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**Effects of Abomasal Nematode Parasites *In Vivo*
and *In Vitro***

A thesis presented
in partial fulfilment of the requirements
for the degree of
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Abstract

During abomasal parasitism, parietal cells are inhibited and may be lost, although the exact mechanism is not known. The present *in vivo* and *in vitro* experiments investigated whether the hypoacidity was linked to the host's inflammatory response and whether they are directly targeted by parasites.

Pathophysiological consequences of abomasal parasitism, particularly the effects on parietal cells were studied in different immunological settings in high-fleece weight lambs (HFW) and their unselected controls (C). Lambs were infected with a single dose of *Ostertagia circumcincta* L₃ at the age of 6.5 months (Experiment 1) or 4.5 months (Experiment 2). The older lambs also received further doses of L₃ weekly following primary infection. Lambs were killed on Day 94 or Day 8 or 28 respectively. Serum gastrin and pepsinogen concentrations, blood eosinophils, abomasal pH, paracortical cells in wool follicles (wool quality), FEC and worm burden were monitored. Antibody levels were measured in Experiment 1 and lymphocyte types in abomasal lymph nodes and food intake in Experiment 2.

Tissue eosinophil and parietal cell counts, mucosal thickness and general histopathological changes in abomasal tissues from lambs in Experiment 2 were studied along with tissues collected from randomly bred lambs between five and 30 days after infection with *O. circumcincta* L₃ or six to 72 hours after transplantation of adult *O. circumcincta*.

HFW lambs exhibited resilience, but its expression differed with age. Paracortical cells in wool follicles were lower and increased later in both experiments, yet FEC was higher in older HFW lambs. The rise in abomasal pH was delayed and serum pepsinogen concentrations were higher in older HFW lambs. Generally, blood eosinophils tended to be higher in controls and cytokine responses suggested a lower Th2 response in HFW lambs. In Experiment 2, tissue eosinophils were lower in HFW sheep on Day 8. Resilience in HFW lambs appeared to be based on a down-regulated

inflammatory response and the expression of resilience, which was dependent on full immune responsiveness with age. Additional bases for resilience may be the ability to delay gastric hypoacidity and a higher food intake before and during parasitism as seen in the younger HFW lambs.

No conclusive answer could be given to the question of hypoacidity being secondary to inflammation, although there was a strong link between inflammation and raised abomasal pH associated with vacuolation and loss of parietal cells. Tissue eosinophils were correlated with abomasal pH in all lambs. Parietal cell loss coincided with hypoacidity and inflammation. Blood eosinophil levels did not correlate well with abomasal mucosal eosinophils. In tissues collected 30 hours after larval infection, eosinophils appeared chiefly in the tips of folds, but parietal cell numbers were not reduced.

Evidence for a direct effect of parasite excretory/secretory products on epithelial cells was obtained *in vitro* using the HeLa cell test system for vacuolating activity (neutral red uptake). Adult products were more potent than L₃ chemicals.

These experiments support roles for both the host inflammatory response and parasite chemicals in inhibiting and damaging parietal cells.

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List of abbreviations

µg	Microgram
µM	Micromolar
µm	Micrometre
Ab	Antibody
ACh	Acetylcholine
ALN	Abomasal Lymph Node
ANOVA	Analysis of Variance
AP	Aminopyrine
AR	Amphiregulin
AT	Adult Transplant
ATV	Antibiotic-Trypsin-Versene solution
BSA	Bovine Serum Albumin
C	Control flock
cAMP	Cyclic Adenosine Monophosphate
CCK	Cholecystokinin
CCK-A/B	Cholecystokinin Receptor type A or B
CEM	Complete Essential Medium
CNS	Central Nervous System
CGRP	Calcitonin Gene Related Peptide
conA	Concavalin A
DAG	Diacyl Glycerol
e.p.g.	eggs per gram
EBSS	Earle's Balanced Salt Solution
EC	Enterochromaffin

ECL	Enterochromaffin-like
EDTA	di-sodium Ethylene Diamine Tetra-Acetate
EGF	Epidermal Growth Factor
ES	Excretory/Secretory products
FBS	Fetal Bovine Serum
FEC	Faecal Egg Count
g	Gram
<i>g</i>	Gravitational force
Gal	Galanin
GIP	Gastric Inhibitory Peptide
Gm-CSF	Granulocyte-macrophage Colony-Stimulating Factor
GRP	Gastrin Releasing Peptide
GRPR1	Gastrin Releasing Peptide Receptor type 1
h	Hour
H2	Histamine receptor type 2
H&E	Haematoxalin & Eosin
<i>H. pylori</i>	<i>Helicobacter pylori</i>
<i>H. contortus</i>	<i>Haemonchus contortus</i>
HB-EGF	Heparin Binding-Epidermal Growth Factor
HBSS	Hank's Balanced Salt Solution
HDC	Histidine Decarboxylase
HFW	High Fleece Weight flock
I.A.	Immature Adult
i.m.	intramuscular
i.v.	intravenous
IFN- γ	Interferon-gamma

Ig	Immunoglobulin
IL	Interleukin
IP ₃	Inositol 1,4,5-triphosphate
kg	Kilogram
l	Litre
L ₃	Third-stage larva
L ₄	Fourth-stage larva
L ₅	Fifth stage larva
LI	Larval infection
LT	lymphotoxin
M	Molar
M3	Muscarinic receptor type 3
MEM	Minimal Essential Medium
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimetre
MMC	Mucosal Mast Cell
mRNA	Messenger Ribonucleic Acid
MWCO	Molecular Weight Cut-Off
n	Number
<i>N. brasiliensis</i>	<i>Nippostrongylus brasiliensis</i>
NA	Noradrenalin
NHE2	Sodium/Hydrogen Exchanger 2
NK	Neurokinin
nm	Nanometers
NR	Neutral Red

<i>O. circumcincta</i>	<i>Ostertagia circumcincta</i>
<i>O. leptospicularis</i>	<i>Ostertagia. leptospicularis</i>
<i>O. ostertagi</i>	<i>Ostertagia ostertagi</i>
OD	Optical Density
P follicle	Primary follicle
p.i.	Post infection
PACAP	Pituitary Adenylate Cyclase-Activating Peptide
PAC-R1	Pituitary Adenylate Cyclase-Activating Peptide Receptor type 1
PAS	Periodic Acid Schiff
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PG	Prostaglandin
PHI	Peptide Histidine Isoleucine
PKA	Protein kinase A
PKC	Protein kinase C
PLC	PhospholipaseC
PP	Pancreatic Polypeptide
PSN	Penicillin-Streptomycin-Neomycin
<i>reg</i>	Regenerating gene
RO water	Distilled water
S follicle	Secondary follicle
s.c.	subcutaneous
SD	Standard Deviation
SEM	Standard Error of the Mean
SP	Substance P
SPSS	Statistic Package for Social Scientists

SST	Somatostatin
SST2	Somatostatin receptor subtype 1
<i>T. axei</i>	<i>Trichostrongylus axei</i>
<i>T. colubriformis</i>	<i>Trichostrongylus colubriformis</i>
TAE	Tris Acetate EDTA
TGF- α	Transforming Growth Factor-alpha
Th	T helper cell
TNF- α	Tumor Necrosis Factor-alpha
v/v	volume per volume
VIP	Vasoactive Intestinal Polypeptide
w/v	weight per volume

Introduction

The agricultural industry, especially pastoral farming, plays an important role in the New Zealand economy. Since livestock are almost exclusively kept on pasture in New Zealand, parasites, especially internal helminths, have a significant impact on the outcome of the sheep and beef and dairy industries.

The alarming number of reports of drench resistance, the increasing interest in organic farming and the recognition of the long-term impact of agricultural chemicals on the environment are all incentives to develop alternative strategies to the current chemical anthelmintics. High concentrations of anthelmintics are excreted in the faeces onto the pasture where free-living nematodes and other soil and pasture dwellers are also being affected by highly potent chemicals. Further, there is a significant incidence of anthelmintic resistance amongst the common nematodes infecting ruminants in New Zealand, as is the case elsewhere in the world. This alone has highlighted the need to supplement current drenches with other measures to minimise the use of these chemicals to slow the rate of development of resistance.

Alternative strategies to drenching include better management to minimise larval intake, exploiting the genetic variation in susceptibility of animals to parasites and to their impact on productivity, the use of plants containing natural anthelmintics as fodder, trapping the free-living stages of nematodes by fungi and the development of vaccines against the parasites. Another alternative would be biological control by interfering with parasite establishment, metabolism or reproduction. This approach will depend on a much more detailed understanding of the host-parasite interaction, parasite biology and the pathophysiological basis for resistance, resilience and susceptibility of hosts.

The objective of the present experiments was to study the responses of sheep of different genetic backgrounds to infection with abomasal parasites in order to learn more about the communication between the parasite and the host tissues and the relative importance of host inflammatory responses and the

excretory/secretory products of the worms in causing abomasal dysfunction. The effects of infection with *Ostertagia circumcincta* or *Haemonchus contortus* in outbred sheep are well described: inhibition of gastric acid secretion, hypersecretion of gastrin, increased levels of circulating pepsinogen, loss of functioning parietal cells, inflammation characterised by large numbers of eosinophils and mucous cell hyperplasia in the infected abomasum. What is still unknown is whether the parasites actively inhibit acid secretion to promote their survival, if so, how do they do it and could this be a target for biological control of abomasal nematodes.

Alternatively, it may be more practical to breed for resistant or resilient hosts to reduce the impact of the parasites with a minimum of chemical intervention. Whereas it has been established that resistance has an immunological basis, there seems to be no information on the physiological basis for resilience to parasitism. If resistance and resilience are not opposite extremes of the same phenomenon, then selection may provide a way for the incorporation of both traits into a breeding programme. The experiments described in this thesis are also aimed at establishing the underlying reasons for different host responses to abomasal parasites and from these studies also to gain a better understanding of the pathophysiological processes initiated by the parasites.

Chapter 1

Literature review

1.1 Life Cycle of Abomasal Nematode Parasites

The most common abomasal trichostrongylid parasites of sheep, *Haemonchus contortus* and *Ostertagia (Teladorsagia) circumcincta*, and of cattle, *Ostertagia ostertagi* and *Trichostrongylus axei* (Urquhart *et al.*, 1987), all have a similar direct life cycle. The length of the cycle varies with the parasite species and is influenced by the physiological status of the host, as well as by the strain of parasite and its population dynamics.

Fertilised eggs are passed in the faeces and develop to infective, ensheathed third stage larvae (L₃) in the environment (Silverman & Campbell, 1959; Waller & Donald, 1970). After ingestion by the host, the larvae exsheath in the rumen, initiated possibly by components of the bicarbonate buffer system (Sommerville, 1957, 1964; Rogers & Sommerville, 1960, 1963, 1968; Hertzberg *et al.*, 2002) as well as by exsheathing fluid secreted by the parasite (Rogers & Sommerville, 1960). The exsheathed L₃ pass down to the abomasum, enter the gastric glands and pits and undergo further development.

Within the gastric glands, the parasites grow and may moult twice to become immature adult worms before emerging, they may emerge as fourth stage larvae (L₄) (Sommerville, 1954, 1963) and complete their development in the abomasal lumen, or alternatively some enter a hypobiotic state as L₄ (Sommerville, 1954, 1963; Armour *et al.*, 1966; Michel, 1976; Armour & Duncan, 1987). Generally, *H. contortus* emerge from ovine glands after 2-4 days (Stoll, 1943; Sommerville, 1963; Christie *et al.*, 1967; Hunter & MacKenzie, 1982), *O. circumcincta* after 5-6 days (Sommerville, 1954; Dash, 1985) and *O. ostertagi* after 16-21 days in cattle (Jennings *et al.*, 1966; Fox *et al.*, 1987). Larval development is influenced by the size of the infective dose (Dunsmore, 1960; Elliott, 1974a, b; Durham &

Elliott, 1976), the presence of adult worms (Michel, 1971) and the immune status of the host (Michel, 1970; Stear *et al.*, 1995).

Parasite population dynamics are complex. A greater infective dose of L₃ will not necessarily produce a larger parasite population or greater pathological effects (Elliott, 1974a, b; Fleming, 1988). Parasite development was slowed when large numbers of *O. circumcincta* L₃ were administered (Elliott, 1974a, b) and egg laying was delayed by host resistance to *H. contortus* (Silverman & Patterson, 1960) or *O. circumcincta* (Stear *et al.*, 1995). An existing worm population can also influence the number of larvae establishing, the proportion entering an arrested state and also the fecundity and survival of the population (Michel, 1971). Egg production per *Ostertagia* female worm was reduced at high population density, so that faecal egg count (FEC) did not accurately reflect the number of worms present (Anderson *et al.*, 1965; Barger *et al.*, 1985; Barger, 1986; Stear *et al.*, 1995; Stear & Bishop, 1999).

1.2 Abomasal Structure and Function

The ruminant abomasum is the equivalent of the monogastric stomach. It consists of two main parts, which can be distinguished functionally, histologically and on gross morphology. The glands in both areas of the abomasum are invaginations of the surface epithelium into the lamina propria, ending in numerous blind tubules. The fundus secretes acid (Hersey & Sachs, 1995), pepsinogen (Hirschowitz, 1989; Samloff, 1989), bicarbonate, mucins (Garner *et al.*, 1984) and intrinsic factor (McKay & McLeay, 1981; Lorenz & Gordon, 1993). The antrum secretes mucus and pepsinogen (Samloff, 1989) and is a regulator of acid secretion through its release of the hormone gastrin (Walsh, 1988; Dockray & Gregory, 1989).

1.2.1 Antrum

1.2.1.1 Gland Structure and Cell Types

The majority of antropyloric glands consist of a single pit, an isthmus and a gland. Some tubular units have more than one gland per pit, probably resulting

from division during the growing phase (Lee, 1985b). Typically, the glands are short, the pits are very long and the progenitor zone is located in the isthmus at the junction of the pit and gland (Lee, 1985a, b; Lee & Leblond, 1985a, b).

The predominant cells in the antral glands are mucous cells. There are also endocrine cells, chiefly the gastrin-secreting G cells (Creutzfeldt *et al.*, 1971; Walsh, 1988; Dockray *et al.*, 1996) and the adjacent somatostatin-secreting D cells (Larsson *et al.*, 1979; Larsson, 2000). G cells may be located principally in the upper, middle or lower part of the gland, depending upon the species (Polak, 1986). In sheep, G (and D) cells have been reported throughout the gland (Bunnett, 1984) or are more numerous close to the gland base (Scott *et al.*, 1998b). There are also small numbers of other endocrine cells, including histamine-secreting enterochromaffin-like (ECL) cells and serotonin-secreting enterochromaffin (EC) cells (Rubin, 1972; Scott *et al.*, 1998b).

Proliferation of antral mucosal cells appears to be under the control of the Epidermal Growth Factor (EGF) family of peptides, which includes Transforming Growth Factor (TGF)- α . EGF stimulates the proliferation of G cells and reduces the number of D cells (Vinter-Jensen *et al.*, 1995), and increases gastrin gene expression (Ford *et al.*, 1997).

1.2.1.2 Secretion of Gastrin

Gastrin is a peptide hormone which is synthesised and stored in the G cells in the antrum of the stomach (Walsh, 1988; Dockray *et al.*, 1996; Sawada & Dickinson, 1997) or abomasum (Reynolds *et al.*, 1984, 1991). Gastrin is an important co-ordinator of acid secretion associated with feeding and the arrival of food in the stomach, which stimulate release of gastrin from the G cells through neural, chemical, paracrine and endocrine pathways (Walsh, 1984, 1988; Dockray & Gregory, 1989; Schubert & Makhlof, 1992). Amidated gastrins and several other molecular forms of gastrin are trophic agents for the fundus (See Section 1.2.2.2).

Classically, gastrin secretion during feeding is divided into cephalic, gastric and intestinal phases and the control mechanisms involved in these phases are categorised as neural, hormonal, paracrine and chemical. The principal

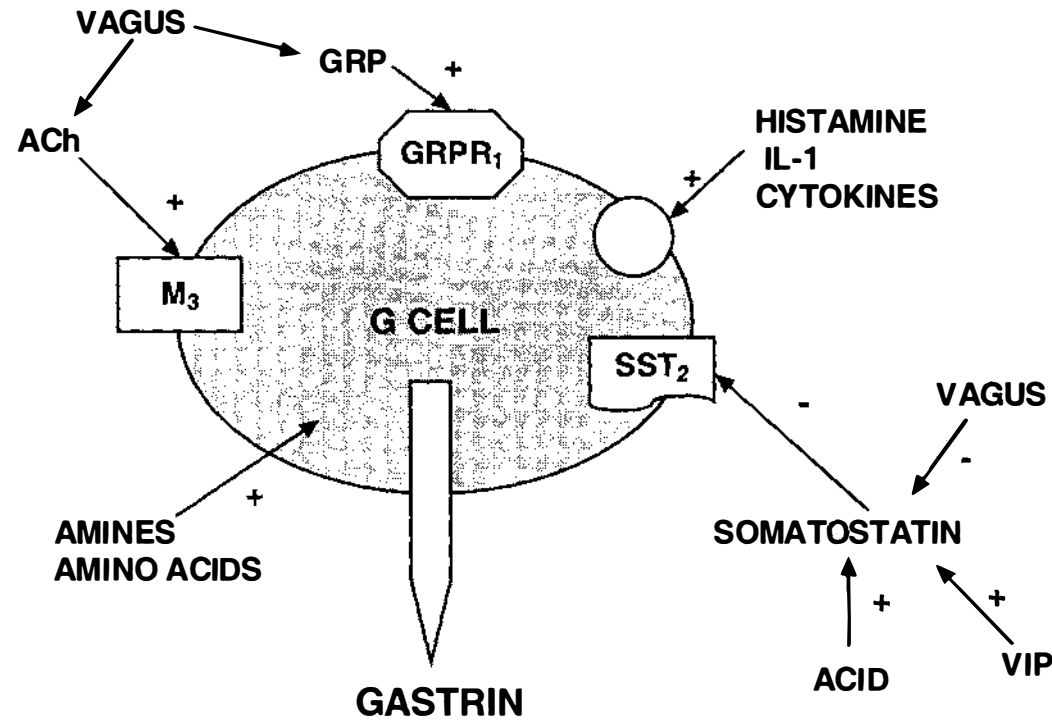


Figure 1.1. Principal physiological and inflammatory regulators of gastrin secretion by the G cell. Somatostatin is a tonic paracrine inhibitor of the G cell, exerting continuous restraint on gastrin secretion. The vagus mediates control from the central nervous system. ACh: acetylcholine; GRP: gastrin releasing peptide; IL: interleukin; VIP: Vasoactive Intestinal Polypeptide.

pathways and receptors involved are represented diagrammatically in Figure 1.1. The complex interaction of regulatory mechanisms involving G, D, parietal and ECL cells is summarised in Figure 1.7. A key regulator of the G cell is the negative feedback loop through the release of the inhibitor somatostatin (SS) from antral D cells (Schubert, 1997). SS is a tonic inhibitor of gastrin secretion (Saffouri *et al.*, 1979; McIntosh *et al.*, 1991) and many physiological changes in gastrin secretion are mediated through either an increase or decrease in SS release. For example, vagal stimulation stimulates gastrin secretion, in part by inhibiting SS release (Debas & Carvajal, 1994; Koop *et al.*, 1997; Weigert *et al.*, 1998), and, conversely, gastrin secretion is inhibited by Vasoactive Intestinal Polypeptide (VIP)-containing nerves which release SS (Saffouri *et al.*, 1984a; Holst *et al.*, 1993; Schubert & Makhlouf, 1993).

The cephalic phase of gastrin secretion is the response to the sight, smell or taste of food (Dockray & Tracy, 1980a, b; Hirschowitz & Fong, 1990). The vagi mediate this response, directly by the release of Gastrin Releasing Peptide (GRP) at the G cell (Schubert *et al.*, 1985; Weigert *et al.*, 1993), and indirectly through cholinergic neurones, which inhibit SS release by the D cells (Debas & Carvajal, 1994). These vagal pathways have been demonstrated in dogs in response to feeding (Dockray & Tracy, 1980a) and sham feeding (Teppermann *et al.*, 1972; Hirschowitz & Fong, 1990), and by nerve stimulation in sheep (Bladin *et al.*, 1983) and calves (Adrian *et al.*, 1983). A cephalic phase may be relatively unimportant in ruminants at pasture or when fed *ad libitum*, in which fluctuations in serum gastrin concentrations are small (Reynolds *et al.*, 1991). Sheep on restricted feeding regimens show greater variations in serum gastrin levels, with peak responses coinciding with feeding times (McLeay & Titchen, 1970; Lawton *et al.*, 1996).

In the gastric phase, gastrin secretion is stimulated by amino acids (Richardson *et al.*, 1976; Konturek *et al.*, 1977b; Feldman & Grossman, 1980; Lichtenberger *et al.*, 1982a; Taylor *et al.*, 1982; DelValle & Yamada, 1990), peptones (Saffouri *et al.*, 1984b; Schubert *et al.*, 1992) and amines (Lichtenberger *et al.*, 1982b; DelValle & Yamada, 1990). Amino acids act directly on the G cell (DelValle &

mainly by inhibiting SS release (Saffouri *et al.*, 1984b; Schubert *et al.*, 1992). Gastrin release is stimulated by moderate gastric distension (Debas *et al.*, 1975; Schiller *et al.*, 1980). At low pressures, gastrin secretion is inhibited by increased SS release via VIP neurones, but increasing distension progressively recruits cholinergic neurones, SS is reduced and gastrin release is increased (Schubert & Makhoul, 1993).

Acid feedback plays a key role in controlling gastrin release and thereby acting as a brake on acid secretion. Gastrin secretion is increased when gastric pH is raised by infusion of alkaline solutions (Becker *et al.*, 1973; Smith *et al.*, 1975), by removing the fundus (Alumets *et al.*, 1979; Håkanson *et al.*, 1982) or by the pharmacological inhibition of acid release (Allen *et al.*, 1986; Dimaline *et al.*, 1991; Dockray *et al.*, 1991). Conversely, lowering the gastric pH reduces the response of the G cell to stimulants (Becker *et al.*, 1973; Hirschowitz & Gibson, 1979; Dockray & Tracy, 1980b; Konturek *et al.*, 1995). The inhibitory effects of high gastric acidity are mediated by increased SS secretion (Schusdziarra *et al.*, 1978; Schubert *et al.*, 1988) via sensory Calcitonin Gene Related Peptide (CGRP)-containing nerves acting on the D cell (Ren *et al.*, 1992; Manela *et al.*, 1995).

The passage of gastric contents into the duodenum initiates the inhibitory intestinal phase of gastric secretion. It is still not clear whether acid secretion is inhibited mainly by direct effects on the parietal cell or also indirectly by inhibiting gastrin release. Low pH in the duodenum stimulated the secretion of the hormones secretin (Konturek *et al.*, 1977a) and cholecystokinin (CCK) (Konturek *et al.*, 1995) and inhibited acid secretion (Konturek *et al.*, 1977a), possibly directly (Kleibecker *et al.*, 1984; You & Chey, 1987). In contrast, the presence in the duodenum of fat, but not casein or glucose, inhibited gastrin secretion (indirectly via increased antral SS secretion), probably through stimulation of secretin or CCK secretion by the duodenum (Schusdziarra *et al.*, 1978).

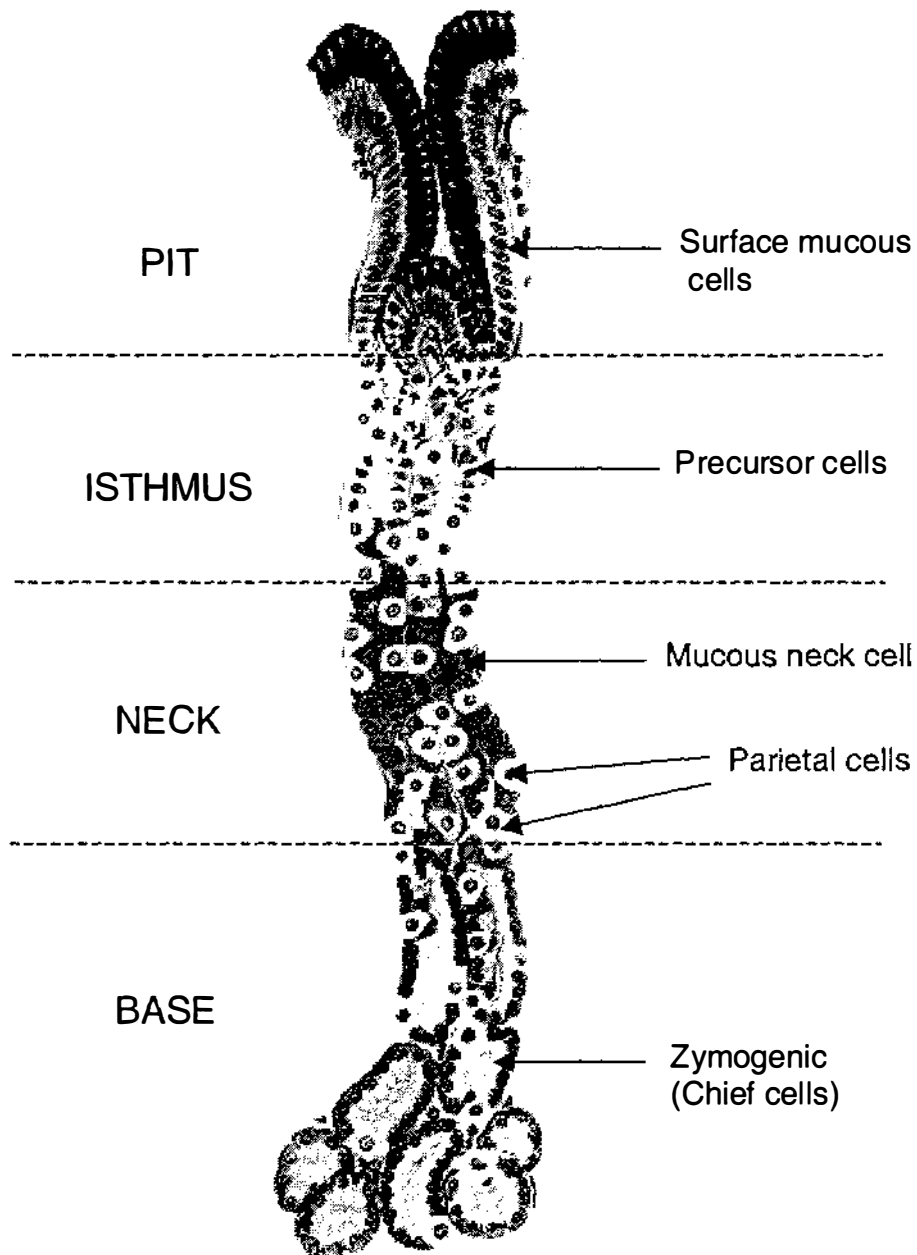


Figure 1.2. The structure of the pit-gland unit in the fundic mucosa. The isthmus contains stem cells and committed precursors of the pit, zymogenic and parietal cell lines. Modified after Ham (1957).

1.2.2 Fundus

1.2.2.1 Gland Structure and Cell Types

Several glands, or zymogenic units, each consisting of an isthmus, neck and base, open into a pit, then to the gastric lumen (Stevens & Leblond, 1953; Karam & Leblond, 1992) (Figure 1.2). The pit contains surface mucous cells (pit cells) and a few parietal cells, whereas the gland contains most of the parietal cells, as well as chief cells, mucous neck cells and endocrine cells (Murray, 1970; Ross & McCracken, 1971; Ross *et al.*, 1971; Rubin, 1972; Karam & Leblond, 1992).

Extensive studies in mice of the renewal of the gastric epithelium by Karam & Leblond (1992; 1993a-d) and Karam (1993) have led to a model which is generally applicable to other mammalian species. A pool of stem cells in the isthmus is thought to generate undifferentiated precursor cells that differentiate into surface mucous cells, mucous neck and zymogenic cells (chief cells), parietal cells and endocrine cells (Karam & Leblond, 1992, 1993a-d, 1995; Karam, 1993; Bjerknes & Cheng, 2002). Nomura *et al.* (1998) and Goldenring *et al.* (2000), however, have proposed further progenitor zones in the glands. As the precursor cells differentiate and mature, the surface mucous cells and some parietal cells migrate upward into the pit and the other cell types migrate down into the gland.

The entire surface epithelium is renewed within 3-6 days in most adult mammals (Willems, 1991), as the surface mucous cells of the pit cell lineage have an average turnover time of 3 days (Karam & Leblond, 1993b). Most downward migrating precursor cells of the pepsinogen-secreting zymogenic cell lineage become mucous neck cells. They reside for 7-14 days in the neck and later differentiate during their further migration towards the gland base into mature chief (zymogenic) cells. Their turnover time is 194 days in mice (Karam & Leblond, 1993c). Hanby *et al.* (1999) recently suggested that mucous neck cells form a discrete cell lineage on the basis that they produce peptides with a luminal protective function (Jeffrey *et al.*, 1994; Alison *et al.*, 1995). Unlike pre-pit, pre-neck and zymogenic cells, the pre-parietal cells are not capable of self-replication by mitosis and have a short turnover time in the mouse of 2.6 days,

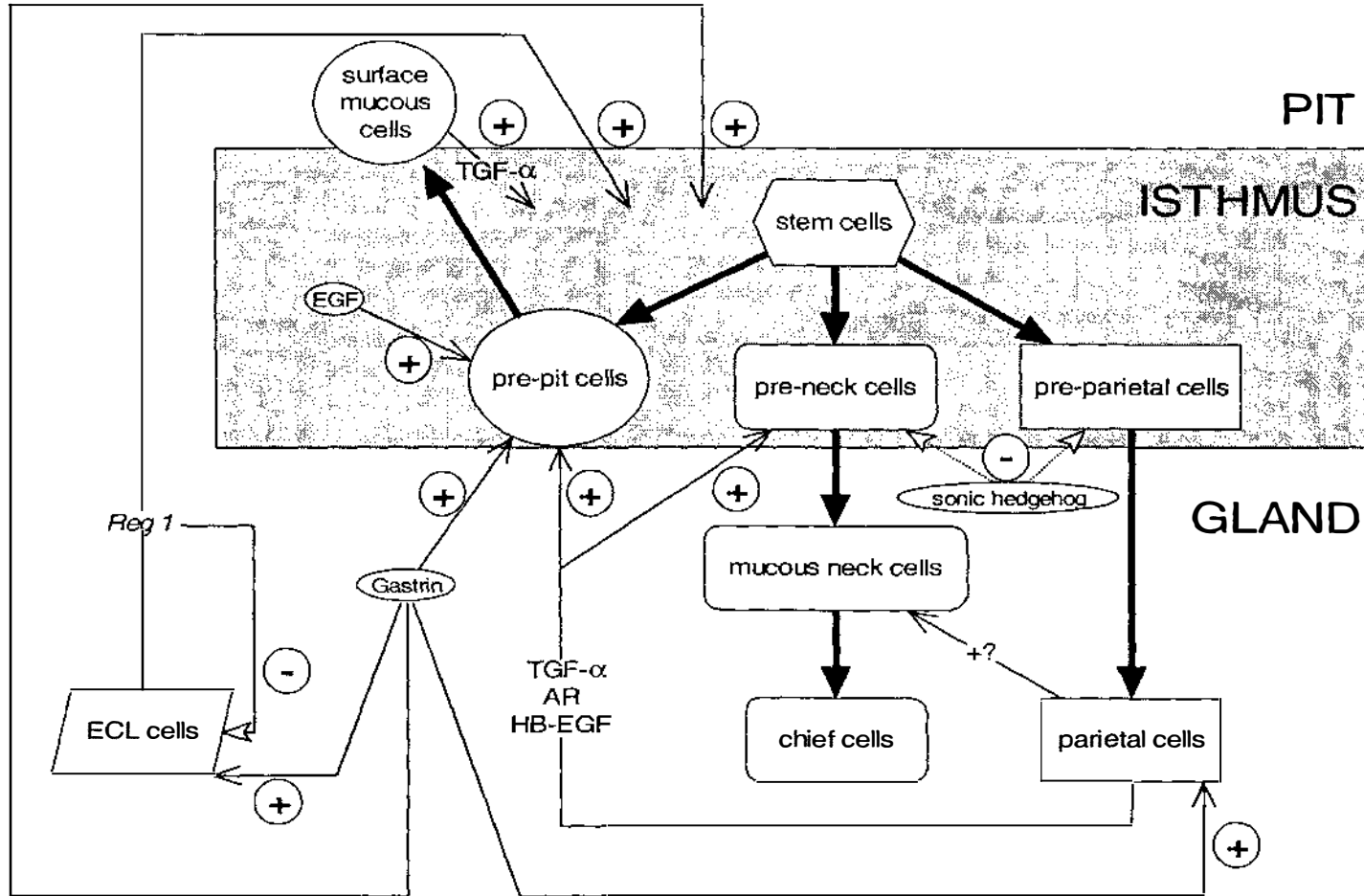


Figure 1.3. Trophic agents and their proposed site of action in the fundic pit-gland unit. AR: amphiregullin; ECL: enterochromaffin-like cell; EGF: epidermal growth factor; HB-EGF: heparin binding-epidermal growth factor; *Reg 1*: product of the *Reg* gene; TGF- α : Transforming Growth Factor- α .

so that mature parietal cells are replaced in 54 days in the mouse (Karam & Leblond, 1992; Karam, 1993). Parietal cells gradually lose their ability to secrete acid during their downward migration to the base of the gland (Coulton & Firth, 1983; Helander & Sundell, 1984; Karam & Forte, 1994; Karam *et al.*, 1997b) and eventually degenerate and are lost through necrosis and apoptosis (Karam, 1993).

1.2.2.2 Trophic Agents

There is a growing list of trophic agents known to be involved in maintaining a stable population of epithelial cells in the fundic gland and in repairing damaged areas. Gastrin and the EGF family of peptides are the best known of these growth factors and the parietal cell is an organisational centre for the mucosa. Sonic hedgehog protein has been proposed as a negative proliferative agent for gland, but not pit, cells (van den Brink *et al.*, 2001) and to be involved in gastric epithelial differentiation (van den Brink *et al.*, 2002). These agents interact to regulate the proliferation of precursor cells, their differentiation into specialised cells and their migration along the pit-gland unit (reviewed by Dockray (1999), Giraud (2000) and Dockray *et al.* (2001)). The current model proposed to explain the actions of key agents is illustrated in Figure 1.3. Not included in the diagram, but known to have specific trophic effects in the fundus, are the trefoil peptides (Giraud, 2000), basic fibroblast derived growth factor (Folkman *et al.*, 1991) and hepatocyte growth factor (Takahashi *et al.*, 1995; Kinoshita *et al.*, 1997; Yoshizawa *et al.*, 2000).

Functioning parietal cells require the presence of gastrin. Gastrin also stimulates the self-replication of ECL cells (Tielemans *et al.*, 1989, 1990; Ryberg *et al.*, 1990a-c). In transgenic mice, in the absence of gastrin (Koh *et al.*, 1997; Friis-Hansen *et al.*, 1998) or the gastrin receptor (Nagata *et al.*, 1996; Langhans *et al.*, 1997), some parietal cells were present, but many lacked the proton pump and none secreted acid. Gastrin stimulates the migration of parietal cells and, although it does not alter their life span (Kirton *et al.*, 2002), it probably affects their secretory activity, which is reduced at lower positions in the gland (Coulton & Firth, 1983; Karam *et al.*, 1997b). A stable parietal cell population may be maintained by controlling the maturation, not generation, of

parietal cells. The β -subunit of the proton pump is a possible control site, as it is required for parietal cell maturation (Scarff *et al.*, 2001; Franic *et al.*, 2001).

Gastrin may act indirectly as a trophic agent for parietal cells, since mature parietal cells, which possess the gastrin receptor, are unable to divide (Karam, 1993) and there are few, if any, gastrin receptors on cells in the proliferative zone (reviewed by Kinoshita & Ishihara (2000)). Asahara *et al.* (1994) were unable to detect CCK_B receptors in the proliferative zone and Nakajima *et al.* (2002a, b) demonstrated them only on the pit-cell precursor. Gastrin has been proposed to act indirectly on proliferative cells by upregulating the production of Reg 1 α protein, the product of the Regenerating (*Reg*) gene, which is expressed by ECL cells in rats (Asahara *et al.*, 1996) and both ECL and chief cells in humans (Higham *et al.*, 1999). Reg protein expression by ECL cells was stimulated by gastrin and was positively correlated with the proliferation of gastric mucosal cells (Fukui *et al.*, 1998). Hypergastrinaemia may also cause pit elongation (Konda *et al.*, 1999) through increased expression of gastrin receptors on pit-cell precursors in the isthmus (Nakajima *et al.*, 2002b).

The parietal cell has a regulatory role in the maturation and differentiation of the zymogenic cell line from mucous neck cells to chief cells. There are fewer chief cells when the number of mature parietal cells is reduced, as well as in many, but not all, conditions in which parietal cells are dysfunctional. Mucous cell hyperplasia and loss of mature chief cells accompanied necrosis of parietal cells after exposure to ammonia (Hagen *et al.*, 1997) and loss of mature parietal cells in transgenic mice (Li *et al.*, 1995a, 1996; Canfield *et al.*, 1996; Karam *et al.*, 1997a; Schultheis *et al.*, 1998). Acid production does not appear to be the critical signal for zymogenic cell maturation, since disruption of acid secretion by deletion of the β -subunit of the proton pump (Franic *et al.*, 2001), chronic treatment with H⁺-K⁺-ATPase inhibitors (Karam & Forte, 1994; Goldenring *et al.*, 2000) or autoimmune gastritis (Judd *et al.*, 1999) prevented maturation of chief cells, but this was not so with dysfunctional parietal cells due to the lack of expression of the α -subunit (Spicer *et al.*, 2000).

The EGF peptides, amphiregulin (AR), TGF- α , and heparin-binding EGF (HB-

epithelium (Beauchamp *et al.*, 1989; Murayama *et al.*, 1995; Tsutsui *et al.*, 1997). EGF itself may be synthesised in the stomach in injured areas (Beauchamp *et al.*, 1989; Tarnawski *et al.*, 1992; Konturek *et al.*, 1997), although most of the EGF necessary for maintenance of normal gastric tissues and repair of damaged areas is provided by saliva; hence sialodenectomy resulted in atrophy of the gastric mucosa (Skinner *et al.*, 1984) or delayed gastric ulcer healing (Olsen *et al.*, 1986). Acutely, EGF or TGF- α inhibits acid secretion (Wang *et al.*, 1993), although this may not be a physiological action (Chew *et al.*, 1994). Chronic administration of TGF- α has the opposite effect of increasing acid secretion (Chew *et al.*, 1994), as it up-regulates expression of the proton pump (Kaise *et al.*, 1995).

AR (Tsutsui *et al.*, 1997), HB-EGF (Murayama *et al.*, 1995) and TGF- α are expressed in parietal cells, and lesser amounts of TGF- α are also present in the surface mucous cells (Beauchamp *et al.*, 1989; Murayama *et al.*, 1995; Tsutsui *et al.*, 1997). In excess, these peptides inhibit the differentiation of parietal and mature chief cells (Sharp *et al.*, 1995) and promote mucous cell hyperplasia (Chen *et al.*, 1993; Rutten *et al.*, 1993). Gastrin increases the production of HB-EGF, its mRNA and its promoter (Tsutsui *et al.*, 1997; Miyazaki *et al.*, 1999), TGF- α mRNA (Wang *et al.*, 2000) and AR mRNA (Tsutsui *et al.*, 1997).

In older transgenic mice over-expressing gastrin, hyperplasia of the fundic mucosa, particularly of parietal and ECL cells, progressed to a gradual loss of parietal cells, pit hyperplasia and atrophy of the glands (Wang *et al.*, 1999), probably related to the increased release of EGF peptides (Wang *et al.*, 2000). Pit elongation may also be the result of gastrin inducing the expression of gastrin receptors on pit-cell precursors in the isthmus (Nakajima *et al.*, 2002b).

