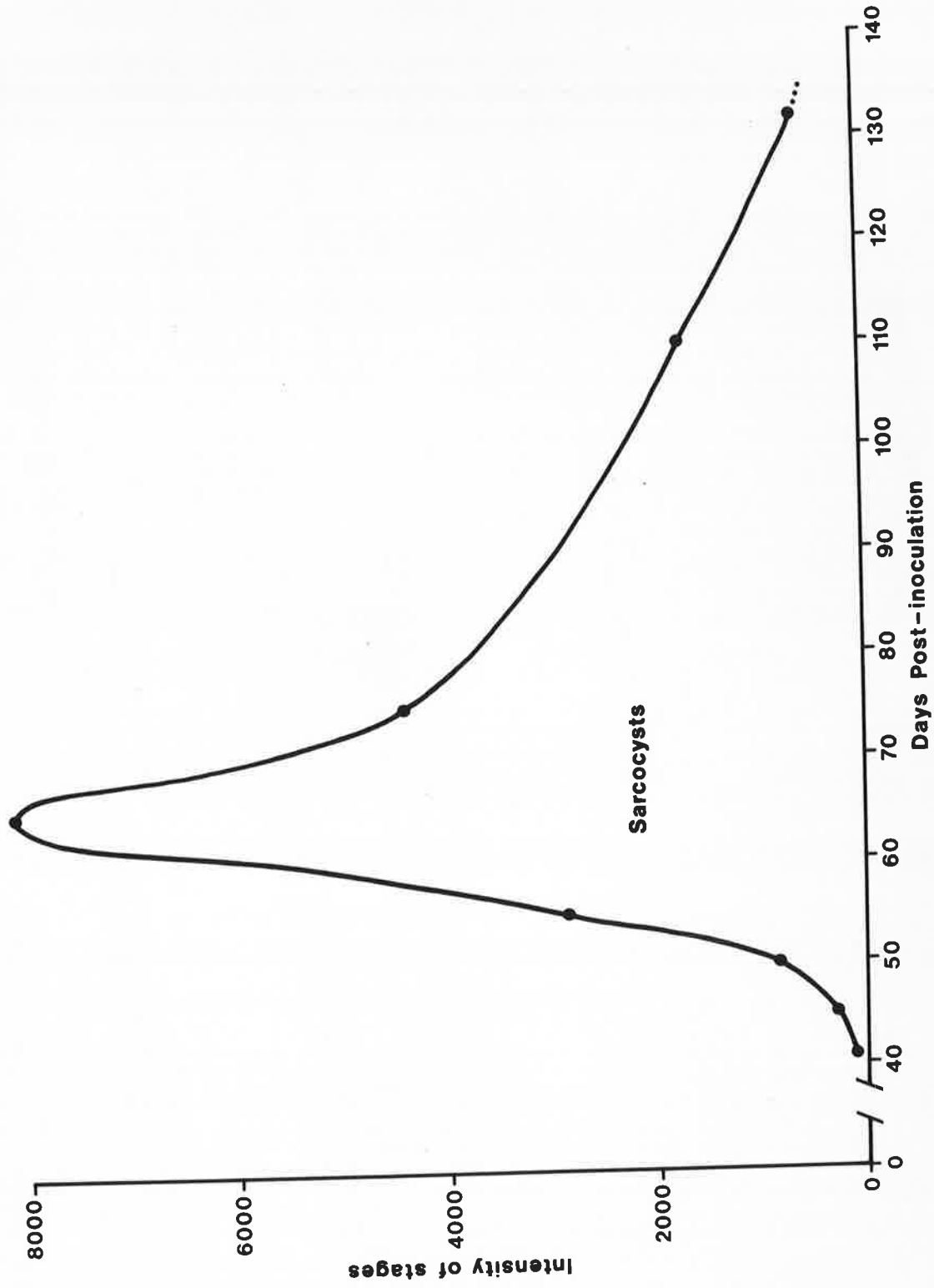


FIGURE 53. Intensity of infection of the lambs by the sarcocyst stages of development of *S. tenella* (derived from stereological calculations and corrected to a standard infective dose).

28.4 OBSERVATIONS ON HISTOCHEMISTRY

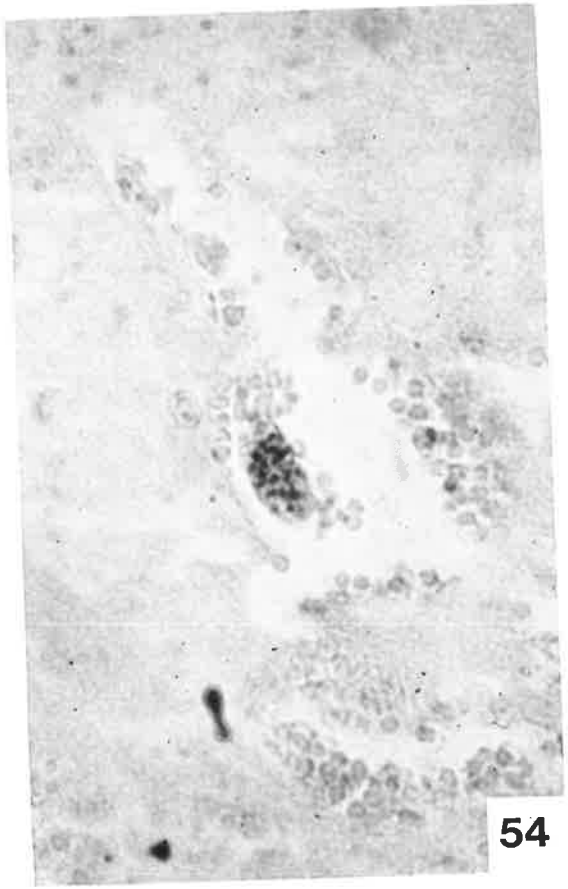
The microscopic examination of host tissues subjected to the various histochemical stains employed provided general information on the staining affinities of the different merogonous stages of development of the parasite.

The 3 merogonous stages detected within the tissues between 6 & 36 dpi exhibited specific staining by the alkaline Giemsa-colophonium method. Such staining was deep purple in colour, dense and granular in appearance and seemed to be specific for the enclosed merozoites (see Figure 54). However, because the stages were extremely small in size, it was not possible at the light microscope level to determine whether certain cellular elements were stained. No metachromatic cytoplasmic granules were observed within any parasitic stages when the neutral Giemsa stain was employed.

Upon staining sections with Periodic acid Schiff's (PAS) reagent, red PAS positive elements were observed within the 2 meront stages detected between 6 & 34 dpi (see Figure 55). These elements did not appear to be associated with specific structures within the meronts even though they were relatively discrete granules. No collagenous or reticulin fibres were found within any meront stages of the parasite when Van Gieson's and Krajian's stains were used.

No bacterial contaminants were found within the tissues of the lambs at any period of infection; as detectable by the Gram and Zil Nielson stains. However, specific staining was observed within the first 2 meront stages of the parasite when the Gram's stain was employed. This staining did not indicate the presence of Gram positive bacilli. It was found to be attributable to the basic fuchsin counterstain used in the technique which stained the merozoite nuclei red (see Figure 56).

The histochemistry of the sarcocyst stages of the parasite have already been adequately reported in the literature (cf. General Introduction).



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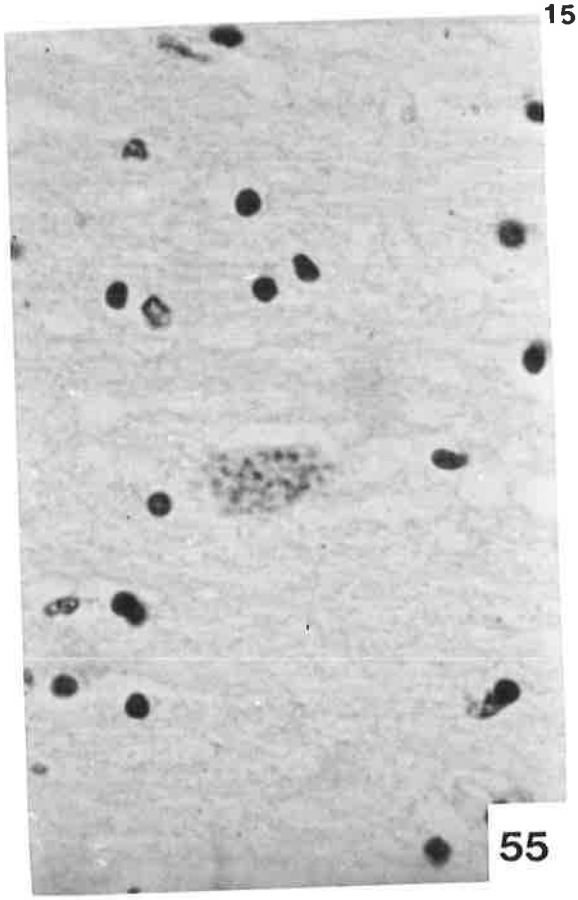

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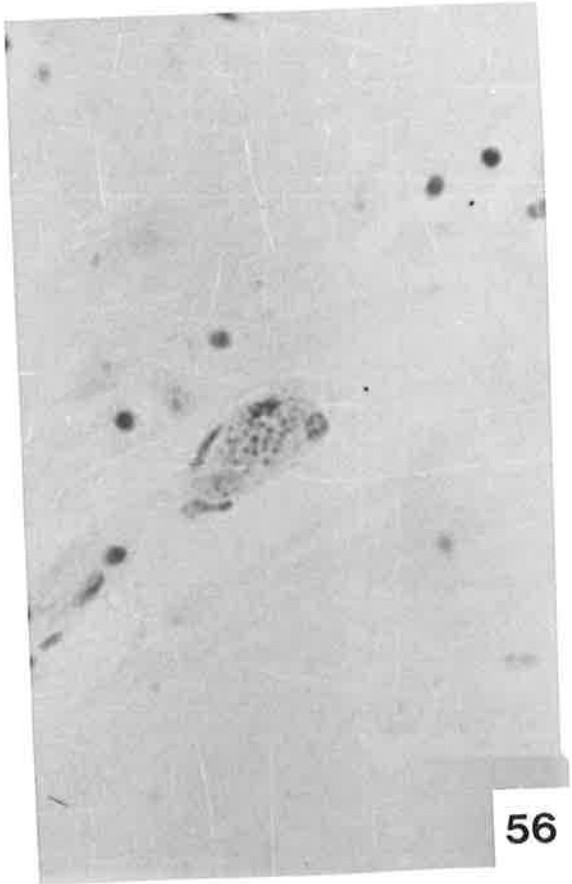

56

Histochemistry of developmental stages of *S. tenella*.
(examples shown are second generation meronts within cerebral capillaries at 25 dpi)

FIGURE 54. Merozoites exhibiting specific staining (actually deep purple in colour) with alkaline Giemsa-colophonium stain.

FIGURE 55. Meront containing PAS positive granules (actually red in colour) after staining with Periodic acid Schiff's reagant.

FIGURE 56. Merozoite nuclei stain darkly (actually red in colour) with basic fuchsin counterstain employed in Gram's stain.

(all ∿ 800 X mag.)

29.0 FLUORESCENT EXAMINATION

Upon specialized microscopic examination of the frozen sections stained with the fluorescent-labelled antibodies, various structures were detected within the tissues of the experimental lambs at different times after infection. Observations made on the specificity of the staining reactions and the sites of reactivity are presented below.

29.1 SPECIFICITY OF REACTION

The Indirect Fluorescent-antibody staining technique readily detected cyst-like stages within the muscle fibres of lambs from 41-134 dpi. These stages were easily identified as developing sarcocysts of the parasite because of the relatively large size and characteristic morphology. Significant fluorescence occurred predominantly over the surface of the cystozoites and some was occasionally observed along the interior of the cyst walls. However, the fluorescence observed for these antigenic stages of the parasite was rather weak in comparison to that observed for the cystozoites on the test slides used in the IFAT.

On examining sections from the lambs sacrificed between 6 & 36 dpi, considerable amounts of localised fluorescence were observed within the tissues. However, the majority of this fluorescence had to be attributed to nonspecific background fluorescence, and sometimes even autofluorescence of the tissues. Much difficulty was encountered in the identification of discrete structures exhibiting fluorescence due to their extremely small size; ranging from 2-15μm in diameter. Furthermore, no internal morphological elements were evident within such structures to aid in identification. It became necessary therefore to compare these sites of reactivity with the sites of infection by the merogonous stages as determined from the histological studies previously described.

29.2 SITES OF REACTIVITY

The exact sites of fluorescent reactivity were identified within the tissues whenever possible. The 2% methylene-blue background stain aided considerably in this identification. The sites so detected were compared to the sites of infection by the merogonous stages of the parasite as determined from the histological examination of the fixed tissue specimens. Only at certain times after infection were sites of reactivity found to be analogous to the sites of infection detected histologically (see Table 25).

TISSUE	DAYS POST-INOCULATION							
	6	12	17	23	25	28	31	36
	Meronts I			Meronts II				Meronts III
(11) Liver			-		-	+	-	-
(14) Heart (apex)					-	-		
(19) Thymus					-		+	
(21) Bronchial L.N.					+	+		-
(22) Prefemoral L.N.		-		-	-			
(24) Mes. L.N. (il.)	-				+	-	+	
(27) Kidney	-	-	-	-	-	+	-	
(30) Cerebrum				+	-	-	-	
(31) Cerebellum				-	+	-		
(39) Lesser diaphragm			-		-	-		
(43) Post. pectoral	-						+	
(46) Ant. semimembr.					+			
	53	55	35	14	29	02	04	09
	LAMB NO.							

(+) Site of fluorescent reactivity analogous to merogonous site of infection
(-) Only nonspecific background fluorescence detected

TABLE 25. Sites of infection by merogonous stages as detected by the examination of Indirect Fluorescent-antibody stained sections.

Fluorescent reactive structures were only detected within the tissues of the experimentally-infected lambs sacrificed between 23 & 31 dpi; i.e., at the times of infection by the second merogonous stages of the parasite. No recognizable structures exhibited fluorescence between 6-19 dpi nor at 36 dpi which were the periods of infection by the first and third merogonous stages. The sites of infection in which fluorescent second stage meronts were detected were confined to the endothelial cells of hepatic sinusoids and the capillaries within the thymus, lymph nodes, renal glomeruli, white matter of the cerebrum and cerebellum and those within the skeletal musculature.

The fluorescent examination was initially employed as an immunological technique to confirm that the stages found within the host tissues were definitely those of a species of *Sarcocystis*. Confirmation was gained that the second merogonous and developing sarcocyst stages of development found from 21-34 dpi and 41-134 dpi were those of *S. tenella* (i.e., the infecting organism). Unfortunately the large degree of nonspecific background fluorescence found within the tissues rendered this technique ineffectual in many instances. Therefore, although the first and third stage meronts occurring from 6-19 dpi and at 36 dpi were not detected upon fluorescent examination, this does not necessarily imply they were not stages of *S. tenella*. This is further explored in the discussion presented in Section 32.2.

30.0 IMMUNOSEROLOGICAL EXAMINATION

The host antibody responses to infection were examined using the Complement Fixation Test (CFT) and the Indirect Fluorescent-antibody Test (IFAT) for *Sarcocystis spp*. The immunoserological titres of the lambs, as determined by the use of both tests every 5 days during the course of infection, are given in Appendix IV (Tables 1 & 2). All 5 un-infected control lambs remained negative to both the CFT and the IFAT.

30.1 COMPLEMENT FIXATION TEST

The results of the CFT were plotted as the reciprocal titre to infection against the post-inoculation times (see Figure 57). The experimentally-infected lambs which were not sacrificed early after infection all seroconverted from a negative (i.e., zero or nonsignificant titre) to a positive titre indicative of infection approximately 10 dpi. The intensity of their CFT-antibody response then rapidly increased to a peak at 30 dpi and gradually declined thereafter although still maintaining a positive titre at 134 dpi. Variation from the mean antibody response was usually within a single double-dilution. All the lambs were negative to the CFT prior to their experimental infection except for the 3 lambs which had been allowed to suckle to obtain natural colostrum. These lambs had low levels of complement-fixing antibodies which were probably maternal colostral antibodies.

30.2 INDIRECT FLUORESCENT-ANTIBODY TEST

The results of the IFAT were similarly plotted against the post-inoculation time (see Figure 58) and the seroconversion of the experimental lambs to a titre indicative of infection was observed to occur at approximately 25 dpi. The IFAT-antibody titres then steadily increased until reaching a relatively constant plateau level around 75 dpi which was maintained through to the last lamb examined at 134 dpi. As for the CFT, the 3 lambs which had suckled had low levels of IFAT-antibodies which were also probably maternal colostral antibodies.

