

THE ECOLOGY AND PATHOLOGY OF TRICHOSTRONGYLUS TENUIS
(NEMATODA), A PARASITE OF RED GROUSE (LAGOPUS LAGOPUS SCOTICUS)

A thesis submitted to the University of Leeds in fulfilment for
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The red grouse, Lagopus lagopus scoticus

ABSTRACT

Trichostrongylus tenuis is a nematode that lives in the caeca of wild red grouse. It causes disease in red grouse and can cause fluctuations in grouse populations. The aim of the work described in this thesis was to study aspects of the ecology of the infective-stage larvae of T.tenuis, and also certain aspects of the pathology and immunology of red grouse and chickens infected with this nematode.

The survival of the infective-stage larvae of T.tenuis was found to decrease as temperature increased, at temperatures between 0-30 C, and larvae were susceptible to freezing and desiccation. The lipid reserves of the infective-stage larvae declined as temperature increased and this decline was correlated to a decline in infectivity in the domestic chicken. The occurrence of infective-stage larvae on heather tips at caecal dropping sites was monitored on a moor; most larvae were found during the summer months but very few larvae were recovered in the winter. The number of larvae recovered from the heather showed a good correlation with the actual worm burdens recorded in young grouse when related to food intake. Examination of the heather leaflets by scanning electron microscopy showed that each leaflet consists of a leaf roll and the infective-stage larvae of T.tenuis migrate into the humid microenvironment provided by these leaf rolls.

Scanning electron microscopy showed that the adult nematodes burrowed into the mucosa as well as lying on its

surface and that the caecal mucosa of heavily infected grouse became disrupted in areas of nematode activity. The caecal mucosa of lightly infected grouse exhibited little damage and the caecal mucosa of grouse treated with an anthelmintic and shot 5-6 months later was similar to that of lightly infected birds. Some of the nematodes from these treated birds were covered in rosette-shaped cells which have been tentatively identified as adherent lymphocytes. The cuticle of adult T.tenuis was superficially annulated but did not possess cuticular ridges, as described in some other trichostrongyle nematodes.

Primary and challenge infections with T.tenuis were established in the domestic chicken and these reached patency but nematodes were expelled in blood-stained balls of mucus and all adult nematodes had been expelled from the birds 30 days after dosing with infective-stage. Following trickle doses of larvae, there was a rise and then a fall in nematode egg output but larvae administered later in the trickle infection appeared to fail to establish. Light and scanning electron microscopy showed haemorrhagic lesions and blood spots on the caecal mucosa of infected chickens and nematodes were found to burrow beneath mucus secreted on the mucosal surface. There were significant increases in the proportions of circulating leucocytes in infected chickens, but only on certain days of infection. No antibodies to T.tenuis were detected in the blood of infected chickens. There was a decrease in the length of the caeca of infected chickens during the period when the

nematodes were being expelled from the caeca. Nematode egg output continued to rise during an infection in young red grouse and there was no expulsion of nematodes from these infected birds.

Infective-stage larvae that had been attenuated by cobalt 60 irradiation stimulated some degree of resistance to challenge infection in the domestic chicken but not in the red grouse. It was concluded that immunization with irradiated larvae would be of little use in the control of T.tenuis in red grouse.

To the players and supporters of Aldershot Football Club

"This is not a book to be tossed aside lightly. It is to be thrown with great force...." (Dorothy Parker)

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CHAPTER 1

GENERAL INTRODUCTION

1.1. INTRODUCTION TO THE TETRAONIDAE

The red grouse Lagopus lagopus scoticus belongs to the Tetraonidae, which is a sub-family of the Phasianidae. The Tetraonidae have similiar general features to other members of the Phasianidae but have the following distinguishing characteristics (Short, 1967); tarsi partly or fully feathered; nostrils covered by feathers; lateral pectinations on the toes; spurless feet; bill covering and nails shed yearly; and a swollen eye comb above the eye. The Tetraonidae may be further divided into 6 genera; Centrocercus Swainson 1831; Dendragapus Elliot 1864; Lagopus Brisson 1760; Tetrao Linnaeus 1758; Bonasa Stephens 1819; and Tympanuchus Gloger 1842. This classification is shown in Fig. 1.1.

1.1.1. THE GENUS LAGOPUS

The genus Lagopus Brisson 1760 is divided into 3 separate species; Lagopus lagopus (Linnaeus) 1758, the willow ptarmigan; Lagopus mutus (Montin) 1776, the rock ptarmigan; and Lagopus leucurus (Richardson) 1831, the white-tailed ptarmigan. The willow ptarmigan breeds on treeless tundra, moors, bogs and heaths across northern Eurasia; south to circa 55 N latitude in the west, to 49 N latitude in the Russian Steppes, and to 47 N latitude in Mongolia (Johnsgard, 1983). In North America this species is restricted to the circumpolar tundra and mountain ranges (Cramp & Simmons, 1980). The species Lagopus lagopus may be further subdivided into a number of distinct

sub-species. The red grouse, Lagopus lagopus scoticus, is a sub-species unique to the British Isles and Lagopus lagopus hibernicus is a sub-species which inhabits the heaths and moors of Ireland. The rock ptarmigan, unlike the willow ptarmigan, survives in parts of Scotland, in the northern Pyrenees, and from Scandinavia and the northern Urals, east through Siberia, to the Bering sea (Johnsgard, 1983). The white-tailed ptarmigan inhabits North America, its distribution conforming closely to that of the alpine tundra from central Alaska, south to Vancouver Island, and along the Rocky mountains from British Columbia to northern New Mexico (Johnsgard, 1983).

1.1.2. THE RED GROUSE

Distribution

The red grouse, Lagopus lagopus scoticus, is exclusively confined to the heather dominant uplands of the British Isles. Large tracts of heather moorland in Scotland support populations of red grouse, these populations being concentrated in 4 main areas; the Southern uplands, the moorlands between the Scottish borders and the Pentland Hills; the west coast highlands and islands; the central highlands, stretching from Loch Lomond, eastwards to Aberdeen; and the North West Highlands, from Glen Mor, northwards (Sharrock, 1976). In the north of England, the heather moors of the Pennines support most of the English population. This Pennine stronghold of the red grouse can be divided into three main regions; the peak district,

Fig. 1.1. The classification of extant species of grouse and ptarmigan (from Johnsgard, 1983).

Family Phasianidae: pheasant like birds

Subfamily Tetraonidae: grouse and ptarmigan

Genus Centrocercus Swainson 1831

1. C.urophasianus (Bonaparte) 1828: sage grouse

Genus Dendragapus Elliot 1864

1. D. obscurus (Say 1923): blue grouse
2. D. canadensis (Linnaeus) 1758: spruce grouse
3. D. falcipennis (Hartlaub) 1855: sharp-winged grouse

Genus Lagopus Brisson 1760

1. L.lagopus (Linnaeus) 1758: willow grouse
2. L. mutus (Montin) 1776: rock ptarmigan
3. L.leucurus (Richardson) 1831: white-tailed ptarmigan

Genus Tetrao Linnaeus 1758

1. T.urogallus (Linnaeus) 1758: capercallie
2. T.parvirostris (Bonaparte) 1856: black-billed capercallie
3. T.tetrix (Linnaeus) 1758: black grouse
4. T.mlokosiewiczi (Taczanowski) 1875: Caucasian black grouse

Genus Bonasa Stephens 1819

1. B.umbellus (Linnaeus) 1776: ruffed grouse
2. B.bonasia (Linnaeus) 1758: hazel grouse
3. B.sewerzowi (Przewalski) 1876: black-breasted hazel grouse

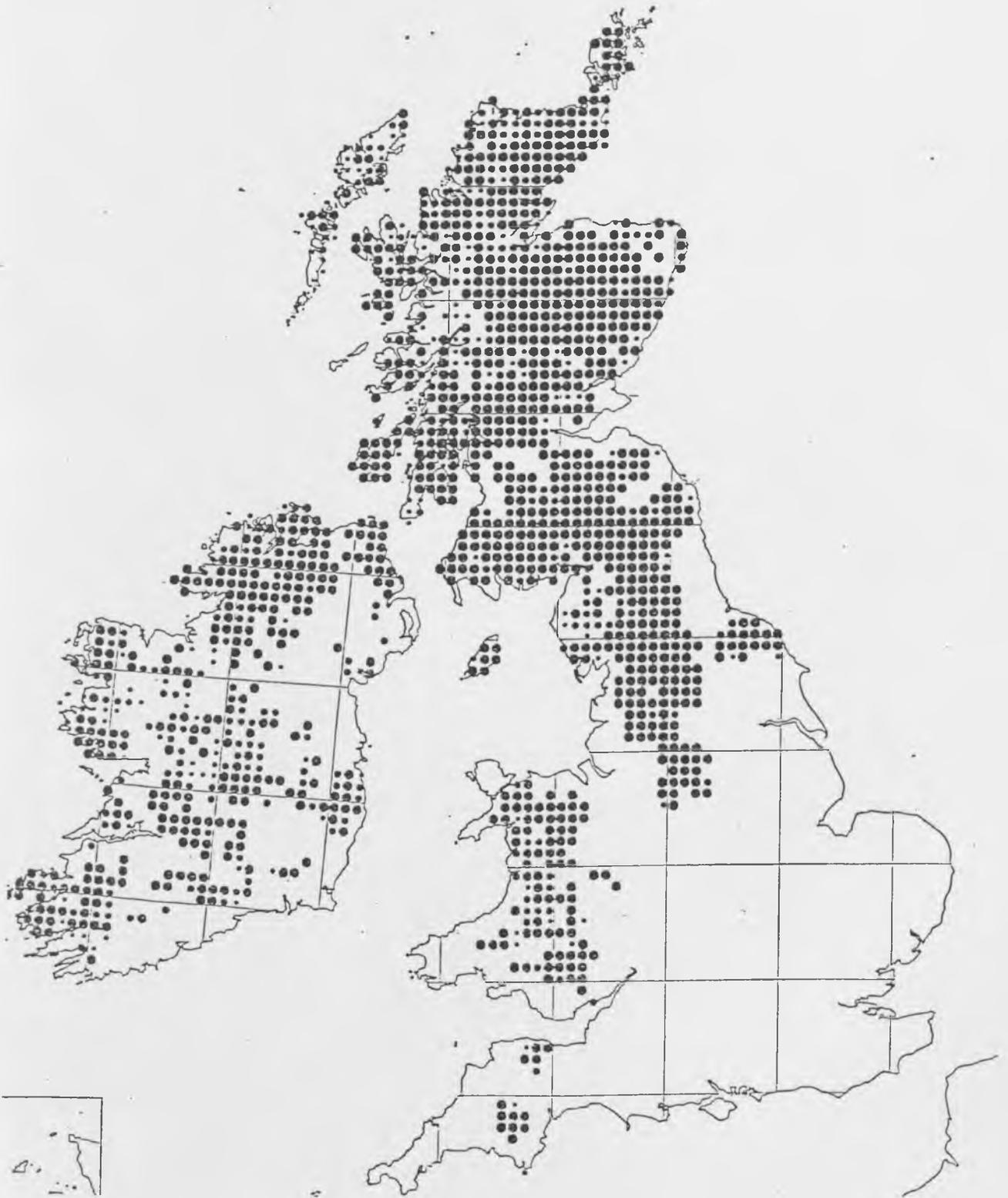
Genus Tympanuchus Gloger 1842

1. T.cupido (Linnaeus) 1758: pinnated grouse
2. T.phasianellus (Linnaeus) 1758: sharp-tailed grouse

which includes parts of Staffordshire, Derbyshire and South Yorkshire; the southern dales, stretching from Leeds and Bradford, northwards across North Yorkshire to Wensleydale; and the northern dales stretching from Wensleydale, across County Durham, Northumberland and parts of Cumbria to the Scottish borders (Hudson, 1986a). Red grouse also inhabit the trough of Bowland moors to the west of the pennines and the heathland plateau of North Yorkshire to the east, with a small population in the Lake district. In other parts of England the red grouse is sparsely distributed. Although it was once common in parts of Wales, to as far south as Glamorgan (Hudson, 1906) and across the bogs and heaths of central Ireland, where the sub-species Lagopus lagopus hibernicus is the indigenous race (Witherby, Jourdain, Ticehurst and Tucker, 1941), populations in these areas have declined and remain at a low density. There have been several attempts to introduce the red grouse to the north or to the south of its natural range but none of these were particularly successful. For example, between 1858 and 1883 birds were introduced onto the Shetland Islands (Lovat, 1911) while at around the same time the red grouse was introduced onto the heather moorlands of Dartmoor and Exmoor (Coward & Barnes, 1975). Only very low numbers of red grouse remain in these areas today. Other attempts at introduction have occurred in Surrey, Norfolk and at Icklingham, Suffolk (Lovat, 1911) and on the continent in the Ardennes region of Belgium and in the Eifel region of West Germany (Witherby, Jourdain, Ticehurst & Tucker, 1941). Although red grouse became established in these areas immediately following introduction, in the long term

Fig. 1.2. The distribution of the red grouse in Great Britain
(from Sharrock, 1976).

- 33+ birds/km²
- 6-22 birds/km²
- 1-2 birds/km²



such introductions failed and no grouse exist in these regions today. The current distribution of the red grouse in Great Britain is shown in Fig. 1.2.

Physical Characteristics

Red grouse differ from nominate Lagopus as follows. Wings are never white and the entire plumage is uniform red-brown, darker than nominate Lagopus, and on some birds, almost purple-black. The all dark plumage is relieved only by white under wing-coverts in winter and a grey bloom on the undersurface of the flight feathers. In winter, however, many Scottish birds show a distinct white barring and even patches of white on the flanks and underbelly (Cramp & Simmons, 1980). In the autumn, an adult cock red grouse weighs on average between 650-750 g and an adult hen between 550-650 g, although in March and April, prior to egg laying, hen grouse may increase considerably in weight (Watson & Miller, 1976). Red grouse, like all ptarmigan, differ from other species of grouse in that their feet are feathered to the bases of their toes (depending on the time of year) and their upper tail coverts extend to the tips of their tails.

Ageing techniques

The red grouse is a fast growing, short-lived bird, which rarely survives beyond 2 years of age. The average mortality of grouse is about 65% per year, whether sportsmen shoot them or not (Watson & Miller, 1976). A

population of grouse may be conveniently divided into two age groups; the young grouse, those which are the produce of the current breeding season; and old or adult grouse, those which are greater than 1 year old (Watson & Miller, 1976). The most reliable way to age red grouse in late summer is to compare the shape of the two outermost primary feathers with the rest of the primary feathers on the wing. Rounded tips on the outermost primaries indicate an old bird while those of a young bird are more pointed. Examination of the toe nails is another way of ageing the birds. Old grouse shed their toe nails between July and September while young grouse do not. A scar across the top of the new nail, showing where the old nail was formerly attached, thus indicates an old bird. The shape of the toe nail may also give an indication of the age of the bird. Young grouse have long, smooth, sharp nails while the nails of old birds are blunter and thicker (Watson & Miller, 1976).

Digestive Physiology

In common with most other Galliformes, the red grouse has two blind-ending sacs or caeca at the junction of the small and large intestine. The caeca are believed to be involved in the break-down and digestion of cellulose and other fibrous materials such as lignin (Moss & Parkinson, 1972; 1975; Gasaway, 1976; Moss, 1977) and it seems likely that this digestion is performed by micro-organisms within the caeca. Although few microorganisms occur in the small intestine of red grouse, many micro-organisms occur in the

caeca (10^8 - 10^{14} /g caecal contents) (Fantham, 1910; McBee & West, 1969; Hanssen, 1979). These include spirochaetes, flagellates, amoebae, small gram-negative rods, and a few Escherichia coli. The biochemical properties and physiological significance of these microorganisms has yet to be studied directly but cellulose fermentation is known to take place in the caeca of tetraonids (McBee & West, 1969; Gasaway, 1976). The length of the gut of gallinaceous birds is known to vary, depending upon the bird's hormones, gut microflora, parasite load, rhythm of feeding (Moss, 1972) and on the composition of diet and the amount of food eaten (Moss & Trenholm, 1987). Leopold (1953) examined the intestinal morphology of gallinaceous birds in relation to food habits and showed that the browsing galliformes of the grouse family, such as the red grouse, have longer caeca than seed-eating quail, partridge, pheasant and turkey. It was suggested that such morphological adaptations enable the grouse to survive on a diet which consists predominantly of heather (Jenkins, Watson & Miller, 1963; Savory, 1975) which is a low quality bulk food, while Sibley (1981) suggested that red grouse retain digesta for long periods of time in this large caeca, so enabling the efficient digestion of the highly fibrous heather. In a later paper, Moss & Trenholm (1987) showed that the length of the gut of red grouse, and their ability to digest fibrous food, increases when the intrinsic digestibility of their diet drops below a certain predictable point.

1.1.3. RED GROUSE MANAGEMENT

The management of a moor for red grouse involves the control of predators and the management of the heather-dominated vegetation. The regular burning of strips or patches of heather rejuvenates and maintains the heather stand and produces a mixture of young heather, which is required by the grouse for food, and older heather, which is required for cover, protection, and nest building. The diverse vegetation produced by such moor management not only benefits the red grouse but is beneficial to many other species of birds, plants and animals which inhabit the uplands.

The red grouse is highly regarded as a sporting bird and grouse shooting is an important land use on many of Britain's moorlands. Under the favourable conditions which can be produced by the management of the grouse and of the heather on a moor, red grouse can reach autumn densities of up to 750 birds per km (Hudson & Watson, 1985). The surplus of birds so produced may then be harvested by shooting. A well-managed grouse moor of 1620 hectares in the North of England may produce on average 1000 brace per season, giving a financial return of 3-4% before tax (Hudson & Watson, 1985). If the red grouse population declines on a commercially run estate, the management of the moor and the harvesting of birds becomes uneconomic. The grouse moor may then change to an alternative land use, such as afforestation or sheep grazing, both of which are supported by generous subsidies and both of which result in

the production of a far less diverse ecosystem. To halt this change in land use in the uplands requires the continuation of red grouse shooting as a commercial interest, which in turn requires the maintenance of high population densities of red grouse on the moors.

1.2. POPULATION CYCLES IN RED GROUSE.

The suitability of the red grouse for population research is now widely recognised. A number of studies have examined the population biology of the red grouse, with the publications of Jenkins, Watson & Miller (1963; 1967) and Watson, Moss, Rothery & Parr (1984) documenting the research on Scottish populations of red grouse, and that of Hudson (1986a) the research on English populations.

The numbers of several species of mammals and birds fluctuate in "cycles", a term applied to population changes in which the successive maxima or peaks occur at regular intervals (Lack, 1954). A number of studies have examined fluctuations in the numbers of red grouse (Mackenzie, 1952; Jenkins, Watson & Miller, 1963; Watson & Moss, 1980; Watson, Moss, Rothery & Parr, 1984) and several have shown that the numbers of red grouse fluctuate in a cyclic fashion, with years of grouse abundance often followed by years of scarcity (Watson & Moss, 1979; Potts, Tapper & Hudson, 1980; Hudson, Dobson & Newborn, 1985).

1.2.1. HYPOTHESES FOR POPULATION CYCLES

Chitty (1954; 1960), following a study of the fluctuations in the population of the vole (Microtus agrestis), proposed that all species are capable of regulating their own population densities without destroying the renewable resources of their environment or requiring enemies or bad weather to keep them from doing so. Under appropriate circumstances, an indefinite increase in population density is prevented through a deterioration in the quality of the population. It was proposed that population cycles were due to intrinsic factors within the population itself, such as the changing genetic composition of individuals, and not to the action of extrinsic physical factors, such as climate. Thus at high densities, natural selection favours aggressive individuals with a low reproductive rate, whilst at low densities natural selection favours the less aggressive individuals with a higher reproductive rate. This hypothesis has been cited as the reason for the fluctuating populations of red grouse (Watson & Moss, 1980).

An alternative hypothesis as to the cause of population cycles has been proposed by Lack (1954). Following studies on a 4 year cycle in the population of the lemming (Lemmus lemmus and Dicrostonyx spp), a 4 year cycle in the population of the vole (Microtus spp), and a 10 year cycle in the population of the Varying Hare (Lepus americanus), it was suggested that the dominant rodent interacts with its vegetable food source and its predators to produce a

predator-prey oscillation. A decline in the numbers of rodents can result in a decline in the numbers of predatory birds and mammals and subsequently cause an increase in the numbers of gallinaceous birds. The prime cause of these population cycles was suggested as either food shortage or predation. This hypothesis was applied to fluctuations in the red grouse population and it was proposed that the population cycles of red grouse were caused primarily by the joint effects of starvation and parasitism. However, the irregular nature of these fluctuations suggests that the severity of these factors may be influenced by variations in either winter snowfall (affecting the availability of heather) or by spring rainfall (affecting chick survival).

Further evidence to support this hypothesis has been developed from the mathematical modelling of populations (Anderson, 1978; Anderson & May, 1978; Anderson, 1979; Anderson & May, 1979). Such models are able to predict the effect of a parasitic species on a host population. It is now believed that a parasitic species has the potential to regulate or suppress host population growth, either by a direct effect on host survival and reproduction; by causing the host to become more susceptible to predation; or by reducing the competitive fitness of the host (Anderson, 1979).

Several studies have attributed fluctuations in the red grouse population to parasitism by the nematode Trichostrongylus tenuis. Lovat (1911) first suggested that

this trichostrongyle nematode may be the prime cause of "grouse disease" epidemics but it was not until much later that the significance of parasitism in the population cycles of red grouse was established. Potts, Tapper & Hudson (1984) produced a simulation model based on the analysis of bag records from grouse moors in the north of England and concluded that red grouse cycles could be caused by the effects of T.tenuis, if a series of stochastic variables, such as the uptake of worms, were incorporated. In a later paper, Hudson, Dobson, & Newborn (1985) further tested the hypothesis that parasitism was a key element in influencing the pattern of fluctuations in red grouse numbers and proposed that grouse populations could be divided into two types. Cyclic populations, associated with moors with high rainfall and high worm burdens, and non-cyclic populations, in areas with a relatively low rainfall and low worm burdens. Hudson (1986b) provided further evidence of the importance of parasitism by demonstrating that breeding success in hen grouse was significantly greater in birds that had been treated with an anthelmintic and carried reduced worm burdens, compared with untreated birds carrying normal worm burdens. He demonstrated experimentally that this reduction in host fecundity was due to the effects of T.tenuis and could be the cause of the cyclic changes in the red grouse population.

1.2.2. PARASITES AND RED GROUSE

Lovat (1911) produced the first comprehensive survey of

the parasites of red grouse and recorded a number of protozoan parasites in the blood of grouse (Leucocytozoon lovati, Haemoproteus masoni, and Spirochaeta lagopodis); a number of protozoa in the alimentary tract (Trichomonas eberthi, Spirochaeta lovati, and Monocystis sp); 3 species of tapeworm in the small intestine (Davainea urogalli, Davainea cesticeilus, and Hymenolepis microps); and a range of ectoparasites (the bird lice Gonoides tetraonis and Nirmus cameratus; the grouse fly Ornithomyia lagopodis; and the mites Aleurobius farinae and Gamasus coleoptratorum). However, these parasites were not recorded as causing high mortality within the grouse population.

The major disorder in grouse was termed "grouse disease". The works of Klein (1892), Cobbold (1873), Farquharson (1874) and Macdonald (1883) document the early studies on the nature and cause of the disease. At that time two hypotheses existed as to the cause of the disease. It was thought to be caused either by an acute infectious pneumonia, characterised by the presence of a specific bacillus of the Bacillus coli group in the lungs, which resulted in the death of well-conditioned birds (Klein, 1892), or by an infection of the caeca of grouse with thousands of nematodes, which brought about irritation of the caecal mucosa and emaciation and subsequent death of birds (Cobbold, 1873). These two hypotheses appeared to account for the finding of both well-conditioned and emaciated dead grouse on many moors. However, throughout the course of Lord Lovat's enquiry not a single case of epidemic pneumonia was recorded while the nematode was

found in most of the grouse examined and was found to be associated with a significant weight loss in heavily infected birds. The conclusion of this study was for the first time to categorically state that the so-called "grouse disease" was caused by the nematode Strongylus pergracilis (Cobbold) syn. Trichostrongylus tenuis.

1.3. TRICHOSTRONGYLUS TENUIS

Strongylus pergracilis was found by Cobbold (1873) to be identical to Strongylus tenuis (Eberth 1861) which had been recovered from the caecum of the goose Anser cinerea. Later, Nagaty (1932) suggested that both species should be classified under the newly formed genus Trichostrongylus (Looss) and called Trichostrongylus tenuis (Mehlis 1846) Raillet and Henry 1909.

Adult T.tenuis inhabit the caeca of a range of avian hosts. Although T.tenuis was originally described in Europe from the ring necked pheasant (Mehlis, 1846), it has subsequently been recorded in a range of hosts including the bobwhite quail (Stoddard, 1931; Cram, 1925), the English partridge (Portal & Collinge, 1932), the turkey and guinea fowl (Clapham, 1935; Owen, 1951) and in various species of ducks and geese (Avery, 1966; Owen, 1951; Enigk, Dey-Hazra & Batke, 1975). There have been several recordings of T.tenuis in the domestic chicken and these are documented in Chapter 6. However, by far the most frequently recorded host for T.tenuis in Great Britain is the red grouse, Lagopus lagopus scoticus, where high levels

of infection are common.

The life cycle of T.tenuis is direct and involves no intermediate hosts (Fig. 1.3). Adult nematodes in the caeca produce eggs which are passed out in the caecal droppings (Fig. 1.4). These eggs develop through two, bacterial-feeding, larval stages into an ensheathed, non-feeding, third-stage larva, which is the infective stage. This larva migrates out of the caecal droppings and onto the surrounding vegetation. Larvae may then ascend the heather plants and are ingested by red grouse when they feed upon the heather tips. The larvae then pass to the caeca where they develop into adults.

1.4. OBJECTIVES

There were two main objectives at the commencement of this research. Firstly, to study the ecology of the infective-stage larvae of T.tenuis. This included an examination of the ability of these stages to develop, survive, migrate and infect the red grouse when subjected to different regimes of temperature and moisture; an examination of the effect of ageing on the lipid food reserves of the infective-stage larvae; and also an examination of the ability of the larvae to establish an infection in grouse and in chickens (which it was hoped would prove to be a readily available source of parasite material). Secondly, this research set out to examine certain aspects of the pathology and immunology of birds infected with T.tenuis as this could have a bearing on the

reasons for the decline in red grouse populations.

Fig. 1.3. The life cycle of Trichostrongylus tenuis in the red grouse.



Grouse

Heather

Eggs in
caecal droppings

Infective
third-stage larva

Bacteria-feeding
first-and second-stage
larva

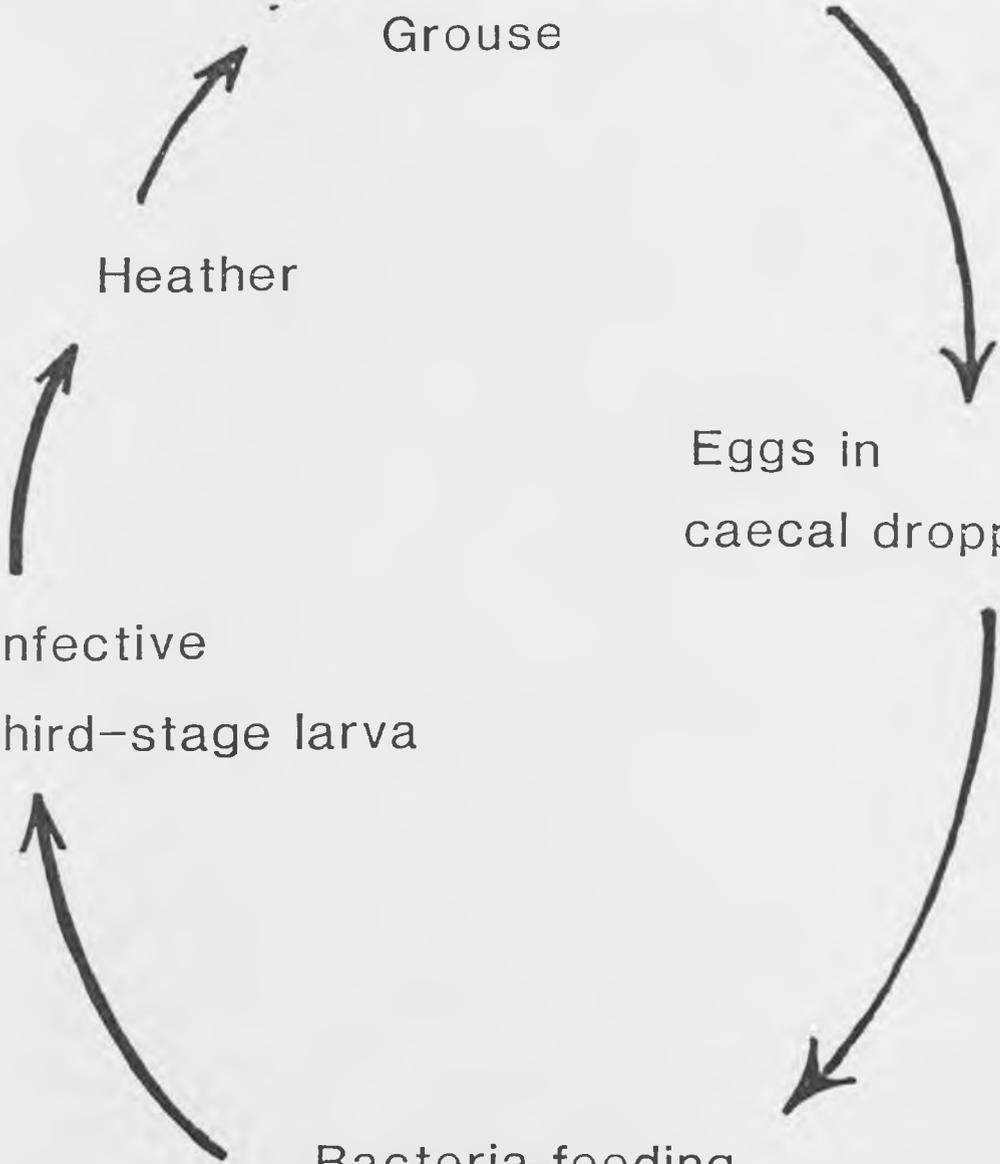


Fig. 1.4. The droppings of a red grouse. Note the dark brown, semi-solid, caecal droppings (c) in which nematode eggs are passed; and the lighter coloured fibrous droppings (f).



CHAPTER 2

THE EFFECT OF TEMPERATURE, FREEZING AND DESICCATION ON
THE SURVIVAL OF THE INFECTIVE-STAGE LARVAE OF T.TENUIS

SUMMARY

Infective-stage larvae of T.tenuis were incubated at a range of temperatures and the percentage survival recorded. Larvae incubated at 0 C survived for over 6 months, while those incubated at 30 C survived for only 10 weeks. The effect of sub-zero temperatures on the survival of the infective-stage larvae of T.tenuis was also monitored. Larvae survived for up to 4 weeks at -5 C; for up to 3 weeks at -10 C; and for 1 week at -15 C. Infective-stage larvae were found to be very susceptible to desiccation, surviving for up to 7 weeks at 80% relative humidity (R.H.); for up to 6 weeks at 60% R.H.; but for only 2 weeks at 35% and 25% R.H. The effect of temperature and humidity on the infective-stage larvae on the moor was discussed in relation to the epidemiology of T.tenuis in red grouse.

INTRODUCTION

The development, survival and transmission of trichostrongyle nematodes involves the interaction of a number of factors (Andersen, Levine & Boatman, 1970; Gibbs, 1972). Firstly, there is a range of factors in the initial host that can influence the biological processes of the individual parasites which are involved in the production of the next infective-stage of the life-cycle. Secondly, since transmission involves a period of time when the infective-stages are external to the host, external environmental factors can be an important influence on the development, viability and transmission of the free-living stages. Thirdly, when the infective-stages enter a new host, parasite/host interactions may influence the establishment, reproduction and survival of the parasite. Thus, the effective transmission of trichostrongyle nematodes, whose life-cycle involves a free-living larval stage outside the host, depends in part upon a number of environmental factors which may affect this free-living stage (Callinan & Westcott, 1986).

The bionomics of trichostrongyle nematodes of livestock has been widely studied and the literature reviewed by Gordon (1957), Crofton (1963), Levine (1962), Kates (1965) and Michel (1976). The survival and rate of development of larvae on the pasture is partially dependent on temperature and available moisture. Effects of temperature have been reviewed by Lucker (1941), Wallace (1961) and Crofton (1963). In general, the development of the free-living

stages of trichostrongyle nematodes outside the host is enhanced by an increase in temperature but their survival time is decreased. The optimum temperature for development is between 20-30 C (Gibbs, 1972). More recently, the effect of temperature on the survival of the infective-stage larvae of trichostrongyle nematodes has been examined by Wharton (1981a) and Boag & Thomas (1985) and the effects of sub-zero temperatures on survival by Wharton, Young & Barrett (1984). The effect of desiccation on the survival of the free-living stages of trichostrongyle nematodes has also been studied (Prasad, 1959; Andersen & Levine, 1968; Ellenby, 1968; Todd, Levine & Whiteside, 1970; Wharton, 1982).

The effect of such environmental factors on infective-stage larvae of trichostrongyle nematodes varies, depending upon the species of nematode. These differences in the ecology of the free-living stages have been used to explain and interpret the different epidemiological patterns of several trichostronglye nematodes (Gibson & Ever ett, 1967; Boag & Thomas, 1970; Callinan, 1979).

A number of other factors may influence the survival of the free-living stages of trichostrongyle nematodes. The immediate environment of an organism, and the conditions that prevail there, constitute the micro-environment of that organism. The micro-environment of the free-living stages of most trichostrongyle nematodes is considered to be the layer between the ground surface and the top of the pasture vegetation (Andersen, Levine & Boatman, 1970). The

temperature and available moisture within the micro-environment will influence the development and survival of the free-living stages. The temperature at ground level is influenced by a number of factors, such as the heat exchange near to the ground, the condition and type of soil, the slope of the land, and the type and amount of vegetation cover. Soils that favour the growth of forage plants may also improve transmission (Gibbs, 1972). Crofton (1948) found that the distribution pattern of the infective-stage larvae of Trichostrongylus retortaeformis is associated with the conformation of the plants on which the larvae develop and Knapp (1964) showed that the infective-stage larvae of Haemonchus contortus survived better on plots seeded with clover than on plots seeded with grass. Dinnik & Dinnik (1958; 1961) found that direct sunlight may affect the survival of the infective-stage larvae of H. contortus. The extent of the contamination of the pasture with faeces may also affect transmission; it has been shown that the distribution of sheep faeces on a pasture approximates the negative binomial (Donald, 1967) and that the build up of larvae on a pasture is essentially exponential (Crofton & Whitlock, 1964). The consistency of the faeces may affect transmission; Michel (1957) and Rose (1960) showed that liquid or semi-solid faeces are easily spread by mechanical or biological agents and appear to facilitate the dispersal of larvae more than solid faeces. The microflora and fauna associated with the faecal mass, soil and vegetation may also assist in the dispersal of infective-stage larvae (Robinson, 1962); or alternatively, may reduce their numbers

by predation (Deschiens, 1939; Dolfus, 1946; Roubaud & Deschiens, 1941; Parnell & Gordon, 1961). A range of host factors, such as the grazing and feeding habits of the host, can also affect transmission. Finally, individual aspects of the parasite's life cycle, such as the generation time (Crofton, 1963) and the fecundity of the females (Kates, 1947), can have an effect on the epidemiology of trichostrongyle infection.

To date, little information is available concerning the effects of temperature and desiccation on the free-living, infective-stage larvae of T.tenuis. Lovat (1911) noted that water was essential for the development of the eggs of T.tenuis and that a coiling behaviour and loss of motility occurred when water surrounding the infective-stage larvae was evaporated. He suggested that the migration of the larvae of T.tenuis to the tips of the heather plants occurs only when mist, rain or dew are present. Cram & Cuvillier (1934) showed that both the eggs and infective-stage larvae of T.tenuis showed a marked resistance to cold when moisture was present, while eggs in fresh caecal droppings remained viable after incubation for 5 days at 7 C but after 35 days at this temperature, eggs failed to develop when returned to a warmer environment. Infective-stage larvae, however, were still viable after 4 months, following incubation at an average temperature of -3 C (range +3 C to -20 C).

It has been proposed, in a theoretical model developed by Hudson, Dobson & Newborn (1985), that the ability of the

infective-stage larvae of T.tenuis to survive desiccation is an important factor in the epidemiology of T.tenuis in red grouse. This model postulates that population cycles in red grouse in the North of England may be due to parasitism by T.tenuis and that the effect of parasitism is greater in areas of high rainfall than in areas of low rainfall. It was proposed that low humidity in areas of low rainfall reduced the survival of the free-living stages of the nematode, so reducing the build up of nematode burdens in the red grouse and the subsequent deleterious effects on the birds which this causes. Conversely, in areas of high rainfall humidity is higher, the survival of the free-living stages of T.tenuis is greater and consequently, parasitism has a greater effect on the host population. To further test this hypothesis, detailed data on the effect of desiccation on the survival of the infective-stage larvae of T.tenuis was required.

The aim of the work described in this chapter was to examine the effect of temperature and desiccation on the survival of the infective-stage larvae of T.tenuis, under controlled laboratory conditions, and then to attempt to relate the results to the conditions which could be experienced by the infective-stage larvae on the moor.

MATERIALS AND METHODS

Collection of Trichostrongylus tenuis infective-stage larvae

Freshly deposited caecal droppings from red grouse were collected from a moor in upper Swaledale, North Yorkshire, (NGR NY 930 020) and were mixed with moist granulated charcoal (B.O.C. Ltd) and placed on moist filter paper lying in a petri dish. The cultures were incubated at 27 C for 7 days (Wilson, 1979) and were kept moist by the addition of a few drops of water when required. Infective-stage larvae were separated from the culture medium by means of a Baermann apparatus. Larvae were stored at 4 C until required, but for no longer than 7 days.

Effect of temperature on the survival of the infective-stage larvae of T.tenuis

Approximately 200 infective-stage larvae were placed into 35 mm high x 20 mm diameter tubes in 25 ml of distilled water. Each tube was wrapped in a black plastic covering, to prevent any algal growth in the larval suspensions. Tubes were incubated at each of the following temperatures; 0, 5, 10, 15, 20 and 30 C, in thermostatically cooled incubators (Gallenkamp Compenstat incubator). Tubes were removed from the incubators every 7 days, up to 11 weeks, after which they were removed every 14 days. The contents was examined microscopically and the number of motile infective-stage larvae were recorded; the tubes were then returned to the appropriate incubation temperature. At each inspection, a portion of the water was withdrawn and

fresh distilled water added or, in the case of those incubated at higher temperatures, the water level in each tube was topped up to compensate for any evaporation loss.

Effect of sub-zero temperatures on the survival of the infective-stage larvae of *T.tenuis*

Approximately 100 infective-stage larvae (the actual number of larvae used in each case was known) were placed in a drop of water onto each of 75 15 mm diameter discs of filter paper (Whatman No 1) and the surplus water was allowed to evaporate at room temperature until the filter paper was moist throughout, but no surface droplet of water could be seen. 25 filter paper discs were placed in each of 3 petri dishes. All 75 samples of infective-stage larvae were then incubated at 0 C for 3 hours, followed by 3 hours at -5 C. 50 samples were then moved to -10 C for 3 hours, after which 25 samples were moved to -15 C. 25 groups of larvae were thus incubated at -5 C, -10 C and -15 C after gradual cooling to those temperatures. To ascertain the survival times at each temperature, 5 discs of filter paper containing larvae were removed every 7 days from each temperature and brought back to 0 C and then to room temperature.

Recovery of infective-stage larvae from filter paper

Filter paper discs were rinsed thoroughly in tap water, in order to remove any adherent larvae, chopped into small pieces and washed again to remove any larvae within the filter paper. The residue was then examined microscopically and the number of motile infective-stage

larvae recorded.

Effect of desiccation on the survival of the
infective-stage larvae of T.tenuis

Approximately 100 infective-stage larvae (the actual number of larvae used in each case was known) were placed in a drop of water onto 15 mm diameter discs of filter paper (Whatman No 1) and the surplus water was allowed to evaporate at room temperature, until the filter paper was moist throughout but no surface droplet of water was visible. Discs were then placed in a petri dish and transferred into glass desiccators maintained at either 100%, 80%, 60%, 35% or 25% relative humidity (RH) by means of glycerol solutions placed within the desiccators (Grover & Nichol, 1960). 50 discs were incubated at 100% RH; 50 at 80% RH; 50 at 60% RH; 25 at 35% RH; and 25 at 25% RH. All desiccators were maintained at 5 C and the humidity was checked every day by means of a hair hygrometer. 5 discs were removed from each desiccator every 7 days, for a period of up to 10 weeks in the case of larvae incubated at 100%, 80% and 60% RH or for a period up to 5 weeks in the case of those incubated at 35% and 25% RH. At the same time discs of filter paper onto which had been placed approximately 100 infective-stage larvae (the actual number of larvae used in each case was known) were kept moist throughout the duration of the experiment by the addition of drops of water so that a meniscus was formed over the surface of the filter paper. 5 of these control discs were removed every 7 days. Infective-stage larvae were recovered from the filter paper discs as described

previously.

Analysis of results

The percentage of infective-stage larvae surviving under each condition was recorded. The mean 50% survival times were calculated for larvae incubated at 0, 5, 10, 15, 20 and 30 C and the relationship between 50% survival time and temperature was compared by means of regression analysis.

RESULTS

Effect of temperature on the survival of the infective-stage larvae of T.tenuis

Approximately 200 infective-stage larvae were incubated at each of the following temperatures; 0, 5, 10, 15, 20 and 30 C. The results are shown in Figs. 2.1a, b and c. Most infective-stage larvae had died by 47 weeks at 0 C; by 41 weeks at 5 C; by 25 weeks at 10 C; by 21 weeks at 15 C; by 19 weeks at 20 C; and by 9 weeks at 30 C. The survival of each group of infective-stage larvae incubated at each temperature was calculated as 50% survival times (S50) (Fig. 2.2) and in all cases a good linear correlation was observed ($r=0.9740$). The mean 50% survival time for larvae incubated at 0 C was 12.2 weeks; 10.6 weeks at 5 C; 7.7 weeks at 10 C; 6.4 weeks at 15 C; 6.6 weeks at 20 C; and 2.3 weeks at 30 C.

Effect of sub-zero temperatures on the survival of the infective-stage larvae of T.tenuis

Most larvae had died by 4 weeks at -5 C; by 3 weeks at -10 C; and by 1 week at -15 C (Fig. 2.3). Since the survival time at these sub-zero temperatures is so low, this data was not converted to survivorship data expressed as 50% survival times.

Effect of desiccation on the survival of the
infective-stage larvae of T.tenuis

The numbers of infective-stage larvae that survived at each humidity are given in Fig. 2.4. Those incubated at 100% relative humidity (RH) showed little decline in survival over the ten weeks incubation period; however, most larvae had died by 7 weeks at 80% RH; by 6 weeks at 60% RH; and by 2 weeks at 35% and 25% RH.

Fig. 2.1a. The effect of temperature on the survival of the infective-stage larvae of T.tenuis.

(●) 0 C

(▲) 5 C

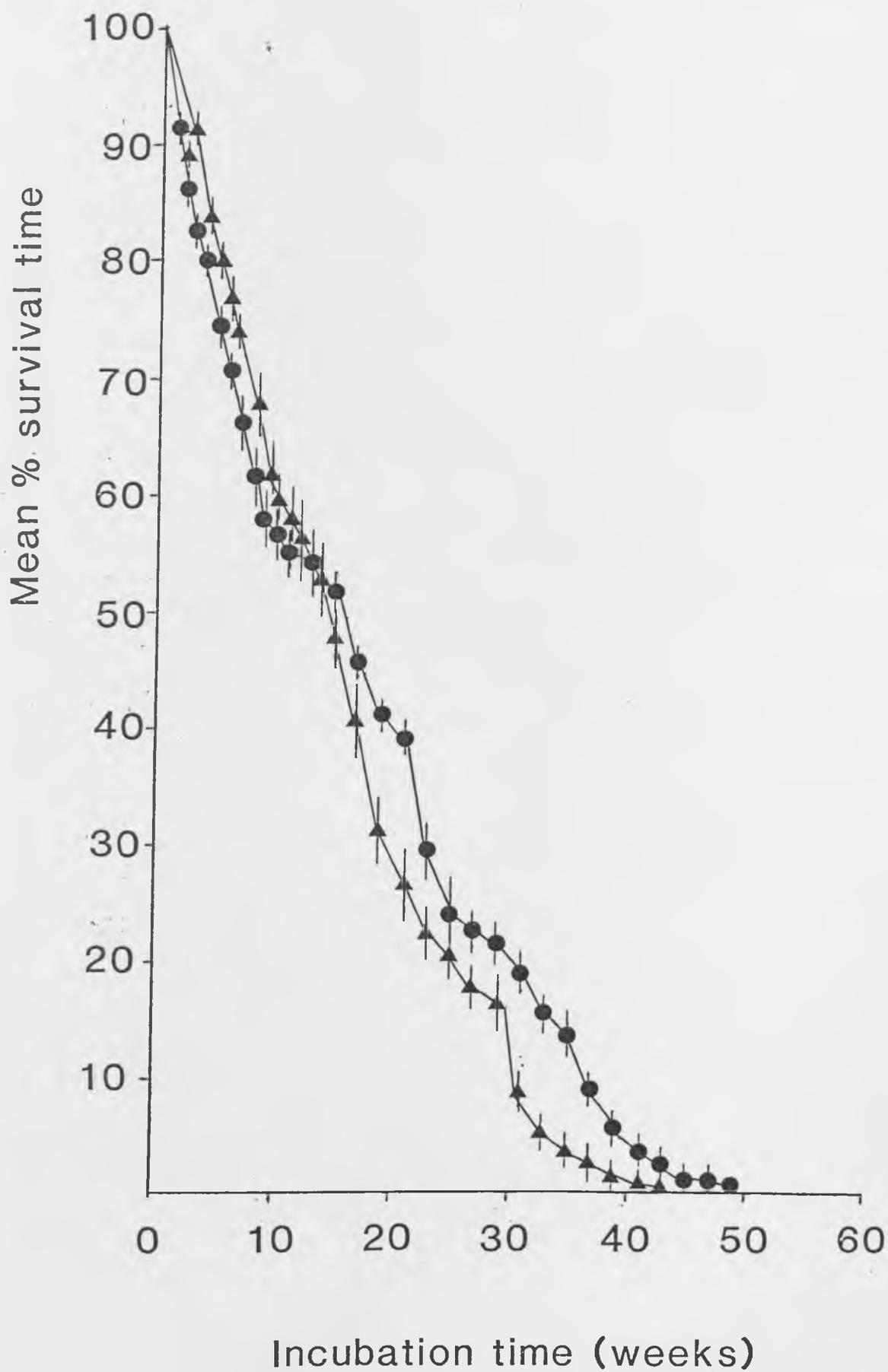


Fig. 2.1b. The effect of temperature on the survival of the infective-**st**age larvae of T.tenuis.