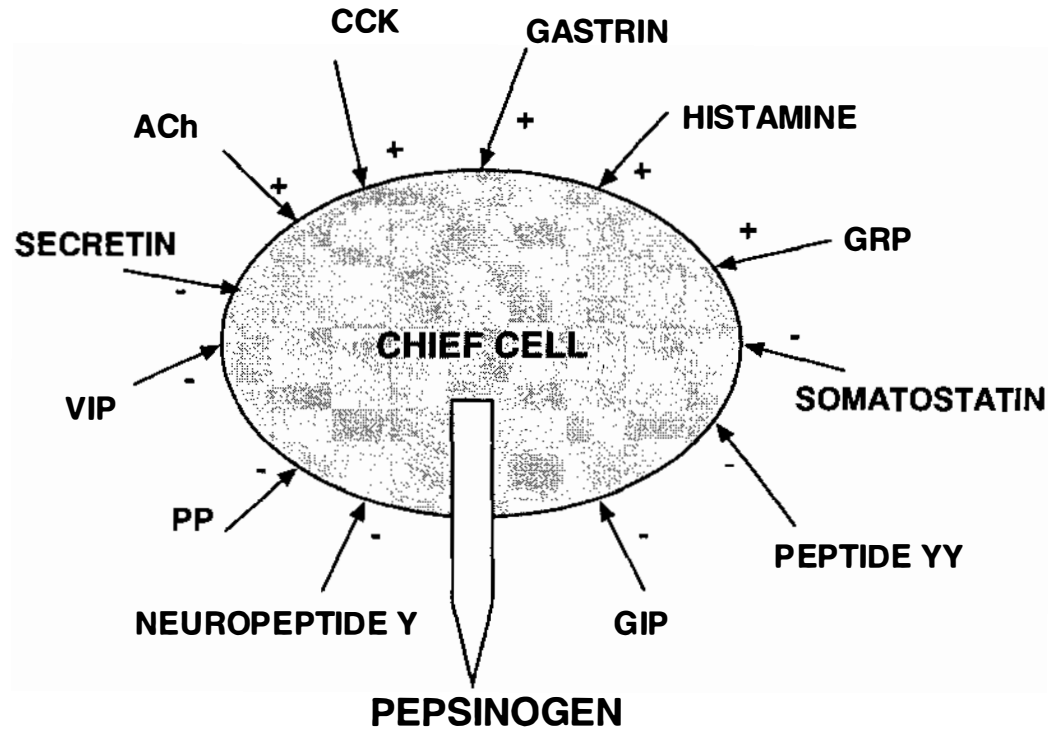


Figure 1.4. Regulation of pepsinogen secretion by the chief cell. ACh: acetylcholine; CCK: cholecystinin; GIP: gastric inhibitory peptide; GRP: gastrin releasing peptide; PP: pancreatic polypeptide; VIP: Vasoactive Inhibitory Polypeptide.

1.2.2.3 Secretion

1.2.2.3.1 Pepsinogen and the Chief Cell

Pepsinogen, in its two isoforms I and II, is an inactive pre-protease, which is secreted into the gastric lumen and transformed into the active aspartic protease, pepsin, by exposure to pH <2 for pepsinogen I and pH >3 for pepsinogen II. It is reversibly inactivated at about pH 5 and irreversibly inactivated at pH 7-8 (Hersey, 1989, 1994; Samloff, 1989). Pepsinogen and acid secretion are usually stimulated and inhibited in parallel *in vivo* and *in vitro*, although they may be independently released (Hersey *et al.*, 1983; Sandvik *et al.*, 1987a, b; Basson *et al.*, 1988; Ruiz *et al.*, 1993; Blandizzi *et al.*, 1999).

Pepsinogen is synthesised and stored as granules in the surface mucous cells, mucous neck cells and chief cells in the bovine fundic epithelium, but only in the latter two cell types in the ovine fundus (Yamada *et al.*, 1988; Cybulski & Andren, 1990; Scott *et al.*, 1998c, 1999). Following physiological or external chemical stimuli, there is a biphasic release of the pepsinogen into the gland lumen: an initial peak is followed by sustained secretion for a prolonged period (Hirschowitz, 1984; Hersey, 1989). Exocytosis of the pepsinogen-containing granules, recorded directly by video microscopy, results in depletion of apical granules during the initial peak secretion, but no further net loss of granules during the sustained phase of secretion (Tao *et al.*, 1998).

Secretion of pepsinogen is regulated through positive and negative feedback mechanisms, which vary considerably in importance in different species (Hirschowitz, 1991; Gritti *et al.*, 2000). Figure 1.4 is a summary of the numerous peptides and neurotransmitters which have been shown *in vivo* or *in vitro* to modify pepsinogen secretion in at least one species (Raufman *et al.*, 1983; Hersey, 1989; Hirschowitz, 1991; Raufman & Singh, 1991; Raufman, 1992).

CCK and gastrin mediate pepsinogen release in monogastric species (Hersey *et al.*, 1983; Lanas *et al.*, 1994; Blandizzi *et al.*, 1999) by binding to CCK_A and CCK_B receptors respectively (Lin *et al.*, 1992; Blandizzi *et al.*, 1999). Vagal release of pepsinogen is believed to be both direct through acetylcholine (ACh) and indirect via histamine release from the ECL cells. Cholinergic agonists

stimulated pepsinogen secretion by tissues from ruminants (McKellar *et al.*, 1990b) as well as laboratory animal species (Samloff, 1971; Koelz *et al.*, 1982; Raufman *et al.*, 1983; Chew & Brown, 1986). Histamine may not be an important physiological regulator of pepsinogen secretion in many species (Hersey, 1989; Hirschowitz, 1991) because of the variable responses it evokes: pepsinogen secretion was stimulated in peptic cells of humans (Lanas *et al.*, 1994) and rats (Sandvik *et al.*, 1987b), but not guinea pigs (Basson *et al.*, 1988), dogs (Hirschowitz, 1991) or rabbits (Koelz *et al.*, 1982; Hersey *et al.*, 1983). SS directly inhibited pepsinogen release induced by cholinergic agonists and CCK peptides in rat chief cells (Tani & Tanaka, 1990; Tanaka *et al.*, 1993). EGF stimulated pepsinogen release in a concentration-dependent manner by guinea pig chief cells (Fiorucci *et al.*, 1996).

1.2.2.3.2 Histamine and the ECL Cell

ECL cells synthesise and store histamine (Rubin & Schwartz, 1979; Kubota *et al.*, 1984; Håkanson *et al.*, 1994; Prinz *et al.*, 1999; Bordi *et al.*, 2000), the principal mediator of gastrin-stimulated acid secretion (reviewed by Dockray (1994), Schubert (1997) and Lindstrom *et al.* (2001)). They also produce and store Chromogranin A, which is considered to be involved in the packaging of secretory granules (Håkanson *et al.*, 1994) and is frequently used as a marker for ECL cells in the fundus. ECL cells are located in the basal half or third of the fundic mucosa, in close contact with chief and parietal cells (Simonsson *et al.*, 1988; Håkanson *et al.*, 1994; Kamoshida *et al.*, 1999), consistent with the role of histamine as a paracrine stimulant of parietal cells (Debas, 1977; Soll & Walsh, 1979; Waldum *et al.*, 1991; Black, 1993) and possibly also of peptic cells (Sandvik *et al.*, 1987b; Lanas *et al.*, 1994).

Gastrin is a potent stimulant of histamine secretion during a meal. The binding of gastrin to CCK_B receptors leads to exocytosis of the histamine-containing vesicles (Prinz *et al.*, 1999; Bordi *et al.*, 2000) and also induces the activation of histidine decarboxylase (HDC) and HDC expression (Håkanson *et al.*, 1994; Chen *et al.*, 1998). Gastrin is also a trophic agent for the ECL cells, hypergastrinaemia leading to ECL cell hyperplasia (See Section 1.2.2.2).

During feeding, increased histamine release is a principal mediator of the

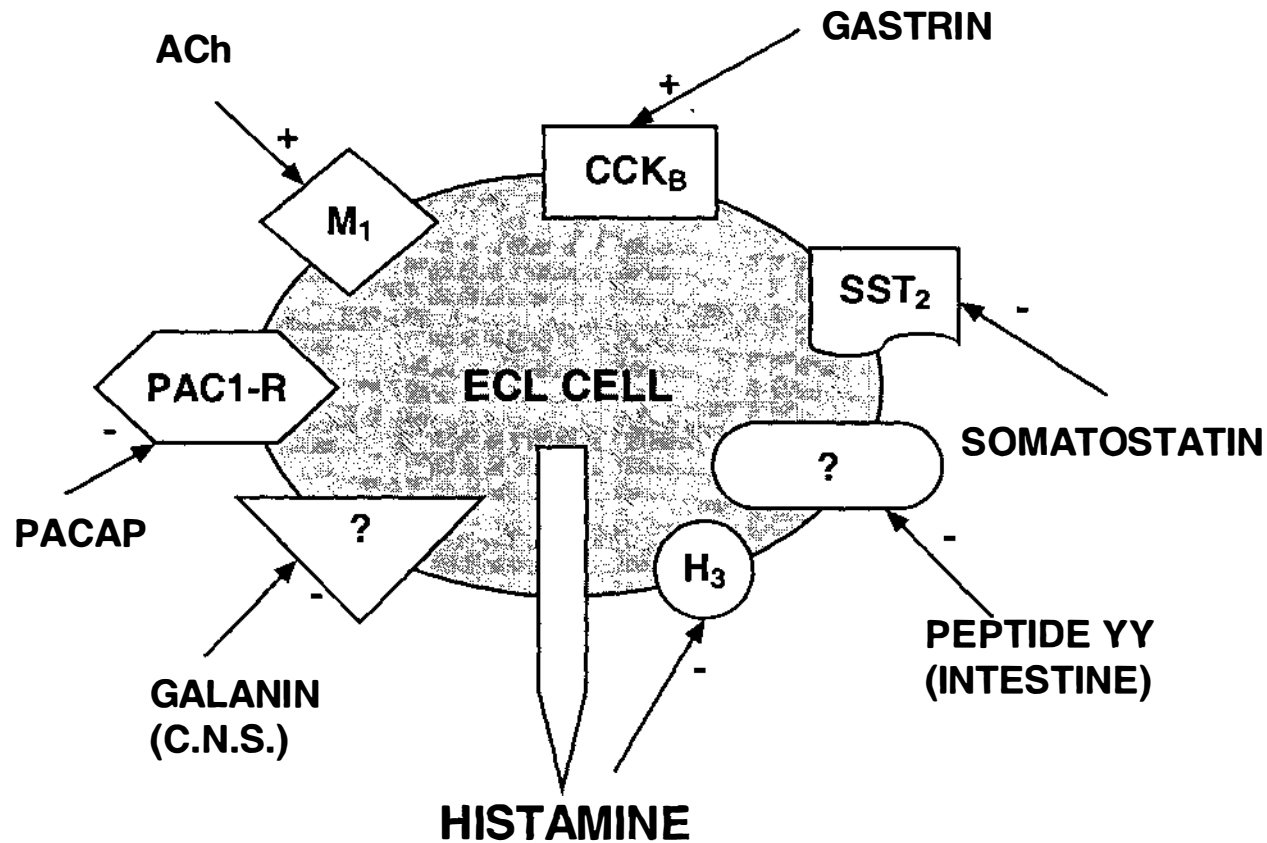


Figure 1.5. Regulation of histamine secretion by the enterochromaffin-like (ECL) cell. The receptor sub-types which have been identified on the ECL cell are shown. ACh: acetylcholine; C.N.S.: central nervous system; CCK: cholecystinin; PACAP: pituitary adenylate cyclase-activating peptide.

increased acid secretion (See Section 1.2.3.1) and the ECL cell is also a point at which negative feedback acts as a brake on acid secretion. Receptors and their ligands, which stimulate or inhibit the ECL cell (reviewed by Lindstrom *et al.* (1997), Prinz *et al.* (1999) and Håkanson *et al.* (2001)), are shown in Figure 1.5. Although hypergastrinaemia is the main stimulant for the ECL cells, cholinergic nerves are probably also additional sources of stimulation, however, the presence of muscarinic receptors (Prinz *et al.*, 1993; Sandor *et al.*, 1996; Athmann *et al.*, 2000) and whether their sub-type is M₁ or M₂ (Sandvik *et al.*, 1988; Hollande *et al.*, 1993; Sandor *et al.*, 1996) remain contentious issues. The vagal neurotransmitter may not only be ACh, but also pituitary adenylate cyclase-activating peptide (PACAP), a member of the secretin/glucagon/VIP family of peptides (Zeng *et al.*, 1999; Sandvik *et al.*, 2001).

Histamine secretion is restrained by an autocrine inhibition through the ECL cell H₃ receptors (Bado *et al.*, 1991; Kidd *et al.*, 1996), as well as by a negative feedback loop with SS secretion from adjacent D cells (Prinz *et al.*, 1993; Zaki *et al.*, 1996), either through a simple loop (Grabau *et al.*, 1999) or a more complex dual loop with the D cell (Vuyyuru *et al.*, 1997). The neuropeptides, galanin (Rossowski & Coy, 1989; Lindstrom *et al.*, 1997; Zeng *et al.*, 1998) and peptide YY (Zeng *et al.*, 1997) are respectively proposed as mediators of central and intestinal inhibition of acid secretion, acting via the ECL cell.

1.2.3 Acid Secretion and the Parietal Cell

The proton pump, an H⁺-K⁺-ATPase, is responsible for gastric acid secretion by the parietal cell (Ganser & Forte, 1973; Sachs *et al.*, 1976; Forte & Lee, 1977; Urushidani & Forte, 1987). The H⁺-K⁺-ATPase has two subunits, α and β (Reuben *et al.*, 1990; Hall *et al.*, 1991; Bamberg *et al.*, 1992), both of which are involved in acid secretion. The α subunit contains the active transport site (Bamberg *et al.*, 1992; Spicer *et al.*, 2000) and the β subunit is required for both membrane docking and cessation of acid secretion after removal of the stimulant (Reuben *et al.*, 1990; Chow & Forte, 1995; Courtois-Coutry *et al.*, 1997; Scarff *et al.*, 1999). H⁺ are generated by the enzymatic activity of carbonic anhydrase, which catalyses the reaction of water and carbon dioxide to form carbonic acid. Dissociation of carbonic acid provides the H⁺ for the

proton pump and one bicarbonate ion diffuses through the basolateral membrane of the parietal cell into the extracellular fluid (reviewed by Hersey & Sachs (1995)).

1.2.3.1 Regulation of Acid Secretion

Acid secretion *in vivo* is regulated by the continuous interaction of numerous mechanisms acting at the central, peripheral and cellular levels (reviewed by Soll & Walsh (1979), Hirschowitz (1989), Schubert (1991) and Hersey & Sachs (1995)), analogous to the control of gastrin secretion (See Section 1.2.1.2). In relation to feeding, there are cephalic, gastric and intestinal phases, gastric secretion being increased by psychic stimuli or food in the stomach and being inhibited by efflux of contents into the duodenum and negative feedback from secreted acid. The peripheral control mechanisms acting in the stomach can be neural, hormonal, paracrine or chemical. These are rather artificial distinctions in many instances, as some peripheral local regulatory mechanisms, such as distension-induced acid secretion, involve central reflexes (Richardson *et al.*, 1976; Soares *et al.*, 1977; Schiller *et al.*, 1980) and chemical stimuli may act through neural elements (Ramos *et al.*, 1992).

Gastric acid secretion increases during a meal (Fordtran & Walsh, 1973; Richardson *et al.*, 1976), but is more continuous in ruminants than in monogastric species (Hill, 1955, 1960; McLeay & Titchen, 1970, 1974). The sight, smell, taste or thought of food are central stimuli for gastric acid secretion involving the hypothalamus and medulla oblongata (Hersey & Sachs, 1995; Wank, 1999). The presence of food in the stomach maintains acid secretion. Gastric distension acts through vago-vagal reflexes (Soares *et al.*, 1977; Hirschowitz, 1989), while luminal chemicals initiate local reflexes and release of hormones and paracrine agents (reviewed by Schubert (1998) and Dockray (1999)).

The most powerful stimuli of acid secretion are gastrin, histamine and ACh, which have interdependent and potentiating effects on the parietal cells (reviewed by Hersey & Sachs (1995)). Acid secretion is chiefly inhibited by SS, a paracrine agent which is secreted by fundic D cells (Chew, 1983; Schubert, 1998; Dockray, 1999). The principal effector agents on the parietal cell are

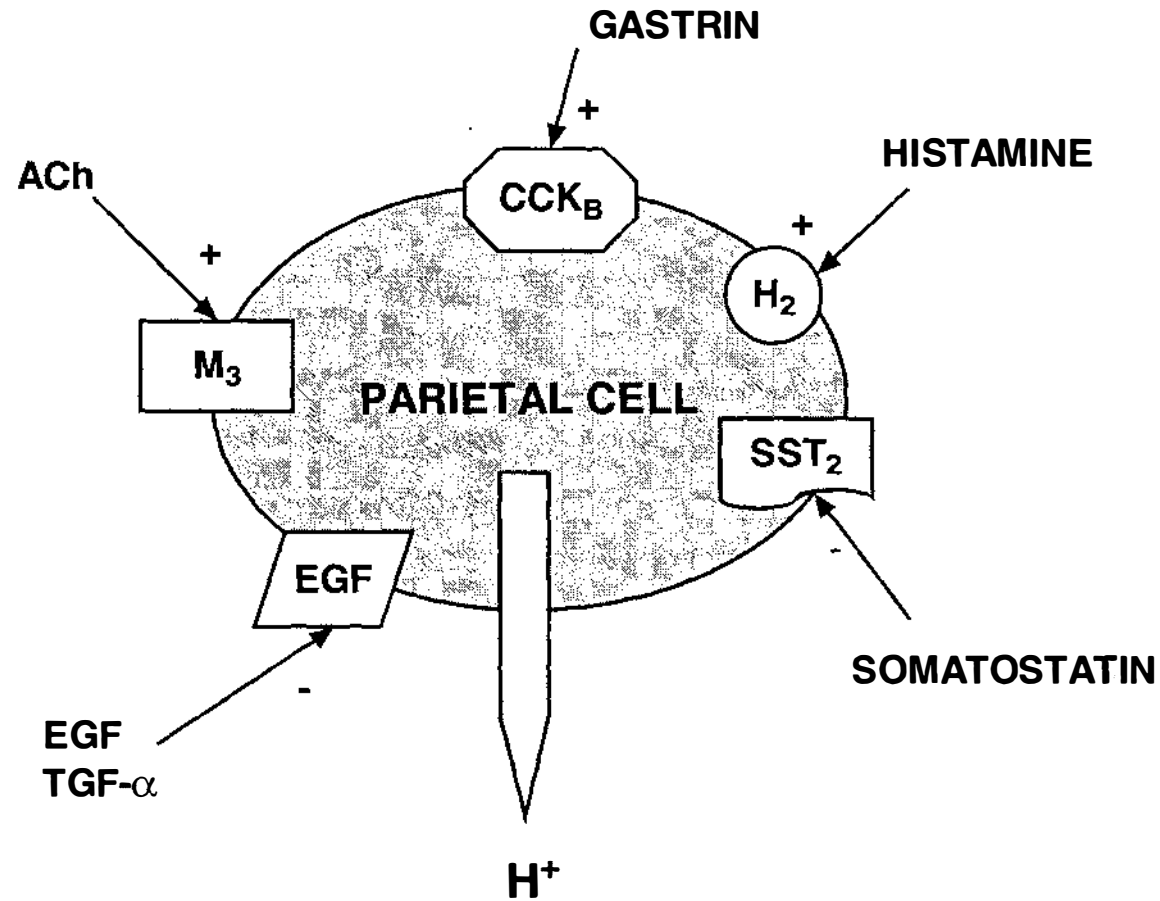


Figure 1.6. Regulation of acid secretion by the parietal cell. The receptor sub-types which have been identified on the ECL cell are shown. Ach: acetylcholine; EGF: epidermal growth factor; TGF- α : transforming growth factor- α ; CCK: cholecystokinin

shown in Figure 1.6.

1.2.3.2.1 Acetylcholine

Cholinergic afferent vagal pathways to the stomach mediate the effects of distension (reviewed by Hirschowitz (1989)) and some local chemical stimuli (reviewed by Cervero (1994)). The ACh released by the postganglionic fibres of the enteric nervous system then acts directly on parietal cells, or indirectly through the release of gastrin from G cells and histamine from ECL cells or by inhibiting the release of SS from D cells (reviewed by Hersey & Sachs (1995)). Although muscarinic M_3 receptors have been identified on the parietal cell (Pfeiffer *et al.*, 1990; Wilkes *et al.*, 1991; Kajimura *et al.*, 1992), there is still considerable debate whether cholinergic stimulation of acid secretion is mediated directly through these receptors or indirectly by releasing histamine from the ECL cell (Urushidani & Forte, 1997) in the mainly *in vitro* experiments which address this issue.

M_3 -receptors bind to G proteins and activate phospholipase C (PLC), which in turn produces inositol 1,4,5-triphosphate (IP_3) (Pfeiffer *et al.*, 1990) and diacylglycerol (DAG). IP_3 is responsible for releasing intracellular Ca^{2+} ($[Ca^{2+}]_i$) from stores, while DAG triggers protein kinase C (PKC) (Urushidani & Forte, 1997). Carbachol, an ACh agonist, also increased the influx of extracellular Ca^{2+} into rabbit parietal cells and glands (Negulescu & Machen, 1988a, b, 1993; Negulescu *et al.*, 1989).

1.2.3.2.2 Gastrin

Gastrin, the principal stimulant of acid secretion during a meal (Blair *et al.*, 1987; Kovacs *et al.*, 1989), acts mainly by increasing histamine release from ECL cells and, to a lesser extent, directly stimulates parietal cells (reviewed by Schubert (1994, 1997, 1998)). Binding studies and cloning and characterisation of the gastrin receptors identified them as CCK_B receptors (Soll *et al.*, 1984; Kopin *et al.*, 1992; Zavros & Shulkes, 1997).

The intracellular signalling pathway activated by gastrin is coupling of CCK_B receptors to G_q proteins producing IP_3 and DAG, the former releasing Ca^{2+} from intracellular stores and the latter activating PKC (Urushidani & Forte,

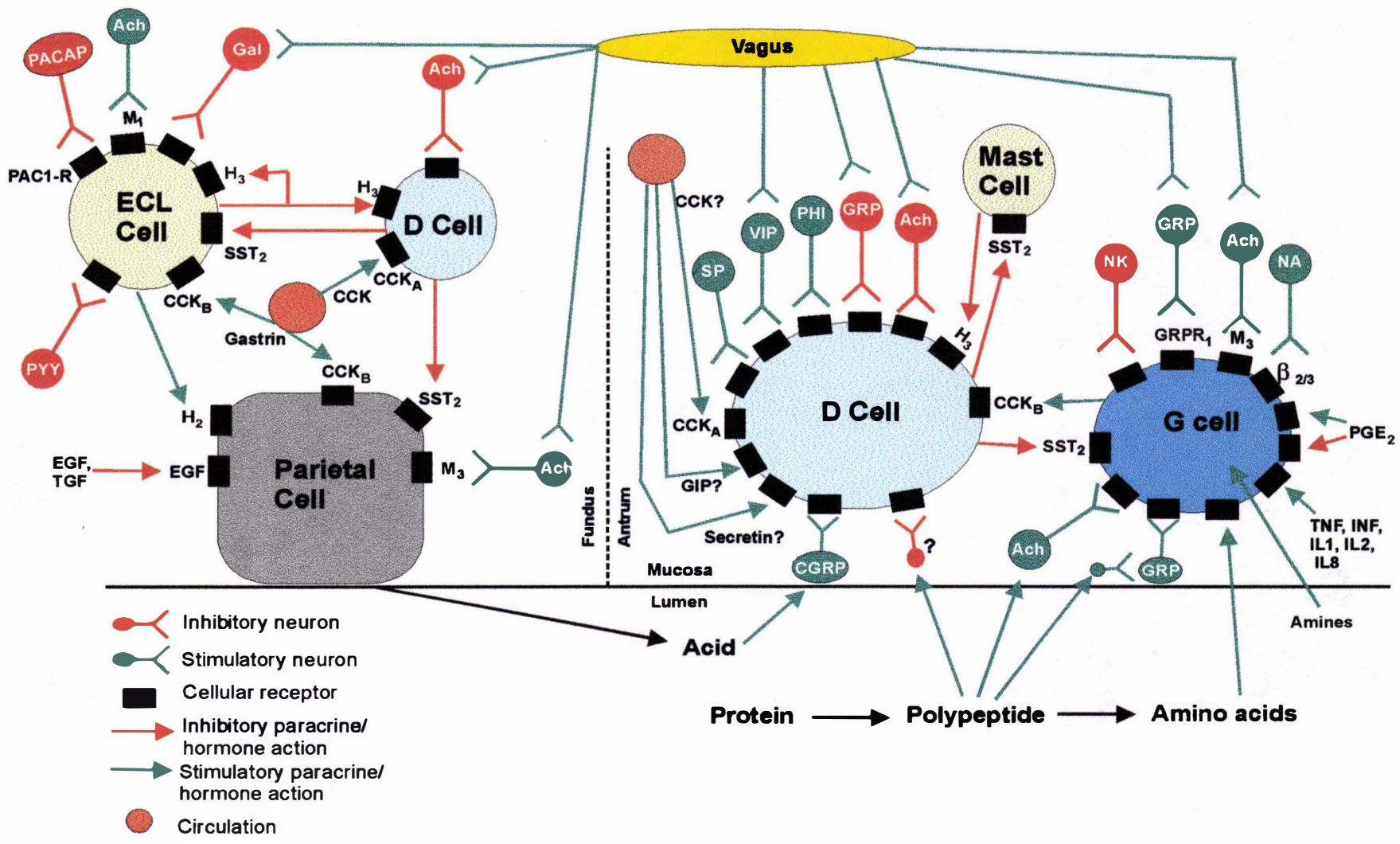


Figure 1.7. Overview of the cellular control of acid secretion. The factors controlling acid and gastrin secretion are shown. All abbreviations are contained in the List of Abbreviations. Modified after Simcock (2000).

1997; Wank, 1998). Gastrin potentiates the action of histamine and ACh, both of which act via the cAMP pathway (Soll, 1978b, 1980, 1982; Chew & Hersey, 1982). Li & Mardh (1996) postulated that gastrin augments the cAMP pathway by increasing intracellular Ca^{2+} levels or may even require the presence of intracellular cAMP to be effective (Geibel *et al.*, 1995; Li *et al.*, 1995b).

1.2.3.1.3 Histamine

Histamine is a potent stimulant of acid secretion both *in vivo* and *in vitro*. A variety of indices of *in vitro* acid secretion have been used to establish histamine as a stimulant in dispersed cells enriched in parietal cells (Soll, 1978a, b), rodent glands (Berglindeh *et al.*, 1976; Berglindeh, 1977; Chew *et al.*, 1980; Chew, 1986; Merkelbach *et al.*, 2002) or parietal cells of canine or rodent origin (Wollin *et al.*, 1979; Chew *et al.*, 1980, 1989; Soll, 1980; Sandvik *et al.*, 2001). Histamine-stimulated acid secretion has been successfully blocked by specific H_2 blocking agents in a variety of species *in vitro* (Soll, 1978b; Soll & Wollin, 1979; Gespach *et al.*, 1982), as well as *in vivo* (Black *et al.*, 1972; Hirschowitz & Molina, 1983; Scott *et al.*, 1993; Xue *et al.*, 2001). Acid secretion has been stimulated by histamine and blocked by ranitidine in both non-parasitised (Grabau *et al.*, 1999) and parasitised sheep (Hertzberg *et al.*, 2000). However, acutely isolated gastric glands from sheep responded weakly to histamine (Merkelbach *et al.*, 2002), while bovine glands did not respond at all (McKellar *et al.*, 1990a).

The H_2 receptor is a G protein-coupled receptor which activates adenylate cyclase leading to the production of PKA via cAMP (Hersey & Sachs, 1995; Urushidani & Forte, 1997). Histamine also raised $[\text{Ca}^{2+}]_i$ levels in rabbit gastric glands (Chew, 1986; Chew & Brown, 1986; Negulescu & Machen, 1988a; Negulescu *et al.*, 1989; Athmann *et al.*, 2000), suggesting that histamine is a full agonist, at least in rabbit parietal cells (Urushidani & Forte, 1997).

The cellular controls of acid, histamine and gastrin secretion by the parietal, ECL and G cell respectively are shown in Figure 1.7.

1.2.3.2 Ultrastructure and the Secretory Process

The characteristic feature of the resting mammalian parietal cell is the small content of secretory canaliculi, but large number of tubulovesicles (Helander & Hirschowitz, 1972; Ito & Schofield, 1974; Forte *et al.*, 1981), which contain the proton pump (Forte & Lee, 1977; Smolka *et al.*, 1983; Urushidani & Forte, 1987). The apical membrane, which has short microvilli, has distinctive invaginations from the lumen into the cell to form the secretory canaliculi (Helander & Hirschowitz, 1974; Ito & Schofield, 1974; Forte *et al.*, 1981), which are lined by polymerized actin microfilaments (Black *et al.*, 1982; Forte *et al.*, 1998; Agnew *et al.*, 1999). The mitochondria are large and abundant (Fryklund *et al.*, 1990; Jiang *et al.*, 2002), indicating a high demand for oxidative energy to secrete acid (Berglindh *et al.*, 1976).

When stimulated, parietal cells undergo dramatic morphological changes, which involve enormous cytoskeletal changes to reach a secreting state. The apical plasma membrane expands, microvilli are elongated and large canalicular spaces develop, whereas the number of tubulovesicles declines (Helander & Hirschowitz, 1972; Ito & Schofield, 1974; Forte *et al.*, 1981; Vial *et al.*, 1985; Ogata & Yamasaki, 2000). Immunostaining located the pump in the resting cell within the tubulovesicles and during secretion in the vesicles of the apical membrane (Smolka *et al.*, 1983; Urushidani & Forte, 1987).

The characteristic morphological changes associated with parietal cell stimulation and the re-arrangement back to the resting stage led to two hypotheses of secretion. Forte & Lee (1977) and Forte & Yao (1996) proposed the membrane recycling hypothesis: in an activated oxyntic cell the incorporation/fusion of H⁺-K⁺-ATPase rich tubulovesicles into/with the apical plasma membrane precedes proton secretion (Hanzel *et al.*, 1991; Scott *et al.*, 1993; Agnew *et al.*, 1999) and is accompanied by high conductance for K⁺ and Cl⁻ in the apical surface (Wolosin & Forte, 1981, 1984; Reenstra & Forte, 1990). Ezrin, a binding protein, may be a link (Okamoto & Forte, 2001) between the signalling pathway and phosphorylation-dependent modulation of the apical actin cytoskeleton (Urushidani *et al.*, 1989; Hanzel *et al.*, 1991). On removal of

the stimulus and in the following resting phase, gastric proton pumps are re-requested into tubulovesicles (Forte & Lee, 1977; Forte & Yao, 1996).

An alternative hypothesis is the osmotic flow and coiled tubule hypothesis, which either assumes either a network of tubules (Berglindh *et al.*, 1980) or coiled tubules (Pettitt *et al.*, 1995, 1996) of H⁺-K⁺-ATPase rich compartments contiguous with the apical membrane. In both hypotheses, osmotic expansion is considered to be the force behind the morphological transition of stimulated parietal cells. The results of Agnew *et al.* (1999) support the membrane-recycling hypothesis, as the proton pump was located on the apical membrane in stimulated parietal cells despite elimination of osmotic forces by pump inhibitors or protonophores.

1.3 Abomasal Parasitism

Abomasal nematodes have an adverse effect on host growth and production through impaired metabolism and inducing a negative nitrogen balance (Parkins *et al.*, 1973; Coop & Kyriazakis, 1999). *H. contortus* has the additional effect of feeding on blood, with resulting anaemia (Rowe *et al.*, 1988; Le Jambre, 1995). The metabolic consequences of gastrointestinal parasitism result from anorexia, loss of protein into the gut lumen in leaked interstitial fluid, shed epithelial cells and mucus and the cost of tissue repair and mounting an immune response (Parkins & Holmes, 1989; Fox, 1993, 1997; Coop & Kyriazakis, 1999).

Within the infected abomasum, secretory activity is disrupted, gastritis is evident and there are changes in the cell composition of the glands. Developing abomasal nematodes cause nodules around infected glands, mucous cell hyperplasia, superficial epithelial damage. Eventually, the tissue is fully restored. More recent studies involving the transplantation of adult worms have demonstrated the importance of this stage of development in the disease (Anderson *et al.*, 1985; McKellar *et al.*, 1986, 1987; Lawton *et al.*, 1996; Simpson *et al.*, 1997; Scott *et al.*, 1998a, b, 2000). Major changes in abomasal secretion are associated with the presence of parasites in the abomasal lumen,

either adult worms or immature stages, depending on the length of the histotrophic phase. Acid secretion is reduced and there are increased serum gastrin and pepsinogen concentrations (Anderson *et al.*, 1965, 1976; Armour *et al.*, 1966; Jennings *et al.*, 1966; Ritchie *et al.*, 1966; Ross *et al.*, 1968; McLeay *et al.*, 1973).

1.3.1 Histopathology

Larvae enter the glands causing local lesions, the nodules. These have at their centre developing larvae in dilated glands, which are often lined by a flat epithelium containing few secretory cells. Surrounding glands show mucous cell hyperplasia with fewer parietal and chief cells (Armour *et al.*, 1966; Durham & Elliott, 1976; Elliott & Durham, 1976; Scott *et al.*, 1998a). Initially, the lesions are in a localised area where larvae are developing, but after the parasites emerge, there is more widespread mucosal hyperplasia, mucous cell hyperplasia and parietal cell loss in non-nodular areas (Ross *et al.*, 1968, 1969; Snider *et al.*, 1983; Scott *et al.*, 1998a, 2000). The depth and weight of the fundic mucosa both increase (Anderson *et al.*, 1988; Fox *et al.*, 1993; Scott *et al.*, 1998a), much of it due to the enlargement of the pits (Scott *et al.*, 1998a, c). The mucosa may slough, causing the nodules to be pitted. By Day 35 after *O. circumcincta* infection, the majority of worms have been lost and the mucosa has a more normal appearance (Armour *et al.*, 1966).

Similar generalised non-nodular histological changes are seen after adult *O. circumcincta* transfer (Scott *et al.*, 1998a, 2000) and in sheep infected with *H. contortus* (Scott *et al.*, 1998c). There is widespread loss of parietal cells as early as one day after transfer of adult *O. circumcincta* (Scott *et al.*, 2000) and parietal cell numbers can be halved after eight days (Scott *et al.*, 1998a). Along with the rapid decrease in abomasal acid secretion, the parietal cells develop dilated canaliculi and/or degenerative changes typical of necrosis (Scott *et al.*, 2000).

The accompanying inflammatory response is rapid, even in a primary infection, and some components occur more rapidly in previously exposed animals (Salman & Duncan, 1985; reviewed by Miller (1996) and Balic *et al.* (2000)).

Infiltration of neutrophils and eosinophils is followed by increasing numbers of lymphocytes, mast cells and later by the presence of globular leucocytes, the intraepithelial mast cells (Armour *et al.*, 1966; Scott *et al.*, 1998a, 2000). Neutrophils, lymphocytes and eosinophils also began accumulating 1-2 days after adult transfer and were present in large numbers after eight days (Scott *et al.*, 1998a, 2000).

The role of parietal cell dysfunction and altered expression of specific trophic agents in the development of mucous cell and foveolar (gastric pit) hyperplasia in gastric parasitism may be explained by studies of other gastropathies and from transgenic mice models. The trophic agents involved in maintaining normal gland architecture are discussed in Section 1.2.2.2. In summary, parietal cells are a source of TGF- α and other EGF peptides and are recognised as an organisational centre in the fundic mucosa, in some way controlling the maturation of zymogenic cells. Thus, parietal cell loss impairs chief cell maturation and causes expansion of the mucous neck cell population. Parietal cell dysfunction raises gastric pH, stimulating gastrin secretion, hence hyperplasia of the fundic mucosa, particularly of parietal and ECL cells.

The inhibition and loss of parietal cells may be an important trigger for mucous cell hyperplasia and loss of mature chief cells when immature mucous neck cells fail to mature because of the absence of the correct signals from parietal cells. Similar cell changes are seen in transgenic mice lacking parietal cells (Li *et al.*, 1995a, 1996; Canfield *et al.*, 1996; Karam *et al.*, 1997a; Schultheis *et al.*, 1998) and in isolated gastric glands incubated with ammonia, in which chief cells became apoptotic after the parietal cells were killed (Hagen *et al.*, 1997).

The role played by hypergastrinaemia in the morphological changes in parasitised tissues is still unclear, particularly its effect on parietal cell numbers. Gastrin stimulates ECL cell replication, so that ECL cell hyperplasia is a common feature of conditions in which serum gastrin levels are high (Tielemans *et al.*, 1989, 1990; Ryberg *et al.*, 1990a-c), although no significant change in ECL cell density was found in *O. circumcincta*-infected sheep by Scott *et al.* (1998b). Gastrin is a general trophic agent for the fundic mucosa (Johnson, 1977) and stimulates the migration of parietal cells, although it does

not alter their life span (Kirton *et al.*, 2002). Hypergastrinaemia very commonly occurs in infected sheep (Anderson *et al.*, 1976; Lawton *et al.*, 1996; Scott *et al.*, 1998b) and is a probable causative agent for the thicker mucosa and generation of new parietal cells in the isthmus during parasitism (Scott *et al.*, 1998a). Serum gastrin concentration was positively correlated with mucosal depth in areas with intact foveoli in calves infected with *O. ostertagi* and *T. axei* (Snider *et al.*, 1988).

In addition to gastrin, other trophic agents, particularly the EGF peptide family, are likely to be involved in the histopathological changes of abomasal parasitism. This is suggested by the role these peptides are believed to play in other similar gastropathies, such as Ménétrier's disease in humans. Snider *et al.* (1983) first pointed out the similarity of the histopathology of abomasal parasitism to that in Ménétrier's disease, gastritis in which there are giant folds made up of foveoli in which expression of TGF- α is increased (Dempsey *et al.*, 1992). Transgenic mice over-expressing TGF- α have an extreme pathology very similar to Ménétrier's disease, with giant foveoli, loss of mature parietal and chief cells, but not their precursors, dilated glands and mucous neck cells replacing chief cells (Takagi *et al.*, 1992, 1997; Sharp *et al.*, 1995). Increased TGF- α expression also accompanies the foveolar and mucous cell hyperplasia associated with mucosal healing after injury (Konturek *et al.*, 1997).

Gastrin increases the production of several EGF peptides (Tsutsui *et al.*, 1997; Miyazaki *et al.*, 1999; Wang *et al.*, 2000), excessive amounts of which inhibit the differentiation of parietal and mature chief cells (Sharp *et al.*, 1995) and promote mucous cell hyperplasia (Chen *et al.*, 1993; Rutten *et al.*, 1993). The gradual loss of parietal cells, pit hyperplasia and atrophy of the glands in older transgenic mice over-expressing gastrin (Wang *et al.*, 1999), has been attributed to the increased release of EGF peptides (Wang *et al.*, 2000). There may be a similar sequence of events in the parasitised abomasum: hypergastrinaemia and physical damage to the tissue may both upregulate TGF- α expression and promote foveolar hyperplasia and further inhibit parietal cells.

1.3.2 Pathophysiology

The length of the histotrophic phase determines when abomasal secretion begins to be disrupted, as luminal parasites, either adult worms or immature stages, appear to be responsible for the majority of the pathophysiological changes. These are first seen in sheep infected with *H. contortus* after 2-4 days (Simpson *et al.*, 1997), after 5-6 days with *O. circumcincta* (Anderson *et al.*, 1976, 1981; Lawton *et al.*, 1996) or 16 days in cattle infected with *O. ostertagi* (Jennings *et al.*, 1966; Fox *et al.*, 1987). Abomasal dysfunction was attributed to tissue damage during emergence (Jennings *et al.*, 1966) until it was shown that secretion is also affected when adult worms are directly transferred into recipient animals. After transplantation of adult *O. ostertagi* (McKellar *et al.*, 1986, 1987), *O. circumcincta* (Anderson *et al.*, 1985; Lawton *et al.*, 1996; Simpson *et al.*, 1999; Scott *et al.*, 1998a, b, 2000) or *H. contortus* (Simpson *et al.*, 1997), abomasal pH and serum gastrin and pepsinogen concentrations became elevated within a day, except in the calves of McKellar *et al.* (1987), in which only serum pepsinogen concentrations increased.