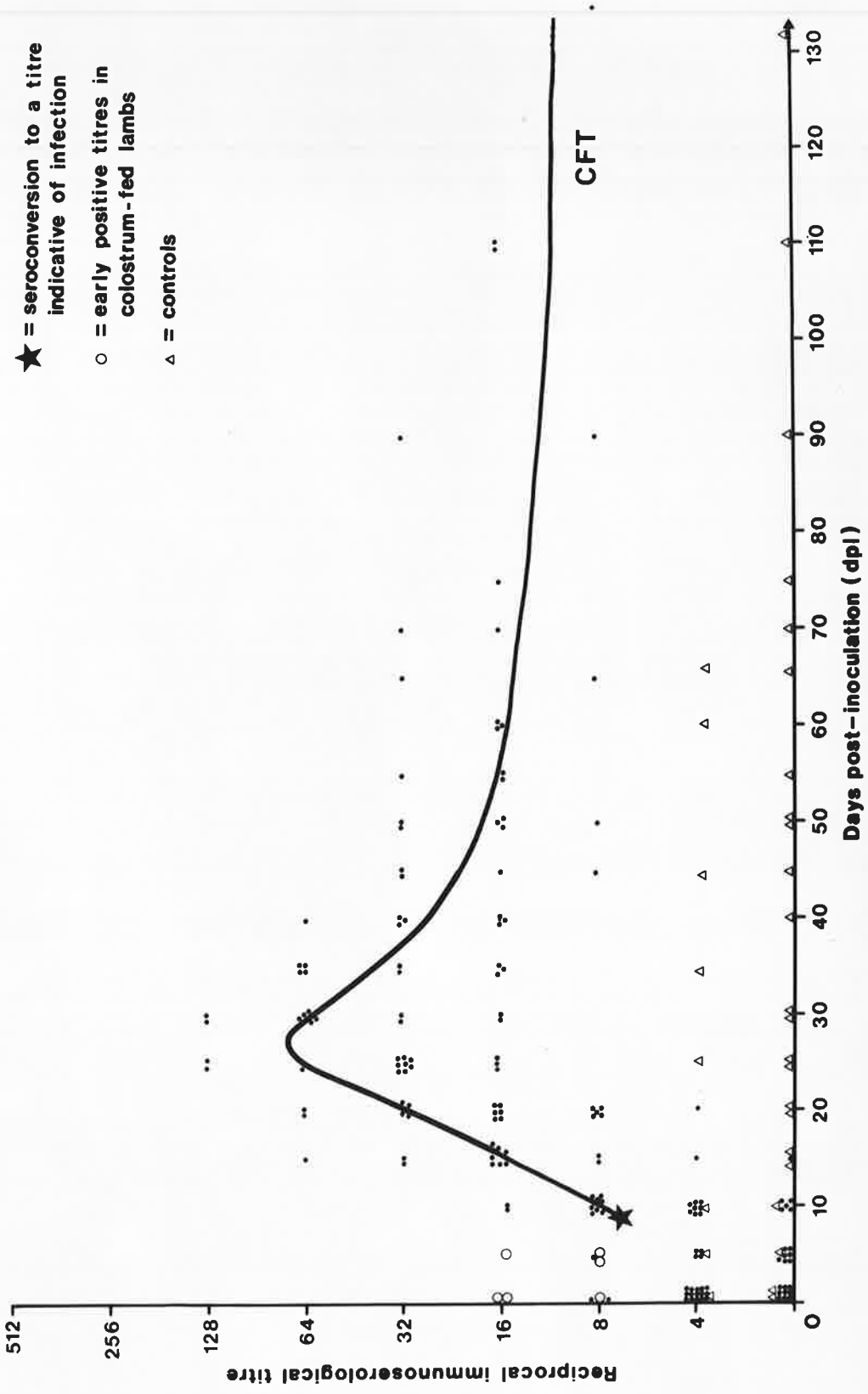

FIGURE 57. Intensity of the CFT antibody response of the lambs following infection by *S. tenella* (mean response indicated).

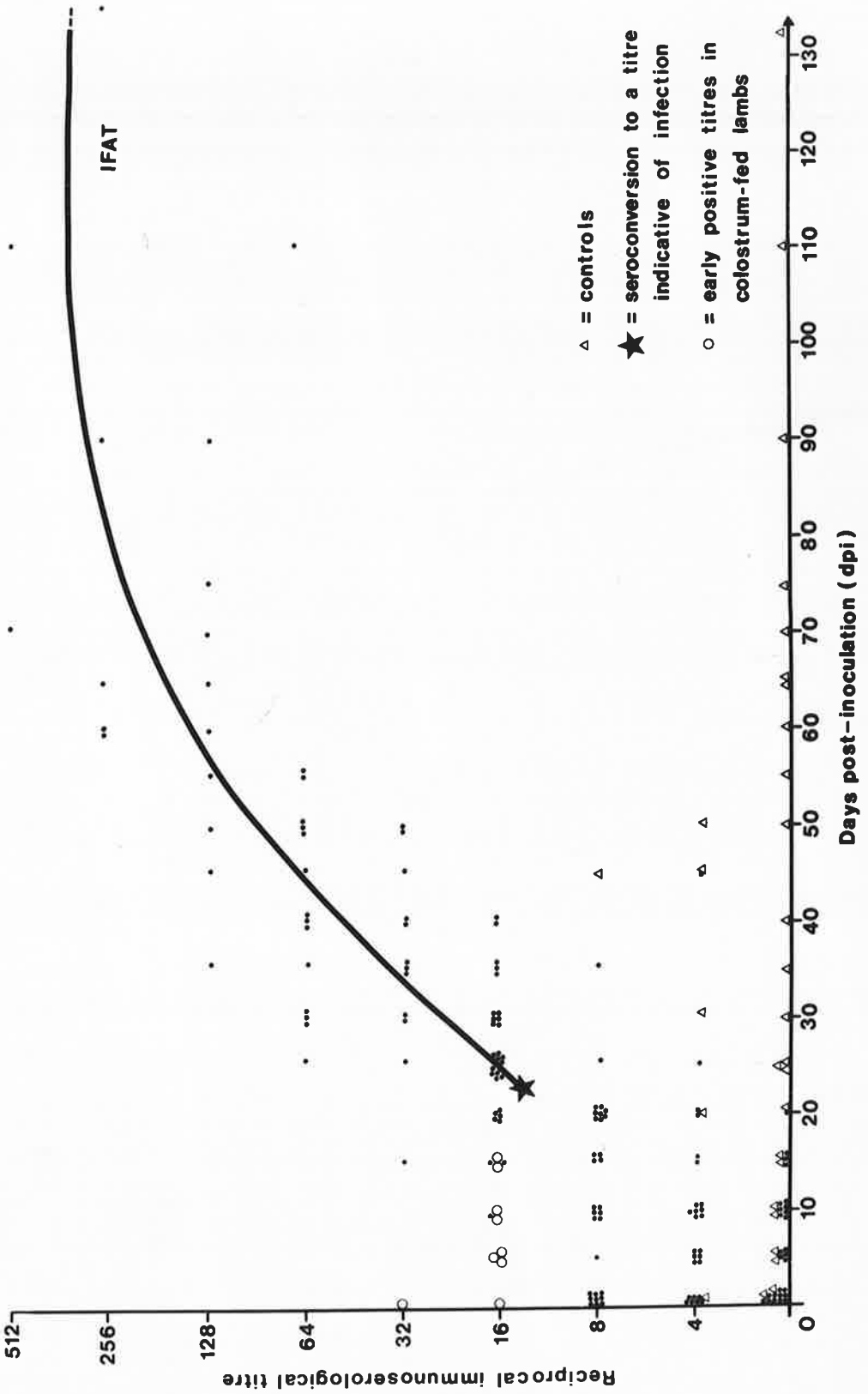

FIGURE 58. Intensity of the IFAT antibody response of the lambs following infection by *S. tenella* (mean response indicated).

31.0 GENERAL OBSERVATIONS

Four of the experimentally-infected lambs died between 27 & 36 dpi and a further 5 lambs became quite ill around 30 dpi. Uniform symptoms were observed to suggest the development of a disease syndrome in these lambs as compared to the general good health of the 5 negative control lambs.

31.1 DISEASE SYNDROME

The 4 lambs that died had been infected with 0.25-0.5 million sporocysts and they had developed an acute and severe disease syndrome approximately 5 days prior to their deaths (see Table 26). The symptoms observed always included marked anorexia, weight loss, elevated body temperatures, a developing anaemia and finally, partial recumbance. The 5 lambs which became ill and then recovered, had also been infected with 0.25-0.5 million sporocysts and they exhibited similar symptoms of disease which were less severe in degree. Although other experimental lambs did not show any obvious disease symptoms, they did lose their appetites and subsequently lost weight from 20-30 dpi.

LAMB NO.	INFECTIVE DOSE (no. sporocysts)	APPEARANCE OF SYMPTOMS	RESULT	
36	0.25 million	21 dpi	Died	27 dpi
02	0.5 "	26 "	Died	28 "
04	0.5 "	26 "	Died	31 "
00	0.5 "	30 "	Died	36 "
29	0.5 "	23 "	Sacrificed	25 "
17	0.25 "	31 "	Recovered	41 " (sacrificed 45 dpi)
22	0.25 "	29 "	Recovered	35 " (sacrificed 55 ")
13	0.25 "	29 "	Recovered	39 " (sacrificed 65 ")
23	0.25 "	30 "	Recovered	43 " (sacrificed 75 ")

TABLE 26. Appearance of disease symptoms in the experimentally-infected lambs.

- Although patho-physiology was not incorporated as part of this thesis, various observations on haematological and biochemical parameters were maintained as parts of the overall project being carried out in this laboratory. Within the scope of this thesis, several of these parameters were examined to determine whether any relationships existed between them and the various stages of develop ment of the parasite.

A significant relationship was found between the haematocrits of the infected lambs and the merogonous stages of parasite development. The haematocrits (or percentage packed cell volumes) of the lambs are given in Appendix IV (Table 3) for each examination carried out every 5 days during the course of infection. They were also plotted against the post-inoculation times as shown in Figure 59. The packe cell volumes (PCV's) of the infected lambs started to decrease from their prior values around 10 dpi; i.e., the time of infection by the first merogonous stages of the parasite. The PCV's decreased dramatically thereafter until approximately 30 dpi. This was during the time of infection by the second merogonous stages and also at the time when some lambs exhibited symptoms of disease and a few even died. After 30 dpi, the PCV's of the remaining lambs steadily increased almost back to normal levels as determined from those of the un-infected control lambs. During this period of infection, the lambs contained only the developing sarcocyst stages of the parasite and they seemed to be recovering from any disease symptoms.

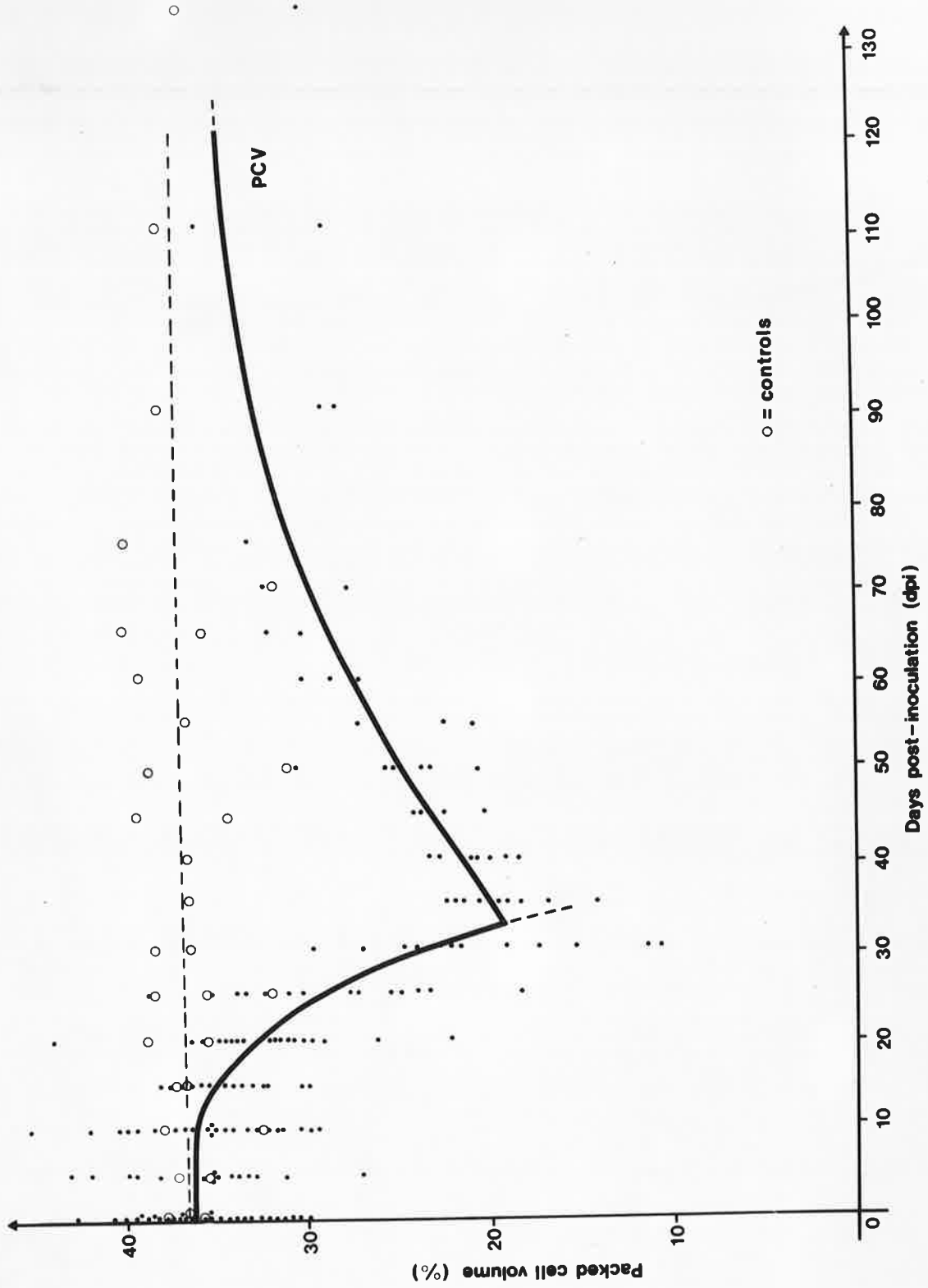

FIGURE 59. Haematocrits (packed cell volumes) of the lambs following infection by *S. tenella* (mean response indicated).

DISCUSSION

During the following discussion, it is important to remember that these studies on the development of the microscopic sarcocyst infections were performed in specific-pathogen-free (i.e., 'Sporozoa-free') lambs. No other similar studies have previously been carried out anywhere else in the world. Those which have been reported in recent literature were not performed in proven 'Sporozoa-free' animals. The majority of such studies have also been made on other species of the parasite in other intermediate hosts. The following observations made on the species of *Sarcocystis* concerned are pertinent to the discussion.

The speciation of the Sarcosporidia in sheep and cattle is based upon transmission studies which revealed a definitive host specificity for certain sarcocysts. Only microscopic sarcocysts from sheep are transmissible to dogs (Rommel *et al.* 1974) and this species is known as *Sarcocystis tenella* (junior synonym *S. ovicanis*). The complete life cycle for this species has been confirmed by several cyclic transmissions in sheep and dogs raised under specific-pathogen-free conditions (Ford 1975). Only certain sarcocysts from cattle are transmissible to dogs (Rommel *et al.* 1974) and this species is usually referred to in the literature as *Sarcocystis fusiformis* (synonym *S. bovicanis*).

An intermediate host specificity for the infective sporocysts obtained from members of the Canidae has also been demonstrated. The sporocysts obtained from dogs and coyotes which had been fed bovine microscopic sarcocysts were apparently not infective to sheep (Rickard & Munday 1975, Fayer *et al.* 1976a); nor were the sporocysts from coyotes which had been fed microscopic sarcocysts from mule deer (Hudkins & Kistner 1977). Alternatively, the sporocysts from dogs which had been fed ovine sarcocysts were reported to be not infective to cattle (Gestrich *et al.* 1975).

From these transmission studies, it appears that the Sarcosporidial infections are very host specific, therefore; analogies made in the following discussion between developmental stages of the parasite found in sheep and those in cattle are actually being made between different species of *Sarcocystis*.

32.0 DEVELOPMENT OF INFECTION

The development of the sporozoan parasite *Sarcocystis tenella* in the experimentally-infected lambs was found to be very definite in terms of the chronological stages of development and the sites of infection within the tissues. From the time of experimental infection, the various developmental stages found in the life cycle of the parasite and their sites of infection were as follows;

- the remnants of the infective sporocysts found in the gastro-intestinal tract,
- the 3 merogonous stages of development found within the endothelial cells of selected parts of the circulatory system, and lastly;
- the developing sarcocyst stages found within the striated muscle fibres.

The sequence of development of *S. tenella* found within the experimentally-infected lambs is shown diagrammatically in Figure 60 and this provides a focal point for the following discussion.