(■) 10 C

(◆) 15 C

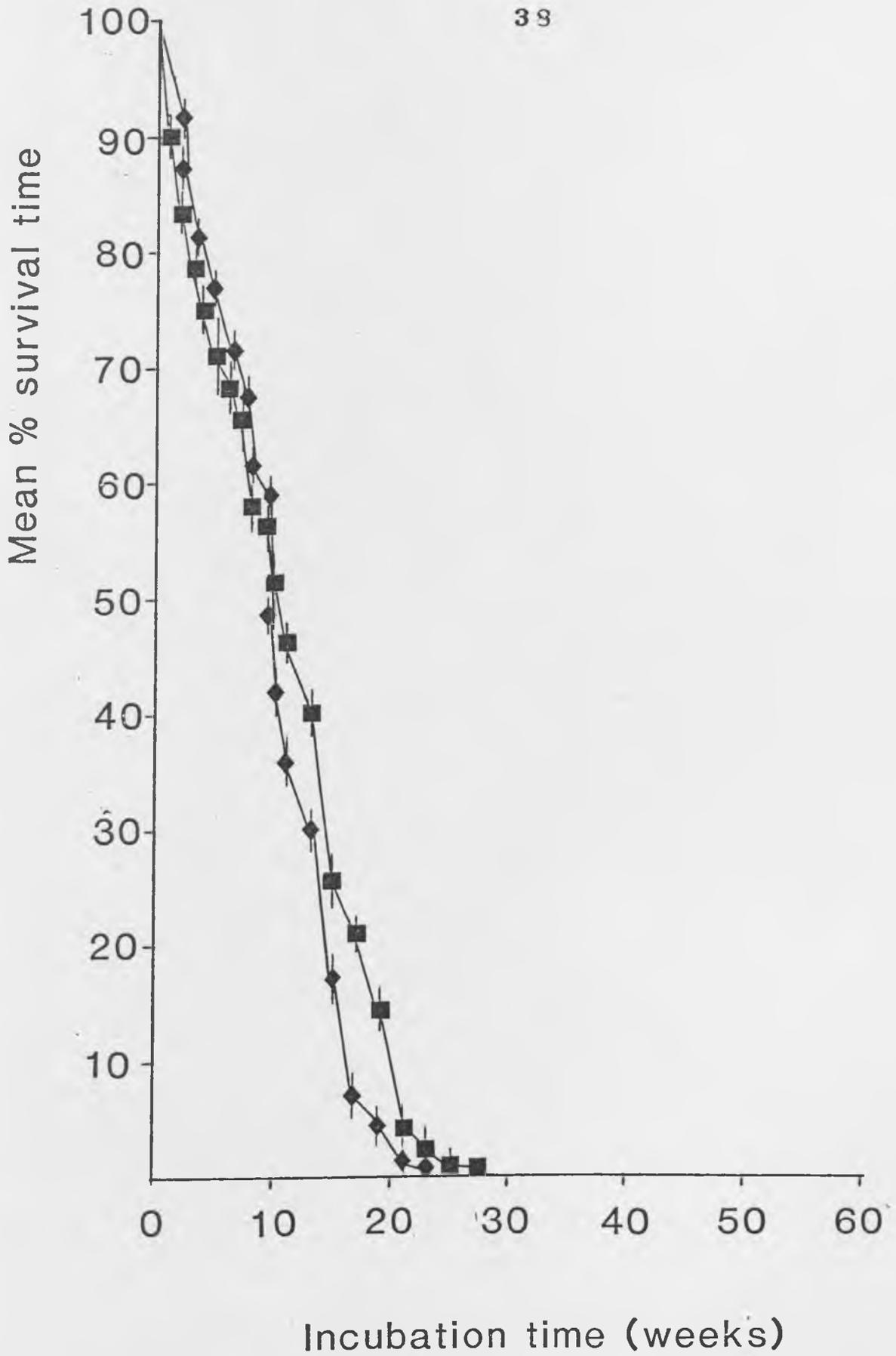


Fig. 2.1c. The effect of temperature on the survival of the infective-stage larvae of T.tenuis.

(Δ) 20 C

(\diamond) 30 C

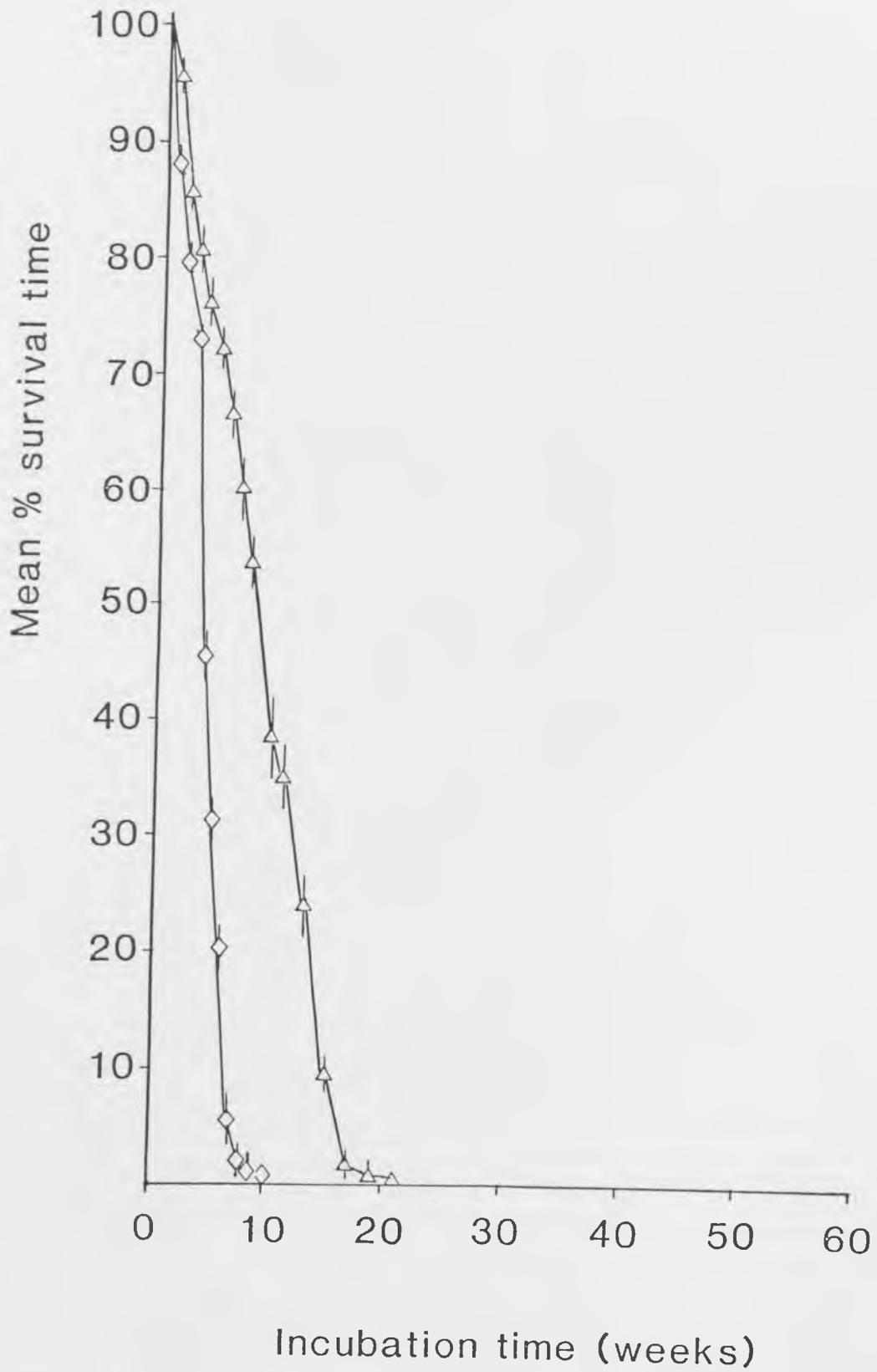


Fig. 2.2. The mean 50% survival times (S 50) of the infective-stage larvae of T.tenuis.

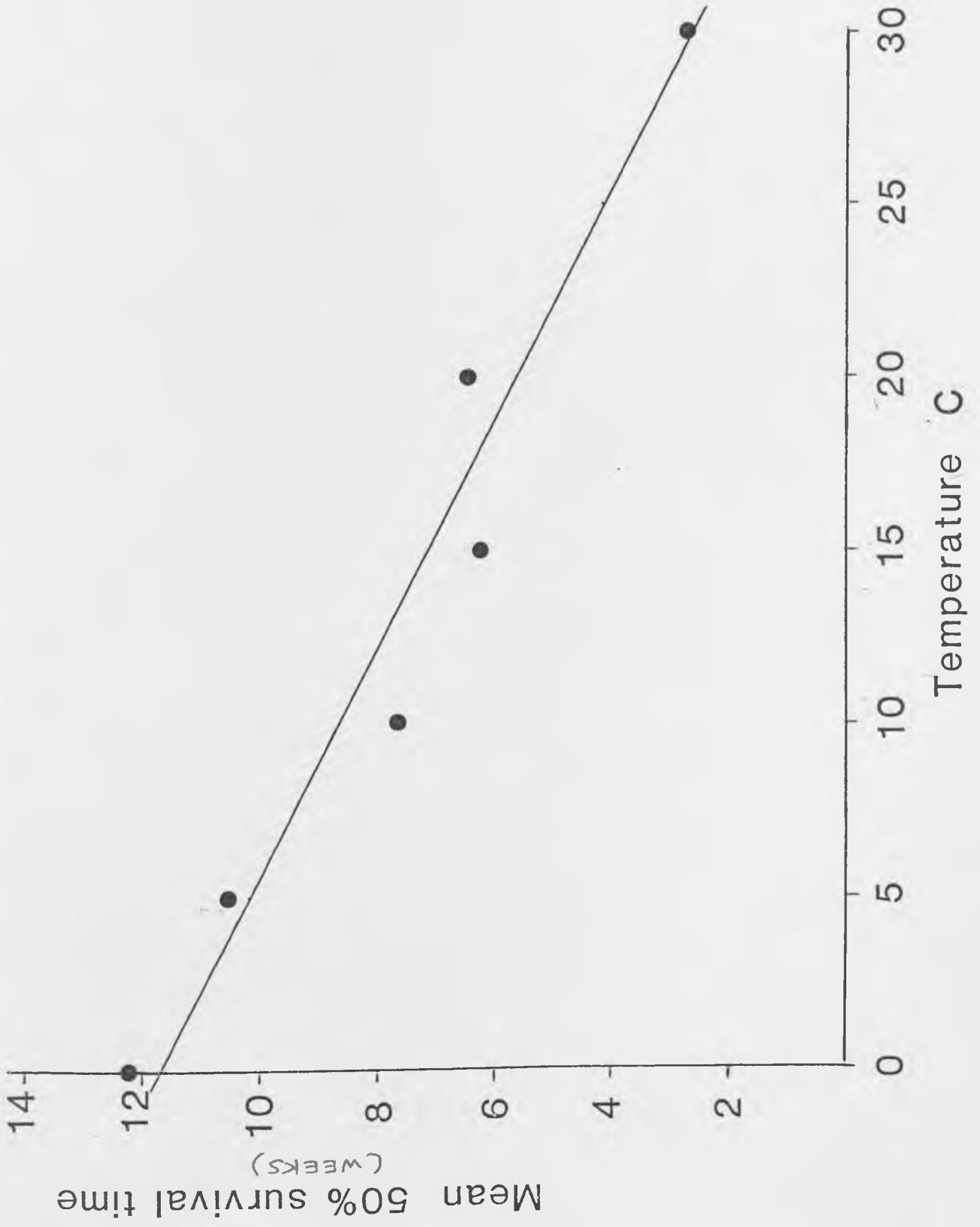


Fig. 2.3. The effect of sub-zero temperatures on the infective-stage larvae of T.tenuis.

(▲) -5 C

(●) -10 C

(■) -15 C

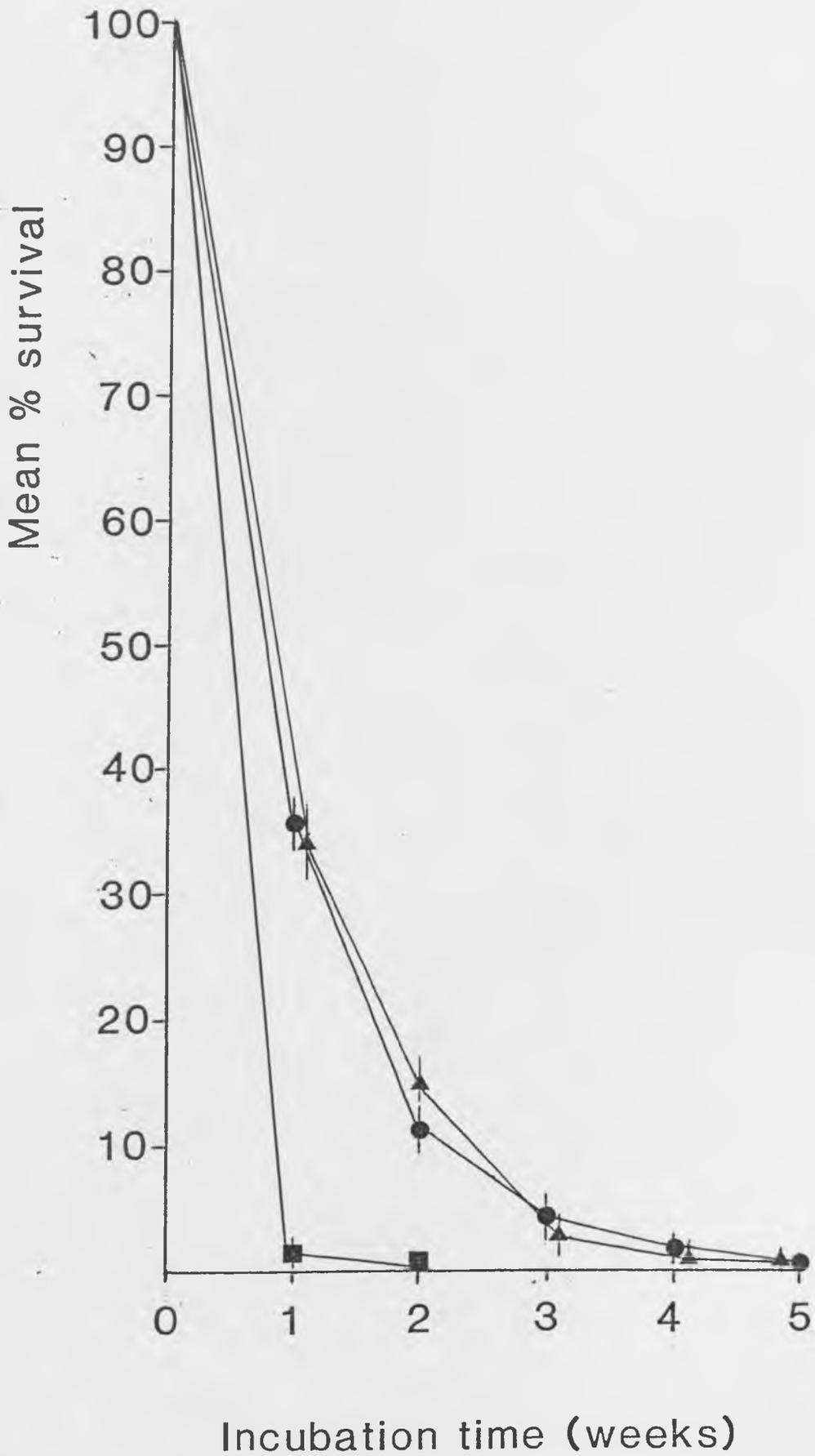
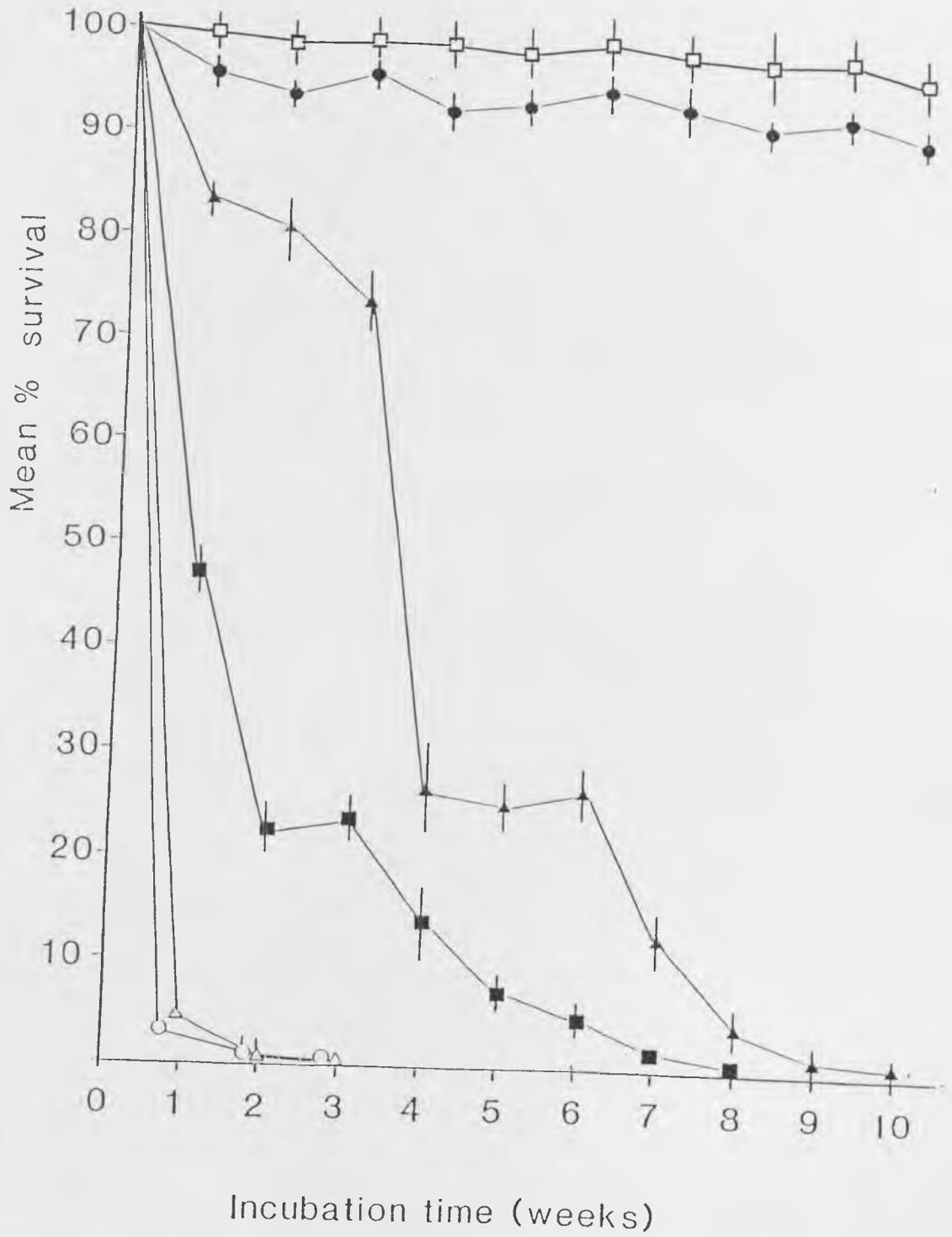


Fig. 2.4. The effect of desiccation on the infective-stage larvae of T.tenuis.

- (□) Controls
- (●) 100% R.H.
- (▲) 80% R.H.
- (■) 60% R.H.
- (△) 35% R.H.
- (○) 25% R.H.



DISCUSSION

Temperature may affect the infective-stage larvae of trichostrongyle nematodes in a number of ways. Firstly, temperature has a direct effect on the survival of the infective-stage larvae. Prasad (1959) examined the effects of both temperature and humidity on the free-living stages of Trichostrongylus retortaeformis and found an increased tolerance of the infective-stage larvae to lower temperatures than to higher temperatures. All larvae were killed after incubation in tap water at 0 C for 5-6 weeks all larvae were killed; 90% of the larvae survived for 13 weeks at 5C; 74% survived at 15 C; 53% survived at 20 C; 37% survived at 25 C; and 19% survived at 30 C. Infective-stage larvae incubated at 35 C survived for less than 2 weeks while those incubated at 40 C survived for only 10-12 days. Todd, Levine & Boatman (1976) found that one percent or more of the infective-stage larvae of Haemonchus contortus survived for 256 days at 4 C; for 128 days at 20 C; for 64 days at 25 C and 35 C; and for only 4 days at 45 C. In a more recent study, Boag & Thomas (1985) examined the effect of temperature on the survival of the infective-stage larvae of a number of species of nematode, and expressed survival as median survival times. The median survival times at 5 C for H.contortus, Cooperia oncophora, T.colubriformis, T.axei, Ostertagia circumcincta and O.ostertagia were in excess of 500 days. T.axei, T.colubriformis and T.retortaeformis were found to differ in their median survival times, T.colubriformis surviving for a longer period of time at all temperatures (5, 10, 15,

20, 25 and 30 C,) than either T.axei or T.retortaeformis. Hence, there are intrinsic differences in the survival of the infective-stage larvae of trichostrongyle nematodes over a range of temperatures. In most studies, the larvae of Ostertagia sp have been recorded as the most resistant to changes in temperature. T.tenuis however, has a much shorter survival time at 0, 5, 10, 15, 20, and 30 C than most other trichostrongyle species. The median survival times at 5 C of T.axei, T.colubriformis, and T.retortaeformis are approximately 550, 708 and 315 days respectively, compared with a 50% survival time at 5 C of approximately 40 days for T.tenuis. At 30 C, T.axei, T.colubriformis, and T.retortaeformis survive for approximately 78, 95 and 31 days, respectively, compared with a 50% survival time of only 14 days for T.tenuis at this temperature.

Secondly, temperature has an effect on the rate of development of the eggs and larval stages of trichostrongyle nematodes. Silverman and Campbell (1957) examined the embryonic and larval development of H.contortus, under a range of temperature and moisture conditions, and found that the developmental rate of the eggs and the larval stages varied with temperature. At a constant temperature of 21.7 C, and with adequate moisture and temperature, freshly passed H.contortus eggs required 5 days before the first of them reached the third, infective, larval stage. Wang (1966) examined the effects of temperature and cultural methods on the development of the free-living stages of T.colubriformis and showed that eggs

hatched and developed to infective-stage larvae at temperatures between 10 and 30 C, with the rates of embryonation, hatching, and larval development generally increasing with temperatures up to 30 C. Mirzayans (1969) followed the development of the eggs of T.axei at constant temperatures of 5, 10, 15, 20, 27, 30 and 35 C and found that eggs developed only to first-stage larvae at 5 C, and that the rate of development increased from 10 C to 27 C, with 27 C being the optimum temperature for development. Wilson (1979) found that the optimum temperature for the development of eggs to infective-stage larvae of T.tenuis was 27 C.

Thirdly, temperature may affect the behaviour of the infective-stage larvae of trichostrongyle nematodes. This aspect has been examined in relatively few studies, compared with the many publications on the influence of temperature on larval survival and development. Wharton (1981a) investigated the effect of temperature on the behaviour of the infective-stage larvae of T.colubriformis and found that larvae responded to sudden or gradual changes in temperature by a coiling response, such behaviour reducing the rate of water loss from the larvae and so reducing the risk of desiccation. This coiling behaviour in response to desiccation was not examined in this study. However, scanning electron microscopy of the infective-stage larvae of T.tenuis (Chapter 4) has shown that they have the capability to coil, but whether this is a response to desiccation is not known.

Finally, differences in the survival, development and behaviour of the infective-stage larvae of trichostrongyle nematodes, in response to temperature, cause differences in the ecology of infective-stage larvae of these nematodes and these differences have been used to interpret their different epidemiological patterns. The ecology of free-living stages of trichostrongyle nematodes has been extensively studied (Rogers, 1939; Kates, 1950; Gibson & Everett, 1967; Callinan, 1978a, b) and is further examined in Chapter 4.

The effect of desiccation and sub-zero temperatures on the infective-stage larvae of trichostrongyle nematodes has also been studied. Ellenby (1968) found that the ensheathed larvae of H.contortus survive desiccation better than the exsheathed forms. Andersen & Levine (1968) found that the desiccation of the infective-stage larvae of T.colubriformis was beneficial to their survival at temperatures below freezing and at 35 C and 50 C. However, Schmidt, Todd & Levine (1974) found that infective-stage larvae in non-desiccated control groups at 20 C or 30 C survived for longer periods of time than larvae incubated at 50% or 70% RH, at the same temperatures. Wharton (1982) studied the survival of the free-living stages of T.colubriformis after desiccation and found that infective-stage larvae survived prolonged exposure to desiccation at 33-98% RH at 20 C, with 50% survival times of between 58-164 days at this humidity. The infective-stage larvae of T.tenuis appear to be highly susceptible to desiccation and survive for up to 8 weeks at

80% RH and for up to only 2 weeks at 25% RH.

The effect of sub-zero temperatures on the survival of the infective-stage larvae of trichostrongyles has not been examined to any great extent. Wertejuk (1959) found that laboratory-reared infective-stage larvae of Ostertagia sp, H.contortus, Trichostrongylus sp, Oesophagostomum venulosum, Chabertia ovina, and Cooperia sp could overwinter on pasture in Poland when the lowest temperature was -28.1 C and that the surviving larvae of H.contortus and O.circumcincta were subsequently able to infect and develop to maturity in lambs. Anderson & Levine (1968) showed that after desiccation, half or more of the infective-stage larvae of T.colubriformis survived for more than 1 day at -95 C and -28 C in tap water, whereas only 2 to 6% of non-desiccated larvae in a control group survived; and in groups of larvae incubated at -10 C, approximately half of both the desiccated larvae and the non-desiccated controls were alive after 1 day. In a later paper, Andersen, Levine & Boatman (1970) showed that the infective-stage larvae of T.colubriformis can survive freezing conditions on the pasture. Wharton, Young & Barrett (1984) indicated that the ability to survive sub-zero temperatures may be an important factor in the overwintering of many free-living nematodes and the free-living stages of parasitic nematodes. They showed that the infective-stage larvae of T.colubriformis and the fourth-stage larvae of Ditylenchus dipsaci are freezing-susceptible but can avoid freezing by supercooling.

Temperature and humidity on the grouse moor are determined by a range of environmental factors and these factors will determine the mortality rates of the infective-stage larvae of T.tenuis. Although weather conditions determine the temperature and humidity on the moor, the survival of eggs, pre-infective-stage larvae and infective-stage larvae is influenced by the conditions within a specific micro-environment in which the larvae exists, that is, the caecal droppings in the case of the eggs and pre-infective-stage larvae, and the environment surrounding the caecal droppings, especially the surrounding vegetation, in the case of the infective-stage larvae. Micro-environmental differences are most likely to occur with changes in humidity as opposed to temperature. The 50% survival times of infective-stage larvae of T.tenuis exposed to a range of temperatures between 0 C and 30 C are relatively low compared to those of other trichostrongyle species. Such low survival, however, may be compensated for by the high nematode burdens in the adult grouse, and subsequent high nematode egg output from infected birds throughout the year. Thus, large numbers of larvae may occur on the moor when conditions are suitable for their development. During the summer months, when environmental conditions are more suitable for larval survival, many infective-stage larvae will develop, will disperse onto the vegetation, and will be available for ingestion by grouse. During the winter months, when the temperature on many moors is low and often falls below freezing for long periods, nematode eggs will not develop

and any infective-stage larvae present will be killed.

Changes in humidity may have a more significant effect on the survival of T.tenuis larvae than changes in temperature, since infective-stage larvae are highly susceptible to desiccation. Pre-infective stage larvae develop within the protective micro-environment of the caecal faeces and are thus protected against gross changes in humidity, while those infective-stage larvae that migrate to the heather leaflet will be similarly protected against gross changes in humidity, as the larvae remain in the leaf roles of the heather plants (Chapter 4). Migration between the caecal faeces and the heather leaflet is the most likely phase of the life-cycle where desiccation could have a significant effect on the survival of the infective-stage larvae, since a film of moisture is required for this migration and the larvae could be exposed to drying conditions during this migration. During the hot summer months on a grouse moor a decrease in humidity may significantly decrease larval migration and survival, and subsequently decrease the transmission of the infective-stage larvae of T.tenuis to the red grouse.

Hudson, Dobson & Newborn (1985) suggested that parasitism was a key element in the pattern of fluctuations in grouse numbers and suggested that populations could be divided into cyclic and non-cyclic types, cyclic populations being associated with greater nematode burdens and higher rainfall than non-cyclic populations. In this model it was proposed that low humidity reduces the survival of the

infective-stage larvae, resulting in lower levels of infection and reduced effects on host fecundity than in non-cyclic populations. By modelling, it was shown that the interaction of a long-lived, free-living stage of the life cycle of the nematode and a parasite-induced reduction in fecundity is an important factor in producing the cycles in grouse density. This work has shown that the infective-stage larvae of T.tenuis are highly susceptible to desiccation and even to relatively high humidity, thus the lower rainfall experienced on non-cyclic moors probably results in lower humidity and consequently poorer survival of the infective-stage larvae and a lower worm burden on these moors.

CHAPTER 3

FOOD RESERVES, AGEING AND INFECTIVITY OF THE
INFECTIVE-STAGE LARVAE OF T.TENUIS

SUMMARY

The amount of lipid in infective-stage larvae of T.tenuis kept for up to 10 weeks at temperatures between 0 C and 30 C was determined every 2 weeks by scanning microdensitometry. The lipid reserves were found to decline more sharply as the incubation temperature increased. This decline in lipid reserves was correlated to a decline in infectivity of the larvae in the domestic chicken. Larvae incubated at 15 C, 20 C or 30 C lost their infectivity faster than larvae incubated at 10 C, which lost their infectivity faster than larvae incubated at 0 C or 5C. The effect of a decline in lipid reserves and in infectivity of the infective-stage larvae is discussed with reference to the behaviour of the larvae and the establishment of the parasite in red grouse.

INTRODUCTION

The longevity of the infective-stage larvae of trichostrongyle nematodes is of epidemiological significance and some of the environmental factors affecting larval survival have been discussed in the previous chapter. However, the infective-stage larva of trichostrongyles is a non-feeding stage and, presumably, the food reserves which have accumulated within the larva during its development will limit its ability to survive for long periods of time (Nwosu, 1979).

The infective-stage larvae of parasitic nematodes contain large amounts of lipid (Frayha & Smyth, 1983) which are stored mainly in the intestinal cells but also in other tissues (Zeletzki, 1965; Kozar & Seniuta, 1974; Rubin, 1977; Rubin & Trelease, 1975). There is a progressive loss of these lipid reserves during the life of the infective-stage larva and this is indicative of physiological ageing (Rogers, 1939; Croll & Matthews, 1973). The physiological age of the infective-stage larvae is of importance since it is a major factor determining the infectivity of the larva. Chronological age is not of such great importance, since larvae may exist for a short period of time but in conditions which may cause them to age rapidly in a physiological sense (Rogers, 1939).

The rate of utilisation of these lipid reserves is a major factor determining larval longevity (Nwosu, 1979).

The rate of activity and the rate of lipid utilisation have been correlated with ageing in the infective-stage larvae of Ancylostoma caninum (Giovannola, 1936; Rogers, 1939; Croll & Matthews, 1973); Haemonchus contortus (Rogers, 1940); Necator americanus (Payne, 1922, 1923; Cort, 1925; Giovannola, 1936) and Nippostrongylus brasiliensis (Haley & Clifford, 1958, 1960). The age of infective-stage larvae of parasitic nematodes has also been shown to be closely correlated to their infectivity (Roger, 1939; 1940; Elliot, 1954).

The infective-stage larvae of T.tenuis develop from free-living first-stage and second-stage larvae that feed upon bacteria within the caecal droppings. During this time, the developing larvae synthesise the food reserves which provide the energy source for the non-feeding, infective-stage larvae. Energy thus stored is used as the larvae migrate out of the faecal pat and onto the surrounding vegetation (Hudson, 1986a). If conditions are suitable, they then migrate onto the tips of young heather plants and are ingested by red grouse, when these birds feed upon the heather (Lovat, 1911).

Presumably the infective-stage larvae ingested throughout the year by red grouse on the moor have been present on the heather for different periods of time, have experienced different temperature regimes and are of different chronological and physiological ages. Consequently, they will have varying capacities to cause an infection in the red grouse. Since the infectivity of the larvae will be a

major factor influencing the numbers of adult nematodes which become established in the grouse, data on the ageing and infectivity of larvae is of importance in our understanding of the epidemiology of T.tenuis in red grouse.

The work described in this chapter aimed to examine the effect of temperature on the physiological ageing, as measured by the decline in lipid content, of the infective-stage larvae of T.tenuis and then to relate the physiological age of the larvae to their infectivity in a model host, the domestic chicken.

MATERIALS AND METHODS

Collection of Trichostrongylus tenuis infective-stage larvae

The collection and culture of infective-stage larvae used for this work was as described in Chapter 2.

Ageing Procedure

Infective-stage larvae were collected from laboratory cultures and were used immediately. Six groups, each of 5000 larvae in 25 ml of distilled water in 50 ml plastic beakers, were incubated in temperature-controlled incubators (Gallenkamp Compenstat Incubator) at either 0, 5, 10, 15, 20 or 30 C for periods of up to 10 weeks. Each week, half of the distilled water was withdrawn, and fresh distilled water added; at higher temperatures, fresh distilled water was also added to compensate for loss due to evaporation.

Experimental birds

Chickens in this experiment were of the same type, the same age and were maintained under the same conditions as described in Chapter 6.

Infection procedure

After incubation for the desired period of time, batches of 500 infective-stage larvae were removed from groups of larvae incubated for either 0, 2, 4, 6, 8 or 10 weeks at either 0, 5, 10, 15, 20 or 30 C and were administered

orally to chickens. Each chicken was given 500 larvae and there were 5 chickens in each group.

Recovery of worms

Chickens were killed 9 days post-infection and worms were recovered from both caeca, as described in Chapter 6.

Staining procedure for neutral lipid reserves

0.7 g Oil Red O (G.T. Gurr, Michrome No 21600) was dissolved in 200 ml of analar absolute propan-2-ol (BDH) and left overnight. The stain was then filtered and 180 ml were diluted with 120 ml of distilled water before being left overnight at 4 C and filtered again. The stain was filtered a third time before use; it could be stored for up to 8 months at room temperature.

Approximately 100 larvae were removed from the groups of larvae kept at each temperature, at the same time that larvae were removed for the infectivity experiments i.e. at 0, 2, 4, 6, 8 or 10 weeks of incubation. The larvae were placed in a flat bottomed watch glass and excess water was removed from around them by means of a microcapillary tube (Drummond). Boiling stain was added immediately. A glass cover was placed on the watch glass and the specimens were kept for 30 minutes at 55 C on a hot plate. This stains the neutral lipid reserves of the larva a bright reddish-orange colour. Larvae were then transferred from the stain, by means of a microcapillary tube, into a watch glass containing equal volumes of glycerol and 70% ethanol. The specimens were removed from this watch glass after the

alcohol had evaporated and were passed through glycerol before mounting in glycerol on grease-free slides.

Measurement of neutral lipid reserves

A scanning microdensitometer (Vickers M86) was used to quantify the stained neutral lipid reserves in the infective-stage larvae. This technique offered a satisfactory method for the measurement of heterogeneously distributed stain within the nematodes (Rost, 1980; Storey, 1981). A measurement beam, of a selected wavelength, measures the relative absorbance of light by the stain in the specimen in a particular light path. During measurement, this beam scans progressively throughout the field of view and an integral is obtained that is proportional to the total amount of stain present in an area within the objective field. Thus, this technique allowed whole mounts of stained larvae to be individually and rapidly measured.

The scanning frame size of the instrument was set at $x=2$, $y=3$ and a standard mask slit of 4.5×11.5 mm was used, which, in effect, limited the area of measurement with a 20 objective to $180 \times 470 \mu\text{m}$. The larva mounted on the slide was placed on the specimen stage so that the image of the larva just fitted the virtual image of the mask slit. A wavelength of 517 nm gives the highest optical density reading for the stained specimens (Croll, 1972) and this was used in all measurements. A spot size of 2 and a slit width of 70 were found to be optimal for this work. Before measurement of the lipid content of the larvae the scanning

spot was placed to the side of the larva within the unmasked area and the optical density of this light path was set to a relative optical density of zero. The area containing the larvae was then scanned 5 times and the accumulated total integral was obtained for each larva examined. Readings were taken from 20-30 individual larva from each group of larvae at each temperature. The amount of lipid present in the larvae is expressed as microdensitometer units.

RESULTS

Effect of temperature on lipid content

Photographs of infective-stage larvae stained with oil red 'O' after incubation at 15 C for 2 and 8 weeks are shown in Figs. 3.1a and 3.1b. The mean lipid content of larvae incubated at a range of temperatures for 0 to 10 weeks is given in Fig. 3.2. The amount of stainable lipid present in the larvae declined over the ten week period, this decline being dependent upon the incubation temperature. Larvae incubated at 0 C showed a relatively slow decline throughout the first four weeks of incubation, after which lipid levels decreased rapidly. In larvae incubated at 5, 10, 15, 20 and 30 C there was a rapid decline in lipid levels over the first two weeks of incubation, followed by a slower decline up to 10 weeks. This decline was most marked in larvae incubated for 10 weeks at 30 C; the stainable lipid remaining after 10 weeks at this temperature barely being sufficient to give a reading on the microdensitometer.

Effect of temperature on the infectivity of larvae in the domestic chicken

The results of the experiments conducted to determine the infectivity of larvae aged for different periods of time at different temperatures are shown in Fig. 3.3. Infectivity declined over a ten week period at all temperatures. The decline in infectivity was correlated to the lipid levels of the infective-stage larvae at all temperatures (Fig.

3.4) (0 C, $r = 0.8160$, $p < 0.02$; 5 C, $r = 0.9323$, $p < 0.001$;
10 C, $r = 0.9436$, $p < 0.001$; 15 C, $r = 0.9436$, $p < 0.001$; 20
C, $r = 0.9780$, $p < 0.001$; 30 C, $r = 0.9648$, $p < 0.001$).

Fig. 3.1a. Infective-stage larva of T.tenuis stained with oil red 'O' following incubation at 15 C for two weeks. Note the large amount of stainable lipid (sl) in the intestinal cells. Note also the characteristic serrated cuticle (c) of the ensheathed larva.

Fig. 3.1b. Infective-stage larva of T.tenuis stained with oil red 'O' following incubation at 15 C for eight weeks. Note the considerable depletion of stainable lipid (sl).

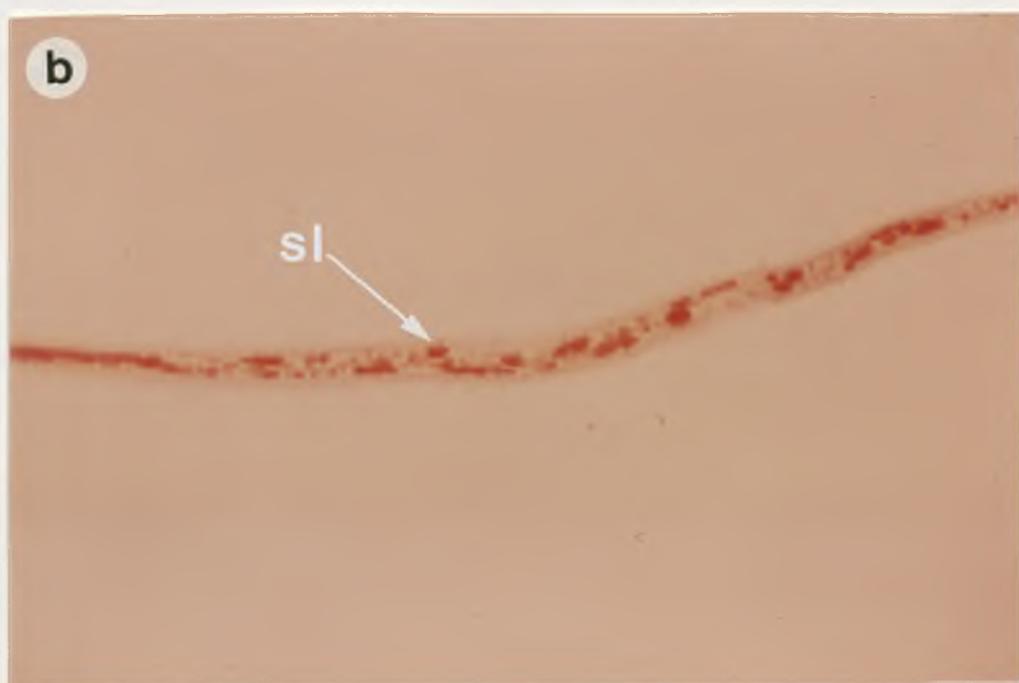
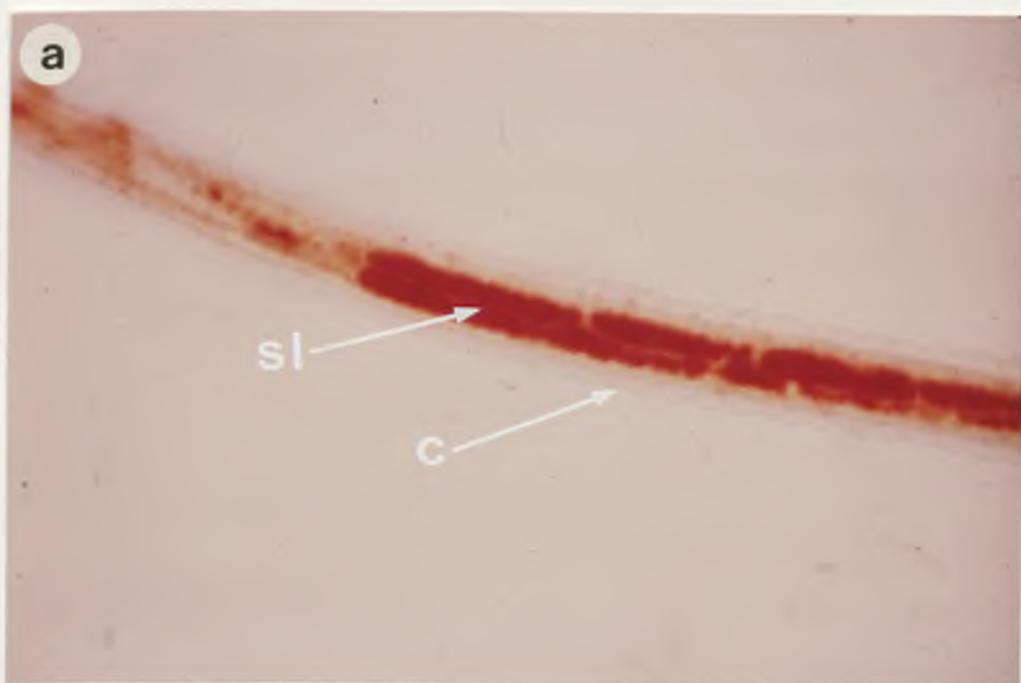


Fig. 3.2. The amount of lipid, stained with oil red 'O', in infective-stage larvae of T.tenuis incubated at different temperatures for periods of time up to 10 weeks.

(●) 0 C

(▲) 5 C

(■) 10 C

(▼) 15 C

(◆) 20 C

(△) 30 C

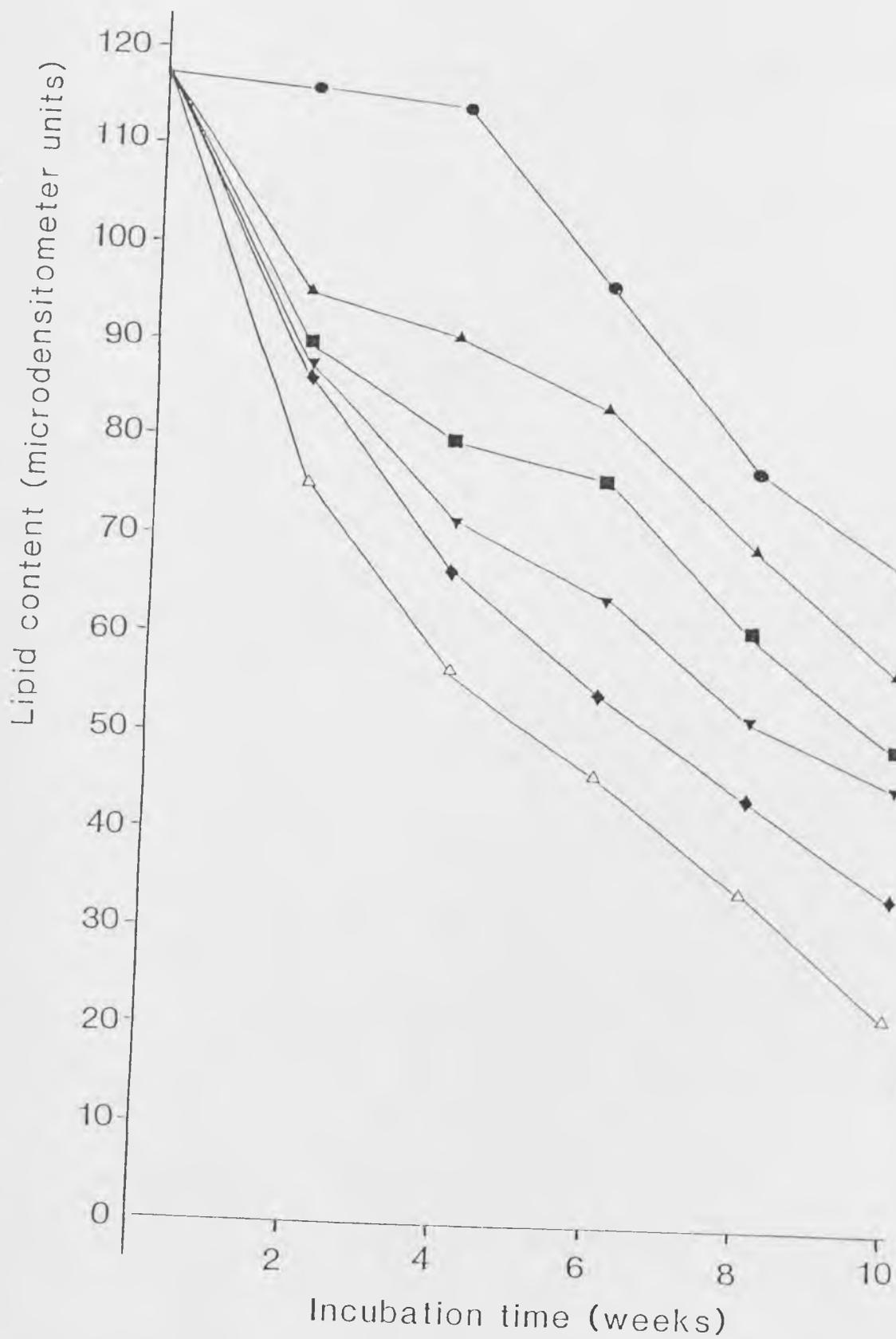


Fig. 3.3. The effect of temperature on the infectivity of the infective-stage larvae of T.tenuis.