1.3.2.1 Hyperpepsinogenaemia

Pepsinogen is mainly released into the gastric lumen, though there are measurable concentrations in the serum of non-parasitised ruminants (Lawton *et al.*, 1996; Simpson *et al.*, 1997). The serum level may depend on the rate of pepsinogen secretion, as the serum pepsinogen concentration in humans is correlated with both the basal and stimulated rates of pepsinogen secretion (Waldum *et al.*, 1978).

Elevated serum pepsinogen levels are an indicator of gastritis in humans with *Helicobacter pylori* infection or peptic ulcers (Samloff *et al.*, 1975; El Nujumi *et al.*, 1992; Biasco *et al.*, 1993; Hunter *et al.*, 1993) and have been used to assess the severity and location of the inflammation, either in the fundus or antrum, according to the pepsinogen isoform in the blood (Bodger *et al.*, 2001). Very low serum pepsinogen levels are considered to be an indication of severe gastric atrophy (Borch *et al.*, 1989; Kekki *et al.*, 1991; Kokkola *et al.*, 2002). Hyperpepsinogenaemia in ruminants is most commonly caused by parasitic abomasal nematodes: in sheep by *O. circumcincta* (McLeay *et al.*, 1973;

Lawton *et al.*, 1996; Simcock *et al.*, 1999), *Ostertagia leptospicularis* (Hertzberg *et al.*, 1995, 2000) or *H. contortus* (Simpson *et al.*, 1997) and in cattle by *O. ostertagi* (Jennings *et al.*, 1966; Armour *et al.*, 1979; Berghen *et al.*, 1987; Fox *et al.*, 1987, 1988a, 1989b; Pitt *et al.*, 1988; McKellar *et al.*, 1987; Wiggin & Gibbs, 1987; Hilderson *et al.*, 1991). There are greater increases in serum pepsinogen concentration in immune animals, even if the adult worm burden is small (Yakoob *et al.*, 1983).

Hyperpepsinogenaemia may precede other pathophysiological changes (Fox *et al.*, 1987; Hilderson *et al.*, 1991; Hertzberg *et al.*, 1995), particularly after the transplantation of adult worms (McKellar *et al.*, 1987; Lawton *et al.*, 1996; Scott *et al.*, 2000), although in the majority of animals, these parameters increase simultaneously at the time of predicted emergence of parasites from the glands (Fox *et al.*, 1987, 1989b; Lawton *et al.*, 1996; Scott *et al.*, 2000). Serum pepsinogen concentrations peak and remain elevated while parasites are present in the abomasum (Armour *et al.*, 1966; Shoo & Wiseman, 1986; Fox *et al.*, 1987; Lawton *et al.*, 1996; Hertzberg *et al.*, 2000; Scott *et al.*, 2000). Several infected “low-responder” sheep have been reported, in which serum pepsinogen levels did not increase during infection (Coop *et al.*, 1977; Lawton *et al.*, 1996; Simpson *et al.*, 1997; Simcock *et al.*, 1999; Hertzberg *et al.*, 2000).

The widely accepted cause of increased serum pepsinogen levels is a “leaking” abomasal mucosa, which allows back-diffusion from the gastric lumen. Macromolecules are normally prevented from moving across epithelial sheets by *zonulae occludentes* (Farquhar & Palade, 1963), but this barrier breaks down during inflammation. At the beginning of the emergence of adult parasites in calves, Murray (1969) and Murray *et al.* (1970) observed the loss of tight junctions between surface epithelial cells and progressively increasing intercellular spaces containing an amorphous electron-dense, proteinaceous material, which they assumed to be pepsinogen. Baker *et al.* (1993) found an increased interstitial fluid-to-blood pepsinogen concentration gradient, which they believed to be sufficient to raise serum pepsinogen levels, but this was augmented by increased pepsinogen production as well as an increased epithelial and capillary permeability.

The greatly increased turnover of albumin and calculated leakage from the plasma also supported the leakage of pepsinogen (Holmes & MacLean, 1971). However, systemically administered horseradish peroxidase, which is similar in molecular weight to pepsinogen, but which may not necessarily be able to pass from the blood to the gastric lumen, did not appear in the gastric contents at the same time as serum pepsinogen levels were raised and chief cells were depleted of granules (Stringfellow & Madden, 1979). These authors therefore concluded that pepsinogen is directly released into the circulation by retrograde secretion and not taken up from the gastric contents through a damaged vascular system.

It has been suggested that, to provide the amounts of pepsinogen appearing in the circulation during abomasal parasitism, higher rates of pepsinogen secretion are needed, stimulated by the concurrent hypergastrinaemia (Fox *et al.*, 1989c). This was based on the treatment of parasite-naïve calves with omeprazole, which increased both serum pepsinogen and gastrin levels (Fox *et al.*, 1989c), although this result was not replicated in a later experiment in which there was no increase in blood pepsinogen (Fox *et al.*, 2002). Other experiments also do not show the necessity for a raised gastrin level for hyperpepsinogenaemia. When Scott *et al.* (2000) used omeprazole to suppress parietal cell activity in parasite-naïve lambs, serum pepsinogen levels did not rise above pre-treatment levels despite a distinct hypergastrinaemia. As well, elevated pepsinogen levels have been reported without a rise in serum gastrin in the serum of individual parasitised sheep (Simcock *et al.*, 1999) or calves infected with *O. ostertagi* (Xiao *et al.*, 1991). However, inflammatory cytokines have been shown to increase pepsinogen secretion *in vitro* (Fiorucci *et al.*, 1995a, b; Serrano *et al.*, 1997).

Excretory/secretory products (ES) have been suggested as an initiator of increased pepsinogen release by parasitised tissues. ES derived from adult *O. ostertagi* or *O. circumcincta* stimulated the release *in vitro* from dispersed bovine and ovine gastric glands respectively, whereas incubation with ES originating from L₃ was ineffective (McKellar *et al.*, 1990b). There was no evidence of cell damage. Later, Scott & McKellar (1998) confirmed that ES of

adult *O. circumcincta* stimulated pepsinogen release, but only by abomasal mucosal sheets from previously infected sheep. A parasympathomimetic agent was suggested, as atropine reduced the effect of the ES and also reduced plasma pepsinogen levels during infection with either adult worms or L₃ *O. circumcincta* (Mostofa & McKellar, 1989).

1.3.2.2 Hypoacidity

The inhibition of gastric acid secretion is believed to reduce pepsinogen activation (Horak *et al.*, 1965; Jennings *et al.*, 1966), increase gastrin secretion (Anderson *et al.*, 1976, 1981, 1985; Fox *et al.*, 1987, 1988a, b; Lawton *et al.*, 1996), enhance survival of greater numbers of microbes (Jennings *et al.*, 1966; Nicholls *et al.*, 1987; Simcock *et al.*, 1999) and have marked effects on tissue growth and differentiation in parasitised animals.

The presence of parasites in the abomasal lumen is associated with raised abomasal pH, which increases at the time of parasite emergence from the gastric glands: 2-4 days in sheep infected with larval *H. contortus* (Christie, 1970; Nicholls *et al.*, 1987, 1988; Simpson *et al.*, 1997); 5-6 days with *O. circumcincta* (Armour *et al.*, 1966; Anderson *et al.*, 1976, 1981; Lawton *et al.*, 1996), 15-17 days with *O. leptospicularis* (Hertzberg *et al.*, 1995, 1999b, 2000), or 16 days in cattle with *O. ostertagi* (Jennings *et al.*, 1966; Fox *et al.*, 1987). The increase in pH is even more rapid, within the first day, after transfer of adult *H. contortus* (Simpson *et al.*, 1997), *O. ostertagi* (McKellar *et al.*, 1986), or *O. circumcincta* (Anderson *et al.*, 1985; Lawton *et al.*, 1996; Simpson *et al.*, 1999; Scott *et al.*, 2000). Comparison of different studies indicates that the numbers of adult worms transplanted influences the magnitude of change in pH (Lawton *et al.*, 1996; Simpson *et al.*, 1999; Scott *et al.*, 2000). The low number of worms transplanted may, therefore, explain the unchanged pH in calves receiving only 9,000 adult *O. ostertagi* (McKellar *et al.*, 1987) and small increases in sheep given 9,000 *H. contortus* (Simpson *et al.*, 1997).

Parietal cells are lost (Scott *et al.*, 1998a, 2000), but those remaining appear capable of functioning despite being inhibited by the presence of the parasites. Even when abomasal pH is highest, in many animals, the pH of the contents can decrease during feeding (Lawton *et al.*, 1996) or after injection of histamine

(Hertzberg *et al.*, 1999b). There is also a very rapid drop in abomasal pH immediately after anthelmintic treatment and abomasal pH often decreases to below pre-infection levels, suggesting there is a large functional population of parietal cells which is inhibited by the parasites but capable of responding rapidly after parasite removal (Simpson *et al.*, 1997; Scott *et al.*, 2000).

There is usually at least partial recovery of acid secretion despite continued exposure to the parasites (Lawton *et al.*, 1996). After a single larval infection, abomasal pH usually peaks rapidly, before decreasing to near normal levels around patency (Lawton *et al.*, 1996). As transplantation of the worms from these sheep into other animals will inhibit acid secretion in the recipients, recovery of secretory function would appear to be a host adaptation: parietal cells may become refractory to the inhibitor and/or there may be increased generation of new parietal cells in the isthmus (Scott *et al.*, 1998a).

Parasite ES may be directly responsible for the parietal cell inhibition, as the presence of the worms is essential for acid inhibition. Both recovery following parasite removal and the rise in abomasal pH after adult worm transfer can be very rapid (Anderson *et al.*, 1985; Dakkak & Daoudi, 1985; Simpson *et al.*, 1997; Scott *et al.*, 2000). Two *in vivo* experiments more directly support a role for ES products: abomasal pH increased in sheep in which adult worms were confined in porous bags, although after the worms had died (by 16 hours) (Simpson *et al.*, 1999) and intramuscular administration of extracts of *O. ostertagi* inhibited acid secretion in rats (Eiler *et al.*, 1981).

Worm ES may directly inhibit acid secretion by parietal cells. Merkelbach *et al.* (2002) demonstrated that *H. contortus* ES reduced the uptake of histamine-stimulated ¹⁴C-aminopyrine by rabbit gastric glands *in vitro*. This is an indirect technique used to monitor acid secretion in isolated parietal cells and glands (Berglindeh *et al.*, 1976; Berglindeh, 1977; Chew *et al.*, 1980; Chew, 1986). Rikihisa & Hammerberg (1982) reported that *Ostertagia* ES reduced the oxygen consumption of rat gastric mucosal cells, also an indicator of gastric acid secretion (Berglindeh *et al.*, 1976). Parietal cell inhibition by ES may be indirect, by inhibiting histamine secretion by the ECL cells (Hertzberg *et al.*, 1999a). A preliminary report by these authors indicates that adult *H. contortus*

ES products inhibit both basal and gastrin-stimulated pancreatic secretion by cultured ECL cells.

Alternatively, worm chemicals could act by stimulating inflammation and release of cytokines such as interleukin (IL)-1 β and Tumour Necrosis Factor (TNF)- α , both of which are potent inhibitors of the parietal cell (Robert *et al.*, 1991; Beales & Calam, 1998) and IL-1 also of the ECL cell (Prinz *et al.*, 1997). The timing of the influx of inflammatory cells matches that of parietal cell inhibition: neutrophils, lymphocytes and eosinophils began accumulating 1-2 days after adult transfer and were present in large numbers after eight days (Scott *et al.*, 1998a, 2000).

1.3.2.3 Hypergastrinaemia

Increased blood gastrin levels are seen in ruminants grazing on pasture contaminated with mixed nematode species (Enterocasso *et al.*, 1986) or following experimental infections. Hypergastrinaemia occurs during primary, challenge or trickle infections of sheep with *O. circumcincta* (Anderson *et al.*, 1976, 1981, 1985, 1988; Lawton *et al.*, 1996; Scott *et al.*, 1998a, 2000; Simcock *et al.*, 1999), *H. contortus* (Blanchard & Wescott, 1985; Nicholls *et al.*, 1985, 1988; Simpson *et al.*, 1997) or *O. leptospicularis* (Hertzberg *et al.*, 1995, 2000) and in cattle after a single or trickle infection with *O. ostertagi* or *T. axei* (Fox *et al.*, 1987, 1988a, 1989a, 1993; Snider *et al.*, 1988; Xiao *et al.*, 1991, 1992; Purewal *et al.*, 1997). Hypergastrinaemia rapidly follows the transfer of adult *H. contortus* (Simpson *et al.*, 1997), *O. ostertagi* (McKellar *et al.*, 1987) or *O. circumcincta* (Anderson *et al.*, 1985; Lawton *et al.*, 1996; Simpson *et al.*, 1999; Scott *et al.*, 2000).

After single L₃ infections, blood gastrin levels increase when late larval stages or young adult worms first emerge into the abomasal lumen, coinciding with the rise in abomasal pH and, in most animals, also with the increase in serum pepsinogen concentration. Nevertheless, Simcock *et al.* (1999) and Hertzberg *et al.* (2000) both reported that there were sheep which had normal serum gastrin levels, despite marked abomasal hypoacidity, following larval infection. Within 24 hours of transplantation of adult worms, serum gastrin increased to

levels equivalent to those seen with adult emergence after larval infection (Lawton *et al.*, 1996; Simpson *et al.*, 1997; Scott *et al.*, 1998a, b).

Hypergastrinaemia may solely be caused by abomasal hypoacidity or, more likely, it is initially caused by hypoacidity and later sustained by inflammation and/or parasite ES. The initial increase in serum gastrin has been attributed to removal of acid feedback on the G cells, the natural brake on gastrin secretion (Becker *et al.*, 1973; Schubert *et al.*, 1988; Holst *et al.*, 1993) (Section 1.2.1.1), because serum gastrin often initially rises in parallel with abomasal pH (Fox *et al.*, 1988a, 1993; Nicholls *et al.*, 1988; Lawton *et al.*, 1996; Simpson *et al.*, 1997). In some studies involving cattle infected with adult *O. ostertagi* (McKellar *et al.*, 1987) or sheep infected with *H. contortus* L₃ (Nicholls *et al.*, 1985) or *O. circumcincta* (Anderson *et al.*, 1981, 1985), elevated serum gastrin levels were reported before any rise in abomasal pH could be detected. In general, this conclusion was reached when the threshold for hypoacidity was considered to be pH 4, far higher than a value two standard deviations above the mean pH for pre-infection values in most animals (Lawton *et al.*, 1996).

Serum gastrin levels usually peak during the course of a single infection and remain moderately elevated despite abomasal pH returning to near pre-infection levels (Lawton *et al.*, 1996; Simpson *et al.*, 1997; Simcock *et al.*, 1999). Other stimulants, such as inflammatory mediators or parasite ES, must be involved in sustaining these elevated serum gastrin levels or when there is no rise in abomasal pH (Anderson *et al.*, 1976, 1981, 1985; Scott *et al.*, 2000). Although there is no direct evidence that inflammation is responsible for causing hypergastrinaemia during abomasal parasitism, inflammatory mediators have been shown to increase gastrin secretion both *in vitro* and in human infections with *H. pylori*. In these patients, the G cell is more sensitive to its normal physiological stimulants, bombesin/GRP (Graham *et al.*, 1991; Beardshall *et al.*, 1992; Gibbons *et al.*, 1997) and gastric alkalinsation (Jensen *et al.*, 1987; El Nujumi *et al.*, 1998) and less sensitive to inhibition by acidification (Jensen *et al.*, 1987). Further, *in vitro* gastrin release by rabbit or canine antral G cells or human antral fragments or by the isolated rodent stomach was stimulated by histamine (Bado *et al.*, 1994), Interferon (IFN)- γ , IL-

2 (Lehmann *et al.*, 1996), IL-1 β (Weigert *et al.*, 1996) or TNF- α (Beales *et al.*, 1996, 1997a; Lehmann *et al.*, 1996; Weigert *et al.*, 1996).

Some cytokines may cause hypergastrinaemia by inhibiting SS release from D cells (Beales *et al.*, 1997b; Calam, 1998). Alternatively, hypergastrinaemia has been reported to cause a reduced number of D cells, which may maximise acid secretion by releasing the inhibition by SS of the G cell and parietal cell (Zavros *et al.*, 2002a-c). These authors observed that infection of mice with *H. pylori* or *Acinetobacter lwoffii* caused gastritis, increased serum gastrin concentrations and G cell numbers and decreased numbers of D cells (Zavros *et al.*, 2002a). Suppression of acid secretion by gastrin deficiency or omeprazole treatment caused bacterial overgrowth in the stomach, gastritis and hypergastrinaemia (Zavros *et al.*, 2002b, c) and reduced numbers of D cells, except in gastrin deficient mice (Zavros *et al.*, 2002c).

McLeay *et al.* (1973) and McKellar *et al.* (1987) suggested that abomasal parasites directly raise serum gastrin levels. This was not confirmed by *in vitro* studies in which ES derived from different developmental stages of *O. circumcincta* (Lawton *et al.*, 2002) or *H. contortus* (Haag, 1995) were tested on ovine antral fragments obtained from either parasite-naïve or previously infected sheep.

The above *in vitro* studies produced a novel finding that gastrin release *in vitro* was inhibited by both soluble products from abomasal microbes (Lawton, 1995; Simcock, 2000) and abomasal contents with a raised pH from parasitised sheep (Simcock, 2000). These microbial gastrin inhibitors may explain the observation of Lawton *et al.* (1996) that serum gastrin concentration and abomasal pH initially increased in parallel, but in some parasitised sheep, when abomasal pH increased above pH 5.5, serum gastrin levels returned to near basal levels, only to increase again when abomasal pH subsequently declined. Anderson *et al.* (1985) also noted unexplained decreases in serum gastrin concentration unrelated to abomasal pH. Simcock *et al.* (1999) monitored bacterial populations during parasitism and were unable to correlate serum gastrin levels with abomasal anaerobic bacterial numbers, although specific species of microbes, not the total population, may be critical. Tissue gastrin

depletion by hypersecretion is unlikely to be the cause in many cases because it occurs too rapidly and without prolonged hypergastrinaemia.

The reason for these abrupt decreases in serum gastrin in parasitised sheep remains unexplained, but could in some cases be caused by damage to G cells by the inflammatory reaction. This may explain why serum gastrin levels do not increase during parasitism in some animals. Physical damage to the G cells may be the reason for the absence of hypergastrinaemia in animals in which the majority of larvae were present in pyloric glands, resulting in massive antral thickening and inflammation (Simcock *et al.*, 1999).

Prolonged high rates of secretion of gastrin required to sustain the observed serum gastrin levels during abomasal parasitism of sheep and cattle (Fox *et al.*, 1987, 1988a; Nicholls *et al.*, 1988; Lawton *et al.*, 1996; Simpson *et al.*, 1997; Simcock *et al.*, 1999; Hertzberg *et al.*, 2000) are probably the prime reason for reduced tissue gastrin (Fox *et al.*, 1993; Purewal *et al.*, 1997; Scott *et al.*, 1998b), in spite of increased gastrin synthesis (Purewal *et al.*, 1997). However, physical damage to G cells due to larval development in pyloric glands (Simcock *et al.*, 1999) or inflammation cannot be ruled out as contributing factors. Calves exposed to *O. ostertagi* for 28 days after larval infection (Fox *et al.*, 1993; Purewal *et al.*, 1997) and sheep for 8 days after adult transplantation of *O. circumcincta* (Scott *et al.*, 1998b) both had a diminished number of G cells, which stained weakly with gastrin antiserum. In these sheep, there were also fewer antral D cells, which stained weakly with SS antiserum (Scott *et al.*, 1998b), consistent with the reduced synthesis of SS when the gastric pH is elevated (Brand & Stone, 1988).

1.3.2.4 Parasite Excretory/Secretory Products

Chemicals, collectively called ES, which are released by abomasal nematodes into their environment, include chemotaxins, metabolic end-products, enzymes, immunomodulators and growth factors. The proposed roles of ES in abomasal pathophysiology have been discussed above: a parietal cell inhibitor (Merkelbach *et al.*, 2002), an inhibitor of the ECL cell (Hertzberg *et al.*, 1999a), a stimulant of pepsinogen release from previously exposed tissue (Scott & McKellar, 1998), but no gastrin stimulant (Haag, 1995; Lawton *et al.*, 2002).

Excreted metabolites include free fatty acids (Bryant, 1993), propan-1-ol, acetate, propionate, small amounts of ethanol, lactate and succinate (Ward, 1974; Ward & Huskisson, 1978; Ward *et al.*, 1981), polar and non-polar lipids (Kapur & Sood, 1991) and ammonia (Barrett, 1981). Of these, ammonia is potentially involved in the pathophysiology of the parietal cell because of its known cytotoxicity and ability to reduce ¹⁴C-aminopyrine accumulation (Hagen *et al.*, 1997), although generally in higher concentrations than measured in *H. contortus* ES by Merkelbach *et al.* (2002). Possible cellular effects of other worm excretory substances appear not to have been examined.

Enzymes reported in the ES of a range of gastrointestinal helminths include acetylcholinesterase (Lee & Hodsden, 1963; Ogilvie *et al.*, 1973; Knox & Jones, 1990; Griffiths & Pritchard, 1994; Huby *et al.*, 1999a), cysteine, aspartic and metalloproteases (Karanu *et al.*, 1993), elastase (Knox & Jones, 1990), N-acetyl- α -D-glucosaminidase and an acid phosphohydrolase (Gamble & Mansfield, 1996). Acetylcholinesterase is higher in the ES of adult than larval stages (Knox & Jones, 1990) and has been suggested to prevent peristalsis and parasite expulsion (Opperman & Chang, 1992), inhibit mucus secretion (Philipp, 1984) or alter the immune response to the parasite (Rhoads, 1984). ES proteolytic enzymes may aid penetration of host tissues (Matthews, 1977), act as an anticoagulant (Hotez & Cerami, 1983; Knox & Jones, 1990), degrade protein for ingestion (von Brand, 1974) or inactivate complement and cytokines (Leid *et al.*, 1987).

Schallig *et al.* (1994) demonstrated in the ES of adult *H. contortus* at least 15 polypeptides, with molecular weights ranging from 10 to >100 kDa, which induced an immune response in infected sheep, as demonstrated by specific immunoglobulin G (IgG) levels and lymphocyte proliferation. Other ES components may be involved in immunomodulation (Lightowers & Rickard, 1988; Klesius, 1993) and stimulation of cell proliferation (Huby *et al.*, 1995, 1999b).

Parasite ES chemotaxins may actively recruit granulocytes. Eosinophil chemotactic factors have been found in *O. ostertagi* (Klesius *et al.*, 1986) and *Anisakis* larvae (Tanaka & Torisu, 1978), *Ascaris* adults (Tanaka *et al.*, 1979),

Shistosoma japonicum eggs (Owhashi & Ishii, 1982) and adults (Horii *et al.*, 1984), *Fasciola* spp. (Horii *et al.*, 1986), *Taenia taeniaeformis* metacestodes (Potter & Leid, 1986), recombinant epitopes from *Dirofilaria immitis* (Owhashi *et al.*, 1996) and *Hymenolepis nana* cysticercoids (Niwa *et al.*, 1998). Neutrophil chemotaxins have been reported from *Ascaris* adults (Tanaka *et al.*, 1979), recombinant epitopes from *Dirofilaria immitis* (Owhashi *et al.*, 1996) and from *F. hepatica* (Jefferies *et al.*, 1996), *S. japonicum* (Horii *et al.*, 1984) and *Onchocerca volvulus* adult parasites (Rubio de Krömer *et al.*, 1998). Abomasal nematodes may deliberately, or inadvertently, use recruited granulocytes to damage parietal cells, reduce acid secretion and thus prevent pepsinogen being activated.

1.3.2.5 Metabolic Disturbances

The effects of gastrointestinal parasitism on the metabolism of the host animal include reduced intake of nutrients, due to loss of appetite, increased protein loss across the gut wall in secreted mucus, through leakage across the inflamed mucosa, and increased shedding of epithelial cells and the increased requirements to repair the tissues and to mount an immune response (Parkins & Holmes, 1989; Fox, 1993; Coop & Kyriazakis, 1999). Of these, reduced food intake is considered to be one of the most detrimental effects of gastrointestinal parasitism (Symons, 1985; Coop & Kyriazakis, 1999).

1.3.2.5.1 Loss of Appetite

Reduced appetite or anorexia is a prominent feature of exposure of humans and animals to a diverse range of pathogens (reviewed by Plata-Salamán (1998) and McCarthy (2000)). In ruminants, such pathogens can be viral (Davies, 1991; Odeon *et al.*, 1999), bacterial (West, 1997; Soliman *et al.*, 2001) or parasites which infest the skin (Corba *et al.*, 1995), other organs (Boon *et al.*, 1984; Ferre *et al.*, 1994; West, 1997) or cells (Fitzgerald, 1980; Dausgchies *et al.*, 1998). Given the diversity of pathogens and the range of organs they infect, there are likely to be universal mechanisms of anorexia, rather than organ- and pathogen-specific processes. The cytokines, IFN- γ , IL-1 IL-2 and TNF- α , which consistently cause anorexia in both experimental manipulations and in clinical cases when given centrally or peripherally and also induce the

release of endogenous cytokines, are likely candidates (reviewed by Plata-Salamán (1998)). Kyriazakis *et al.* (1998) have suggested that anorexia should be viewed as a disease-coping mechanism, akin to fever during bacterial and viral diseases, and to look for functional explanations for its occurrence.

There are numerous reports of reduced food intake in ruminants infected with abomasal or intestinal nematodes: in calves infected with *O. ostertagi* (Fox *et al.*, 1987, 1989a) and in sheep infected with *O. circumcincta* (Horak & Clark, 1964; Sykes & Coop, 1977; Simcock *et al.*, 1999), *Trichostrongylus colubriformis* (Kimambo *et al.*, 1988; Dynes *et al.*, 1994; van Houtert *et al.*, 1995; Kyriazakis *et al.*, 1996), *H. contortus* (Pradhan & Johnstone, 1972; Abbott *et al.*, 1986) or with a mixed infection (Bown *et al.*, 1991; Knox & Steel, 1999). Individual animals are not affected equally by an experimental infection, some not at all (Fox *et al.*, 2001).

Hoste (2001) believed that the extent of inappetence was in part related to the level of infection. A threshold for the development of anorexia was determined by the size of the weekly dose of *T. colubriformis* L₃ in weaners lambs (Steel *et al.*, 1980). Such a threshold was indirectly confirmed by Coop *et al.* (1977, 1985) and Symons *et al.* (1981), who noted a threshold level for demonstrable effects on live-weight gain of weekly doses of *O. circumcincta* L₃.

Many local and systemic mechanisms have been proposed to explain the anorexia of parasitism. A direct effect of the parasites on the motility of the gastrointestinal tract is one such mechanism specific for gastrointestinal parasites. Feed intake is depressed in ruminants by a reduced flow of digesta, abnormal gut motility or distension of the reticulo-rumen, abomasum or intestine (Grofum & Phillips, 1978; Grofum, 1979). Reduced duodenal bulb motility and decreased abomasal emptying have been described in lambs infected with *T. axei* (Bueno *et al.*, 1975), reduced abomasal motility in lambs infected with *H. contortus* (Bueno *et al.*, 1982) and slowed flow of digesta and decreased abomasal volume in parasitised sheep (Rowe *et al.*, 1988; Dynes *et al.*, 1994) and calves (Fox *et al.*, 1989a). The mediator may be gastrin, as raised circulating secretin, gastrin and CCK levels lower feed intake in

ruminants (Grofum, 1981) and gastrin inhibits reticulo-ruminal motility (Carr *et al.*, 1970) and delays gastric emptying (Bell *et al.*, 1977).

Elevated serum gastrin levels were strongly correlated with depressed food intake in calves which had been trickle infected with *O. ostertagi* (Fox *et al.*, 1989a, b) and in parasite-free calves treated with the acid inhibitor, omeprazole (Fox *et al.*, 1989c). This was a direct effect of hypergastrinaemia, as there was no effect on food intake of omeprazole in parasitised calves (Fox *et al.*, 2002). Hypergastrinaemia is also responsible for the anorexia during omeprazole treatment in chickens as a gastrin-inhibitor prevented the loss of appetite during omeprazole treatment (Campbell *et al.*, 1991). Hypergastrinaemia may also be involved in central satiety signalling (Dynes *et al.*, 1998), as the central blockade of CCK_A receptors increased food intake in lambs infected with *T. colubriformis*. However, gastrin binds poorly to CCK_A receptors and peripheral blockade of CCK_B receptors failed to prevent anorexia in infected lambs (Dynes *et al.*, 1998). In addition, the loss of appetite in sheep infected with *O. circumcincta* occurred independently of elevated serum gastrin (Simcock *et al.*, 1999; Fox *et al.*, 2001).

More universal mediators of anorexia in parasitism or infections in which there is no hypergastrinaemia e.g. in intestinal parasitism, may be pain or other sensory input from the gut, central satiety signals or inflammatory cytokines. There is no clear mediator of anorexia in parasitised sheep at this stage. Fox *et al.* (2001) could find no evidence in individual sheep infected with *O. circumcincta* that anorexia was linked to changes in either serum gastrin or leptin concentration, or was mediated by gastric nerves, but did not examine the role for inflammatory mediators.

The peptide hormone leptin is a regulator of appetite, interacting at the hypothalamic centres with insulin, glucocorticoids and signals from the stomach (reviewed by Jeanrenaud & Rohner-Jeanrenaud (2001)). Leptin is secreted by adipocytes, the placenta and gastric epithelial cells and is important in maintaining energy balance and reproduction, as well as in appetite control (Bado *et al.*, 1998; Keisler *et al.*, 1999). Leptin can induce anorexia and loss of body fat in lean and genetically obese mice (Pelleymounter *et al.*, 1995; Weigle

et al., 1995) and down-regulates the expression of neuropeptide Y, a potent stimulant of appetite during negative energy balance (Leibowitz, 1991; White, 1993) which acts at the hypothalamus (Schwartz *et al.*, 1998; Henry *et al.*, 1999).

Leptin was implicated in the suppression of neuropeptide Y in anorexic rats infected with *Nippostrongylus brasiliensis*, as leptin gene-expression was upregulated, although corticotropin-releasing factor and cytokines may also have played a part (Horbury *et al.*, 1995). The same laboratory (Roberts *et al.*, 1999), however, questioned the involvement of leptin in the early phase of anorexia in *N. brasiliensis*-infected obese Zucker rats, in which the leptin receptor is defective (Tartaglia, 1997), as they still were anorexic and had hyperleptinaemia (Hardie *et al.*, 1996).

Cytokines and hypergastrinaemia may both suppress appetite through the release of leptin. Adipocytes express the CCK_B receptor (Attoub *et al.*, 1999) and are likely to be responsive to gastrin. TNF- α depresses appetite in humans (Holden & Pakula, 1996) and Grunfeld *et al.* (1996) observed in hamsters an upregulation of the leptin gene in adipose tissue in response to TNF- α and IL-1, which both, as well as IL-6, depress food intake in laboratory animals (reviewed by Plata-Salamán (1998) and McCarthy (2000)). However, leptin was not the cause of anorexia in sheep suffering from acute endotoxaemia (Soliman *et al.*, 2001).

1.3.2.5.2 Metabolism and Production

Gastrointestinal nematodes impair growth and the production of milk and wool (Poppi *et al.*, 1990; Holmes, 1993; Sykes, 1994; Van Houtert & Sykes, 1996). Reduced feed intake is considered the major factor contributing to impaired nutrition in sheep infected with abomasal parasites (Sykes & Coop, 1977; Fox *et al.*, 1989a), whereas reduced efficiency of nutrient utilisation is important in intestinal infections (Sykes & Coop, 1976). Generally, protein lost into the abomasal contents can be recovered in the small intestine (Rowe *et al.*, 1988), but mixed infections can have more severe effects, as there is less opportunity for compensatory absorption distal to the affected part of the gut.

A negative nitrogen balance is caused more by endogenous loss of protein into the digestive tract than through general malabsorption (Symons & Jones, 1970; Poppi *et al.*, 1986; Rowe *et al.*, 1988; Bown *et al.*, 1991). The reduced retention of protein in sheep with either an intestinal or mixed abomasal and intestinal nematode infection is due to increased protein N at the ileum (4-5 grams/day), much of which is subsequently fermented in the hind-gut, absorbed as ammonia and excreted as urea (Poppi *et al.*, 1986; Kimambo *et al.*, 1988; Rowe *et al.*, 1988; Bown *et al.*, 1991). Sources of ileal protein are mucosal leakage of plasma protein (Parkins & Holmes, 1989), sloughed epithelial cells and an intensified mucoprotein production, caused by local pathological changes (Poppi *et al.*, 1986; Bown *et al.*, 1991).

Increased protein synthesis in the parasitised gastrointestinal tract has a considerable energy cost (Sykes & Coop, 1976, 1977). In *T. colubriformis*-infected guinea pigs, protein synthesis was enhanced by 24% in the small intestine and by 70% in large intestine (Symons & Jones, 1983). The normal contribution of the gastrointestinal tract to whole body protein synthesis is 32-45% in cattle (Lobley *et al.*, 1980) and sheep (MacRae, 1993), and it has been calculated that an extra 50 grams of protein may be required in parasitised sheep (Coop & Kyriazakis, 1999).

Parasitism creates deficiencies of specific amino acids for peripheral tissues. Yu *et al.* (2000) studied leucine fluxes in lambs subclinically trickle-infected with *T. colubriformis* and found a 30% reduction in the amount of leucine available to peripheral tissues because of increased removal by the gastrointestinal tract. Wool production, which needs large amounts of sulphur-containing amino acids, particularly cysteine, can be severely reduced by parasitism (Barger, 1973; Barger *et al.*, 1973; Barger & Southcott, 1975; reviewed by Donald (1979)). This is consistent with observed effects of nutrition on wool growth, which showed that protein supply was more important than energy supply (reviewed by Black (1987) and Reis *et al.* (1992)).

1.4 Host Factors in Abomasal Parasitism

Individual host animals harbour different worm burdens, reject worms at different rates, modulate the fecundity of the worms and vary in the deleterious effects of parasitism they suffer. Some are able to maintain productivity during parasitism and are classed as "resilient" (Riffkin & Dobson, 1979; Albers *et al.*, 1987; Bisset & Morris, 1996; Bisset *et al.*, 2001), while others harbour fewer worms and in general have lower FEC ("resistant"). Animals can therefore be considered as resistant, susceptible or resilient to a parasite infection. Underlying susceptibility/resistance to parasitism is the immune status of the animal, which can be affected by age, reproductive state, nutritional or environmental stress, artificial suppression or genetic background. Whether the immune response is primarily innate or adaptive will depend on previous interactions with the nematode (or antigen).

1.4.1 Age and Nutritional Status

Susceptibility to parasitism changes during the lifetime of the animal. Milk-fed lambs (Zeng *et al.*, 2001) and calves (Satrija *et al.*, 1991) usually have low gastrointestinal parasite burdens, which may in part be due to maternal antibodies ingested in colostrum and milk. Passive immunisation in lambs has been demonstrated against infection by bacteria (Mukkur *et al.*, 1998) and helminths (Andrews *et al.*, 1995; Dempster *et al.*, 1995). Neonatal lambs are able to mount protective immune responses to bacterial and viral antigens (Soulsby *et al.*, 1981), though ruminants younger than six months display a lower resistance to bacterial (Weiss *et al.*, 1986) or helminth infection (Smith *et al.*, 1985; Watson *et al.*, 1994). Immunity develops in older animals and is influenced by sex, males being more susceptible than females (Barger, 1993), and by the nutritional status (van Houtert *et al.*, 1995; Datta *et al.*, 1999; Kahn *et al.*, 2000; Sykes & Coop, 2001). During the periparturient period, parasite immunity may be reduced in ewes, manifest as higher FEC (O'Sullivan & Donald, 1970; Coop *et al.*, 1990) and can be moderated by increased dietary protein intake and body protein reserves (Donaldson *et al.*, 1998; Houdijk *et al.*, 2001).

The effects of gastrointestinal nematodes on nitrogen metabolism have been discussed in Section 1.3.2.5.2. The competition for available nitrogen for tissue repair in the face of increased losses in the gut, growth, reproduction and acquisition and expression of immunity has been put into a partitioning framework, which highlights changing priorities at different times in the animal's lifetime (Coop & Kyriazakis, 1999). Manipulation of host nutrition, especially by increasing protein intake, will improve resistance or resilience (Bown *et al.*, 1991; Coop *et al.*, 1995; van Houtert *et al.*, 1995; Stear *et al.*, 2000), but not necessarily have the same effect in young and old animals with different metabolic priorities (Kambara *et al.*, 1993). Increased protein intake enhances immunity to parasites in sheep (Shaw *et al.*, 1995) and mice (Ing *et al.*, 2000), expressed as greater concentrations of mast cell proteases and increased numbers of blood eosinophils, although specific or non-specific antibody levels may not be raised (van Houtert *et al.*, 1995).

1.4.1 Innate and Acquired Immunity

Immunity to larvae results in their failure to establish or in arrested development (hypobiosis), whereas immunity to adult worms manifests itself as expulsion of the worms or altered worm morphology, smaller size or decreased fecundity. A protective immune response requires repeated infections, varies with the species of nematode and is partly age-dependent (reviewed by Miller (1984), Smith (1988), Watson & Gill (1991) and Balic *et al.* (2000)).

The innate immune response to a primary infection is characterised by increased numbers of CD4⁺ cells (Kambara & McFarlane, 1996), lymphocytes, eosinophils and mucosal mast cells (MMC) in the gastrointestinal mucosa (Charleston, 1965; Armour *et al.*, 1966; Salman & Duncan, 1984; Pfeffer *et al.*, 1996). In most host-parasite systems studied, the ability to mount a T helper type 2 (Th2) response is linked to worm rejection (Nawa *et al.*, 1994; Else & Finkelman, 1998). In immune hosts, parasites induce greater MMC hyperplasia, the appearance of intraepithelial mast cells (globule leucocytes), eosinophilia, increased secretion of mucus containing antiparasitic substances and the production of parasite-specific antibodies, particularly IgE, IgG₁ and mucosal IgA (reviewed by Smith (1988), Meeusen (1999), Balic *et al.* (2000),

Gasbarre *et al.* (2001) and MacDonald *et al.* (2002)).

It is still not clear which of these components of the immune response are responsible for worm rejection and which contribute to pathophysiological changes in the host. In many rodent models, but not all, MMC are crucial effector cells in expulsion of nematode parasites e.g. MMC are essential for expelling *Strongyloides ratti* (Abe & Nawa, 1988), *Strongyloides venezuelensis* (Crowle & Reed, 1981; Lantz *et al.*, 1998) and *Trichinella spiralis* (Urban *et al.*, 2000), but not *N. brasiliensis* (Crowle & Reed, 1981; Urban *et al.*, 2000) or *Trichuris muris* in mice (Madden *et al.*, 1991; Betts & Else, 1999; Koyama & Ito, 2000). In sheep, globule leucocytes are more strongly related to nematode expulsion than are MMC (Douch *et al.*, 1986; Stear *et al.*, 1995). Mast cells are a potent source of pro-inflammatory cytokines, including TNF- α , proteinases, cytokines, histamine, leukotrienes and prostaglandins which may contribute to the inflammatory process and tissue damage (Galli *et al.*, 1989).

Eosinophils contain potent chemicals, including eosinophil-specific peroxidase, major basic protein and lysosomal hydrolases and when activated can produce oxygen-derived metabolites, prostaglandins, leukotrienes and cytokines (reviewed by Meeusen (1999) and Balic *et al.* (2000)). They are therefore capable of inflicting damage on both parasites and host tissues. It is still uncertain whether eosinophils contribute to protection against gastrointestinal parasites, although they have antiparasitic activity *in vitro* (reviewed by Meeusen & Balic (2000)) and the greater eosinophilia in immune animals (Stevenson *et al.*, 1994) could contribute to worm rejection (Rainbird *et al.*, 1998; Meeusen, 1999). On the other hand, the enhanced eosinophilia in immune animals may contribute to tissue damage. Larsen *et al.* (1994) observed increased numbers of mucosal eosinophils in Merino sheep with diarrhoea but low worm burdens after grazing contaminated pasture.