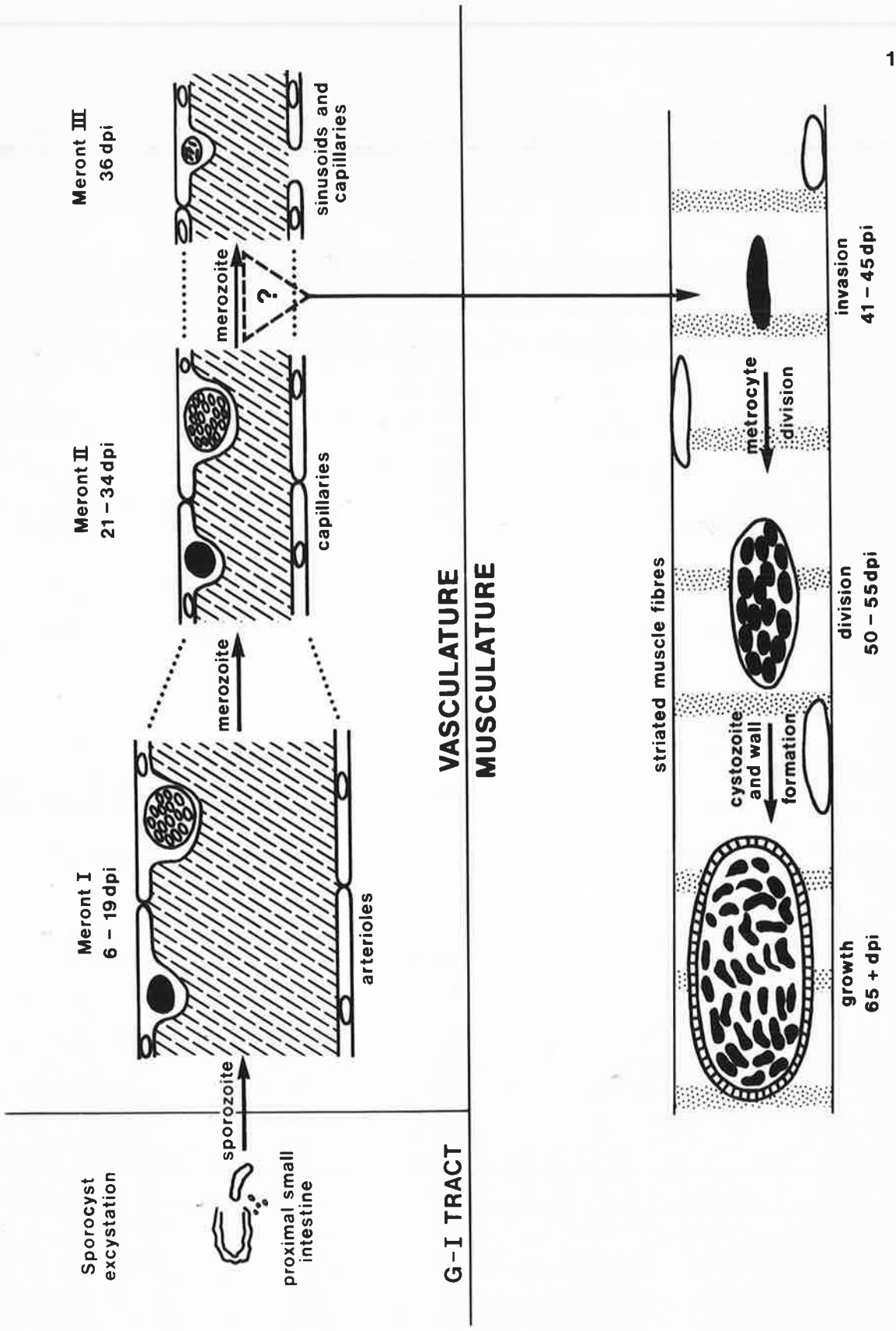

FIGURE 60. Diagrammatical representation of the stages of development of *Sarcocystis tenella* (junior synonym *S. ovicanis*) found in the experimentally-infected SPF lambs .

32.1 INFECTIVE SPOROCYSTS

Very little information could be conclusively determined about the mode and route of infection within the lambs from the sporocyst stages given in the inoculum to the first merogonous stages found within the tissues 6 days post-inoculation (dpi). This was primarily due to the difficulty encountered in the detection and recognition of any parasitic stages during this period of infection. However, certain conclusions were drawn from the detection of some sporocysts, and especially their remnants, within the gastro-intestinal (G-I) tracts of infected lambs from 1.3-72.0 hours post-inoculation (hrs pi).

(a) Intact sporocysts

Whole sporocysts containing 4 sporozoites and a residuum were recovered from G-I content sampled from lambs 1.3-24.0 hrs pi. Only very few of these intact sporocysts were recovered from the lambs even though they had been infected with 2.0 million sporocysts. Those recovered were undamaged indicating they had not received or responded to any excystation stimuli from the host. This could reflect their passage through the host too rapidly for any stimuli to be effective or that these sporocysts did not respond to any stimuli as they were not viable (or infective). However, because excysted sporocysts were found at similar times of infection thereby demonstrating their infectivity, it suggests that the intact sporocysts recovered at these times were not infective. This non-viability of some of the sporocysts may have been naturally-occurring or it may have resulted from the conditions imposed by the technique used to harvest them, or even to store them. Because the numbers of intact sporocysts recovered were too few to effectively quantitate, the degree of non-viability in the inoculation given to the lambs was regarded as insignificant. However,

research must be performed to critically determine the effects that the various laboratory techniques employed have on these stages of the parasite if the percentage viability of the inoculations is to be determined.

(b) Sporocyst remnants

The remains of sporocysts, composed of the ruptured sporocyst walls sometimes enclosing a residuum, were found predominantly within gastric pits and intestinal crypts of lambs examined from 1.3-72.0 hrs pi. The detection of such remnants indicated that these sporocysts were infective and had undergone excystation thereby releasing their contained sporozoites. Because no sporocyst remnants (nor intact sporocysts) were detected within lambs examined after 72 hrs pi, it appears that excystation of the viable sporocysts and the consequent initiation of infection had occurred within 3 days of inoculation; and even as early as 1.3 hrs pi. The sites of the sporocyst remnants found within the lambs suggested that excystation of the sporocysts had occurred predominantly in the abomasum and proximal small intestines of the hosts. Because these remnants were usually firmly entrapped or wedged within the bases of the pits and crypts, it is thought that these locations were not anomalous resulting from the deposition of such stages further along the G-I tract after their excystation elsewhere. These postulated sites of excystation are also comparable to those found for other coccidian parasites; such as certain *Eimeria spp.* (cf. Pellèrdy 1965).

Fayer & Leek (1973) experimentally examined the excystation of *Sarcocystis fusiformis* sporocysts obtained from dogs. They found that either a carbon dioxide or a reducing agent stimulus was required prior to incubation in a trypsin/bile

solution for excystation of the sporocysts to occur. These requirements for excystation are essentially the same as those described for similar stages of other coccidian parasites (Jackson 1962). Such treatment of sporocysts was reported to result in a lateral fracture and subsequent rupture of the sporocyst walls towards one pole of the sporocysts; thereby facilitating the release of the contained sporozoites. Some of the sporocyst remnants detected within the lambs examined in this study were likewise observed to have ruptured at one end only; thus suggesting similar excystation. However, much more research has to be carried out to determine the excystation stimuli required for *S. tenella* sporocysts. Preliminary observations performed within this laboratory have indicated that the stimuli for such excystation do appear to be similar to those required for other species (as adapted to the relevant host species) but they are more complex in character than formerly described (Ford 1976 pers. comm.).

(c) Sporozoites

Once the sporozoites have been released from the sporocysts within the G-I tract, it is not known how or where they penetrate or invade the host tissues. No freed sporozoites were detected within any tissues or cells; nor in any blood or lymphatic vessels of the G-I tract at this stage of infection Considerable difficulty was encountered in the identification of potential parasitic stages in these locations due to the presence of mitotic figures in the epithelia and micro-organisms in the lumena of the G-I tract (both in the size range expected for such stages). However, it can be said with relative certainty that no enteric multiplicative stage of the parasite occurred within the lambs following experimental infection with *S. tenella* sporocysts. It appears that the

sporozoites themselves affect entry into the host vascular system and parasitise the endothelial cells of arterioles without undergoing any prior multiplicative stage. The mode and route of such entry is not known but requires more critical studies utilising specialised techniques to identify the organisms.

32.2 MERONT GENERATIONS

Three distinct merogonous stages of the parasite were detected within vascular endothelial cells in the experimentally-infected lambs examined between 6 & 36 dpi. This was the period of infection following the disappearance of sporocyst stages in the G-I tract and before the appearance of developing sarcocyst stages in the musculature. The merogonous stages occurred at discrete time intervals after infection and were found within specific locations within the vasculature supplying various organs and tissues of the host. Each stage was also morphologically distinctive as were their contained elements (i.e., their merozoites). Various observations made on the distribution, appearance and intensity of such stages are discussed for each stage separately, as follows.

(a) First merogonous generation

The first merogonous stages of the parasite were detected in the lambs examined from 6-19 dpi and they were confined to the endothelial cells of arterioles within most organs and tissues except those of the nervous, endocrine and reproductive systems. Similar morphological stages in sheep have only been observed once before during this period of infection; within the endothelia of arterioles in one lamb at 15 dpi (Munday *et al* 1975). Other than this single report, research workers have not examined developing *Sarcocystis* infections in sheep prior

to 24 dpi. No stages have been detected within biopsy material taken before 20 dpi from the tissues of calves experimentally-infected with *S. fusiformis* (Fayer & Johnson 1974). In fact, most of the previous observations made on the pre-sarcocyst stages in the development of *Sarcocystis spp.* infections have been made on experimental animals which had died or developed obvious gross and clinical signs of disease. The observations made within this thesis on the first merogonous stages detected within the lambs actually represent the first conclusive evidence that this stage of development occurs at this time for a species of *Sarcocystis*; that of *S. tenella*.

The first stage meronts detected in the lambs from 6-9 dpi were homogeneous in appearance and were undifferentiated in that they did not contain any discernible internal elements. From 12-19 dpi, the merogonous stages detected had undergone differentiation (or division) and now contained numerous distinct zoites. Using the terminology of Levine (1971), these zoites are known as merozoites which are formed by the divisional process called merogony (i.e., a form of schizogony).* From their appearance, the zoite stages were certainly not sporozoites (formed in sporocysts) nor gametes (formed in gametocytes, alternatively known as gamonts). The merogonous stages detected in the lambs from 6-19 dpi therefore represent the first asexual multiplicative generation of the parasite following infection. However, the exact type of asexual division occurring within these meronts (fission or endogeny)** cannot

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* Although the term schizogony is often limited to merogony (formation of daughter cells termed merozoites), under the terminology proposed by Levine (1971) it was broadened to incorporate sporogony (formation of sporozoites) as well as gametogony (formation of gametes).

** See footnote at bottom of Page 180.

be positively known without performing ultrastructural studies to determine the mechanism of merozoite (or daughter cell) formation.

In discussing the intensities of the various developmental stages of the parasite found in the lambs, it must be noted that the intensity values were stereologically calculated and expressed per arbitrary unit volume of host tissue examined. These values therefore represent the intensity of 'detection' of such stages rather than the actual intensity of 'infection'. As such, the intensity values cannot be extrapolated back to the actual infective doses of sporocysts given to the lambs to determine the relative success of infection. However, they do allow tentative comparisons to be made between the detected levels of infection in individual lambs because the stereological calculations were standardised for each animal. A minimum level of detection was employed in the histological examination of the tissues from each lamb (i.e., the minimum area of any section examined was 1.0 cm^2) and the intensity values calculated from the stereological formula were adjusted to a uniform infective dose of 0.5 million sporocysts.

The first merogonous stages of the parasite detected in the lambs from 6-19 dpi were found to peak in intensity at 15 dpi thereby indicating the stage at which maximum multiplicatior of the parasite was occurring. The intensity of these stages at this time was approximately 4 times greater than the intensities observed for the sporocyst remnants detected within the G-I tracts of lambs examined from 1.3-72.0 hrs pi. As each infective sporocyst contained 4 sporozoites, it is quite feasible that each sporozoite formed a first stage meront. However, without performing rigidly controlled

infection studies with known numbers of sporocysts of proven viability and then somehow determining the actual number of meronts formed within the infected animals, such an observation can only be speculative.

The first merogonous generation of the parasite was relatively labile in that it only occurred during the discrete time interval of 6-19 days following infection. After 19 dpi, no first stage meronts were detected in the lambs within the endothelial cells of arterioles. The disappearance of these stages at this time indicates the climax of this first asexual division of the parasite and that the enclosed merozoites had attained maturity and affected release from the parasitised host cells. Whether the actual mechanism of merozoite release was passive or active in nature is not known but it may simply have resulted from the rupture of the host cell due to extreme distention. Because the meronts invariably distended the host endothelial cells into the lumena of the arterioles, the merozoites most probably passed into the blood stream of the host. It then follows that they would next come into contact with the host micro-circulatory (or capillary) networks; which is exactly where the next merogonous generation of the parasite was detected.

Unfortunately, no free merozoites were detected within any blood samples taken from lambs at this time after infection to confirm that they were passed into the blood stream. Because the blood samples were also subjected to specialised histochemical and fluorescent examinations, it is not thought that the merozoites were not recognized or overlooked in the samples because of their very small size. Rather, this negative result appears to simply reflect the greater volume of blood that need be examined to detect such stages of the parasite.

It is interesting to note the lack of success found in the Indirect Fluorescent-antibody staining technique for the detection of the first stage meronts. This result initially suggested that these stages did not exhibit any antigenicity to the immunoglobulin groups utilized in the fluorescent-labelled stain. However, this was not the case for all classes of immunoglobulins (derived from a known infected sheep) were included in the stain. Furthermore, these stages of the parasite must have acted as an antigen at some period because an antibody response to infection was observed in the lambs at this time (as detected by the Complement Fixation Test). It is therefore anomalous that no first stage meronts were detectable upon fluorescent examination whereas antibodies to infection were detected upon immunoserological examination. In other words, it appeared that these stages of the parasite did not exhibit any detectable antigenicity to one test whereas they must have to have elicited an antibody response from the host as detected by another test. This immunological enigma remains to be resolved and it appears to be biological in origin rather than technological for each reagent and technical procedure was thoroughly checked and found in good order. Whether it reflects a complex interaction between the parasite and the host during the development of infection is not known as yet.

(b) Second merogonous generation

The second merogonous stages detected in the lambs from 21-34 dpi were found within the endothelial cells of capillaries in all host organs and tissues except those of the reproductive systems. Morphological stages presumed to be those of *Sarcocystis spp.* have previously been detected at

similar times of infection in other animals infected with *Sarcocystis sp.* obtained from dogs. Stages have been found within, or in close proximity to, capillary endothelia in experimentally-infected lambs at 24 & 25 dpi (Gestrich *et al.* 1974, 1975, Munday *et al.* 1975) and also in experimentally-infected calves between 26 & 33 dpi (Fayer & Johnson 1973, 1974, Mehlhorn *et al.* 1975c, Johnson *et al.* 1975). However, none of this reported research had rigidly precluded the possibility of previous host infection by *Sarcocystis spp.* nor concomitant infections by other genera of Sporozoa; for the experimental animals used were not proven to be 'Sporozoa-free' as were those used in this study. This thesis therefore presents the first conclusive evidence that these morphological stages found within the host tissues are actually developmental stages of species of *Sarcocystis*.

Some differences in the sites of infection within the hosts were observed between the previous reports and those made in this study. No second stage meronts were detected within the reproductive systems of the experimentally-infected lambs.* However, similar stages had been found within the testes of experimentally-infected calves (Fayer & Johnson 1974). It is important that future research determine whether this merogonous generation of the parasite does involve the reproductive organs to any great extent for this may influence the potential reproductivity of the host. This is further discussed in Section 33.2. The other

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* Subsequent research performed within this laboratory has detected second stage meronts within the endothelial cells of capillaries in the uterine submucosa and the ovarian stroma of an experimentally-infected lamb at 25 dpi (Phillips 1978 pers. comm.).

sites of infection by the second merogonous stages detected within the lambs included all other previously reported sites of infection found in the other experimental animals. Some meronts were also found in previously unreported sites of infection; namely, within the capillary endothelial cells in the rumen, reticulum, abomasum, colon and also in the pituitary gland. Furthermore, previous research has not detected merogonous stages so widely distributed throughout the tissues of individual animals as was found within this study.

The second merogonous stages detected from 21-23 dpi were homogeneous in appearance and not differentiated into discrete internal elements. Those detected from 25-34 dpi had undergone differentiation and contained numerous distinct zoites. Using the terminology proposed by Levine (1971), these zoites are also known as merozoites having arisen by the divisional process of merogony. The second stage meronts therefore represent the second asexual multiplicative generation of the parasite following infection. Certain observations made on these stages were useful in suggesting the type of asexual division occurring within them (i.e., fission or endogeny)*. No marked invaginations were observed in the membrane around the periphery of the meronts; which is usually suggestive of division by fission. Furthermore, the palisade- and rosette-like arrangements of the merozoites

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* Fission may be defined as the formation of daughter cells by the splitting of the maternal cell into two equal parts (binary fission) or more than two equal parts (multiple fission). Endogeny may be defined as the formation of daughter cells by internal budding within the maternal cell producing two individuals (endodyogeny) or more than two individuals (endoploygeny).

occasionally observed within the meronts was suggestive of their division by endogeny; by analogy with the division observed for similar life cycle stages of *Toxoplasma gondii* (Wildfuhr 1966, Sheffield & Melton 1968). Meronts found within experimentally-infected calves at similar times of infection have previously been suggested as dividing by repeated endodyogeny (Mehlhorn *et al.* 1975c) and also by endopolygeny (Pacheco & Fayer 1977). Further ultrastructural studies have to be performed and critically interpreted before the actual divisional process can be determined for this asexual multiplicative generation of the parasite.

Quite large variations were observed in the size of the merozoites found within the various experimental animals at this time after infection. The second generation merozoites detected within the experimental lambs had the mean dimensions of 1.6 µm in length by 0.5 µm in width; which is similar to the merozoite sizes measured from the previous report of such stages in lambs (Gestrich *et al.* 1974). The size of the merozoites detected in calves infected with *Sarcocystis sp.* obtained from dogs were reported to range from 5.4-8.0 µm in length by 2.0-3.0 µm in width (Fayer & Johnson 1974, Gestrich *et al.* 1975, Mehlhorn *et al.* 1975c). This variation in merozoite size was too large and consistent to be accounted for by preparation artefacts and it therefore must have some biological significance the exact nature of which is unknown as yet. Whether it simply reflects a size variation between the species of *Sarcocystis* examined (as seemingly apparent from the above research) has yet to be conclusively determined.