- (●) 0 C
- (▲) 5 C
- (■) 10 C
- (▼) 15 C
- (◇) 20 C
- (○) 30 C

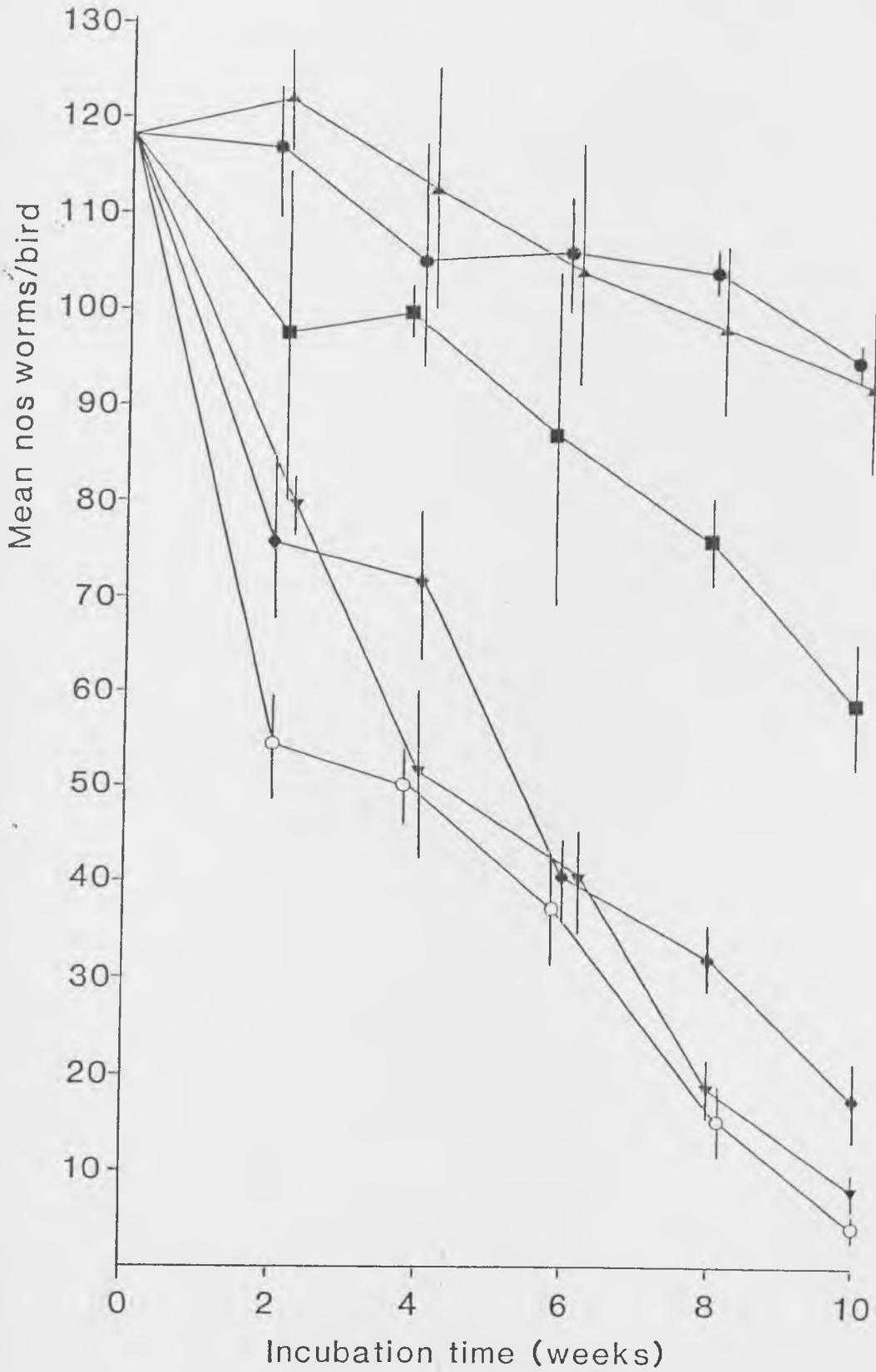
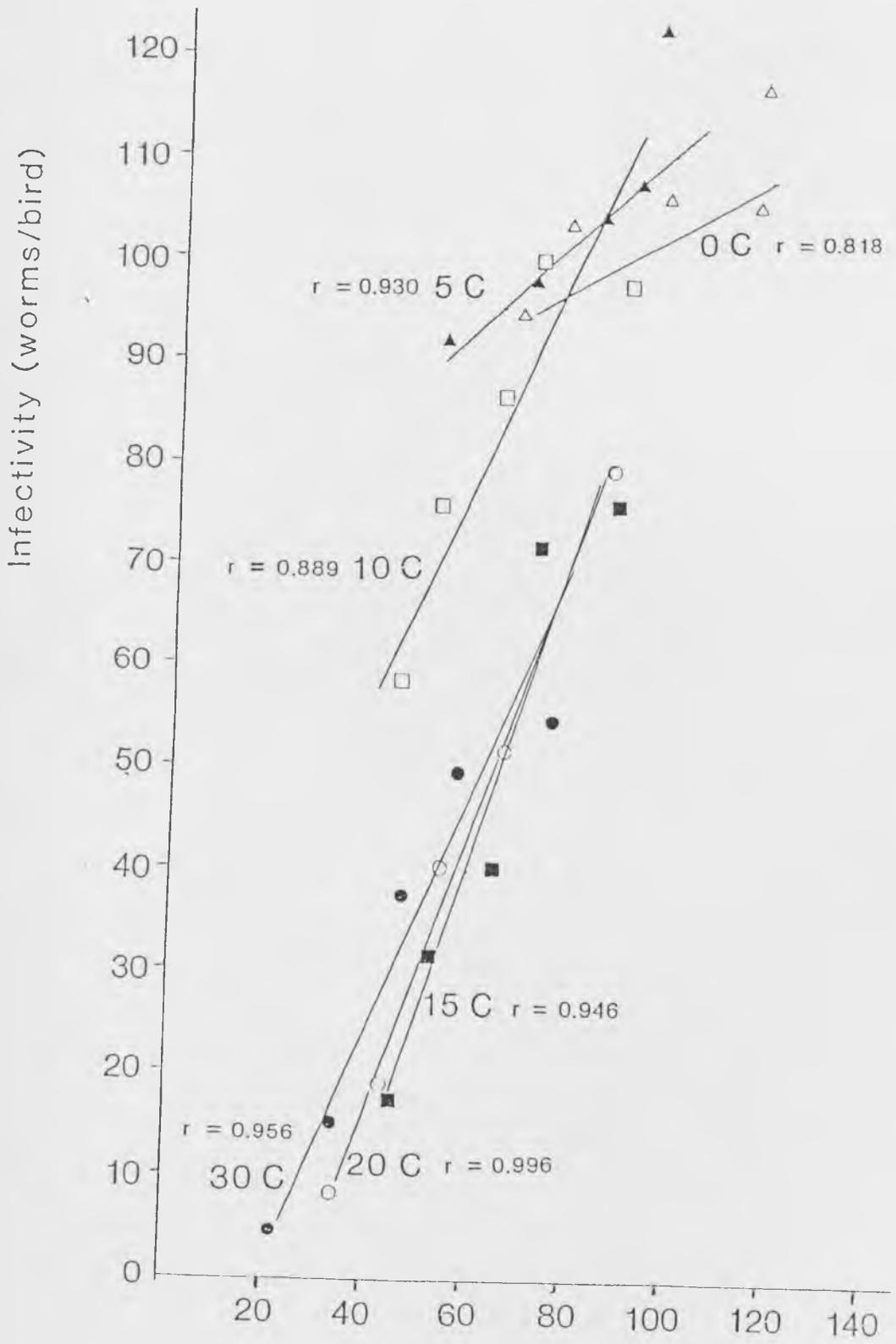


Fig. 3.4. The correlation between lipid reserves of the infective-stage larvae of T.tenuis incubated at a range of temperatures for different periods of time and their infectivity in the domestic chicken. Lines fitted by regression analysis.

- (Δ) 0 C
- (▲) 5 C
- (□) 10 C
- (■) 15 C
- (○) 20 C
- (●) 30 C



Lipid reserves (microdensitometer units)

DISCUSSION

Croll & Matthews (1973) suggested that there are three fundamental changes which occur during the ageing of the infective-stage larvae of the hookworm Ancylostoma tubaeforme; these are changes in lipid utilization, changes in the metabolism of the larvae, and changes in larval behaviour. Such changes have also been shown to occur during ageing in the larvae of a number of other species of parasitic nematode. Several authors have described the decline of lipid or fat reserves with time in the infective-stage larvae of animal parasitic nematodes (Payne 1922; 1923; Giovannola, 1936; Rogers 1939; 1940; Wilson 1965; Barrett, 1968; 1969; Clark, 1969) and of plant-parasitic nematodes (Van Gundy, Bird & Wallace, 1967; Storey, 1981). The results from the work with T.tenuis larvae are similar to the results obtained by these authors as it has been shown that the age of the infective-stage larvae is related to their lipid content, and that a decline in lipid content occurs quicker at higher than at lower temperatures. Other workers (Rogers, 1939; 1940; Elliot, 1954) have shown that the lipid content and age of parasitic nematode larvae are closely correlated with their infectivity. McKenna (1973) showed that after storage for 160 days at 5 C, the infective-stage larvae of H.contortus have a considerably higher infectivity in lambs than larvae stored for the same period of time at 21 C; while Mallet & Kerboeuf (1985) demonstrated that the infective-stage larvae of T.colubriformis, when stored at 24 C, maintained a relatively high infectivity for lambs

(68%) over the first 6 weeks of storage, but infectivity then declined rapidly to only 12 % after 22 weeks of storage. A decline in lipid reserves has also been correlated to a decline in infectivity in plant parasitic nematodes. Storey (1981) demonstrated that the utilization of neutral lipid was correlated to a decrease in the levels of invasion of tomato seedlings by Globodera rostochiensis.

A series of metabolic changes are known to occur during the ageing process of larval nematodes. Barrett (1969) detected a high respiratory quotient (R.Q.) in infective-stage larvae of Strongyloides ratti, indicating that lipid was the main metabolite during the ageing process. This work also showed that as the infective-stage larvae of S.ratti aged, their infectivity decreased sharply, and that this decrease in infectivity was accompanied by an exponential decrease in the rate of activity of the larvae. Costello & Grollman (1958) suggested that the metabolic rate of infective-stage larvae was inversely related to their longevity, with lipid levels declining relatively slowly at low temperatures due to a relatively low metabolic rate, while at higher temperatures, a higher metabolic rate results in a rapid decline of stored neutral lipid and a consequent reduction in longevity.

The loss of lipid is only one of the many physiological factors involved in the process of ageing and loss of infectivity in nematodes. Van Gundy, Bird & Wallace (1967) reported that esterase and acid phosphatase enzymes had

lower activity in aged than in fresh larvae of the plant-parasitic nematode Meloidogyne javanica; while Gershon & Gershon (1970) and Erlanger & Gershon (1970) detected a decrease in enzyme activity with increasing age in the nematode Turbatrix aceti, under axenic conditions. In a later paper, Zeelon, Gershon & Gershon (1973) found a decrease in activity of isocitrate lyase in T.aceti and Caenorhabditis briggsae with increasing age. Wilson (1965) found changes in both the lipid levels and the nitrogen content of infective-stage larvae of Nippostrongylus brasiliensis when they were aged at a constant temperature. Nwosu (1978b) found age-related changes in esterase and acetylcholinesterase (ACHE) activity in the infective-stage larvae of Ancylostoma tubaeforme, with activity decreasing as the age of the larvae increased. Presumably, similar physiological changes occur during the ageing of the infective-stage larvae of T.tenuis. It may be a combination of these factors, together with the decline in the lipid reserves, which causes the infective-stage larva to age physiologically and subsequently lose its infectivity.

The decline in lipid reserves, and the related loss of infectivity in infective-stage larvae, is possibly related to changes in locomotory activity. Croll & Matthews (1973) suggested that there is not a direct 'cause and effect' correlation between the lipid content of larvae and their physiological condition, and argued that it was unsatisfactory to estimate the physiological age of larvae using lipid content alone, without reference to their

energy expenditure. It was demonstrated that locomotory activity of the infective-stage larvae of A.tubaeforme declined with age and Nwosu (1978a) quantified lipid changes in the infective-stage larvae of A.tubaeforme after measured periods of activity, and demonstrated that 'excessive activity' resulted in a significant depletion of lipid reserves. However, larvae stored under constant conditions, similiar to those in this work, are believed to be relatively inactive (Croll & Al-Hadithi, 1972).

On the moor, changes in the behaviour of the larvae, brought about either by changes in temperature or physiological age, will have an important effect on the transmission of the nematode. At low temperatures, the infective-stage larvae will be less mobile and will remain in or near the caecal droppings. They will thus retain their lipid reserves and in doing so may retain their viability and infectivity for the host for a longer period of time. Under such conditions, they are less likely to ascend the heather plants and consequently may not be ingested by the next host. At higher temperatures, however, the larvae will be more active and this will enable them to migrate out of the caecal droppings and ascend surrounding vegetation, including heather plants, and in so doing, they will make themselves available for ingestion by the grouse. The penalty for this activity is that such larvae will use up their lipid reserves more quickly, and thus will have a reduced chance of establishing an infection once eaten by the grouse. Presumably, the larvae require a minimum amount of energy,

in the form of lipid reserves, to enable them to carry out exsheathment and migration in the host before they begin to replenish their food reserves by feeding within the host. If the lipid remaining within the larvae, following exsheathment and migration is below this critical level, then the larvae may fail to establish. The rapid decline of lipid reserves in the infective-stage larvae over the first two weeks of incubation could be related to their higher motility at this stage of their existence. The more gradual decline in the lipid reserves after the second week could indicate that the larvae have become less motile. On the moor, following suitable environmental conditions for development and migration, many infective-stage larvae could have reached the heather leaflets within their first two weeks of existence and reduced motility thereafter would be advantageous as it would tend to keep the larvae within the heather leaflet and conserve their energy reserves for the next stage of the life cycle. The larvae therefore have to reach a balance between using up their food reserves during migration to reach a suitable location on the heather, and conserving their food reserves to enable them to exist on the heather until they can successfully develop to maturity after they are eaten by a grouse. Clearly, many older larvae are alive and capable of motility after several weeks at temperatures above 10 C, but their ability to infect the grouse will have declined by 40% if they have been kept at 10 C and by 80% to 90% if the larvae have been kept at 15 to 20 C for 10 weeks.

As yet, no detailed information is available on the rates

of migration of the infective-stage larvae of T.tenuis from the caecal droppings to the heather plants, on the motility of the larvae as they age at different temperatures, or on how long infective-stage larvae remain viable and infective on the moor. It has been suggested in Chapter 4, that the infective-stage larvae of T.tenuis migrate away from the caecal pat, where they develop, and disperse into the vegetation surrounding the caecal droppings on the moor, since very low numbers of larvae were found in the immediate vicinity of the caecal pat, even though the caecal droppings themselves have been shown to contain many thousands of nematode eggs. Although this migration onto the heather plant is essential for the efficient transmission of T.tenuis, as red grouse are highly selective feeders and will feed only on certain stands of heather, it may mean that the larvae ingested have reduced lipid levels and subsequently have a reduced potential to infect the grouse.

A high level of infectivity in the infective-stage larvae of parasitic nematodes will lead to increased establishment of the nematode in the host, but does not necessarily indicate a subsequently high nematode egg production from the nematodes which become established. Mallet & Kerboeuf (1985) demonstrated that infectivity of the infective-stage larvae of T.colubriformis stored for between 0-6 weeks at 24 C was significantly greater than that of larvae stored for up to 22 weeks at the same temperature, but the females resulting from the larvae stored for up to 6 weeks produced 200 epg/1000 female nematodes, while those resulting from

larvae stored for 9 weeks, produced 400 epg/female nematode. Thus, there was a higher production of eggs by adults established from larvae with reduced infectivity.

It must not be forgotten that all infectivity experiments in this work were conducted using laboratory-reared domestic chickens. This was because of difficulties encountered in obtaining and maintaining red grouse in the laboratory. The course of T.tenuis infection in the domestic chicken is somewhat different from that in the red grouse (Chapter 6). However, this should not affect the infectivity aspects of the life cycle since the larvae of T.tenuis do become established in chickens. It appears to be mainly in the immunological response that the two hosts differ in their response to the nematode.

A range of factors, other than the lipid food reserves in the larvae, may affect larval vigour and their degree of retention of infectivity. Hansen, Oonyawaongse & Ackert (1951) suggested that the humid air and water content of the culture media in which the larvae develop may affect the rate of development and viability of the eggs of Ascaridia galli, while Todd, Crowds & Wyant (1952) suggested that incubation temperature and the nature of the egg-containing medium play a role in determining the infectivity of A.galli eggs. Haley & Clifford (1960) suggested that the vigour of the infective-stage larvae of N.brasiliensis is dependent upon the temperature, moisture and substrate medium in which the larvae develop, while Wang (1970) demonstrated that microorganisms in the culture

media effect the development of the infective-stage larvae of T.colubriformis. It is possible that the infectivity of the infective-stage larvae of T.tenuis is dependent not only on the lipid reserves within the larvae, but also upon other factors, such as the nature and abundance of the bacterial food source available to the pre-infective larval stages or upon the consistency of the caecal faeces, with liquid or semi-solid faeces perhaps facilitating the migration of the infective-stage larvae onto the heather, and in doing so, enabling the larvae to conserve lipid reserves.

This work has shown that temperature has a significant effect on the lipid reserves of the infective-stage larvae of T.tenuis and on the subsequent ageing and loss of infectivity of these larvae. However, the infectivity of the larvae is only one of a complex of factors that may affect the efficiency of transmission of T.tenuis to red grouse. The density, distribution and survival of the infective-stage larvae on the moor are also important factors that affect the transmission of T.tenuis and these aspects of the epidemiology of T.tenuis in red grouse are examined in the next chapter.

CHAPTER 4

THE OCCURRENCE OF THE INFECTIVE-STAGE LARVAE OF
T.TENUIS ON HEATHER AND A SCANNING ELECTRON MICROSCOPE
STUDY OF THE INFECTIVE-STAGE LARVA

SUMMARY

The occurrence of the infective-stage larvae of T.tenuis on the tips of heather plants growing beside caecal droppings of red grouse was examined over a 12 month period on a moor in Upper Swaledale, North Yorkshire. Relatively high numbers of larvae were found in the months from May to August but very few larvae were recovered during the winter months. The actual numbers of larvae recovered were low but, when taken in context with the large amount of heather a grouse consumes in a day, the estimated number of larvae that could be ingested by a young grouse prior to the shooting season showed a good correlation with the actual recorded worm burdens in shot, young grouse.

Scanning electron microscopy of heather plants, following the deposition of infective-stage larvae at the base of the plants, showed that some larvae migrate into the leaf rolls of the heather plant and are thus within a protective micro-environment. The opportunity was also taken to study, by means of scanning electron microscopy, the external structure of the infective-stage larva of T.tenuis, and to compare it with that of other trichostrongyle larvae. It was shown that the infective-stage larva of T.tenuis has the ability to form coils and that the external structure of the larva is similar to that of T.colubriformis larvae.

INTRODUCTION

Survival of the infective-stage larvae of T.tenuis under a range of conditions of temperature and humidity has been described in Chapter 2. Although such data, when used in conjunction with available climatic data, may be used to predict the occurrence of infective-stage larvae on the moor, it is no substitute for the actual sampling of larvae on the moor at different times of year. The availability of the infective-stage larvae of T.tenuis to grouse is important for an understanding of the epidemiology of the disease it causes in red grouse, as the ingestion of infective-stage larvae during feeding is the only way in which larvae may enter and subsequently infect the host. The infective-stage larvae of T.tenuis develop from nematode eggs which have been passed out in the caecal droppings of infected birds and so the distribution of infective-stage larvae is presumably closely associated with the distribution of caecal droppings on the moor. Hudson (1986a) found that the distribution of caecal droppings on a moor was aggregated and suggested that the distribution of infective-stage larvae is similarly aggregated, subsequently causing an aggregated distribution of the adult nematodes within the grouse population. The aim of the work described in this chapter was to examine the numbers of infective-stage larvae of T.tenuis on heather at caecal dropping sites on a moor throughout the year and so find the times of the year when the infective-stage larvae of T.tenuis are available to the

grouse.

A scanning electron microscope study of the leaf tips of heather plants (Calluna vulgaris), following the deposition of infective-stage larvae at the base of the plants, was also carried out as part of this study, in an attempt to detect the location of the larvae on the heather tips. At the same time, a scanning electron microscope study of the infective-stage larvae themselves was carried out, in order to study the external structure and morphology of the larvae and to compare it with that of other trichostrongyle nematodes.

MATERIALS AND METHODS

Study area

The work to detect infective-stage larvae of T.tenuis on heather was done in the Botchergill area of Gunnerside moor, Upper Swaledale, North Yorkshire (NGR NY 930 020), between September 1985 and October 1986.

Location of caecal dropping sites

Each month, ten freshly deposited caecal droppings from red grouse were located and the position of the droppings marked with 1.5 m long bamboo canes, labelled from 1-10, so that the individual sites could be located at a later date.

Caecal egg counts

Approximately 0.5 g of each caecal dropping was taken from each site and the number of nematode eggs per gram of caecal faeces was calculated by means of the modified McMaster technique, as described by Gordon & Whitlock (1939).

Sampling of heather for infective-stage larvae

The remainder of the caecal droppings were then left on the moor where they gradually disintegrated. 8 weeks later, when the caecal droppings at each site had disintegrated, approximately 20-30 g of heather shoots from the tips of heather plants, from a random sample within 20 cm of each site, were removed. Each sample of heather was

weighed and any nematodes present were extracted by means of a Baermann apparatus. After 24 hours any nematodes present in the neck of the Baermann apparatus were collected and concentrated by centrifugation into a volume of 10 ml of water. A drop of Lugol's iodine was added to the resultant suspension. A few drops of sodium hypochlorite were added prior to microscopic examination of the sample; this facilitates examination, since any free-living larvae are decolourised, whereas trichostrongyle larvae retain the stain (Bairden, Duncan & Armour, 1981). The numbers of trichostrongyle larvae present in each sample were then counted and expressed as the number of infective-stage larvae per gram of fresh weight of heather sampled. It should be noted that sheep also graze this moor and there is a possibility that some of the trichostrongyle larvae collected were of species parasitic in sheep. However, care was taken to avoid areas where sheep had been grazing and had deposited faeces.

Inoculation of heather plants with infective-stage larvae

Two heather plants (Calluna vulgaris) were obtained from Plantland (Leeds) Ltd garden centre, York Road, Leeds, and were placed beneath an inverted plastic fish tank. The soil at the base of each plant was then inoculated with approximately 50,000 infective-stage larvae of T.tenuis and the plants were left at room temperature (day temperature 20 C, night temperature 14 C). Both plants were lightly sprayed with water from a mystifier, every 8-12 hours, in order to maintain a moist foliage and atmosphere around the plants. After 3 weeks, the top 1 cm tips of the plants

were removed and examined by means of scanning electron microscopy.

Scanning electron microscopy of the tips of heather leaflets

The tips of the heather plants were placed immediately into 10% phosphate-buffered formalin (pH 7) for 24 hours. Samples were then dehydrated in ascending concentrations of acetone, dried in a Polaron critical point drier, coated with gold/palladium in a sputter coater, and examined using a Cam-scan 3-30 scanning electron microscope operating at 10 kV.

Scanning electron microscopy of infective-stage larvae

Infective-stage larvae which were to be examined by means of scanning electron microscopy were obtained from fresh laboratory cultures and placed into 10% phosphate-buffered formalin (pH 7) for 24 hours. A sample of approximately 100 larvae was then transferred into a polythene beam capsule (Agar Scientific Ltd), the lid of which had been removed and the capsule top was resealed with 10 μ m plankton net (Henry Simon Ltd). This technique allowed the larvae to become immersed in acetone during dehydration and critical point drying, whilst containing them in an easily manageable container. The larvae were dehydrated in ascending concentrations of acetone and dried in a Polaron critical point drier. They were then removed from the beam capsule and tipped onto a disc of resin-coated photographic paper (Kodak Veribrom) which had been slightly wetted.

This provided both an adhesive surface and a clear background to facilitate microscopic examination. The paper disc was then stuck onto a specimen stub, coated in gold/palladium in a sputter coater, and examined using a Camscan 3-30 scanning electron microscope operating at 10 kV.

RESULTS

The occurrence of infective-stage larvae of *T.tenuis* on heather plants around caecal dropping sites

The number of nematode eggs in the caecal droppings and the number of infective-stage trichostrongyle larvae recovered from heather at each site at each month during the study are given in Table 4.1. The highest mean nematode egg output occurred in December 1985, at a mean of 45745 eggs per gram (epg) of caecal droppings and the lowest in January 1986, at a mean of 20795 epg of caecal droppings. Although the sites of 10 caecal dropping deposits were located and marked out each month, only 8 sites were relocated from the caecal droppings marked out in November 1985 and June 1986 and only 4 sites relocated from the caecal droppings marked out in September 1986. This was because the bamboo canes marking the sites tended to be knocked down during the 8 weeks between their erection and the sampling of heather at the sites, presumably by sheep rubbing up against them. In September 1986, the unusually strong winds associated with storm 'Hurricane Charlie' blew many of the canes to the ground. However, although nematode eggs were detected throughout the year, infective-stage larvae were not detected during every month. A mean of 0.025 larvae per gram fresh weight of heather was found in November 1985 and no larvae were found during months of December 1985 and January, February and April 1986, although a mean of 0.018 larvae was found in March 1986. There was an increase in the number of larvae

Table 4.1. Mean monthly nematode egg output in caecal droppings and the mean numbers of infective-stage larvae of *T.tenuis* recovered at the caecal dropping sites throughout a 12 month period.

Month/year	Mean no. of eggs/gram of caecal droppings	Sample size	Mean no. of infective-stage larvae/gram fresh weight of heather
November 1985	41,400 ± 7395	8	0.025 ± 0.015
December 1985	45,745 ± 6465	10	0
January 1986	20,795 ± 1954	10	0
February 1986	26,665 ± 3772	10	0
March 1986	31,285 ± 3889	10	0.018 ± 0.011
April 1986	31,035 ± 5270	10	0
May 1986	31,930 ± 4970	10	0.229 ± 0.156
June 1986	33,338 ± 4638	8	0.616 ± 0.448
July 1986	34,334 ± 4995	10	0.126 ± 0.067
August 1986	39,585 ± 3053	10	0.183 ± 0.095
September 1986	40,513 ± 9284	4	0.052 ± 0.033
October 1986	44,530 ± 7382	6	0

on the heather in May and June 1986, with a mean of 0.229 larvae per gram of heather occurring in May and a mean of 0.616 larvae in June. Numbers of larvae on the heather tips began to fall in July 1986, with a mean of 0.126 larvae per gram of heather, a mean of 0.183 larvae in August and a mean of 0.052 larvae present in September. No larvae were detected on the heather tips in October 1986.

Structure of heather leaflets

Heather leaves consist of numerous leaflets attached to a central stem (Figs. 4.1a and 4.1b). When the leaflets were examined by scanning electron microscopy the outer surface of each leaflet was found to possess spike-like projections and each leaflet was made up of a distinct leaf roll which formed a crevice along the longitudinal axis of the leaflet (Fig. 4.2a). This crevice is on the ventral surface of the leaflet. Hair-like structures, the surfaces of which are not smooth but are covered in small nodules, arise at the edges of the crevice and extend across the opening (Fig. 4.2b). Characteristic plant stomata, surrounded by pairs of guard cells, were observed within the leaf roll (Fig. 4.2b). Some of the infective-stage larvae which had been placed at the base of the heather plant 3 weeks previously were found on the uppermost leaves of the heather plants. Figs. 4.3a, b show one of these larva entering a longitudinal crevice formed by the leaf roll.

Structure of the infective-stage larva

Infective-stage larvae of T.tenuis, which had been obtained from fresh cultures and which had been examined by

means of scanning electron microscopy were well preserved and showed only slight collapse of structure. All the larvae examined were found in a tightly coiled posture with the body of the larvae contained within 3.5 clockwise turns. No larvae were observed in a straight posture. The cuticular sheath of the infective-stage larva is marked by striations, approximately $0.77\mu\text{m}$ apart and the sheath has a slightly wrinkled appearance. The lateral surfaces possessed prominent alae which appeared to be formed by lateral extensions of the cuticle. The cuticular striations do not pass across the lateral alae. The posterior end of the sheath tapers to a point and the alae appear to terminate approximately $50\mu\text{m}$ before the end of the sheath (Figs. 4.4a and 4.4b).

Fig. 4.1a. Scanning electron micrograph of a heather leaf made up of several leaflets or leaf rolls. Note the leaf roll (lr), and the crevice running along the longitudinal axis of the leaf roll (c).

Scale bar 1000 μm

Fig. 4.1b. Scanning electron micrograph of a heather leaf made up of several leaflets. Note the longitudinally running crevice along the length of the leaflet (c) and spike-like structures projecting from the surface of the leaflets (s).

Scale bar 300 μm



Fig. 4.2a. Scanning electron micrograph of a single heather leaflet. Note the crevice running along the leaflet (c) and the elongated plant hairs along the edges of the crevice which extend across its opening (h).

Scale bar $100\mu\text{m}$

Fig. 4.2b. Scanning electron micrograph of the inside of a heather leaflet. Note the plant hairs (h) extended across the opening of the crevice; and plant stomata (st) surrounded by guard cells (gc).

Scale bar $30\mu\text{m}$

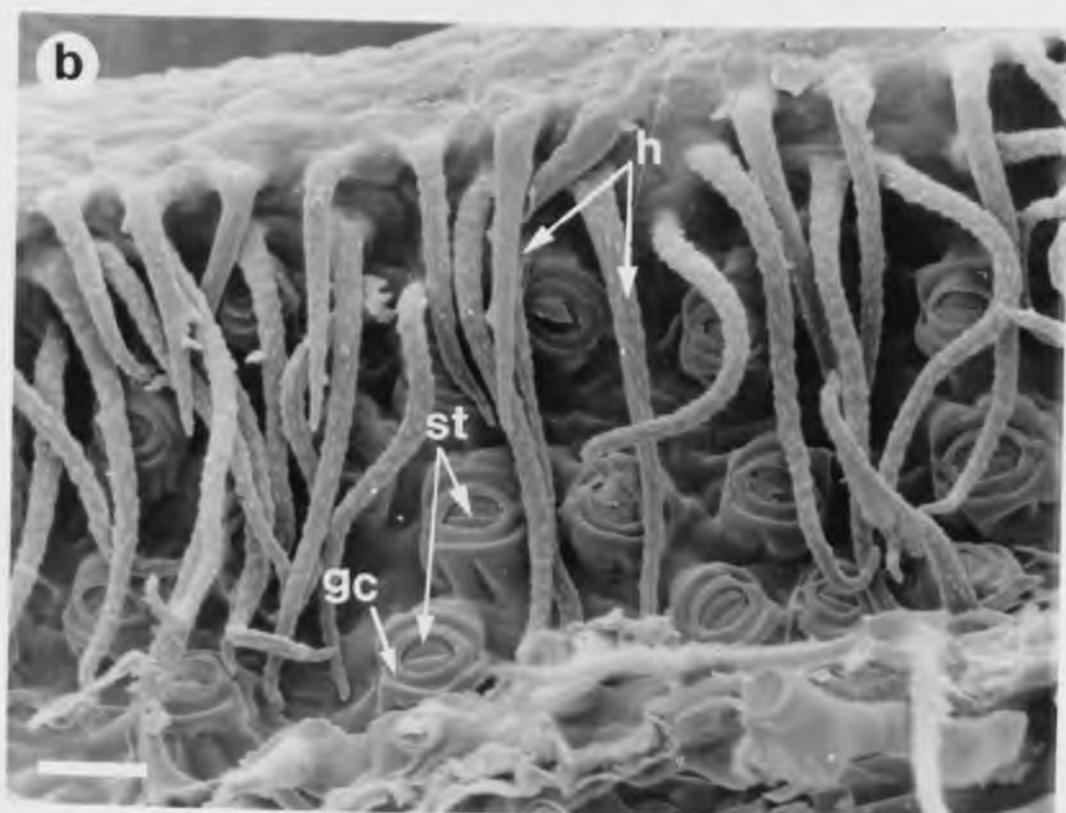
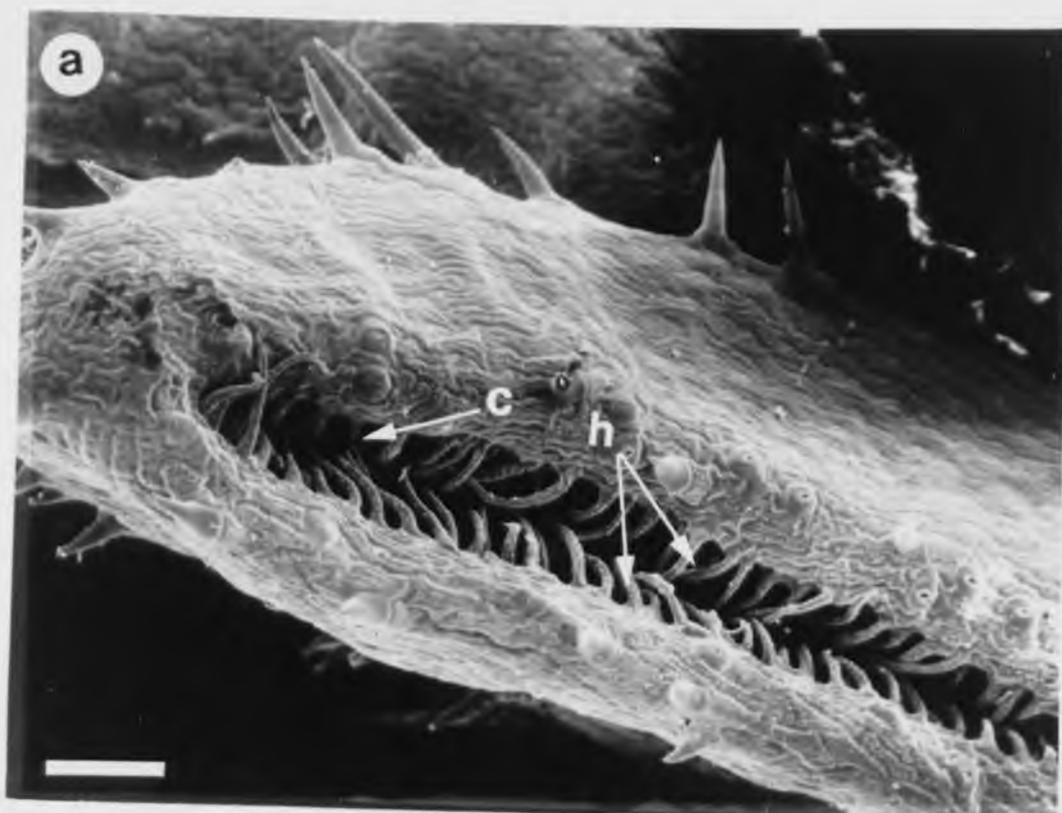


Fig. 4.3a. Scanning electron micrograph of a single heather leaflet with the crevice closed. Note the infective-stage larvae of T.tenuis (isl) entering the crevice (c) of the leaflet.

Scale bar 30 μ m

Fig. 4.3b. Scanning electron micrograph of a single heather leaflet. Note the infective-stage larva (isl) entering the crevice (c) formed by the leaf roll.

Scale bar 30 μ m

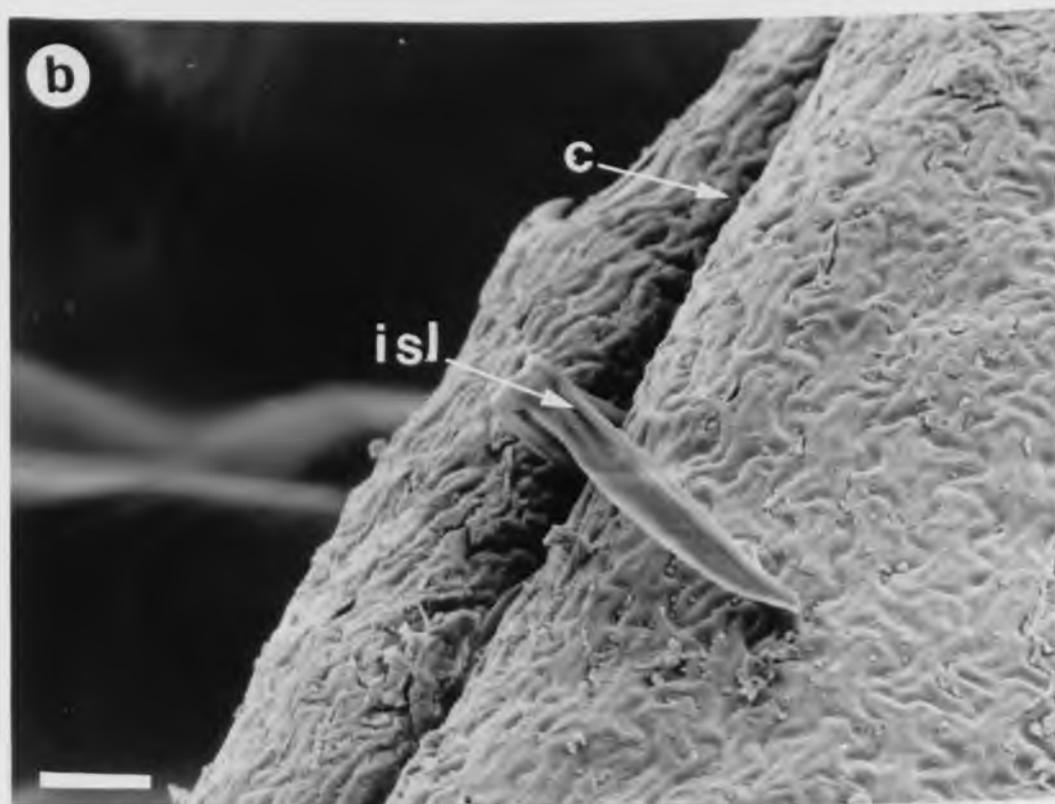
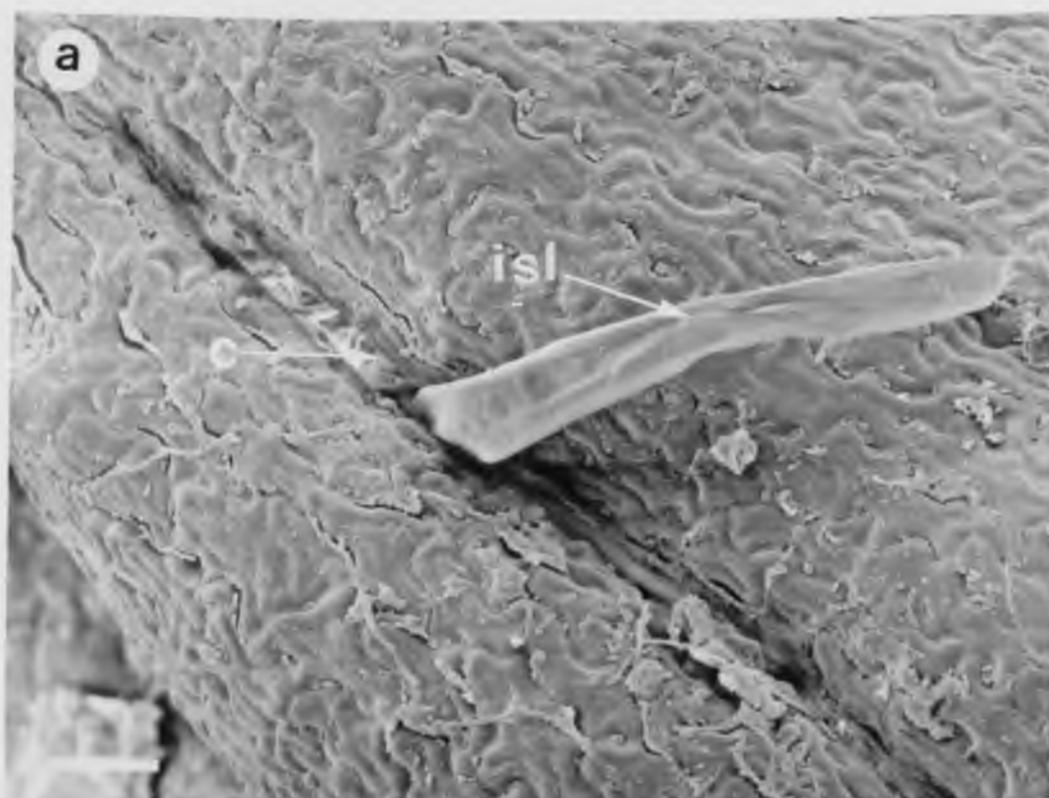
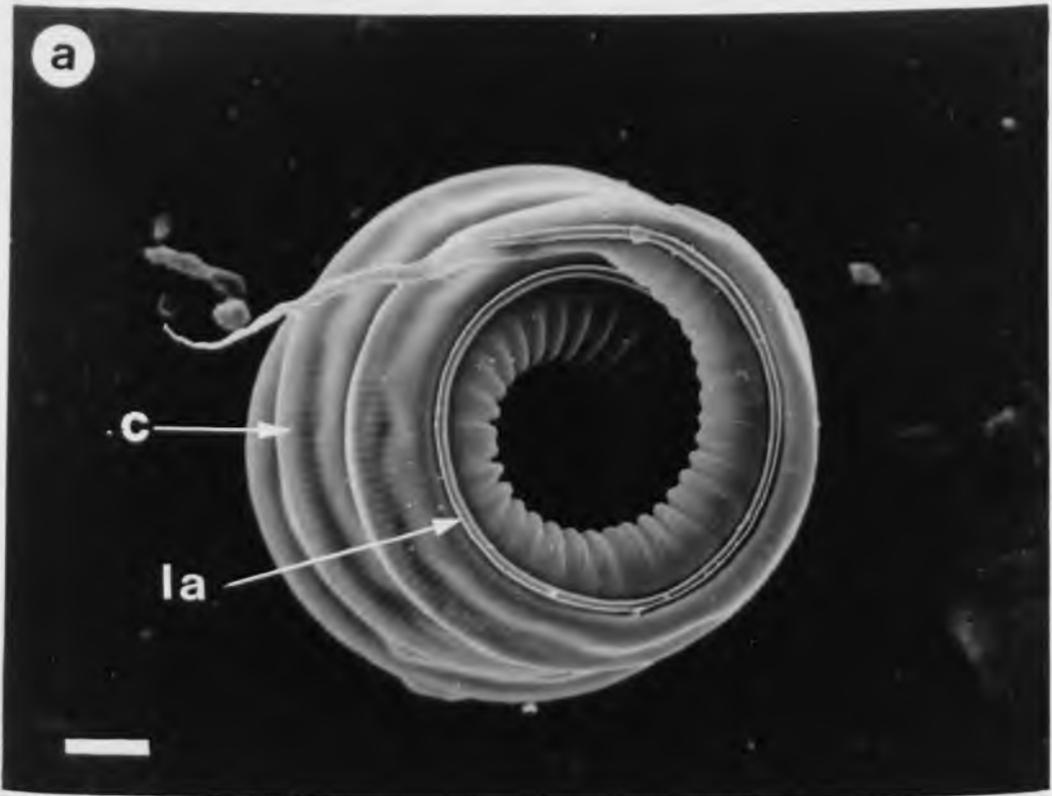


Fig. 4.4a. Scanning electron micrograph of an infective-stage larva of T.tenuis. Note the coiled position, the superficial annulations of the cuticle (c) and one of the lateral alae (la).

Scale bar 10 μ m

Fig. 4.4b. Scanning electron micrograph of an infective-stage larva of T.tenuis. Note the superficial annulations of the cuticle (c) and one of the lateral alae (la).

Scale bar 10 μ m



DISCUSSION

The survival on pasture of the free-living stages of a number of species of parasitic nematode have been examined in several studies because detailed information on the ecology of these free-living stages is important in the development of control strategies against economically important nematodes (Donald, 1968). One of the most practical procedures employed for obtaining information on this subject is to graze parasite-free animals on pastures that have been naturally or experimentally contaminated with nematode eggs and then later examine these animals for parasites (Kates, 1950). Several studies on the survival of the free-living stages of trichostrongyles have been determined in this way. Griffiths (1937) observed survival of some T.colubriformis larvae over the winter period in Canada, and Baker (1939) reported survival of larvae of T.colubriformis for up to one year on pasture in New York state. Shorb (1942) and Kates (1943) observed only low survival of T.colubriformis larvae over the winter in Beltsville, Maryland, as did Swales (1940b) in Eastern Canada. However, Hawkins, Cole and Kline (1944) reported substantial survival of larvae after 4.5 months exposure on the pasture from September to January in Michigan. Seghetti (1948) showed that the main acquisition of a number of species of trichostrongyles by lambs on the ranges of Eastern Montana occurred during the period of relatively heavy rainfall in May and June; and that later in the summer, when rainfall was less than 1 inch in 2

months, most of the free-living stages were dead after exposure for 10 days on the pasture. More recent studies in England have shown that the overwintered larvae of T.colubriformis may survive until the months of May to July in the South-East of England (Gibson & Everett, 1967) and until the months of April and May in the North-East of England (Boag & Thomas, 1970). Anderson, Levine & Boatman (1970) studied the survival of infective-stage larvae of T.colubriformis on pasture in Urbana, Illinois over a one year period and found that larvae placed on experimental plots during late autumn, winter, or early spring, usually survived better than those exposed during the warm summer months and that, in general, the lower the temperature the greater the survival of the infective-stage larvae.

The survival on pasture of the free-living stages of Haemonchus contortus has also been examined in detail. Ransom (1906; 1907; 1908; 1910) first studied the free-living larvae of H.contortus under natural conditions in the field in Washington D.C, and suggested that the infective-stage larvae are highly resistant to freezing and drying. He found that they may survive repeated freezing and thawing for up to 12 weeks and survive drying for up to 35 days. Boughton & Hardy (1935; 1936; 1937; 1938) found that the infective-stage larvae survived for up to 1 year on pasture in Texas, while Baker (1939) found relatively long survival of free-living larvae on pasture in New York. Other workers, however, have suggested that the free-living larvae of H.contortus survive on pasture for a much shorter period of time. Dikmans & Andrews (1933) and Sarles (1943)

found little or no survival of larvae over the winter period on pasture in Beltsville, Maryland; Doll & Hull (1946; 1948) observed no survival of larvae during the summer months on contaminated plots in Kentucky; Swales (1940a) found that larvae did not survive the winter in Eastern Canada; and Goldsby & Eveleth (1947) reported no survival of larvae after winter exposure in North Dakota. In Australia, Kauzal (1936; 1937) stated that 1-2 months of continuous dry weather greatly reduces the number of H. contortus larvae on pastures and may completely eradicate them.