The protective CD4⁺ lymphocyte response to gastrointestinal nematodes is a Th2 response in sheep (Gill *et al.*, 2000), but a more mixed Th0 type in cattle (Gasbarre *et al.*, 2001). The Th1 response, directed against intracellular pathogens, is distinguished from the Th2 type on function and on the cytokine profiles associated with it. Characteristic cytokines are IL-4 for the Th2 type

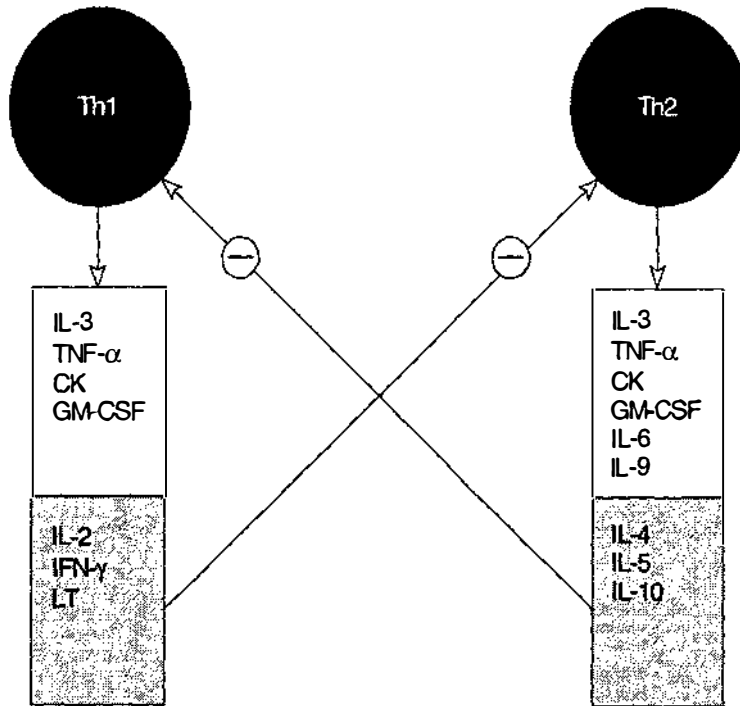


Figure 1.8. Principal cytokines secreted by Th1 and Th2 CD4⁺ cells. Those in the shaded boxes inhibit the proliferation of the other cell population CK: chemokines; GM-CSF: granulocyte-macrophage colony-stimulating factor; IFN- γ : interferon-gamma; IL: interleukin; LT: lymphotoxin; TNF: tumour necrosis factor. Adapted from Mosmann & Sad (1996).

and IFN- γ for Th1 (Figure 1.8). The mutually inhibitory Th1 and Th2 paradigm is an oversimplification, as in addition to these two, there are several sub-sets of regulatory T cells (T_r) cells, which secrete IL-10 and/or TGF- β (reviewed by McGuirk & Mills (2002)). T_r cells have an immunosuppressive role and may be involved in down-regulation of immunity by parasites to promote their survival.

Polarisation of CD4⁺ T cells towards either a Th1 or Th2 response is influenced by the dose of antigen and the duration of T cell receptor engagement, which collectively are called “strength of stimulation” (reviewed by Boyton & Altmann (2002)). In a Th2 environment, naïve T cells would be selected from the pool on the basis of low affinity receptors, as Th1 responses appear to require shorter T cell receptor activation times and higher affinity receptors than do Th2 responses. This may explain the different responses in hosts to single large doses of larvae or prolonged trickle infections with low numbers of larvae (Bancroft *et al.*, 2001).

1.4.3 Genetic Resistance, Resilience and Susceptibility

Resistance to parasitism is the ability of an animal to maintain a lower worm burden compared with other members of its flock or herd, when similarly challenged, and is the opposite of susceptibility (Gray, 1991). Other authors include in the definition of resistance the ability to suppress the establishment of parasites, reduce their growth and eliminate the parasite burden (Albers *et al.*, 1987; Woolaston & Baker, 1996; McEwan *et al.*, 1997). Resistance may have a cost to the host: Riffkin & Dobson (1979) found an inverse relationship between growth rate and reactivity to *H. contortus* antigens. On the other hand, resilience is the ability to withstand the effects of parasitism or “resistance to the effects of infection” (Clunies Ross, 1932) and therefore suffer lesser effects on animal productivity (Albers *et al.*, 1987; Bisset & Morris, 1996).

Whether naturally infected on pasture or artificially infected indoors, some breeds of cattle, sheep and goats are naturally more resistant to ectoparasites such as ticks (Francis, 1966; Seifert, 1971; Turner & Short, 1972; Utech *et al.*, 1978; Wambura *et al.*, 1998), endoparasites such as *Babesia* (Francis, 1966) and *Trypanosoma* spp. (Trail *et al.*, 1991) or gastrointestinal nematodes

(Loggins *et al.*, 1965; Zajac *et al.*, 1990; Pralomkarn *et al.*, 1997; Peña *et al.*, 2000). Sheep breeds identified as resistant include Florida Native (Loggins *et al.*, 1965; Courtney *et al.*, 1984; Zajac *et al.*, 1990; Amarante *et al.*, 1999), Barbados Blackbelly (Courtney *et al.*, 1984), St. Croix (Courtney *et al.*, 1984; Gamble & Zajac, 1992), Red Maasai (Preston & Allonby, 1978; Mugambi *et al.*, 1997; Wanyangu *et al.*, 1997), Scottish Blackface (Altaif & Dargie, 1978), Rhön (Gauly & Erhardt, 2001) and Merinos d'Arles (Gruner *et al.*, 1992).

Selective breeding for resistance to parasitism within a flock is usually based on low FEC, as it is the only direct selection tool available without sacrificing stock. Alternatively, resistant animals can be identified through genotypic markers (Altaif & Dargie, 1978; Schwaiger *et al.*, 1995; Beh & Maddox, 1996; Buitkamp *et al.*, 1996; McEwan *et al.*, 1997) or the nature of the immune response (Outteridge *et al.*, 1985; Wedrychowicz *et al.*, 1992; Gill, 1994; Pernthaner *et al.*, 1995a, b; Douch *et al.*, 1996; Kanobana *et al.*, 2001). Concerns have been expressed about the reliability of FEC as a measure of resistance, particularly its repeatability under field conditions (Kelly & Gray, 1995) and the effects of worm species in the natural nematode challenge (Douch *et al.*, 1996) and of nutrition (Preston & Allonby, 1978). Morris *et al.* (1995) found that under field conditions low FEC had a moderately high heritability of 0.23 when assessed once, but 0.35 when an average of two challenge periods was used.

Resistance has an immunological basis, as low FEC is associated with an enhanced immune response in sheep (Kimambo *et al.*, 1988; Gray *et al.*, 1992; Douch *et al.*, 1995; Bisset *et al.*, 1996; Stear *et al.*, 1996). Although circulating and tissue eosinophil levels increase in parasitised animals, peripheral eosinophil counts have only limited use as a measure of resistance, as the association of eosinophilia with resistance and low FEC was apparent in some studies (Dawkins *et al.*, 1989; Buddle *et al.*, 1992; Stear *et al.*, 2002) but not others (Pernthaner *et al.*, 1995a; Bisset *et al.*, 1996; Woolaston *et al.*, 1996; Kanobana *et al.*, 2001).

A more desirable trait may be resilience to parasitism, the ability of an animal to maintain productivity without anthelmintic treatment (Riffkin & Dobson, 1979;

Albers *et al.*, 1987; Bisset *et al.*, 1994, 2001; Douch *et al.*, 1996). Ideally, resilient animals should be identified under the most severe challenge, which is impractical in a commercial setting (Bisset *et al.*, 2001). Instead, selective drenching of affected animals is often used and the most resilient animals identified from the age when they first need drenching and the number of drenches required in a given period. Unlike resistance, which appears to be associated with an enhanced inflammatory response of eosinophils, MMC and globular leucoctyes (Bisset *et al.*, 1996), the mechanisms underlying resilience do not appear to have been established. An important question to be addressed is whether resistance and resilience represent the opposite extremes of immune responsiveness.

1.5 Conclusions

Although the pathophysiology, immune responses and morphological effects of abomasal parasitism are well described, the underlying interaction between the parasite and host remain unknown. There are important unresolved issues concerning the roles of the parasite and the host in causing the pathological changes and the benefits and detrimental effects to either or both partners. Questions remaining to be answered include the following: are the parietal cells reversibly inhibited or does recovering the ability to secrete normal amounts of acid require the generation of new parietal cells; is the inflammatory response involved in initiating, or only exacerbating, the pathological effects of the parasites; are the cellular changes in infected glands the result of physical stretching and separation of cell-cell junctions; does parasite ES play a role in the loss of parietal cells?

The experiments described in the following Chapters were designed to address some of these questions. It was hoped that in addition to furthering the understanding of abomasal parasitism, this naturally occurring model of gastric pathology might give insights into the normal relationships between trophic agents and mucosal cells in gastric glands. Abomasal parasitism was studied in genetically different hosts to investigate the physiological basis for susceptibility and resilience to parasitism. A particular focus was the

relationship between secretory, morphological and inflammatory reactions in sheep selected for higher wool production, by inference “resilient” animals, which might prove a useful model in which to define the roles of host and parasite in the parasitic relationship.

Chapter 2

Pathophysiological effects of infection with *Ostertagia circumcincta* in sheep selected for high fleece weight

2.1 Introduction

Genetic selection of sheep better able to cope with parasitism is a valuable tool in controlling parasitism in livestock, particularly as the incidence of drench resistance is increasing (reviewed in Section 1.4.3). One approach, to breed for resistance to gastro-intestinal nematodes by selecting animals which harbour lower worm burdens, has the important benefit of less pasture contamination (Bisset *et al.*, 2001). Another, less common, strategy is breeding for resilience, defined by Albers *et al.* (1987) as the "ability to maintain a relatively undepressed production level while infected, or by Bisset & Morris (1996) as "growth rate compared to flock peers in relation to a standard challenge".

Since 1956, Massey University has maintained a Romney flock, which has been selected for high yearling greasy fleece weight and a control flock derived from the same base flock. The selected high-fleece weight (HFW) flock appears to conform to the above definitions of resilience to parasitism, as it has been bred selectively for higher productivity, but has higher FEC and worm burdens than the control (C) flock when naturally infected with nematodes grazing on contaminated pasture (Howse *et al.*, 1992; Williamson *et al.*, 1995b) or artificially infected when kept indoors (Williamson *et al.*, 1994, 1995a). Similar observations of higher FEC have come from other flocks selected for fleece weight (McEwan *et al.*, 1992) or higher fertility (Watson *et al.*, 1986), leading to the suggestion that high fleece weight and susceptibility to parasitism may be genetically linked.

Blair *et al.* (1985) reported the responses to selection from 1956 to 1979 of the Massey University HFW flock of 70-80 ewes, which were mated annually to 4 selected rams. In addition to the cumulative differential in greasy fleece weight of 5 kg in the HFW flock, clean fleece weight, staple length, mean fibre diameter, clean scoured yield, weaning weight and liveweight also increased, but wool quality number and crimp frequency decreased. Selection for HFW was associated with an increase in the frequency of the HbB allele, but did not change the proportions of animals with high or low potassium erythrocytes (Pijls *et al.*, 1988). McCutcheon *et al.* (1993) confirmed that at 16 months-of-age HFW rams had a greater average body weight, a heavier and longer carcass, but decreased muscularity and a greater hind leg weight at the expense of the shoulder.

The HFW and C flocks also have developed some differences in metabolism, not apparent at all ages or in all studies. Higher wool production in HFW animals was not the result of greater feed intake or digestibility (McClelland *et al.*, 1986; Thomson *et al.*, 1989), but HFW animals on average have plasma thyroxine, urea and creatinine concentrations lower than C animals under controlled feeding conditions (McCutcheon *et al.*, 1987; Clark *et al.*, 1989; Thomson *et al.*, 1989; Sun *et al.*, 1991). HFW animals had a higher rumen fractional outflow rate for lignin and a higher ruminal molar proportion of acetate and lower proprionate (Thomson *et al.*, 1989). Creatinine clearance (glomerular filtration rate) was higher (McCutcheon *et al.*, 1987; Thomson *et al.*, 1989) and urea clearance lower in HFW animals (Thomson *et al.*, 1989).

Other studies of the HFW flock have confirmed differences in wool characteristics as a result of the selection process. Antram *et al.* (1991) observed greater mid-side wool growth and wool sulphur output in HFW rams for most of the observation period from 4-14 months-of-age. Wool samples from 11 body sites of 14 month-old rams revealed greater wool growth and lower wool sulphur concentrations in HFW animals (Sun *et al.*, 1991).

Howse *et al.* (1992) were the first to establish in the field that the HFW flock had higher FEC than the C flock with naturally acquired infections. Despite the higher FEC, six month-old HFW animals maintained higher clean wool growth

and ram lambs had greater liveweight gains than C animals over a 42-day period; a similar difference was apparent between other animals from the two flocks given albendazole capsules. A later study by Williamson *et al.* (1995b) over two successive seasons confirmed the higher FEC in the HFW flock during natural exposure to parasites, but lower serum gastrin levels and little evidence of greater production depression in C than in HFW sheep. Indoors. HFW and C male sheep were infected at 15 months-of-age with a mixture of *H. contortus*, *O. circumcincta* and *T. colubriformis* larvae by Williamson *et al.* (1994, 1995a). In the HFW sheep, there was a higher FEC and numbers of adult worms of both abomasal species, but no difference in *T. colubriformis* burdens. There was no group difference in serum IgG1 to *H. contortus* larvae, or in abomasal MMC numbers, but an inverse relationship between abomasal MMC and numbers of each of the abomasal species in HFW animals, but not in the C sheep.

Whereas an enhanced inflammatory response of eosinophils, MMC and globular leucocytes appears to be associated with resistance to parasitism (Bisset *et al.*, 1996), little is known about the mechanisms underlying resilience, or whether resistance and resilience represent the opposite extremes of immune responsiveness. The Massey HFW and C flocks may offer the opportunity to study abomasal parasitism in resilient animals and to compare pathophysiological responses in hosts of different genetic backgrounds. This may also clarify the relationships between the inflammatory reactions to, and pathophysiological effects of, abomasal nematodes. Experiments were conducted in two successive years on groups of parasite-naïve lambs from the two flocks which were infected for the first time with *O. circumcincta* L3. The pathophysiological changes in these animals are reported below and morphological effects of the parasitism in Chapter 3.

2.2 Material and Methods

2.2.1 Experimental Design

In **Experiment 1**, at 6.5 months-of-age, four parasite-naïve lambs (two male, two female) from each of the Romney HFW and C flocks were infected intraruminally, initially with 50,000 L₃ *O. circumcincta*, followed by weekly doses of 10,000 L₃ from Days 35-70 p.i. The lambs were euthanased 24 days after the last infection (Day 94 p.i.). C lambs were #1-4 and HFW lambs were #5-8. Females were #3, #4, #5 and #8.

In **Experiment 2**, at 4.5 months-of-age, eight parasite-naïve male lambs from each of the HFW and C flocks were infected with 50,000 L₃ *O. circumcincta*. Four animals per group were euthanased on Day 8 p.i. and the remaining eight animals were euthanased on Day 28 p.i. C lambs were #9-12 and 17-20 and HFW lambs were #13-16 and 21-24.

2.2.2 Animals

2.2.2.1 Housing and Feeding

Lambs were brought indoors at 2-4 days-of-age to be reared nematode free. Those from the control flock were selected at random, while the lambs from the HFW flock were chosen from the remainder after breeding stock had been selected. The lambs were initially reared on a commercial milk replacer (200g/l; Anlamb, New Zealand Dairy Co, Te Puke, NZ) and later weaned on to the experimental diet of lucerne chaff and lucerne pellets. Water was available *ad libitum*.

After they had undergone surgery, the sheep were housed individually in metabolism crates until the end of the experiment. Fresh food, consisting of 500g lucerne pellets and a weighed excess of lucerne chaff and *ad libitum* water was provided each day at 9.30 a.m. Usually all pellets were consumed. Daily food intake (fresh weight) was recorded for sheep only in **Experiment 2**.

2.2.2.2 Abomasal Cannulation

An abomasal cannula was surgically implanted into each animal under general anaesthesia at 5.5 months-of-age in **Experiment 1** and at 4 months-of-age in **Experiment 2**. Anaesthesia was induced by i.v. thiopentone (10mg/kg bodyweight, Thiovet[®] C-vet, Leyland, UK) and maintained on flurothane (0.75%) mixed with oxygen. The abdomen was accessed through the upper rear quadrant of the right flank. Teflon abomasal cannulae with a thread and screw plug (locally manufactured by AgResearch Ltd, Palmerston North, NZ) were inserted into the abomasum, as far as possible from the pylorus. The cannulae used in **Experiment 1** were 7.0cm long, inner diameter 2.0cm and outer diameter 2.4cm. In **Experiment 2**, the same cannulae were used for eight animals and smaller cannulae (length 7.0cm, inner diameter 1.4cm and outer diameter 1.9cm) were used for the other eight lambs. The cannulae were exteriorised through the right flank 3cm behind the last rib, mid-way between ventral and dorsal lines. Ketofen (Parnell Laboratories, Auckland, NZ, 3mg/kg) was administered i.m. after surgery and prophylactic antibiotics (Streptopen[®]; Pitman-Moore, U.S.A., 3ml i.m. daily) were given for 3 days post-operatively. The lambs were allowed to recover from surgery for 4 weeks before **Experiment 1** and for 2 weeks before **Experiment 2**.

2.2.2.3 Necropsy

Lambs were euthanased by captive bolt and exsanguination. In **Experiment 1**, all sheep were killed on Day 94 except sheep #4 and #7, which were killed on Days 62 and 64 respectively, due to leakage of contents around the abomasal cannulae. In **Experiment 2**, Lambs #9-16 were necropsied on Day 8 p.i. and sheep #17-24 on Day 28 p.i.

The abdomen was opened and the abomasum tied off at both ends and removed from the animal. Abomasal lymph nodes (ALN) were first collected from the greater curvature of the abomasa of the lambs in **Experiment 2** (#9-24). For all lambs, a small incision was made in the pylorus and the abomasal contents were collected in a measuring cylinder. The abomasum was then opened along the greater curvature and washed with 0.9% saline. The

washings were added to the abomasal contents and a 10% sub-sample was mixed with an equal volume of 10% formalin for worm counting.

The abomasum was cut in half and one half was placed on ice until it was frozen and stored at -20° C for worm counting. From the other half of the abomasa of the lambs in **Experiment 2** (#9-24), two tissue samples were collected, one from each of the larger fundic folds, and placed in Bouins fluid for later histological examination (reported in Chapter 3).

2.2.3 Experimental Sample Collection

In **Experiment 1**, blood and abomasal fluid samples were collected twice a day, 1h before, and 3h after, fresh food was offered, on Days -14, -12 and -8 and each day from Day 6 until Day 24 p.i. Between Days 1-5 and Days 25-35, daily blood and abomasal fluid were taken only before feeding. The frequency of sampling was reduced to twice weekly from Day 35 until Day 73, thereafter weekly until necropsy on Day 94. In **Experiment 2**, blood and abomasal fluid were collected daily 1h before fresh food was offered. Pre-infection samples were taken on Days -5, -3 and -1 and, after infection, daily from Day 1 to either Day 8 (Sheep #9-16) or Day 28 p.i. (Sheep #17-24).

Skin biopsies were taken from each animal during surgery for the abomasal cannulation and thereafter weekly until euthanasia to determine the percentage of paracortex in wool follicles.

Faecal samples were collected from each animal before infection to confirm the absence of infection. In **Experiment 1**, samples were collected on Days -14, -12 and -8 p.i., daily from Day 15-35 and thereafter twice a week. A faecal sample was also taken immediately after euthanasia on Day 94. In **Experiment 2**, faecal samples were taken daily from Day 16 onwards.

2.2.4 Abomasal Contents

About 20ml of abomasal contents were collected by spontaneous flow from the opened cannula into a 50ml plastic tube (Labserv, Auckland, NZ). The pH was

measured with a PHM82 Standard pH Meter (Radiometer, Copenhagen, Denmark).

2.2.5 Blood Samples

Blood samples (5ml) were collected by jugular venipuncture into plain evacuated tubes (NZvet, Auckland, NZ), allowed to clot at room temperature and centrifuged at 2000g for 20min. The serum was separated and stored at -20°C for later determination of serum pepsinogen and gastrin concentrations and antibody titres. Additional blood samples (2ml) were collected before feeding into evacuated Lithium-Heparin tubes (NZvet, Auckland, NZ) for immediate determination of blood eosinophil concentrations.

2.2.5.1 Serum Pepsinogen

Serum pepsinogen concentrations were measured using a modification of the method of Berghen *et al.* (1987) (Appendix 2.3).

2.2.5.2 Serum Gastrin

Serum gastrin was measured by two comparable radioimmunoassays. Samples from **Experiment 1** were assayed using the method of Simpson *et al.* (1993), based on the method of Hansky & Cain (1969) and employing Ab74 (Appendix 2.4.1). Samples from **Experiment 2** were assayed by the method of Ciccotosto & Shulkes (1992) and employing Ab1296 (Appendix 2.4.2).

2.2.5.3 Blood Eosinophils

A 0.2ml sample of whole blood was transferred into a tube containing 0.8ml of Carpentier's eosinophil counting solution (1ml of 2% Eosin Yellow and 1.5ml of CaCO₃ saturated in 40% formaldehyde made up to 50ml with distilled H₂O). This cell suspension was kept at room temperature for 30min and then counted in a Neubauer haemocytometer.

2.2.5.4 Serum Antibody Titres

Total titres of immunoglobulins specific for ES antigens of either exsheathed L₃ or adult *O. circumcincta* were determined in serum samples using an ELISA similar to that of Douch *et al.* (1994) (Appendix 2.5). Immunoglobulin isotypes

were determined using an ELISA and monoclonal antibodies against sheep IgG1, IgG2, IgA and IgM (the gift of Dr. Ken Beh, McMaster Laboratory, CSIRO, Australia (Beh, 1987, 1988) (Appendix 2.5). Both assays were performed on serum collected from sheep in **Experiment 1** on Days 12 and 8 before infection and on the following days after infection: 7, 11, 14, 18, 21, 24, 28, 31, 35, 38, 42, 49, 56, 63, 70, 73, 80, 87 and 94. Assays were carried out with the assistance of Mr. Richard Green in the laboratories of AgResearch Ltd, Wallaceville, NZ.

2.2.6 Abomasal Lymph Node Cytokine Profiles

The expression of cytokine genes by lymphoid cells derived from ALN was determined in **Experiment 2**. ALN from lambs #9-16 (euthanased on Day 8 p.i.) and lambs #17-24 (euthanased on Day 28 p.i.). Expressions of the cytokine genes for IL-4, IL-5, IL-10, IL-13, IFN- γ and TNF- α were determined after mitogenic (Concavalin A) or antigenic (*O. circumcincta* L₃ antigen) stimulation. These analyses were conducted by Dr A. Pernthaner in the laboratories of AgResearch Ltd, Wallaceville, NZ (Appendix 2.6).

2.2.7 Wool Follicles

2.2.7.1 Skin Biopsies

An area of fleece approximately 10cm x 10cm situated over the last rib on the midside of each animal was clipped to skin level and 2ml of 2% Lignocaine hydrochloride (Virbac Laboratories, Auckland, NZ) was injected s.c. into the centre of the shaved patch. After 5min, a skin biopsy was taken adjacent to the site of injection using a 10mm trephine punch.

Skin biopsies were fixed in Bouins fluid for up to 12 h before removal to 70% (v/v) ethanol for storage until histological processing. Samples were routinely processed for paraffin wax embedding: they were dehydrated in 70% ethanol, cleared in chloroform and two changes of xylene and infiltrated with paraffin wax at 59°C, according to standard schedule on a TP1050 automatic tissue processor (Leica Jung, Wetzlar, Germany). The samples were embedded on a Tissue-Tek® embedding console (Miles Scientific, Chicago, USA) and then

sectioned on a rotary microtome at 5 μm thickness transverse to the plane of the follicle. The sections were floated in a waterbath at 40°C, attached to microscope slides and oven-dried at 60°C. Sections were dewaxed and hydrated using a standard method. They were then oxidised with performic acid (30 min at room temperature), stained with 1% (w/v) methylene blue to distinguish areas of orthocortical and paracortical cells (Clarke & Maddocks, 1965; Orwin *et al.*, 1984), and mounted using DPX mounting medium.

2.2.7.2 Paracortex in Wool Follicles

Sections were selected containing primary (P) and secondary (S) follicles at the level of the sebaceous gland in the skin and were examined using a light microscope. Follicle counts were made at 40x magnification within five fields for each skin sample using computer-aided image analysis (Sigma Scan, Jandel Scientific, Corte Madera, USA) and a graphic digitising tablet (Sketchmaster, GTCO Corporation, Columbia, USA). Primary and secondary follicle densities were then estimated and individual P and S follicles were assessed for the presence or absence of paracortical cells. Numbers of P and S wool follicles containing paracortex were pooled for each group and expressed as percent of the total number of wool follicles counted.

2.2.8 Parasitology

2.2.8.1 Parasite Cultures

Infective L₃, used for intraruminal infection of the sheep, were obtained from cultures of faeces from sheep infected with a pure strain of *O. circumcincta* and had a viability greater than 98% at the time of infection. Larval culture is described in Appendix 1.1.

2.2.8.2 Faecal Egg Counts

Faecal floats were used to detect the presence of eggs, and if these were positive, faecal egg counts per gram of faeces (e.p.g.) were determined using the modified McMaster method of Stafford *et al.* (1994) (Appendix 1.2).

Table 2.1. Pre-infection values for abomasal pH, serum gastrin and pepsinogen and blood eosinophil content for parasite-naïve lambs used in Experiments 1 and 2. The upper limits of the normal values, defined as mean plus two standard deviations, are shown.

Parameter	Experiment 1			Experiment 2		
	Mean	SD	Mean + 2 SD	Mean	SD	Mean + 2 SD
Abomasal pH	2.35	0.35	3.05	2.62	0.29	3.20
Serum gastrin (pM)	53	18	89	39	15	69
Serum pepsinogen (iU)	0.36	0.15	0.66	0.36	0.19	0.75
Blood eosinophils (count/ μ l)	162	120	402	266	455	1176

2.2.8.3 Worm Counts

Worm counts were carried out on 10% of the abomasal contents and on 10% of a pepsinogen-HCl-digestion of half of the abomasum (Appendix 1.3). The numbers of eggs *in utero* in 50 female worms per sample of abomasal contents were counted for **Experiment 2**.

2.2.9 Data Presentation and Statistical Analyses

Group data are presented as mean \pm SEM. The upper limits of the normal ranges for abomasal pH, serum pepsinogen, serum gastrin and blood eosinophil count were calculated as 2 SD above the mean values of all pre-infection samples for each experiment (Table 2.1). Elevated levels were defined as values above the upper limits for two consecutive days. For food intake, the lower limit was calculated as 2 SD below the mean values of all pre-infection samples for Experiment 2.

Data were analysed using SPSS (Statistical Package for Social Scientists) version 11.0 (SPSS Inc., Chicago, USA). In **Experiment 1**, most data for the primary (to Day 35 p.i.) and trickle infection (Days 35-94) were analysed separately. Because of the small number of data points, wool follicle data, FEC and correlations involving FEC were analysed for the whole experimental period. In **Experiment 2**, data were analysed separately for Days 1-8 (n=8 per group) and Days 9-28 (n=4 per group).

Due to the small group sizes, data were examined for normality using a “predicted value versus residuals” model. If necessary, data were then log transformed; this was required for FEC [$\log_{10}(\text{FEC}+1)$], abomasal pH, serum pepsinogen concentration (Days 35-94 only) and serum gastrin concentration and blood eosinophil counts for **Experiment 1** and for FEC [$\log_{10}(\text{FEC}+1)$], abomasal pH, cytokine gene expression and blood eosinophil counts for **Experiment 2**. Differences between groups were calculated using a General Linear Model for Repeated Measures. Pearson’s or Spearman correlations were used to relate data. Relative cytokine gene expression was compared by two-way ANOVA followed by Bonferroni’s post-test for group and time differences and the effects of stimulants by non-parametric t-tests.

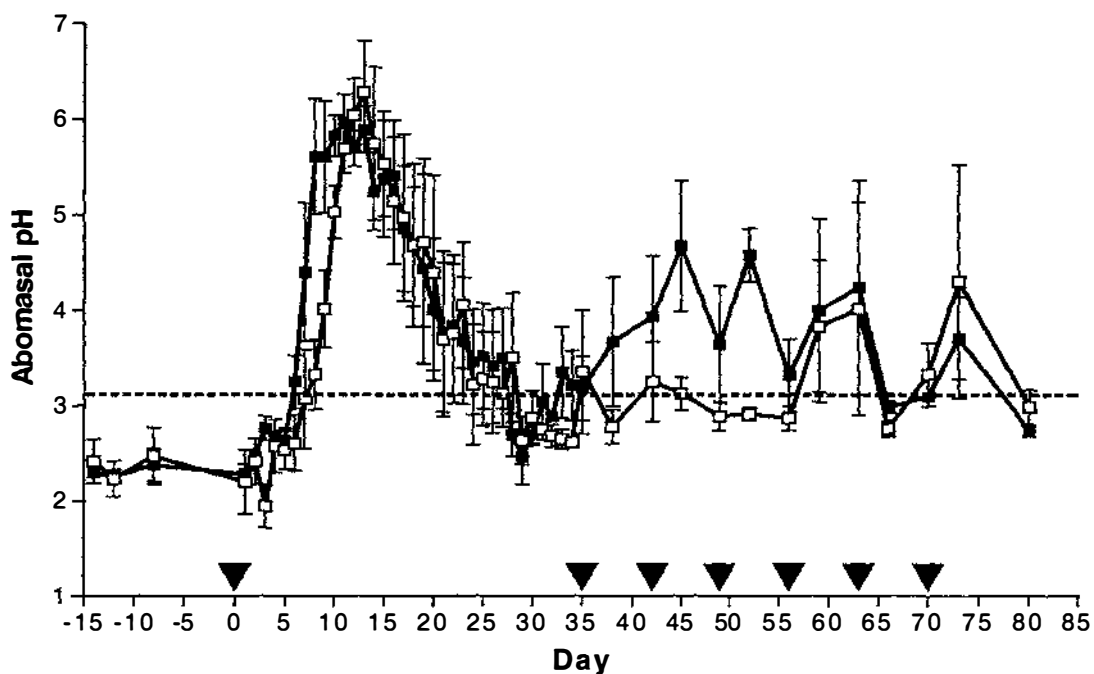


Figure 2.1. Abomasal pH (mean \pm SEM) in high fleece weight (HFW) and control (C) lambs which were infected at Day 0 with 50,000 L₃ *O. circumcincta* and at Days 35, 42, 49, 56, 63 and 70 with 10,000 L₃ *O. circumcincta*. HFW: \square ; C: \blacksquare ; administration of L₃: \blacktriangledown . The horizontal dotted line represents 2SDs above the mean value determined from uninfected lambs (pH =3.05).

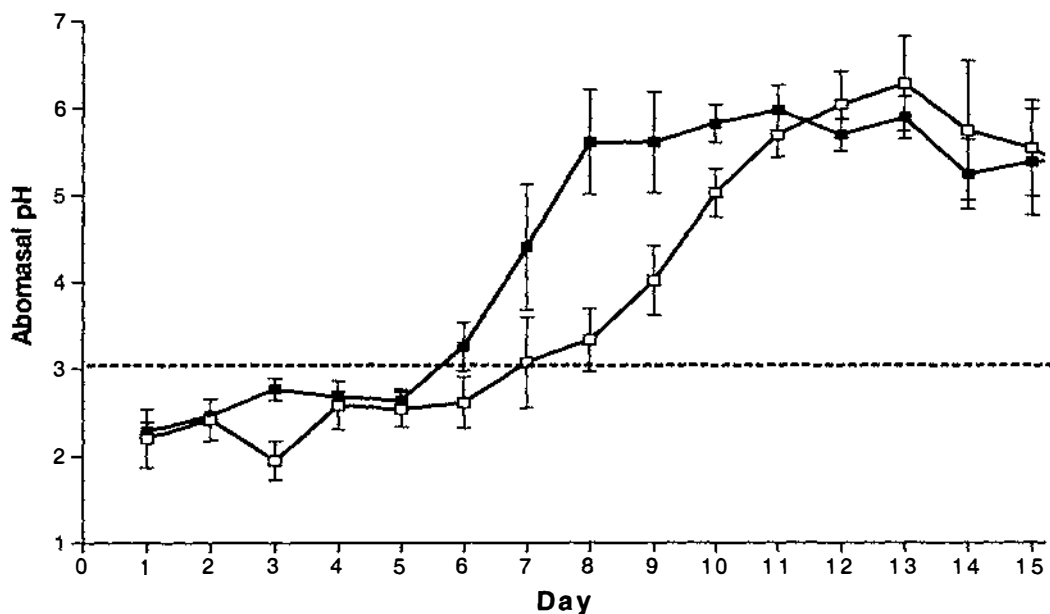


Figure 2.2. Abomasal pH (mean \pm SEM) in high fleece weight (HFW) and control (C) lambs on Days 1-15 after infection on Day 0 with 50,000 L₃ *O. circumcincta*. The group mean was lower in the HFW group on Day 8 ($p < 0.05$) and approached significance on Day 9 ($p = 0.075$) and Day 10 ($p = 0.071$). HFW: \square ; C: \blacksquare . The horizontal dotted line represents 2SDs above the mean value determined from uninfected lambs (pH =3.05).

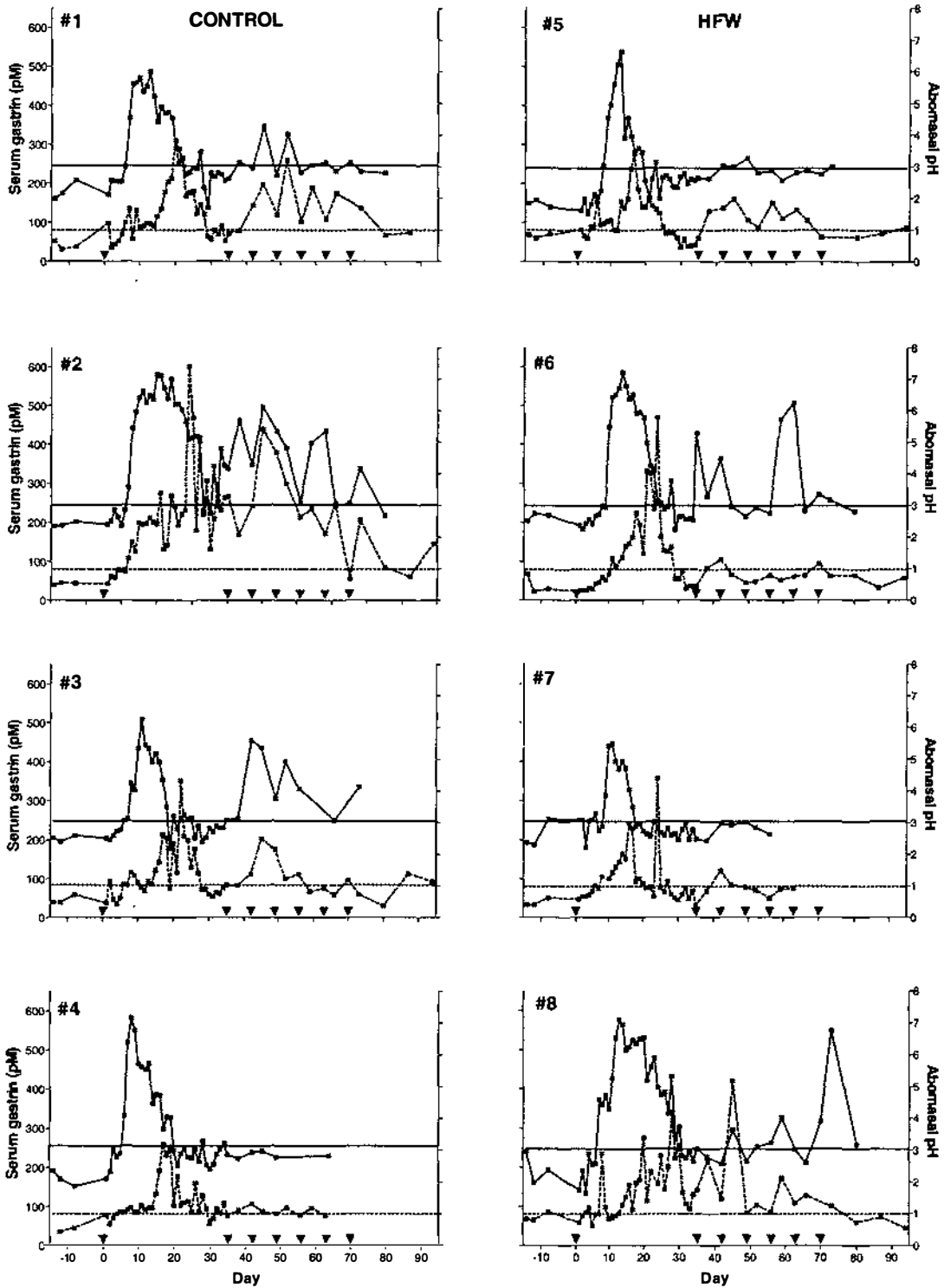


Figure 2.4. Abomasal pH and serum gastrin concentrations in individual lambs after infection at Day 0 with 50,000 L_3 *O. circumcincta* and at Days 35, 42, 49, 56, 63 and 70 with 10,000 L_3 *O. circumcincta*. Left panel: lambs in control (C) group; right panel: lambs in high fleece weight (HFW) group; abomasal pH: — serum gastrin concentration - - - - ; administration of L_3 : ▼. The horizontal solid lines represent 2SDs above the mean value determined from uninfected lambs (pH =3.05) and dotted lines represent 2SDs above the mean values determined from uninfected lambs (89 pM).