The infection of lambs by the second merogonous stages of the parasite achieved a peak in intensity at 27 dpi. This peak was relatively high in comparison to that detected for the other merogonous stages indicating that this second merogonous generation was very prolific. Tentative comparisons may be made between the intensity values observed for the first and second stage meronts to gain a general indication of the success of sequential infection. Each first stage meront produced between 18 & 28 merozoites (average number of 24) which were most probably released into the blood stream of the host. If each merozoite was successful in establishing a second stage meront, an approximate 24-fold increase in the intensity of infection would have been observed for the successive merogonous generation. However, only an approximate 7-fold increase in intensity was observed. This does not necessarily reflect poor success on the part of the parasite in the establishment of its next asexual multiplicative stage. Rather, it is more suggestive of a phenomenon common to many parasites; that of excessive proliferation to ensure their survival (cf. Noble & Noble 1971). Some first generation merozoites may not have been successful in the maintenance of infection because they encountered unfavourable conditions or locations which were not conducive to their further development. Whether certain host immune responses contributed to this restriction of successful parasitism is not positively known although a host antibody response to infection (detected by the CFT) was observed to be at its height at this time of infection. Much more exacting immunological techniques have to be employed to determine the complex interactions between host and parasite.

No second stage meronts were detected in lambs after 34 dpi and those found from 28-34 dpi were mature in appearance in that they contained numerous distinct merozoites. These meronts also distended the host endothelial cells such that they bulged into the lumena of the capillaries, and in some cases to the apparent occlusion of the vessel. It appears that the second generation merozoites are also released into the host circulation once having attained maturity. Unfortunately, no free merozoites were detected within peripheral blood samples taken from the lambs at this time. However, several zoite stages have previously been detected within blood smears taken from an experimentally-infected calf at 29 dpi (Gestrich *et al.* 1975). These stages were certainly not cystozoites presumably derived from sarcocysts accidentally ruptured during sampling; as has been occasionally reported beforehand (Keymer 1963). Furthermore, only merogonous stages (and no sarcocysts) were detected within the calf and their contained merozoites were similar in dimensions and appearance to those detected within the blood. This single report of such stages in blood could be considered as experimental proof of the route of infection by the merozoites between successive generations but it must be subject to further confirmation through more critical studies. The actual mode of merozoite transport within the blood is not known nor is it known whether such stages are transmissible or infective to other hosts. Such a finding may account for the apparent transmission of *Sarcocystis sp.* infection from human blood to mice and spermophiles (Sibalic 1975), but without working with proven SPF animals, nothing conclusive can be determined.

Although the Indirect Fluorescent-antibody staining technique did not reveal any second generation merozoites in blood samples taken from the infected lambs, it did elicit fluorescent reactive sites within the tissues which could be demonstrated to be second stage meronts of the parasite. This merogonous generation therefore does exhibit detectable antigenicity and react with the immunoglobulin groups used in the fluorescent-labelled stain. Because total immunoglobulins were used in the stain, it is not known whether this antigenicity relates to any specific immunoglobulin class. These stages of the parasite must also have acted as an antigen for host antibody responses to infection (detected by the CFT and the IFAT) were observed at this time. Further research must be performed to determine the immunological specificity of these antigen-antibody reactions.

Certain histochemical stains were found to exhibit a specific staining affinity for discrete elements within the three merogonous stages of the parasite. The alkaline-Giemsa colophonium stain appeared to stain only the merozoites deep purple in colour. Although various cytoplasmic organelles have varying affinities for different components of the dye stuff (methylene blue and its lower metachromatic homologues azure A or B, plus eosin), it was not possible at the light microscopic level to determine whether the staining observed was restricted to specific merozoite organelles. The Periodic acid Sciff's (PAS) reagent was observed to stain relatively discrete granules within the meronts. This stain is specific for certain molecular groups (i.e., neutral mucoploysaccharides, muco- and glyco-proteins, sphingo- and glyco-lipids and glycogen) in that the aldehyde groups created by periodic acid oxidation stain

red with Schiff's reagent. PAS positive granules were readily observed within the meronts found in frozen tissue sections stained with PAS reagent. They were also present within meronts detected in fixed tissue sections even though many of the lipids and most of the glycogen was leached out of the sections during processing (i.e., during dehydration in alcohol and clearing in chloroform). Although this observation narrows the specificity of the PAS staining reaction, it is not known as yet which of the other molecular groups composed the PAS positive granules within the meronts. The basic fuchsin counter-stain employed in the Gram's stain appeared to be selective for the nuclei of the merozoites as only they stained red. The aldehyde and sulphuric acid groups of specific proteins and mucopolysaccharides stain with basic fuchsin; and in this instance, the highly sulphated polynucleotides forming the nuclei of the merozoites had stained; i.e., the DNA (deoxy-ribonucleic acid).

It is important that further histochemical studies be performed and categorized for the various developmental stages of *Sarcocystis spp.*, as well as for the other closely-related genera of Sporozoa which parasitize the tissues of the host. In this manner, some histochemical criteria may be found to aid in the differential diagnosis of experimentally- and naturally-occurring infections by sporozoan parasites within the various animal hosts. In fact, some of the previous reports in the literature on tissue Sporozoa may possibly have been infections by *Sarcocystis spp.* rather than those to which they had been attributed by the authors (e.g., *Toxoplasma gondii*). This particularly appears to be the case with what had been termed 'Dalmeny disease' in cattle; presumed to be caused by

an unidentified protozoan (Corner *et al.* 1963, Lainson 1972). These unidentified organisms were described as parasitizing the vascular endothelial cells in various tissues taken from cattle which exhibited severe gross and clinical signs of disease. They were also very similar in appearance to the unidentified organisms found within the central nervous systems of some sheep (Hartley & Blakemore 1973, McErlean 1974). These organisms found in the cattle with 'Dalmeny disease' and also in the sheep were identical in size and appearance to the second stage meronts of the *Sarcocystis sp.* detected within the tissues of the lambs examined in this study. They were also in similar sites of infection within the tissues. It is therefore thought that these unidentified organisms were actually those of *Sarcocystis spp.* and that the syndrome termed 'Dalmeny disease' was actually associated with such infection.

(c) Third merogonous generation (?)

Third stage meronts were detected within the endothelial cells of hepatic sinusoids and lymph node capillaries in the two lambs examined at 36 dpi. These meronts were morphologically different from the preceding merogonous stages; being more restricted in location, smaller in size and containing fewer zoites which were larger in size and different in appearance and shape. No similar morphological stages have previously been found within the tissues of any animals examined for experimental or naturally-occurring infections. The report of their occurrence given in this thesis therefore represents the first such report that this stage of development occurs for a species of *Sarcocystis*. However, the biological significance of this merogonous stage of the parasite is difficult to determine due to their limited sites of infection, their

occurrence at a specific time after infection and their very low intensity of infection.

No undifferentiated third stage meronts were detected within the tissues of the lambs prior to 36 dpi; and at this time, only differentiated meronts were found which contained several distinct zoites. As for the preceding two merogonous generations of the parasite, the third stage meronts represent an asexual multiplicative stage of the parasite where merozoites were formed by the process of merogony. Unfortunately, the exact type of asexual division occurring within these meronts cannot be conclusively determined without performing ultrastructural studies to ascertain the mechanism of merozoite formation. The intensity of infection of the lambs by the third stage meronts was very low in comparison to that detected for the first, and particularly, the second stage meronts. It seems anomalous that so few third stage meronts were formed from the many merozoites produced by the prolific second merogonous generation (average number of 30 merozoites per meront). Therefore, whether these stages actually represent a discrete proliferative generation of the parasite essential for their continued development or were merely stages of the parasite undergoing division within extra-muscular locations and not strictly essential for their continued development is not positively known as yet. The significance of the occurrence of these third stage meronts at this time can only be speculative at present, but the two logical alternatives are as follows.

i) Discrete parasitic generation.

The third stage meronts may be an extremely proliferative generation of the parasite which is essential for the development of the sarcocyst stages within the musculature. Such a generation may have been extremely labile and undergone rapid division resulting in a high turnover of the meronts. This could explain why so few meronts were detected and also why they appearred to occur only at a certain time after infection. However, if this was the situation, it is not known why these meronts were restricted to specific locations within the tissues of the host when the preceding two merogonous generations were much more widespread throughout the host tissues. Nor it is known how the third generation merozoites could subsequently affect entry into the entire striated musculature from these specific locations.

ii) Uncommon parasitic stages.

The meronts detected in the lambs at 36 dpi may have been stages of the parasite which were not strictly essential for the development of the sarcocysts within the musculature. They may have arisen from second generation merozoites being fortuitously present within extramuscular locations which were conducive to their further merogonous division but not to sarcocyst development. These uncommon stages may have been solicited by changes in the host's physiology at this time; for the various host responses to infection observed within the lambs were quite marked at this stage of infection. If this was the case, it could explain why so few stages were detected, why they only occurred within specific locations and also why they were only detected at a certain time after infection.

However, without performing precise transmission studies orientated towards the time of infection at which these merogonous stages were found, very little can be conclusively determined about the actual biological significance of this stage of the parasite. For the moment, the latter alternative appears to be the more feasible as it suggests that the developing sarcocysts within the musculature were derived from the second generation merozoites associated with the microcirculation of the host. Such speculation appears quite logical when comparing the distribution and intensity of the second stage meronts within the lambs with those of the developing sarcocyst stages. This is further discussed in Section 32.3 for the development of the sarcocysts.

The third stage meronts were not considered to represent a resting or dormant form of the parasite; as has been postulated for certain stages of another sporozoan parasite. It was recently reported that the oocysts of some *Isospora spp.* obtained from dogs and cats gave rise to single intracellular zoites within various extra-intestinal tissues (e.g. lymphoid tissues) when fed to mice (Frenkel 1974) and also to chickens (Markus 1975). In some instances, such stages apparently persisted for up to one year and they were tentatively termed 'dormozoites' (Markus 1976). From these few reports, it was postulated that such stages may be linked to the subsequent relapse of infection which is observed for some sporozoan parasites. However, the third stage meronts found for the species of *Sarcocystis* examined in this study did not appear to be at all persistent nor dormant. They were only detected at a certain time after infection and they were definitely undergoing merogonous division and forming several distinct merozoites.

32.3 SARCOCYST DEVELOPMENT

The developing sarcocyst stages of the parasite detected within the striated muscle fibres of lambs between 41 & 134 dpi exhibited three distinct morphological stages in their development. From the time of their detection within the musculature, these stages represented the initial invasion of the individual muscle fibres by the parasite, the rudimentary division of such intracellular stages and lastly, the further division and growth of the characteristic sarcocysts. Various observations made on the appearance and intensity of these three different stages are discussed below.

(a) Invading stages

Parasitic stages were first observed within individual muscle fibres of a lamb at 41 dpi. These stages were centrally located within the muscle fibres, ovoid to elongate in shape and appearred as homogeneous basophilic bodies which did not contain any discernible internal elements. These bodies are considered to represent single primary parasitic cells which had successfully invaded the musculature of the host. No other stages of the parasite (i.e., meronts or characteristic sarcocysts) were detected within any other host tissues at this time of infection; nor at 45 dpi.

Developing sarcocyst stages have formerly been described within the musculature of experimentally-infected sheep examined at 41 & 42 dpi (Mehlhorn *et al.* 1975d, Munday *et al.* 1975). However, such stages were morphologically different from those detected within the lambs examined in this study at this time of infection in that the former appearred to be more mature. They were described as being ovoid in shape and containing distinguishable cells as well as distinct zoites. Such cells, as found and described in larger and older sarco-

cysts, have been termed metrocytes (Sènaud 1967, Mehlhorn & Scholtyseck 1973) and under the terminology proposed by Hoare (1972), the zoites are known as cystozoites (cf. General Introduction). These differences in the morphology of the parasitic stages found at similar times after infection may indicate that the sequential development of the parasite is not so chronologically discrete as it appears. However, it must be noted that this study utilized many more lambs which were proven to be 'Sporozoa-free' prior to their experimental infection whereas the previous studies were performed on fewer animals which were not proven to be 'Sporozoa-free'. Variations in the sequential development of this species of the parasite may have also been influenced by the environmental physiological and immunological parameters pertaining to the experimental animals being different between the various studies.

The invading stages of the parasite detected in the lambs examined in this study also differred from those found in experimentally-infected calves examined between 40 & 48 dpi (Fayer & Johnson 1974, Gestrich *et al.* 1975). The stages found in the calves exhibited a distinct thin cyst wall whereas those detected within the lambs did not contain a discernible cyst wall at this stage of their development. In these instances, such morphological differences could simply reflect variable structural characteristics between the developmental stages of the different species of *Sarcocystis* examined in the studies.

The actual route and mode of penetration of the parasite into the striated muscle fibres at, or prior to, this time of infection is not known as yet. At the light microscopic level, there appearred to be no evidence of any cellular membrane

damage or of any cytoplasmic organelle disorganization within any of the parasitised muscle fiblres. However, ultrastructural studies will have to be critically performed to provide the necessary information to determine the mechanism of muscle fibre invasion by the parasite as well as to determine the effects that these parasitic stages have on the fine structure of the host tissues.

The intensity of infection by the invading stages of the parasite within the musculature of the lambs was relatively high in comparison to that detected for any of the preceding merogonous generations (i.e., 2-14 times higher). These differences in intensity were most probably even greater for some of the invading stages in the musculature may not have been detected or recognized due to their very small size and their weak staining affinity. The significance of these intensity differences are discussed later in this Section.

(b) Dividing stages

Intracellular parasitic stages which appearred to have undergone division were detected within the striated muscle fibres of lambs examined at 50 & 55 dpi. These stages were very elongate in shape and contained discrete internal bodies in the form of numerous spherical to ovoid shaped cells. These cells lacked any resemblance to recognizable zoite structure and they contained diffuse basophilic elements which suggested that their nuclei were undergoing division. The cells were also very similar in appearance to the metrocytes which had previously been described within larger and more mature sarcocysts (Sènaud 1967, Mehlhorn & Scholtyseck 1973). The parasitic stages detected within the host muscul-

ature at this time of infection are therefore considered to represent developing sarcocysts containing numerous metrocytes which are undergoing rudimentary division prior to the occurrence of zoite formation.

This rudimentary division of the metrocytes was asexual in nature as there was no evidence of gamete formation which is indicative of sexual division. Unfortunately, the exact type of asexual division occurring within the metrocytes could not be determined from these light microscopic studies. From electron microscopic studies on similar parasitic stages found in experimentally-infected calves at 48 & 62 dpi (Heydorn *et al.* 1975a, 1975b), it was suggested that metrocytes divide by the process of endodyogeny thereby forming two daughter cells within each metrocyte. However, although such division certainly appears to be endogenous in nature, further ultrastructural studies must be performed to confirm whether it is endodyogeny and not endopolygeny. Furthermore, it must be ascertained whether the metrocyte division occurring within the stages found in lambs at this time of infection is of a similar nature.

Differences in morphology were also observed at this time of infection between the parasitic stages detected in the lambs examined in this study and those described in other experimental animals employed in previous studies. Developing sarcocysts found in the musculature of an experimentally-infected sheep at 60 dpi were reported to contain ovoid young zoites (Munday *et al.* 1975) and those detected within experimentally-infected calves examined at 48 & 62 dpi were described as containing distinct zoites as well as dividing metrocytes (Heydorn *et al.* 1975a, 1975b). Furthermore, stages

found in other experimentally-infected calves between 40 & 54 dpi exhibited a distinct cyst wall (Fayer & Johnson 1974, Gestrich *et al.* 1975). The dividing stages found in the lambs examined in this study at 50 & 55 dpi did not contain zoites and were not bounded by a distinct cyst wall. Such differences in morphology could reflect differences between the various species of the parasite examined as well as variations in their development due to the different physiological and immunological make-ups of the various hosts employed. However, much more research must be carried out to determine exactly why these differences in the morphology of the parasite occurred between the various infection studies.

The intensity of infection of the lambs by the dividing stages of the parasite was approximately 2-5 times greater than that found for the invading stages. This increase in intensity may simply have resulted from an increased level of detection of the parasitic stages due to their larger size and deeper basophilic-staining affinity. Further discussion on the intensity of sarcocyst infection is given later in this Section.

(c) Characteristic sarcocysts

The developing sarcocysts detected within the musculature of lambs between 65 & 134 dpi were very characteristic in appearance. They were bounded by a distinct cyst wall and contained numerous cystozoites as well as some metrocytes. The morphology of muscle sarcocysts has been intensively studied quite recently and such stages have been described quite comprehensively in other experimentally-infected sheep examined at 63, 81 & 104 dpi (Mehlhorn *et al.* 1975d, Munday

et al. 1975, Heydorn & Gestrich 1976) as well as in experimentally-infected calves examined at 76 & 150 dpi (Mehlhorn *et al.* 1975b, 1975c). However, various observations made on the different morphological characteristics of the sarcocysts are discussed below.

i) Cyst wall

Between 65 & 134 dpi, a distinct cyst wall was observed around all of the sarcocysts detected within the musculature of the lambs examined in this study. Approximately 80% of the sarcocysts had a very definite light microscopic appearance; which consisted of a thick primary cyst wall with pronounced radial striations. The remainder of the sarcocysts appearred to be bounded by a thin primary cyst wall which did not have apparent radial striations. It must be remembered that all of these sarcocysts belong to the species *Sarcocystis tenella* (junior synonym *S. ovicanis*) for this was the only parasite species introduced into the SPF lambs.