Survival of the free-living stages of Ostertagia species on pasture has also been well documented. Fallis (1938) indicated that the larvae of Ostertagia sp could survive the winter on pastures in Ontario and Furman (1944) found that larvae of O. circumcincta survived all summer on irrigated pasture which had a high moisture content but that they were rapidly killed on non-irrigated pasture in California. Griffiths (1937) found that in Eastern Canada sufficient O. circumcincta larvae survived on pasture from October to May to cause an infection the following spring. Hawkins, Cole & Kline (1944) reported considerable survival of Ostertagia sp on pasture in Michigan from September to January and Goldsby & Eveleth (1947) reported survival of Ostertagia sp in North Dakota from December to June. Callinan (1978) found that larvae of O. circumcincta failed to survive on pasture over the winter months in Western Victoria, Australia. More recent studies have examined the ecology of the free-living stages of Ostertagia sp in the

British Isles. Boag & Thomas (1970) indicated that the larvae of O.circumcincta overwinter on pasture in the North-East of England and that this overwintering population may produce a significant infection the following spring. Similar results were obtained by Gibson & Everett (1972) in the South-East of England. Waller & Thomas (1978) found that larvae of Ostertagia sp survived well on pasture over the winter in the North-East of England and that the overall level of parasitism was related to different climatic conditions, the amount of rainfall being the most critical factor affecting survival. Bairden, Armour & McWilliam (1985) showed that considerable numbers of infective-stage larvae of O.ostertagi survived for at least 18 months on pastures in south-west Scotland, even when such pastures were maintained free from grazing animals.

The survival on pasture of the free-living stages of a number of trichostrongyle nematodes has thus been examined in various parts of the world and under various climatic regimes. It is now believed that infective-stage larvae of most trichostrongyle nematodes have the potential to survive on pasture for considerable periods of time, depending upon the weather conditions in that area. In many cases, infective-stage larvae can survive during the winter months and later become a major source of infection to animals that subsequently graze on the pasture during the following spring and summer.

However, data from the current study indicates that the

infective-stage larvae of the nematode T.tenuis, unlike the infective-stage larvae of some trichostrongyle species, have a limited potential to survive during the winter months. Laboratory experiments on the effects of sub-zero temperatures and desiccation on the survival of the infective-stage larvae of T.tenuis (Chapter 2) have indicated that larvae are highly susceptible to freezing and drying. Data obtained from Leeming weather centre, North Yorkshire (NGR 4306 4890) shows that between 1951 and 1981, the average coldest day in January was below freezing and the average coldest night between November and March was below freezing, ranging from -4.3 C in November to -7.4 C in January. Conditions on the moor in upper Swaledale, North Yorkshire, at an altitude of 1400 feet above sea level, will presumably be as cold, if not considerably colder, than those recorded at Leeming, and the exposure to such cold temperatures will cause severe mortality amongst infective-stage larvae remaining on the moor over the winter months. The detrimental effect of freezing and low temperatures on the infective-stage larvae of T.tenuis may, however, be decreased if the moor is covered in snow. Anderson, Levine & Boatman (1970) found that the larvae of T.colubriformis survived best during times of year when low temperatures prevailed, especially when the experimental plots were covered in snow.

The number of infective-stage trichostrongyle larvae detected on the heather appears to be very low. However, the low numbers of larvae recovered from the heather during this study must be taken in context with the amount of

heather a grouse consumes. A red grouse consumes an average of 40 g of heather per day throughout the year (R. Moss, Institute of Terrestrial Ecology, Banchory, Scotland, pers comm). Thus the total number of larvae which may be ingested by the red grouse can be calculated on a daily basis and projected further to the expected larval intake per month. The expected daily and monthly intake of infective-stage larvae by a red grouse feeding upon 40g of heather per day is given in Table 4.2. In November 1985 the estimated monthly intake was about 30 larvae. During December 1985 and January, February and April 1986 no larvae were detected on the heather, although during March 1986 larvae were present and the estimated monthly intake was about 73 larvae. During May 1986, the estimated monthly intake was about 284 larvae; in June 1986, about 739 larvae; in July 1986, about 156 larvae; in August 1986, about 227 larvae; and in September 1986, about 62 larvae. No larvae were detected on the heather during October 1986. There was a good correlation between the estimated number of worms in young grouse, based upon the number of larvae detected on the heather, and the actual worm burdens recorded in shot, young red grouse (Fig. 4.5).

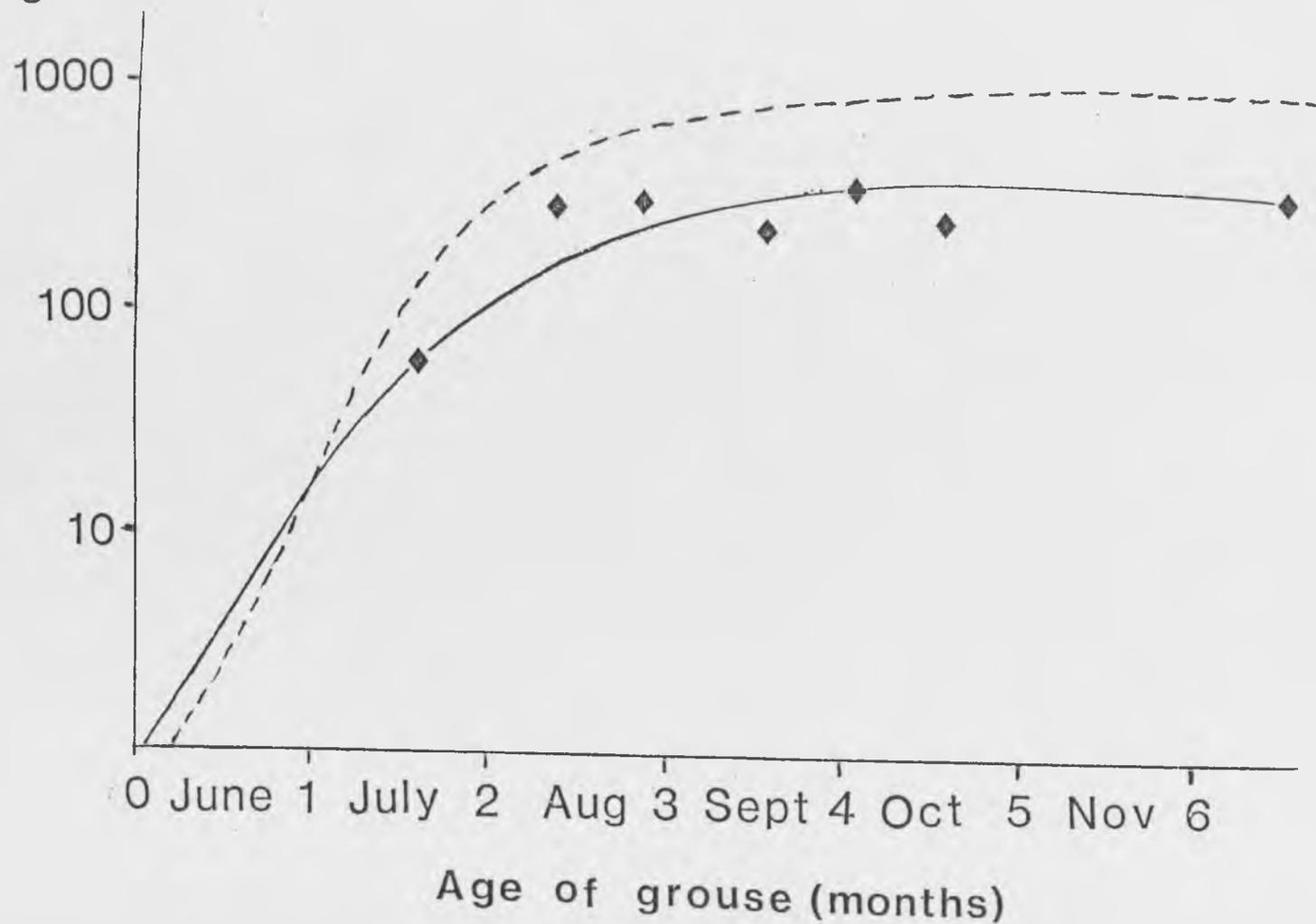
The estimated intake of larvae by red grouse has been based upon the assumption that a single grouse consumes an average of 40g of heather per day. It must be stressed, however, that this figure is an estimate. The red grouse consumes different amounts of food at different times of year. Savory (1975) measured the daily food intake of captive red grouse fed on a diet of 'maintenance' pellets

Table. 4.2. The estimated daily and monthly intake of infective-stage larvae of *T.tenuis*, based on a single grouse consuming 40 g fresh weight of heather per day.

Month/year	Estimated no of larvae consumed/day	Estimated no of larvae consumed/month
November 1985	1.00	30.00
December 1985	0	0
January 1986	0	0
February 1986	0	0
March 1986	2.36	73.16
April 1986	0	0
May 1986	9.16	283.96
June 1986	24.64	739.20
July 1986	5.04	156.24
August 1986	7.32	226.92
September 1986	2.08	62.40
October 1986	0	0

Fig. 4.5. Worm burdens in young red grouse, estimated from the number of infective-stage larvae recovered from heather at caecal dropping sites (---) and the actual worm burdens recorded in young red grouse (◆) (Hudson, 1986a). Actual worm burdens recorded during June and July from birds which were accidentally killed during counts of grouse chicks and worm burdens recorded between August and November from shot grouse.

Geometric mean
worm burden
(Log scale)



and heather and found that food intake was positively correlated to both body weight and day length and negatively correlated to air temperature. Grouse ate more during their main summer moult and hen grouse ate most during egg laying and incubation of the eggs during April and June. However, changes in the general activity of the captive grouse were not measured in that study and such changes would presumably influence the food requirement of the birds. Lance (1983) showed that the feeding of grouse continued later into the morning as the breeding season approached. These results are similiar to my own observations on wild red grouse, which have indicated that grouse appear to feed much more vigo rously, and for a longer period of time, in late spring, prior to egg laying and in October, prior to the onset of winter. Since infective-stage larvae are ingested by grouse when they consume heather tips contaminated with larvae, presumably the total number of larvae ingested not only depends upon the density of larvae on the heather tips, but also on the amount of heather consumed by the grouse. Thus, many more larvae than estimated may be ingested by hen grouse in the month of May, when food intake is at a peak, and simil arly in June, when many larvae are present on the heather and food intake is still relatively high. Conversely, during the months of August and September, although many larvae are still present on the heather, food intake is much lower and consequently, fewer larvae than estimated may be ingested.

The estimated levels of infection in red grouse were calculated on the surmise that every larva ingested will develop into an adult nematode. However, as demonstrated in Chapter 3, the infectivity of the infective-stage larva depends upon the amount of stored lipid reserves within that larva. It is probable that after migration of the larvae from the caecal faeces to the heather tips the lipid reserves of the larvae are considerably depleted. As there will be further loss of lipid as the larvae ages, whilst waiting to be consumed, many larvae may fail to produce an infection in the grouse, even if they are ingested. It was impossible to determine the lipid levels of the larvae that were obtained from the heather because of the very low numbers of larvae that were recovered from the heather samples. Other factors may also affect the numbers of adult nematodes which become established in the grouse and these were not taken into account when estimating the worm burdens in this work. Shaw (1987) described a number of factors which affect the establishment rate of T.tenuis in red grouse, including variations in the physiological status of the bird and whether the birds were dominant or subordinate. Also, any resistance to T.tenuis in red grouse may affect the ability of the larvae to infect the grouse, although available evidence indicates that the grouse has little or no resistance to T.tenuis infection.

The work described here is the first attempt to relate the numbers of larvae recovered from the heather stand to worm burden in wild red grouse which have been shot on the moor. Hudson (1986a) has shown that there is an increase

in worm burdens in young grouse during August and September, followed by relatively stable worm burdens for at least two months. However, inspection of worm burdens in the following autumn indicated that there is a further period of increased infection during, or after, the winter. The data from the current study is compatible with the suggestion that the numbers of adult T.tenuis increase in young grouse during August and September (Hudson, 1986a), since infective-stage larvae occur in relatively high numbers during these months. Worm burdens may also increase prior to this time, during the months of May, June and July. However, since grouse are not shot until August and thus worm burdens in shot grouse cannot be calculated until this time, it is difficult to demonstrate an early summer increase in worm burdens. Little infection can be expected to occur during the months of October to April, since few larvae are present on the heather at this time, and so any later infection must be derived from an alternative source.

An increase in the numbers of adult nematodes in the caeca during the winter months could be due to the presence of infective-stage larvae, which have become arrested in the tissues of the grouse after infection in late summer or the autumn, restarting development into adult nematodes during the winter. Contrary to the widely prevailing view that parasitic nematodes in the normal definitive host develop to adulthood within a relatively restricted and characteristic prepatent period, it is now apparent that prolonged interruption of development is a common

alternative (Schad, 1977). Arrested development of nematodes has been defined by Michel (1974) as a temporary cessation of development at a precise point in early parasitic development, where such an interruption contains a facultative element, occurring only in certain hosts, under certain circumstances or at certain times of year, and often affecting only a proportion of the worms. This phenomena has been described in sheep infected with Haemonchus contortus (Connan, 1968; 1971; 1975; Blitz & Gibbs, 1972; Waller & Thomas, 1975); with Trichostrongylus sp. (Michel, 1952; Reid & Armour, 1972; Eysker, 1978); with Nematodirus filicollis (Reid & Armour, 1972); and with Cooperia oncophora (Anderson, Armour, Jennings, Ritchie & Urquhart, 1975). It has also been described in cattle infected with Ostertagia ostertagia (Connan, 1968; Armour, 1970; Reid & Armour, 1972; Michel, Lancaster & Hong, 1973; 1975). The reason for arrested development is a controversial subject. It may be induced either by external environmental factors, such as unfavourable environmental conditions (Connan, 1968; Anderson, 1972; Armour & Bruce, 1974); by host factors, such as any natural or acquired resistance in the host (Donald, Dineen, Turner & Wagland, 1964; Dineen, Donald, Wagland & Offner, 1965); by parasite-related factors, such as the genetic strain of the nematode (Michel, Lancaster & Hong, 1973; Waller & Thomas, 1975); by the presence of adult worms in the host, the removal of adult worms stimulating the recruitment of a new population from a reservoir of arrested larvae (Michel, 1952; Gibson, 1953; Michel, 1971); or by the relative numbers of worms present in a single invasion, the size of

the dose influencing the proportion of larvae that become arrested (Dunsmore, 1960; Michel, Lancaster & Hong, 1975).

It is possible that a proportion of the infective-stage larvae of T.tenuis that are picked up from the heather by red grouse between May and September enter this arrested stage and are not detected in the worm counts taken during the shooting season of that year. These larvae may develop to adults later on in the winter and so could cause an increase in the nematode burdens in grouse, even though few, or no, infective-stage larvae are available on the heather at that time of year. During this study, grouse caeca were not available outside the shooting season and so worm numbers in the birds during this winter period could not be recorded.

The data from this study have shown that although large numbers of nematode eggs are present in the caecal droppings of red grouse throughout the year, the numbers of infective-stage larvae within a radius of 20 cm of the caecal dropping site are very low throughout the year. This could be due to the low survival rate of the eggs and infective-stage larvae caused by unfavourable environmental conditions, such as freezing in the winter or drying in the summer. Alternatively, the low levels of infective-stage larvae detected on heather at the caecal dropping sites could be due to migration of larvae away from the area where the droppings had been deposited; the larvae may travel further than 20 cm; or most may remain in the surface mat of vegetation with relatively few managing to

reach, and ascend, the heather plants. McGladdery (1985) showed that the infective-stage larvae of T.tenuis migrate upwards onto the heather tips but it is possible that many larvae ascend other vegetation at the site and are not eaten by the grouse.

Several studies have examined the migration of trichostrongyle nematode larvae from the faecal droppings to the parts of the herbage that are eaten by the host (Taylor, 1938; Rogers, 1940; Kauzal, 1941; Dinaburg, 1944). Crofton (1954) concluded that the vertical migration of infective-stage larvae of trichostrongyles may be described in terms of normal larval movements without any reference to geotropism or special receptors in the larvae. However, a number of other studies have shown that the ability of the infective-stage larvae to migrate vertically can be affected by certain environmental factors. Rees (1950) examined the migration of the infective-stage larvae of Haemonchus contortus on experimental plots of Lolium perenne, in relation to meteorological and micrometeorological factors, and found that temperature, humidity and light intensity influenced vertical migration. Silanwanga & Todd (1964) found that only 2-3% of infective-stage larvae of trichostrongyles ascended blades of grass, even when conditions were considered favourable for migration, and that most larvae remained at the base of the vegetation. Larval migration was found to be higher on fescue grasses than on brome grasses. They concluded that the external morphology of the grass influences the migration of infective-stage larvae. Tall fescue blades,

which have a highly ribbed upper surface, are easily wetted and retain moisture better than smooth brome blades and so facilitate larval migration.

The infective-stage larvae of T.tenuis must migrate to the tips of heather plants if they are to infect the grouse. There, many of them may enter the crevices of the leaflets. Once within the crevice of the leaflet of the heather plant the larva is within a protective microenvironment. Inside the crevice it will be protected by the high humidity within the leaf roll, produced as the plant transpires, and this may allow longer survival. It will also be protected against harmful ultra-violet rays from the sun and protected against heavy rain which may wash the larvae off the heather if they were on the surface of the heather leaflet.

This work has shown for the first time that the infective-stage larvae of T.tenuis have the ability to form coils. Coil formation has been described in the infective-stage larvae of 12 species of trichostrongyle nematode (Wharton, 1982). In T.colubriformis, coiling is initiated by an increase in temperature and by the restriction of lateral movement during the evaporation of water films from around the larvae (Wharton, 1981a; 1981b). Coiling is thought to be important in desiccation survival as it reduces the surface area of the larvae and therefore the rate of water loss from that larva (Womersley, 1978). Since the larvae of T.tenuis examined by scanning electron microscopy in this study were exposed to neither

temperature extremes nor to desiccation prior to fixation, the coiling response is most likely to be a response to the formalin fixative or to the acetone used during dehydration. It has been shown however, that the infective-stage larvae of T.tenuis does have the ability to form coils and this behaviour may occur when the larvae are subjected to desiccation in the field. There have been few studies, using scanning electron microscopy, to examine the external morphology and structure of the larvae of parasitic nematodes, although Hendrix, Wagner, Bemrick, Schlottauer & Stromberg (1984) used scanning electron microscopy to examine the surface architecture of the third-stage larvae of Dirofilaria immitis. Wharton (1982) used scanning electron microscopy to examine the coiled posture of the infective-stage larvae of T.colubriformis and results were similiar to those obtained with T.tenuis. Both species have the ability to form coils, possess a cuticular sheath marked by prominent striations, and have prominent lateral alae. It is interesting to note that preparation of the infective-stage larvae of T.colubriformis for scanning electron microscopy using the same techniques as those used for T.tenuis (fixation in 4% glutaraldehyde and 2% Osmium tetroxide) followed by critical point drying), resulted in the preservation of larvae in a straight posture compared with a coiled posture with T.tenuis. However, in both cases surface preservation was good and the degree of collapse limited.

CHAPTER 5

THE EFFECT OF T.TENUIS ON THE CAECAL MUCOSA OF YOUNG,
OLD AND ANTHELMINTIC-TREATED WILD RED GROUSE

SUMMARY

The caecal mucosa of immature and adult grouse infected naturally with Trichostrongylus tenuis was examined by means of scanning electron microscopy and compared with adult grouse which had been treated with an anthelmintic. The caecal mucosa of young red grouse which carried low worm burdens showed little damage to the longitudinal plicae of the mucosal wall. The caeca from adult grouse, most of which carried high worm burdens, showed localized depression of plicae, atrophy and cell disruption in areas of nematode aggregation. Caeca from adult birds treated with an anthelmintic showed a similiar caecal structure to lightly infected caeca from young birds. Some of the adult nematodes in the caeca of treated birds were covered in adherent rosette-shaped structures, which gave the surface of the nematode a ruffled appearance. It is suggested that these structures may be adherent leucocytes. It is concluded that the normal functioning of the caeca is probably affected by heavy infections of T.tenuis.

INTRODUCTION

Cobbett & Graham-Smith (1911) described, for the first time, in detail the pathological changes that occurred in the caeca of red grouse infected with T.pergracilis (synonym of T.tenuis). In grouse free from nematodes, the caecal mucosa showed characteristic ridges and depressions of mucosa, termed longitudinal plicae, which are covered in numerous villi. These villi consist of a central core of vessels surrounded by sub-epithelial tissue and lymphoid cells. In heavily infected grouse, the caecal ridges and villi were markedly increased in size, causing a chronic inflammation of the caeca, leading to fibrosis and large quantities of mucus were present within the gut lumen. Adult nematodes were observed within the infected caeca and were aggregated in the proximal regions, where they were found both in the lumen and between the villi, in some cases having penetrated into the deepest portions of the crypts.

Since this early work, our understanding of the gross pathological and histopathological changes to gut structure brought about by parasitic infections has been increased by developments in microscopical techniques. The scanning electron microscope, with its flexible range of magnifications, increased depth of field and high resolution (Toner & Carr, 1968; Grey, 1972) has provided a useful tool with which to study the surface morphology of the gut. Several studies have used the scanning electron

microscope to examine damage to the mucosa of the alimentary tract caused by trichostrongyle nematodes in mammalian hosts. Barker (1973) described a marked flattening of the mucosa and atrophy of the duodenal villi in lambs infected with Trichostrongylus colubriformis; Martin & Lee (1980) described a reduction of the duodenal villi to flattened plate-like structures in lambs infected with Nematodirus battus; and Nicholls, Lee & Sharpe (1985) examined damage to the abomasum of sheep infected with Haemonchus contortus and described a more localized effect, with mucosal flattening and the disruption of gastric pits occurring in areas where nematodes were feeding.

Similar studies have not been conducted on nematode infections in birds, although scanning electron microscopy has been used to examine changes in the surface morphology of the alimentary tract of birds infected with protozoan parasites. Witlock, Lusbaugh, Danforth & Ruff (1974) described erosion and sloughing of cells in the caeca of chickens infected with Eimeria tenella; and Wilkins & Lee (1974) described severe atrophy of the longitudinal ridges of the caecal mucosa of turkeys infected with Histomonas meleagridis.

The prevalence of T.tenuis in red grouse has been examined in several studies. Wilson (1983) found 99% of wild red grouse carried T.tenuis, with old birds carrying 30 times as many nematodes than young birds. The prevalence of T.tenuis in red grouse in the north of England has been examined by Hudson, Dobson & Newborn

(1985). A total of 3370 grouse from 46 estates were examined and infection was found in 99.47% of the grouse. All of the old grouse examined were infected. Unlike trichostrongyle infections in other hosts, each of these studies has shown that nematode burdens in the red grouse increase with the age of the grouse and consequently adult grouse carry greater nematode burdens than young grouse (mean adult = 2291.48; mean young = 378.40) (Hudson, 1986a).

The object of this study was, for the first time, to examine by means of scanning electron microscopy, changes in the caecal mucosa brought about by infection with a trichostrongyle nematode in an avian host, and to discover the effect that the treatment of the birds with an anthelmintic has on the mucosal structure.

MATERIALS AND METHODS

Caecal samples were collected from wild red grouse shot in upper Swaledale, North Yorkshire (NGR NY 930 020) between 12th August and 10th December in 1984. Birds were picked at random from the day's bag and aged as either adult (greater than 1 year old) or immature (3-7 months old) according to plumage characteristics, claw scars and relative skull strength (Watson & Miller, 1976).

As part of a long-term study of T.tenuis in red grouse, a number of wild adult hen birds were caught at night, between December and March 1984, and treated orally with 2 ml of Nilverm (levamisole hydrochloride 1.5% w/v BP Vet, I.C.I. Ltd). Numbered tags were fitted to each bird and when tagged birds were shot after the 12 August, caeca were taken to count the number of nematodes present, to examine the effect of anthelmintic treatment on the structure of the caecal mucosa and to compare it with that of untreated adult birds.

The two caeca were removed from each gut sample. One caecum was cut open longitudinally and washed with water over a 210 μ m sieve. The residue, including the nematodes, was then mixed with 300 ml of water and sub-sampled to estimate the number of nematodes per bird, as described by Wilson (1983).

The second caecum was placed into 10% (v/v) phosphate-buffered formalin (pH 7) within an hour after death. From the middle of each caecum a 0.5 cm section was removed and cut open to expose the caecal mucosa. Each sample was dehydrated in ascending concentrations of acetone and dried in a Polaron critical point drier. Specimens were mounted on a specimen stub; coated with gold/palladium in a Polaron E5000 sputter coater; and examined by means of a Camscan 3-30 BM scanning electron microscope operating at 10kV.

RESULTS

The caecal mucosae of 44 wild immature grouse were inspected; 66% had infections of fewer than 100 nematodes and 9% greater than 500 nematodes. The caecal mucosa of young birds with relatively low levels of infection (greater than 100 nematodes but fewer than 500 nematodes) had well defined ridges or plicae running longitudinally along the caecum (Fig. 5.1a). The surface of these ridges was composed of blunt tips of short villi (Fig. 5.1b). The surface of the caecum was not extensively obscured by mucus or caecal contents and few nematodes were observed in the specimens.

Caeca from 36 wild adult birds were examined; 64% of the birds carried infections greater than 2000 nematodes and 47% greater than 3000 nematodes. In wild adult grouse carrying heavy infections (greater than 2000 worms), the nematodes were found to burrow deeply into the caecal mucosa and a localized flattening and disruption of the plicae occurred in areas where nematodes were burrowing (Figs. 5.2a and 5.2b). Nematodes appeared aggregated within the caeca, large scale disruption of the mucosal surface occurring in areas of high aggregation, and the longitudinal plicae were often much reduced in these regions (Figs. 5.3a and 5.3b). Nematodes were sometimes found burrowing between the ridges of caecal villi (Fig. 5.4a) but the surface of the mucosa was often obscured by a coating of mucus, bacteria and caecal digesta, and nematode eggs were frequently found embedded in this material (Fig.

5.4b). In some cases, despite frequent washing to remove adherent caecal content, the surface of the caecal mucosa was obscured by layers of caecal digesta; nematodes were observed burrowing into this coating (Figs. 5.5a and 5.5b). In some cases the nematodes had left tracks or impressions on this caecal material (Fig. 5.5a).

The structure of the adult nematode was examined briefly. The head of the adult nematode bears a tri-radiate mouth and two large lateral amphids (Fig. 5.6a). The posterior end of the male nematode has a characteristic copulatory bursa and a copulatory spicule, surrounded by the folds of the bursa. Rod-shaped bacteria were observed attached to the copulatory spicule (Fig. 5.6b). The tail of the female tapers quickly to a point (Fig. 5.3b). The surface of the nematode lacks the longitudinal cuticular ridges found in several other trichostrongyles but is superficially annulated (Figs. 5.2a; 5.3b; 5.4a; 5.5a, b). The position of the lateral cords is marked by a depression.

Adult grouse which had been treated with levamisole hydrochloride several months previously carried fewer nematodes than untreated adult grouse (geometric mean treated = 865 (n=10); untreated = 2032 (n=20); one-tailed t test, $p < 0.001$). The structure of the caecal mucosa of treated grouse was similar to that of lightly infected young grouse (Fig. 5.7a). Localised flattening and disruption of the mucosa, as observed around groups of nematodes in untreated adult grouse, was not seen in any of the 10 treated birds inspected. Some of the nematodes from

the caeca of birds that had been dosed with anthelmintic were covered in clusters of rosette-shaped structures (Fig. 5.7b, 5.8a and b, 5.9a and b) which were adherent to the cuticle and gave the surface of the nematode a lace-like appearance (Fig. 5.8a). These structures were present only on some of the nematodes and did not occur on the surface of every nematode present in the dosed birds. On closer examination, the adherent structures appeared to be of two distinct types, those with a highly ruffled surface (Fig. 5.7b) and those with a bumpy, uneven, granular surface. These structures were patchily distributed on the surface of the cuticle and the cuticular annulations were clearly visible in the areas not covered by these structures (Fig. 5.9a).

Fig. 5.1a. Scanning electron micrograph of the caecal mucosa of a young red grouse infected with between 100-500 nematodes to show well defined longitudinal ridges or plicae (lp). Scale bar 1000 μ m

Fig. 5.1b. Higher magnification of plicae to show blunt tips of caecal villi (cv). Scale bar 30 μ m

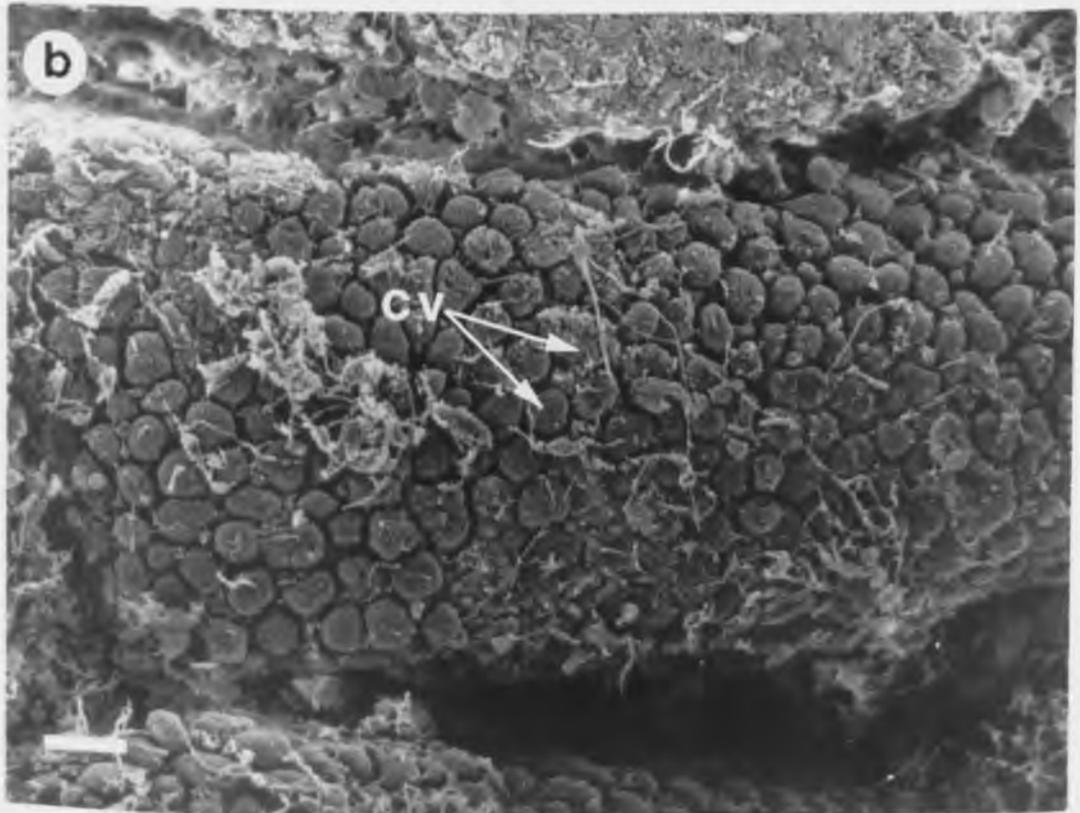
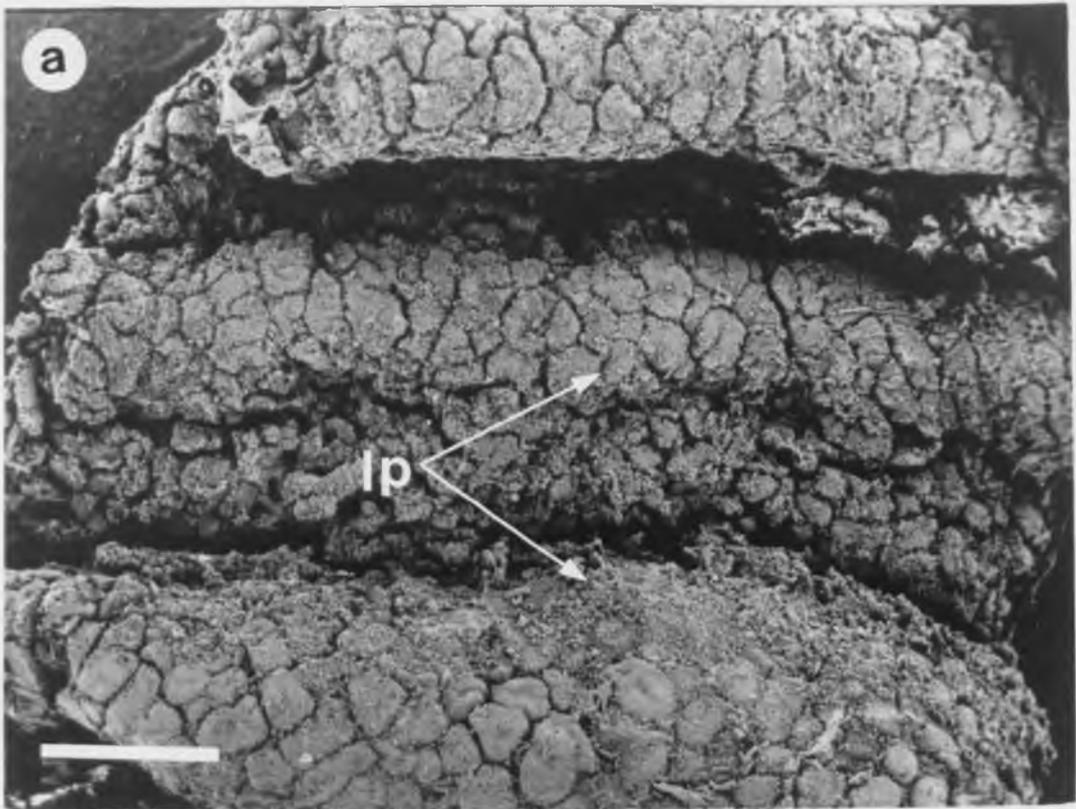


Fig. 5.2a. Scanning electron micrograph of the caecal mucosa of an adult red grouse infected with more than 2000 nematodes to show the burrowing activity of the nematodes (n). Note the large amount of surface mucus (m); parasite eggs (e); and copulatory bursa (cb). Scale bar $100\ \mu\text{m}$

Fig. 5.2b. Scanning electron micrograph to show localized flattening of the caecal mucosa of an adult red grouse infected with more than 2000 nematodes. Note muscularis externa (me); depressed plicae (lp); and aggregation of nematodes (n). Scale bar $300\ \mu\text{m}$

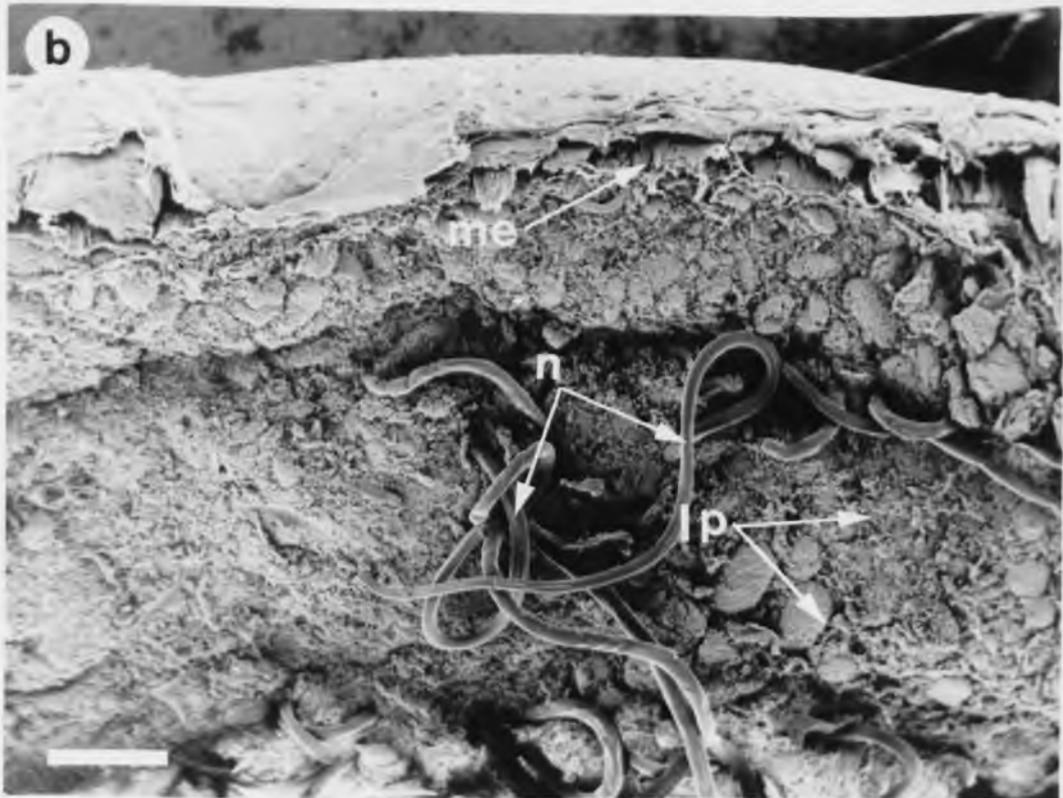
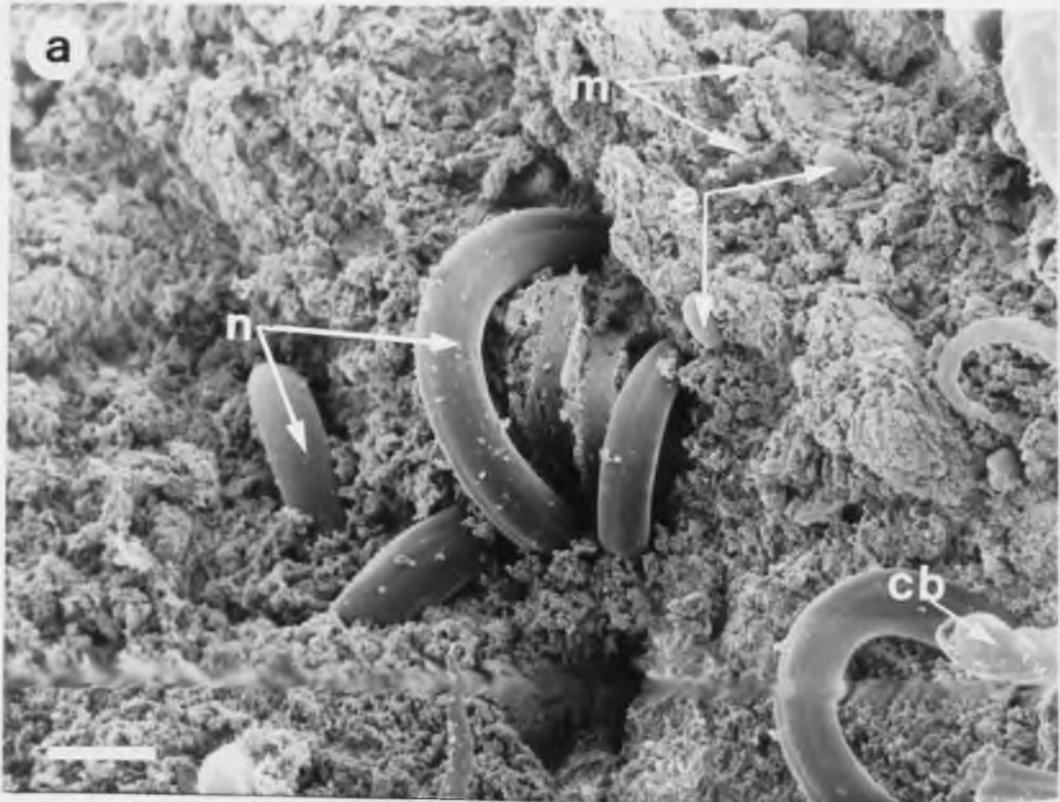


Fig. 5.3a. Scanning electron micrograph of the caecal mucosa of an adult red grouse infected with more than 2000 nematodes. Note widespread burrowing activity of the nematodes (n), and associated disruption of the longitudinal plicae (lp). Scale bar 1000 μ m

Fig. 5.3b. Scanning electron micrograph of the caecal mucosa of an adult red grouse infected with more than 2000 nematodes. Note aggregation of nematodes (n), and depressed plicae where nematodes are burrowing (lp). Scale bar 300 μ m

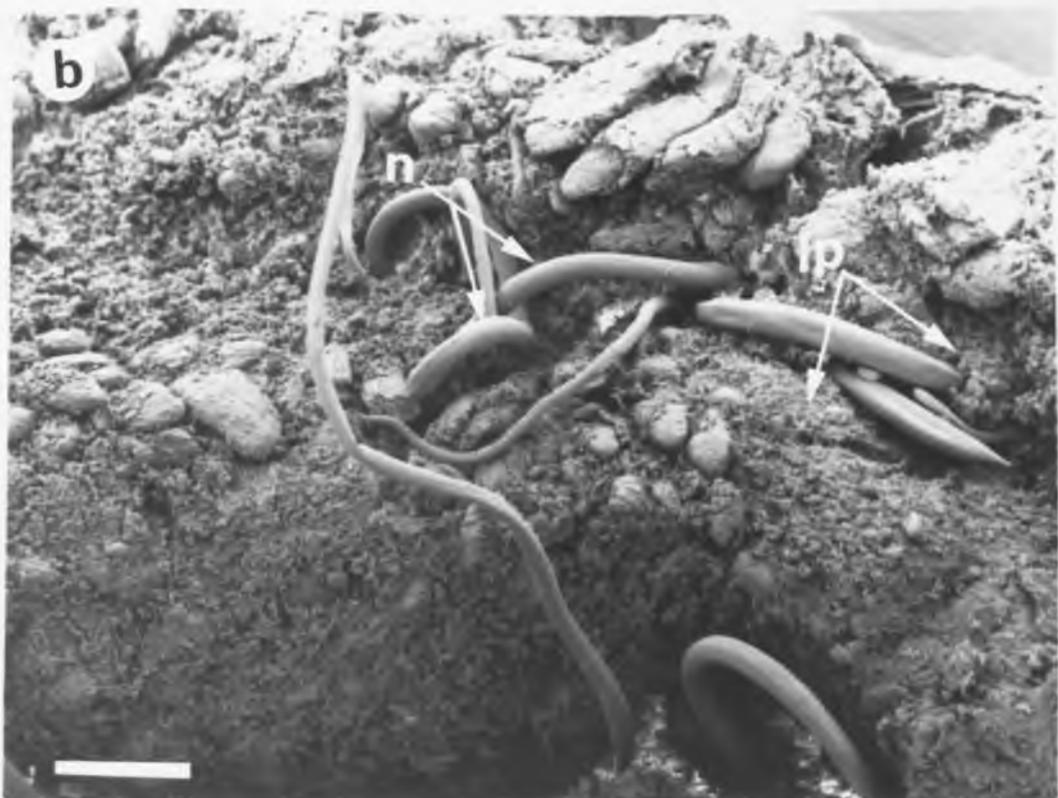
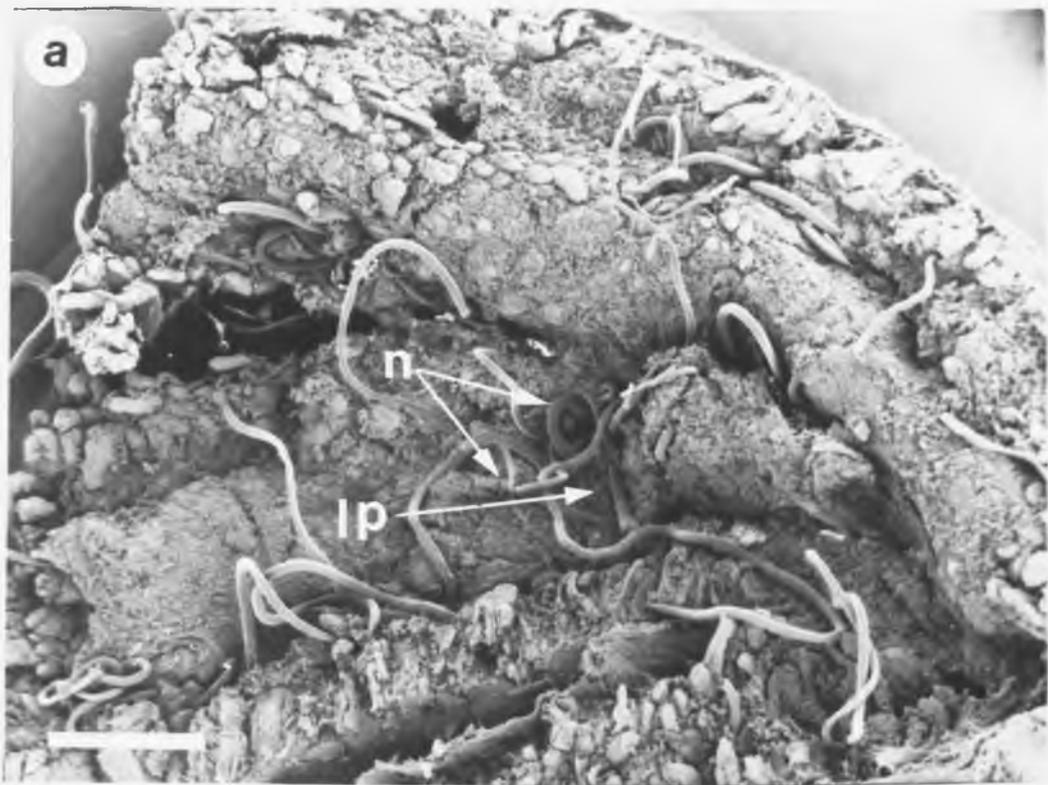


Fig. 5.4a. Scanning electron micrograph of the caecal mucosa of an adult red grouse infected with more than 2000 nematodes to show an adult nematode (n) burrowing into the longitudinal plicae (lp). Scale bar $100\mu\text{m}$

Fig. 5.4b. Scanning electron micrograph of the caecal mucosa of an adult red grouse infected with more than 2000 nematodes to show nematode eggs (ne) surrounded by caecal digesta (cd). Scale bar $100\mu\text{m}$