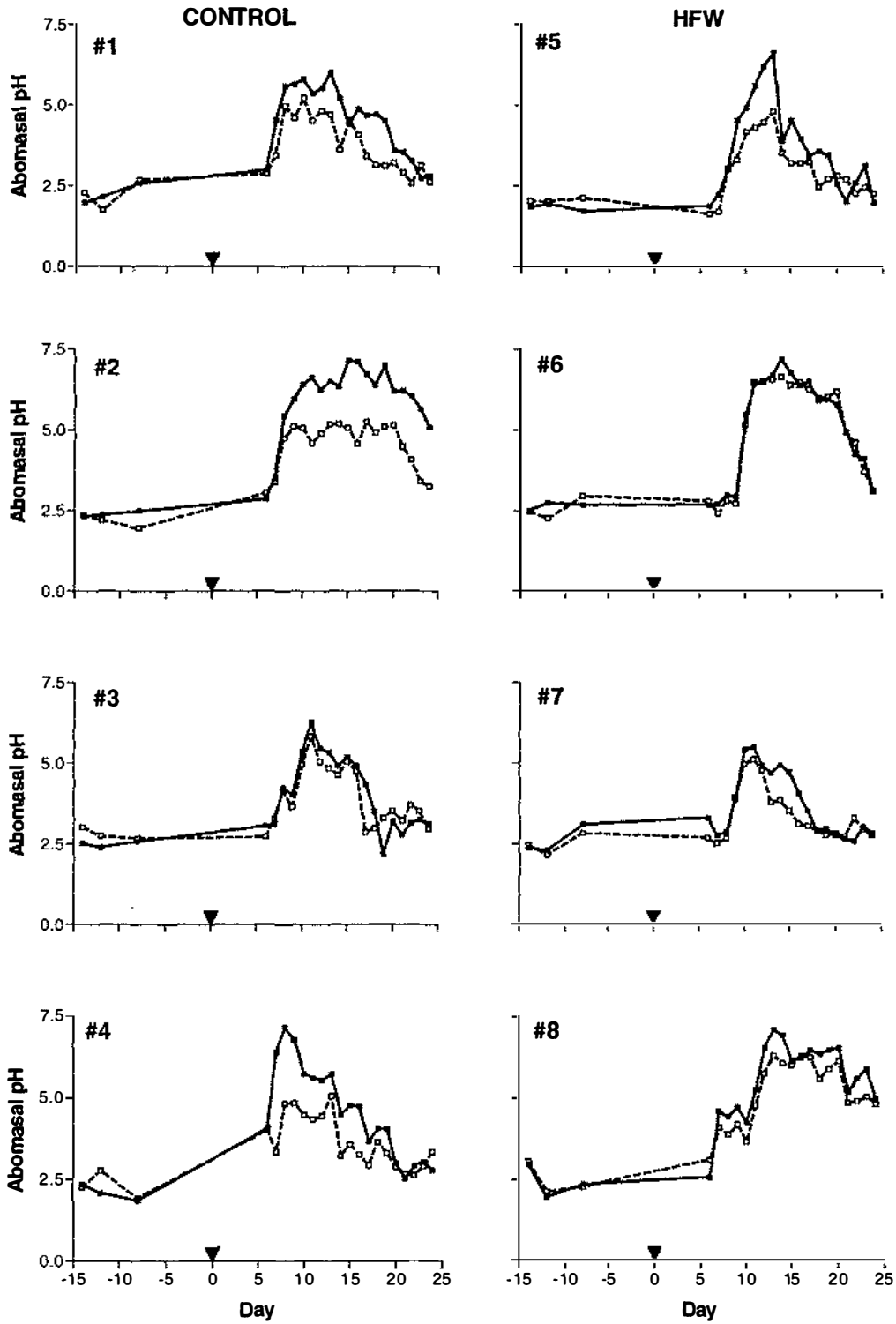


Figure 2.3. Abomasal pH 1 hour before, and 3 hours after, feeding in individual high fleece weight (HFW) and control (C) lambs, which were infected on Day 0 with 50,000 *L3* *O. circumcincta*. Left panel: C group; right panel: HFW group; before feeding: ■; after feeding: □; administration of *L3*: ▼.

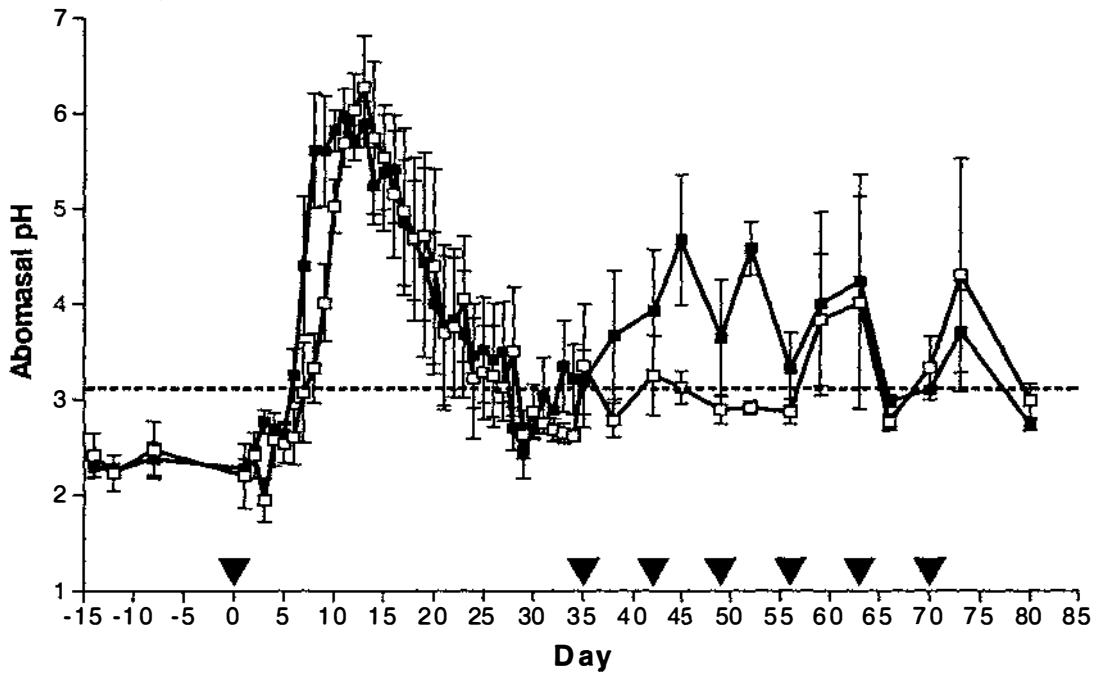


Figure 2.1. - Abomasal pH (mean \pm SEM) in high fleece weight (HFW) and control (C) lambs which were infected at Day 0 with 50,000 L₃ *O. circumcincta* and at Days 35, 42, 49, 56, 63 and 70 with 10,000 L₃ *O. circumcincta*. HFW: \square ; C: \blacksquare ; administration of L₃: \blacktriangledown . The horizontal dotted line represents 2SDs above the mean value determined from uninfected lambs (pH =3.05).

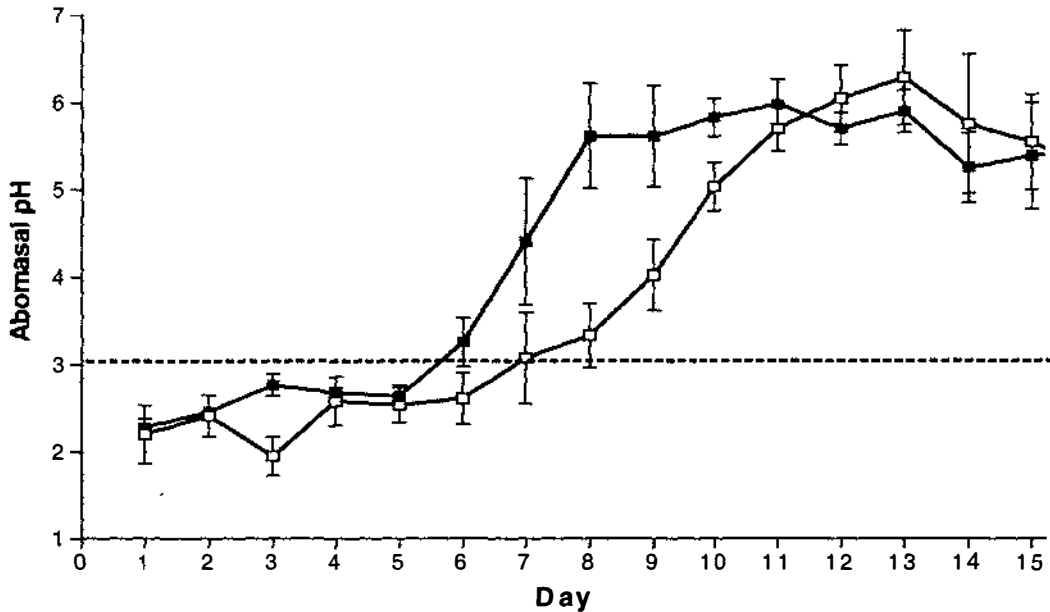


Figure 2.2. Abomasal pH (mean \pm SEM) in high fleece weight (HFW) and control (C) lambs on Days 1-15 after infection on Day 0 with 50,000 L₃ *O. circumcincta*. The group mean was lower in the HFW group on Day 8 ($p < 0.05$) and approached significance on Day 9 ($p = 0.075$) and Day 10 ($p = 0.071$). HFW: \square ; C: \blacksquare . The horizontal dotted line represents 2SDs above the mean value determined from uninfected lambs (pH =3.05).

2.3 Results: Experiment 1

2.3.1 Abomasal pH

Following the initial infection, abomasal pH in both groups increased abruptly between Days 5-10, rapidly reached peak values and thereafter declined to pre-infection values around Day 25 (Figure 2.1). The mean responses of the two groups differed significantly in the time of the initial onset of hypochloridia: C animals from Day 7, compared with Day 9 in HFW animals (Figure 2.2). Over the whole period Days 0-35, the group mean pH did not differ significantly, but was lower in the HFW group on Day 8 ($p < 0.05$) and approached significance on Days 9 ($p = 0.075$) and 10 ($p = 0.071$).

Feeding lowered abomasal pH in most lambs during the period of greatest hypoacidity (Figure 2.3). For the period Day -5 to Day 26 after the initial infection, abomasal pH was significantly lower 3h after presenting fresh feed than before feeding in three animals from each group ($p < 0.05$). The exceptions were sheep #6 (HFW) and #3 (C). Four individual sheep had a sustained period of elevated abomasal pH (#1, #2, #6 and #8), while, in the other four sheep (#3, #4, #5 and #7), abomasal pH peaked on a single day (Day 13, 11, 11 and 8 respectively) (Figure 2.4). There were often abrupt declines in abomasal pH over 1-2 days followed by a period of only slightly raised values around pH 3. The length of the period of elevated pH was unrelated to the experimental grouping.

During the subsequent trickle infection from Day 35 onward, four of the seven sheep for which there were adequate data had elevated abomasal pH (Figures 2.1 and 2.4), often peaking to high levels 2-3 days after administration of L_3 .

2.3.2 Serum Gastrin

Group mean serum gastrin concentrations did not differ significantly between the two groups for the duration of the experiment (Figure 2.5). During the primary infection, individual lambs generally showed the same overall response of a steady increase from Day 6 onwards, reaching peak values between Days 16-21 (Figure 2.4). Two exceptions were sheep #8 (HFW) and sheep #2 (C),

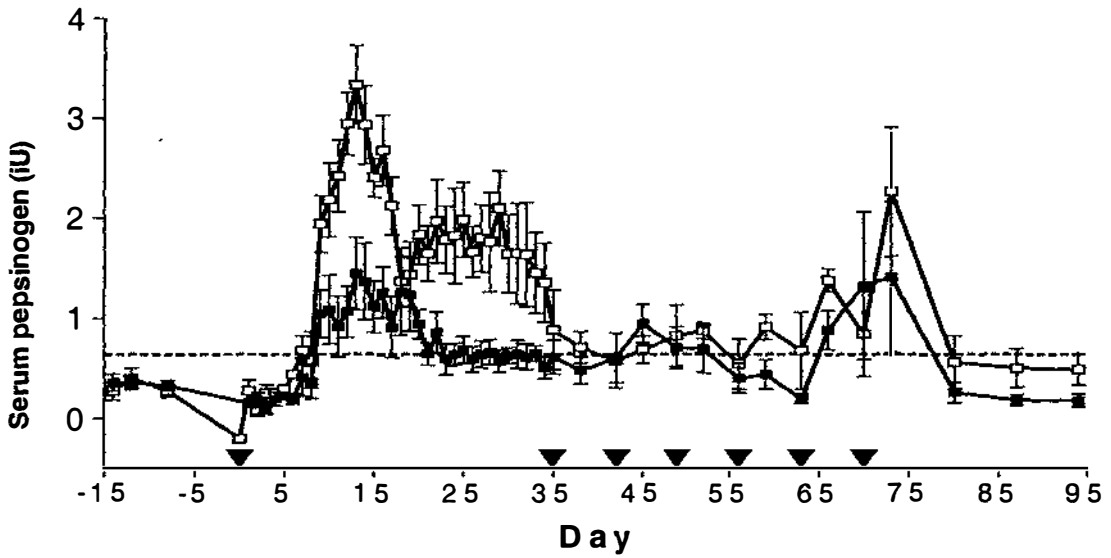


Figure 2.6. Serum pepsinogen concentration (mean \pm SEM) in high fleece weight (HFW) and control (C) lambs which were infected at Day 0 with 50,000 *L*₃ *O. circumcincta* and at Days 35, 42, 49, 56, 63 and 70 with 10,000 *L*₃ *O. circumcincta*. HFW: \square ; C: \blacksquare ; administration of *L*₃: \blacktriangledown . The horizontal dotted line represents 2SDs above the mean value determined from uninfected lambs (0.66 iU).

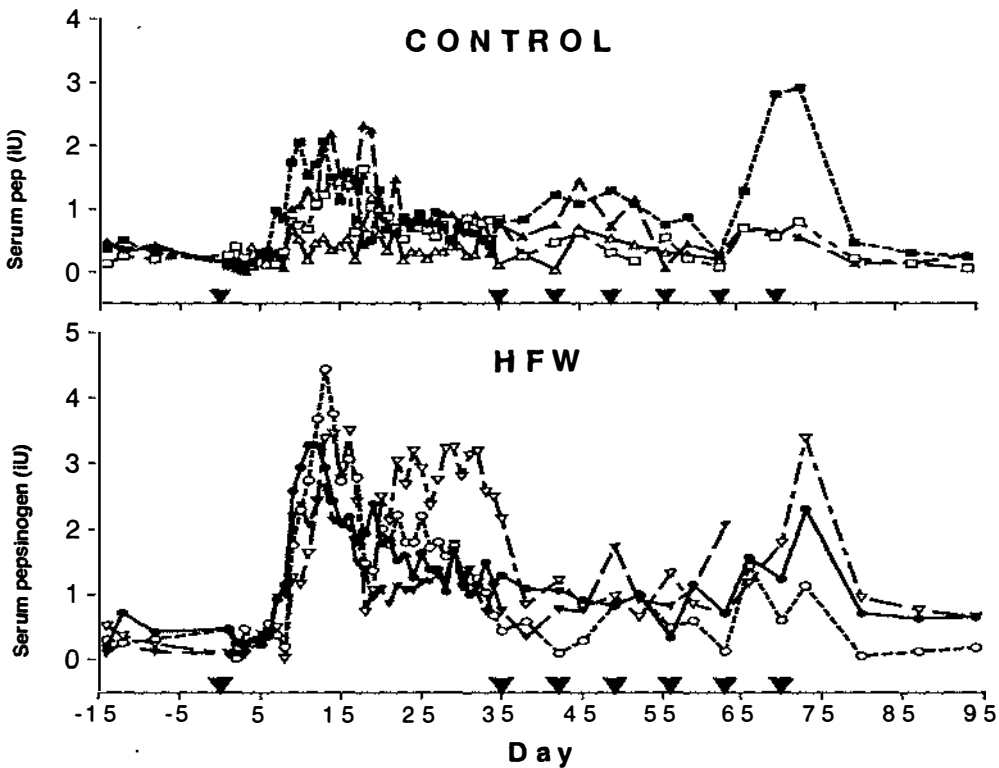


Figure 2.7. Serum pepsinogen concentration in individual lambs after infection at Day 0 with 50,000 *L*₃ *O. circumcincta* and at Days 35, 42, 49, 56, 63 and 70 with 10,000 *L*₃ *O. circumcincta*. Top: lambs in control (C) group; bottom: lambs in high fleece weight (HFW) group. Lamb #1: \blacksquare ; #2: \square ; #3: \blacktriangle ; #4: \triangle ; #5: \bullet ; #6: \circ ; #7: \blacktriangledown ; #8: \triangledown ; administration of *L*₃: \blacktriangledown .

which both had markedly elevated serum gastrin concentrations from Day 25-35. During the trickle infection, all lambs generally had serum gastrin concentrations under 200pM, including three (#4, #6 and #7) in which it was around or below 100 pM. The exception was sheep #2, in which serum gastrin concentrations were much higher (Figure 2.4).

The relationship between changes in serum gastrin concentration and abomasal pH was remarkably consistent between animals (Figure 2.4). The most striking feature was the steady increase in serum gastrin to a later peak value around Day 21, which contrasted with the abrupt increases in abomasal pH over the period Days 6-10 to a peak at about Day 15. During the trickle infection, changes in serum gastrin concentration mostly, or partly, mirrored those in abomasal pH in six sheep (#1, #2, #3, #5, #7 and #8). In the two other sheep, serum gastrin concentrations were independent of abomasal pH, with sheep #6 showing no elevated gastrin levels. Administration of larvae was occasionally followed by elevation of serum gastrin concentration.

2.3.3 Serum Pepsinogen

Changes in serum pepsinogen concentration after infection followed a similar time course in the HFW and C groups (Figure 2.6). The group mean for the HFW sheep was higher than in the C group from Day 1-35 ($p < 0.01$) and on individual days from Day 9 until Day 17 ($p = 0.05-0.001$) and again from Day 20 to Day 30 ($p = 0.05-0.001$).

Data from Individual sheep show the similarity of the timing of responses to infection (Figure 2.7), except in Sheep #4, which was a "low-responder" that showed much smaller changes in serum pepsinogen concentration than the other animals. The initial abrupt increase in serum pepsinogen on Days 7-9 was followed by maximum values between Days 11-19, ending with a rapid decline to moderately elevated levels until Day 35. In the late phase of the subsequent trickle infection, further increases were associated with L_3 administration, but there were no differences between HFW and C groups. All animals had low serum pepsinogen levels by Day 80 p.i. Unusual events were the late increase in serum pepsinogen concentration following initial infection in

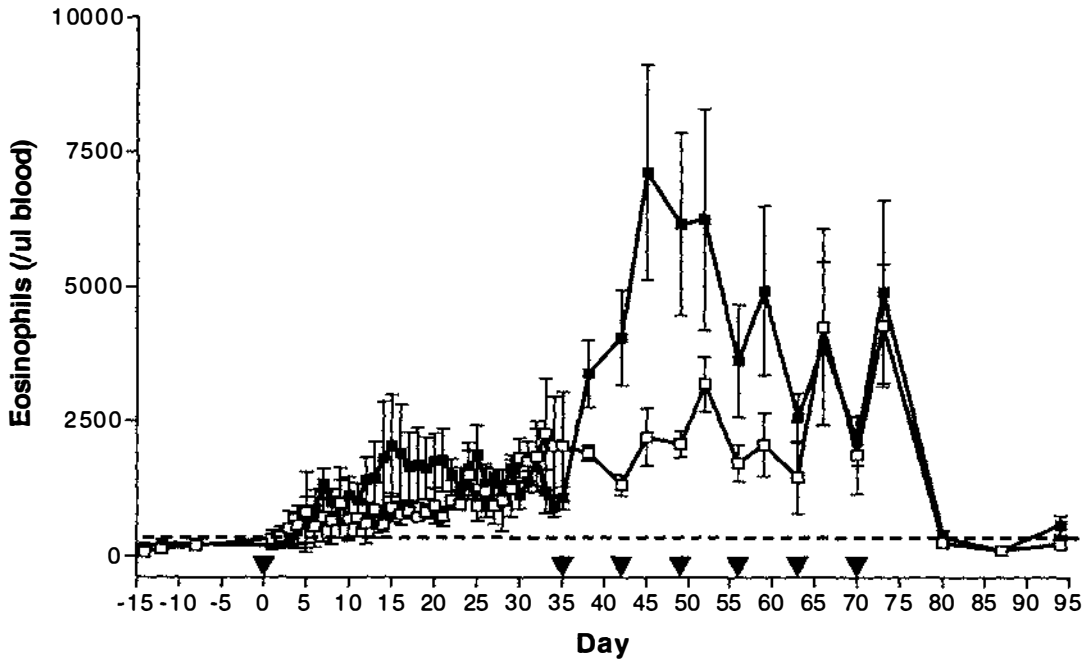


Figure 2.8. Blood eosinophil counts (mean \pm SEM) in high fleece weight (HFW) and control (C) lambs which were infected at Day 0 with 50,000 L₃ *O. circumcincta* and at Days 35, 42, 49, 56, 63 and 70 with 10,000 L₃ *O. circumcincta*. HFW: \square ; C: \blacksquare ; administration of L₃: \blacktriangledown . The horizontal dotted line represents 2SDs above the mean value determined from uninfected lambs (402cells/ μ l).

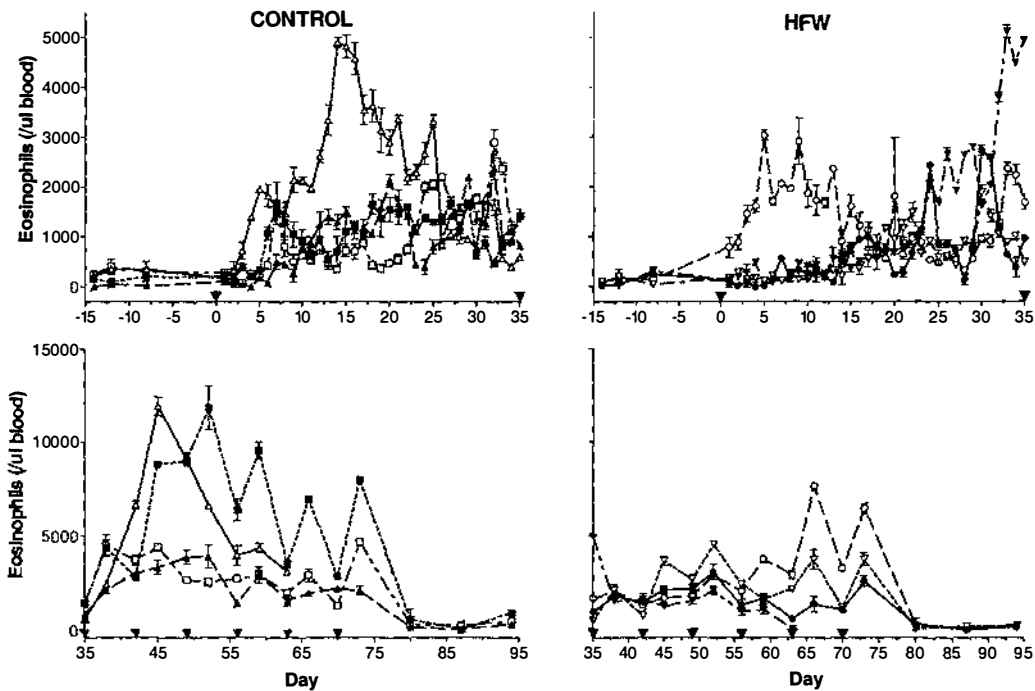


Figure 2.9. Blood eosinophil counts in individual lambs after infection at Day 0 with 50,000 L₃ *O. circumcincta* and at Days 35, 42, 49, 56, 63 and 70 with 10,000 L₃ *O. circumcincta*. Left panel: lambs in control (C) group, top: Days 1-35 p.i., bottom: Days 35-94 p.i.; right panel: lambs in high fleece weight (HFW) group, top: Days 1-35 p.i., bottom: Days 35-94 p.i. Lamb #1: \blacksquare ; #2: \square ; #3: \blacktriangle ; #4: \triangle ; #5: \bullet ; #6: \circ ; #7: \blacktriangledown ; #8: \triangledown ; administration of L₃: \blacktriangledown .

Sheep #8 and the very high levels in Sheep #1 (C) and #8 (HFW) late in the trickle infection.

Serum pepsinogen concentration was positively correlated over Days 1-35 with abomasal pH in HFW lambs ($r^2=0.33$, $p<0.001$) and in C lambs ($r^2=0.19$, $p<0.001$) but not during Days 35-94. There were no correlations between serum pepsinogen concentration and either blood eosinophil count or FEC.

2.3.4 Blood Eosinophils

Blood eosinophil counts after the initial infection generally increased moderately in comparison with the high levels seen during the subsequent trickle infection (Figure 2.8). Overall, the group means were higher in the C than HFW group during Days 35-94 ($p<0.05$), but not on Days 1-35. Individual days on which the group means were lower for the HFW group were Days 38, 45 and 49 ($p<0.05$) and Day 42 ($p<0.01$).

Serum pepsinogen concentrations and blood eosinophil counts on Days 1-35 were significantly correlated in both groups, although the r^2 values were low: C group, $r^2=0.13$, $p<0.001$; HFW, $r^2=0.10$, $p<0.001$. For Days 35-94, there was no significant correlation for the HFW group, but for the C group $r^2=0.21$, $p<0.001$.

Following initial infection, with the exception of lamb #6, HFW sheep had later increases in blood eosinophil counts (on about Day 15 p.i.) than those in controls (about Day 5) (Figure 2.9). Both sheep #4 (C) and #6 (HFW) had rising eosinophil counts by Day 2 p.i. and very high counts up to Day 15 for their respective groups. Lamb #8 was unusual in showing a late rise in blood eosinophil counts in this period, whereas in the other animals counts were declining by Day 35 p.i.

The trickle infection increased blood eosinophil counts, with each dose of L_3 appearing to evoke an increase in blood eosinophils. In the C group, there were moderate increases in Sheep #2 and #3, but very large and rapid increases in Sheep #1 and #4. Moderately raised eosinophil counts were observed in all HFW lambs, with the highest counts being recorded from sheep

Table 2.2. Worm counts (L_3 , L_4 , immature adults (I.A.), adult female worms and adult male worms) in high fleece weight (HFW) and control lambs which were infected at Day 0 with 50,000 L_3 *O. circumcincta* and at Days 35, 42, 49, 56, 63 and 70 with 10,000 L_3 *O. circumcincta* and killed on Day 94 after infection. a: killed on Day 62; b: killed on Day 64.

Group	Lamb	L_3	L_4	I.A.	Female	Male	Total
Control	#1	0	0	10	0	0	10
	#2	0	10	10	10	0	30
	#3	0	40	360	240	250	890
	#4 ^b	140	20	50	20	40	270
HFW	#5	0	40	360	430	190	1020
	#6	0	0	10	50	10	70
	#7 ^a	1640	1100	840	360	290	4230
	#8	0	0	10	30	0	40

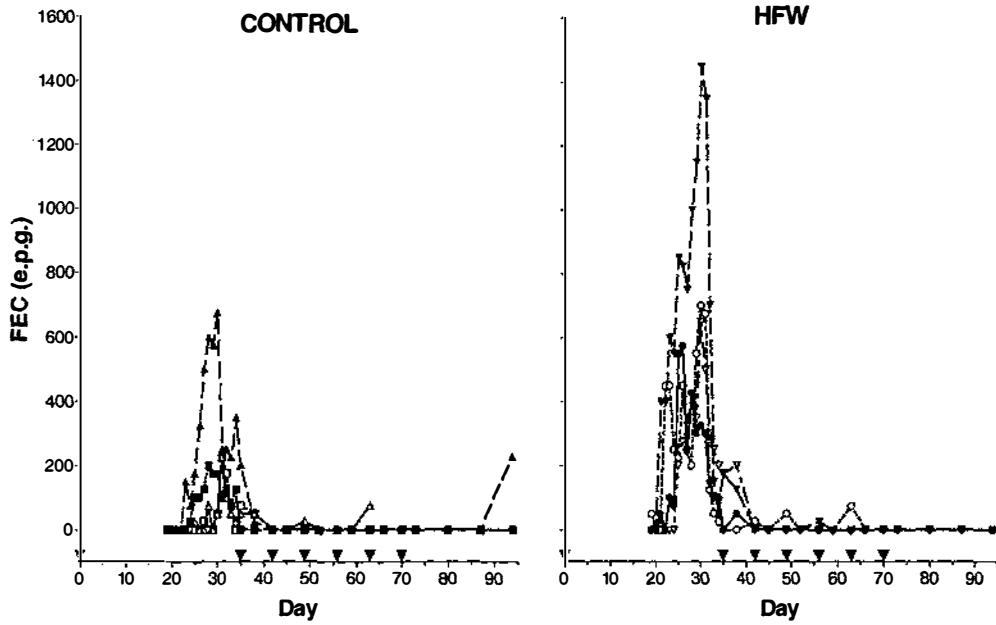


Figure 2.11. Faecal egg counts in individual lambs after infection at Day 0 with 50,000 L₃ *O. circumcincta* and at Days 35, 42, 49, 56, 63 and 70 with 10,000 L₃ *O. circumcincta*. Left panel: lambs in control (C) group; right panel: lambs in high fleece weight (HFW) group. Lamb #1: ■; #2: □; #3: ▲; #4: △; #5: ●; #6: ○; #7: ▼; #8: ▽; administration of L₃: ▽.

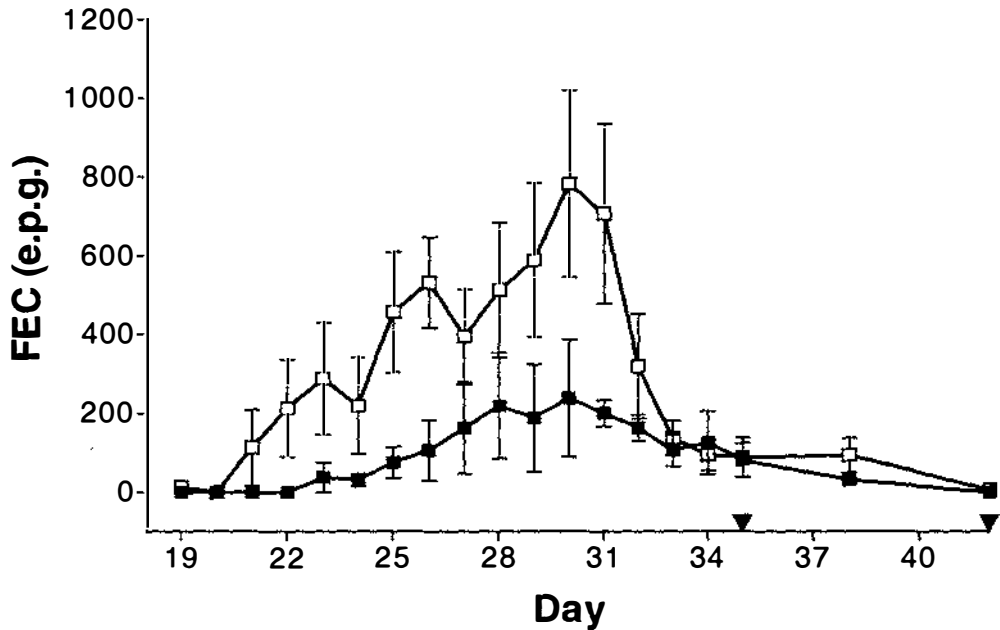


Figure 2.12. Faecal egg counts (mean \pm SEM) in high fleece weight (HFW) and control (C) lambs which were infected at Day 0 with 50,000 L₃ *O. circumcincta* and at Days 35, 42, 49, 56, 63 and 70 with 10,000 L₃ *O. circumcincta*. HFW: □; C: ■; administration of L₃: ▽.

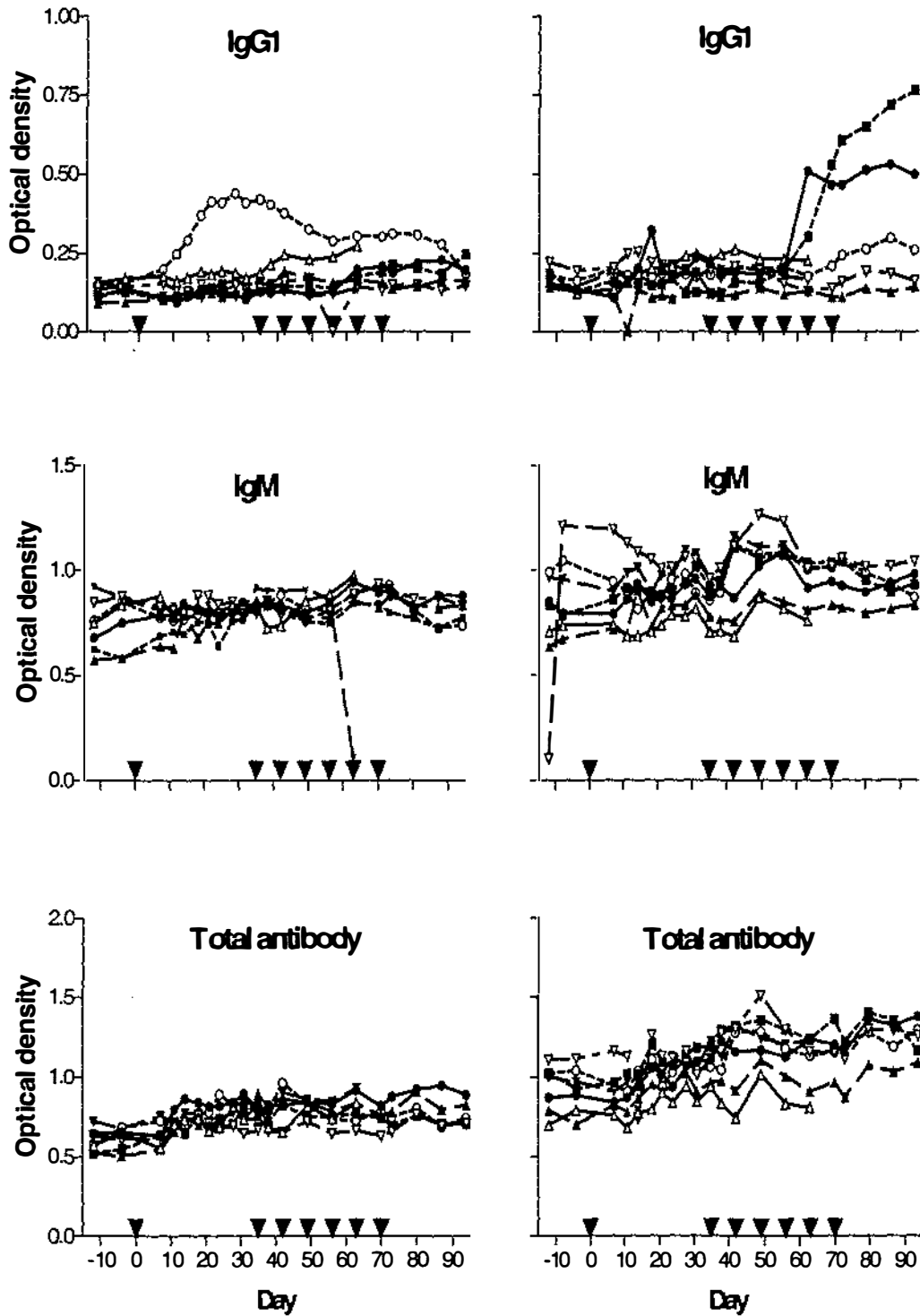


Figure 2.10. IgG1, IgM and total immunoglobulins specific for adult and L₃ *O. circumcincta* in individual lambs after infection at Day 0 with 50,000 L₃ *O. circumcincta* and at Days 35, 42, 49, 56, 63 and 70 with 10,000 L₃ *O. circumcincta*. Left panel: adult worm antigens; right panel: L₃ antigens. Lamb #1: ■; #2: □; #3: ▲; #4: △; #5: ●; #6: ○; #7: ▼; #8: ▽; administration of L₃: ▼.

#6, particularly after administration of L₃ on Day 63. By Day 80, 10 days after the last dose of L₃, all animals had low levels of blood eosinophils.

2.3.5 Serum Antibody Titres

Antibody titres for IgG1, IgM and total immunoglobulin specific for ES antigen derived from *O. circumcincta* larvae or adult worms showed only small increases after infection when tested within serum diluted 1/2000. Therefore, serum dilutions of 1/100 were used to determine IgG1, IgM and total antibody levels against larval and adult ES antigen. However, responses were still weak and there were no consistent trends or group differences (Figure 2.10). Antibody titres for IgG2 and IgA, at a dilution of 1/20, showed no responses to infection.

2.3.6 Faecal Egg Count

Eggs were first detected in the faeces of one HFW sheep (#6) on Day 19 and in the faeces of all four animals by Day 25, whereas the earliest detection of eggs in a control sheep (#3) was on Day 23 and eggs were found in the faeces of all four animals only by Day 30 (Figure 2.11). Cumulative eggs shed were significantly higher in the HFW sheep ($p < 0.001$) (Figure 2.12).

All lambs in the HFW group shed eggs between Days 25-34, while all control animals shed eggs only between Days 30-33 (Figure 2.11). The highest FEC of 1450 e.p.g was recorded in Sheep #7 (HFW) and the other HFW animals had maximum FEC of 500-700 e.p.g. The maximum FEC in control sheep was 675 e.p.g in Sheep #3 and in the other three sheep the peak values did not exceed 250 e.p.g. Eggs were detected only in the faeces of a few sheep and in very small numbers from Day 42 onward. There were no significant correlations between FEC (Days 19-94) and either blood eosinophil count or serum pepsinogen concentration in either C or HFW group.

2.3.7 Worm Counts

Worm counts are recorded in Table 2.2. There was no difference in worm counts between groups. By Day 94, worm counts were generally low in both

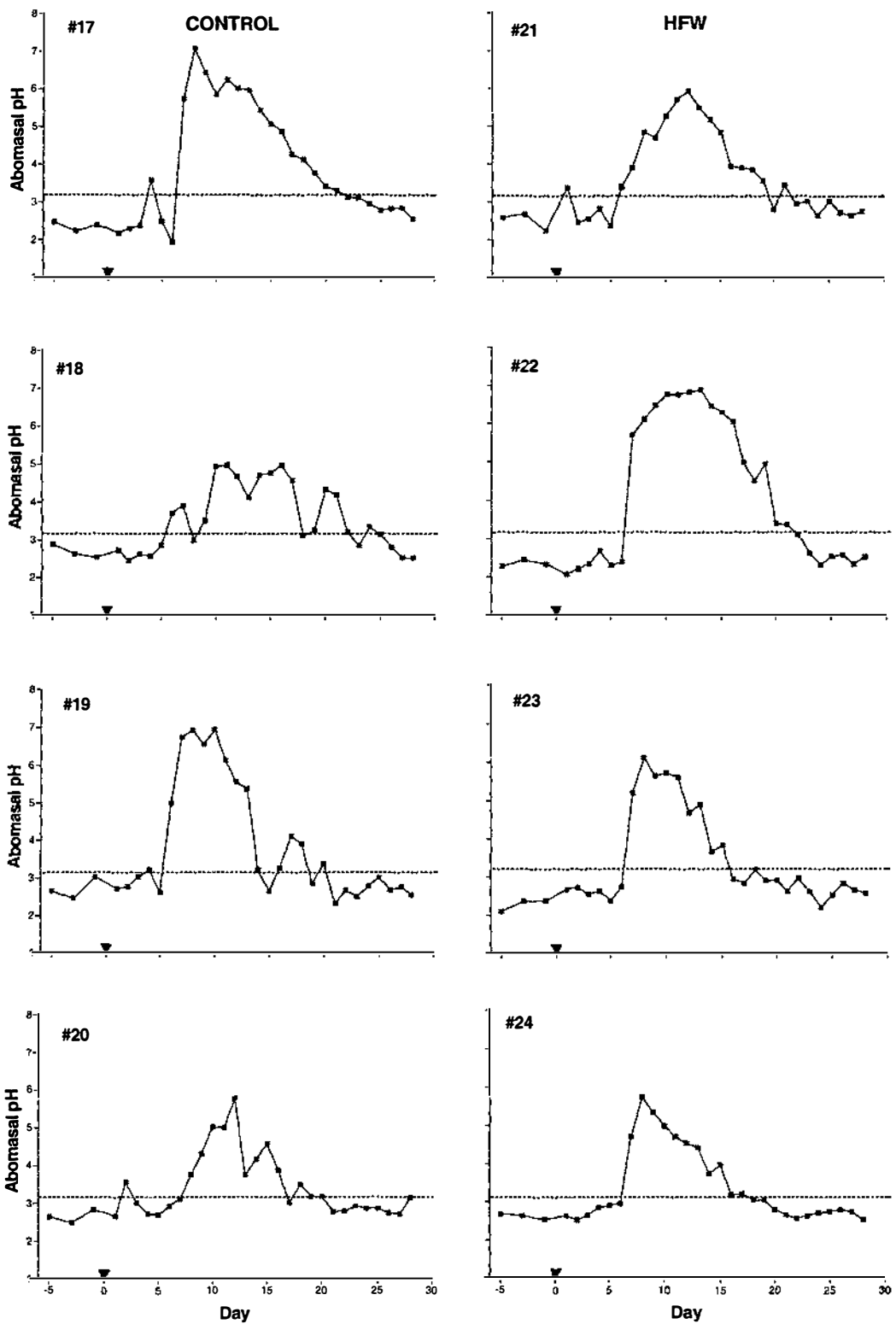


Figure 2.17. Abomasal pH in individual lambs after infection at Day 0 with 50,000 L₃ *O. circumcincta*. Left panel: lambs in control (C) group; right panel: lambs in high fleece weight (HFW) group. Administration of L₃: ▼. The horizontal dotted lines represent 2SDs above the mean value determined from uninfected lambs (pH = 3.20).