None of the sarcocyst walls detected within the lambs resembled any of those previously reported within calves which had been infected with *Sarcocystis spp.* sporocysts obtained from dogs, cats or humans (reviewed by Mehlhorn *et al.* 1976). However, thick radially-striated cyst walls have previously been reported for *S. tenella* sarcocysts in sheep (Mehlhorn *et al.* 1975d) and ultrastructural studies have revealed that the striations result from regularly-folded 'palisade-like' protusions of the primary cyst wall (Mehlhorn *et al.* 1976). These appearances under the light and electron microscopes are now generally considered to be quite characteristic for this species

of the parasite in sheep. It was also recently noticed that these protusions occasionally lie flat against the surface of the sarcocyst (Hartley 1977 pers. comm.); thereby giving the light microscopic appearance of a thin non-striated cyst wall; as was observed for some of the sarcocysts detected within the experimentally-infected lambs. This appearance is similar to that of the microscopic sarcocysts which are thought to develop into the macroscopic sarcocysts of the ovine species *Sarcocystis gigantea* (junior synonym *S. ovifelis*) (Bergman & Kinder 1975). However, the ultrastructure of the walls of these sarcocysts was vastly different; for the primary cyst walls contained numerous 'cauliflower-like' protusions (Mehlhorn *et al.* 1976). This means that although the cyst walls of *S. tenella* sarcocysts are ultrastructurally characteristic, no absolute definitive criteria can be employed at the light microscopic level to differentiate between cysts of the different species of the parasite in sheep due to their occasionally identical appearances.

ii) Metrocytes

Several cellular bodies (or metrocytes) were usually found around the internal periphery of the sarcocysts detected within the lambs between 65 & 134 dpi. These cells appearred to be undergoing division for they did not have very discrete nuclei and only contained diffuse basophilic elements. Dividing metrocytes have previously been reported within developing sarcocysts in sheep at similar times after experimental infection (Mehlhorn *et al.* 1975d). Although this asexual division of the metrocytes

certainly appears to be endogenous in nature, the exact mode of such division cannot be determined without performing critical ultrastructural studies.

These residual metrocytes within the sarcocysts may represent the stages of the parasite which facilitate the continued development and persistence of the characteristic sarcocysts and their zoites. However, nothing is known about the many factors which must be involved in the development and maintenance of these sarcocysts. Whether there are limitations to the development of the sarcocysts is not known although some indications were gained from tentative comparisons between naturally-occurring and experimental infections. The microscopic sarcocysts detected within the slaughter sheep examined in the factorial-design survey (cf. Section II) appearred to have an upper limit to their size range; occurring at approximately 70µm in width. The largest sarcocyst observed within the experimentally-infected lambs was found at 134 dpi and measured 36µm in width. From these observations it seems that the microscopic sarcocysts of *S. tenella* are capable of achieving a size greater than half their apparent limit within 5 months of infection. However, it is not known whether the growth of the sarcocysts was uniform or variable during this time; nor what factors may have influenced such growth. Further infection studies must be carried out over longer time scales to determine the maintenance mechanisms of the parasitic cysts within the host tissues. In this manner, the significance of the residual metrocytes on the persistence of the sarcocysts may be determined.

iii) Cystozoites

The numerous zoites observed within each of the sarcocysts found in the lambs between 65 & 134 dpi were uniform in size, shape and appearance. They were very characteristic for such stages of the Sarcosporidia and under the terminology proposed by Hoare (1972), they are best known as cystozoites (cf. General Introduction). The cystozoites appear to be formed by asexual division of the metrocytes and such division was regarded as being merogonous in nature. The process of sarcocyst formation was therefore considered to be a form of extra-intestinal merogony where the cystozoites are formed by asexual merogonous division of the basic metrocytic cells. This means that the 'sarcocysts' and 'cystozoites' could alternatively be named 'meronts' and 'merozoites'. However, the former terminology is still more applicable to the characteristic tissue cyst stages of the parasite and it also distinguishes such stages from those of the preceding merogonous generations of the parasite occurring in association with the host vasculature. From the time of infection, the parasite therefore undergoes a series of asexual merogonous divisions within specific tissues of the intermediate host. The last group of such divisions are slightly more complicated that their predecessors in that metrocytes are firstly formed which then undergo repeated division before finally producing the characteristic cystozoites.

The actual mode of asexual division occurring in the formation of the cystozoites appearred to be by the process of endogeny rather than by fission. However,

exacting ultrastructural studies must be performed and critically interpreted to determine the actual mechanism of cystozoite formation. Early ultrastructural research on the muscle cysts of various *Sarcocystis spp.* suggested that the cystozoites were formed within the sarcocysts by binary fission (Sènaud 1967) whereas more recent studies suggested they were formed from the metrocytes by repeated endodyogeny (Heydorn *et al.* 1975a, 1975b, Mehlhorn *et al.* 1975a, 1975d). Upon critical examination of the accompanying electron micrographs, the recent studies certainly appeared to indicate endogenous division but the evidence in favour of division by endodyogeny and not by endopolygeny was not given convincingly. Division of tissue cyst stages by endodyogeny is regarded by some authors as quite characteristic for the tissue Sporozoa, and it has previously been reported for other genera; such as *Toxoplasma* (Frenkel 1974), *Besnoitia* (Sènaud *et al.* 1974) and *Frenkelia* (Scholtyseck *et al.* 1973). However, the actual divisional processes of endogeny must be more completely understood and much more research must be performed on the many developmental stages of all the tissue Sporozoa before any broad generalizations can be made.

The intensity of infection of the lambs by the characteristic sarcocysts was observed to achieve an extremely high peak at 65 dpi. This peak in intensity was approximately 55 times greater than that observed for the second merogonous generation of the parasite and approximately 400 times greater than that found for the third stage meronts. Various observations made on these different stages of the parasite

tend to suggest that the intramuscular sarcocysts become established directly from the second merogonous generation of the parasite rather than from the third stage meronts. Some discrepancies between the intensities of infection by the various stages would be expected due to the lower levels of detection of the small meront stages as opposed to that of the much larger and more recognisable sarcocyst stages. However, the large increases in the intensity of infection from the meront stages to the sarcocyst stages are best accounted for by the apparently greater proliferation of the second stage meronts. These meronts were very prevalent, widely distributed throughout the host tissues and each produced an average number of 30 merozoites; whereas the third stage meronts were fewer in number, restricted in their locations within the host and only produced an average of 8 merozoites. Such differences in the numbers, sites and proliferation of the meront stages suggest that the sarcocyst stages are formed from the second generation merozoites rather than from those of the third stage meronts. This tends to confirm the concept of the third stage meronts as being uncommon parasitic stages instead of being a true multiplicative generation of the parasite. However, such discussion can only be speculative for it is not known whether such stages were extremely labile and therefore representative of a rapid, but effective, proliferative generation. Much more research must be performed to enlighten this situation as well as to determine the actual mechanism of muscle fibre invasion by the relevant merozoite stages of the parasite.

The intensity of infection by the characteristic sarcocysts was observed to rapidly decline after 65 dpi and appearred to progress towards a relatively lower, but more stable, level of infection around 134 dpi. This decrease in intensity indicated that not all the invading and dividing sarcocyst stages were successful in their maintenance and development into characteristic sarcocysts. This implies that the intensity of microscopic sarcocyst infections is limited to an extent as some developing sarcocysts apparently disappear resulting in a reduction in intensity. The disappearance of some developing cysts may have resulted from either their regression or their destruction. Some cysts may have regressed due to the maintenance of a biological equilibrium on the part of the parasite; e.g., as may occur in a 'crowding effect' similar to that observed for some helminths (cf. Ford 1967 p.324). Alternatively, some cysts may have been destroyed as a result of certain responses to infection on the part of the host. Some host responses observed in the lambs at this time of infection were quite marked and could be indicative of a host-parasite interaction which was partially detrimental to the continued development of the parasite (discussed further in Section 33.0). However, further research must be carried out to determine whether microscopic sarcocyst infections are limited in their intensity to a great degree and also to determine the actual mechanism of such limitation.

Some tentative comparisons may be made between the intensities of sarcocyst infections found in the experimentally-infected lambs and those detected in the slaughter sheep examined in the factorial-design survey. Such comparisons

cannot be made directly, but must be subject to data transformations in regard to their base units. The intensity values measured for the experimental lambs (i.e., number of stages per cc of all tissues examined) must be transformed so as to become compatible to the intensity values measured for the slaughter sheep (i.e., number of sarcocysts per cc of striated muscle only). The average intensity of infection found for the slaughter sheep was 2,410 cysts/cc muscle; whereas that transformed for the experimental lambs found to be infected with characteristic sarcocysts between 65 & 134 dpi was 1,020 cysts/cc muscle. This indicates that the experimental infections performed in this study could be regarded as being almost biological in intensity when compared to naturally-occurring infections. However, the upper limits for the intensities of infection were markedly different between the experimental and natural infections. The intensities found in the experimental lambs ranged from 150-2,260 cysts/cc muscle whereas those found in the slaughter sheep had a much greater range from 70-98,210 cysts/cc muscle. This suggests that the apparent limitation to the development of sarcocyst infections found in the experimental lambs was not as great as it appearred. However, it must be remembered that the experimental lambs were subjected to a single primary infection only; whereas the slaughter sheep must have been exposed to continuous or repeated challenge infections. Until more is known about the precise epidemiology and development of sarcocyst infections, comparisons between laboratory and field infections can only be made speculatively.

Microscopic sarcocysts were detected within the cerebrum and cerebellum of experimentally-infected lambs between 55 & 111 dpi. These sarcocysts did not have distinct cyst walls and their contained metrocytes, and in some cases cystozoites, were not as developed as their counterparts found within sarcocysts in the musculature. The sarcocysts also appearred to be in close association to the host vasculature within the nervous tissue. The occurrence of sarcocysts in these extramuscular locations were not considered to be experimental artefacts; but rather, to be just spasmodically-occurring sites of infection. Microscopic sarcocysts have previously been reported within the central nervous systems of field sheep (Hilgenfeld & Punke 1974, Hartley & Blakemore 1974) and also in another experimentally-infected sheep (Munday *et al.* 1975). The latter report also observed neuroglial aggregations around the sarcocysts which was suggestive of an immune cellular response by the host to infection. No nuclear aggregations were detected in association with any of the sarcocysts in the nervous tissue of the lambs examined in this study. However, further research must be performed to determine whether the low frequency of detection of sarcocysts within nervous tissue may be attributable to the host responses to infection in these locations or whether such locations are simply not conducive to the continued development of the sarcocysts.

33.0 HOST RESPONSES TO INFECTION

The various host responses observed during the development of the *Sarcocystis tenella* infections in the experimental lambs could be divided into those occurring during two distinct periods following infection. These periods are colloquially referred to as the acute and chronic phases of infection and they were closely related to the time intervals at which different developmental stages of the parasite were detected. The acute phase of infection occurred prior to 36 dpi during which time only the merogonous stages of the parasite were found. The chronic phase of infection was observed after 41 dpi when only the developing sarcocyst stages were detected within the lambs. Various observations made on the host antibody responses to infection, as determined by the various immunoserological tests employed, were very informative of such phases of infection; as were the observations made on the gross and haematological responses to infection.

33.1 HOST ANTIBODY RESPONSES

All of the colostrum-deprived SPF lambs were negative to the Complement Fixation Test (CFT) and the Indirect Fluorescent-antibody Test (IFAT) for *Sarcocystis spp.* prior to their experimental infection. However, the three lambs which had been allowed to suckle from their mothers to obtain natural colostrum (as per experimental design) were just positive to the CFT and the IFAT. These reactions were considered to result from low levels of maternally-derived antibodies being passed to the lambs in the colostrum. These antibodies were not very persistent and disappearred within 5-10 days of birth. The efficacy of these antibodies in preventing or even reducing the experimental infections appearred to be very poor because all three lambs became infected with relatively high levels of infection. However, at

this stage of our knowledge, much more critical studies have yet to be performed to determine the actual significance, let alone the efficacy, of the colostral antibodies in the transference of any immunity from parent to progeny.

All of the experimentally-infected lambs seroconverted to positive titres indicative of infection in the CFT and the IFAT at varying times after infection. Some of the lambs also exhibited very low titres to these tests prior to, or at, the time of experimental infection. These reactions were not indicative of infection by *Sarcocystis spp.* but were considered to have resulted from the presence of low levels of non-specific serum antibodies within some of the lambs. The specific serum antibodies which were formed by the lambs against infection by *S. tenella* are best discussed separately for each of the immunoserological tests employed; as follows.

(a) Complement Fixation Test

Experimental lambs seroconverted from negative (i.e., zero or non-significant) to positive titres in the CFT for *Sarcocystis spp.* between 9 & 15 dpi. The complement-fixing antibodies were then observed to peak in intensity at approximately 30 dpi and gradually disappear thereafter. These results represent the first quantitative reports on the sequential development of serum antibodies formed by the host against infection by a species of *Sarcocystis*. Previous immunoserological studies have only been qualitative in nature or have only been performed at isolated times after infection. Seroconversion in the CFT had previously been reported much later after infection in one experimentally-infected lamb; i.e., at 41 dpi (Munday & Corbould 1974);

whereas previous studies carried out in this laboratory on other 'Sporozoa-free' lambs (cf. Ford 1975) recorded CFT titres to experimental infection at 28 dpi which were similar to those found at this time in the lambs examined in this study.

The times of infection at which CFT antibodies were detected in the lambs corresponded very well with those times of infection at which the pre-sarcocyst stages of the parasite were found. Complement-fixing antibodies appearred during the time of infection by the first merogonous generation of the parasite, peaked in intensity at the time of infection by the second merogonous generation and then gradually disappearred thereafter when only developing sarcocyst stages were found. It appears that the CFT measures initial, short-lived antibodies formed by the lambs against infection by the merogonous stages of the parasite. It is not known which elements of these merogonous stages acted as antigens to have elicited the host antibody response detected by the CFT. Merogonous stages of the parasite have also recently been found to elicit an antibody response in infected calves as detected by the Indirect Haemagglutination Test (Frelier *et al.* 1977). Exacting immunological studies must be carried out to determine the specificity of the antigen-antibody reactions occurring between the parasite and the host at this time of infection.

The CFT antibody response exhibited by the experimental lambs appearred to be quite informative of the acute phase of infection found in the lambs at this time. Relatively high CFT antibody titres occurred at the time of infection when the other gross and haematological host responses observed

in the lambs were indicative of an acute phase of infection. This apparent relationship initially suggested that the CFT may be a good laboratory test to use for the clinical diagnosis of acute Sarcosporidial infections; such as could be employed in screening programmes on livestock. However, the CFT results were gained from examinations performed on experimental animals which were subjected to single primary infections only. These animals were not exposed to continuous or repeated challenge infections as must occur in the field. It is not yet known whether a CFT antibody response occurs to any large degree against these subsequent challenge infections; therefore, the CFT alone cannot be employed for the detection of acute phases of infection in field animals.

It was observed that the complement-fixing antibodies occurred at maximum intensity at approximately the same time after infection as the establishment of the developing sarcocysts. Unfortunately, it is not yet definitely known whether these two events were related. However, the cessation of multiplicative stages within the host vasculature system and the subsequent invasion of the muscle fibres by the parasite may be related to the presence of specific host antibodies. Tissue cyst formation by the parasite may have been induced when its asexual merogonous proliferation was retarded. The mechanism of such retardation could reflect a protective response by the parasite to antibody production by the host. A similar concept for the instigation of cyst formation has previously been tentatively postulated for another genus of the tissue Sporozoa; that of *Toxoplasma* (Matsubayashi & Akao 1963). However, this concept assumes that the humoral antibody response to infection by the host was solely and directly

responsible for the instigation of tissue cyst formation. At present, it is not even known whether the serum antibodies detected were protective in nature nor whether they were very active against the merogonous stages of the parasite. Many other factors may also directly or indirectly influence the parasite resulting in the onset of cyst formation. Further immunological studies must be performed to examine the complex interactions between host and parasite to determine their actual significance on the occurrence of cyst formation.

Some general indications on the specificity of the complement-fixing antibodies detected against *Sarcocystis sp.* may be gained from analogies made with another genus of the tissue Sporozoa. Intensive studies performed on *Toxoplasma* infections in humans (Braveny *et al.* 1974) and pigs (Shirahata *et al.* 1974) found that an initial antibody response to infection was due to the formation of antibodies in the IgM fraction of the immunoglobulins which were detectable by the CFT and the IgM-IFAT. Because *Toxoplasma* and *Sarcocystis* are closely related taxonomically and both undergo asexual merogonous division prior to tissue cyst formation, it is highly probable that the initial antibody response detected by the CFT for *Sarcocystis spp.* resulted from the formation of IgM (macroglobulin 19S) antibodies. This would be very similar to that known for many bacterial, viral and protozoan infections in animals which produce IgM antibodies in the early stages of infection (cf. Weir 1973). Preliminary research performed in this laboratory has indicated that the host serum antibodies detected by the CFT at this time of infection are predominantly IgM antibodies (O'Donoghue 1977 unpublished data); but these observations must be subject to further confirmation.