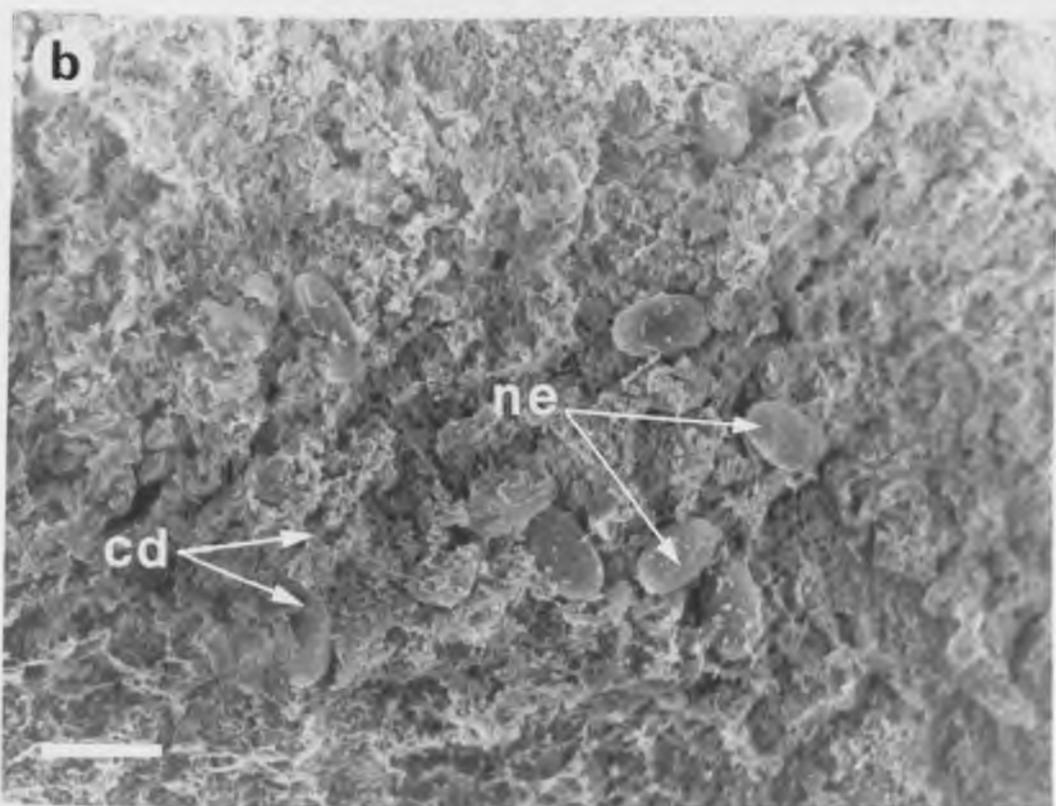
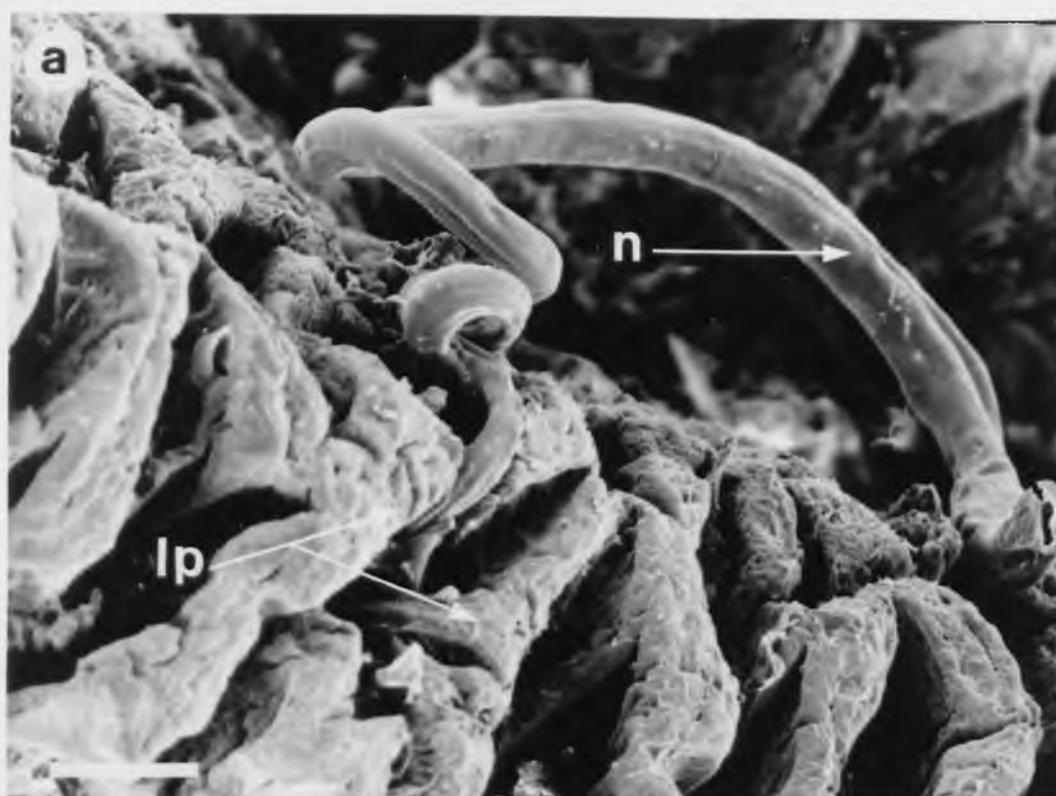


Fig. 5.5a. Scanning electron micrograph of the caecal mucosa of an adult red grouse infected with more than 2000 nematodes to show the nematodes (n); burrowing into the caecal digesta (cd); and impressions or tracks of nematodes upon the caecal content (nt). Scale bar 300 μm

Fig. 5.5b. Scanning electron micrograph of the caecal mucosa of an adult grouse infected with more than 2000 nematodes. Note nematodes (n) burrowing into the caecal digesta (cd) and causing cracks or fissures to appear in it. Scale bar 100 μm

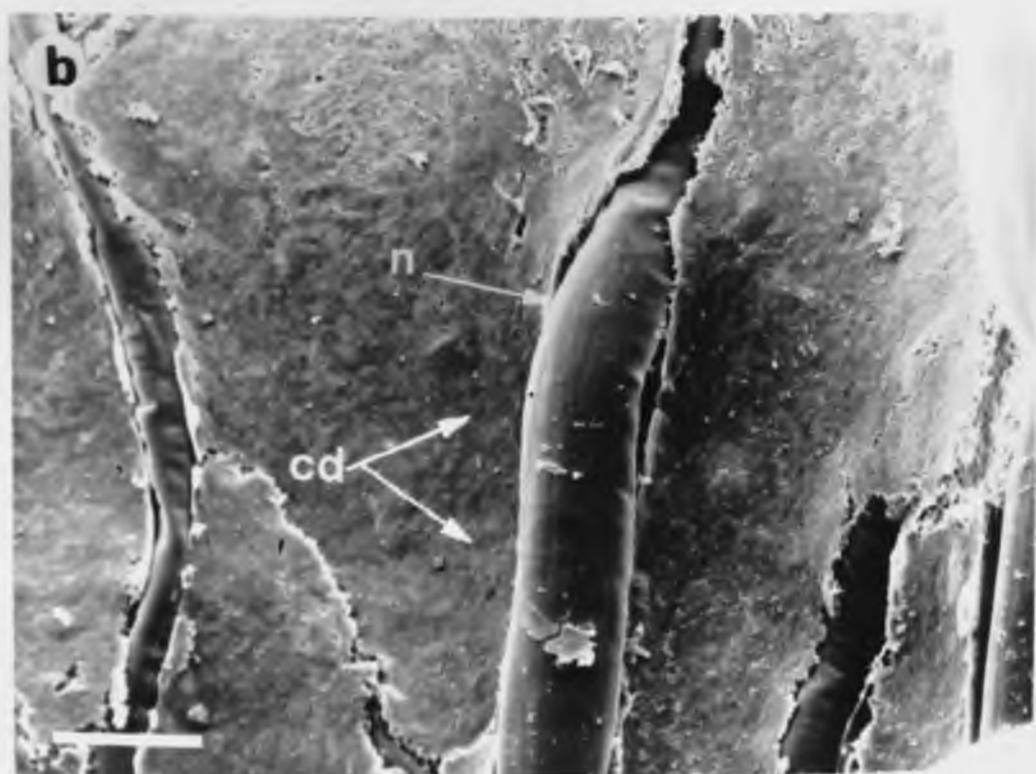
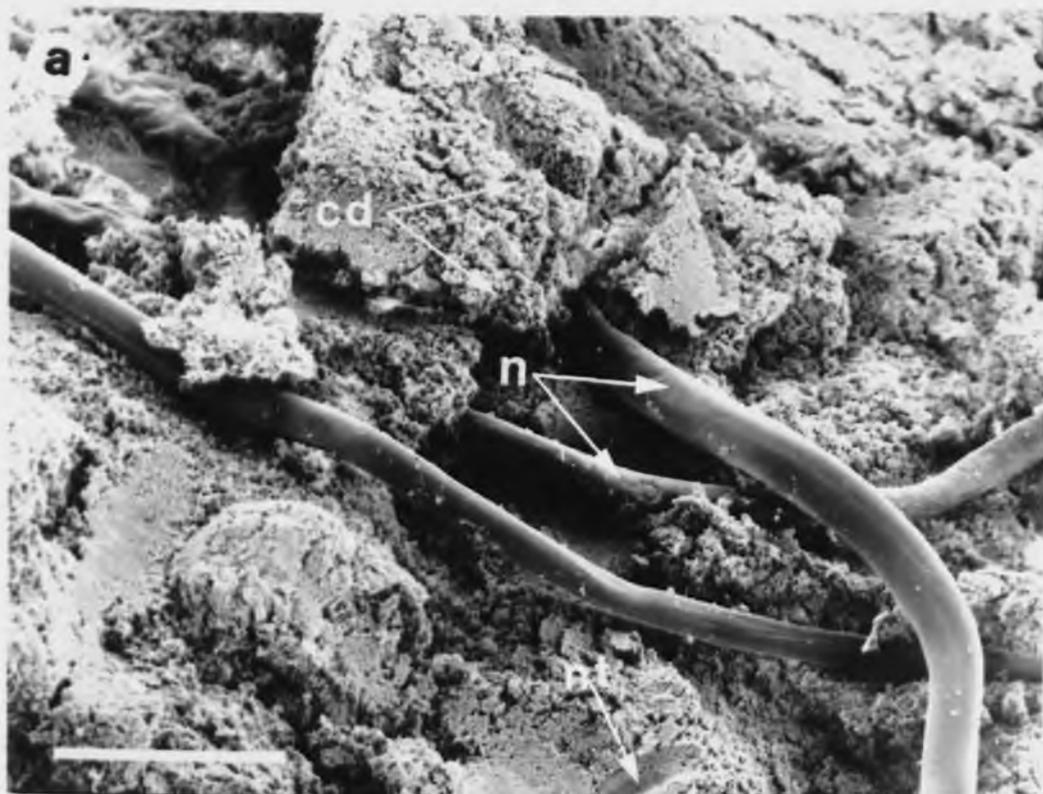


Fig. 5.6a. Scanning electron micrograph of the anterior end of an adult nematode to show the characteristic tri-radiate mouth (trm); and large lateral amphids (la). Scale bar $10\mu\text{m}$

Fig. 5.6b. Scanning electron micrograph of the posterior end of a male adult nematode to show the copulatory bursa (cb); the tip of the copulatory spicule (cs); and adherent bacteria (b). Scale bar $10\mu\text{m}$

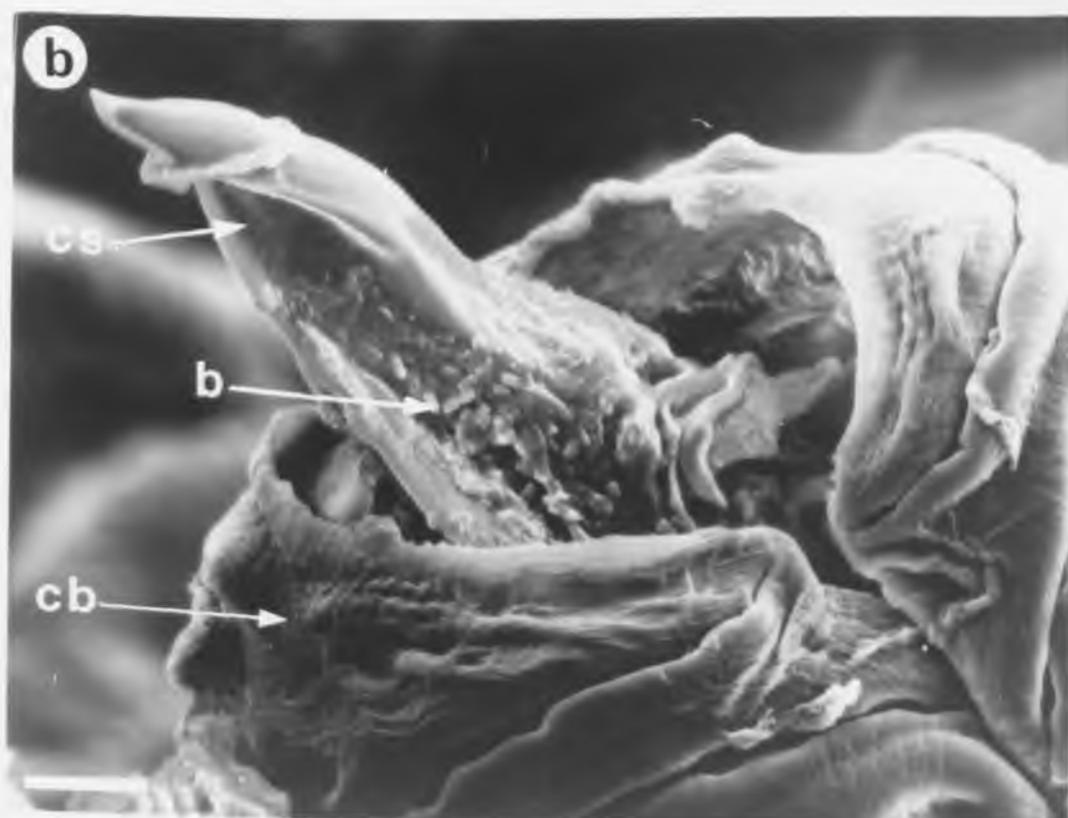
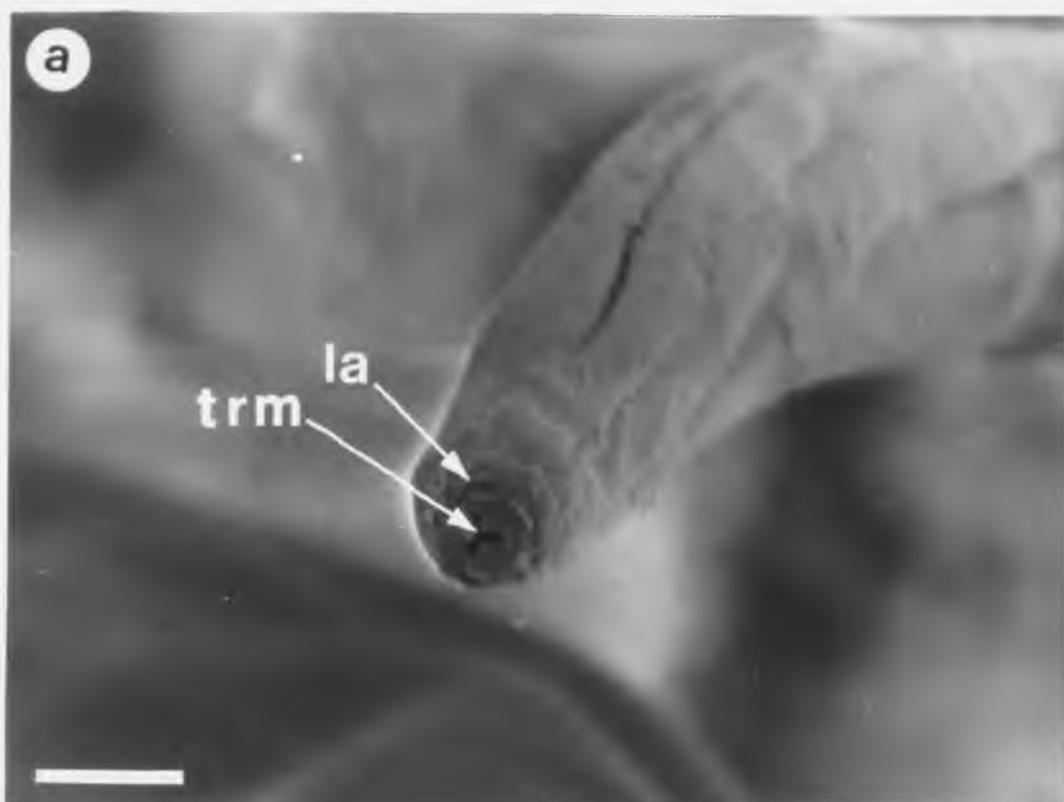


Fig. 5.7a. Scanning electron micrograph of the caecal mucosa of an adult red grouse which had been treated 5-6 months previously with the anthelmintic levamisole hydrochloride. Note the normal appearance of the longitudinal plicae (lp) and the absence of nematodes and nematode damage. Scale bar 1000 μ m

Fig. 5.7b. Scanning electron micrograph of the cuticular surface of a nematode from the caeca of a red grouse that had been treated with levamisole hydrochloride 5-6 months previously. Note the rosette-shaped structures (rs) adherent to the nematode cuticle (c). Scale bar 10 μ m

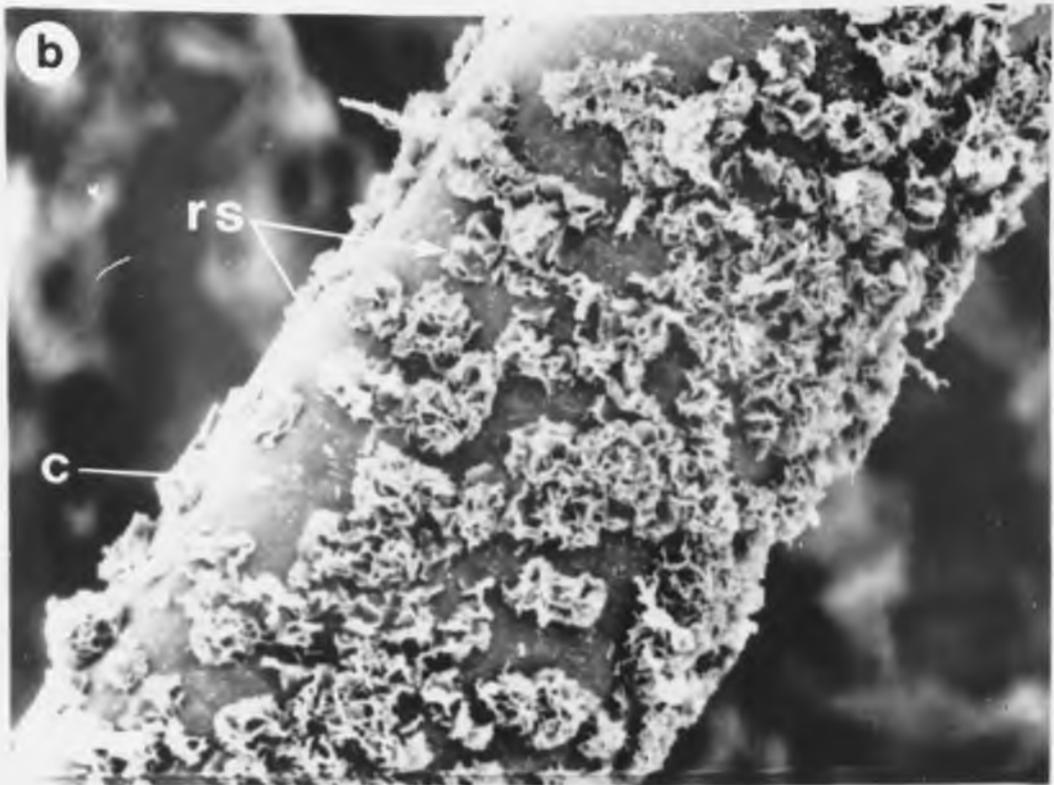
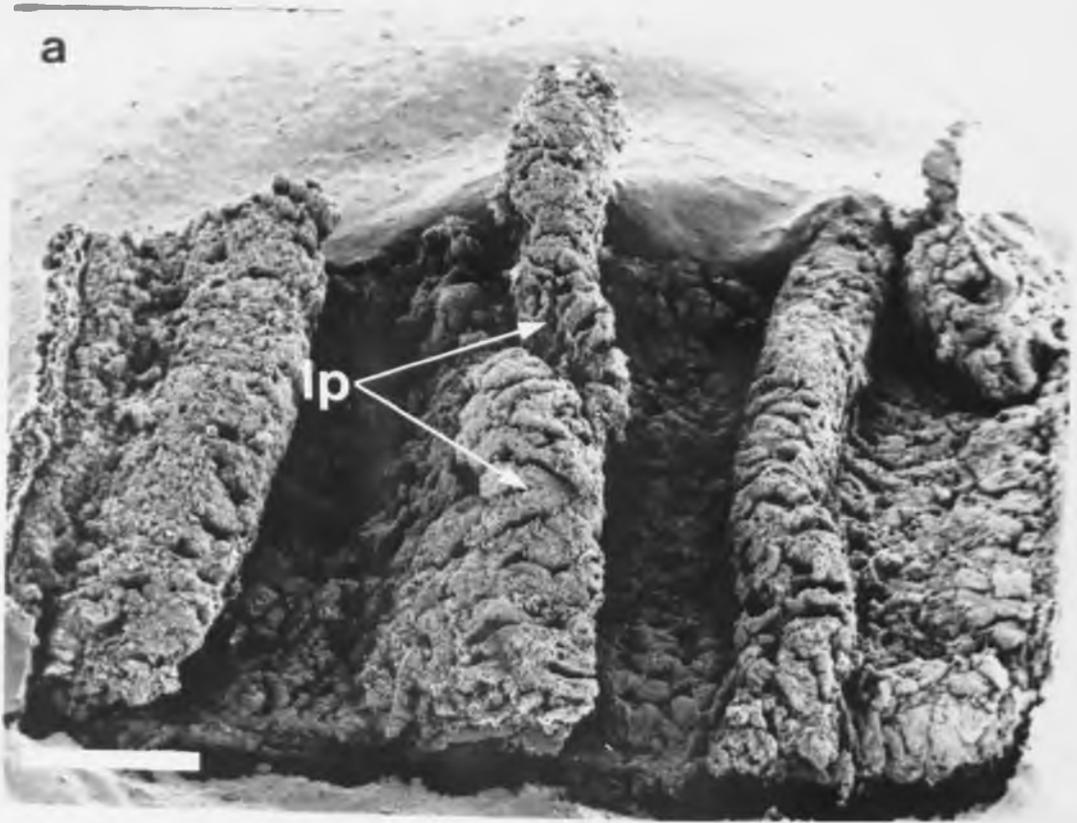


Fig. 5.8a. Scanning electron micrograph of adult nematodes from the caeca of a red grouse that had been treated with levamisole hydrochloride 5-6 months previously. Note adult nematodes (n) and parts of the cuticular surface of the nematodes covered in adherent rosette-shaped structures (rs) and debris, producing a lace-like appearance on the surface of the cuticle. Scale bar 100 μm

Fig. 5.8b. Scanning electron micrograph of an adult nematode from the caeca of a red grouse that had been treated with levamisole hydrochloride 5-6 months previously. Note the rosette-shaped structures adherent to the cuticle (rs) and superficial annulations across the cuticle (ca). Scale bar 30 μm

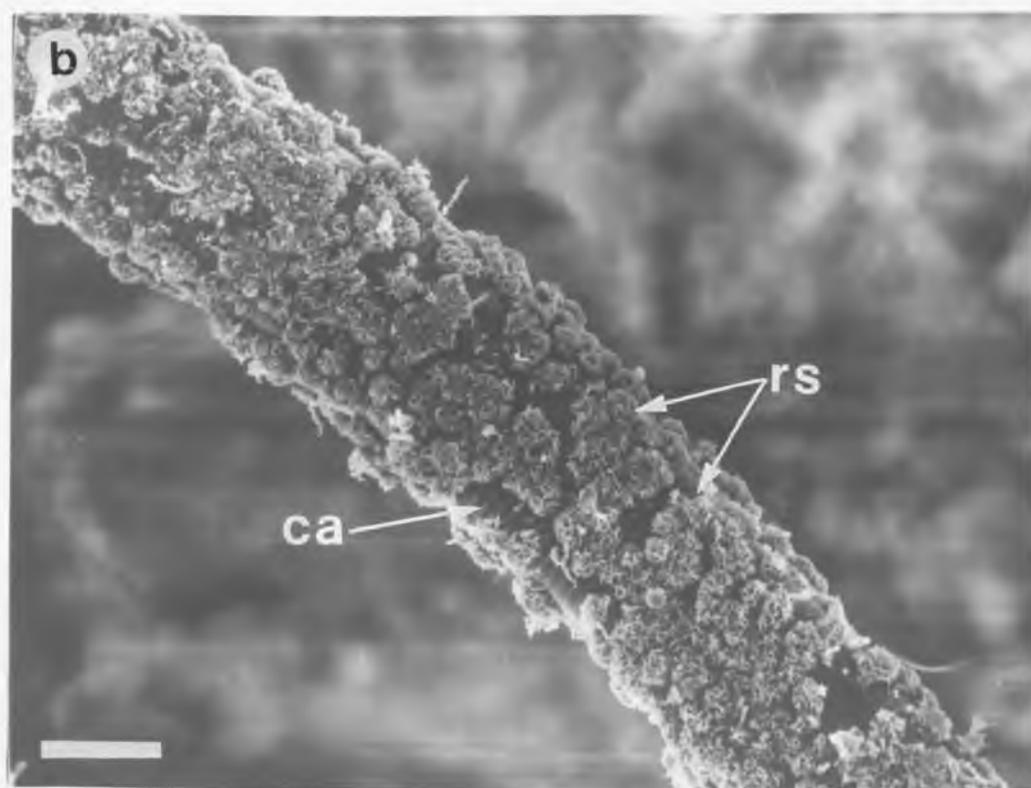
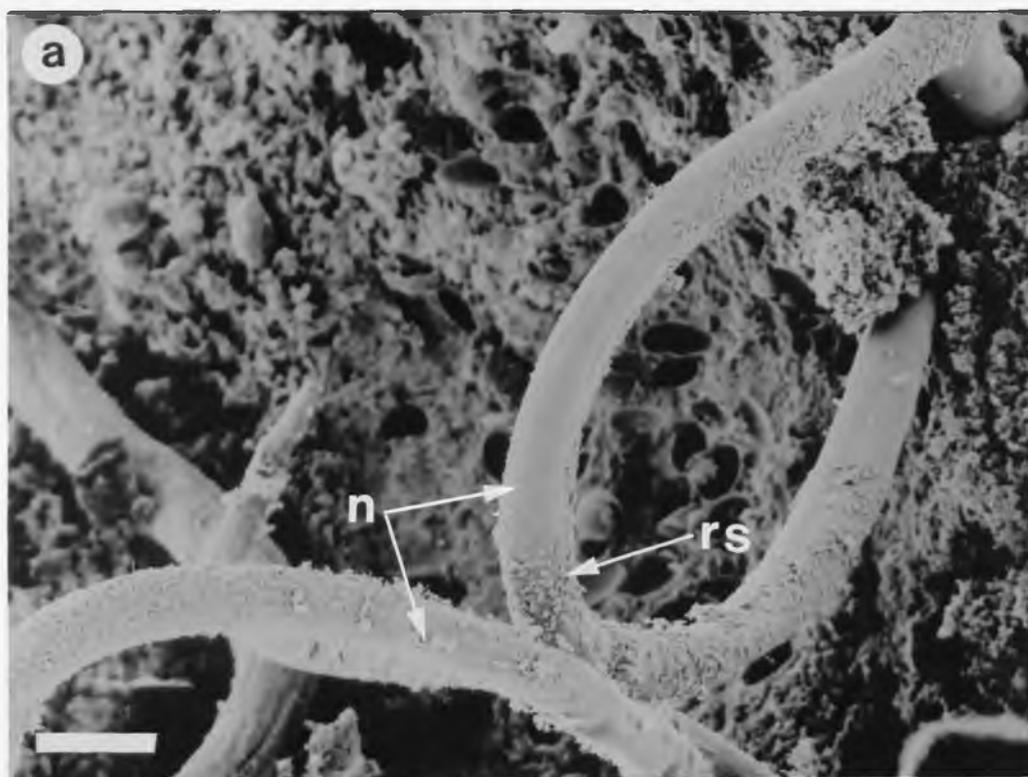
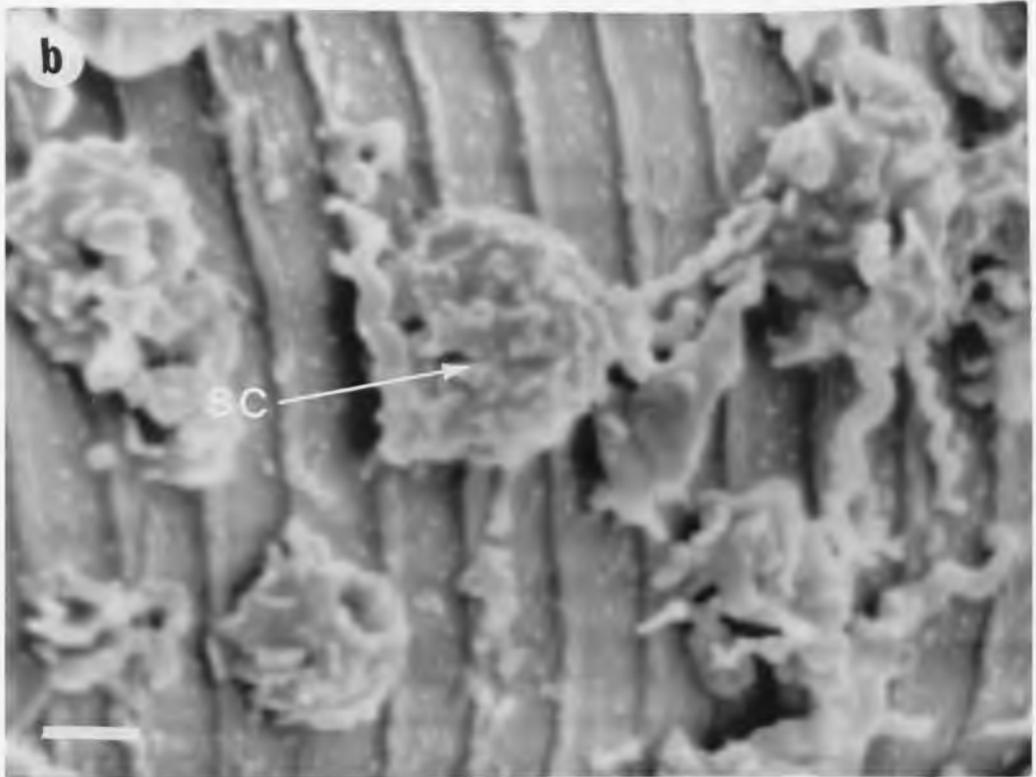
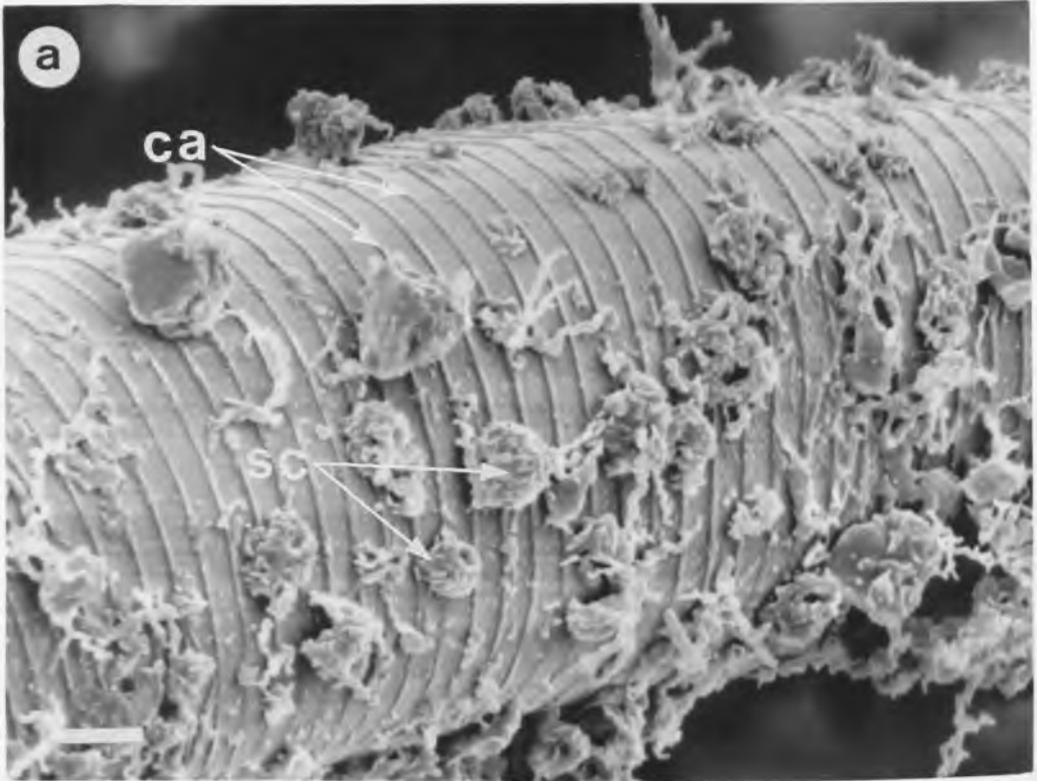


Fig. 5.9a. Scanning electron micrograph of an adult nematode from the caeca of a red grouse that had been treated with levamisole hydrochloride 5-6 months previously. Note the spherical-shaped cells (sc) with a highly ruffled surface adherent to the cuticle and superficial annulations across the cuticle (ca). Scale bar $10\ \mu\text{m}$

Fig. 5.9b. Scanning electron micrograph of the cuticle of an adult nematode from the caeca of a red grouse that had been treated with levamisole hydrochloride 5-6 months previously. Note the spherical-shaped cells (sc) adhering to the cuticle. Scale bar $3\ \mu\text{m}$



DISCUSSION

Scanning electron microscopy of the caeca from heavily and lightly infected grouse in this study has shown that the nematodes burrow into the caecal mucosa, often with their heads and tails free in the lumen, but with large parts of their body coiled through tissue. This confirms the findings of Wilson (1911). The flattening effect of the nematodes on the caecal mucosa tends to be localized in areas where the nematodes aggregate and is similar to the reduction in villus profile that occurs in lambs infected with Nematodirus battus (Martin & Lee, 1980). In the case of N.battus in lambs (Martin & Lee, 1980) and Nippostrongylus brasiliensis in rats (Symons, 1976) this reduction in villus profile is thought to be associated with an immunological response to the nematodes. The red grouse, however, seems to lack resistance to T.tenuis and, unlike N.battus and N.brasiliensis, which are quickly expelled from their respective hosts when these are given large doses of larvae (Mapes & Coop, 1972; Ogilvie, 1969), adult T.tenuis appear to remain within the caeca throughout the life of the grouse. It is possible that adult nematodes are expelled from the grouse but are subsequently replaced as more infective-stage larvae are ingested during feeding. However, this is unlikely since the numbers of adult nematodes present in the caeca build up during the life of the bird. It is unlikely, therefore, that the localized reduction in villus profile is due to an attempt to reject the nematode from the red grouse. Coop, Angus & Mapes (1973) suggested that the presence of mature N.battus

on the surface of the duodenal mucosa of infected lambs, may cause direct damage to the epithelial cells on the villi, resulting in a loss of these cells into the lumen of the gut and consequent reduction of the villus profile. Similarly, direct damage to the mucosa by T.tenuis may cause the localized flattening of caecal villi which was observed in this study. Alternatively, such flattening of the caecal mucosa may be due to an allergic or hypersensitive reaction. In such cases, the normal secondary response to contact with a previously experienced antigen is an exaggerated reaction that may result in tissue damage and the inflammation of infected tissue (Wakelin, 1984). Such hypersensitivity reactions may be classified into four distinct types. Type I, referred to as an immediate or anaphylactic hypersensitivity reaction, is dependent upon interactions between reaginic antibodies, usually IgE, and amine-containing cells; Type II are antibody-dependent, cytotoxic phenomena, resulting in the lysis of cells; Type III are antigen-antibody complex-mediated reactions; and Type IV are delayed hypersensitivity reactions, initiated by T-cells as opposed to antibodies, and may initiate the formation of granuloma. In red grouse infected with T.tenuis, any one of these reactions may occur and subsequently cause the disruption, inflammation and flattening of the caecal mucosa in infected birds. However, further study is required into the detailed pathophysiological and immunological effects of T.tenuis on red grouse before the type of reaction can be categorised.

Large infections of T.tenuis have been shown to cause an increase in the size of the caeca (Hudson, 1986a) and the pathological effects of the nematode on the caecal mucosa may interfere with the normal digestion of heather. Atrophy of the caecal mucosa is clearly associated with the presence of nematodes since it occurred only in those areas where nematodes were aggregated and was not found in anthelmintic-treated grouse which carried relatively few nematodes. Any pathophysiological effects on the bird could be caused by a decrease in the absorptive area of the affected parts of the caecum, a reduction in brush border enzyme activity and/or loss of plasma across the lesion.

It is important to note that there appeared to be marked recovery of the caecal mucosa in adult birds that had been treated with levamisole hydrochloride between December and March and this was apparent when the birds were shot in August or September of that same year, 5-6 months after treatment. It is possible that this effect was due to a low re-infection rate of the red grouse after dosing. However, current research into the epidemiology of the disease (Chapter 4) has shown that infective larvae, although not present on the heather during the winter months, occur in greater numbers in the late spring and summer, when there is a rise in numbers of infective larvae on the heather tips and a consequent increase in infection of the red grouse at this time of year.

The cuticle of nematodes from some birds which had been treated treated with levamisole hydrochloride 5-6 months

previously was covered in clusters of rosette-shaped structures. Levamisole hydrochloride is a fast, short-acting drug which is rapidly absorbed from the gastro-intestinal tract (Janssen, 1976) and causes paralysis and passive elimination of nematodes. Enigk & Dey-Hazra (1971) and Gualalp & Mayilmayil (1971) showed that adult T.tenuis in pheasants and in geese are susceptible to levamisole hydrochloride. Levamisole not only eliminates adult nematodes from the host, it is also known to enhance immune reactions in some hosts. Fischer, Oi, Kelley, Podgore, Bass, Wagner & Gordon (1974) demonstrated that levamisole enhanced immunological protection against bacterial and viral pathogens in immunologically immature rats. Lods, Dujardin & Halpern (1975) concluded that levamisole has no effect on antibody production in healthy subjects but Levo, Rotter & Ramont (1975) concluded that levamisole may affect cell-mediated immune reactions, normalizing the function of phagocytes and T-lymphocytes when their function is depressed. It is interesting to note that the rosette-shaped structures found adhering to the cuticle of some nematodes were found only on nematodes in grouse which had been treated with levamisole hydrochloride. Red grouse show no apparent resistance to T.tenuis and perhaps levamisole hydrochloride had stimulated the immune system of red grouse infected with T.tenuis, so that immunity had developed against the nematode and the rosette-like cells, which appeared to be macrophages, were attacking the surface of the nematode as part of a cell-mediated response to the presence of the nematode.

Parasitic nematodes have a tough, rigid cuticle composed of collagen-like proteins. It might be expected that this cuticle would be intrinsically resistant to immune attack (Butterworth, 1984). However, a number of immune effector mechanisms have been described that are active against parasitic nematodes. The adherence of eosinophils to the surface of the cuticle of nematodes in vitro has been demonstrated with N.brasiliensis (McLaren, Mackenzie & Ramalho-Pinto, 1977; Mackenzie, Jungery, Taylor & Ogilvie, 1981); Ascaris suum (Ziprin & Jeska, 1975); Dictyocaulus viviparus (Knapp & Oakley, 1981); Nematospiroides dubius (Chaicumpa & Jenkin, 1978); Litomosomoides carinii (Bagai & Subrahmanyam, 1970). However, such adherence has not yet been demonstrated in vivo. It has been suggested that, although eosinophils act against a wide range of nematodes in vitro, and appear from histological studies to be extensively involved in nematode destruction in vivo, neutrophils may be more active against ensheathed larvae (Butterworth, 1984). However, Mackenzie, Taylor, Jungery & Ogilvie (1981) reported the adherence in vitro of leucocytes, in the presence of complement or antibodies, to the surface of various stages in the life-cycle of Trichinella spiralis and N.brasiliensis. The authors noted considerable differences between the different types of leucocytes, in the time of onset of adherence to the worm surface, in the length of time the leucocytes remained attached, and also in the way they attached. Mast cells adhered for a short time, did not flatten and did not degranulate; neutrophil adherence ceased after 2-24 h;

macrophages adhered permanently to the surface of the worms, did not flatten and retained their integrity, while eosinophils adhered within minutes to the surface of the worms, flattened and subsequently degranulated. Such eosinophils flattened onto the surface of the worms become very closely adherent, so that each cell received a large area of contact between its surface and the cuticle of the worm. No other cells flattened to this extent. After more than 3 h in culture with pure populations of eosinophils, the surface of the worms had a 'lacy' appearance. This was due to membranous material left on the nematode cuticle and presumably consisted of cellular fragments of eosinophils left after the eosinophils had degranulated. Macrophages interacted with the surface of the parasite later to phagocytose eosinophil debris. It is possible that the rosette-shaped structures with a ruffled surface described in the current work, which produced a similar lace-like effect on the cuticular surface of several adult T.tenuis from red grouse, are the remains of degranulated eosinophils while the more spherical-shaped structures with an uneven, granular surface could be phagocytic macrophages. Macrophages are known to be highly effective against some nematodes (Butterworth, 1984) and play a vital role in the induction, regulation and expression of immune responses (Wakelin, 1984). The consequences of such adherence to the parasitic nematodes has been described by Mackenzie, Ramalho-Pinto, Taylor & Ogilvie (1980) and include the killing of infective larvae of T.spiralis by eosinophils and infective larvae of N.brasiliensis by the monocyte population. However, it is possible that both

structures described are of the same type but their appearance has been altered during the fixation and processing of samples for electron microscopy.

It is interesting to note that only some of the nematodes observed in the caeca from dosed grouse were covered in adherent leucocytes. It is possible that only nematodes of a certain age were affected in this way or, alternatively, adherence of leucocytes could occur at a certain time after infection with these individuals when they become susceptible to attack by host cells. The grouse in this study were examined 5-9 months after dosing with levamisole hydrochloride and this seems to be a long time for the drug to continue any enhancing effect on the immune system; this needs further investigation. If the adherent cells are leucocytes then this is the first time that adherence of these cells to the surface of a parasitic nematode in vivo, has been recorded. To provide conclusive evidence that the structures observed using scanning electron microscopy were adherent leucocytes, transmission electron microscopy or histopathological examination of sections of nematodes bearing the rosette-shaped structures is required. However, since only a very small proportion of nematodes in this study were covered in these structures, and this material was used for scanning electron microscopy, this was not possible. It will be necessary to obtain nematodes from dosed grouse at a time when a greater proportion of nematodes are covered in these structures, to provide further evidence as to the suggestion that the rosette-shaped structures on the

surface of the nematodes are adherent leucocytes.

Adult worm survival in the red grouse has been estimated at 54% with the nematodes surviving for up to 2 years (Hudson, 1986a). Adult birds which have experimentally reduced worm burdens by treatment with levamisole gain weight faster and are more likely to survive to the following shooting season than grouse with normal worm burdens (Hudson, 1986a). Treated birds produce larger clutches of eggs; these eggs increased hatching success and there is improved chick survival and this may result in a seven-fold increase in the production of young grouse (Hudson, 1986b). Such an improvement in the survival, condition, and fecundity of grouse with artificially reduced worm burdens could be, in part, due to the recovery of the caecal tissue following the elimination of the nematodes from the caeca

The cuticle of adult T.tenuis was superficially annulated and did not possess longitudinal ridges which are present on several adult Trichostrongylesp (Lee, 1965; Lichtenfels, 1971; Lichtenfels, 1974; Lichtenfels, 1977; Lichtenfels & Pilitt, 1983). Lee (1969) suggested that the cuticular ridges play an important role in both locomotion through the host gut and in abrasion of the host intestinal cells. Adult T.tenuis lack such cuticular ridges and thus differ from these other trichostrongyles.

It is concluded that T.tenuis damages the caecal mucosa of red grouse during its burrowing and feeding activity and

that this damage may lead to poor condition and poor breeding success of hen red grouse which in turn will contribute to the fall in the numbers of grouse available for harvesting each year.

CHAPTER 6

THE COURSE OF AN INFECTION WITH T.TENUIS IN THE
DOMESTIC CHICKEN

SUMMARY

The course of both primary and secondary infections with T.tenuis in the domestic chicken was investigated. Primary infections were established after the administration of single or trickle doses of infective-stage larvae. The worm burden in the caeca was highest after a single dose of 500 infective-stage larvae; this gave a mean of 87 nematodes per bird on days 8-9 of infection; 20 nematodes on day 14; and 0 on day 28 of infection. Following trickle doses of either 60, 100, 200, 300, 400 or 500 infective-stage larvae, there was a rise and then a fall in nematode egg output in all groups over a period of 36 days. In chickens given a primary dose of 500 infective-stage larvae followed 30 days later by a single secondary dose of 500 infective-stage larvae, the mean worm burden rose to 57 nematodes on day 9 of infection, then fell to 18 nematodes on day 15 and to 2 on day 30 of infection. Light microscopy of caecal mucosa showed a reddening of the mucosa of infected birds and scanning electron microscopy showed changes in the caeca of infected birds, with the caecal surface being covered in a layer of mucus from 12 days after infection. Balls of blood-stained mucus, some of which contained nematodes, were observed in the caecal droppings from day 9 of infection onwards. Following administration of 500 infective-stage larvae, a maximum of only 0.88% of larvae was recovered from the alimentary tract of infected birds between days 1 and 3 of infection. During a primary infection with 500 infective-stage larvae, there was a significant increase in the number of

circulating eosinophils between days 10 and 26 of infection; in the number of circulating monocytes between days 12 and 20 of infection; and in the number of circulating heterophils between days 18 and 20 of infection; but no circulating antibodies were detected in the blood of infected chickens. The effect on the gross structure of the caecum of chickens infected with 500 infective-stage larvae was also studied. There was a significant decrease in the length of the caeca of infected birds, compared with uninfected controls, between days 12 and 15 of infection. This corresponded to the time when nematodes were being expelled from the caeca. The course of both single and trickle infections with T.tenuis in grouse chicks was compared with that in chickens. Unlike the course of infection in the chicken, there was a gradual increase in nematode egg output from the grouse chicks throughout the duration of the experiment.

INTRODUCTION

There have been several recordings of T.tenuis in the domestic chicken. Cram & Wehr (1934) recorded the domestic chicken Gallus Gallus domesticus as a host for T.tenuis and Cram & Cuvillier (1934) established an infection with T.tenuis in domestic chickens from infective-stage larvae of pheasant origin and reported that chickens infected in this way lost the infection after 3 months. In Scotland, Morgan & Wilson (1938) examined a total of 1113 chickens, ranging from 6 months to 2 years of age, and found an infection with T.tenuis in only 22 of the birds examined, such an infection usually comprising 1-2 worms per bird and on this basis they suggested that a partial immunity to the parasite existed in chickens. Owen (1951) examined a total of 267 adult chickens (over 12 weeks of age) and 26 young chickens (2-12 weeks of age) and recorded T.tenuis in 3% of the older birds, with a mean worm burden of 4.6 worms, and found only one of the younger chickens was infected. More recently, Radhakrishnan & Ebrahimian (1975) reported T.tenuis in 90% of chickens examined in the Fars province of Iran but gave no indication of the level of infection in these birds.

The following work was initially undertaken to establish infections of T.tenuis in the domestic chicken in an attempt to study certain aspects of its pathology and life cycle in an easily maintained laboratory-reared host, since red grouse proved to be difficult to maintain under

laboratory conditions, were unavailable for several months of the year, and in captivity develop caeca of different length and structure and are thus not comparable with wild red grouse (Moss & Trenholm, 1987). However, the results proved to be very different from those obtained with red grouse (Wilson, 1983) and were explored further.

MATERIALS AND METHODS

Experimental birds

Male chickens (Sussex cross breeds), obtained as one-day-old birds from Mytholmroyd Hatcheries, Hebden Bridge, West Yorkshire, were used in all the experiments. Doses of infective-stage larvae to establish primary infections of T.tenuis were administered when birds were two weeks old. They were kept in standard laboratory chicken cages at a temperature of 26-28 C on a 16 h light/8 h dark cycle and were fed chick starter crumbs (without added coccidiostat) and water (with added riboflavin) ad lib. In the case of experiments involving a secondary infection, and therefore birds older than two weeks of age, birds were maintained in wire coops at 16-18 C.