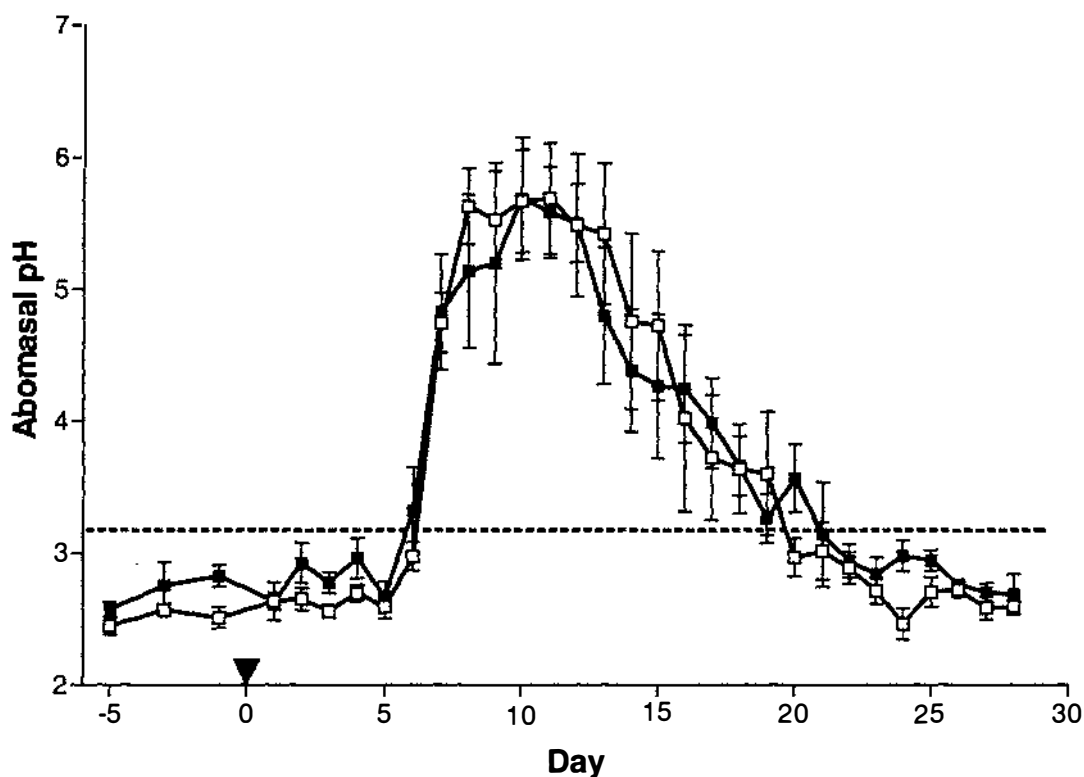


Figure 2.15. Abomasal pH (mean \pm SEM) in high fleece weight (HFW) and control (C) lambs which were infected at Day 0 with 50,000 L₃ *O. circumcincta*. HFW: \square ; C: \blacksquare ; administration of L₃: \blacktriangledown . The horizontal dotted line represents 2SDs above the mean value determined from uninfected lambs (pH = 3.20).

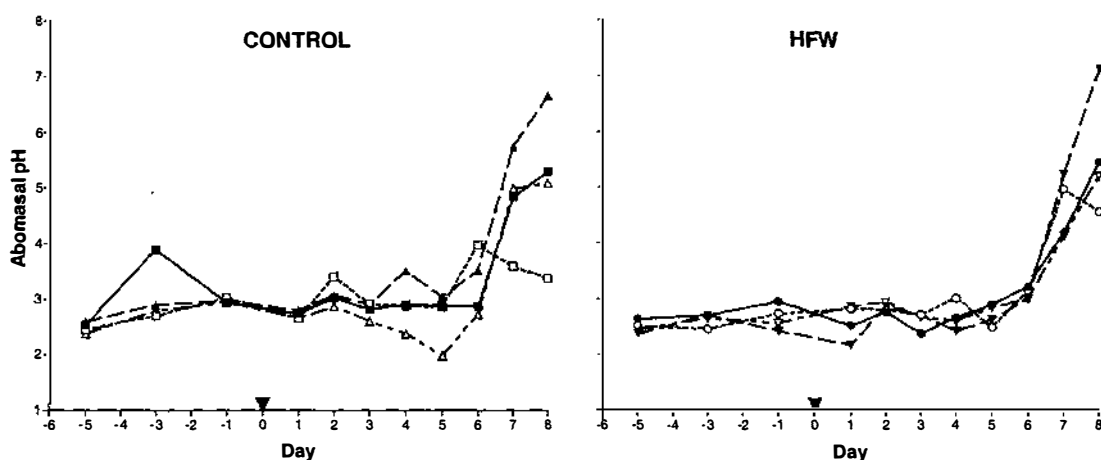


Figure 2.16. Abomasal pH in individual lambs after infection at Day 0 with 50,000 L₃ *O. circumcincta*. Left panel: lambs in control (C) group; right panel: lambs in high fleece weight (HFW) group. Lamb #9: \blacksquare ; #10: \square ; #11: \blacktriangle ; #12: \triangle ; #13: \bullet ; #14: \circ ; #15: \blacktriangledown ; #16: \triangledown ; administration of L₃: \blacktriangledown .

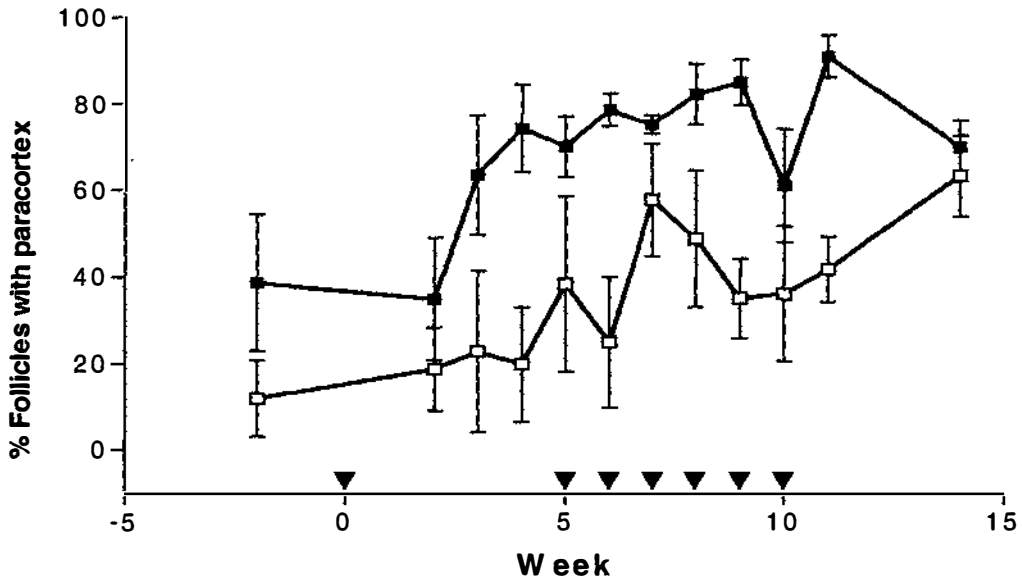


Figure 2.13. Percentage of wool follicles containing paracortex (mean \pm SEM) in high fleece weight (HFW) and control (C) lambs which were infected at Day 0 with 50,000 L₃ *O. circumcincta* and at Days 35, 42, 49, 56, 63 and 70 with 10,000 L₃ *O. circumcincta*. HFW: \square ; C: \blacksquare ; administration of L₃: \blacktriangledown .

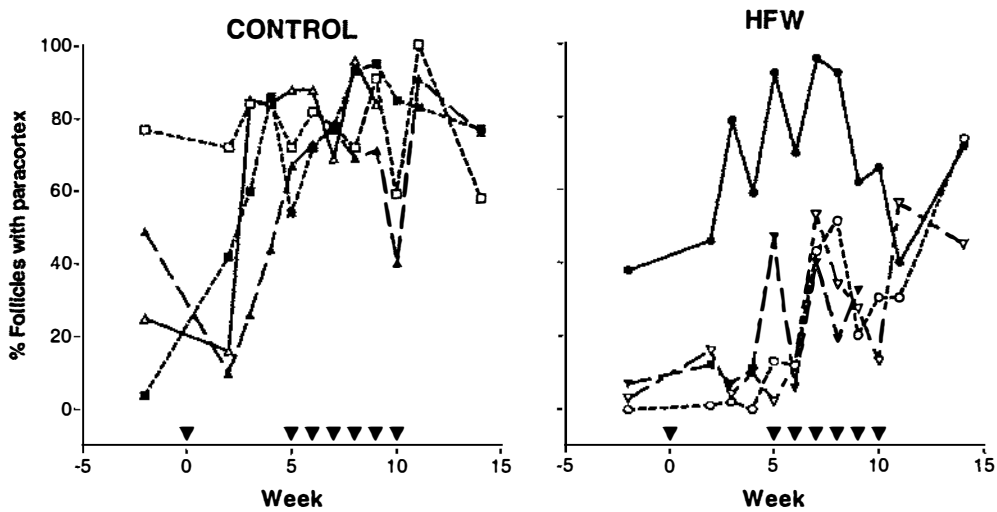


Figure 2.14. Percentage of wool follicles containing paracortex in individual lambs after infection at Day 0 with 50,000 L₃ *O. circumcincta* and at Days 35, 42, 49, 56, 63 and 70 with 10,000 L₃ *O. circumcincta*. Left panel: lambs in control (C) group; right panel: lambs in high fleece weight (HFW) group. Lamb #1: \blacksquare ; #2: \square ; #3: \blacktriangle ; #4: \triangle ; #5: \bullet ; #6: \circ ; #7: \blacktriangledown ; #8: \blacktriangledown ; administration of L₃: \blacktriangledown .

groups. The highest worm count of 4230 was obtained from sheep #7 and consisted mainly of larval stages (L₃ and L₄). In all remaining sheep, there were worm counts of fewer than 1020 parasites in the abomasal contents and digests.

2.3.8 Wool Follicles

Prior to infection, 12% of wool follicles in HFW animals contained paracortical cells, compared with 39% in group C animals (Figure 2.13). Parasitism caused a rapid increase in the proportion of follicles containing paracortex in the C group, but a more gradual increase in the HFW group, reaching similar values by 14 weeks p.i. Over the whole period, fewer wool follicles in HFW animals contained paracortex than in the C group ($p < 0.001$). There were significant differences between the groups at Weeks 4 and 6 ($p < 0.05$) and at Weeks 9 and 11 ($p < 0.01$) and was approaching significance at Week 8 ($p = 0.058$).

Individual sheep within each group showed similar effects of parasitism, with the exception of #5 (HFW), whose wool follicles before and after infection resembled sheep from the C group (Figure 2.14). Sheep #2 had an unusually high proportion of wool follicles containing paracortex before infection, but similar data after infection to other sheep in the C group. Visual assessment of the proportion of the follicle occupied by paracortex was made independently by two observers to be 0-10% in the HFW and 10-20% in the C groups respectively pre-infection, increasing with time to 50-70% of the follicles in both groups at the end of the experiment.

2.4 Results: Experiment 2

2.4.1 Abomasal pH

There were no significant differences between the mean abomasal pH for HFW and C groups overall, or at any time point (Figure 2.15). The abomasal pH in most individual lambs was raised by Day 7 (Figure 2.16 and 2.17), as were the group means. Sheep #10 was the only one of the eight lambs killed on Day 8 p.i. not to show a markedly elevated abomasal pH. Of the eight animals

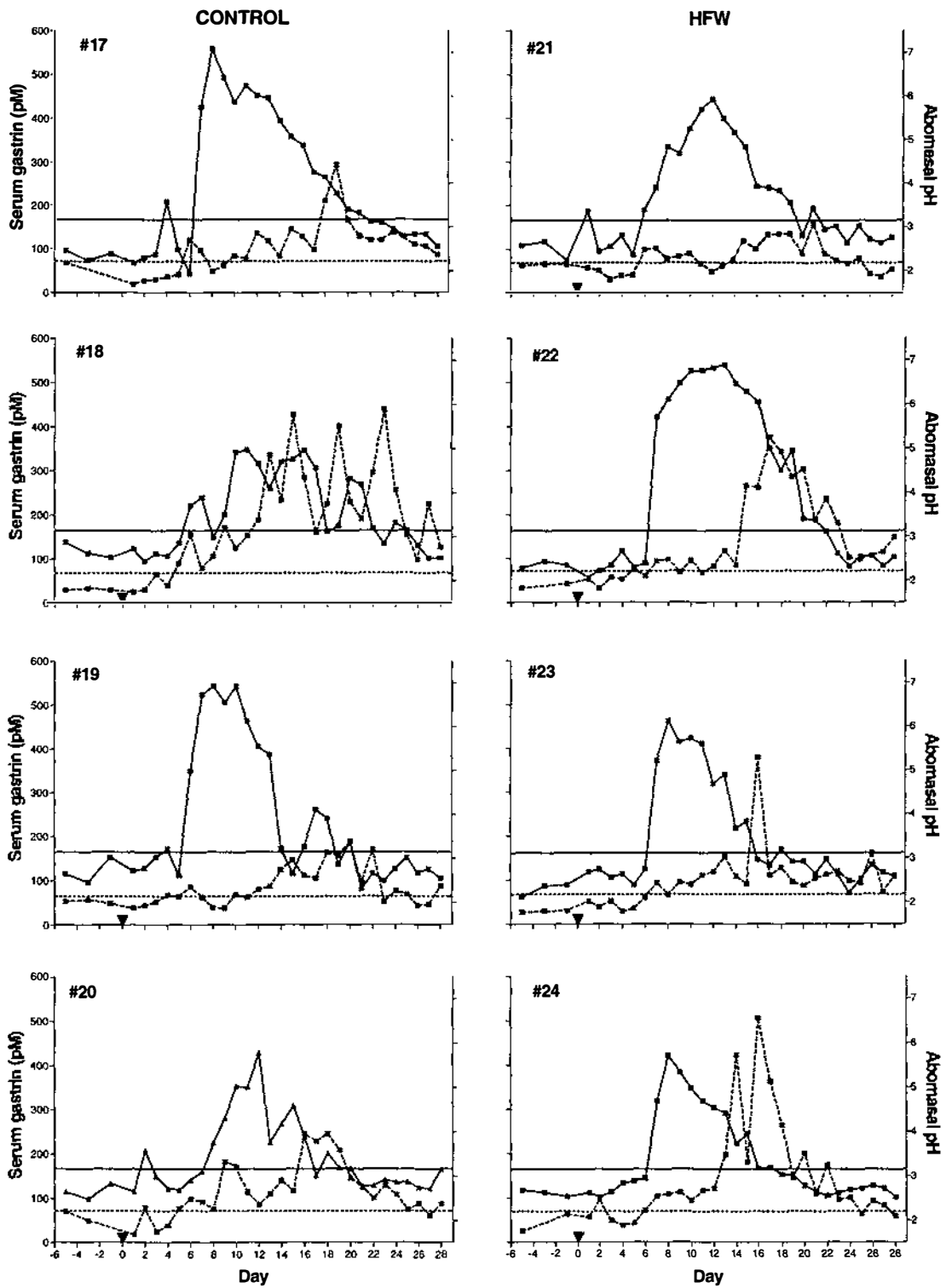


Figure 2.20. Abomasal pH and serum gastrin concentration in individual lambs after infection at Day 0 with 50,000 L₃ *O. circumcincta*. Left panel: lambs in control (C) group; right panel: lambs in high fleece weight (HFW) group; abomasal pH: — ; serum gastrin concentration ---- ; administration of L₃: ▼. The horizontal dotted lines represent 2SDs above the mean value for gastrin (69 pM) and the horizontal solid lines represent 2SDs above the mean value for pH (3.20), both determined from uninfected lambs.

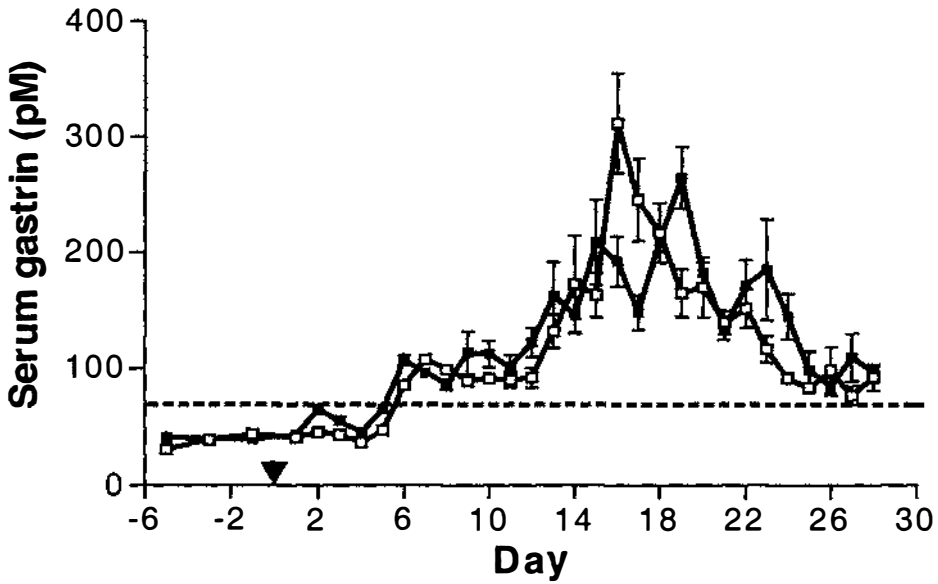


Figure 2.18. Serum gastrin concentration (mean \pm SEM) in high fleece weight (HFW) and control (C) lambs which were infected at Day 0 with 50,000 L₃ *O. circumcincta*. HFW: \square ; C: \blacksquare ; administration of L₃: \blacktriangledown . The horizontal dotted line represents 2SDs above the mean value determined from uninfected lambs (69pM).

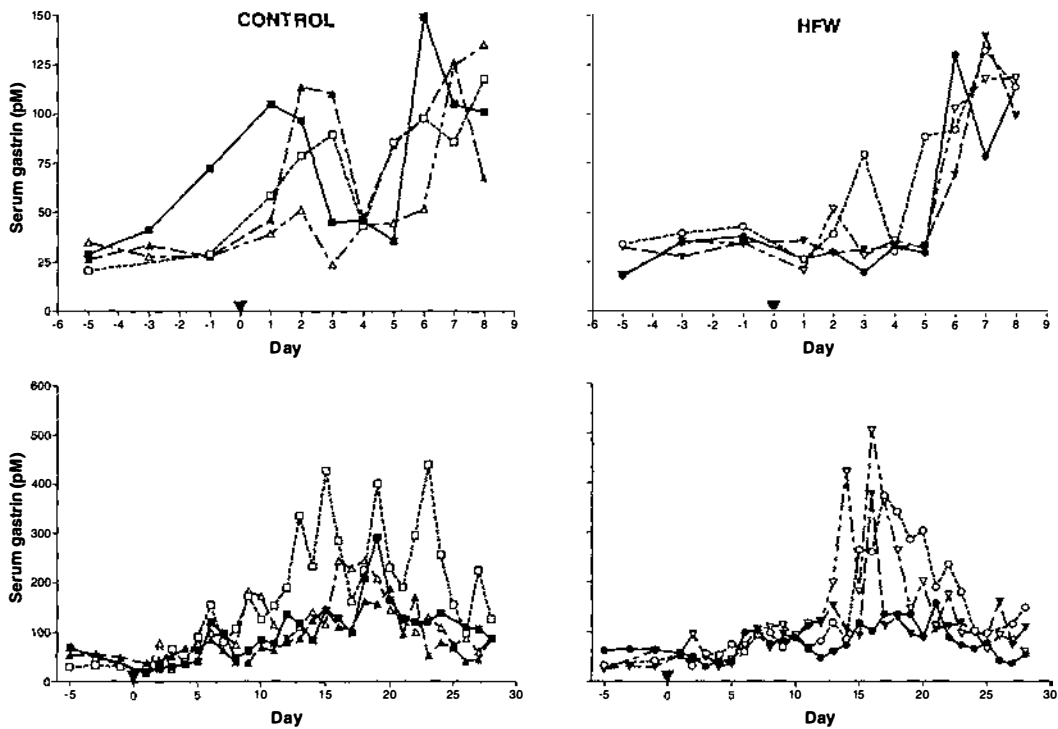


Figure 2.19. Serum gastrin concentration in individual lambs after infection at Day 0 with 50,000 L₃ *O. circumcincta*. Left panel: lambs in control (C) group; right panel: lambs in high fleece weight (HFW) group; top: lambs killed on Day 8 p.i.; bottom: lambs killed on Day 28 p.i. Top: lamb #9: \blacksquare ; #10: \square ; #11: \blacktriangle ; #12: \triangle ; #13: \bullet ; #14: \circ ; #15: \blacktriangledown ; #16: \triangledown . Bottom: lamb #17: \blacksquare ; #18: \square ; #19: \blacktriangle ; #20: \triangle ; #21: \bullet ; #22: \circ ; #23: \blacktriangledown ; #24: \triangledown ; administration of L₃: \blacktriangledown . The horizontal dotted lines represent 2SDs above the mean value determined from uninfected lambs (69pM).

studied for 28 days p.i., two control (#18 and #20) and one HFW (#21) lamb were unusual in not showing a very abrupt increase in abomasal pH, but instead a steady increase to Day 10 p.i. Unrelated to their grouping, individual lambs had sustained peak values above pH 4.5 (#17, #19, #21, #22 and #23) for at least 7 days or for a maximum period of 4 days (#18, #20, #24). Abomasal pH was in the normal range by at least Day 25 and earlier in several sheep (Figure 2.17).

2.4.2 Serum Gastrin

There were no differences in mean serum gastrin concentration between HFW and C groups either overall for the whole duration of the experiment or on individual days (Figure 2.18). In individual lambs, elevated serum gastrin levels were observed the first time between Days 6-8 and peak values occurred between Days 13 and 23 (Figure 2.19). In three animals (#9, #10 and #11) serum gastrin concentration was clearly elevated on Day 2 or 3, decreased, before subsequently increasing again. In several other animals, a smaller rise also occurred at this time. Substantial increases in serum gastrin concentration occurred mainly during the second half of the infection from about Days 14-18 (Figure 2.20). There was only moderate hypergastrinaemia in lambs #19 and #21 and serum gastrin concentration had returned within normal limits in lambs #19, #20 and #24 before euthanasia on Day 28. In the other animals, serum gastrin remained elevated.

A comparison of the time courses of the changes in serum gastrin concentration with those in abomasal pH in sheep #17-24 showed that in six of the eight animals the marked early peaks in abomasal pH were not accompanied by similar peaks in serum gastrin concentration (Figure 2.20). In these sheep, the major elevation in serum gastrin coincided with the decline in abomasal pH to about pH 5. Sheep #18 (and to a lesser extent Sheep #20) was exceptional in the closely associated changes in the two parameters during the early period after infection.

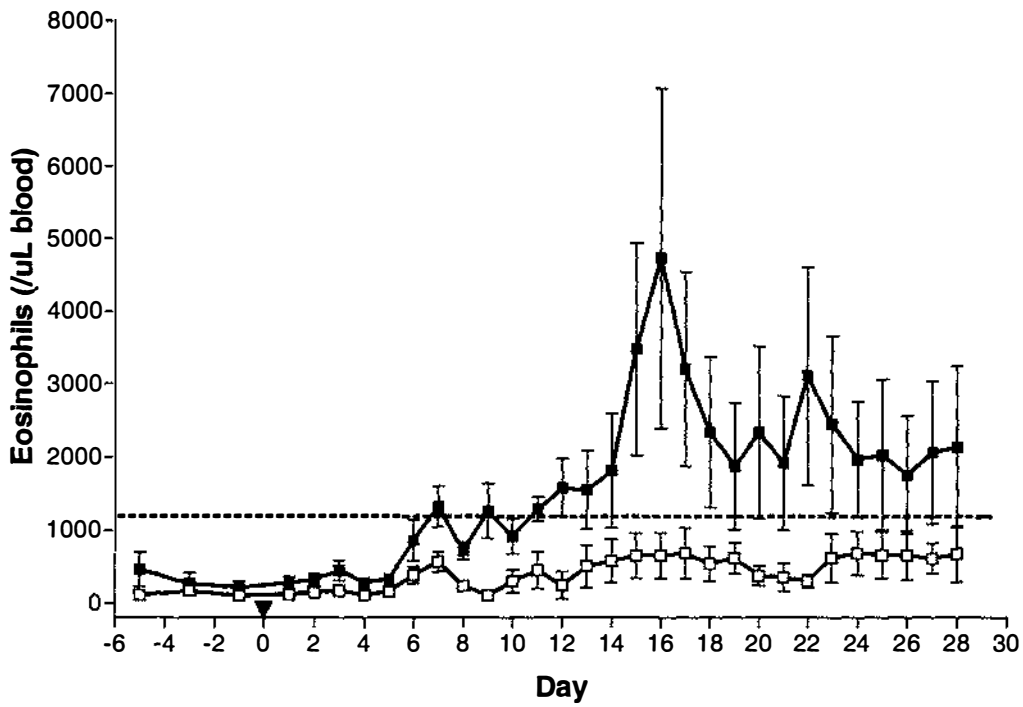


Figure 2.23. Blood eosinophil counts (mean \pm SEM) in high fleece weight (HFW) and control (C) lambs which were infected at Day 0 with 50,000 L₃ *O. circumcincta*. HFW: \square ; C: \blacksquare ; administration of L₃: \blacktriangledown . The horizontal dotted line represents 2SDs above the mean value determined from uninfected lambs (1176cells/ μ l).

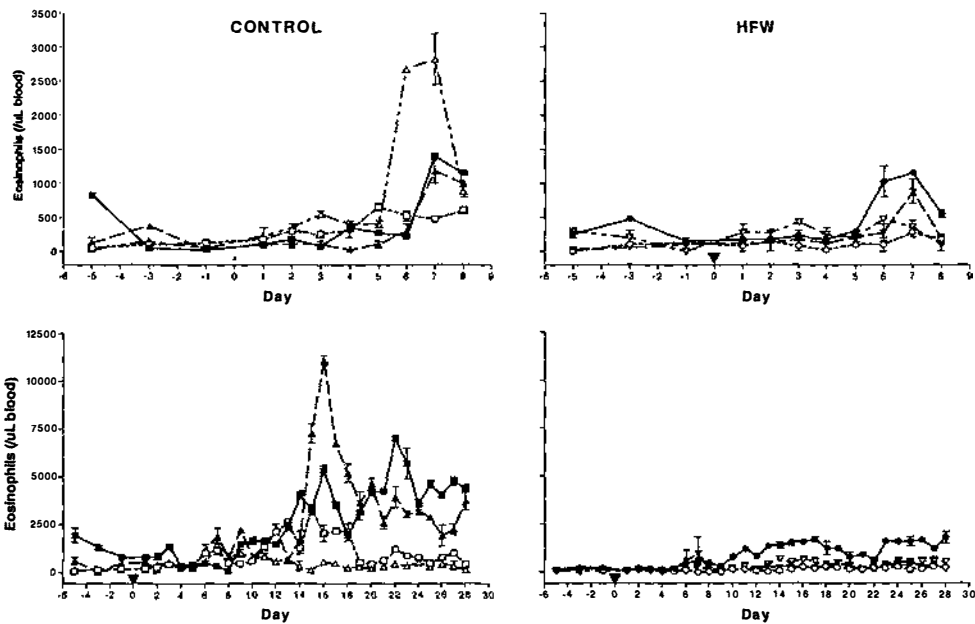


Figure 2.24. Blood eosinophil counts in individual lambs after infection at Day 0 with 50,000 L₃ *O. circumcincta*. Left panel: lambs in control (C) group; right panel: lambs in high fleece weight (HFW) group; top: lambs killed on Day 8 p.i.; bottom: lambs killed on Day 28 p.i. Top: lamb #9: \blacksquare ; #10: \square ; #11: \blacktriangle ; #12: \triangle ; #13: \bullet ; #14: \circ ; #15: \blacktriangledown ; #16: \triangledown . Bottom: lamb #17: \blacksquare ; #18: \square ; #19: \blacktriangle ; #20: \triangle ; #21: \bullet ; #22: \circ ; #23: \blacktriangledown ; #24: \triangledown ; administration of L₃: \blacktriangledown .

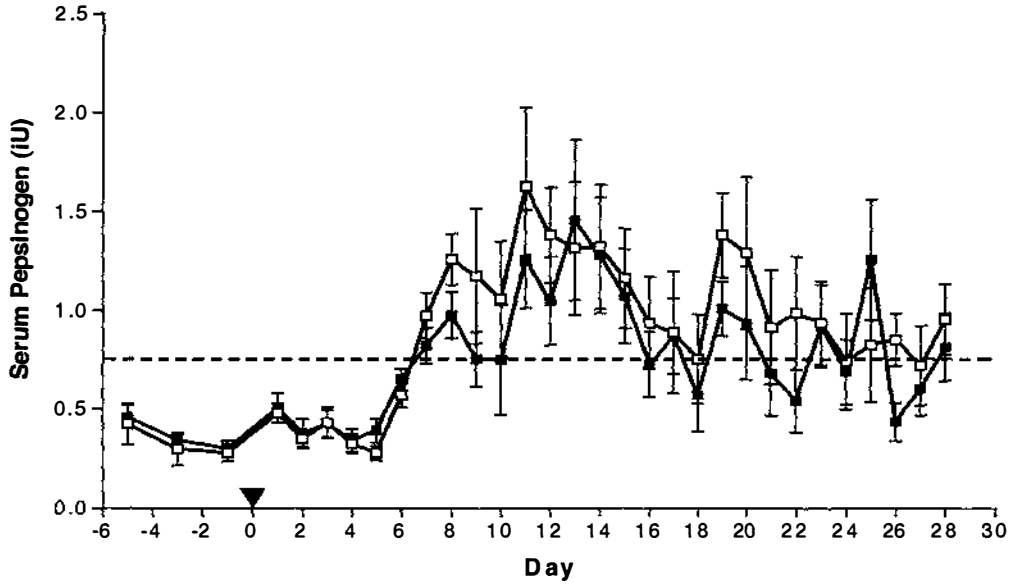


Figure 2.21. Serum pepsinogen concentration (mean \pm SEM) in high fleece weight (HFW) and control (C) lambs which were infected at Day 0 with 50,000 *L*₃ *O. circumcincta*. HFW: \square ; C: \blacksquare ; administration of *L*₃: \blacktriangledown . The horizontal dotted line represents 2SDs above the mean value determined from uninfected lambs (0.75iU).

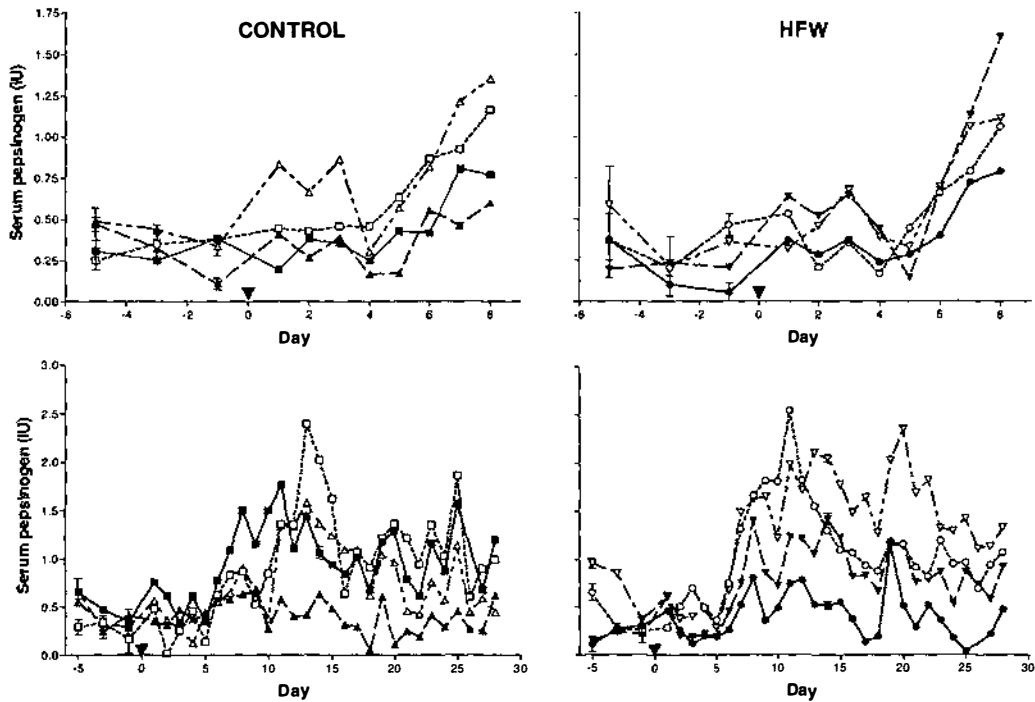


Figure 2.22. Serum pepsinogen concentration in individual lambs after infection at Day 0 with 50,000 *L*₃ *O. circumcincta*. Left panel: lambs in control (C) group; right panel: lambs in high fleece weight (HFW) group; top: lambs killed on Day 8 p.i.; bottom: lambs killed on Day 28 p.i Top: lamb #9: \blacksquare ; #10: \square ; #11: \blacktriangle ; #12: \triangle ; #13: \bullet ; #14: \circ ; #15: \blacktriangledown ; #16: \triangledown . Bottom: lamb #17: \blacksquare ; #18: \square ; #19: \blacktriangle ; #20: \triangle ; #21: \bullet ; #22: \circ ; #23: \blacktriangledown ; #24: \triangledown ; administration of *L*₃: \blacktriangledown .

2.4.3 Serum Pepsinogen

There were no differences in mean serum pepsinogen concentration between HFW and C groups either overall for the whole duration of the experiment or on individual days (Figure 2.21). Maximum serum pepsinogen levels seen in both groups in Experiment 2 were similar to those in sheep in the control group, not the HFW group, in Experiment 1 (Figure 2.6).

The 16 sheep had widely differing levels after infection (Figure 2.22) and two sheep (#19 and #21) could be considered as low-responders. Even in these sheep there were small increases in serum pepsinogen concentration at the times of marked changes in the other animals. Generally, there were elevated levels by Day 7 or 8, peak values around Days 11-14 and an abrupt decrease on Day 18, rising again the following day (Figure 2.21). Serum pepsinogen concentration remained elevated until the experiment was terminated on Day 28 p.i.

Serum pepsinogen and abomasal pH were weakly correlated in the HFW group ($r^2=0.22$, $p<0.001$) and in the C group ($r^2=0.16$, $p<0.001$).

2.4.4 Blood Eosinophils

Over Days 0-8 p.i., the group mean blood eosinophil count was higher in group C than in the HFW group ($p<0.05$) and was approaching significance for the period Days 9-28 p.i. ($p=0.058$) (Figure 2.23). Mean blood eosinophil counts were lower in the HFW than C group on Days 1, 5, 9-11 and 22 ($p<0.05$) and was approaching significance on Day 12 p.i. ($p=0.059$). The counts in individual sheep (Figure 2.24) showed marked variation between animals in the C group, but relatively uniform low levels in the HFW group. Some sheep had increasing counts as early as Day 1-2 p.i., there was a peak from Days 5-8 in most animals and the highest counts were seen from Day 12 onward. Sheep #17 and #19 had the highest counts from group C and Sheep #21 had the highest counts in the HFW group from Day 11 onwards. Blood eosinophil count and serum pepsinogen concentration were correlated only in sheep from group C ($r^2=0.05$, $p<0.01$). There was no correlation between blood eosinophil count and tissue eosinophil density (data in Chapter 3).

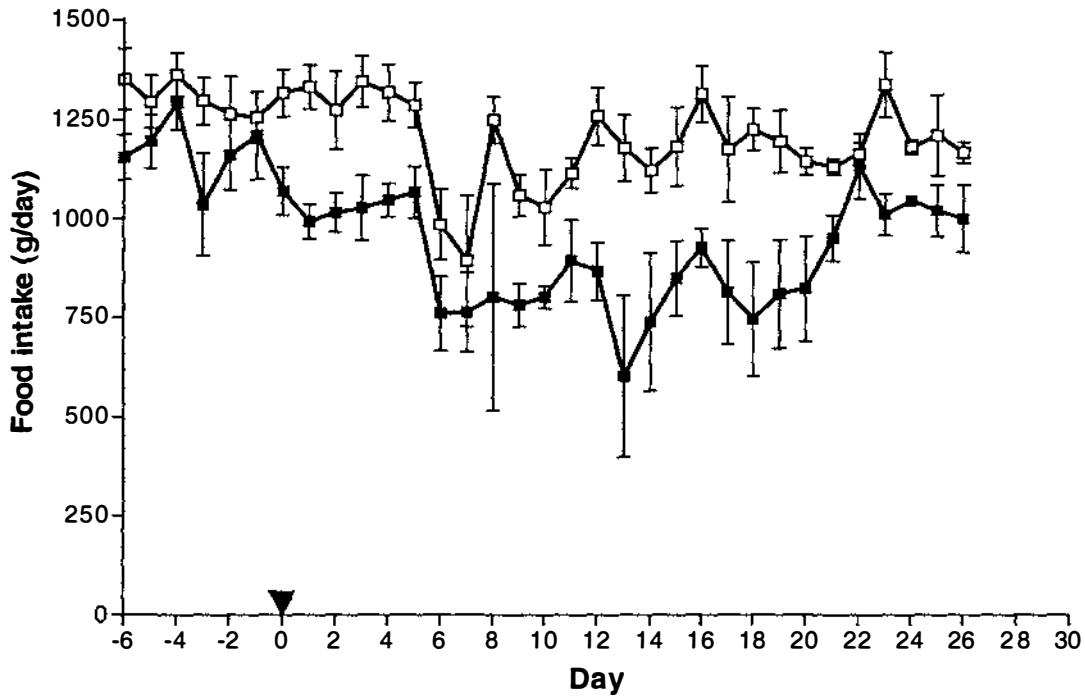


Figure 2.28. Daily food intake (mean \pm SEM) in high fleece weight (HFW) and control (C) lambs which were infected at Day 0 with 50,000 L₃ *O. circumcincta*. HFW: \square ; C: \blacksquare ; administration of L₃: \blacktriangledown . Data from HFW #24 omitted.

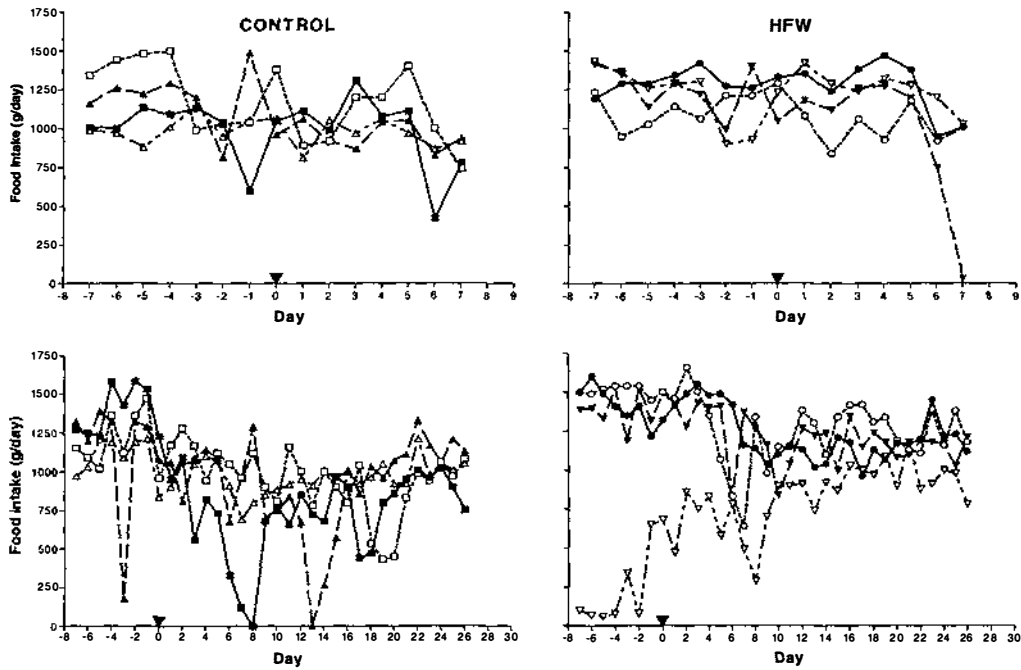


Figure 2.29. Daily food intake in individual lambs after infection at Day 0 with 50,000 L₃ *O. circumcincta*. Left panel: lambs in control (C) group; right panel: lambs in high fleece weight (HFW) group; top: lambs killed on Day 8 p.i.; bottom: lambs killed on Day 28 p.i. Top: lamb #9: \blacksquare ; #10: \square ; #11: \blacktriangle ; #12: \triangle ; #13: \bullet ; #14: \circ ; #15: \blacktriangledown ; #16: \triangledown . Bottom: lamb #17: \blacksquare ; #18: \square ; #19: \blacktriangle ; #20: \triangle ; #21: \bullet ; #22: \circ ; #23: \blacktriangledown ; #24: \triangledown ; administration of L₃: \blacktriangledown .