(b) Indirect Fluorescent-antibody Test

Experimentally-infected lambs seroconverted to positive titres in the IFAT for *Sarcocystis spp.* between 21 & 28 dpi. The intensity of the IFAT antibody response then steadily increased until approximately 75 dpi, became relatively stable at this time and was maintained at a plateau level until the last examination at 134 dpi. These results represent the first quantitative reports on the sequential development of serum antibodies detectable by the IFAT which were formed by a host against a species of *Sarcocystis*. Previous studies utilizing the IFAT have been qualitative in nature and were performed predominantly on experimental animals which had been fed muscle sarcocyst stages of the parasite; i.e., on potential definitive hosts. Limited success was reported for the IFAT in the examination of such infections in cats, chimpanzees, humans and rats (Markus *et al.* 1974, Tadros *et al.* 1974, Aryeetey & Piekarski 1976). The IFAT has also been used with good success on some animals which had been fed sporocyst stages of the parasite; i.e., on potential intermediate hosts such as cattle and mice (Markus 1973, Wallace 1973). Other studies have shown the absence of a cross-reaction between the IFAT for *Sarcocystis* and that for *Toxoplasma* (Fulton & Voller 1964, Markus 1973, O'Donoghue 1976 unpublished data); thereby demonstrating the relative specificity of this immunoserological test.

The development of the IFAT antibody response in the lambs corresponded very closely to the times of infection by certain developmental stages of the parasite. Antibodies detected by the IFAT appearred at the time of infection by the second merogonous generation of the parasite. These

antibodies steadily increased to maximum intensity during the period of establishment and development of the muscle sarcocysts and was then maintained at this level for some time. It appears that the IFAT measures secondary, longer-lived antibodies formed by the lambs against the later stages of development of the parasite. This host antibody response must have been elicited by the second merogonous stages of the parasite and then been maintained by the developing sarcocyst stages. This suggests that common antigenic sites exist between these different developmental stages of *Sarcocystis*; which is similar to that found for the developmental stages of other sporozoan parasites (cf. Noble & Noble 1971). However, further immunological studies must be performed to determine which elements of these parasitic stages acted as antigens to have elicited and maintained the IFAT antibody response observed in the lambs.

The IFAT antibody response detected in the experimental lambs appearred to best reflect the chronic phase of infection. High IFAT antibody titres were found at the times of infection when other gross and haematological host responses indicated that most of the lambs had recovered from the acute phase of infection. These lambs did not exhibit any obvious gross or clinical signs of disease although they were chronically infected with developing sarcocyst stages of the parasite. The IFAT may therefore be a good laboratory test to use for the diagnosis of chronic Sarcosporidial infections. This observation appears to be confirmed by the results of the IFAT in detecting infections in the slaughter sheep examined in the factorial-design survey (cf. sero-epidemiological results discussed in Section 19.3). A significant positive correlation was found

between the prevalence of infections in the slaughter sheep as determined by the IFAT and by the microscopic examination of histological sections. This result demonstrates that the IFAT can be used as a qualitative test to detect sarcocyst infections in field animals; i.e., those with chronic infections. However, it cannot be used as a quantitative test to indicate the actual status of infections within individual animals. Little is known about the longevity or persistence of the IFAT antibodies to infection and nothing is known about the IFAT antibody response to subsequent challenge infections. Until further research provides this necessary information, the direct application of the IFAT must remain restricted to the qualitative assessment of infections.

The intensity of the developing sarcocysts found within the host musculature was observed to rapidly decline at the time of infection when the IFAT antibody response reached maximum intensity. Unfortunately, it is not yet definitely known whether these two events were related. However, some tentative postulates may be made about the existence of a relationship between them. The disappearance of some developing sarcocysts at this time of infection may have been influenced by the presence of specific antibodies within the host; i.e., by a humoral immune response. It did not appear to be related to a cellular immune response as nuclear aggregations were only very rarely found in association with developing sarcocysts. The IFAT antibodies formed against infection may have interacted with some of the developing sarcocyst stages resulting in a direct or indirect inhibition to their further development. However, the subsequent disappearance of these stages, presumably by their regression or their destruction,

is difficult to envision without the occurrence of some sort of phagocytic cellular response by the host. Furthermore, the apparent persistence of the fully-developed sarcocysts within the host musculature could indicate that the IFAT antibodies were not very effective against these tissue cyst stages of the parasite. Such stages were bounded by a definite cyst wall which was relatively thick and radially-striated in appearance. This cyst wall may have acted as an effective barrier against the postulated antibody attack. However, such discussion must remain strictly speculative for it is not yet known whether the IFAT antibodies were even protective in nature nor whether they were very active against these developmental stages of the parasite. Very exacting immunological studies must be carried out to determine the actual host immune responses to infection and their influence on the parasite.

Comparisons made between the host antibody responses to infections by *Sarcocystis* and *Toxoplasma* provided some general indications on the molecular specificity of the IFAT antibodies. These comparisons between the two closely related genera of the tissue Sporozoa are quite valid as both parasites form tissue cysts after having undergone asexual merogonous division. Studies performed on *Toxoplasma* infections in pigs (Shirahata *et al.* 1974) and humans (Karim & Ludlam 1975) found that a secondary antibody response to infection, as detectable by the IFAT, resulted from the formation of antibodies in the IgG fraction of the immunoglobulins. It is therefore highly probable that the IFAT antibodies detected against *Sarcocystis* infections in the experimental lambs were actually IgG (globulin 7S) antibodies. Preliminary studies carried out in this laboratory have demonstrated that these antibodies were predominantly from the

IgG fraction of the immunoglobulins; as detected by the IgG-IFAT (O'Donoghue 1977 unpublished data). Further research must be undertaken to verify these preliminary findings as well as to examine the antibody responses to infection in other host animal species.

33.2 OTHER HOST RESPONSES

The host responses detected by gross and clinical examinations of lambs during the course of experimental infection could be allocated to two separate phases of infection. Symptoms indicative of an acute disease syndrome developed in lambs during the early stages of infection; i.e., during the period of parasite proliferation within the host vasculature system. Most lambs subsequently recovered from this disease syndrome during the later stages of infection; i.e., during the chronic phase of infection by the tissue cyst stages of the parasite within the host musculature. Although patho-physiology was not incorporated as part of this thesis *per se*, some general discussion is given below on various observations made during the acute and chronic phases of infection.

(a) Acute phase of infection

The disease syndrome observed in the experimentally-infected lambs occurred between 21 & 30 dpi. It was relatively acute and severe in nature and appearred to be characterized by the obvious gross symptoms of anorexia, weight loss, elevated body temperatures and a developing anaemia. A clinically-detected symptom was also indicative of a developing anaemia, in that the haematocrits of the infected lambs were very depressed at this time of infection. This acute disease syndrome proved to be lethal in some instances; thereby demonstrating that *Sarcocystis tenella* infections are more

important than previously thought as they can be pathogenic to the host during their development. This acute disease is best known as sarcocystosis; as proposed for a similar disease previously detected in experimentally-infected calves (Fayer & Johnson 1974).

Symptoms indicative of sarcocystosis were observed in the lambs at the time of infection when the second merogonous generation of the parasite was undergoing marked proliferation within capillary endothelial cells of the host. Considerable localised damage must occur to these capillaries when the second generation merozoites are released from the meronts within the parasitised cells. The gross and clinical host responses observed to infection at this time could indirectly result from such damage occurring to the host micro-circulatory system. Previous histopathological studies performed on experimentally-infected calves have indicated that localised damage to capillaries does occur in association with the proliferative merogonous stages of the parasite (Johnson *et al.* 1974, 1975). Haematological observations carried out on other experimentally-infected calves also suggested that an oligocythemic anaemia may be a characteristic clinical symptom of sarcocystosis (Mahrt & Fayer 1975). Comprehensive studies are presently underway within this laboratory to determine the exact patho-physiological changes occurring within experimentally-infected SPF lambs during this acute phase of infection (Ford & Phillips 1978 pers. comm.).

At present, cases of acute sarcocystosis have only been found in experimentally-infected lambs and none have been reported in lambs or sheep from the field. However, field reports have previously been made on what was described as

an outbreak of Dalmeny disease, probably sarcocystosis, in dairy cattle (Meads 1976) and a clinical outbreak of sarcocystosis in dairy calves (Frelier *et al.* 1977). The disease symptoms described in conjunction with these reports were very similar to those observed in the various experimentally-infected animals. It is therefore highly probable that cases of acute sarcocystosis naturally-occurring in field sheep have not been detected or diagnosed *per se*.

Sarcosporidial infections have also recently been linked to instances of spontaneous abortion in cattle. An acute illness, supposed to be sarcocystosis, was described as being responsible for the abortion observed in experimentally-infected, pregnant cows between 41 & 69 dpi although no parasitic stages were detected in any of the aborted foetuses (Fayer *et al.* 1976b). However, parasitic stages closely resembling the merogonous stages of *Sarcocystis* have recently been detected within the brains of two stillborn calves and also in the placentas of another four aborted foetuses (Munday & Black 1976). It appears that the parasite not only can produce severe disease in the initial stages of infection, but may also be passed transplacentally from parent-to-progeny and sometimes result in spontaneous abortion. This situation is very similar to that known for another genera of the tissue Sporozoa which can also be transmitted congenitally and sometimes cause abortion; i.e., *Toxoplasma* (cf. Frenkel 1973). Such being the case, Sarcosporidial infections are much more important than previously thought as they can not only be pathogenic to the host, but also appear to affect the reproductivity of the host. These infections must reduce the potential, and therefore the commercial, productivity of these domesticated host animals. The economic impact of such

reductions in productivity cannot be estimated as yet. Future research must be orientated towards providing information which may eventually be used for the control of these infections Preliminary studies on pharmaceutical control have indicated that the application of a coccidiostatic drug during the course of experimental infection in calves appearred to reduce the severity of disease; as determined by comparisons with untreated, infected controls (Fayer & Johnson 1975). However, much more research must firstly be performed to determine the actual effects the parasite has on the host before the efficacy of drug treatment can be positively ascertained.

It is important to remember that all previous research has been performed on experimental animals which were not proven to be 'Sporozoa-free'. This implies that the observations made on host responses could also be attributable to concomitant infections by other undetected organisms. The resulting interactions between host responses to these various infections would be extremely difficult to assign to individual organisms. Future research on the exact host responses to Sarcosporidial infections must perforce be carried out on proven 'Sporozoa-free' animals. Futhermore, the severity of the host responses to infection must be related to the level of infection; i.e., the number of infective sporocysts. At present, quite large variations in lethal doses have been found; ranging from 0.1-1.6 million sporocysts (Phillips 1978 pers. comm., Gestrich *et al*. 1974). Studies must be performed to determine the many factors which may have resulted in these apparent variations in the lethal dose required; such as percentage viability of inoculum, efficacy of inoculation, etc.

(b) Chronic phase of infection

The experimental lambs surviving the acute disease syndrome slowly recovered during the later stages of infection. These lambs were chronically infected with the tissue cyst (or muscle sarcocyst) stages of the parasite and no obvious gross or clinical host responses to such infection were detected. This period of infection is 'classically' known in the literature as Sarcosporidiosis.

Very little can be said about the host responses to infections by sarcocysts for very little is known. Although much research has been performed on these parasitic stages, almost no work has been carried out on the integrity of the infected host animals. A few previous reports made on suspected host reactions have described a myositis supposedly related to infection (cf. Freudenberg 1956, Reiten *et al.* 1966). However, the actual effects that such infections have on the various host animal species are not yet known.

In conclusion, it is apparent that much more research must be carried out on the many interactions occurring between the host and the parasite during the development of infections; particularly on the host responses to infection and their significance on the potential productivity of the host.

APPENDICES

The appendix: that oft but not useless appendage

Anonymous

APPENDIX I.

SUMMARY OF ANALYSES OF PARAMETERS

The results of the analyses of each parameter measured in the factorial-design survey are summarized in this appendix.

Analyses of variance (Tables 1 - 8)

The variances (s^2) of the main effects and the 2 X and 3 X interactions are given with their corresponding number of degrees of freedom (d.f.). All non-contributory variances (nc) have been pooled within the residual variance and are deleted from the summaries except for those found for main effects.

Chi-squared Test (Table 9)

The Chi-squared value (χ^2) is given for each main factor with the corresponding number of degrees of freedom (d.f.).

The levels of statistical significance are shown as follows:-

ns	=	$p>0.05$ (not significant)
*	=	$0.05>p>0.01$
**	=	$0.01>p>0.001$
***	=	$0.001>p$

APP. I
TABLE 1.

Prevalence of macroscopic sarcocysts in sheep

Mean = 0.403(equivalent to 6.71% infected)

Summary of Analysis of Variance

Source of Variation		d.f.	s^2	F-test sig.
MAIN EFFECTS				
Age	A	1	23.36	***
Sex	B		nc	
Breed	C	1	0.44	ns
Location	D	5	1.91	***
Season	E	2	1.51	**
INTERACTIONS				
2 X:	A x C	1	0.44	ns
	A x D	5	1.91	***
	A x E	2	1.51	**
	B x D	5	0.63	*
	C x E	2	0.47	ns
3 X:	A x B x D	5	0.63	*
	A x C x E	2	0.47	ns
	B x C x D	5	0.69	*
	B x C x E	2	0.55	ns
RESIDUAL		105	0.27	

APP. I
TABLE 2.

Prevalence of microscopic sarcocysts in sheep

Mean = 5.59 (equivalent to 93.17% infected)

Summary of Analysis of Variance

Source of variance		d.f.	s^2	F-test sig.
MAIN EFFECTS				
Age	A	1	4.34	**
Sex	B		nc	
Breed	C	1	5.06	**
Location	D	5	0.76	ns
Season	E	2	20.67	***
INTERACTIONS				
2 X:	A x B	1	0.84	ns
	A x D	5	4.26	***
	A x E	2	6.09	***
	B x C	1	0.84	ns
	C x E	2	3.56	***
	D x E	10	1.01	*
3 X:	A x B x E	2	0.63	ns
	A x C x D	5	1.89	**
	A x D x E	10	3.76	***
	B x C x E	2	0.63	ns
	C x D x E	10	0.63	ns
RESIDUAL		84	0.45	

APP.I
TABLE 3. Prevalence of CFT antibodies to *Sarcocystis* in sheep

Mean = 5.62 (equivalent to 93.74% positive)

Summary of Analysis of Variance

Source of Variation		d.f	s^2	F-test sig.
MAIN EFFECTS				
Age	A	1	2.51	**
Sex	B		nc	
Breed	C		nc	
Location	D	5	0.71	ns
Season	E	2	0.69	ns
INTERACTIONS				
2 X:	A x D	5	0.57	ns
	D x E	10	0.67	*
3 X:	A x D x E	10	0.54	ns
	B x C x D	5	0.71	ns
	B x C x E	2	0.53	ns
RESIDUAL		103	0.32	

APP. I
TABLE 4.

Prevalence of IFAT antibodies to *Sarcocystis* in sheep

Mean = 5.81 (equivalent to 96.88% positive)

Summary of Analysis of Variance

Source of Variation		d.f.	s^2	F-test sig.
MAIN EFFECTS				
Age	A	1	0.56	*
Sex	B		nc	
Breed	C		nc	
Location	D		nc	
Season	E	2	1.27	***
INTERACTIONS				
2 X:	A x D	5	0.28	*
	B x E	2	0.40	*
	C x D	5	0.21	ns
	C x E	2	0.51	ns
	D x E	10	0.19	ns
3 X:	A x B x D	5	0.22	ns
	A x C x D	5	0.25	*
	A x D x E	10	0.44	***
	B x D x E	10	0.15	ns
	C x D x E	10	0.15	ns
RESIDUAL		76	0.10	

APP. I
TABLE 5.

Intensity of sarcocyst infection in sheep

Mean = 22.92 (equivalent to 2.41 X 10^3 cysts/cc muscle)

Summary of Analysis of Variance

Source of Variation		d.f.	s^2	F-test sig.
MAIN EFFECTS				
Age	A	1	310.29	ns
Sex	B		nc	
Breed	C		nc	
Location	D	5	346.73	*
Season	E	2	4439.18	***
INTERACTIONS				
2 X:	A x B	1	251.75	ns
	A x C	1	957.39	*
	A x D	5	251.22	ns
	A x E	2	388.83	ns
	C x E	2	344.37	ns
	D x E	10	338.66	*
3 X:	A x B x C	1	232.77	ns
	A x C x D	5	244.43	ns
	A x C x E	2	643.78	*
	A x D x E	10	189.04	ns
	C x D x E	10	310.66	*
RESIDUAL		86	143.02	

APP. I
TABLE 6.

Intensity of CFT antibodies to *Sarcocystis* in sheep

Mean = 3.60 (approximately equal to a titre of 1:26)

Summary of Analysis of Variance

Source of variation		d.f.	s^2	T-test sig.
MAIN EFFECTS				
Age	A	1	230.23	***
Sex	B		nc	
Breed	C		nc	
Location	D	5	2.06	ns
Season	E	2	15.03	***
INTERACTIONS				
2 X:	A x C	1	1.67	ns
	A x D	5	9.76	***
	A x E	2	3.38	ns
	B x D	5	2.36	ns
	B x E	2	4.30	ns
	C x D	5	2.13	ns
	C x E	2	8.75	**
	D x E	10	3.06	*
3 X:	A x B x C	1	3.13	ns
	A x B x D	5	5.20	**
	A x C x D	5	6.86	***
	A x C x E	2	5.52	*
	A x D x E	10	7.57	***
	B x C x D	5	1.59	ns
	B x C x E	2	3.58	*
	B x D x E	10	1.95	ns
	C x D x E	10	3.50	*
RESIDUAL		773	1.54	

APP. I
TABLE 7.