Grouse chicks were reared and maintained as follows. Two clutches of grouse eggs were removed from the nests of grouse, approximately 1 week after laying, from a moor in upper Swaledale, North Yorkshire (NGR NY 930 020). Eggs were incubated in a still air cabinet incubator (Brinsea Ltd) until hatching. Grouse chicks were subsequently reared in a purpose built, solid bottomed pen and fed on a diet of high protein chick crumbs (Preston Farmers Ltd) and heather, cut from an outcrop of heather growing in an area free from grouse and therefore, presumably, free from T.tenuis larvae. Grouse chicks were reared in this way by Mr D.Newborn, Swale Farm House, Satron, Gunnerside, North Yorkshire. At 3 weeks of age, grouse chicks were transferred to the Animal House Unit, Dept of Pure & Applied Biology, University of Leeds and were maintained

under the same conditions as the chickens in the previous experiments, with the exception that the diet of grouse chicks continued to be supplemented with heather. These grouse chicks did not produce nematode eggs in their caecal droppings until after experimental infection. The small number of chicks (6) was all that could be made available for this experiment and this limited the number of replicates.

Collection of Trichostrongylus tenuis infective-stage larvae

The culture and collection of infective-stage larvae has been described in Chapter 2.

Infection procedure

Chickens and grouse chicks were given oral doses of infective-stage larvae of T.tenuis, suspended in a small volume of water (0.5-1.0 ml).

Caecal egg counts

Fresh caecal droppings from each group of birds were removed each day and the number of nematode eggs in each deposit was counted after salt flotation and estimation of the number of eggs present, using standard McMaster counting slides as described by Gordon & Whitlock (1939). The counts were expressed as the mean number of eggs per gram of caecal droppings.

Recovery of worms

Chickens were killed with carbon dioxide and their caeca removed. One caecum was cut along its length, everted, and the contents washed over a 180 μ m sieve. Worms collected on the sieve were washed into a plastic beaker and allowed to settle to the bottom of the beaker. The supernate was removed and the worms were decanted onto a petri dish, onto which a counting grid had been etched. The worms were counted under a low power dissecting microscope and the sex of each individual worm noted. The total numbers of worms present in each bird was obtained by counting the number of each sex removed from one caecum and multiplying by two. This provided a reasonably accurate count of the total nematode burden, since personal observation has indicated that the nematodes are almost equally distributed between the two branches of the caeca. The caecal wall was examined for lesions by means of a low power dissecting microscope.

Scanning electron microscopy

The second caecum was placed immediately into 10% phosphate-buffered formalin (pH 7) and left for 24 h. Cylinders of tissue, up to 1.0 cm in length, were removed from the mid-region of the caecum, slit longitudinally to expose the caecal mucosa and washed gently in buffered formalin to remove any adherent caecal content. Each piece of caecum was then frozen at -10 C and freeze-dried in a Chem Lab S.B.4 freeze drier. The freeze-dried samples were mounted on a specimen stub, coated with gold/palladium in a Polaron E5000 sputter coater and examined by means of a Camscan 3-30 scanning electron microscope operating at 10

kV. Caeca from uninfected birds of the same age as the experimental birds were processed and examined in the same way as the experimental birds, to act as controls.

Examination of the alimentary tract for infective-stage larvae

The alimentary tract was removed and divided into 7 sections; oesophagus and crop; gizzard; small intestine (anterior 300 cm); small intestine (middle 300 cm); small intestine (posterior 100 cm); caeca; and rectum. Each section was cut open, everted, and the contents washed into a 50 ml plastic beaker. The residue was then examined and the number of infective-stage larvae recorded. The remaining wall of each section of alimentary tract was chopped into small pieces and placed into 50 ml glass beakers. 20 ml of 1% pepsin/0.5% HCl was added and the mixture incubated at 37 C for 10-12 hours. The resultant digested material was examined by means of a dissecting microscope and the number of infective-stage larvae recorded.

Examination of caecal droppings for infective-stage larvae

Each day the caecal and faecal droppings were collected from each dosed bird and were placed in a 100 ml beaker. 100 ml of tap water was added to the droppings and the solution was stirred and left for 2-3 hours to allow the breakdown of the droppings. A 10 ml sample was then removed from the solution and placed in the bottom of a 50 ml test tube. 10 ml of sucrose solution (20 g sucrose in

50 ml distilled water) was placed below the suspension of faeces using a pasteur pipette. The mixture was centrifuged at 100 g for 5 minutes. Any larvae present were concentrated at the interface between the sucrose solution and the suspension of faecal material and were removed with a pasteur pipette.

Determination of the proportion of circulating blood leucocytes

Blood samples were taken by piercing the leg of each chicken with a sterile lancet and placing a drop of blood on a clean microscope slide. A blood smear was then made by drawing the drop of blood across the slide. Blood smears were air dried and were stained using Wright's stain. A total of 200 leucocytes were examined in each smear and the total numbers of lymphocytes, heterophils, monocytes, basophils and eosinophils were recorded and expressed as a percentage of the total leucocytes examined.

Detection of circulating antibodies

Collection of serum

Blood was extracted by cardiac puncture (by Mr D. Pedley) and placed in a non-heparinised tube and incubated at 37 C to allow the formation of a blood clot. The clot was discarded. The remainder of the sample was centrifuged at 3100 g for 10 minutes and the supernatant serum was removed and stored at -20 C until required.

Preparation of antigens

Two different antigens were prepared: a larval antigen made from 22000 infective-stage larvae of T.tenuis and an adult nematode antigen made from 100 adult nematodes. Larval and adult antigens were prepared in the same way.

Nematode larvae or adults were washed in phosphate-buffered saline (PBS) and added to 2% (w/v) Nonidet P40 in phosphate-buffered saline (PBS) and 1 mM phenylmethylsulfonylfluoride (PMSF) solution. The suspension was homogenised in a Jencons glass homogeniser, centrifuged at 15000 g for 10 minutes and the supernate was stored at -20 C until required for electrophoresis.

Electrophoresis

Electrophoresis was carried out using the method of Grabar & Williams (1953). 1% buffered agarose gels were cast onto microscope slides. Two 10 μ l wells and a central trough were cut in the gels. One well was filled with 5 μ l bromophenol blue, to monitor the progress of electrophoresis; the second well was filled with 10 μ l of larval or adult nematode homogenate; and the central trough was filled with 100 μ l of serum taken from an infected chicken. Two samples of sera were prepared from each chicken so that electrophoresis could be repeated a second time. Electrophoresis was carried out on the cooling plate of an LKB 2117 Multiphor (LKB, Croyden) at 5 V/cm for 1 hour. Once electrophoresis was complete, the gels were stained for protein.

Staining of gels

Slides were removed from the cooling plate and laid on a flat surface. A sheet of 82 x 13 mm Whatman 3 MM filter paper was placed on the gel surface and this was covered with 5 layers of paper towels in order to absorb moisture. The gel was then pressed by laying a glass plate on the paper towels and placing a 2.5 kg weight on the glass plate. The weight and paper towels were removed after 10 minutes and the gel was soaked in 0.1 M NaCl for 30 minutes. The gel was then pressed and soaked in 0.1 M NaCl twice more and then pressed again before rehydration in distilled water for 10 minutes. The gel was pressed once more and then dried at 37 C for 2 h. Dried gels were stained with 0.3% Coomassie Blue R250 (Sigma) in destaining solution [distilled water: ethanol: acetic acid; 9:9:2 (v:v:v)] for 15 minutes. They were then placed in destaining solution and continually agitated until excess stain was removed, then rinsed in distilled water and dried. Any antigen/antibody complex precipitated during electrophoresis would be revealed by the Coomassie Blue stain.

Determination of the length and weight of the caeca

The caeca from each chicken were examined and compared in the following sequence:

1. Each set of caeca was placed on a white cardboard background and photographed with a Pentax spotmatic camera and extension tube.
2. Each individual caecum was separated from any adherent portions of small intestine and weighed on a Mettler

PI 65 balance.

3. Individual caeca were measured and caecal length recorded.
4. Nematode burdens in the caeca of infected birds were recorded.
5. The remaining caecal wall was weighed on a Mettler PI 65 balance.
6. The caecal wall was placed in a glass vial, dried in an oven for 48 hours at 80 C, and the dry weight recorded on a Mettler PI 65 balance.

Statistical analysis of results

The results were analysed by the non-parametric Wilcoxon test or the Students t-test. A value of $P < 0.05$ was considered to be significant.

RESULTS

The course of infection in chickens given varying doses of infective-stage larvae

Chickens, in groups of five, received single doses of 100, 500, 1000, 2000 or 3000 larvae. The results are shown in Fig. 6.1. Doses of 100 and 500 larvae produced the highest nematode egg output, while nematode egg output was lower in the groups given 1000 and 2000 larvae. Chickens given 3000 larvae failed to produce any nematode eggs. Maximum egg production in birds given 100 or 500 larvae occurred about day 12 post-infection but egg production fell rapidly after that and egg production had virtually ceased by day 26 in all groups.

Effect of trickle doses of infective-stage larvae on nematode egg production

Chickens, in groups of five, received single doses of 20 larvae per day to establish cumulative doses of 60, 100, 200, 300, 400 or 500 larvae. Birds receiving 60 larvae were given larvae between days 1-3 of infection inclusive; 100 larvae, between days 1-5 inclusive; 200 larvae, between days 1-10 inclusive; 300 larvae, between days 1-15 inclusive; 400 larvae, between days 1-20 inclusive; and 500 larvae, between days 1-25 inclusive. The results are shown in Fig. 6.2. All birds in groups given trickle doses of larvae produced nematode eggs, and a rise and fall in nematode egg production was observed in all groups throughout the course of infection. Nematode egg output in chickens given cumulative doses of 100, 200 or 300

infective-stage larvae were similar, but differed from those given cumulative doses of 60, 400 or 500 larvae, where nematode egg output was much lower.

Adult worm burden during a primary infection

One hundred and forty chickens were each given a single oral dose of 500 infective-stage larvae. Ten birds were killed every alternate day from days 2 to 28 post-infection and the adult worms were collected from one caecum and the result doubled to obtain the number of nematodes per bird. The results are shown in Fig. 6.3. The number of adult nematodes in the caeca increased rapidly after day 4 post-infection and reached a peak at about day 8. There was then a fall in the number of nematodes present, until by day 20 there were very few individuals present. Adult nematodes were observed in the caecal droppings of infected birds from about day 9 post-infection. These expelled nematodes were often embedded in balls of mucus which also contained small clots of blood and pieces of caecal mucosa (Fig. 6.5a). The sex ratio of male to female T.tenuis was found to alter throughout the course of the infection (Fig. 6.3). Females were initially more numerous than males but there was a gradual increase in the percentage of male nematodes in the caeca between days 6-18 post-infection (Fig. 6.4). However, from day 20 onwards, the total number of nematodes present in the caeca was so low (less than 5 nematodes per bird) it was not meaningful to express the proportion of male and female nematodes present in the

Fig. 6.1. The effect of different single doses of infective-stage larvae of T.tenuis on nematode egg output in the domestic chicken.

- (▲) single dose of 100 infective-stage larvae
- (●) single dose of 500 infective-stage larvae
- (◇) single dose of 1000 infective-stage larvae
- (△) single dose of 2000 infective-stage larvae

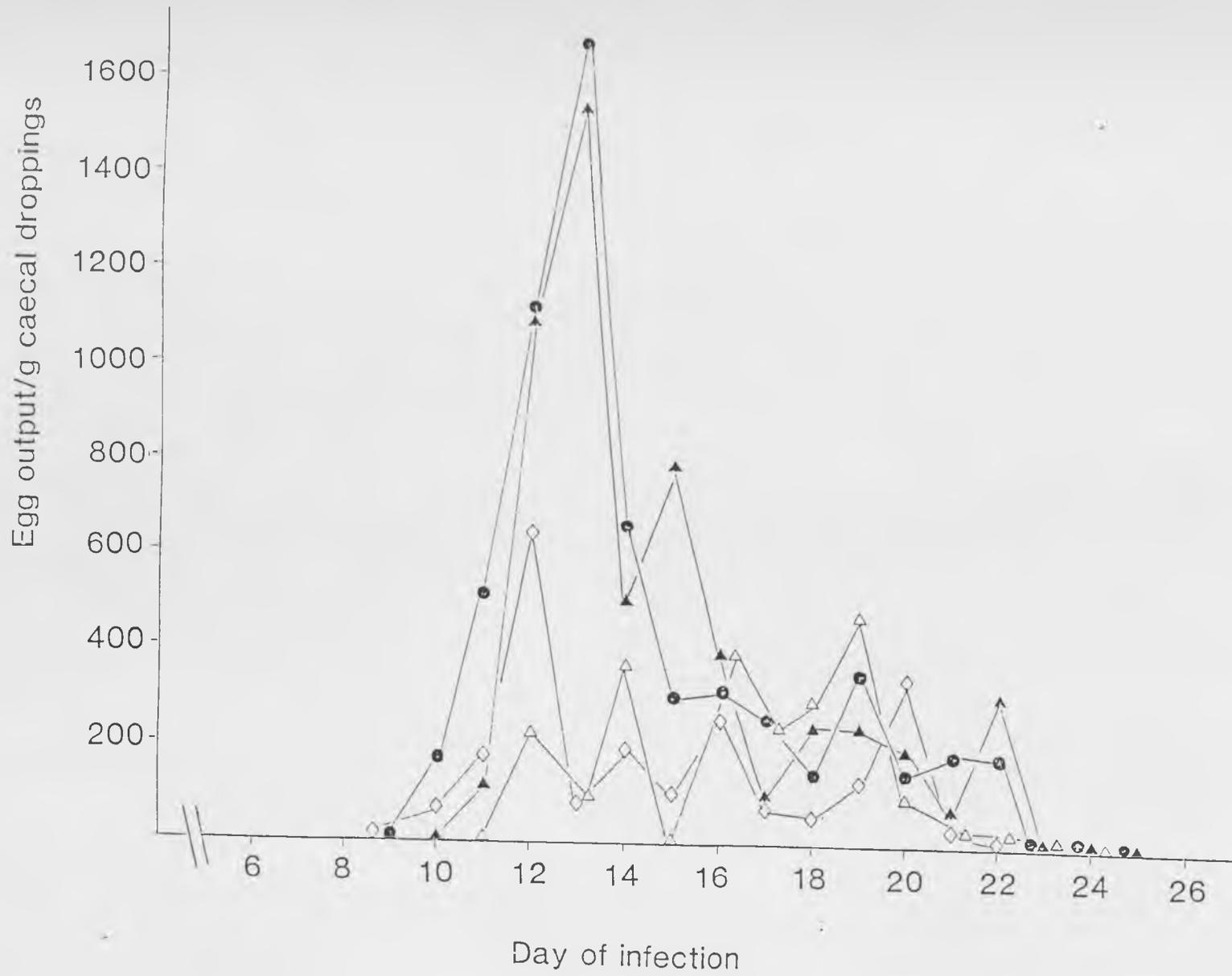


Fig. 6.2. The effect of trickle doses (20 larvae per day for up to 25 days) of infective-stage larvae of T.tenuis on nematode egg output in the domestic chicken.

- (●) cumulative dose of 60 larvae
- (■) cumulative dose of 100 larvae
- (○) cumulative dose of 200 larvae
- (▼) cumulative dose of 300 larvae
- (△) cumulative dose of 400 larvae
- (□) cumulative dose of 500 larvae

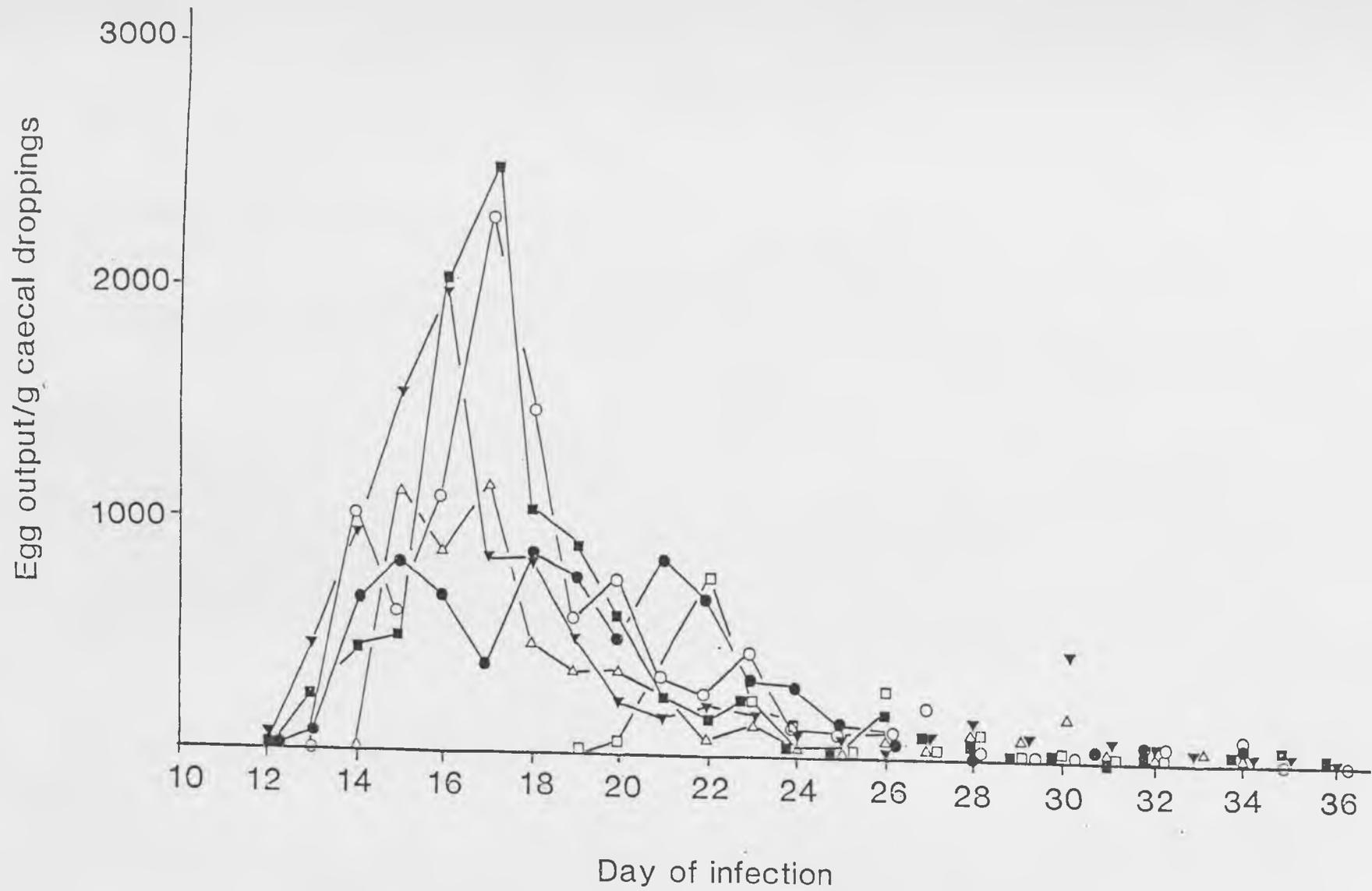


Fig. 6.3. The number of adult T.tenuis which became established in the caeca of the domestic chicken after a single dose of 500 infective-stage larvae.

- (■) total worm burden
- (□) number of females
- (△) number of males

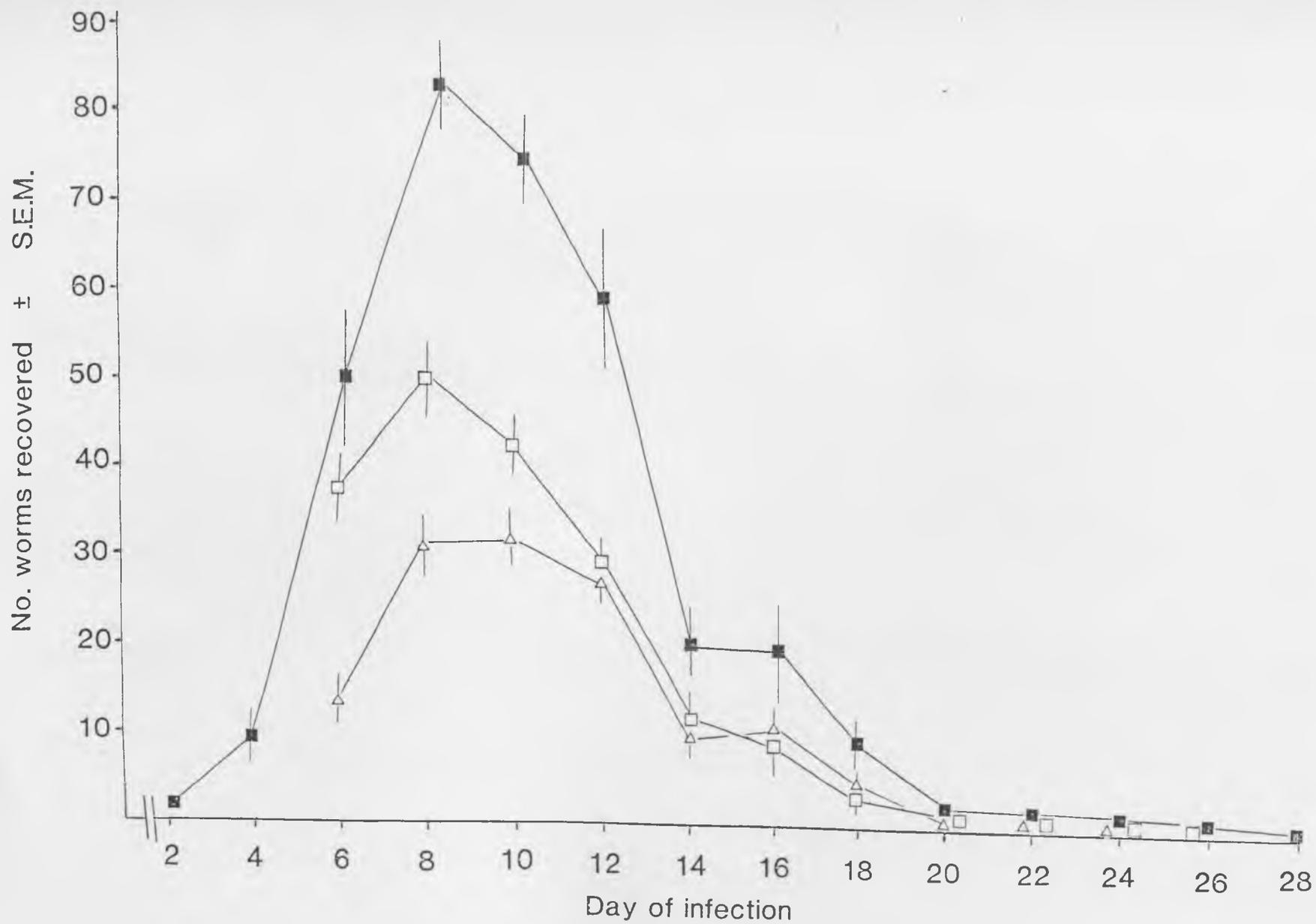
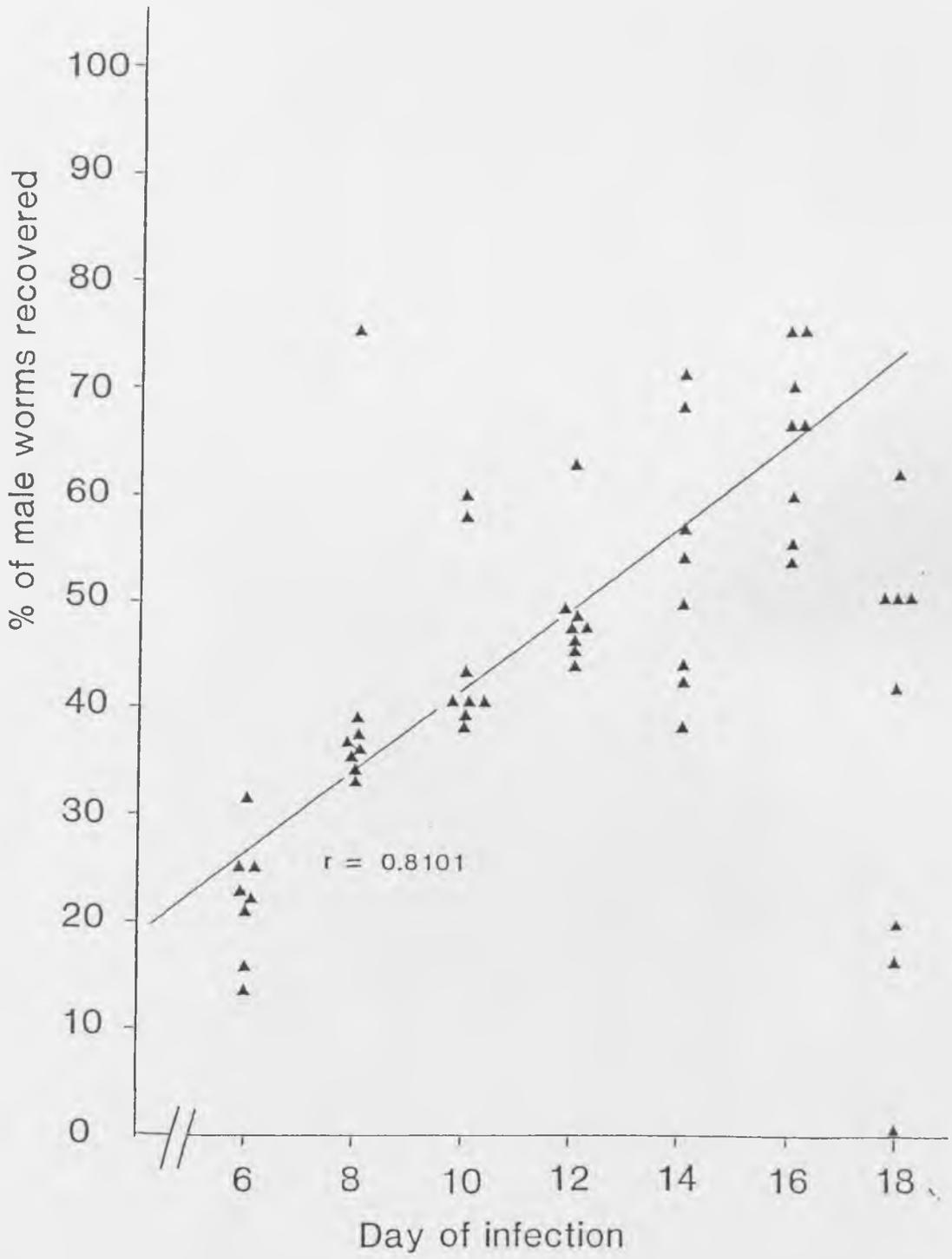


Fig 6.4. The percentage of adult male nematodes recovered from the caeca of the domestic chicken between days 6 to 18 post-infection, following a single dose of 500 infective-stage larvae of T.tenuis. Line fitted by regression analysis.



caeca as a percentage.

Pathological changes in the caeca produced by infection with *T.tenuis*

The caeca of forty-two chickens given a single inoculum of 500 larvae were examined at intervals of two days, from day 2 to day 28 post-infection, by means of a dissecting microscope and by scanning electron microscopy. From day 6 to day 30 post-infection, there were numerous haemorrhagic lesions present on the mucosa (6.5b) and from days 12 to 28, the mucosa was usually covered by a layer of mucus which masked the ridges of the mucosa (Fig. 6.6b). These ridges, and the mucosal surface, were clearly visible in the uninfected chickens (Fig. 6.6a). The location of the crypts of the mucosa was revealed by holes in the layer of mucus in infected birds (Fig. 6.6b). The adult nematodes burrowed superficially beneath this layer of mucus (Fig. 6.7a and 6.7b) but were only rarely found within the mucosa itself.

Adult worm burden during a secondary infection

One hundred and fifty chickens were divided into 3 groups of 50 birds. Each bird in groups 1 and 2 was given a single oral dose of 500 infective-stage larvae and all birds in group 3 remained uninfected. Groups of 5 birds from group 1 were then killed at intervals of 3 days, from day 3 to day 30 post-infection, and the adult worm burden from both caeca was recorded. After 30 days, the 50 chickens in group 2 were given a second oral dose of 500 infective-stage larvae and, concurrently, all birds in

group 3 were given a single oral dose of 500 infective-stage larvae, thus establishing a primary infection in group 3 birds. Five birds from each group were then killed at intervals of 3 days, from day 3 to day 30 post-infection, and the worm burden in both caeca was recorded. The same stock of infective-stage larvae was used to infect all chickens in this experiment but was different from that used in the earlier experiment. The larvae were kept at 4 C until used. The results of this experiment are shown in Fig. 6.8. A primary infection became established (positive egg production) in all the chickens of groups 1 and 2 given infective-stage larvae. The course of infection in the re-infected birds (group 2) was similar to that of the primary infection in group 1 birds and to that of the age-matched control birds (group 3), but fewer nematodes became established in the secondary infection. There were, however, no significant differences in the number of nematodes which became established during the primary infection in group 1 birds, in the secondary infection (group 2) and the primary infection in age-matched controls (group 3) ($p > 0.05$, non-parametric Wilcoxon test).

Location in the alimentary tract of infective-stage larvae
1-3 days post-infection

15 chickens were each given a single oral dose of 500 infective-stage larvae of T.tenuis. 5 birds were subsequently killed either 1, 2 or 3 days post-infection. The alimentary tract of each bird was removed and the wall and contents were examined microscopically for the presence

Table 6.1.

The mean number of the infective-stage larvae of T.tenuis in the alimentary tract of the domestic chicken, following a dose of 500 infective-stage larvae, on days 1-3 of infection. (n=5 chickens)

Day	Oesoph- agus	Crop	Gizzard	0-300 cm SMALL	300-600 cm INTESTINE	600-700 cm	Caeca	Rectum	Faecal DROPPINGS	Caecal	% Recovery
1	0	0	0	1.2±0.8	3.0 ± 2.0	1.4 ± 0.98	1.6±1.2	0	0	0	1.44
2	0	0	0	0	1.0 ± 0.5	3.6 ± 1.0	8.4±3.0	0	0	0	2.60
3	0	0	0	0	1.0 ± 0.63	0.2 ± 0.2	13.4±3.4	0	0	0	2.92

of infective-stage larvae. The results are shown in Table 6.1. Very low numbers of infective-stage larvae were found in the alimentary tract; no larvae were found in any of the peptic digests, nor in the caecal or faecal droppings examined. Only 0.32% of the total number of larvae given were found in the caeca 1 day after infection; 1.68% were found 2 days after infection; 2.68% 3 days after infection. The percentage recovery of larvae from the alimentary tract on day 1 was 0.2%; 2.6% on day 2; and 2.92% on day 3. The larvae which were recovered were exsheathed larvae.

Effect of infection on the proportion of circulating blood leucocytes

Two groups, each of 10 chickens, received either a single dose of 500 infective-stage larvae or remained uninfected. Each bird was individually marked and blood samples were taken from each individual chicken on alternate days, between days 0 to 26 of infection, and the proportion of circulating blood leucocytes was determined. The various types of circulating blood leucocytes are shown in Figs 6.9a-e. The proportion of circulating blood leucocytes was found to alter during the course of infection. There was a significant increase ($p < 0.01$, Students t-test) in the numbers of eosinophils found between days 10 and 26 of infection, although this increase was not significant on day 16; a significant increase ($p < 0.01$, Students t-test) in the numbers of circulating monocytes between days 12 and 20 of infection; and in the numbers of circulating heterophils ($p < 0.01$, Students t-test) between days 18 and 20 of infection.

Fig. 6.5a. Photograph of balls of mucus containing adult nematodes which had been expelled from the caecum of a chicken infected with 500 infective-stage larvae of T.tenuis, on day 15 of infection. Note balls of mucus containing clots of blood (b); mucus expelled with faeces (m); and adult nematodes entrapped in the mucus (n).

Fig. 6.5b. Photograph of the caecal mucosa of a 26 day old chicken infected with 500 infective-stage larvae of T.tenuis 14 days previously. Note lesioning and reddening of the caecal ridges (cr) and blood spots on the mucosal surface (bs).

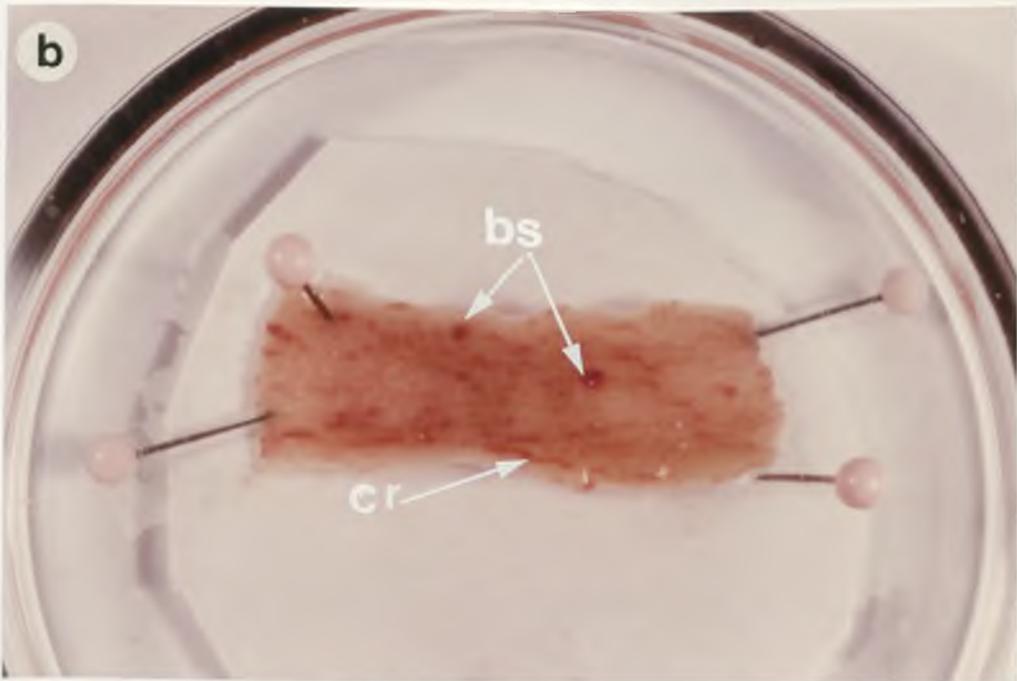
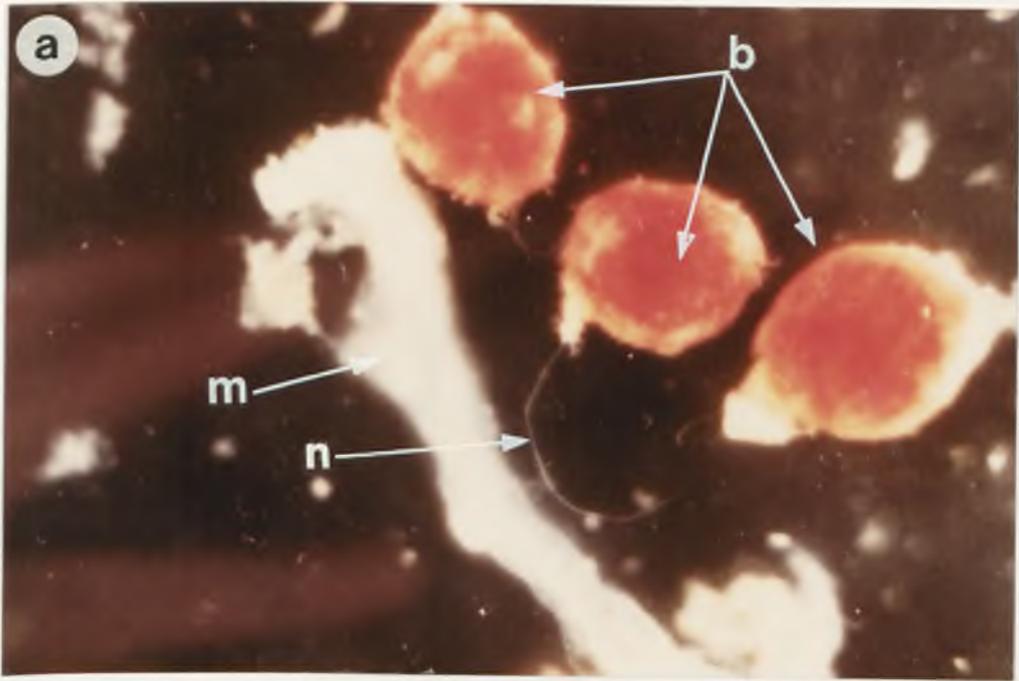


Fig. 6.6a. Scanning electron micrograph of the caecal mucosa of an uninfected 26 day old chicken to show the characteristic ridges of the caecal mucosa (cr) unobscured by mucus. Scale bar $100\ \mu\text{m}$

Fig. 6.6b. Scanning electron micrograph of the caecal mucosa of a 26 day old chicken which had been given 500 infective-stage larvae of T.tenuis 12 days previously. Note caecal mucosa covered in a mucus coat (m); crypt openings penetrating through the mucus coat (c); and bacteria adherent to the mucus coat. Scale bar $100\ \mu\text{m}$

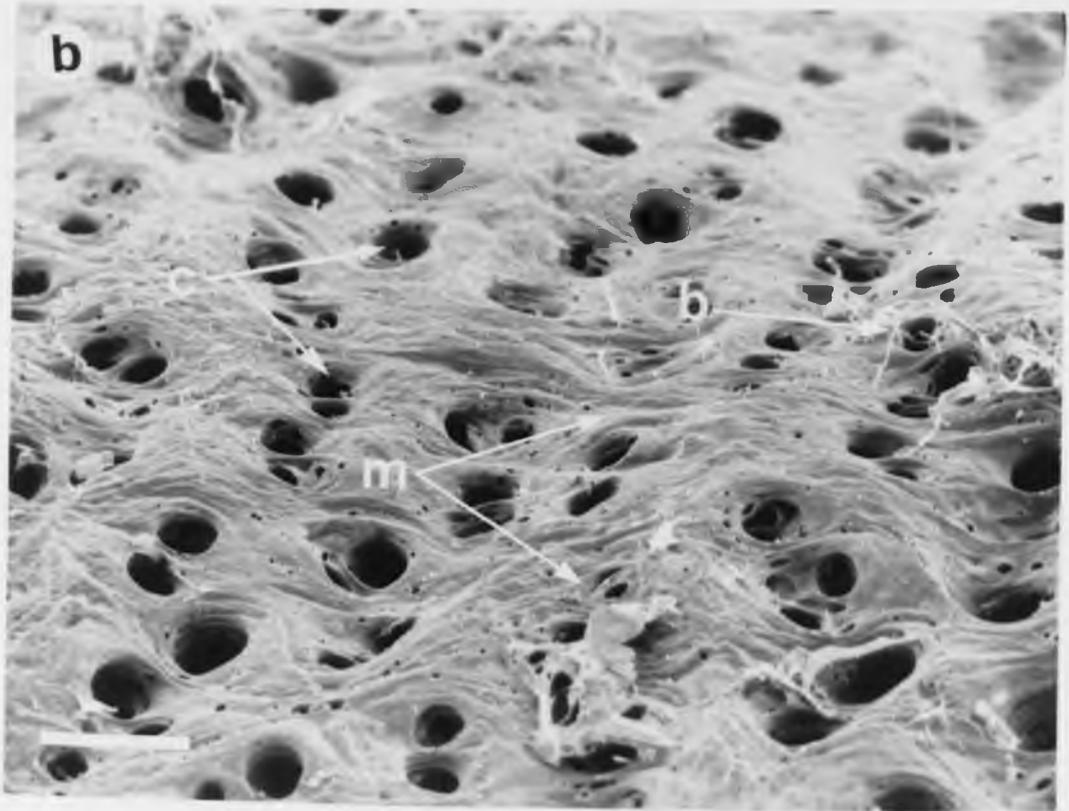


Fig. 6.7a. Scanning electron micrograph of the caecal mucosa of a 26 day old chicken which had been given 500 infective-stage larvae of T.tenuis 12 days previously. Note two adult nematodes (n) burrowing through the mucus coat (m). Scale bar 300 μ m

Fig. 6.7b. Scanning electron micrograph of the caecal mucosa of a 26 day old chicken which had been given 500 infective-stage larvae of T.tenuis 12 days previously. Note part of an adult nematode burrowing across the caecal mucosa (n); mucus around the nematode (m); and crypt openings (c). Scale bar 100 μ m

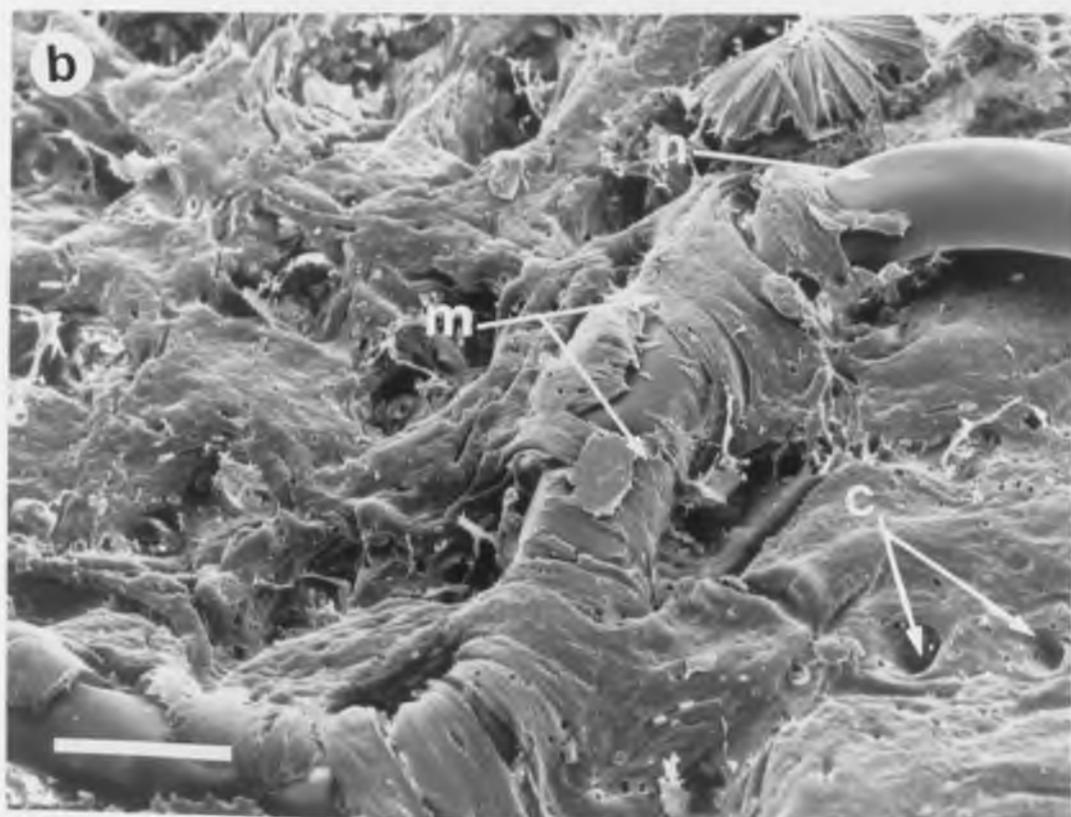
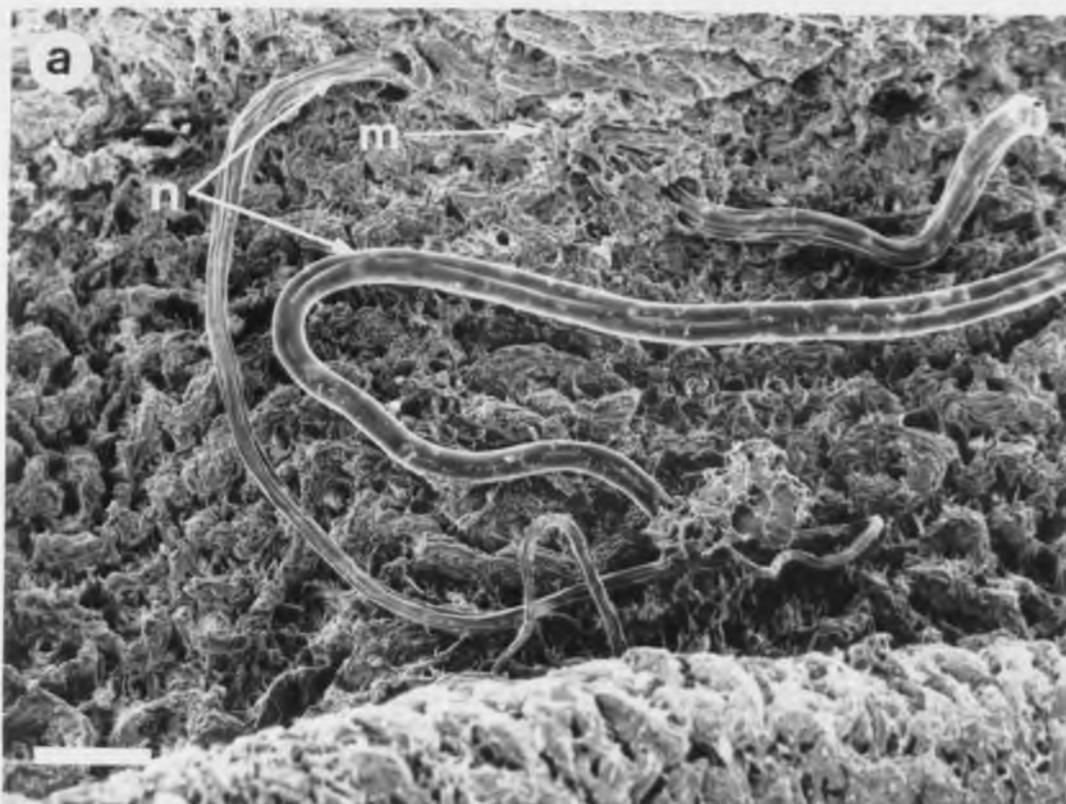


Fig. 6.8. The total numbers of adult T.tenuis which became established in the domestic chicken during a primary infection (group 1) , during a secondary infection (group 2), and in age-matched controls (group 3) given a primary infection . In the primary infections birds were given a single dose of 500 infective-stage larvae; in secondary infections birds were given 500 infective-stage larvae 30 days after the initial dose of 500 infective-stage larvae. Bars indicate standard error of the mean.