Table 2.3. Worm counts (L_3 , L_4 , immature adults (I.A.), adult female worms and adult male worms, eggs *in utero* per female worm (mean \pm SEM) and total worms) in high fleece weight (HFW) and control lambs, which were infected with 50,000 L_3 *O. circumcincta* and killed either on Day 8 or Day 28 after infection.

Group	Lamb	L_3	L_4	I.A.	Female	Male	Total	Eggs <i>in utero</i>
Control Day 8	#9	90	12,720	790	0	0	13,600	
	#10	70	21,950	930	0	0	22,950	
	#11	20	10,610	130	0	0	10,760	
	#12	0	16,590	330	0	0	16,920	
HFW Day 8	#13	220	15,360	710	20	0	16,310	
	#14	20	17,730	650	0	0	18,400	
	#15	0	19,530	120	0	0	19,650	
	#16	40	20,970	640	0	0	21,650	
Control Day 28	#17	0	140	150	1,090	820	2,200	28.7 \pm 1.38
	#18	0	170	540	2,680	1,950	5,340	17.8 \pm 1.28
	#19	0	100	40	210	280	630	34.3 \pm 1.82
	#20	0	10	80	940	650	1,680	28.4 \pm 1.36
HFW Day 28	#21	0	70	50	780	610	1,510	30.8 \pm 1.56
	#22	0	40	30	570	380	1,020	34.6 \pm 1.26
	#23	0	100	60	1,130	800	2,090	26.4 \pm 1.33
	#24	0	130	320	1,940	1,110	3,500	8.7 \pm 0.95

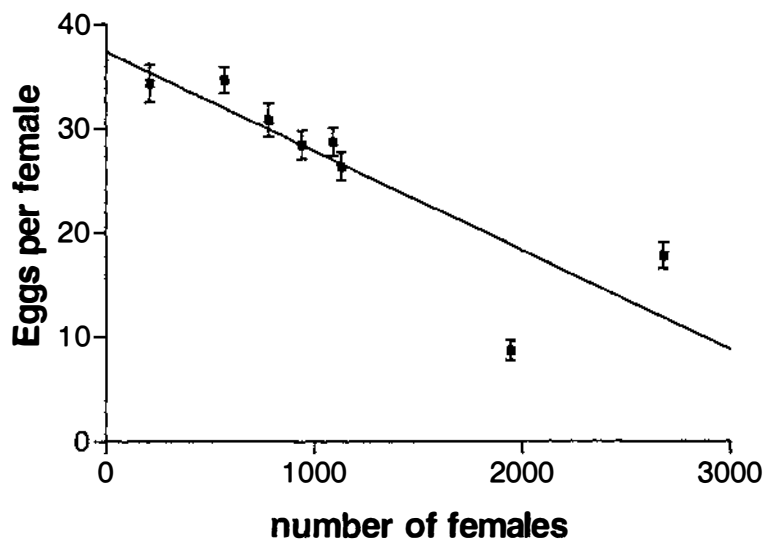


Figure 2.27. Relationship between number of eggs *in utero* per female worm (mean \pm SEM) and number of female worms in both high fleece weight (HFW) and control lambs, which were infected with 50,000 L_3 *O. circumcincta* and killed on Day 28 after infection. The calculated correlation coefficient is $r^2=0.66$, $p<0.01$.

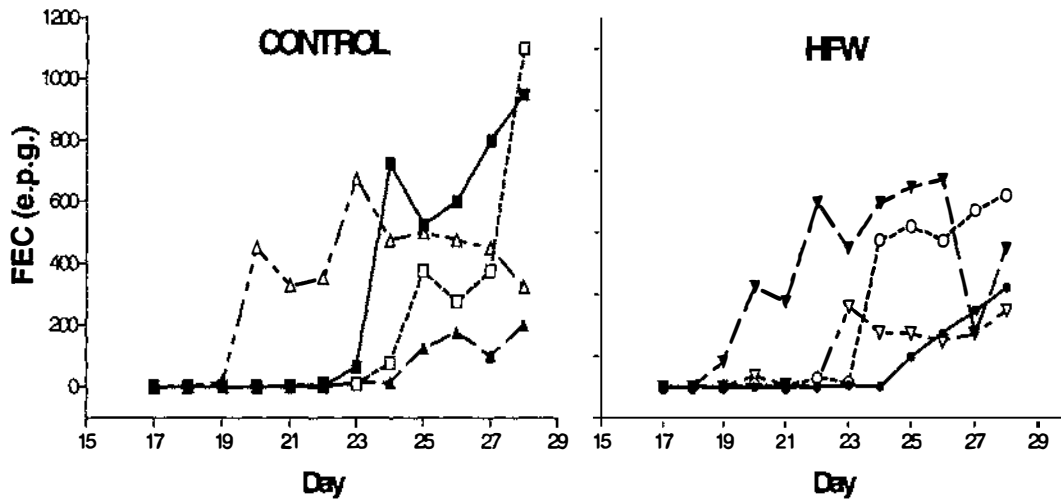


Figure 2.25. Faecal egg counts in individual lambs after infection at Day 0 with 50,000 L₃ *O. circumcincta*. Left panel: lambs in control (C) group; right panel: lambs in high fleece weight (HFW) group. Lamb #17: ■; #18: □; #19: ▲; #20: △; #21: ●; #22: ○; #23: ▼; #24: ▽; administration of L₃: ▼.

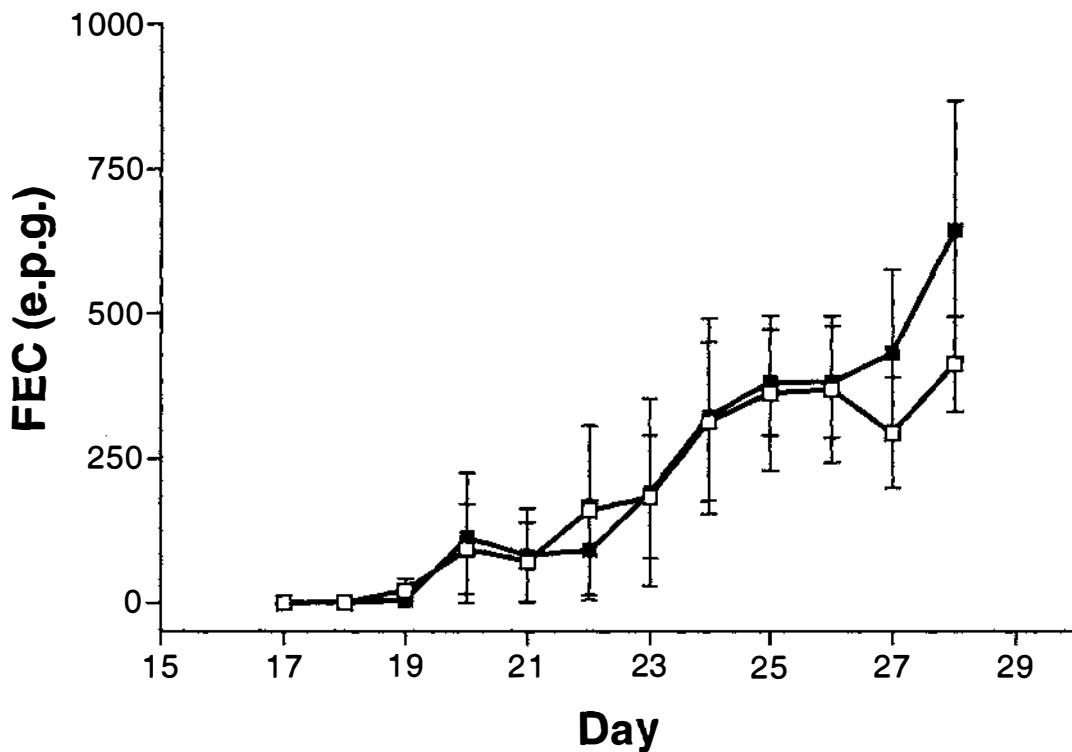


Figure 2.26. Faecal egg counts (mean \pm SEM) in high fleece weight (HFW) and control (C) lambs which were infected at Day 0 with 50,000 L₃ *O. circumcincta*. HFW: □; C: ■; administration of L₃: ▼.

2.4.5 Faecal Egg Count

Eggs were first detected in the faeces of one sheep in each group (#20 and #23) by Day 20 and in the faeces of the other sheep by Days 23-24 (Figure 2.25). FEC continued to rise in most animals until the end of the experiment on Day 28 p.i., except in Sheep #20 and #23. There was no difference in FEC between the two groups (Figure 2.26). FEC was not significantly correlated with either serum pepsinogen concentration or blood eosinophil counts.

2.4.6 Worm Counts

Worm counts are presented in Table 2.3. On Day 8, most parasites were L₄ and numbers did not significantly differ between groups (HFW: $\mu \pm \text{SEM} = 19000 \pm 1120$; C: $\mu \pm \text{SEM} = 16060 \pm 2620$). The HFW as well as control lambs had significantly higher counts on Day 8 than on Day 28 ($p < 0.001$). On Day 28, there were mainly mature female and male parasites and no significant differences between the two groups (HFW: $\mu \pm \text{SEM} = 2055 \pm 529$; C: $\mu \pm \text{SEM} = 2463 \pm 1013$). *In utero* egg counts in Day 28 female worms were similar for females harvested from HFW ($\mu \pm \text{SEM} = 24.58 \pm 5.69$) and control lambs ($\mu \pm \text{SEM} = 27.51 \pm 3.41$). *In utero* egg counts were negatively correlated with numbers of female worms (Figure 2.27). Worm counts were correlated with tissue eosinophil (discussed in Chapter 3) density in both groups ($r^2 = 0.55$, $p < 0.05$ for C and $r^2 = 0.73$, $p < 0.05$ for HFW). There was a strong correlation between worm counts and abomasal pH in HFW lambs ($r^2 = 0.77$; $p < 0.01$), but no such correlation in controls or across groups. Worm count was not correlated with serum pepsinogen concentration.

2.4.7 Food Intake

Data from Sheep #24 were omitted from the group mean and from the statistical analysis because this sheep became anorexic when it was placed in a metabolism crate, although its appetite returned slowly during the experiment. Food intake began decreasing in control lambs by Day 2, whereas HFW animals retained their appetite until Day 5. Both groups displayed a noticeable depression in food intake on Day 6 (Figures 2.28 and 2.29).

Table 2.6. Relative cytokine gene expression by abomasal lymph nodes collected from individual high fleece weight (HFW) and control (C) lambs, infected at Day 0 with 50,000 L₃ *O. circumcincta* and killed on Days 8 or 28. Cytokine production was measured before exposure to antigen (control) and after exposure to Concavalin A (conA) or to *O. circumcincta* L₃ antigen.

Sheep	Stimulant	IL-4	IL-5	IFN- γ	IL-13	TNF- α	IL-10
Day 8 C #9	Control	9.8	43.6	12.5	40.5	3.6	1.0
	ConA	33.8	29.4	43.0	126.2	2.8	12.5
	<i>O.c. Ag</i>	6.2	90.5	14.4	23.0	2.3	1.7
#10	Control	3.0	8.2	44.5	4.3	6.6	4.5
	ConA	38.3	9.4	13.2	12.3	3.6	12.8
	<i>O.c. Ag</i>	4.8	2.1	74.3	1.7	4.1	8.8
#11	Control	7.4	34.9	7.1	17.0	6.6	1.7
	ConA	47.8	79.3	11.4	141.5	5.6	12.8
	<i>O.c. Ag</i>	5.2	8.2	29.3	11.5	11.0	7.4
#12	Control	5.2	2.1	22.6	4.9	5.9	2.1
	ConA	20.5	11.0	2.0	20.0	1.4	5.4
	<i>O.c. Ag</i>	6.4	1.4	59.9	5.8	8.5	5.4
Day 8 HFW #13	Control	8.4	2.4	8.8	5.6	4.9	3.3
	ConA	13.5	12.5	8.6	13.8	17.3	2.9
	<i>O.c. Ag</i>	15.3	5.0	104.7	2.3	13.1	17.1
#14	Control	6.1	2.8	31.2	2.1	1.3	1.9
	ConA	41.8	2.0	1.4	3.1	0.9	8.2
	<i>O.c. Ag</i>	4.0	2.2	5.7	3.6	3.1	3.6
#15	Control	1.0	6.9	1.0	1.7	4.4	2.6
	ConA	21.4	17.4	1.7	14.1	3.2	6.2
	<i>O.c. Ag</i>	1.4	1.2	22.4	1.0	3.0	9.1
#16	Control	24.8	7.3	7.7	21.3	5.2	9.7
	ConA	18.7	31.9	31.1	12.7	4.8	2.1
	<i>O.c. Ag</i>	9.1	5.3	17.0	5.5	5.8	4.5
Day 28 C #17	Control	54.9	3.0	70.5	6.6	8.8	6.8
	ConA	22.8	4.0	2.6	3.1	3.2	5.9
	<i>O.c. Ag</i>	31.1	1.9	86.2	4.6	2.3	16.5
#18	Control	2.7	6.9	1.9	1.6	2.7	1.0
	ConA	15.6	2.1	1.0	1.0	1.3	7.7
	<i>O.c. Ag</i>	4.5	8.1	12.6	2.7	5.9	17.6
#19	Control	15.9	1.7	243.9	14.4	8.9	12.2
	ConA	64.0	8.2	7.2	8.8	7.1	18.6
	<i>O.c. Ag</i>	10.5	1.6	160.3	6.1	3.2	19.4
#20	Control	8.8	8.5	5.0	2.4	4.8	3.0
	ConA	23.9	1.0	1.4	2.3	4.5	11.5
	<i>O.c. Ag</i>	6.0	1.2	38.2	3.0	3.8	19.6
Day 28 HFW #21	Control	43.4	8.2	8.7	13.3	11.9	3.4
	ConA	32.7	3.3	3.5	2.3	4.5	11.0
	<i>O.c. Ag</i>	28.9	6.1	77.7	8.6	4.3	13.1
#22	Control	1.5	157.6	27.5	14.0	3.5	6.5
	ConA	27.9	2.0	4.5	3.3	3.5	5.8
	<i>O.c. Ag</i>	31.2	32.7	13.3	3.4	1.0	7.4
#23	Control	10.0	20.3	3.4	3.6	2.7	1.8
	ConA	28.2	2.2	1.4	2.2	2.3	9.5
	<i>O.c. Ag</i>	15.4	2.1	12.8	3.6	2.1	11.3
#24	Control	28.9	27.0	6.4	8.8	1.6	2.3
	ConA	36.4	3.1	2.2	2.6	3.7	8.8
	<i>O.c. Ag</i>	46.0	7.5	15.6	8.1	4.8	6.9

Table 2.5. Relative cytokine production (mean \pm SEM and median) by abomasal lymph nodes collected from high fleece weight (HFW) and control (C) lambs which were infected at Day 0 with 50,000 L₃ *O. circumcincta* and killed on Day 28. Cytokine production by lymph nodes was measured before exposure to antigen (control) and after exposure to Concavalin A (conA) or to *O. circumcincta* L₃ antigen. Group medians which differ significantly from the relevant control median ($p < 0.05$) are shown *.

Cytokine	Control (mean \pm SEM) (median) n=4		ConA-stimulated (mean \pm SEM) (median) n=4		<i>O. circumcincta</i> antigen-stimulated (mean \pm SEM) (median) n=4	
	C	HFW	C	HFW	C	HFW
IL-4	20.6 \pm 11.8 12.4	21.0 \pm 9.42 19.5	31.6 \pm 11.0 23.4	31.3 \pm 2.02 30.5	13.0 \pm 6.16 8.3	30.4 \pm 6.27 30.1
IL-5	5.03 \pm 1.60 5.0	53.3 \pm 35.0 23.7	3.8 \pm 1.58 3.1	2.7 \pm 0.32 2.7*	3.2 \pm 1.64 1.8	12.1 \pm 6.96 6.8
IFN- γ	80.3 \pm 56.8 37.8	11.5 \pm 5.44 7.6	3.1 \pm 1.42 12.3	2.9 \pm 0.69 2.0	74.3 \pm 32.5 62.2	29.9 \pm 16.0 14.5
IL-13	6.3 \pm 2.9 4.5	9.9 \pm 2.40 11.1	3.8 \pm 1.72 2.7	2.6 \pm 0.25 2.5*	4.1 \pm 0.79 3.8	5.9 \pm 1.40 5.9
TNF- α	6.3 \pm 1.53 6.8	4.9 \pm 2.36 3.1	4.0 \pm 1.22 3.9	3.5 \pm 0.45 3.6	3.8 \pm 0.76 3.5	3.1 \pm 0.90 3.2
IL-10	5.8 \pm 2.46 4.9	3.5 \pm 1.05 2.9	10.9 \pm 2.81 9.6	8.8 \pm 1.09 9.2	18.3 \pm 0.74 18.5*	9.7 \pm 1.51 9.4*

Table 2.4. Relative cytokine production (mean \pm SEM and median) by abomasal lymph nodes collected from high fleece weight (HFW) and control (C) lambs which were infected at Day 0 with 50,000 L₃ *O. circumcincta* and killed on Day 8. Cytokine production was measured before exposure to antigen (control) and after exposure to Concavalin A (conA) or to *O. circumcincta* L₃ antigen. Group medians which differ significantly from the relevant control median ($p < 0.05$) are shown *.

Cytokine	Control (mean \pm SEM) (median) n=4		ConA-stimulated (mean \pm SEM) (median) n=4		<i>O. circumcincta</i> antigen-stimulated (mean \pm SEM) (median) n=4	
	C	HFW	C	HFW	C	HFW
IL-4	6.4 \pm 1.46 6.3	10.1 \pm 5.15 7.3	35.1 \pm 5.67 36.1*	23.9 \pm 6.20 20.1	5.7 \pm 0.39 5.7	7.5 \pm 3.07 6.6
IL-5	22.2 \pm 10.1 21.6	4.9 \pm 1.30 4.9	32.3 \pm 16.3 20.2	16.0 \pm 6.21 15.0	25.6 \pm 21.7 5.2	3.4 \pm 1.02 3.6
IFN- γ	21.7 \pm 8.26 17.6	12.2 \pm 6.60 8.3	17.4 \pm 8.88 12.3	10.7 \pm 7.00 5.2	44.5 \pm 13.7 44.6	37.5 \pm 22.7 19.7
IL-13	16.7 \pm 8.46 40.5	7.7 \pm 4.63 3.9	75.0 \pm 34.2 73.1	10.9 \pm 2.63 13.3	10.5 \pm 4.63 8.7	3.1 \pm 0.96 3.0
TNF- α	5.7 \pm 0.71 6.6	4.0 \pm 1.80 3.0	3.4 \pm 0.88 3.2	6.6 \pm 3.67 3.4	6.5 \pm 2.00 6.3	6.3 \pm 2.37 4.5
IL-10	2.3 \pm 0.76 4.5	6.4 \pm 1.46 6.3	10.9 \pm 1.83 12.8*	4.9 \pm 1.43 4.6	5.8 \pm 1.54 6.4	8.6 \pm 3.09 6.8

Table 2.5. Relative cytokine gene expression (mean \pm SEM and median) by abomasal lymph nodes collected from high fleece weight (HFW) and control (C) lambs which were infected at Day 0 with 50,000 L₃ *O. circumcincta* and killed on Day 28. Cytokine production by lymph nodes was measured before exposure to antigen (control) and after exposure to Concavalin A (conA) or to *O. circumcincta* L₃ antigen. Group medians which differ significantly from the relevant control median ($p < 0.05$) are shown *.

Cytokine	Control (mean \pm SEM) (median) n=4		ConA-stimulated (mean \pm SEM) (median) n=4		<i>O. circumcincta</i> antigen-stimulated (mean \pm SEM) (median) n=4	
	C	HFW	C	HFW	C	HFW
IL-4	20.6 \pm 11.8 12.4	21.0 \pm 9.42 19.5	31.6 \pm 11.0 23.4	31.3 \pm 2.02 30.5	13.0 \pm 6.16 8.3	30.4 \pm 6.27 30.1
IL-5	5.03 \pm 1.60 5.0	53.3 \pm 35.0 23.7	3.8 \pm 1.58 3.1	2.7 \pm 0.32 2.7*	3.2 \pm 1.64 1.8	12.1 \pm 6.96 6.8
IFN- γ	80.3 \pm 56.8 37.8	11.5 \pm 5.44 7.6	3.1 \pm 1.42 12.3	2.9 \pm 0.69 2.0	74.3 \pm 32.5 62.2	29.9 \pm 16.0 14.5
IL-13	6.3 \pm 2.9 4.5	9.9 \pm 2.40 11.1	3.8 \pm 1.72 2.7	2.6 \pm 0.25 2.5*	4.1 \pm 0.79 3.8	5.9 \pm 1.40 5.9
TNF- α	6.3 \pm 1.53 6.8	4.9 \pm 2.36 3.1	4.0 \pm 1.22 3.9	3.5 \pm 0.45 3.6	3.8 \pm 0.76 3.5	3.1 \pm 0.90 3.2
IL-10	5.8 \pm 2.46 4.9	3.5 \pm 1.05 2.9	10.9 \pm 2.81 9.6	8.8 \pm 1.09 9.2	18.3 \pm 0.74 18.5*	9.7 \pm 1.51 9.4*

Table 2.4. Relative cytokine gene expression (mean \pm SEM and median) by abomasal lymph nodes collected from high fleece weight (HFW) and control (C) lambs which were infected at Day 0 with 50,000 L₃ *O. circumcincta* and killed on Day 8. Cytokine production was measured before exposure to antigen (control) and after exposure to Conavalin A (conA) or to *O. circumcincta* L₃ antigen. Group medians which differ significantly from the relevant control median ($p < 0.05$) are shown *.

Cytokine	Control (mean \pm SEM) (median) n=4		ConA-stimulated (mean \pm SEM) (median) n=4		<i>O. circumcincta</i> antigen-stimulated (mean \pm SEM) (median) n=4	
	C	HFW	C	HFW	C	HFW
IL-4	6.4 \pm 1.46 6.3	10.1 \pm 5.15 7.3	35.1 \pm 5.67 36.1*	23.9 \pm 6.20 20.1	5.7 \pm 0.39 5.7	7.5 \pm 3.07 6.6
IL-5	22.2 \pm 10.1 21.6	4.9 \pm 1.30 4.9	32.3 \pm 16.3 20.2	16.0 \pm 6.21 15.0	25.6 \pm 21.7 5.2	3.4 \pm 1.02 3.6
IFN- γ	21.7 \pm 8.26 17.6	12.2 \pm 6.60 8.3	17.4 \pm 8.88 12.3	10.7 \pm 7.00 5.2	44.5 \pm 13.7 44.6	37.5 \pm 22.7 19.7
IL-13	16.7 \pm 8.46 40.5	7.7 \pm 4.63 3.9	75.0 \pm 34.2 73.1	10.9 \pm 2.63 13.3	10.5 \pm 4.63 8.7	3.1 \pm 0.96 3.0
TNF- α	5.7 \pm 0.71 6.6	4.0 \pm 1.80 3.0	3.4 \pm 0.88 3.2	6.6 \pm 3.67 3.4	6.5 \pm 2.00 6.3	6.3 \pm 2.37 4.5
IL-10	2.3 \pm 0.76 4.5	6.4 \pm 1.46 6.3	10.9 \pm 1.83 12.8*	4.9 \pm 1.43 4.6	5.8 \pm 1.54 6.4	8.6 \pm 3.09 6.8

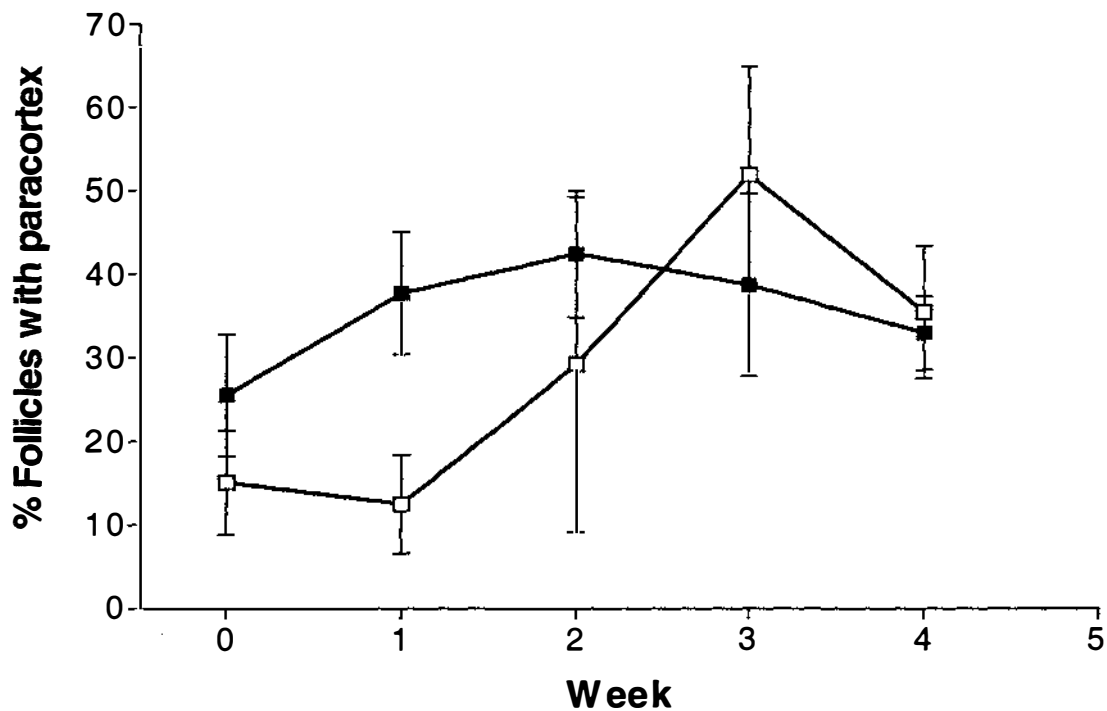


Figure 2.30. Percentage of wool follicles containing paracortex (mean \pm SEM) in high fleece weight (HFW) and control (C) lambs which were infected at Day 0 with 50,000 L₃ *O. circumcincta*. HFW: □; C: ■; administration of L₃: ▼.

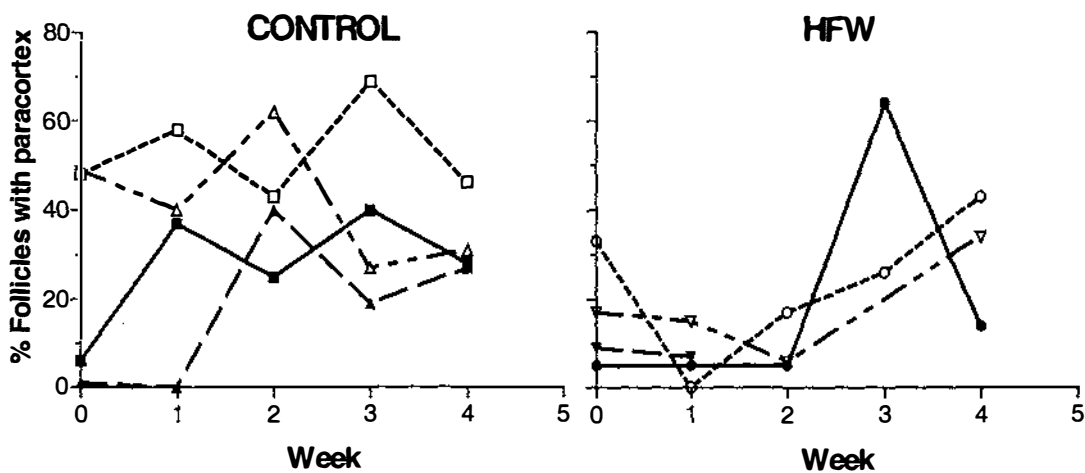


Figure 2.31. Percentage of wool follicles containing paracortex in individual lambs after infection at Day 0 with 50,000 L₃ *O. circumcincta*. Left panel: lambs in control (C) group; right panel: lambs in high fleece weight (HFW) group. Lamb #17: ■; #18: □; #19: ▲; #20: △; #21: ●; #22: ○; #23: ▼; #24: ▽; administration of L₃: ▼.

Food intake was higher overall in the HFW group on Days 1-8 ($p<0.05$) and on Days 9-28 ($p<0.01$). The intakes differed significantly in the two groups on Days 1 and 4 ($p<0.05$) and Days 2-3 ($p<0.01$), but not on Days 5-8. The differences between groups were most marked from Days 8-21, when intake in HFW lambs was only 200g below pre-infection levels, about half the depression of intake seen in the control sheep. Food intake was higher overall in the HFW group on Days 9-28 ($p<0.01$) and there were significant group differences on Days 9, 10, 12, 18, 23 and 24 ($p<0.05$), Day 16 ($p<0.01$) and was approaching significance on Day 21 ($p=0.052$). Animals within each group had similar patterns of intake following infection (Figure 2.29) and in general appetite was below pre-infection levels until the end of the experiment.

2.4.8 Wool Follicles

Wool follicle data are presented in Figures 2.30 and 2.31. Due to the short experimental period, statistical analyses were not carried on the limited data points. Similar trends to those seen in the longer Experiment 1 (Figures 2.13 and 2.14) were emerging: more follicles with paracortex in control animals before infection (particularly Sheep #18 and #20) and an increase after infection in all lambs.

2.4.9 Cytokines

Relative cytokine gene expression by ALN are shown in Tables 2.4-2.6. ConA significantly ($p<0.05$) stimulated IL-4 and IL-10 production by ALN of C lambs killed on Day 8 p.i. and reduced IL-5 and IL-13 production by ALN of HFW lambs killed on Day 28 p.i. *O. circumcincta* antigen significantly ($p<0.05$) stimulated IL-10 production by ALN of C and HFW lambs killed on Day 28 p.i.

Two-way ANOVA compared the data from ALN collected on the two days from the two groups. Higher levels of IL-4 were present later in the infection. Antigen stimulated cells from Day 28 p.i. produced significantly more ($p<0.01$) IL-4 than Day 8 cells. There were no differences between groups C and HFW.

For IL-5, the interaction term for the unstimulated cells was significant ($p<0.05$). At Day 8, the mean value from the C animals was greater (but not significantly

different), however, by Day 28, the HFW ALN produced significantly more IL-5 than those from group C ($p < 0.05$). Cells from Day 8, stimulated with Concavalin A, produced significantly more IL-5 than did Day 28 cells ($p < 0.01$), but there was no difference between groups C and HFW.

At Day 8, Concavalin A-stimulated cells of the C animals produced more IL-10 than did the equivalent HFW cells ($p < 0.05$). For antigen-stimulated cells, IL-10 levels were significantly higher in Day 28 than Day 8 samples ($p < 0.05$), with the largest increases occurring in the C animals. For IL-13, lower levels of the cytokine were produced at Day 28 than Day 8 ($p < 0.01$), whilst in Day 8 ALN, IL-13 levels were higher in the C than HFW ALN ($p < 0.05$).

2.5 Discussion

These experiments, which investigated whether HFW and C lambs would respond differently on first exposure to abomasal nematodes, also addressed several unanswered questions: do HFW and control animals differ in their pathophysiological responses to abomasal parasitism; if so, are HFW lambs resilient? What are the underlying mechanisms for resilience? Is there a relationship between inflammation and the pathophysiological consequences of abomasal parasitism? Is there a direct effect on the parietal cell?

2.5.1 Worm Burdens and FEC in HFW and Control Lambs

Both experiments supported the finding that HFW lambs do differ from control lambs, even when exposed to abomasal parasites for the first time in their lives at the age of 6.5 months. FEC was higher than in C lambs only in the older HFW lambs. The higher egg output by HFW lambs following primary challenge at 6.5 months-of-age was consistent with previous observations in the field (Howse *et al.*, 1992; Williamson *et al.*, 1995b) and after experimental infection (Williamson *et al.*, 1994, 1995a). The earlier and more prolonged detection of eggs in the faeces of HFW lambs indicates either, or all, of a greater worm burden, higher worm fecundity or slower development of resistance. Higher serum pepsinogen concentrations in the HFW lambs may result from greater numbers of worms, causing more abomasal damage. In contrast, there was no

difference in egg output between the HFW and C groups following infection with the same number of L₃ at 4.5 months-of-age, despite sampling procedures, feeding and housing of the animals being identical for the two experiments.

The only apparent differences in the primary infection protocol between Experiments 1 and 2 were the presumed lighter body weight of the younger lambs and the younger age at infection. However, a greater infective dose of L₃ will not necessarily produce a larger parasite population or greater pathological effects (Elliott, 1974a, b; Fleming, 1988) and parasite development may be delayed by administering large numbers of *O. circumcincta* L₃ (Elliott, 1974a, b). Egg production per *Ostertagia* female worm may be reduced at high population density, so that FEC does not accurately reflect the number of worms present (Anderson *et al.*, 1965; Barger *et al.*, 1985; Barger, 1986; Stear *et al.*, 1995; Stear & Bishop, 1999). In Experiment 2, such an inverse relationship between parasite density and eggs per female worm was clearly evident in worms examined on Day 28 p.i. (Figure 2.27).

Another possible explanation for the lower FEC in the older C lambs is that they are genetically more resistant to abomasal nematodes. The lower and shorter egg output might suggest genetic resistance in control sheep. The rams used for breeding the C flock are randomly selected from the closed Massey University flock and are not selected for low FEC. C lambs, therefore, might be expected to behave like outbred sheep. Since the HFW and C flocks have always been grazed together, constant exposure of the C flock to a contaminated pasture may have selected for resistance.

2.5.2 Are the Pathophysiological Effects of Abomasal Parasitism different in HFW and Control Lambs?

At both ages, all lambs showed the typical pathophysiological effects of *O. circumcincta* infection of increased abomasal pH and increased serum gastrin and pepsinogen concentrations, as previously reported (McLeay *et al.*, 1973; Anderson *et al.*, 1976, 1981, 1985; Lawton *et al.*, 1996; Simcock *et al.*, 1999; Simpson *et al.*, 1999; Scott *et al.*, 1998a, b, 2000). Wool follicles were

adversely affected and, where determined, food intake was depressed. Blood eosinophil counts increased and there was evidence of immune stimulation, although antibody responses were weak.

During both primary infections, there were differences in the extent of some of the pathophysiological responses in HFW and C lambs, although these were not consistent at the two ages. In Experiment 1, most group differences observed during the primary infection did not continue during the trickle series, when individual sheep variation predominated and there was no longer a clear distinction between the two groups. Animal variation makes it difficult to detect group differences when numbers of animals are small, however, this is unavoidable when surgical modification and intensive sampling are required. The HFW lambs supplied for these experiments were not selected randomly, as they had been chosen after breeding stock had been selected, as described by Blair *et al.* (1985). In addition, control lambs were also selected after breeding stock had been randomly selected. Thus, any differences between groups are more likely to be under-estimated than over-estimated.

In the older HFW lambs, higher FEC was accompanied by higher serum pepsinogen levels and delayed changes in abomasal pH. These differences were not seen in the lambs infected at 4.5 months-of-age. Food intake was monitored only in the younger lambs; the HFW group was able to maintain a higher food intake during infection. Generally, the HFW group had lower blood (and abomasal mucosal) eosinophil counts during the primary infection, which became more marked during the trickle infection. Lymphocyte cytokine production (Tables 2.4 and 2.5) suggests there may be a lesser Th2 response in HFW lambs than in the C group. Parasite-specific antibodies did not appear to be greatly increased in either group. The differing inflammatory responses and effects on food intake are discussed later as possible mechanisms producing resilience.

Hyperpepsinogaemia around the time of parasite emergence is generally accepted to be the result of a leaky abomasal mucosa due to damage caused by the parasites. Other causes have been proposed, including direct stimulation of the zymogenic cells by parasite ES, greater pepsinogen content

in nodular tissue and stimulation of pepsinogen secretion by cytokines (reviewed in Section 1.3.2.1). Although the higher serum pepsinogen concentrations in the HFW group in Experiment 1 may have resulted from higher worm burdens, which could be inferred from the higher FEC, serum pepsinogen concentration and FEC were not correlated in either experiment. This may be because FEC does not necessarily reflect the worm burden. Alternatively, the large individual differences between sheep in the extent of the increase in serum pepsinogen concentration in both groups in Experiment 2 (Figure 2.22) may relate more to the tissue pepsinogen content, which varies markedly between sheep (Scott & McKellar, 1998), but was not measured in the present experiments. Low responder sheep were identified in Experiment 2 (#19 and #21) as in other studies (Coop *et al.*, 1977; Lawton *et al.*, 1996; Simpson *et al.*, 1997; Simcock *et al.*, 1999; Hertzberg *et al.*, 2000), but only #19 had a low worm burden (Table 2.3).

The delayed inhibition of acid secretion in HFW lambs compared with C lambs in Experiment 1 suggests delayed emergence from the gastric glands. The onset of hypoacidity generally coincides with the expected time of emergence from the glands (Armour *et al.*, 1966; Anderson *et al.*, 1976, 1981; Lawton *et al.*, 1996). In Experiment 2, hypoacidity occurred in both HFW and C lambs at the expected time of Days 5-6 p.i. If the older HFW lambs delayed parasite emergence, it did not affect maturation of the female worms, since two of the HFW lambs were among the first in which eggs were detected in the faeces. Alternatively, parasitised HFW sheep may be able to maintain a physiological gastric pH for a longer period than can C lambs, possibly delaying parasite development or emergence. On the other hand, delayed abomasal hypoacidity may simply reflect a greater capacity to maintain acid production in the presence of nematodes.

In all older lambs, abomasal pH remained moderately elevated during the trickle infection, with periods of very high pH following each dose of L₃. The very low FEC suggests that the presence of adult worms is not necessary for parietal cell inhibition. The most prolonged periods of elevated abomasal pH were seen in two C lambs, contributing to high group mean values from Days

35-55. In most animals, parietal cells remained capable of responding to feeding by lowering abomasal pH, although not to the range of acidity seen in uninfected lambs. Lawton *et al.* (1996) and Hertzberg *et al.* (1999b) also observed that the pH of abomasal contents can decrease during feeding or after injection of histamine respectively, even when abomasal pH was at its highest.

No group differences were detected in serum gastrin concentrations after infection, in contrast to the lower serum gastrin levels in HFW than in C lambs seen in a field infection (Williamson *et al.*, 1995b). In all groups, the peak serum gastrin concentrations were seen later than the peak abomasal pH, as has been previously reported (Lawton *et al.*, 1996; Simcock *et al.*, 1999). No significant group differences were found during the period of delayed acid inhibition in HFW lambs in Experiment 1, although there was a trend towards an earlier rise in C lambs (Figure 2.5). The hypergastrinaemia of abomasal parasitism has been attributed to the initial loss of acid feedback and sustained by inflammation and or parasite ES (reviewed in Section 1.3.2.3). In the present experiments, both HFW and C lambs had decreases in serum gastrin levels when the abomasal pH reached 5.5 or higher, increasing again when the pH dropped to 3.5. This observation had previously been reported (Lawton *et al.*, 1996; Simcock *et al.*, 1999; Simcock, 2000) and may be caused by a microbial gastrin inhibitor which is released into rumen fluid and also in non-acidic conditions in parasitised abomasa (Simcock, 2000).