Intensity of IFAT antibodies to *Sarcocystis* in sheep

Mean = 4.47 (approximately equal to a titre of 1:47)

Summary of Analysis of Variance

Source of variation		d.f.	s^2	F-test sig.
MAIN EFFECTS				
Age	A	1	325.12	***
Sex	B	1	1.19	ns
Breed	C		nc	
Location	D	5	2.82	*
Season	E	2	8.47	**
INTERACTIONS				
2 X:	A x B	1	2.89	ns
	A x C	1	2.89	ns
	A x D	5	2.09	ns
	A x E	2	3.85	*
	B x C	1	2.24	ns
	B x E	2	5.60	**
	D x E	10	1.58	ns
3 X:	A x B x D	5	1.42	ns
	A x B x E	2	4.53	*
	A x C x D	5	1.80	ns
	A x D x E	10	3.84	***
	B x C x D	5	1.26	ns
	C x D x E	10	1.57	ns
RESIDUAL		795	1.16	

APP. I

TABLE 8. Size of microscopic sarcocysts in sheep

Mean = 34.77 µm

Summary of Analysis of Variance

Source of Variation		d.f.	s^2	F-test sig.
MAIN EFFECTS				
Age	A	1	2246.60	***
Sex	B	1	34.46	*
Breed	C	1	17.13	ns
Location	D	5	16.88	ns
Season	E	2	323.79	***
INTERACTIONS				
2 X:	A x C	1	15.63	ns
	A x D	5	21.80	*
	A x E	2	139.41	***
	B x C	1	20.57	ns
	C x D	5	52.68	***
	C x E	2	49.75	**
	D x E	10	14.60	ns
3 X:	A x C x D	5	56.56	***
	A x C x E	2	46.94	**
	A x D x E	10	34.06	***
	B x D x E	10	11.34	ns
	C x D x E	10	55.62	***
RESIDUAL		70	8.58	

APP. I
TABLE 9.

Size of macroscopic sarcocysts in sheep

Mean = 956.74 μm

Summary of Chi-squared Analysis

Source of variation$^{\phi}$		χ^2	d.f.	χ^2 Sig.
MAIN EFFECTS				
Age	A	na		
Sex	B	0.03	1	ns
Breed	C	2.49	1	ns
Location	D	1.37	5	ns
Season	E	10.24	2	**

ϕ = The interactions of the factors are not presented in this summary for there were not enough animals infected with macroscopic sarcocysts for such comprehensive statistical analysis.

na = Analysis not applicable for only aged animals were infected.

APPENDIX II.

LIST OF MEANS OF 2 X INTERACTIONS OF FACTORS

The geometric means of the 2 X interactions of the factors are presented in this appendix for each parameter measured in the factorial-design survey (Tables 1 - 8).

The subdivisions of each factor are as follows:-

(A)	Age	Y = young sheep
		A = aged sheep
(B)	Sex	♀ = ewes
		♂ = wethers
(C)	Breed	M = Merino
		C = Corriedale
(D)	Location	SE= South East
		KI= Kangaroo Island
		LE= lower Eyre Peninsula
		MM= Murray Mallee
		ME= mid-Eyre Peninsula
		PZ= Pastoral Zone
(E)	Season	w = wet period
		m = moist period
		d = dry period

APP. II

TABLE 1. Prevalence of macroscopic sarcocysts in sheep

List of means for 2 X interactions

INTERACTION	$\bar{x}$ (%)	INTERACTION	$\bar{x}$ (%)	INTERACTION	$\bar{x}$ (%)
A x B: Y,♀	0	C x E: M,w	3.47	C x D: M,SE	2.78
A,♀	14.81	C,w	4.17	C,SE	4.17
Y,♂	0	M,m	6.94	M,KI	8.33
A,♂	12.04	C,m	6.25	C,KI	12.50
		M,d	6.94	M,LE	9.73
		C,d	12.50	C,LE	9.73
B x C:♀,M	6.94	A x D: Y,SE	0	M,MM	2.78
♂,M	4.63	A,SE	6.90	C,MM	2.78
♀,C	7.87	Y,KI	0	M,ME	9.72
♂,C	7.41	A,KI	20.82	C,ME	15.28
		Y,LE	0	M,PZ	1.39
		A,LE	19.47	C,PZ	1.39
C x A:M,Y	0	Y,MM	0	D x E: SE,w	2.08
C,Y	0	A,MM	5.62	SE,m	4.17
M,A	11.12	Y,ME	0	SE,d	4.17
C,A	15.74	A,ME	25.21	KI,w	6.25
		Y,PZ	0	KI,m	10.42
		A,PZ	2.82	KI,d	14.58
A xE: Y,w	0	B x D: ♀,SE	4.27	LE,w	6.25
A,w	7.64	♂,SE	2.83	LE,m	12.50
Y,m	0	♀,KI	11.12	LE,d	10.42
A,m	13.19	♂,KI	9.73	MM,w	0
Y,d	0	♀,LE	6.95	MM,m	2.08
A,d	19.44	♂,LE	12.50	MM,d	6.25
		♀,MM	2.78	ME,w	8.33
B x E:♀,w	4.17	♂,MM	2.78	ME,m	10.42
♂,w	3.47	♀,ME	18.06	ME,d	18.75
♀,m	8.33	♂,ME	6.95	PZ,w	0
♂,m	4.86	♀,PZ	1.39	PZ,m	0
♀,d	9.72	♂,PZ	1.39	PZ,d	4.17
♂,d	9.72				

APP. II

TABLE 2. Prevalence of microscopic sarcocysts in sheep

List of means for 2 X interactions

INTERACTION	$\bar{x}$ (%)
A x B: Y,♀	91.67
A,♀	94.91
Y,♂	88.89
A,♂	97.22
B x C: ♀,M	97.69
♂,M	94.91
♀,C	88.89
♂,C	91.20
C x A: M,Y	92.59
C,Y	87.96
M,A	100.00
C,A	92.13
A x E: Y,w	70.84
A,w	90.28
Y,m	100.00
A,m	97.92
Y,d	100.00
A,d	100.00
B x E: ♀,w	81.25
♂,w	79.87
♀,m	98.62
♂,m	99.31
♀,d	100.00
♂,d	100.00

INTERACTION	$\bar{x}$ (%)
C x E: M,w	88.89
C,w	72.23
M,m	100.00
C,m	97.92
M,d	100.00
C,d	100.00
A x D: Y,SE	81.95
A,SE	100.00
Y,KI	97.23
A,KI	100.00
Y,LE	100.00
A,LE	86.12
Y,MM	97.23
A,MM	90.28
Y,ME	80.56
A,ME	100.00
Y,PZ	84.73
A,PZ	100.00
B x D: ♀,SE	94.44
♂,SE	87.52
♀,KI	98.61
♂,KI	98.63
♀,LE	93.06
♂,LE	93.06
♀,MM	91.67
♂,MM	95.83
♀,ME	90.28
♂,ME	90.28
♀,PZ	91.67
♂,PZ	93.07

INTERACTION	$\bar{x}$ (%)
C x D: M,SE	93.06
C,SE	88.89
M,KI	98.61
C,KI	98.61
M,LE	100.00
C,LE	86.11
M,MM	97.22
C,MM	90.28
M,ME	94.44
C,ME	86.11
M,PZ	94.44
C,PZ	90.28
D x E: SE,w	72.92
SE,m	100.00
SE,d	100.00
KI,w	95.84
KI,m	100.00
KI,d	100.00
LE,w	79.17
LE,m	100.00
LE,d	100.00
MM,w	87.50
MM,m	93.72
MM,d	100.00
ME,w	70.84
ME,m	100.00
ME,d	100.00
PZ,w	77.09
PZ,m	100.00
PZ,d	100.00

APP. II
TABLE 3. Prevalence of CFT antibodies to *Sarcocystis* in sheep

List of means for 2 X interactions

INTERACTION	$\bar{x}$ (%)
A x B: Y,♀	96.76
A,♀	91.68
Y,♂	94.91
A,♂	91.21
B x C: ♀,M	94.44
♂,M	94.92
♀,C	93.98
♂,C	91.22
C x A: M,Y	96.30
C,Y	93.98
M,A	93.06
C,A	89.82
A x E: Y,w	93.75
A,w	88.89
Y,m	97.92
A,m	92.36
Y,d	95.83
A,d	93.06
B x E: ♀,w	90.28
♂,w	92.36
♀,m	96.53
♂,m	93.75
♀,d	95.83
♂,d	93.06

INTERACTION	$\bar{x}$ (%)
C x E: M,w	90.97
C,w	91.67
M,m	95.83
C,m	94.44
M,d	97.22
C,d	91.67
A x D: Y,SE	88.89
A,SE	93.07
Y,KI	98.61
A,KI	84.73
Y,LE	97.22
A,LE	91.68
Y,MM	97.22
A,MM	94.46
Y,ME	97.22
A,ME	91.68
Y,PZ	95.83
A,PZ	93.07
B x D: ♀,SE	90.28
♂,SE	91.68
♀,KI	93.06
♂,KI	90.28
♀,LE	95.83
♂,LE	93.07
♀,MM	95.83
♂,MM	95.85
♀,ME	93.06
♂,ME	95.84
♀,PZ	97.22
♂,PZ	91.68

INTERACTION	$\bar{x}$ (%)
C x D: M,SE	91.67
C,SE	90.29
M,KI	91.67
C,KI	91.67
M,LE	95.83
C,LE	93.07
M,MM	97.22
C,MM	94.46
M,ME	95.83
C,ME	93.07
M,PZ	95.83
C,PZ	93.07
D x E: SE,w	81.25
SE,m	95.83
SE,d	95.83
KI,w	93.75
KI,m	93.75
KI,d	95.83
LE,w	91.67
LE,m	97.92
LE,d	97.92
MM,w	91.67
MM,m	91.67
MM,d	91.67
ME,w	95.83
ME,m	97.92
ME,d	89.58
PZ,w	93.75
PZ,m	93.75
PZ,d	95.83

APP. II
TABLE 4 Prevalence of IFAT antibodies to *Sarcocystis* in sheep

List of means for 2 X interactions

INTERACTION	$\bar{x}$ (%)	INTERACTION	$\bar{x}$ (%)	INTERACTION	$\bar{x}$ (%)
A x B: Y,♀	96.30	C x E: M,w	95.14	C x D: M,SE	97.22
A,♀	98.16	C,w	92.36	C,SE	97.24
Y,♂	95.37	M,m	98.61	M,KI	97.22
A,♂	97.69	C,m	98.61	C,KI	98.62
		M,d	97.92	M,LE	97.22
		C,d	98.61	C,LE	94.46
B x C:♀,M	97.69	A x D: Y,SE	94.44	M,MM	98.61
♂,M	96.77	A,SE	100.00	C,MM	94.45
♀,C	96.76	Y,KI	98.61	M,ME	97.22
♂,C	96.30	A,KI	97.23	C,ME	94.46
		Y,LE	95.83	M,PZ	95.83
		A,LE	95.83	C,PZ	100.00
C x A:M,Y	95.83	Y,MM	95.83	D x E: SE,w	91.67
C,Y	95.85	A,MM	97.23	SE,m	100.00
M,A	98.61	Y,ME	93.06	SE,d	100.00
C,A	97.23	A,ME	98.62	KI,w	95.83
		Y,PZ	95.83	KI,m	100.00
		A,PZ	100.00	KI,d	97.92
A x E: Y,w	92.36	B x D: ♀,SE	97.22	LE,w	89.58
A,w	95.14	♂,SE	97.24	LE,m	97.92
Y,m	97.92	♀,KI	97.22	LE,d	100.00
A,m	99.31	♂,KI	98.62	MM,w	95.83
Y,d	97.22	♀,LE	97.22	MM,m	95.83
A,d	99.31	♂,LE	94.46	MM,d	97.92
		♀,MM	98.61	ME,w	91.67
B x E:♀,w	92.36	♂,MM	94.45	ME,m	100.00
♂,w	95.14	♀,ME	95.83	ME,d	95.83
♀,m	100.00	♂,ME	95.86	PZ,w	97.92
♂,m	97.22	♀,PZ	97.22	PZ,m	97.92
♀,d	99.31	♂,PZ	98.62	PZ,d	97.92
♂,d	97.22				

APP. II
TABLE 5 Intensity of sarcocyst infection in sheep

List of means for 2 X interactions

INTERACTION	$\bar{x}$ *
A x B: Y,♀	2.35
A,♀	2.37
Y,♂	2.16
A,♂	2.74
B x C: ♀,M	2.40
♂,M	2.46
♀,C	2.32
♂,C	2.44
C x A: M,Y	2.00
C,Y	2.51
M,A	2.86
C,A	2.26
A x E: Y,w	1.03
A,w	2.01
Y,m	2.21
A,m	2.17
Y,d	3.53
A,d	3.49
B x E: ♀,w	1.31
♂,w	1.73
♀,m	2.10
♂,m	2.28
♀,d	3.67
♂,d	3.35

INTERACTION	$\bar{x}$ *
C x E: M,w	1.45
C,w	1.59
M,m	1.99
C,m	2.39
M,d	3.85
C,d	3.17
A x D: Y,SE	1.92
A,SE	2.56
Y,KI	2.35
A,KI	2.64
Y,LE	2.66
A,LE	2.05
Y,MM	1.90
A,MM	1.65
Y,ME	2.31
A,ME	3.66
Y,PZ	2.39
A,PZ	2.78
B x D: ♀,SE	2.34
♂,SE	2.14
♀,KI	2.18
♂,KI	2.81
♀,LE	2.20
♂,LE	2.52
♀,MM	1.62
♂,MM	1.94
♀,ME	3.25
♂,ME	2.72
♀,PZ	2.58
♂,PZ	2.58

INTERACTION	$\bar{x}$ *
C x D: M,SE	2.46
C,SE	2.02
M,KI	2.42
C,KI	2.57
M,LE	1.96
C,LE	2.76
M,MM	1.88
C,MM	1.67
M,ME	2.94
C,ME	3.02
M,PZ	2.82
C,PZ	2.35
D x E: SE,w	1.22
SE,m	2.29
SE,d	3.21
KI,w	1.75
KI,m	2.65
KI,d	3.08
LE,w	1.97
LE,m	2.45
LE,d	2.67
MM,w	1.18
MM,m	1.72
MM,d	2.43
ME,w	1.54
ME,m	2.28
ME,d	5.13
PZ,w	1.45
PZ,m	1.76
PZ,d	4.55

* = $\bar{x}$ is expressed as no. sarcocysts/cc muscle ($X10^{-3}$)

APP. II

TABLE 6 Intensity of CFT antibodies to *Sarcocystis* in sheep

List of means for 2 X interactions

INTERACTION	$\bar{x}$ *
A x B: Y,♀	4.15
A,♀	3.07
Y,♂	4.04
A,♂	3.14
B x C: ♀,M	3.53
♂,M	3.59
♀,C	3.70
♂,C	3.62
C x A: M,Y	4.01
C,Y	4.17
M,A	3.09
C,A	3.15
A x E: Y,w	3,83
A,w	3.00
Y,m	4.46
A,m	3.25
Y,d	3.99
A,d	3.10
B x E: ♀,w	3.28
♂,w	3.54
♀,m	3.88
♂,m	3.83
♀,d	3.67
♂,d	3.42

INTERACTION	$\bar{x}$ *
C x E: M,w	3.35
C,w	3.48
M,m	3.65
C,m	4.08
M,d	3.67
C,d	3.42
A x D: Y,SE	3.76
A,SE	3.32
Y,KI	4.74
A,KI	2.92
Y,LE	3.89
A,LE	3.43
Y,MM	4.06
A,MM	2.88
Y,ME	3.99
A,ME	3.11
Y,PZ	4.13
A,PZ	3.03
B x D: ♀,SE	3,61
♂,SE	3.47
♀,KI	3.63
♂,KI	4.03
♀,LE	3.60
♂,LE	3.72
♀,MM	3.65
♂,MM	3.29
♀,ME	3.51
♂,ME	3.59
♀,PZ	3.68
♂,PZ	3.48

INTERACTION	$\bar{x}$ *
C x D: M,SE	3.60
C,SE	3.48
M,KI	3.79
C,KI	3.86
M,LE	3.43
C,LE	3.89
M,MM	3.50
C,MM	3.44
M,ME	3.42
C,ME	3.68
M,PZ	3.57
C,PZ	3.59
D x E: SE,w	2.94
SE,m	3.98
SE,d	3.71
KI,w	3.77
KI,m	3.90
KI,d	3.81
LE,w	3.44
LE,m	4.08
LE,d	3.46
MM,w	3.54
MM,m	3.63
MM,d	3.23
ME,w	3.52
ME,m	3.83
ME,d	3.29
PZ,w	3.27
PZ,m	3.71
PZ,d	3.77

* = $\bar{x}$ is expressed as transformed titre

APP. II
TABLE 7 Intensity of IFAT antibodies to *Sarcocystis* in sheep

List of means for 2 X interactions

INTERACTION	$\bar{x}$ *
A x B: Y,♀	3.87
A,♀	4.97
Y,♂	3.86
A,♂	5.16
B x C: ♀,M	4.50
♂,M	4.50
♀,C	4.35
♂,C	4.51
C x A: M,Y	3.85
C,Y	3.87
M,A	5.15
C,A	4.99
A x E: Y,w	3.80
A,w	4.76
Y,m	3.89
A,m	5.28
Y,d	3.90
A,d	5.16
B x E: ♀,w	4.11
♂,w	4.44
♀,m	4.52
♂,m	4.65
♀,d	4.63
♂,d	4.43