- (○) primary infection (group 1)
- (■) secondary infection (group 2)
- (●) primary infection in age-matched controls (group 3)

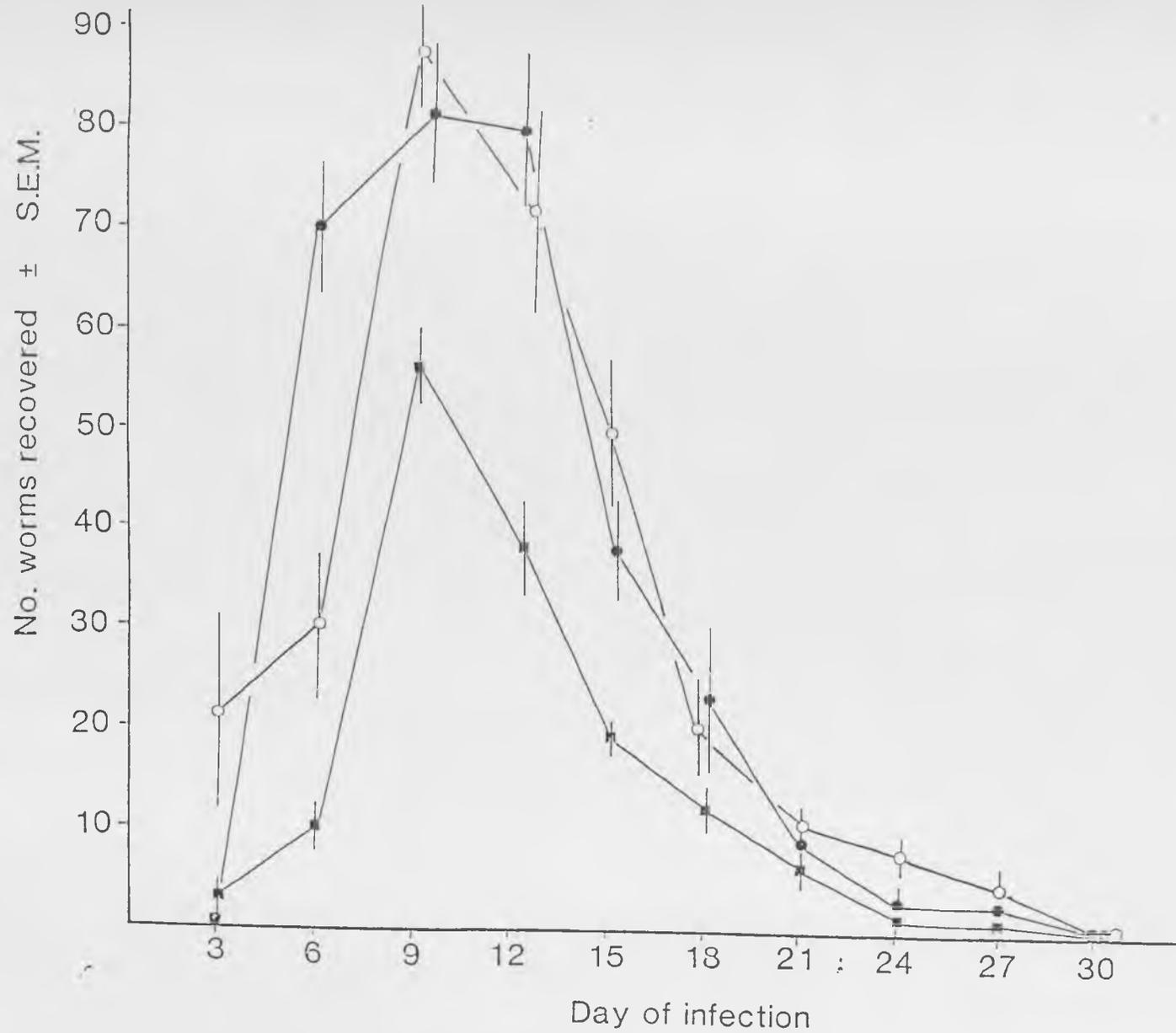


Fig. 6.9a. Blood smear from an infected chicken stained using Wright's stain. Note heterophil leucocyte (h).

Fig. 6.9b. Blood smear from an infected chicken stained using Wright's stain. Note small lymphocyte (l).

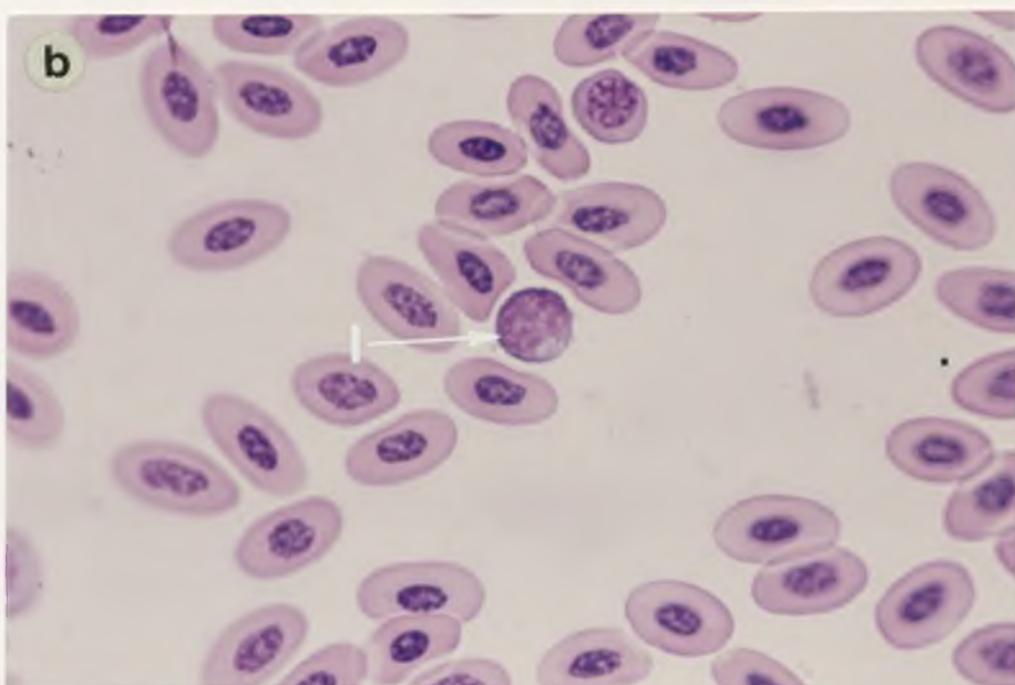
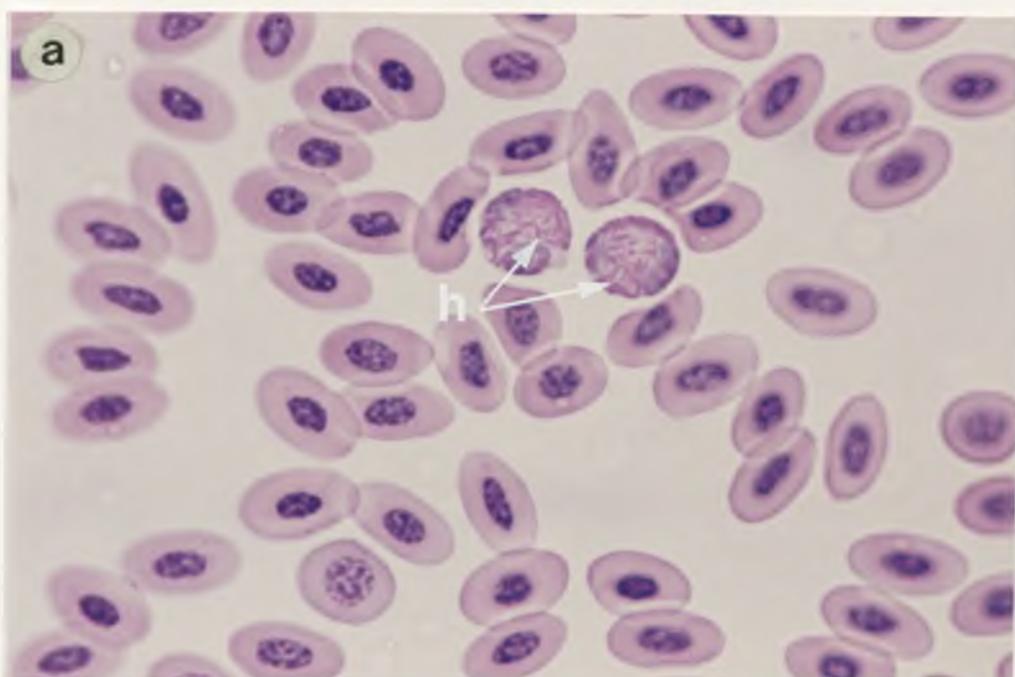


Fig. 6.9c. Blood smear from an infected chicken stained using Wright's stain. Note monocyte (m).

Fig. 6.9d. Blood smear from an infected chicken stained using Wright's stain. Note basophil leucocyte (b).

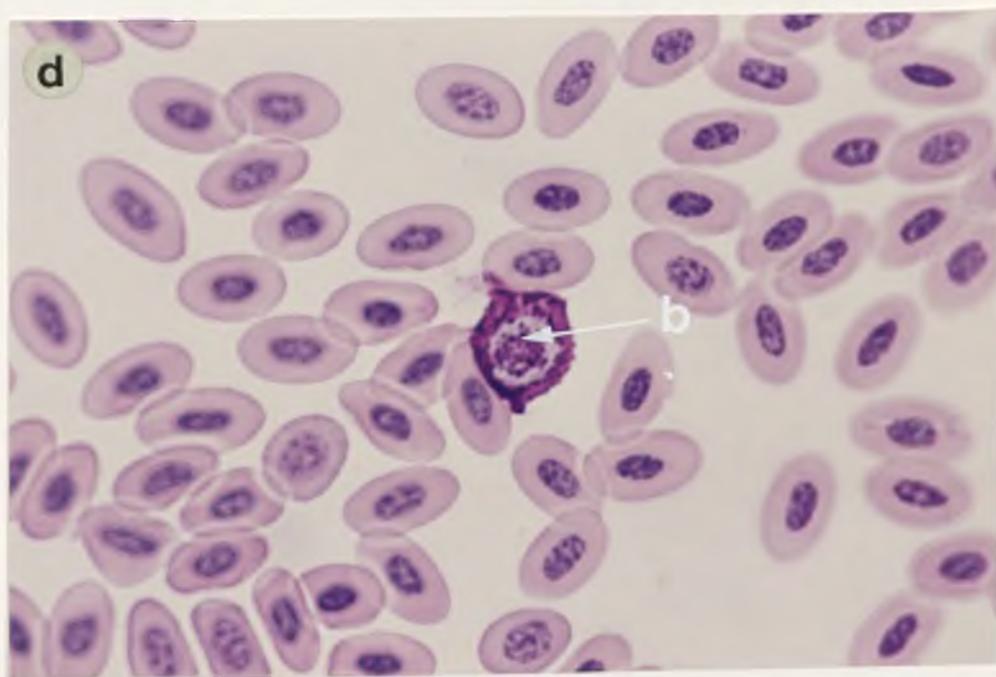
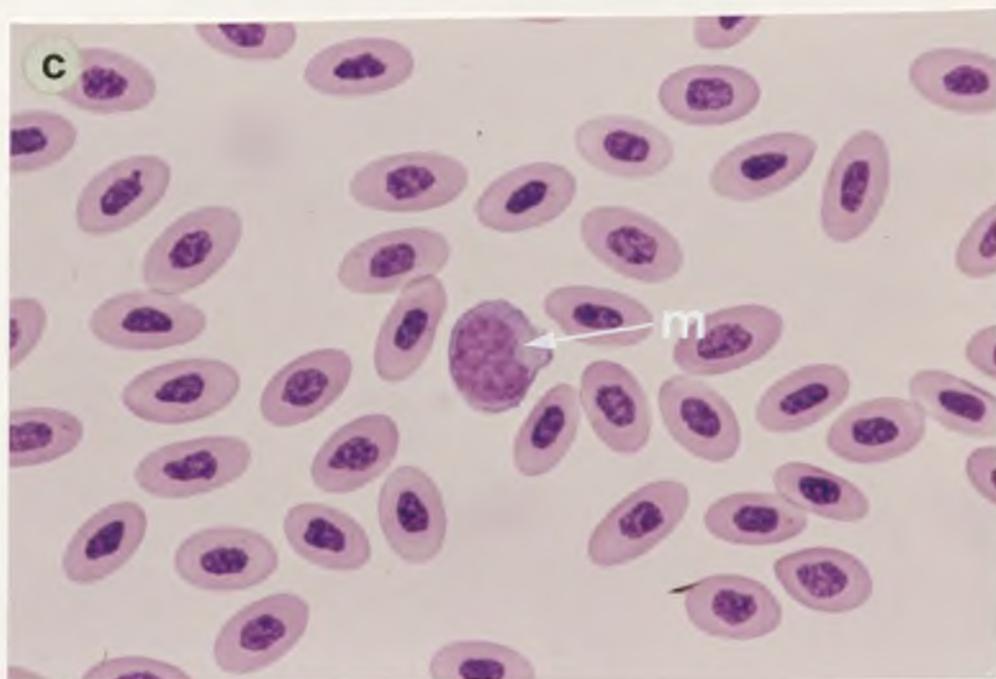
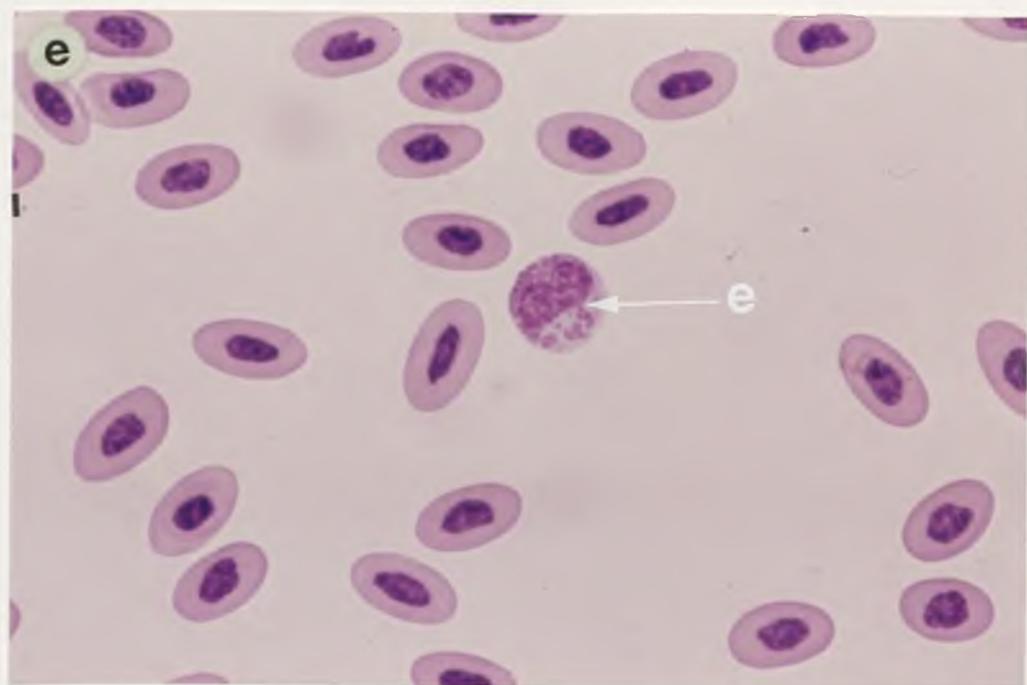


Fig. 6.9e. Blood smear from an infected chicken stained using Wright's stain. Note eosinophil leucocyte (e).



Effect of infection on the production of circulating antibodies

Three chickens were infected with 500 infective-stage larvae of T.tenuis and blood serum was taken from each bird 5, 10 and 15 days post-infection. Serum taken from each bird at each day was used in electrophoresis with both adult worm and larval antigen. No antigen/antibody precipitate was observed with any of the sera or antigens used.

Effect of infection on the length and weight of the caeca

One-hundred and five chickens were divided into 2 groups, group 1 consisting of 50 chickens and group 2 of 55 chickens. Each chicken in group 1 was given a single oral dose of 500 infective-stage larvae of T.tenuis and 5 chickens were subsequently killed every 3 days between days 3 and 30 post-infection. Concurrently, 5 chickens from group 2 were killed between days 0 and 30 post-infection and the caeca treated in the same way as the experimental birds, to act as a group of uninfected controls. The caeca from each chicken in both groups were examined and compared. The appearance of both infected and uninfected control caeca are shown in Figs. 6.10a-k. On days 3 to 6 of infection there was little difference between uninfected and control caeca. On days 9-15, however, infected caeca appeared to be shorter in length and the caecal contents were of a much lighter colour than those from uninfected birds. Between days 18 and 27, there appeared to be recovery of the caeca, so that by day 27 both infected and

uninfected caeca were of similiar appearance. There was a significant decrease ($p < 0.01$, Students t-test) in the length of the caeca of infected birds compared with uninfected birds between days 12 and 15 of infection (Fig. 6.11); a significant decrease ($p < 0.01$, Students t-test) in the weight of the full caeca of infected birds on days 6 and 30 of infection (Fig. 6.12); and a significant increase ($p < 0.01$, Students t-test) in the empty weight of infected caeca on day 21 of infection (Fig. 6.13). There were no significant difference in the dry weight between infected and control caeca (Fig. 6.14).

The course of infection in young grouse chicks

Grouse chicks, in groups of 2, were given either a single oral dose of 500 infective-stage larvae; a single oral dose of 1000 infective-stage larvae; or a single oral dose of 20 larvae per day, between days 1 to 36 of infection, to establish a cumulative dose of 720 larvae per bird. Nematode egg output was monitored as described previously. The results of this experiment are shown in Fig. 6.15. Both single and trickle doses of larvae produced an infection. Egg production commenced between days 8 and 12 of infection and by day 36 of infection egg output was between 5500 and 6500 epg in all groups.

Fig. 6.10a. Uninfected caeca from 14 day old chickens.

Fig. 6.10b. Caeca from 17 day old chickens. Infected caeca
on day 3 of infection (above) and uninfected
controls (below)



Fig. 6.10c. Caeca from 20 day old chickens. Infected caeca on day 6 of infection (above) and uninfected controls (below)

Fig. 6.10d. Caeca from 23 day old chickens. Infected caeca on day 9 of infection (above) and uninfected controls (below)



Fig. 6.10e. Caeca from 26 day old chickens. Infected caeca on day 12 of infection (above) and uninfected controls (below)

Fig. 6.10f. Caeca from 29 day old chickens. Infected caeca on day 15 of infection (above) and uninfected controls (below)

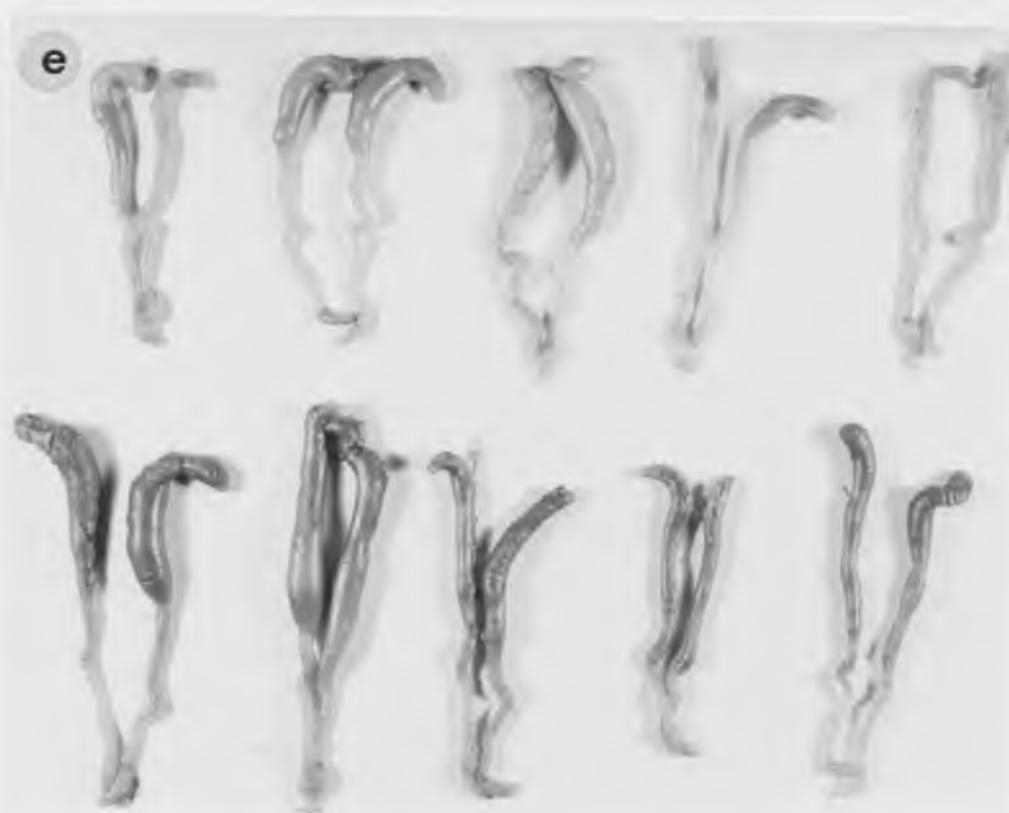


Fig. 6.10g. Caeca from 32 day old chickens. Infected caeca on day 18 of infection (above) and uninfected controls (below).

Fig. 6.10h. Caeca from 35 day old chickens. Infected caeca on day 21 of infection (above) and uninfected controls (below).

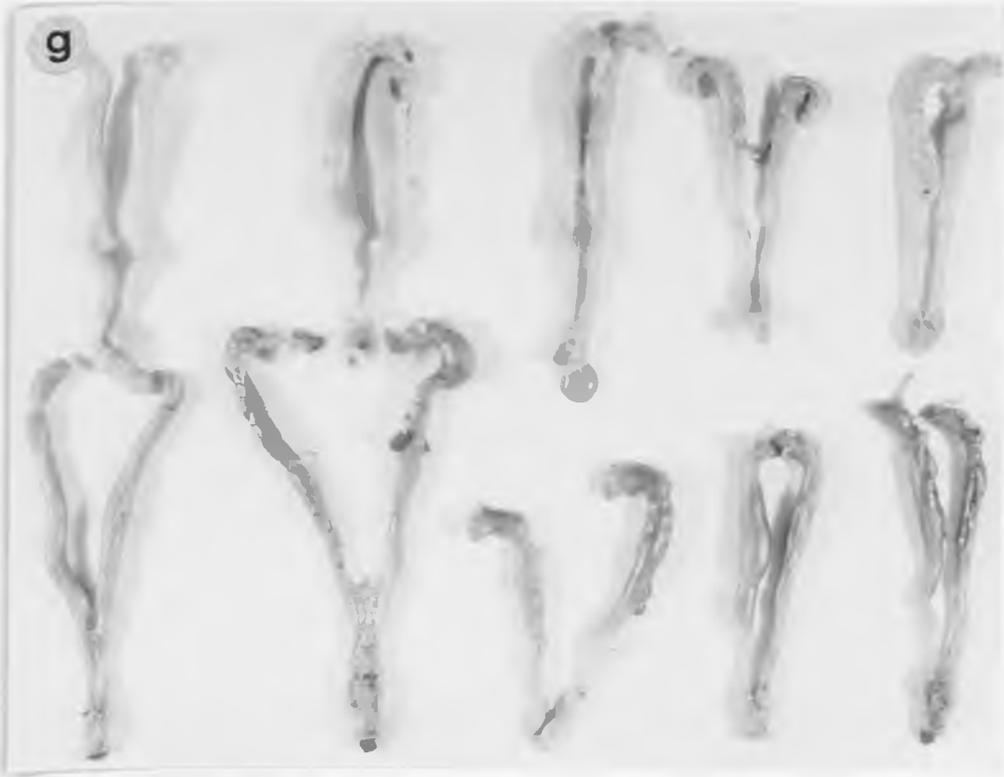


Fig. 6.10i. Caeca from 38 day old chickens. Infected caeca on day 24 of infection (above) and uninfected controls (below).

Fig. 6.10j. Caeca from 41 day old chickens. Infected caeca on day 27 of infection (above) and uninfected controls (below)



Fig. 6.10k. Caeca from 44 day old chickens. Infected caeca on day 30 of infection (above) and uninfected controls (below).



Fig. 6.11. The effect of an infection with 500 infective-stage larvae of T.tenuis on the length of the caeca. Bars indicate standard error of the mean.

(▲) infected

(△) controls

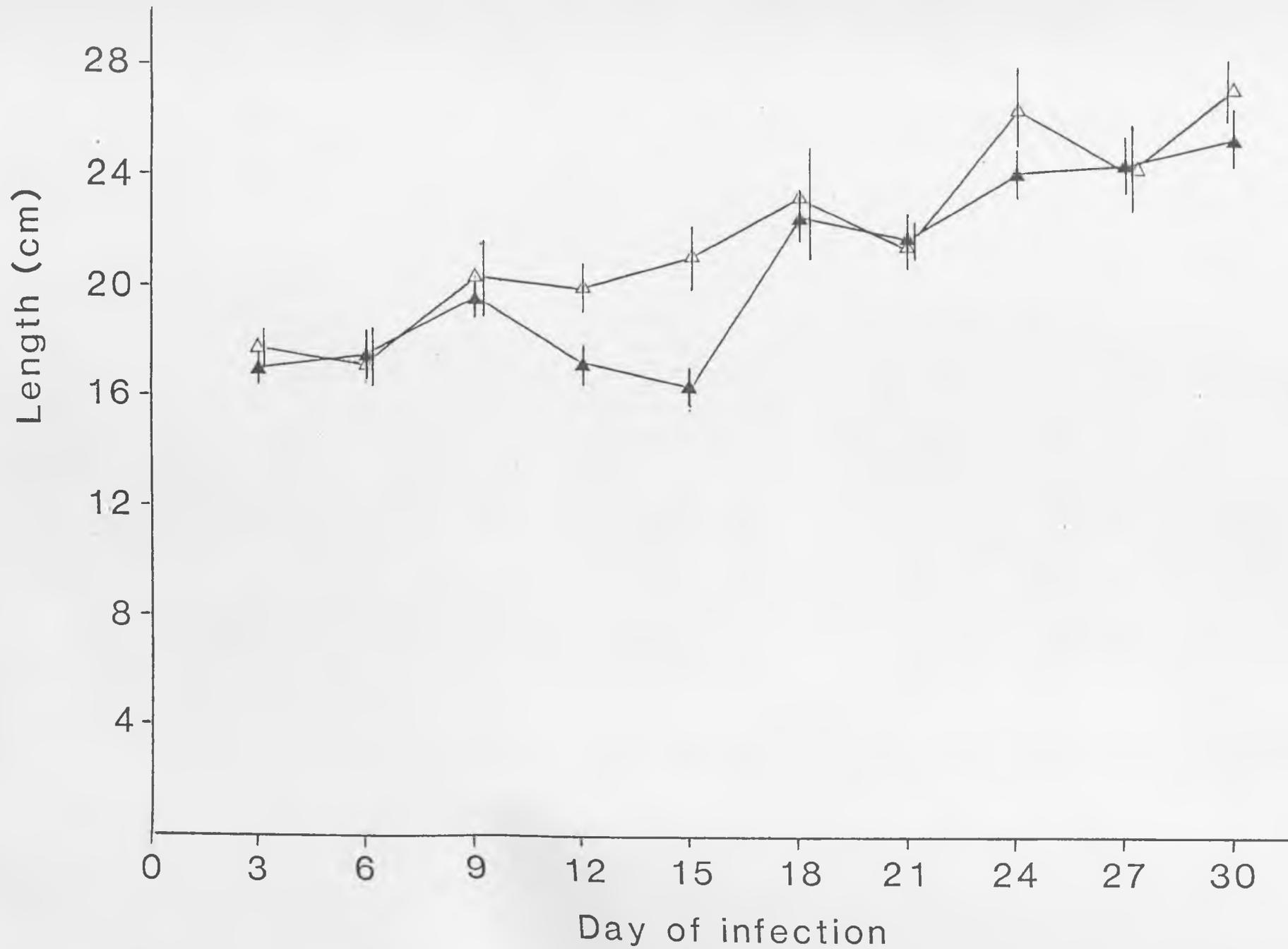


Fig. 6.12. The effect of an infection with 500 infective-stage larvae of T.tenuis on the weight of the caeca when full. Bars indicate standard error of the mean.

(▲) infected

(△) controls

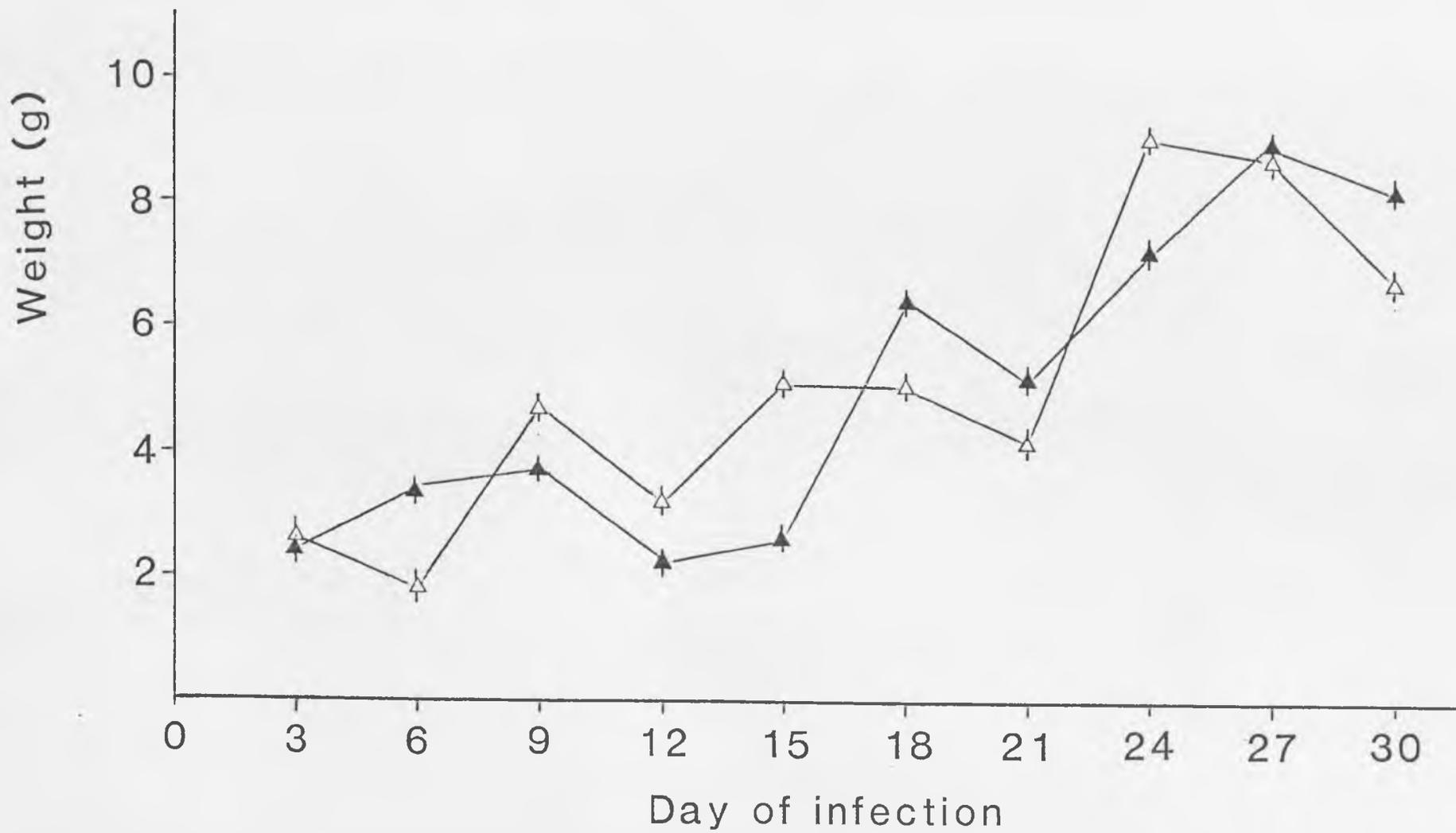


Fig. 6.13. The effect of an infection with 500 infective-stage larvae of T.tenuis on the wet weight of the caecal wall. Bars indicate standard error of the mean.

(▲) infected

(△) controls

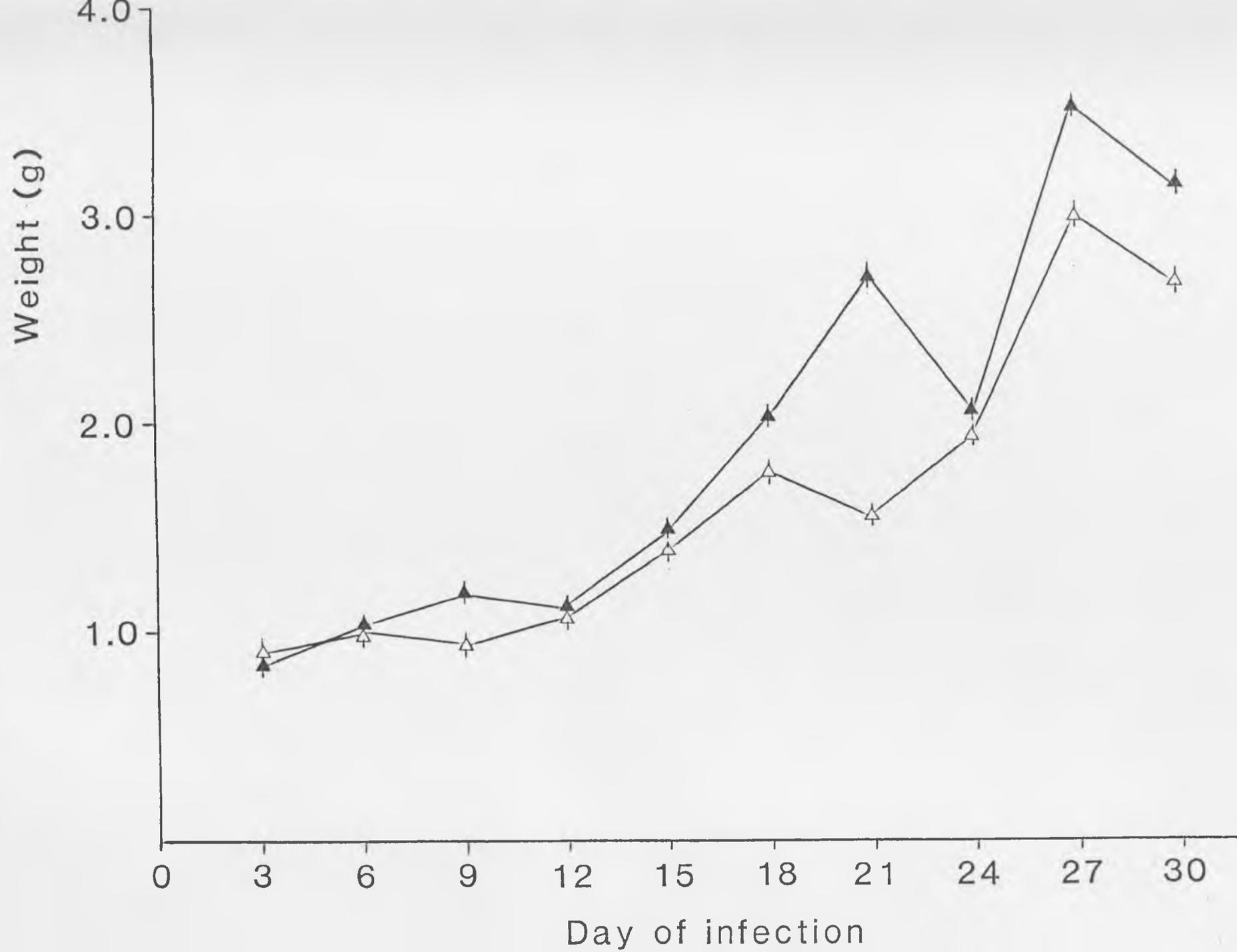


Fig. 6.14. The effect of an infection of 500 infective-stage larvae of T.tenuis on the dry weight of the caeca wall. Bars indicate standard error of the mean.

(▲) infected

(Δ) controls

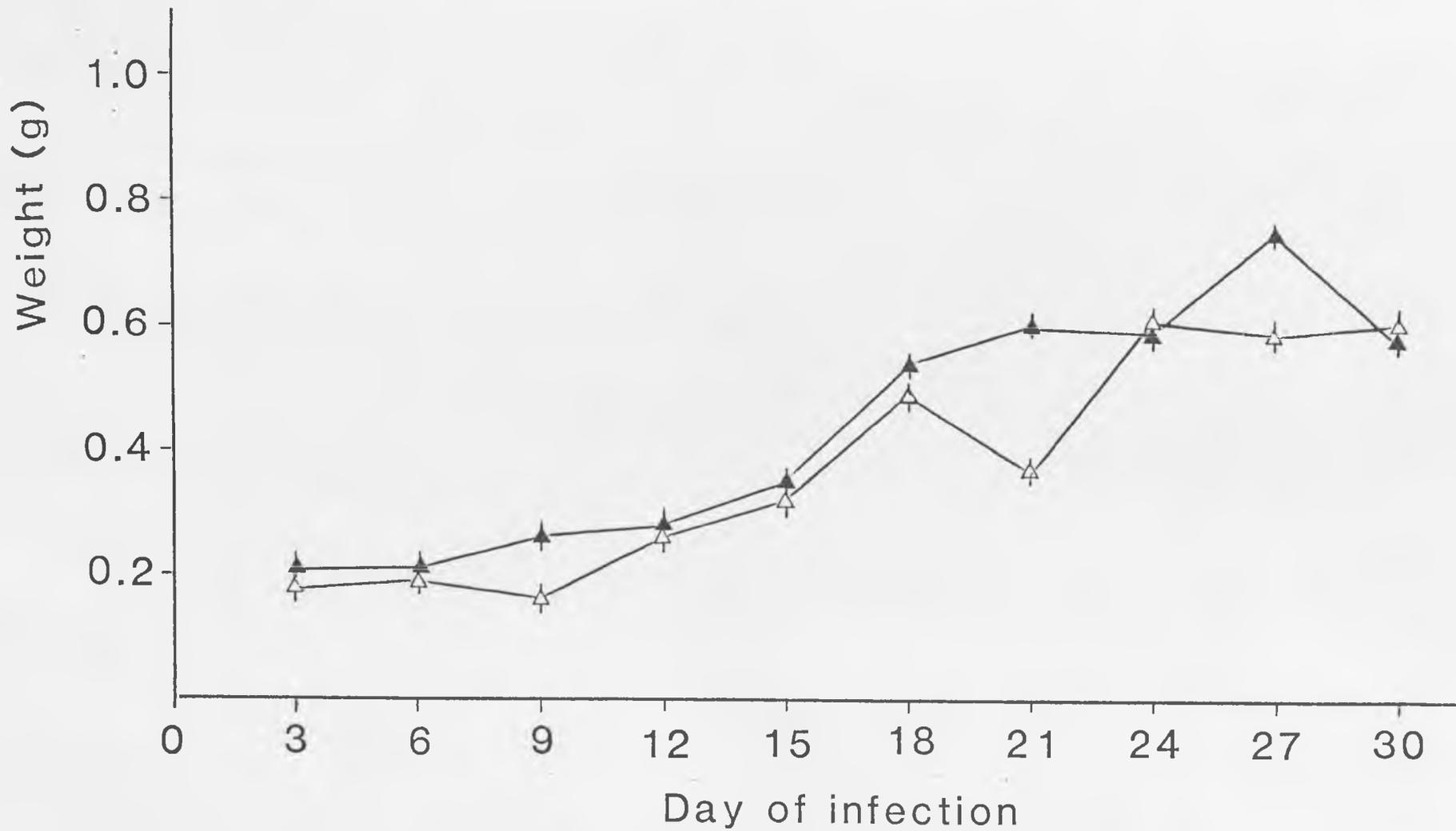
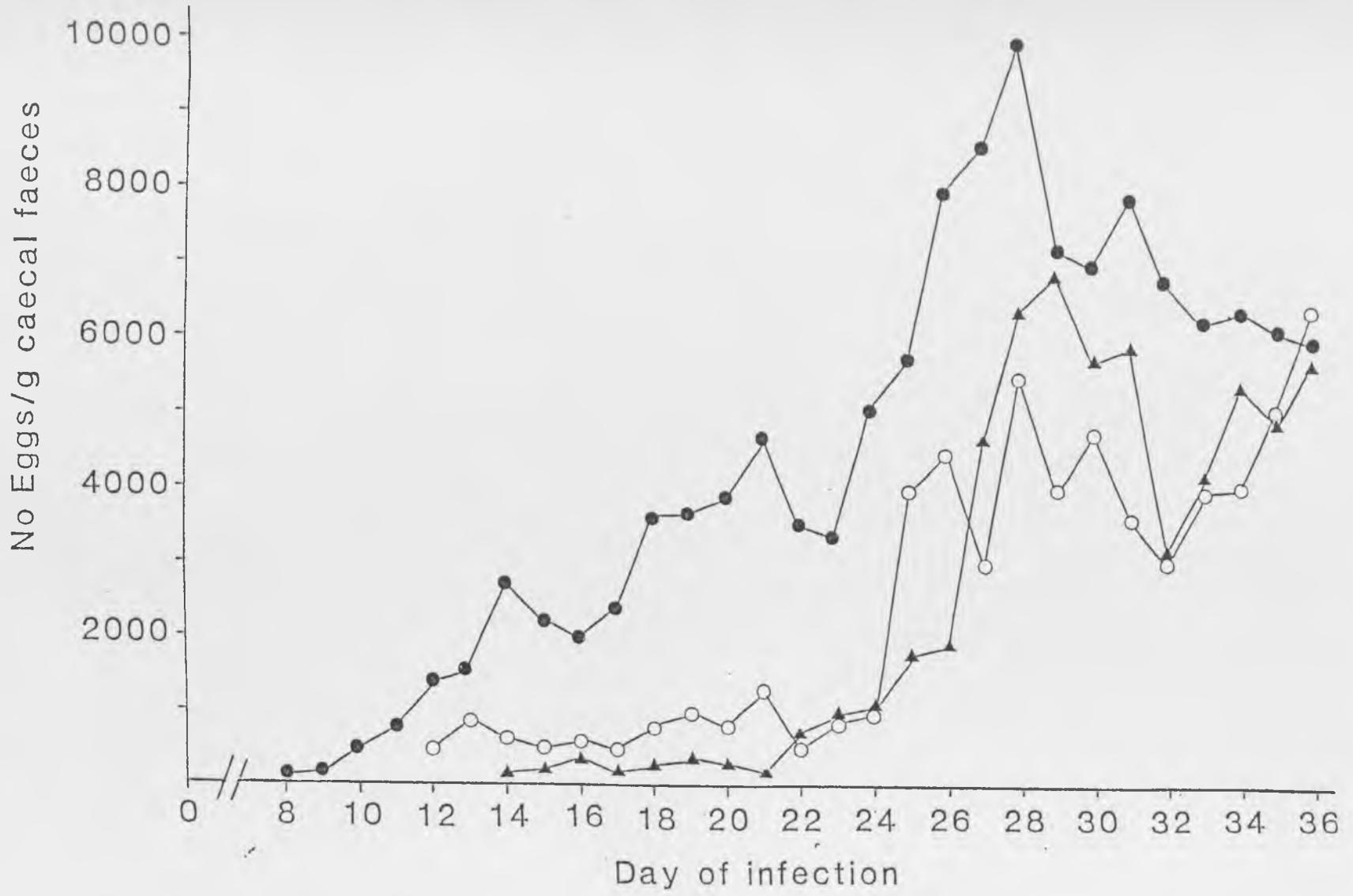


Fig. 6.15. The course of T.tenuis infection in 3 week old red grouse chicks given single and trickle doses of infective-stage larvae.

- (○) Single dose with 500 larvae
- (●) Single dose with 1000 larvae
- (▲) Trickle dose with 20 larvae per day
to give a cumulative dose of 720 larvae



DISCUSSION

This work describes, for the first time, the course of both primary, trickle and secondary infections of T.tenuis in the domestic chicken and the results reveal some interesting differences from information available for red grouse (Wilson, 1979). In wild red grouse, the number of nematodes present in the caeca increases throughout the life of the bird, greater nematode burdens being found in adult grouse than in young grouse (mean nematode burden adult grouse = 2291.48; mean burden young grouse = 378.40) (Potts, Tapper & Hudson, 1984). The lifespan of the adult nematode, once established in the caeca, has been estimated to be between 2-3 years (Hudson, pers comm); this is similar to the expected lifespan of the grouse, which is normally shot within two years. In the young domestic chicken, however, the duration of an infection with T.tenuis is much shorter, being about 30 days, and nematodes are expelled from the host from about day 9 onwards. It would appear, therefore, that unlike the red grouse, the young domestic chicken develops a form of resistance to T.tenuis which results in the infection being rejected.

The course of an infection with T.tenuis in the domestic chicken has never been examined in detail, although several studies have indicated that the domestic chicken is able to eliminate adult nematodes from the caeca. Cram & Cuvillier (1934) established a T.tenuis infection in 18 out of 20 chickens and recorded a number of clinical and pathological observations. During the early stages of infection, mucus

and blood were present in a caecal discharge and the mucosal wall was covered in a layer of blood-stained mucus. However, no deaths occurred from such experimentally induced trichostrongylosis. In birds which were maintained beyond these acute stages of infection, the symptoms disappeared and the caeca gradually resumed its normal appearance. Infection was normally over by 2 months. In several cases, live adult worms were found in the droppings during the second month of infection. Such results are similar to those obtained in the current study, in respect that blood-stained mucus and adult nematodes are expelled from the caeca, and following an infection, the caeca gradually recovers its normal appearance. However, in their study, infection lasted for up to 2 months, compared with approximately 1 month in the current work. Cram & Cuvillier (1934) gave no indication of the number of infective-stage larvae which were given to chickens, nor of the type of chicken used. Also, the chickens which were infected were aged between 2 weeks and 3 months, compared with 2 weeks old birds in the current study. Such differences may account for the longer duration of infection in the earlier study. Both studies have shown, however, that there is an expulsion of nematodes from the caeca, resulting in the termination of an infection. Morgan & Wilson (1938) surveyed the helminth parasites of poultry in Scotland and found infestations of only 1-2 adult T.tenuis per chicken. On the basis of this they suggested that a partial resistance to the nematode occurs. A similar conclusion has been drawn as a result of the findings of the present work.

The pattern of a primary infection with T.tenuis in the domestic chicken, involving a rapid rise followed by a fairly rapid fall in the numbers of eggs passed in caecal droppings and in the numbers of nematodes present, is similiar to that exhibited by several other nematodes that inhabit the alimentary tract of their hosts (Ogilvie & Jones, 1971; Wakelin & Lloyd, 1976; Hannah & Behnke, 1982; Sinski, Jeska & Bezubik, 1983).

Miller (1987) categorized the expulsion of nematodes from the gastrointestinal tract into three types. First, rapid expulsion (RE), which is directed against the infective-stage larvae as they enter the gastrointestinal tract, such expulsion occurring before establishment takes place and with the nematodes being expelled within 24-48 hours after challenge. Secondly, expulsion directed against developing larvae or pre-adults which have already established themselves in the host, but which are expelled before they reach adulthood. Thirdly, a spontaneous cure, wherein the adult nematodes are expelled over a period of several days, weeks or months. The expulsion of T.tenuis from the domestic chicken is most likely to be a spontaneous cure, as no infective-stage larvae or pre-adult nematodes were found in the caecal faeces prior to day 9 of infection, but from day 9 onwards, adult nematodes were expelled from infected birds.

Such expulsion from the host is caused by a protective response in the alimentary tract. With N.brasiliensis in

the rat, expulsion is believed to be caused by an immediate hypersensitivity reaction when antigens derived from the nematode cross-link host antibody molecules which are bound to receptors on the surfaces of mast cells or basophils. This causes the release of potent mediators which bring about immediate physiological changes, such as increased permeability of blood vessels and epithelial membranes or increased contractility of smooth muscle tissue, which in turn results in the expulsion of the nematodes from the gastrointestinal tract (Wakelin, 1984).

To date, three hypotheses exist as to the cause of such an immediate hypersensitivity reaction and the consequent expulsion of N.brasiliensis from the rat. Firstly, amines released from mast cells may damage worms directly or, secondly, intestinal inflammation resulting from such an amine release may produce an environment unsuitable for nematode survival. Thirdly, the leak lesion hypotheses suggests that an amine-induced increase in vascular and epithelial permeability allows the passage of anti-nematode antibodies into the gut lumen. It is possible that the expulsion of T.tenuis from the caeca of the domestic chicken is brought about by such an immediate hypersensitivity reaction. Tests for the presence of circulating antibodies against the antigens of larval or adult T.tenuis proved to be negative in this investigation. This, together with the presence of lesions on the surface of the caecal mucosa suggests that a cell-mediated reaction that induces an inflammatory response is the most likely cause of expulsion. However, this aspect of the

host-parasite relationship needs more detailed study before any conclusion can be drawn.

The vertebrate immune response to infection is an integrated response, involving lymphocytes and their products, as well as a range of other host cells which originate in the bone marrow (Wakelin, 1984). Circulating blood leucocytes are part of this system. Parasitic infections are known to stimulate an increase in the numbers of circulating blood leucocytes in a number of host/parasite systems; with N.brasiliensis in rats (Ogilvie, Hesketh & Rose, 1978); Trichostrongylus colubriformis in guinea pigs (Rothwell & Dineen, 1972; Handlinger & Rothwell, 1981); T.colubriformis in sheep (Gallagher, 1963); T.colubriformis in man (Wallace, Henkin & Mathies, 1956); Trichinella spiralis in mice (Crandell & Crandell, 1972); and with Toxocara canis in dogs (Sugane & Oshima, 1972). A similar association between lymphocytes and helminth infection has been described with nematode infections in birds. Kibakin, Kibakina & Abdurakhaova (1983) detected changes in the ratios of eosinophil and heterophil leucocytes in chickens given Capillaria obsignata ova, while Fantham (1910) described an increase in eosinophil leucocytes, a decrease in the number of erythrocytes, and a decline in the haemoglobin levels of red grouse carrying heavy infections of T.tenuis. Wilson & Wilson (1978) concluded that red grouse infected with T.tenuis develop a mild anaemia and detected a fall in blood haemoglobin levels, no significant changes in the number or size of red blood cells, and an increase in the

number of eosinophils and heterophils present. In their study it was suggested that an increase in the number of heterophils may be a response to stress or, alternatively, a response to bacterial invasion of the caeca which had been initiated by damage caused by the nematodes. However, the increase in the number of eosinophils was attributed directly to the nematode infection. In a later study, Maxwell & Burns (1985) detected blood eosinophilia in 36% of 115 bantams naturally infected with T.tenuis.

In the current study, significant differences were found in the blood leucocyte levels of infected chickens compared those of uninfected chickens, but only on certain days of infection. It may be that although the nematodes are expelled from the birds and a slight resistance to further infection occurs, such a response is not caused by circulating blood leucocytes or substances produced by these cells. The response may be a lymphocyte independent, hypersensitivity reaction. It is surprising that adult bantams infected with T.tenuis produced a significant eosinophilia (Maxwell & Burns, 1985), while two-week-old chickens in the current work produced a significant eosinophilia only on certain days. Bantams used in the former experiment were free-range birds, naturally infected with T.tenuis. No indication was given as to the levels of infection in the bantams, nor to their time of exposure to infection. Perhaps the nematodes were picked up gradually over a long period of time and this constant exposure to T.tenuis larvae may have stimulated the observed eosinophilia, while chickens in the current study were

given a single dose of 500 infective-stage larvae of T.tenuis and therefore may have reacted differently.

It is interesting that an increase in the number of circulating eosinophils and heterophils occurs in red grouse infected with T.tenuis (Wilson & Wilson, 1978) since red grouse appear to show no resistance to infection and, unlike the domestic chicken, nematodes appear not to be expelled. Perhaps an immune reaction is stimulated in the red grouse by T.tenuis infections but the response produced is not sufficiently powerful to remove the nematodes from the caeca or to affect their reproductive ability. It is also possible that the red grouse may have an immune tolerance to T.tenuis infection.

An increase in antibody activity in response to helminth infections is well documented and occurs with protozoan, helminth and ectoparasite infections (Wakelin, 1984). Few studies have examined the antibody levels in birds infected with T.tenuis. Wilson & Wilson (1978) demonstrated that red grouse infected with T.tenuis have increased levels of alpha, beta and gamma globulins and Maxwell & Burns (1985) detected no measurable levels of IgE in the plasma of adult bantams infected with T.tenuis. Burns & Maxwell (1981) have shown that birds with a blood eosinophilia, produced after stimulation with immune horse sera, produced high levels of IgE, and concluded that the absence of IgE in birds infected with nematodes suggests that a state of immune tolerance may exist, in which antibody production ceases or is suppressed. However, in the domestic chicken

there is a definite response to T.tenuis infection resulting in the expulsion of the nematodes, and further work using modern immunological techniques, such as enzyme-linked immunoabsorbant assays, are required to examine at a more detailed level, the possible occurrence of antibodies against T.tenuis in the domestic chicken.

The course of infection with T.tenuis in chickens given trickle doses of larvae appears to be similar to the course of infections derived from single doses since in both cases there was a characteristic rise and fall in nematode egg output over the period of infection. With trickle infections of T.tenuis in the chicken, nematode egg production in all groups had ceased by day 36 of infection. Chickens which received a cumulative dose of 60 larvae were given larvae only up to day 3 of infection, while chickens given a cumulative dose of 500 larvae were receiving larvae up to day 25 of infection. This indicates that larvae given later in the infection had failed to establish or to mature into adults capable of oviposition. This could be due to increased immunological resistance in the host or to the inability of these larvae to establish because of alterations to the caecal environment caused by the pathological changes induced by the existing infection. It is interesting to note that the maximum egg production following a primary infection of 500 infective-stage larvae is 1695 epg compared with a maximum egg production of 788 epg following a trickle infection of 500 infective-stage larvae. This lower egg production during the trickle infection could be due to poorer establishment of larvae

later in the trickle infection.

Slightly fewer nematodes were present in re-infected birds between days 6 to 18 of the secondary infection than in birds given a primary infection but there were no significant differences in the number of nematodes establishing in each group. Jarrett, Jarrett & Urquart (1968) suggested that adult N.brasiliensis become affected by an immunity initiated by the primary infection. Such an immunity may affect the nematodes during the final larval moult or after they have reached the adult stage (Sarles & Taliaferro, 1936; Ogilvie, 1965). Adult N.brasiliensis expelled from a secondary infection are often stunted (Sarles & Taliaferro, 1936; Ogilvie & Hockley, 1968). In the early stages of a primary infection the cytoplasm of the intestinal cells of adult N.brasiliensis contain well organized masses of rough endoplasmic reticulum, but later in the infection the endoplasmic reticulum of these cells becomes disorganized and the cells fill with large amounts of lipid (Lee, 1969). While some degree of resistance to T.tenuis seems to occur in the domestic chicken, as slightly fewer nematodes became established during a secondary infection, this difference was not statistically significant. Unlike N.brasiliensis in the rat, expulsion was not quicker during secondary infection with T.tenuis in chickens.

The numbers of nematodes which became established up to day 6 in birds given a primary infection of 500 larvae, in the experiment carried out to determine the effect of a

secondary infection on the number of nematodes present, differed slightly from the numbers which became established in the first experiment, carried out to determine the numbers of nematodes present in a primary infection. This is difficult to explain since, in both cases, the chickens used were of the same age, where the same breed and same sex, and an inoculum of 500 infective-stage larvae was used in both cases. These differences could be due to different conditions experienced in the culture media during the incubation of the larvae, although these were kept as constant as possible, or to the age of the larvae used, 2-3 day old infective-stage larvae being used in the first experiment to determine the numbers present in a primary infection, and 5-6 day old larvae being used in the second experiment. However, the infective-stage larvae of T.tenuis remain infective if kept at 4 C for up to 40 weeks (Chapter 3). As the numbers of adult nematodes present at day 8 or 9 of infection was similar in both experiments, these differences early in the infection represent normal variation; this serves to emphasise the need for large groups of animals in these experiments.

The course of T.tenuis infection in the domestic chicken is also similiar to that of N.brasiliensis in the rat, in that in both cases the sex ratio of adult nematodes alters during infection, with male nematodes remaining within the host for a longer period of time than female nematodes. This was first described for N.brasiliensis in the rat (Africa, 1931). It has been suggested that expulsion of male or female nematodes at different times is based upon

the maintenance behaviour of the adult nematodes, maintenance behaviour, as defined by Damian (1982), being the long term relationship between the parasite and its host. Brambell (1965) studied the distribution of N.brasiliensis in the rat jejunum and found that at the commencement of infection, most nematodes were found in the anterior region, where male nematodes predominated. When adult nematodes are expelled, male and female nematodes are lost more rapidly from the mid-region of the jejunum than from the anterior regions and consequently a higher proportion of male nematodes remain within the jejunum.

To date, there have been few studies on the distribution of adult T.tenuis in the alimentary tract of the red grouse or the domestic chicken. Lovat (1911) observed that nematodes were aggregated towards the proximal end of the caecum in infected red grouse. Observations on the caeca of infected chickens in this study have suggested that adult nematodes inhabit the mid and proximal regions of the caecum, nematodes being rarely found in the narrow neck. It is possible that male nematodes are more numerous than female nematodes in certain portions of the caecum and if expulsion does occur at different rates from different regions of the caecum, then this could explain why male nematodes remain within the caecum for longer than female nematodes.

It is interesting to note that following a primary infection with 500 infective-stage larvae, very few larvae were recovered from the alimentary tract from days 1 to 3

of infection nor were they found passed out in caecal or faecal droppings. This is difficult to explain, since a dose of 500 infective-stage larvae has been shown to cause an infection in chickens. A maximum of 2.92% recovery of infective-stage larvae is low and the number of larvae recovered is not sufficient to produce the numbers of adult nematodes which are known to develop in the caeca. The rate of recovery should be over 40% if comparisons with the recovery of sheep strongylids are valid (G.Mitchell, Veterinary Investigation Centre, Auchincruive, pers comm). The relative fullness of the crop and gizzard may have an effect on the survival of the infective-stage larvae. For example, if the upper alimentary tract is empty, a sudden flush of HCl in the proventriculus may be harmful to the larvae (A.P.C. McLaren, Veterinary Investigation Centre, Auchincruive, pers comm). This work has shown that the infective-stage larvae of T.tenuis can exsheath outside the caeca and may commence development in the small intestine, prior to establishment in the caeca itself.

Scanning electron microscopy and light microscopy showed some changes to the mucosa of the caeca of chickens given 500 infective-stage larvae of T.tenuis. The main change in the caecum of infected birds was an increase in the amount of mucus over the caecal surface; haemorrhagic lesions were also present on the surface of the caeca from day 6 of infection onwards. Examination of the caecal droppings of the infected birds from day 9 onwards often revealed the presence of nematodes embedded in blood-stained balls of mucus. T.tenuis in the domestic

chicken burrows superficially through the surface mucus coat, in the red grouse in which it burrows deeply into the caecal mucosa (Watson, Lee & Hudson, 1987). The surface of all mucus membranes are protected by a layer of mucus (Clamp, 1978 ; Allen, 1981), the protective character of which lies not only in its characteristic physical and chemical properties, but also in the presence of certain specific elements, mucus being a complex secretion receiving contributions from many types of cell (Jones & Reid, 1978). Mucus consists mainly of mucin glycoprotein but also contains DNA, enzymes, immunoglobulins and other plasma proteins (Forstner, Wesley & Forstner, 1982). Mucus secretions may protect mucus membranes from attack by pathogens and parasites in several ways. It is believed to contain attachment sites for pathogenic bacteria (Lindley, 1980) and is known to contain specific proteins such as lysozyme, lactoferrin and secretory IgA, which play a protective role against microbial attack. Lysozyme, for example, interacts with sialic acid residues (Creth, Bridge & Horton, 1979) thus remaining associated with the mucus layer where it acts against muramic acid-containing bacterial cell walls. The lactoferrin component in mucus is believed to act by complementing with any iron present in secretions, thereby denying it to the pathogenic organism (Emery, 1980). Similarly, specific antibodies are known to act together with mucin to prevent the adherence of enteropathogenic bacteria to the intestinal mucosa (Schrank & Verwey, 1976).

Mucus secretions protect the gastrointestinal tract

against nematode infections primarily by physical entrapment and immobilization of the nematode (Miller, 1987). For example, T.spiralis larvae become entrapped in mucus both in vivo in immune rats, and in vitro (Lee & Ogilvie, 1981). The immune expulsion of N.brasiliensis from the rat intestine is accompanied by goblet cell hyperplasia and an increased turnover of mucin (Miller, Huntley & Dawson, 1981) and it is now believed that, prior to the expulsion of N.brasiliensis from the rat, the nematodes are excluded from their predeliction site between the villi by a layer of mucus, and so become separated from the mucosa (Miller, Huntley & Wallace, 1981). Evidence from the study of T.tenuis in the domestic chicken suggests that mucus trapping and immune exclusion may also occur in this host/parasite relationship. The fact that T.tenuis in the domestic chicken burrows only superficially across the mucosal surface may be due to the secretion of mucus, which could act as a barrier against the free access of the nematode to the mucosa. Such mucus secretion is most likely to be caused by a reaction in the caeca to T.tenuis since it was not seen to occur in uninfected chickens and in the apparently non-immune, normal host, the red grouse. Although the domestic chicken is partially resistant to T.tenuis infection, perhaps the nature of this resistance and the cause of the immune expulsion is different from that of N.brasiliensis in the rat, and requires further study from an immunological point of view. It certainly appears to be different from infections of T.tenuis in red grouse.

Several studies have examined changes in the gut length of gallinaceous species of birds (Leopold, 1953; Moss, 1972; 1974; Pendergast & Boag, 1973), and have shown that one of the major factors influencing gut length is dietary composition and the amount of food eaten. Pulliainen (1976) found willow grouse (Lagopus lagopus lagopus) of lighter body weight tended to have a longer small intestine and caeca and suggested that this may be because the smaller grouse must consume relatively large amounts of food in order to survive hard winters. Pulliainen & Tunkkari (1983) described seasonal changes in the length of the caeca of willow grouse and found that the length of the caeca of male grouse decreased from April/May to a minimum length in June/July and then increased again to a winter maximum, and concluded that the length of the caeca depends upon the amount of fibrous food eaten. However, diet is not the only factor which may influence gut length. Moss (1972) suggested that a number of factors may be significant, including the hormone balance, parasite load, and rhythm of feeding of the grouse. The importance of parasite load in influencing caecal structure was first indicated by Lovat (1911) who described in detail the structure of the caeca of red grouse infected with T.tenuis and concluded that T.tenuis can cause a chronic inflammation and fibrosis in the caecum. However, his work gave no indication of overall changes in length and weight of infected and uninfected caeca. Hudson (1986a) examined a large number of red grouse and found a correlation between caecal length and the level of T.tenuis infection.

Several parasites inhabit the caecum of the domestic chicken and are known to cause pathological changes within the caeca. Gordon & Jordan (1982) described haemorrhagic lesions and necrotic patches on the caecal wall of chickens infected with Eimeria tenella and suggested that in infected birds the digestive functions are poor, body weight is reduced, and proteins and electrolytes are lost via the faeces. They also described the pathological effects of Capillaria obsignata in the caecum of turkeys. These nematodes caused emaciation of infected birds, the production of faeces containing a pinkish coloured mucus and diarrhoea, and the caeca were often distended with fluid. Wilkins (1976) described changes to the caecal mucosa caused in turkeys infected with Histomonas meleagridis but again recorded no change in the overall length and weight of the caeca. Wilson (1979), following dosing of grouse with between 5000 and 35000 infective-stage larvae of T.tenuis found that the caecal faeces of infected grouse were less cohesive than those from uninfected controls and was sometimes passed out as caecal diarrhoea. The caecal faeces of infected birds weighed more than the faeces of uninfected controls. It was suggested that this was due to extra water and mucus in the faeces as a result of poorer water absorption and/or irritation caused by the worms. However, he found no correlation between the length of the caeca and worm burden. With T.tenuis infection in the domestic chicken, the length of the caeca of infected birds is much reduced on days 12 and 15 of infection. Maximum nematode burden occurs in the caeca on days 8 to 9 of infection and most

nematodes are expelled between days 9 and 18. The shortening of the caeca may be associated with this expulsion. Blood, mucus and portions of caecal mucosa are expelled with the nematodes and so the contents of the caeca will presumably be reduced during expulsion. It is interesting to note that after expulsion, from day 18 onwards, there is a recovery in the length of the caeca. The external appearance of the infected caeca was much lighter in colour than uninfected caeca and the caecal droppings produced by infected birds were a light yellow colour and were of a watery consistency while those from uninfected birds were a dark brown colour and of a firmer consistency. This suggests that the digestive functions of the caeca of infected birds could be impaired in some way.

CHAPTER 7

ATTEMPTS TO STIMULATE IMMUNITY TO T.TENUIS IN THE
DOMESTIC CHICKEN AND IN THE RED GROUSE USING LARVAE
ATTENUATED BY COBALT-60 IRRADIATION

SUMMARY

Dosing with infective-stage larvae of T.tenuis attenuated by cobalt 60 irradiation produced a significant degree of protection against a challenge infection in chickens. A mean of 86.2 nematodes became established on day 9 of a primary infection after dosing with 500 normal larvae; a mean of 43.1 nematodes on day 9 of a challenge infection with 500 normal larvae, following a primary infection with normal larvae; and a mean of 34.5 nematodes on day 9 of a challenge infection with 500 normal larvae, following a primary dosing with 500 irradiated infective-stage larvae. Dosing with larvae attenuated by cobalt 60 irradiation in red grouse chicks produced little protection against challenge. Nematode egg output on day 34 of a challenge infection, following a primary infection with 1000 non-irradiated larvae; on day 34 of a challenge infection, following a primary dosing with 1000 irradiated larvae; and on day 34 of a primary infection with 1000 non-irradiated larvae in age-matched controls were all similar. There were no significant differences in worm burdens of wild red grouse which had been given 1000 irradiated larvae and those given 1000 non-irradiated larvae. Since larvae attenuated by cobalt 60 irradiation produced little protective immunity in red grouse, the use of irradiated larvae as a means to control T.tenuis in red grouse would appear to be of little value.

INTRODUCTION

In host/parasite relationships the host can regulate the degree of infection to which it is subject, only through the activities of its immune system, that is, by the development of an acquired resistance to infection (Wakelin, 1984). The host immune system may be triggered if antigenic material of parasite origin is introduced into the host. From as early as 1933, attempts have been made to initiate a satisfactory immune response against nematodes by means of extracts or suspensions of living or dead parasite material (Bachman & Mollina, 1933). If living larvae are used as immunizing agents they may give rise to the disease against which protection is sought. One way to avoid this difficulty is by the introduction of parasite material via an abnormal route. Soulsby (1957) showed that a protective immunity in the guinea-pig against Ascaris lumbricoides, comparable to that produced by a normal infection, could be produced by a subcutaneous injection of embryonated and infective ova. The migration of larvae to the lungs, following a challenge infection was much reduced in a host treated in this way. Similarly, a saline extract of disintegrated ova or a saline extract of larval secretions, when injected subcutaneously, was sufficient to make guinea pigs partially immune to a challenge infection.

Irradiated nematode larvae have been used as immunizing agents against several nematode infections, a suitable dose of radiation damaging the reproductive and moulting ability

of the larvae, without impairing their pathogenic effect (Mulligan, Gordon, Stewart & Wagland, 1961). To date, the administration of a weakened or attenuated strain of infective-stage larvae to induce an immunity has been attempted with a number of nematode species; with Haemonchus contortus in lambs (Jarrett, Jennings, McIntyre, Mulligan, Sharp & Urquhart, 1959); Trichostrongylus colubriformis in lambs and in guinea-pigs (Mulligan, Gordon, Stewart & Wagland, 1961; Gregg, Dineen & Griffiths, 1976); Dictyocaulus viviparus in cattle (Jarrett, Jennings, McIntyre, Mulligan & Urquhart, 1960; Jarrett & Sharp 1963); Ancylostoma caninum in dogs (Miller, 1971; 1978); and with Syngamus trachea in pheasants (Shikhobalova & Paruzhinskaya-Korsak, 1973). However, to date, the only commercially available vaccine made from an irradiation attenuated strain of larvae is Dictol (Glaxovet), made from attenuated D.viviparus larvae, which is currently used to vaccinate cattle against parasitic bronchitis (Jarrett, Jennings, McIntyre, Mulligan & Urquhart, 1957). A vaccine developed to give protection against canine hookworm, Ancylostoma caninum, was introduced for sale in Florida in late 1973, but due to increasing costs, a relatively limited shelf life, and a low volume of sales, this vaccine was discontinued in 1975 (Miller, 1978).

It has been shown in Chapter 6 that the domestic chicken rejects an infection with T.tenuis in 30 days. This suggests that an immune mechanism may be involved in rejection of the nematode. However, red grouse build up the number of T.tenuis present in their caeca throughout

their life, suggesting that there is no immune rejection of the nematode by this host. The use of irradiation attenuated infective-stage larvae to induce an immunity to T.tenuis has never been attempted. The aim of this work was to discover if an irradiated strain of the infective-stage larvae of T.tenuis could stimulate an immunity to a challenge infection in the domestic chicken and also in the normal definitive host, the red grouse.

MATERIALS AND METHODS

Experimental birds

Sussex cross breed chickens were used in the experiments and were maintained as described in Chapter 6. Chickens were 2 weeks old at the start of each experiment. Red grouse chicks were obtained from Mr D.Newborn, Swale farm House, Satron, North Yorkshire and were 2-3 weeks of age at the start of the experiment. Grouse were reared and maintained as described in Chapter 6, with the exception that they were housed in purpose built, wire-bottomed cages, at Swale Farm House, Satron, North Yorkshire.

Collection of Trichostrongylus tenuis infective-stage larvae

The culture of infective-stage larvae and the methods used for infection have been described in Chapter 6.

Irradiation of infective-stage larvae

Infective-stage larvae were irradiated from an orbitron head with a cobalt 60 source, at a dose rate of 1.7 krads (17 grays) per minute. Larvae were given a total dose of 30 krads (300 grays). Irradiated and non-irradiated larvae used in this experiment were from the same culture and were 2-3 days of age. Irradiation was carried out by Dr B.Dixon, Radiobiology Department, Cookridge Hospital, Cookridge, Leeds.

Anthelmintic

Levamisole hydrochloride (1.5% w/v BP Vet, I.C.I Ltd) was

used to remove any adult T.tenuis from grouse given a primary infection with T.tenuis. A dose of 1 ml was administered orally as an aqueous suspension.

Caecal egg counts

Approximately 1 g of fresh caecal droppings from red grouse was collected each day from a pooled sample of droppings of all the grouse in each group. The number of eggs was counted after salt flotation, and estimation of the number of eggs present, using standard McMaster counting slides as described by Gordon & Whitlock (1939).

Recovery of worms

The recovery and counting of worms from the caeca of infected chickens has been described in Chapter 6 and recovery from the caeca of wild red grouse in Chapter 5.

Statistical analysis of results

The results were analysed by either the non-parametric Wilcoxon test or the Students t-test. A value of $P < 0.05$ was considered to be significant.

RESULTS

Effect of a primary infection with irradiated larvae on the adult worm burden during a challenge infection in the domestic chicken

Thirty chickens, in groups of 10, were given either a single dose of 500 non-irradiated infective-stage larvae (Group A); a single dose of 500 30 krad-irradiated infective-stage larvae (Group B); or remained uninfected (Group C). After 30 days, all chickens were given a dose of 500 non-irradiated infective-stage larvae (from the same batch of larvae as those used in the primary infection) and were killed 9 days post-infection. The results are shown in Table 7.1. There were no significant differences between the number of worms establishing in groups A and B ($p > 0.05$, non-parametric Wilcoxon test), but there were significant differences between groups A and C ($p < 0.05$, non-parametric Wilcoxon test) and groups B and C ($p < 0.05$, non-parametric Wilcoxon test)

Effect of a primary infection with irradiated larvae on nematode egg production during a challenge infection in young grouse chicks

3 groups, each of 5 grouse chicks, were given either a single dose of 1000 30 krad-irradiated larvae; a single dose of 1000 non-irradiated larvae; or remained uninfected. All larvae used were 2-3 days old and were from the same batch of larvae. Nematode egg output was monitored daily in each group. All birds were treated with 1 ml levamisole

hydrochloride (1.5% w/v) on day 24. The results are shown in Fig 7.1. There were significant differences in nematode egg production from chickens given a primary dose of irradiated larvae and those given a primary dose of non-irradiated larvae ($p < 0.05$, non-parametric Wilcoxon test). One week after dosing with levamisole hydrochloride, all birds were given a single dose of 1000 non-irradiated larvae and the nematode egg output was monitored daily. The results are shown in Fig. 7.2. There was a significant difference in nematode egg production following a challenge infection, between grouse given a primary dose of irradiated larvae and those given either a primary dose of non-irradiated larvae or in previously uninfected age-matched controls ($p < 0.05$, non-parametric Wilcoxon test)

Effect of a primary infection with irradiated larvae on adult worm burdens in wild young red grouse

100 grouse chicks, of approximately 2-3 weeks of age, were captured from the Botchergill area of Gunnerside moor, upper Swaledale, North Yorkshire (NGR NY 930 020) between 28th May and 4th June 1987. 50 chicks were dosed with 1000 30 krad-irradiated infective-stage larvae and 50 were given a placebo of 1 ml of water. A wing tag was attached to each of the captured birds to distinguish between the two groups. Birds were then released back onto the moor. After August 12th, some of the tagged birds were shot. The caeca were removed and the worm burden in the caeca of each grouse counted. The results are shown in Table 7.2. There were no significant differences in the number of adult

nematodes recovered from the caeca of grouse which had been given irradiated larvae and those which had received a placebo ($p > 0.05$, Students t-test). It should be noted, however, that one of the birds that had received irradiated larvae contained 1440 adult T.tenuis which is much higher than found in the other birds.

Table 7.1.

The effect of a primary dose of 500 normal infective-stage larvae of T. tenuis (Group A), and of a primary dose of 500 larvae which had been treated with 30 krad from a Cobalt 60 source (Group B), on a challenge infection with 500 normal larvae in the domestic chicken. Group C chickens were not given larvae when Groups A and B received their primary doses, but were given 500 normal infective-stage larvae when chickens in Groups A and B received their challenge doses of larvae.

Larvae administered pre-challenge	Worm recoveries (mean \pm SEM) Day 9 post-challenge with 500 normal larvae
A 500 (normal) larvae	43.1 \pm 4.91
B 500 (30 krad) larvae	34.5 \pm 7.00
C No larvae	86.2 \pm 5.84

Fig. 7.1. The numbers of eggs of T.tenuis in caecal faeces of red grouse chicks dosed with 1000 non-irradiated (▲) or with 1000 30 krad-irradiated (■) infective-stage larvae of T.tenuis. Birds were dosed with 1 ml levamisole hydrochloride (1.5% w/v) on day 24 of infection.

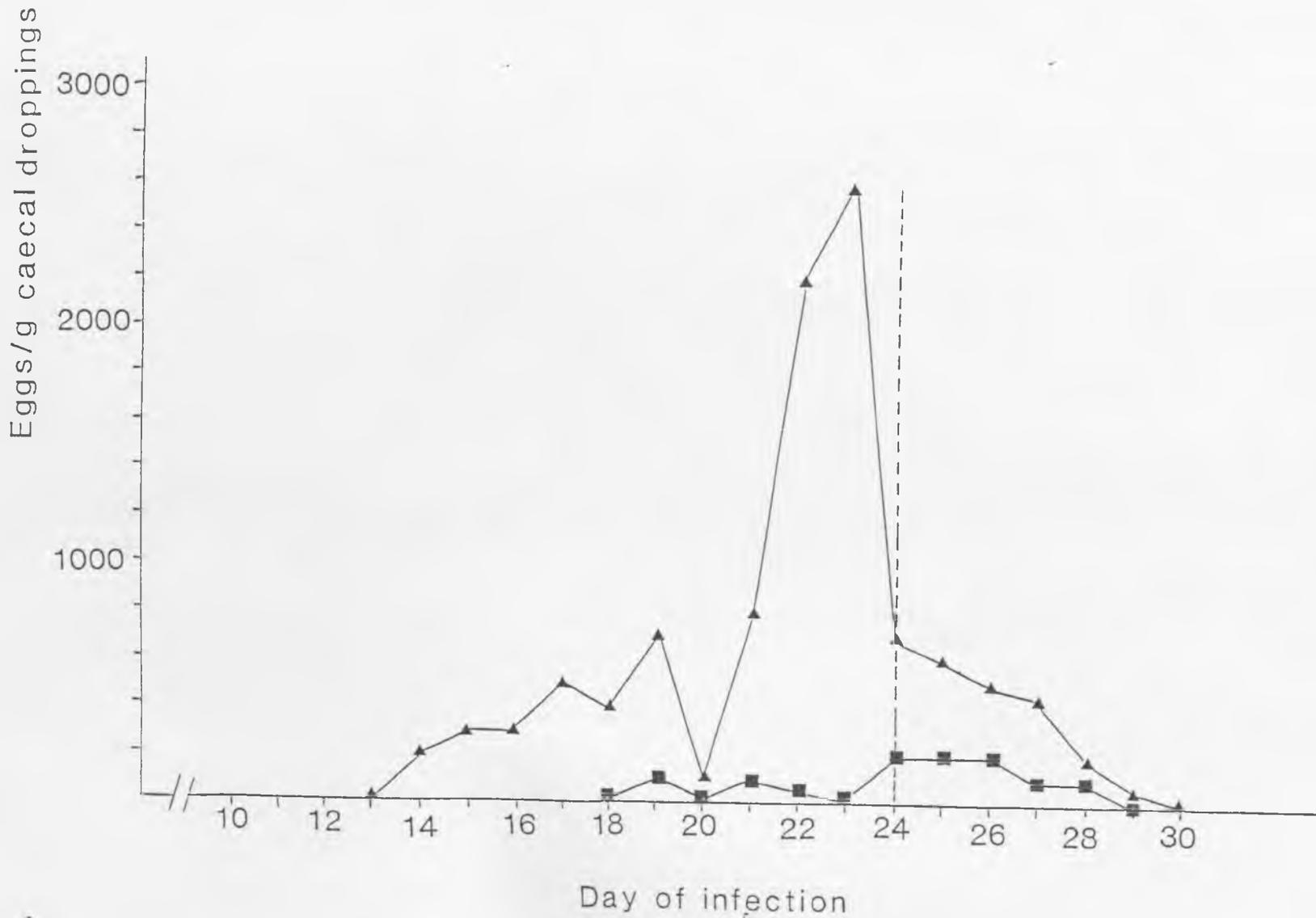


Fig. 7.2. The number of eggs of T.tenuis in the caecal faeces of red grouse chicks dosed with 1000 non-irradiated larvae. The red grouse chicks had previously been given doses of 1000 non-irradiated larvae (▲); 1000 30 krad-irradiated larvae (■); or were age-matched previously uninfected controls (◆).

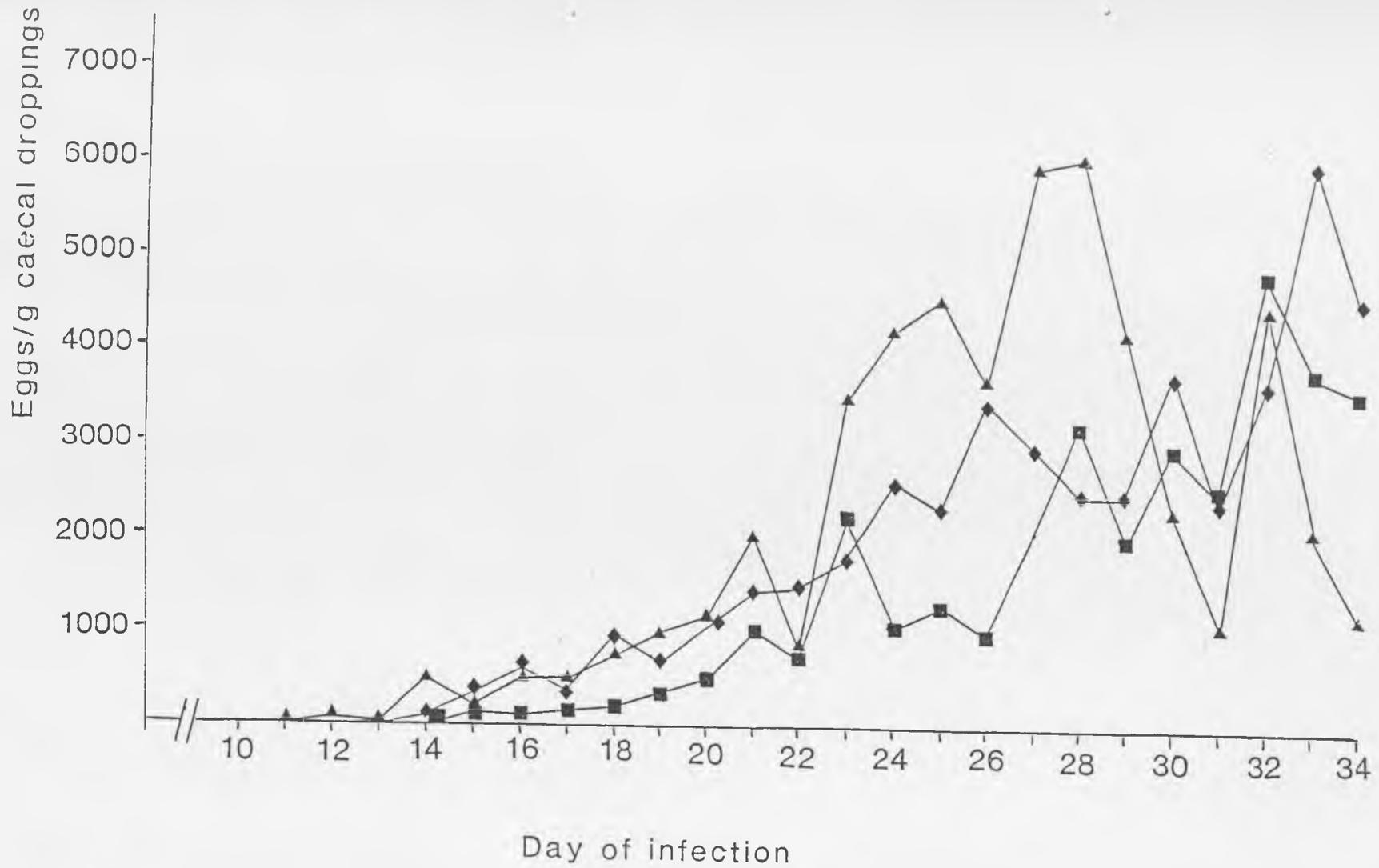


Table 7.2.

The effect of dosing with 1000 krad-irradiated infective-stage larvae of T.tenuis on the worm burdens in wild red grouse shot 2-3 months later.

Worm burden, non-dosed	Worm burden, birds dosed with 1000 Irradiated larvae	
240	120	320
280	220	60
200	40	
140	40	
120	1440	
220	80	
260	60	
160	140	
	80	
Mean = 202 ± 20.51	Mean = 236 ± 123.11	

DISCUSSION

The results of these experiments show that resistance to T.tenuis in the domestic chicken may be stimulated with larvae attenuated by cobalt 60 irradiation, but not in the red grouse. The course of an infection with T.tenuis in the chicken contrasts with that in the red grouse. In the domestic chicken, following a single dose of 500 infective-stage larvae, adult nematodes are expelled from the caeca and the infection is usually over by day 30 (Chapter 6). The red grouse, however, seems to lack resistance to T.tenuis. Even large repeated doses of infective-stage larvae to adult red grouse fail to evoke any resistance to challenge infections (J.Shaw, Institute of Terrestrial Ecology, Banchory, pers comm), and work described in Chapter 6 has shown that in young grouse doses of 500 and 1000 infective-stage larvae result in high nematode egg output from infected birds.

Although nematodes are expelled from the caeca following a primary infection with T.tenuis in the domestic chicken, birds are able to be reinfected and there are no significant differences between the worm burdens of a primary or challenge infection (Chapter 6). However, a dose of 500 30 krad-irradiated larvae resulted in a significant degree of protection against a challenge infection. Although nematode egg output was not monitored during the primary infection with irradiated larvae, the larvae obviously affected the course of a challenge infection. The details of survival and possible

development of irradiated larvae of T.tenuis are not known. However, Hagan, Behnke & Parish (1981) suggested that the protective immunity stimulated by Nematospiroides dubius larvae that had been attenuated by cobalt 60 irradiation may be due to prolonged exposure of the host to larval antigens. 10-30 krads of cobalt 60 irradiation prevents the normal maturation and emergence of larvae. As a result, infected mice are exposed for a prolonged period of time to the larvae and to larval antigens and this is believed to stimulate high levels of protective immunity. It is possible that this occurred in the domestic chickens given irradiated larvae of T.tenuis. In red grouse, however, irradiated larvae appeared not to stimulate any resistance.

Following a single dose with 1000 30 krad-irradiated larvae in red grouse, larvae do develop to adults capable of oviposition. However, egg production is significantly lower than egg production from an infection derived from non-irradiated larvae. This could be due to fewer of the irradiated larvae developing to maturity or a reduced fecundity in those nematodes which do mature. It is interesting to note that following dosing with levamisole hydrochloride, eggs continued to appear for up to 5 days after dosing. This is presumably due to the difference between the time that the nematodes ceased producing eggs in the caeca, and the time that they were detected in the caecal droppings which have been passed to the exterior. There was a significantly lower nematode egg output during a challenge infection with normal larvae, in grouse

previously given irradiated larvae compared with those given normal larvae. However, the nematode egg output during a challenge infection from grouse previously given irradiated larvae appeared to be reduced during the early stages of infection but later increased so that by the end of the experiment (day 34 of infection) egg output was similar to that from an infection in age-matched control birds.

The successful use of irradiated larvae as immunizing agents against nematode infections depends upon the selection of the most effective components from a large number of variables (Mulligan, Gordon, Stewart & Wagland, 1961). The dose of irradiation that larvae are exposed to greatly influences the stimulation of immunity. Hagan, Behnke & Parish (1981) tested various doses of cobalt 60 irradiation on larvae of Nematospiroides dubius and found that doses of between 10 and 30 krads were the most effective in stimulating an immunity to this nematode in mice; while Prowse, Mitchel, Ey & Jenkin (1979) showed that different strains of mice subjected to larvae attenuated by cobalt 60 irradiation developed different levels of resistance to subsequent challenge infections. In the current work, red grouse were dosed with larvae attenuated with 30 krads of cobalt 60 irradiation. It is possible that a higher or lower dose than that used is required to stimulate an immunity to a challenge infection. However, in order to test a range of doses of irradiation, a large number of grouse chicks would be required. Such work would be difficult to carry out since grouse chicks are available

at only certain times of year, are limited in supply, and are difficult to maintain in captivity.

To date, one of the most effective methods to control T.tenuis in red grouse is to dose birds with an anthelmintic. This, however, involves the capture of adult birds and is both laborious and time consuming. If larvae attenuated by cobalt 60 irradiation could be found to stimulate an immunity against further infection, it could result in a less laborious control procedure, since grouse chicks at 2-3 weeks of age are poor flyers and tend to stay in groups and are thus easily captured and dosed. Such a control procedure would obviously be easier to carry out than the oral dosing of adult grouse with an anthelmintic. However, the production, purification, and irradiation of larvae would be both costly and difficult. One of the main problems in developing a vaccine from irradiated larvae is the production of a larval suspension free from contaminating impurities and bacteria. Miller (1978) described the difficulties in producing a 'clean' vaccine against canine hookworm. Even after extensive formalin washings and filtrations, larval suspensions still contained some microorganisms. On the basis that included the conducting of exhaustive microbiological and safety tests to identify any contaminant organisms to prove their safety, the commercial viability and success of any such vaccine was found to be extremely limited. The need for the production of a 'clean' vaccine against canine hookworm was of paramount importance since the administration of the vaccine via a subcutaneous route was found to stimulate

maximum and uniform resistance (Miller, 1965). Even though relatively 'clean' suspensions of vaccine could be produced, they still resulted in a practically unacceptable incidence of abscesses and cellulitis at the point of injection. If a vaccine against T.tenuis infection in red grouse could be developed, it would presumably be administered via an oral route, and so contamination of the vaccine with microorganisms may not be of such great importance. It is significant that Dictol (Glaxovet), the only commercially available vaccine made from an irradiation attenuated strain of infective-stage larvae, is administered via an oral route.

This work has shown that the use of irradiation-attenuated infective-stage larvae of T.tenuis produces little immunity to challenge infection in red grouse, but they do produce some degree of resistance to subsequent infection in the domestic chicken. The use of irradiated larvae as a control measure against T.tenuis in red grouse is thus of little use. The treating of red grouse with an anthelmintic has been proved to be a very successful control procedure (Hudson, 1986b). With the recent improvements in the formulation of the anthelmintic, grouse may now dose themselves when they feed on grit coated with anthelmintic (Hudson, pers comm) and this may prove to be the most efficient method of controlling T.tenuis in grouse.

CHAPTER 8
GENERAL DISCUSSION

GENERAL DISCUSSION

This study has demonstrated a range of factors which can interact and influence the various stages of the life-cycle of T.tenuis, a life cycle which, in parasitological terms, is regarded as a relatively simple, direct life cycle which involves no intermediate host. Sexual reproduction of trichostrongyle nematodes occurs in the host and does not directly augment the parasitic population, because the products of reproduction pass out in the faeces to the exterior (Crofton, 1967). It is here that the infective-stages develop and thus the pasture, or moor in the case of T.tenuis larvae, is of considerable importance as a centre of dispersal and exchange between the host and its parasites. The extent and success of this exchange depends upon the number of infective-stage larvae which develop from the eggs within the faeces and successfully infect the host. This depends on a number of factors. Temperature has a direct effect on the development and survival of the infective-stage larvae of T.tenuis (Chapter 2) and an indirect effect on the ability of these larvae to infect the red grouse, since temperature affects the utilisation of stored food reserves within the larvae (Chapter 3). The behaviour of the larvae in response to adverse conditions may increase their chances of survival, the migration of the larvae into the heather leaflet microenvironment (Chapter 4) protecting larvae from potentially lethal desiccation. However, there are several other factors which may have a significant impact on the dispersal and survival of the infective-stage larvae of

T.tenuis (Chapter 3). For example, the nature and availability of bacteria within the caecal faeces, which provides the food source for the first and second-stage larvae of T.tenuis, may directly affect the survival of larvae by controlling the build up of lipid reserves in the larvae.

Another factor which may affect the dispersal and exchange of infective-stage larvae on the moor is the loss of larvae to nematophagous predators and parasites. The caecal faeces themselves will contain a range of predatory microorganisms and during migration from caecal faeces to heather the larvae will be exposed to microorganisms which inhabit the soil and vegetation. Pandey (1973) described a number of fungi which prey upon trichostrongyle larvae and invertebrates, such as predatory mites, may feed upon trichostrongyle larvae. It is possible that microorganisms cause a significant reduction in the number of infective-stage larvae of T.tenuis and this aspect of the disease ecology requires further study.

It must not be forgotten that some of the factors that affect the infective-stage larva also influence the eggs and first and second-stage larvae which develop within the caecal faeces.

Hudson (1986a) outlined for the first time a series of possible control procedures for T.tenuis in red grouse. Control could be brought in three ways. Firstly, by reducing the effects of the nematode on the breeding

production of hen red grouse. This may be done by reducing the number of worms by chemotherapy or by improving the plane of nutrition of the grouse. Oral dosing of red grouse with the anthelmintic levamisole hydrochloride is now used on some moors to reduce T.tenuis infections in red grouse. The current work has confirmed that this drug reduces the worm burden in wild red grouse and has shown that it allows recovery of damage to the caeca. Further work is required to monitor the worm burdens in both young grouse (less than 4 months old) which have not been treated with an anthelmintic (and which have low worm burdens) and old grouse (greater than 1 year old) which have been treated with an anthelmintic (and therefore have low worm burdens). Since both young and old red grouse inhabit the same area of moor and will feed on the same stand of heather, they should have similar infection rates. If dosed grouse do show a resistance to infection, worm burdens would be lower than in young non-dosed grouse. However, the preliminary work described in this thesis has shown that dosing of wild red grouse with infective-stage larvae that have been subjected to cobalt 60 irradiation has little effect on the worm burdens in wild red grouse (Chapter 7). This work only examined worm burdens in young grouse which had been given irradiated larvae. It would be interesting to examine the worm burdens from these dosed birds in a years time ie. from those birds shot in the next shooting season (August 12th 1988).

Secondly, T.tenuis could possibly be controlled by reducing the production and survival of worm eggs, either

by treating areas where caecal faeces have been deposited or by the drainage of moorland. This work has shown that relatively few larvae occur at the site of the caecal deposit and that larvae migrate into the surrounding herbage, including the protective micro-environment of the heather leaflet (Chapter 4). Thus, treating specific areas with a larvicidal compound would be of little use in controlling T.tenuis. Although the infective-stage larvae of T.tenuis have been shown to be highly susceptible to desiccation, the various developmental stages spend much of their life within a humid microenvironment, the caecal faeces in the case of eggs, first-stage and second-stage larvae and the heather leaflet in the case of the infective-stage larvae.

Thirdly, control could be brought about by reducing the contact between the infective-stage larvae and the red grouse. This may be done by increasing shooting pressure, a reduction in grouse density resulting in reduced worm burdens; improved grazing management of the moor, as sheep grazing on heather presumably ingest T.tenuis larvae thereby reducing their numbers; or by chemoprophylaxis, by introducing a slow-release anthelmintic bolus into the gizzard of grouse. These control measures may prove to be successful but have yet to be evaluated.

This work has shown both the advantages and drawbacks in using a model host for parasitological research. The range of problems involved in the supply and maintenance of red grouse were avoided by using the domestic chicken as a host

for T.tenuis. However, the course of infection in the domestic chicken was found to contrast with that in the normal, definitive host, the red grouse (Chapter 6) and this limited the value of direct comparisons between the two systems. The domestic chicken throws off an established infection with T.tenuis in about 4 weeks, with most adult nematodes being lost after about 2 weeks of infection whereas the red grouse apparently shows little resistance to infection with this nematode (Chapter 6). Further study of the T.tenuis/ domestic chicken system, from an immunological point of view, may give an indication of what is lacking in the T.tenuis/red grouse system. The red grouse is for some reason tolerant to T.tenuis infections. If the reason for this immune tolerance could be found then it may be possible to artificially stimulate immunity to T.tenuis.

In conclusion, this work has increased our understanding of the relationship of T.tenuis with red grouse but at the same time it has indicated the need for further epidemiological studies on the larval stages. Certain aspects of the pathology of infection in both the red grouse and in the domestic chicken have also been studied but the immunological responses to T.tenuis in both these hosts has yet to be fully elucidated. This should prove to be a fruitful area of study but, unfortunately, is beyond the time allowed for this study. It is hoped that, as a result of the work described in this thesis, control procedures for T.tenuis in red grouse may be improved and in doing so, the red grouse and its habitat will remain an

integral part of Britain's unique upland environment.

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