INTERACTION	$\bar{x}$ *
C x E: M,w	4.29
C,w	4.26
M,m	4.63
C,m	4.54
M,d	4.58
C,d	4.49
A x D: Y,SE	3.85
A,SE	5.43
Y,KI	3.83
A,KI	4.99
Y,LE	3.88
A,LE	4.64
Y,MM	3.83
A,MM	4.95
Y,ME	3.88
A,ME	5.28
Y,PZ	3.92
A,PZ	5.12
B x D: ♀,SE	4.63
♂,SE	4.65
♀,KI	4.38
♂,KI	4.44
♀,LE	4.21
♂,LE	4.31
♀,MM	4.42
♂,MM	4.36
♀,ME	4.60
♂,ME	4.56
♀,PZ	4.31
♂,PZ	4.73

INTERACTION	$\bar{x}$ *
C x D: M,SE	4.65
C,SE	4.63
M,KI	4.46
C,KI	4.36
M,LE	4.36
C,LE	4.16
M,MM	4.44
C,MM	4.34
M,ME	4.57
C,ME	4.59
M,PZ	4.51
C,PZ	4.53
D x E: SE,w	4.50
SE,m	4.69
SE,d	4.73
KI,w	4.35
KI,m	4.38
KI,d	4.50
LE,w	3.81
LE,m	4.65
LE,d	4.31
MM,w	4.27
MM,m	4.40
MM,d	4.50
ME,w	4.38
ME,m	4.81
ME,d	4.54
PZ,w	4.35
PZ,m	4.60
PZ,d	4.60

* = $\bar{x}$ is expressed as transformed titre

APP.II
TABLE 8 Size of microscopic sarcocysts in sheep

List of means for 2 X interactions

INTERACTION	$\bar{x}$ (µm)
A x B: Y,♀	30.49
A,♀	38.11
Y,♂	31.17
A,♂	39.37
B x C: ♀,M	34.26
♂,M	36.00
♀,C	34.33
♂,C	34.55
C x A: M,Y	30.84
C,Y	30.82
M,A	39.40
C,A	38.08
A x E: Y,w	32.62
A,w	42.93
Y,m	31.17
A,m	35.19
Y,d	28.70
A,d	38.09
B x E: ♀,w	37.08
♂,w	38.47
♀,m	33.11
♂,m	33.26
♀,d	32.70
♂,d	34.08

INTERACTION	$\bar{x}$ (µm)
C x E: M,w	39.25
C,w	36.30
M,m	33.24
C,m	33.12
M,d	32.89
C,d	33.90
A x D: Y,SE	32.33
A,SE	38.49
Y,KI	30.96
A,KI	40.22
Y,LE	30.95
A,LE	38.47
Y,MM	30.40
A,MM	36.36
Y,ME	28.83
A,ME	39.79
Y,PZ	31.49
A,PZ	39.07
B x D: ♀,SE	35.76
♂,SE	35.06
♀,KI	34.42
♂,KI	32.34
♀,LE	33.61
♂,LE	37.57
♀,MM	32.93
♂,MM	36.49
♀,ME	34.17
♂,ME	34.45
♀,PZ	34.85
♂,PZ	35.71

INTERACTION	$\bar{x}$ (µm)
C x D: M,SE	36.40
C,SE	34.42
M,KI	34.99
C,KI	36.19
M,LE	37.01
C,LE	32.41
M,MM	34.69
C,MM	32.07
M,ME	34.19
C,ME	34.43
M,PZ	33.46
C,PZ	37.10
D x E: SE,w	40.19
SE,m	33.39
SE,d	32.66
KI,w	39.03
KI,m	34.48
KI,d	33.27
LE,w	36.44
LE,m	33.40
LE,d	34.29
MM,w	35.59
MM,m	32.52
MM,d	32.02
ME,w	35.76
ME,m	32.97
ME,d	34.18
PZ,w	39.65
PZ,m	32.26
PZ,d	33.92

APPENDIX III

SUMMARY OF ANALYSES OF ECOLOGICAL FEATURES

The results of the analyses of the ecological features determined for each property involved in the survey are presented in this appendix. For ease of presentation, the ecological features are classified under 3 phases of the life cycle of the parasite; the 'prey', 'predator' and 'dust' phases.

Multiple Regression (Table 1)

The quantitative ecological features were analysed by multiple regression and the multiple correlation coefficient (r) is given for each analysis. The geometric mean ($\bar{x}$) of each feature is also presented.

Chi-squared Tests (Tables 2 - 4)

The qualitative ecological features were analysed by the Chi-squared Test and its value (χ^2) is shown for each analysis with the corresponding number of degrees of freedom (d.f.).

The levels of statistical significance are shown as follows:-

- = $p>0.05$ (not significant)

* = $0.05>p>0.01$

TABLE 1. Summary of regression analyses of quantitative ecological features.

CLASSIFICATION	FEATURE	$\bar{x}$	INTENSITY		SARCOCYST SIZE	
			r	sig.	r	sig.
'PREY'	Tot. no. sheep	2863.7	0.031	-	0.100	-
	No. Merinos	1975.8	0.034	-	0.075	-
	No. Corriedales	833.7	0.094	-	0.075	-
	No. lambs	499.0	0.040	-	0.098	-
	No. adults	2384.6	0.013	-	0.089	-
	No. rams	27.9	0.055	-	0.030	-
	No. ewes	2189.1	0.076	-	0.106	-
	No. wethers	745.4	0.024	-	0.068	-
	Percentage reproduction	81.8	0.085	-	0.001	-
	Reproduction index[1]	19.0	0.020	-	0.027	-
	No. times sheep yarded/year	7.1	0.074	-	0.026	-
	Tot. no. cattle	116.1	0.041	-	0.038	-
	No. times cattle yarded/year	3.3	0.066	-	0.017	-
	Max. diam. of property (miles)	3.6	0.017	-	0.016	-
'PREDATOR'	No. dogs	3.7	0.112	-	0.036	-
	No. neighbours dogs	8.1	0.030	-	0.143	-
	No. ration sheep killed/year	56.9	0.038	-	0.052	-
	No. farm cats	2.9	0.008	-	0.085	-
	Estimated no. foxes	10.9	0.061	-	0.081	-
	Estimated no. feral cats	9.0	0.002	-	0.081	-
'DUST'	Shade facilities[2]	1.4	0.017	-	0.058	-
	Capacity of sheep yards	1119.9	0.012	-	0.006	-
	Watering facilitites[3]	1.4	0.069	-	0.024	-
	Average annual rainfall (ins.)	19.0	0.041	-	0.024	-

[1] Reproduction index $= \frac{\text{No. lambs born}}{\text{Tot. no. sheep}} \times 100$

[2] Shade facilities = No. stands or groups of trees/paddock

[3] Watering facilities = No. troughs or dams/paddock

APP.III
TABLE 2. Summary of analysis of qualitative ecological features

'PREY' phase of life cycle

FEATURES	DIVISIONS	df	INTENSITY χ^2	sig.	CYST SIZE χ^2	sig.
Sheep type	Various breeds	2	0.25	-	0.40	-
Origin of sheep	Various birth-places	2	0.06	-	0.31	-
Lambing season	Various months	2	0.20	-	0.35	-
Cattle type	Various breeds	4	1.45	-	1.28	-
Other stock	Pigs	1	1.43	-	0.23	-
	Horses	1	0.23	-	0.06	-
	Poultry	1	2.30	-	2.70	-
Sheep feed	Graze crops	1	0.09	-	0.09	-
	Graze stubble	1	0.14	-	1.27	-
	Graze native pasture	1	0.01	-	0.01	-
	Supplementary feed	1	0.27	-	0.76	-
	Rotational grazing	1	0.02	-	1.19	-
Sheep yarding	Yarded for treatment	1	0.21	-	0.00	-
	Yarded for marking	1	0.00	-	0.06	-
Sheep treatment	Mineral supplements	1	0.08	-	2.77	-
	Drenched	1	0.00	-	0.26	-
	Vaccinated	1	0.01	-	0.15	-

APP.III

TABLE 3. Summary of analysis of qualitative ecological features

'PREDATOR' phase of life cycle

FEATURES	DIVISIONS	df	INTENSITY χ^2	sig.	CYST SIZE χ^2	sig.
Dogs - Management	Run of Property	1	1.88	-	0.08	-
	Restricted overnight	2	1.89	-	2.83	-
	Control of roving	3	5.84	-	0.24	-
	Use in mustering	3	4.82	-	2.08	-
	Used with lambs	2	3.51	-	1.36	-
Water supply	Various types	3	2.15	-	0.16	-
Food supply	Pet food	2	1.97	-	0.08	-
	Fresh meat	2	3.62	-	0.24	-
	Table scraps	2	2.91	-	2.27	-
	Cooked meat	2	3.72	-	0.08	-
	Refrigerated meat	2	6.62	*	0.51	-
	Frozen meat	2	4.25	-	5.13	-
Defeacation point	Various areas on property	4	5.30	-	4.62	-
Cats - Management	Run of property	2	4.56	-	1.88	-
	Restricted overnight	2	1.94	-	0.28	-
Water supply	Various types	3	1.70	-	3.84	-
Food supply	Pet food	2	1.75	-	1.17	-
	Fresh meat	2	2.57	-	0.79	-
	Table scraps	2	1.07	-	0.48	-
	Prepared meat	2	5.38	-	1.72	-
Defeacation point	Various areas on property	4	1.85	-	2.22	-
Sheep carcasses	Various methods of disposal	(2)	0.72	-	0.19	-
Ration sheep	Types	1	0.56	-	0.14	-
	Site of slaughter	1	0.07	-	1.11	-
	Offal disposal	1	2.61	-	0.19	-
	Trimmings disposal	1	4.55	*	0.00	-
	Accessible to carnivores	1	3.42	-	0.04	-
Supplementary fodder	Accessible to carnivores	1	0.50	-	0.50	-

APP.III
TABLE 4. Summary of analysis of qualitative ecological features

'DUST' phase of life cycle

FEATURES	DIVISIONS	df	INTENSITY χ^2	sig.	CYST SIZE χ^2	sig.
Soil	Various types	3	0.44	-	2.51	-
	Ploughed	1	0.03	-	1.63	-
	Fallow	1	1.42	-	0.63	-
	Fertilized	1	0.83	-	0.30	-
	Topography	2	1.41	-	1.61	-
Cereals	Various types	4	2.12	-	1.72	-
Pastures	Various types	2	1.95	-	0.53	-
Land clearance	Amount cleared	1	2.59	-	0.10	-
Flora	Various types	2	3.12	-	1.96	-
Sheep yards	Temporary - degrees of repair	1	4.39	*	0.74	-
	Permanent - degrees of repair	1	3.99	-	2.71	-
Water	Various sources	3	9.02	*	1.61	-
	Degrees of seepage	2	3.45	-	0.38	-
	Facilities for stock	2	0.11	-	0.21	-

APPENDIX IV

RESULTS OF IMMUNOSEROLOGICAL AND HAEMATOCRIT EXAMINATIONS

The results of the immunoserological and haematocrit examinations of the SPF lambs experimentally-infected with *Sarcocystis tenella* (junior synonym *S. ovicanis*) are presented in this appendix.

Serum antibodies (Tables 1 & 2)

The results of the examinations of the lambs for serum antibodies to *Sarcocystis*, using the Complement Fixation Test (CFT) and the Indirect Fluorescent-antibody Test (IFAT), are given for the various days post-inoculation (dpi) examined. The results are expressed as the reciprocal titre found to infection; where the titres were gained from double-dilutions of serum.

Haematocrit (Table 3)

The results of the haematocrit examinations of whole blood from each lamb (i.e., percentage packed cell volumes) are given for the various days post-inoculation examined.

TABLE 1. CFT antibody titres of experimentally-infected SPF lambs.

LAMB NO'S	DAYS POST-INOCULATION (dpi)																		
	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	90	110	135
Experimental Lambs																			
42	0]																		
51	4]																		
40	4]																		
52	0]																		
50	0	0]																	
53	4	4]																	
49	4	-	0]																
55	4	-	4]																
07	4	4	16	64]															
35	4	-	0	8]															
26	0	-	0	-	4]														
38	4	-	4	-	8]														
14	0	0	4	4	8	32]													
29	4	-	8	-	-	128]													
36	8	-	4	-	32	-]													
02	4	4	0	16	64	128]													
04	0	0	0	0	8	32	128]												
10*	16	8	8	32	16	32	32]												
12*	16	8	16	16	16	16	32	16]											
00	4	0	8	32	64	32	64	16]											
09*	8	16	8	16	16	32	16	32]											
30	0	-	8	-	32	-	-	-	32]										
17	0	8	8	8	16	16	16	32	16	32]									
28	4	-	4	-	32	-	-	-	-	-	16]								
22	8	4	8	16	16	64	128	64	64	16	16	32]							
13	4	0	4	16	32	32	64	64	16	32	32	16	16	8]					
23	4	0	8	16	16	32	64	16	16	8	8	16	16	32	16	16]			
11	4	0	4	16	8	32	64	64	32	-	32	-	16	-	-	-	32	16]	
56	0	-	4	-	8	16	64	64	32	-	16	-	-	-	32	-	8	16	8]
Control Lambs	Equivalent days of age																		
43	4	-	-	0]															
25	-	0	-	-	0	-	0]												
41	-	-	-	-	-	4	-	-	-	4	-	-	-	4]					
08	0	-	0	-	-	0	-	-	0	-	0	-	-	-	0]				
21	0	4	4	0	0	0	0	4	-	0	0	0	4	0	-	0	0	0	0]

] = Approx. time of sacrifice

* = Colostrum-fed lambs (early positive titres may be colostral antibodies)

- = not sampled

TABLE 2. IFAT antibody titres of experimentally-infected SPF lambs.

] = approx. time of sacrifice

* = colostrum-fed lambs (early positive titres may be colostral antibodies)

\- = not sampled

LAMB NO'S	DAYS POST-INOCULATION (dpi)																		
	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	90	110	135
Experimental Lambs																			
42	0]																		
51	8]																		
40	4]																		
52	0]																		
50	4	4]																	
53	8	4]																	
49	0	-	0]																
55	8	-	8]																
07	4	0	4	16]															
35	0	-	0	8]															
26	0	-	4	-	4]														
38	4	-	8	-	8]														
14	4	0	4	4	16	64]													
29	8	-	8	-	-	8]													
36	8	-	0	-	4	-]													
02	0	0	0	0	8	16]													
04	4	0	0	8	16	16	64]												
10*	16	16	4	16	8	32	64]												
12*	32	16	16	16	8	16	16	16]											
00	8	4	0	4	8	16	32	128]											
09*	8	16	16	8	8	16	16	32]											
30	0	-	4	-	16	-	-	-	32]										
17	4	4	4	8	8	4	16	32	16	64]									
28	8	-	16	-	16	-	-	-	-	-	64]								
22	8	8	8	16	16	16	16	64	64	128	32	64]							
13	0	4	4	32	8	16	32	16	64	32	32	128	128	256]					
23	0	0	0	0	8	16	16	16	16	4	64	64	256	128	128	128]			
11	0	4	8	0	8	16	64	32	64	-	64	-	256	-	-	-	128	64]	
56	0	-	8	-	0	16	16	8	32	-	128	-	-	-	512	-	256	512	256]
Control Lambs	Equivalent days of age																		
43	0	-	-	0]															
25	-	0	-	-	0	-	4]												
41	-	-	-	-	-	0	-	-	-	8	-	-	-	0]					
08	4	-	0	-	-	0	-	-	0	-	4	-	-	-	0]				
21	0	0	0	0	4	0	0	0	-	4	0	0	0	0	-	0	0	0	0]

TABLE 3. Packed cell volumes (%) of experimentally-infected SPF lambs.

LAMB NOS.	DAYS POST-INOCULATION (dpi)																		
	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	90	110	130
Experimental lambs																			
42	33]																		
51	37]																		
40	34]																		
52	39]																		
50	38	44]																	
53	37	46]																	
49	41	-	41]																
55	36	-	40]																
07	36	35	31	36]															
35	36	-	36	35]															
26	35	-	37	-	36]														
38	38	-	35	-	34]														
14	43	40	36	32	27	20]													
29	36	-	37	-	-	33]													
36	38	-	34	-	33	-]													
02	38	35	34	35	37	24]													
04	32	27	30	30	31	27	10]												
10*	35	33	31	34	23	26	18]												
12*	30	34	34	37	34	19	13	18]											
00	31	40	34	34	34	31	11	15]											
09*	40	35	30	33	31	28	23	20]											
30	30	-	35	-	36	-	-	-	20]										
17	37	37	37	37	34	34	25	24	24	24]									
28	34	-	32	-	30	-	-	-	-	-	24]								
22	40	37	37	36	33	32	23	17	21	23	23	22]							
13	36	35	35	35	33	36	24	21	21	20	23	27	30	30]					
23	34	33	33	33	34	34	24	23	23	23	25	22	29	32	32	33]			
11	39	34	36	35	30	25	14	10	21	-	30	-	28	-	-	-	28	28]	
56	36	-	47	-	47	38	30	22	19	-	21	-	-	-	28	-	29	36	26]
Control lambs	Equivalent days of age																		
43	37	-	-	37]															
25	-	37	-	-	38	-	36]												
41	-	-	-	-	-	35	-	-	-	34	-	-	-	35]					
08	37	-	38	-	-	32	-	-	36	-	31	-	-	-	31]				
21	37	36	33	36	35	39	38	36	-	39	38	36	39	40	-	40	37	37	36]

] = approx. time of sacrifice

* = colostrum-fed lambs

- = not sampled

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Read not to contradict and confute, nor to believe and take for granted, nor to find talk and discourse, but to weigh and consider

Francis Bacon
(1561-1626)

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