2.5.3 Are HFW Lambs Resilient?

In order to assess where the HFW and C lambs fit in the spectrum of resistance, susceptibility and resilience to parasitism, the definitions need to be clarified. Resilient sheep are considered to be those in which production losses are less evident during parasitism. Albers *et al.* (1987) defined resilience as the “ability to maintain a relatively undepressed production level when infected.” Bisset *et al.* (2001) introduced the term “tolerant” to distinguish between two types of resilience observed in research flocks in New Zealand: lambs with above average growth rate under challenge were called “resilient” and those

maintaining an above average growth rate under challenge despite having an above average FEC were called “tolerant”. In contrast to resilience, resistance to parasitism has been defined as the ability to maintain a lower worm burden compared with other members of its flock or herd, when similarly challenged, and is the opposite of susceptibility (Gray, 1991). The concept of resistance has been extended to include the ability to suppress the establishment of parasites, reduce their growth and eliminate the parasite burden (Albers *et al.*, 1987; Woolaston & Baker, 1996; McEwan *et al.*, 1997). Lambs with below average FEC under challenge are considered resistant and those with below average growth rates and above average FEC are susceptible.

In the present experiments, no direct production parameters, such as wool growth or live weight gain, were monitored and the only parameter relating to production which was followed was the proportion of paracortical cells in the wool follicles, a measure of crimp frequency. This is likely to be a valid production parameter, as abomasal parasitism has been reported to have a detrimental effect on wool quality and wool production (Barger & Southcott, 1975). The proportions of orthocortical and paracortical cells, which make up the inner cells within a wool follicle, can be influenced by the availability of sulphur-containing amino acids, such as methionine, cystine and cysteine (Hynd & Masters, 2002). Increased numbers of paracortical cells are associated with more crimp, one of the criteria for wool quality, because they take up water, swell and therefore straightened the fibre.

Selection for higher greasy fleece weight appears to have also selected for a lower percentage of wool follicles containing paracortex, seen in parasite-naïve lambs before infection (Figures 2.13 and 2.30). After 23 years of selection, the HFW flock produced a heavier greasy fleece, but wool quality number and crimp frequency decreased (Blair *et al.*, 1985). Antram *et al.* (1991) and Sun *et al.* (1991) also reported that the greater wool growth rate in HFW lambs was associated with lower wool sulphur concentrations but higher wool sulphur output. Both Blair *et al.* (1985) and McCutcheon *et al.* (1993) established that the HFW flock at those times were also heavier than the unselected flock and

there were also some differences in metabolism (McCutcheon *et al.*, 1987; Thomson *et al.*, 1989; Sun *et al.*, 1991).

Following primary infection in both experiments, the number of follicles containing paracortex, as well as the amount of paracortex within a follicle, increased earlier in C than in HFW lambs. During Experiment 1, the maximum percentage of paracortical cells in C lambs had reached its maximum by Week 5, whereas a significant increase in the percentage of paracortical cells in HFW lambs, with the exception of one lamb, was not detected until Week 7. This lamb (#5) did not fit within the range of values for the other HFW lambs in either experiment. At least in this flock, there is potential to use the percentage of wool follicles containing paracortex to distinguish between the two groups. It may be useful to select newborn lambs on such a criterion, because a parasitic challenge obscured any differences within weeks.

On the basis of the higher FEC, but more slowly developing effects on wool quality in the present experiment, combined with the previous production data and higher FEC in HFW sheep in the field, there is no reason to doubt that the HFW flock contains resilient animals. These data support the classification of the HFW lambs in the "tolerant" subset of resilient animals as defined by Bisset *et al.* (2001). However, the expression of resilience appeared to be age-dependent, as there were more differences following a primary infection at 6.5 months-of-age than at 4.5 months.

2.5.4 What is the Basis of Resilience in the HFW lambs?

Resilience in the HFW lambs may be the result of modified physiological or metabolic processes associated with selection for wool production, or it may represent an attenuated immune response to parasitism. The difference in food intake between the HFW and C groups, both before and after infection in Experiment 2, suggests there may be a physiological basis for resilience, while the differences in blood eosinophils and ALN cytokine profiles supports an immunological basis.

2.5.4.1 Food Intake

Nutrition has a well-known effect on both wool quality and production. A later deterioration of wool quality after infection in the HFW lambs suggests they can compensate better for the metabolic consequences of parasitism (reviewed in Section 1.3.2.5). This could have been mediated through maintaining a higher food intake than did lambs in the C group. Such a hypothesis has to be considered with caution, since food intake was monitored only during the second experiment. It is well established that adequate nutrition is positively linked to good performance despite a parasitic challenge (reviewed by van Houtert & Sykes, 1996). The higher feed intake of HFW lambs, observed both before and during infection in Experiment 2, may be the reason why HFW lambs produce more wool than C lambs in the field and also may underpin their resilience to parasitism.

Nutrition has a complex effect on both wool quality and production. An inadequate supply of amino acids, particularly those containing sulphur, reduces fibre diameter and hence fibre strength (Reis *et al.*, 1992). Wool from high producing sheep generally contains less ultra-sulphur-containing protein and fewer paracortical cells and high producers are more responsive to an increased plane of nutrition than are low producing sheep (Hynd & Masters, 2002). Mid-side wool of HFW hoggets had lower sulphur contents than that of C hoggets in the field study of Sun *et al.* (1991). In the present experiments, the HFW lambs appeared to maintain a lower level of ultra-sulphur-containing protein and fewer paracortical cells for a longer period of time after infection, along with the higher food intake.

In their nutrient partitioning model, Coop & Kyriazakis (1999) suggested that parasite-naïve hosts invest nutrients primarily to overcome the pathophysiological consequences of their first infection to secure their immediate survival. An increased availability of nutrients would thus enhance resilience, as defined by Albers *et al.* (1987), and not resistance, in the parasite-naïve host. The cause of inappetence during parasitism is not understood yet, but could involve inflammatory cytokines, leptin or gastrin (reviewed in Section 1.3.2.5.1). Until the cause is established, differences in

food intake in genetically divergent flocks can only be speculated upon. Research linking high serum gastrin levels to the reduction in appetite in parasitised calves (Fox *et al.*, 1989a, b) is not supported by the present experiment or an earlier study in lambs by Simcock *et al.* (1999).

2.5.4.2 Immune Response

There also appears to be an immunological component to resilience in the HFW lambs. Blood eosinophils, cytokine production by ALN lymphocytes and serum IgG1, IgM and total Ig were the parameters measured which represented the inflammatory process. Taken together, they support a less intense Th2 response in the HFW lambs during parasitism, although this did not necessarily result in different worm burdens or FEC. Increases in serum pepsinogen concentrations in individual lambs did not match well with changes in blood eosinophils, suggesting the two parameters do not reflect equally the inflammatory response. As discussed above, variations in tissue pepsinogen content may confound the effects of the back diffusion of secreted pepsinogen due to the leaky epithelium. Products of inflammation, such as cytokines and leukotrienes, may elevate serum pepsinogen by stimulating chief cells (Serrano *et al.*, 1997) or by affecting cell-cell contacts (reviewed by Fiocchi, 1997).

Cytokine profiles of ALN lymphocytes from the two groups support a less intense Th2 response to infection (Tables 2.4-2.6). IL-4, IL-5, IL-10 are cytokines typical of a Th2 response (reviewed in Section 1.4.1). IL-4 and IL-10 were significantly elevated by concavalin A in the C lambs and *O. circumcincta* antigen stimulated IL-10 production in both groups. Overall, IL-5 levels tended to be lower for the HFW animals 8 days after infection and this is consistent with the lower peripheral eosinophilia observed in HFW animals. IL-13 is important in mice for resistance to nematodes (Bancroft *et al.*, 1998) and IL-13 levels tended to be lower in HFW animals at Day 8 in particular. Very big differences in IFN in individual lambs in both groups made group differences undetectable or not important.

Specific antibody levels detected at the usual serum dilutions were very low and showed insignificant changes with parasitism, which did not differ between groups (Figure 2.10). Williamson *et al.* (1995b), using an identical test system,

made the same observations in a field study of the Massey University HFW and C flocks. Even when unusually low dilutions of the serum were used, only insignificant changes could be detected. One explanation is that the antigens were not fully recognised by the antibodies in the serum of the sheep, though this seems rather unlikely since the test was able to deliver pre-infection values. A more likely explanation is that neither HFW nor C lambs produced significant levels or changes in the antibodies tested.

Following primary infection at 6.5 months-of-age, increased numbers of blood eosinophils were apparent earlier in C lambs than in HFW lambs, although concentrations were similar overall. One lamb in each group had noticeably higher levels. Blood eosinophils increased further during the ensuing trickle infection, particularly in two C lambs (#1 and #4). Primary infection at the age of 4.5 months resulted, with the exception of one sheep, in distinctly higher numbers of eosinophils in the blood of the C lambs, but a much lower, more homogenous response in the HFW lambs. Despite the difference in blood eosinophil levels, there was no difference in FEC or worm burdens. Because of the overlapping ranges in the two groups, it seems impossible to define diagnostic ranges which could be used to identify resilient animals.

Taken together, these results question the use of blood eosinophil levels as a phenotypic marker for resilience to internal nematodes. Peripheral eosinophilia has been proposed as a marker for resistance, because of its negative correlation with FEC in sheep bred for high or low FEC under parasitic challenge (Buddle *et al.*, 1992) or selected for higher resistance to *T. colubriformis* following oral vaccination with gamma-irradiated larvae (Dawkins *et al.*, 1989). However, other studies of flocks of selected sheep (Pernthaner *et al.*, 1995a, Bisset *et al.*, 1996; Woolaston *et al.*, 1996; Kanobana *et al.*, 2001) concluded that peripheral eosinophilia was of limited use as a marker for genetically resistant animals.

2.5.4.3 Age and Exposure to Parasites

While resilience may be an innate trait with a genetic basis, its expression appears to be age-dependent. There were more differences between the HFW and C lambs following a primary infection at 6.5 months-of-age than at 4.5

months, probably linked to the maturity of the immune system. Lambs are known to be more susceptible to infection of any kind when aged below 6 months, due to immunological hypo-responsiveness, rather than non-efficient exposure to antigens (Colditz *et al.*, 1996). Other studies also have shown that, following a parasite infection, there are differences in parasitological and immunological parameters between younger (less than 6 months-of-age) and older parasite-naïve (Douch & Morum, 1993) or previously exposed sheep (Watson *et al.*, 1994).

In Experiment 1, all lambs acquired resistance to the parasites, evidenced by the very low FEC during the trickle infection. There may have been only small numbers of mature worms, possibly stunted in their development or with a lower fertility, all common observations in sheep resistant against gastrointestinal nematodes (reviewed by Balic *et al.*, 2000). Only small numbers of mature worms were present at necropsy on Day 94 p.i. The much higher numbers of L₃ and L₄ in HFW lamb #7 was caused by administration of infective larvae only one day before euthanasia. Whether eosinophils are involved in worm expulsion remains uncertain. The very low numbers of eggs in combination with the increased levels of circulating eosinophils in lambs in both groups during the trickle series would support such a hypothesis. However, no correlations were observed between FEC and blood eosinophils, neither for C nor for HFW lambs in either experiment.

It is uncertain whether the lambs in Experiment 2 had begun to develop resistance over the 28 day experimental period. There was an estimated 90% elimination of parasites from Day 8 to Day 28 in both HFW and C groups, assuming all lambs had similar L₄ burdens to those in the lambs killed on Day 8. Single *O. circumcincta* infections appear to be short-lived, with many adult worms being eliminated soon after egg laying, as seen here with a still rising FEC. The short life span of adult worms may be due to active removal of the parasites by the host immune system.

The almost complete resistance after the primary infection in the older lambs was very marked, more so than might be expected from a single exposure. Most likely it was caused by maintaining the lambs parasite-naïve until

6.5 months old, an age when they can be considered to be fully immune responsive. The same phenomenon was apparent in sheep in other studies, in which previously parasite-naïve animals older than 6 months also had unusually low egg counts (Douch & Morum, 1993; Hertzberg *et al.*, 1999b). Smith *et al.* (1985) have previously reported a similar observation from experiments in which 10 month-old parasite-naïve lambs proved to be more resistant than 4 month-old parasite-naïve lambs. Is it possible that larvae need to be present in the immature host to prime the host for “acceptance”? If priming does not occur before the host is fully immune responsive, the host may recognise the parasite as a pathogen and prevents it from establishing.

2.5.5 How do abomasal parasites inhibit the parietal cell?

The hypoacidity associated with *O. circumcincta* infection in sheep begins when parasites are present in the lumen after emergence from the gastric glands, usually around Days 5-6 p.i. (Armour *et al.*, 1966; Anderson *et al.*, 1976, 1981; Lawton *et al.*, 1996), or one day after adult worm transplantation (Anderson *et al.*, 1985; Lawton *et al.*, 1996; Simpson *et al.*, 1999; Scott *et al.*, 2000). However, the mechanisms behind it are still not clear. The main question is whether the parasites themselves target the parietal cell directly or whether the hypoacidity is mediated by the accompanying inflammation. Moreover, are parietal cells inhibited or do they die following a parasitic infection?

Several observations in Experiments 1 and 2 support parietal cell inhibition rather than death. Both abrupt increases in abomasal pH of 1-3 units over 24 hours and equally rapid recoveries were common in nearly all sheep. Similar observations were made by Lawton *et al.* (1996) and Scott *et al.* (2000). In addition, sheep regain the ability to acidify abomasal contents within hours of parasites being removed by anthelmintic drenches (Simpson *et al.*, 1997). Functional, but perhaps inhibited, parietal cells are likely to be present in parasitised animals as the maturation of pre-parietal cells takes 2.6 days in mice (Karam & Leblond, 1992; Karam, 1993), seemingly too slow to explain such rapid recovery. However, it is not known what percentage of the whole

parietal cell population need to be actively secreting acid to effectively restore physiological pH in ruminants. Maturation of a smaller number of immature parietal cells, which are further along in their development than pre-parietal cells, might be sufficient.

Whereas there is evidence that parasites may release substances inhibitory to acid secretion in their ES, the effects of inflammation are unclear. Two experiments indicate that parasite chemicals may be capable of reducing acid secretion: *H. contortus* ES decreased the uptake of histamine-stimulated ^{14}C -aminopyrine by rabbit gastric glands *in vitro* (Merkelbach *et al.*, 2002) and inhibited both basal and gastrin-stimulated pancreastatin secretion by cultured ECL cells (Hertzberg *et al.*, 1999a). Differences in the inflammatory responses in HFW and C lambs suggest that these animals might provide data on the link between the loss of parietal cell function and the inflammatory response to the presence of parasites. Eosinophils are capable of inflicting damage on both parasites and host tissues and may be a key factor in parietal cell damage. Therefore, it seems logical to investigate the role of mucosal eosinophils in relation to hypoacidity as well as to circulating eosinophils. Any differences between HFW and C sheep might be expected to clarify any involvement of the immune response in the expression of resilience. These studies are reported in Chapter 3.

Chapter 3

Histopathological changes during abomasal parasitism: Is the parietal cell a target?

3.1 Introduction

The histological changes that occur during abomasal parasitism include hyperplasia of mucous cells and the loss of mature chief and parietal cells (Scott *et al.*, 1998a, 2000). Any loss of parietal cells is of special interest, since parasites are poorly viable at a pH of less than 4.5 (Haag, 1995; Simpson, unpublished data). Therefore, a targeted interruption of parietal cell activity or viability by the nematodes themselves is a possibility.

Previous studies have reported the presence of vacuolated parietal cells in sheep infected with *O. circumcincta* (Scott *et al.*, 2000) or calves with *T. axei* (Ross *et al.*, 1971) although none of these studies established the underlying causal mechanism/s. While the parasites themselves are a likely cause, the inflammation that accompanies infection needs to be considered as well. An inflammatory reaction expresses a variety of aggressive components able to injure the gastrointestinal mucosal layer and its cells (reviewed by Fiocchi, 1997).

Parietal cells regulate key differentiation decisions in the gastric gland and ablation of parietal cells leads to mucosal hyperplasia and loss of chief cells (Li *et al.*, 1996). Clinical pictures of other gastric diseases, in which parietal cells are also lost, thus resemble the changes seen during parasitic infection and a single mechanism may be common to many diseases.

Sheep that are relatively more resistant against gastrointestinal parasite infection display a more pronounced inflammatory reaction within their mucosae. Mast cells, globule leucocytes as well as eosinophils may all play a

protective role against different parasite stages (Rothwell, 1989) and eosinophils themselves have been found to be responsible for at least one mechanism of larval rejection/destruction (Balic *et al.*, 2000). In comparison, the mechanisms underlying resilience must surely be different, since resilient sheep carry a higher worm burden (Albers *et al.*, 1987), but apparently do not suffer.

The experiments reported in this chapter were designed to answer several questions regarding the pathophysiology of abomasal parasitic infections. In Chapter 2, abomasal hypoacidity was reported to be present in both HFW and C animals to varying extents, whilst the two animal types differed in their ability to generate a peripheral eosinophilia. In Experiment 2, tissues had been collected from both HFW and C animals killed 8 and 28 days after infection. These tissues were now used to establish the relative levels of damage incurred by the two animal types and matched to the numbers of infiltrating eosinophils. A link between inflammatory cell infiltrates and loss of ability to secrete acidity was therefore sought as a first step in establishing the exact mechanism underlying resilience.

In addition, tissues were also collected from non-selected animals that had been infected with either infective L₃ or adult *O. circumcincta* and killed at various times post-infection. The precise timing of changes affecting the parietal cell population and the populations of invading inflammatory cells could therefore be established. Evidence of direct effects of the parasites on the parietal cells was also sought. Such evidence might accrue if it could be demonstrated that parietal cell function was affected in situations where inflammation was limited or absent.

3.2 Materials and Methods

3.2.1 Animals

Group HFW: HFW and C lambs (Experiment 2)

Eight parasite-naïve male lambs from each of the HFW and C flocks were infected at 4.5 months-of-age with 50,000 L₃ *O. circumcincta*. Four animals per

group were euthanased on Day 8 p.i. and the remaining eight animals were euthanased on Day 28 p.i. C lambs were #9-12 and 17-20 and HFW lambs were #13-16 and 21-24 (Experiment 2) (fully described in Section 2.2.1)

Group LI: Larval Infection Experiment

Twenty-six parasite-naïve Coopworth lambs, approximately 3 months-of-age, were used. Twenty lambs each received 35,000 *O. circumcincta* L₃ intraruminally and were sacrificed 5, 10, 15, 20 or 30 days after infection. Times of infection were staggered so that one animal was euthanased per day and animals were sacrificed over a period of 7 weeks. Two uninfected control lambs were sacrificed at the start of the experiment and the remaining four controls at the end of the study. Sheep numbers were: Control #25-30, Day 5 #31-34, Day 10 #35-38, Day 15 #39-42, Day 20 #43-46 and Day 30 #47-50.

Group AT: Adult Transplant Experiment

Fifteen parasite-naïve Coopworth lambs, approximately 4 months-of-age, were used. All lambs underwent surgery to implant abomasal cannulae, as described in Section 2.2.2.2. Twelve lambs each received approximately 10,000 adult *O. circumcincta* through the abomasal cannulae and were sacrificed 6h, 12h, 24h and 72h after the transplant. Adult worms were derived from donor sheep, which had been slaughtered 21 days after infection with 100,000 *O. circumcincta* L₃. Pooled abomasal contents of donor sheep, concentrated by sedimentation, were divided into 13 aliquots. Worm numbers were estimated in one aliquot, whilst the remaining 12 were given to the recipient animals within 1 – 2h of collection. Three control lambs were sacrificed at the start of the experiment. Sheep numbers were: control #53-55, 6h p.i. #56-58, 12h p.i. #59-61, 24h p.i. #62-64 and 72h p.i. #65-67.

3.2.2 Abomasal pH and Serum Gastrin

For Group HFW, sample collection and assays have been described in Sections 2.2.3 to 2.2.5. Serum gastrin concentrations and abomasal pH are presented in Figures 2.15 to 2.20.

For Group LI, abomasal fluid for pH measurement and jugular venous blood samples for serum gastrin concentration (Appendix 2.4.1) were collected in a

similar manner to that described in Sections 2.2.4 and 2.2.5. Samples were collected daily for all sheep sacrificed up to Day 15 p.i. Sheep sacrificed on Day 20 p.i. were also sampled on Day 17 p.i., while animals sacrificed on Day 30 p.i. were sampled every second day from Day 15 to Day 27 p.i. All sheep had blood and abomasal fluid taken on the day of necropsy.

For Group AT, the pH of abomasal fluid collected at necropsy was used for correlation with histological parameters.

3.2.3 Necropsy

The protocol for necropsy of Group HFW sheep is described in Section 2.2.2.3. The only difference for the other groups was that, instead of stunning and exanguination, the sheep were euthanased by intravenous injection of a barbiturate (Pentobarb 500[®], NZVet, Auckland, NZ) overdose. Fundic tissue from all sheep was fixed in Bouin's solution. For Group LI, additional tissue was collected from control sheep and those sacrificed on Days 5 and 10 and fixed in 2% glutaraldehyde.

3.2.4 Electron Microscopy

Tissues fixed in 2% glutaraldehyde were routinely processed and embedded in epoxy resin (EPON812[®], SPI supplies, West Chester, USA). Sections 1 μ m thick were also cut and stained with 1% toluidine blue for light microscopic examination. Sections for transmission electron microscopy were cut 70–90nm thick, mounted on 200 mesh copper grids (SPI supplies, West Chester, USA) and stained with 2% uranyl acetate (Sigma-Aldrich, St. Louis, USA) followed by Reynold's lead citrate (Reynolds, 1963). Sections were examined using a Philips 201C transmission electron microscope.

3.2.5 Light Microscopy

3.2.5.1 Tissue Preparation

Gastric mucosa was fixed overnight in Bouin's solution and stored in 70% ethanol (v/v) until further processing. Samples were routinely processed for paraffin wax embedding. They were dehydrated in 70% ethanol, cleared in

chloroform and two changes of xylene and infiltrated with paraffin wax at 59°C, according to the standard schedule on a TP1050 automatic tissue processor (Leica Jung, Wetzlar, Germany). The samples were embedded on a Tissue-Tek® embedding console (Miles Scientific, Chicago, USA) and then sectioned on a rotary microtome at 5µm thickness. The sections were floated in a waterbath at 40°C, attached to microscope slides and oven-dried at 60°C. Sections were dewaxed and hydrated using a standard method.

3.2.5.2 Standard Stains

Paraffin-embedded sections 5µm thick were stained with haematoxylin and eosin (H&E) from all tissues. Additional sections from Group LI sheep (control and Days 5 and 10 p.i.) and Group AT were stained using Luna's method for eosinophils (Luna, 1993). For Group LI sheep, resin-embedded semithin sections of 1µm thickness were stained with 1% toluidine blue.

3.2.5.3 Immunohistochemistry

Dewaxed and hydrated 5µm thick sections were pre-treated with 0.05% saponin (Sigma-Aldrich, St. Louis, USA), washed with phosphate buffered saline (PBS) and exposed to 0.5% bovine serum albumin (BSA) (Fraktion V, Boehringer Mannheim GmbH, Germany). Sections from all tissues were stained for TGF-α. Sections from Group HFW sheep only were stained for eosinophils.

Immunohistochemical staining for TGF-α was done using a mouse monoclonal antibody (IgG_{2a}) (Calbiochem, San Diego, CA, USA) in a 1/500 dilution in PBS containing 0.5% BSA. Immunohistochemical staining for sheep eosinophils was performed using a mouse monoclonal (IgG₁) antibody (the kind gift of Dr Wayne Hein, AgResearch Ltd, Wallaceville, NZ) in a 1/500 dilution in phosphate-buffered saline (PBS) containing 0.01% BSA. The second antibody in each case was an ovine anti-mouse antibody (whole Ig) (Amersham Life Science, Little Chalfont, UK) in a 1/200 dilution in PBS containing 0.5% BSA. The streptavidin-biotin method was used to amplify binding of antibodies using 1/200 dilution of a streptavidin-biotinylated horseradish peroxidase complex (Amersham Life Science, Little Chalfont, UK) in PBS containing 0.5% BSA.

Antibody binding was visualised using a 0.4% (w/v) diaminobenzidine solution containing heavy metal intensification and 0.12% (v/v) hydrogen peroxidase. All sections were counterstained with Mayer's haematoxylin. Some of the TGF- α stained sections from the larval infection experiment were also counterstained with H&E to assess the number of parietal cells (if any) that did not bind the antibody.

3.2.5.4 Eosinophil and TGF- α positive Parietal Cell Counts

Nucleated parietal cells and eosinophils were counted in a 258 μ m wide column of mucosa (a location), from the muscularis mucosae to the luminal surface at 40x magnification using a 1cm² eyepiece graticule. Nucleated parietal cells were counted within the full mucosal depth from base to surface in five locations within each section. When counting parietal cells, nodular areas associated with larval development within glands were avoided. Tissue thickness was also recorded.

Group HFW, two sections from each of two folds and for Groups LI and AT, five sections, one from each of five gastric folds, were examined per animal.

3.2.5.5 TGF- α negative Parietal Cell Counts

From the groups of animals killed on each day in Group LI, TGF- α stained sections counterstained with H&E from two or three animals were used to establish the number of TGF- α negative parietal cells. Parietal cells that were clearly labelled only for H&E were counted and added to the TGF- α (only) counts and expressed as the total parietal cell count. TGF- α negative parietal cells were then expressed as a percentage of the total count. For Group AT, tissues from one or two animals per group were used to establish the number of TGF- α negative parietal cells.

3.2.6 Statistical Analyses

Data are presented mean \pm SEM ($\mu \pm$ SEM).

For Group HFW, data were examined for normality using the Shapiro-Wilk test in the SPSS version 11.0. If necessary, data were log₁₀ transformed. In all

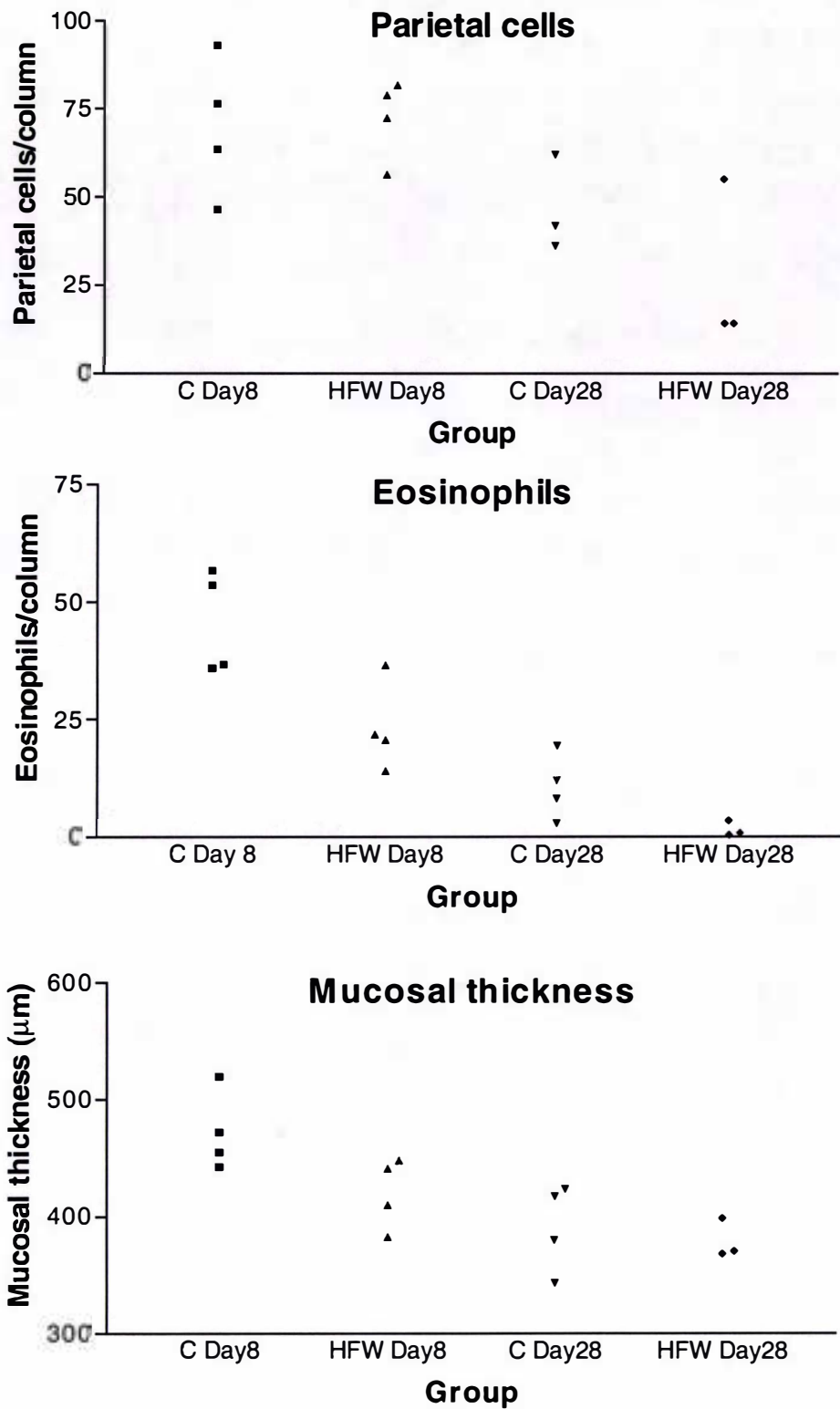


Figure 3.1. Mucosal thickness (μm) and numbers of TGF- α -positive parietal cells and eosinophils per 258 μm wide column of fundic tissue in individual high fleece weight (HFW) and control (C) sheep which were infected on Day 0 with 50,000 *L*₃ *O. circumcincta* and killed on Day 8 or Day 28.

Table 3.1. Parietal cells stained with TGF- α antibody, mucosal eosinophils and mucosal thickness (μm) (mean \pm SEM) in fundic tissue from high fleece weight (HFW) and control (C) sheep which were infected on Day 0 with 50,000 L₃ *O. circumcincta* and killed on Day 8 or Day 28. TGF- α stained parietal cell and eosinophil numbers are expressed in total cell numbers per 258 μm wide column from the mucosal base to lumen.

	Day 8		Day 28	
	C	HFW	C	HFW
TGF- α positive PC	69.90 \pm 9.88	72.30 \pm 5.71	46.73 \pm 7.82	33.60 \pm 19.40
Eosinophils	45.75 \pm 5.50	23.23 \pm 4.77	10.55 \pm 3.50	1.53 \pm 0.94
Mucosal Thickness	472.5 \pm 17.0	420.5 \pm 15.2	391.1 \pm 18.6	379.0 \pm 9.9

cases, correlations between data sets were tested using Pearson's or Spearman's correlations. Correlations were made within the HFW and C groups and for pooled data between mucosal eosinophils and TGF- α positive cell counts, mucosal thickness and abomasal pH, blood eosinophils and serum pepsinogen concentration. One-way ANOVA or Kruskal-Wallis tests were employed to compare groups and days p.i. for TGF- α positive cell counts and mucosal eosinophils. *Post hoc* comparisons were done using Bonferroni's or Dunn's tests. Student's t-test was used to compare between days across groups.

For Groups LI and AT, data were examined for normality using Kolmogorov-Smirnov test. In all cases correlations between data sets were tested using Pearson's correlation. One-way ANOVA was employed to compare groups. *Post hoc* comparisons used Tukey's test. Correlations were made between tissue eosinophils and TGF- α positive cell counts, mucosal thickness and abomasal pH.

3.3 Results

3.3.1 Group HFW

3.3.1.1 Abomasal Parameters

Table 3.1 presents group means for TGF- α positive parietal cell counts, eosinophil counts and mucosal thicknesses for the HFW and control lambs on Days 8 and 28 p.i. The individual mean values for each animal for each parameter are shown in Figure 3.1.

There were no differences in parietal cell numbers between groups by day or between days for each group, however, when data from both groups were pooled, a difference was found between Day 8 and Day 28 p.i. ($p < 0.05$).

Control lambs had significantly more eosinophils in their mucosae than did HFW lambs ($p < 0.05$) on Day 8 p.i., but not on Day 28 p.i. Both groups had significantly more eosinophils on Day 8 p.i. compared with Day 28 p.i. ($p < 0.05$)

HFW, $p < 0.001$ C). Further, a highly significant difference existed across groups by day ($p < 0.001$).

There was no difference in mucosal thickness between groups by day, whereas control lambs, but not HFW lambs, had significantly thicker mucosae on Day 8 p.i. ($p < 0.05$).

There was a very strong correlation between tissue eosinophils and abomasal pH for both the control ($r^2 = 0.72$, $p < 0.01$) and the HFW lambs ($r^2 = 0.99$, $p < 0.001$). The correlation was weaker when all animals were considered together ($r^2 = 0.56$, $p < 0.01$). Tissue eosinophils correlated with worm counts for control ($r^2 = 0.55$, $p < 0.05$) and HFW animals ($r^2 = 0.73$, $p < 0.05$), though the correlation was again weaker when both groups were considered together ($r^2 = 0.4$, $p < 0.05$). Tissue eosinophils correlated with mucosal thickness for controls ($r^2 = 0.53$, $p < 0.05$), but not for HFW lambs. Additionally, tissue eosinophils correlated highly significantly with mucosal thickness across groups ($r^2 = 0.58$, $p < 0.001$). There were no correlations within or across groups between tissue eosinophils and either serum pepsinogen or blood eosinophils.

TGF- α positive parietal cells showed no correlation with tissue eosinophils in either group, though pooled data had a calculated correlation coefficient of $r^2 = 0.33$ with $p < 0.05$. Parietal cell counts did not correlate with abomasal pH in controls or across groups, but they did correlate significantly in HFW lambs ($r^2 = 0.61$, $p < 0.05$).

3.3.1.2 Light Microscopy

3.3.1.2.1 Day 8

A typical observation within sections derived from the HFW lambs and their controls was the great abundance of inflammatory cells and an apparent reduction in the number of TGF- α positive parietal cells in nodular areas compared with non-nodular areas.

Inflammatory cells present were mainly eosinophils and lymphocytes. Large numbers of eosinophils were located in the base of the mucosa, often distributed in bands above the muscularis mucosa, although in some places,

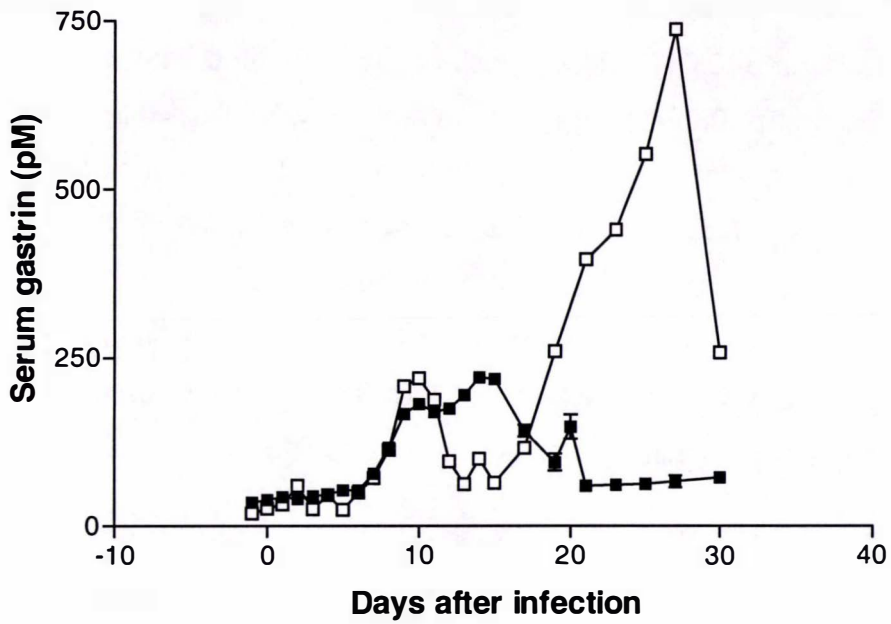


Figure 3.8. Serum gastrin concentration in Sheep #50 and the group mean (mean \pm SEM) for all sheep surviving on each day after infection on Day 0 with 35,000 L3 *O. circumcincta*. Sheep #50: \square ; group mean: \blacksquare .

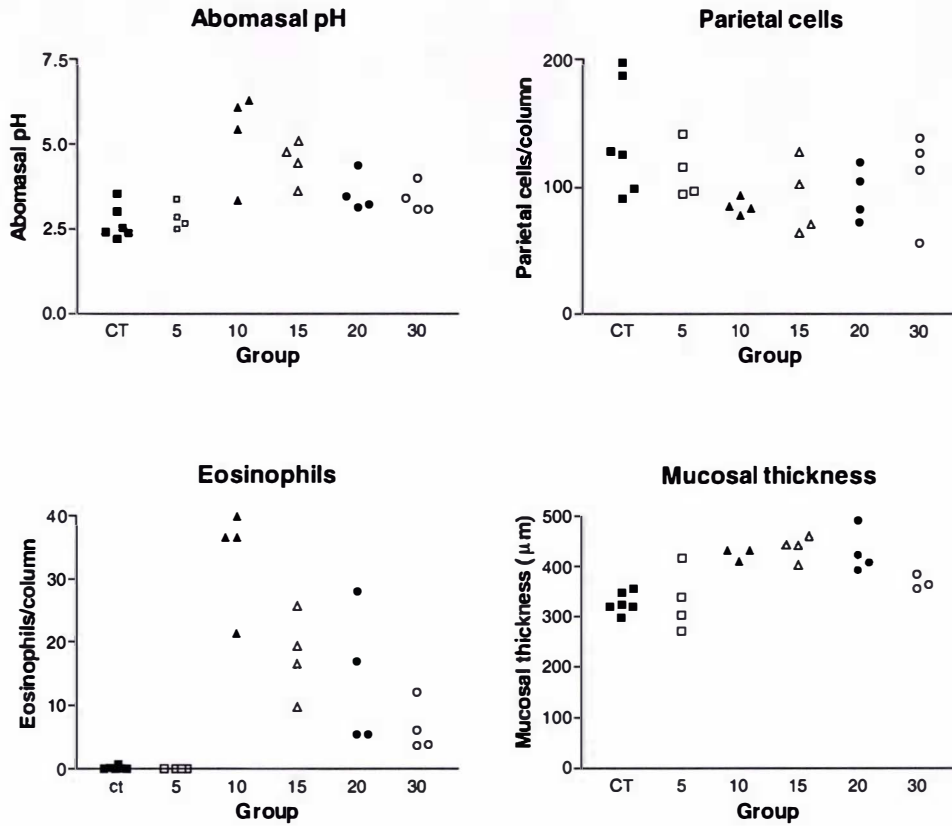


Figure 3.7. Abomasal pH at necropsy, mucosal thickness (μm) and numbers of TGF- α -positive parietal cells and eosinophils per 258 μm wide column of fundic mucosa of individual sheep before (control, CT) and 5, 10, 15, 20 or 30 days after infection with 35,000 L₃ *O. circumcincta*.

Table 3.2. Abomasal pH, parietal cells stained with TGF- α antibody, mucosal eosinophils and mucosal thickness (μm) (mean \pm SEM) in fundic tissue from sheep (Group LI) which were infected on Day 0 with 35,000 L₃ *O. circumcincta* and killed on Days 5, 10, 15, 20 or 30. TGF- α stained parietal cell and eosinophil numbers are expressed as total cell numbers per 258 μm wide column from the mucosal base to lumen.

	Control	Day 5	Day 10	Day 15	Day 20	Day 30
Abomasal pH	2.68 \pm 0.20	2.84 \pm 0.19	5.30 \pm 0.68	4.48 \pm 0.32	3.55 \pm 0.29	3.40 \pm 0.22
TGF- α -positive PC	145.6 \pm 9.9	108.4 \pm 6.2	83.9 \pm 4.2	88.6 \pm 6.7	92.5 \pm 7.4	105.8 \pm 10.7
Eosinophils	0.16 \pm 0.12	0.00 \pm 0.00	33.67 \pm 4.16	17.85 \pm 3.34	13.90 \pm 5.40	6.37 \pm 1.96
Mucosal thickness	328.1 \pm 8.4	332.6 \pm 31.2	425.5 \pm 6.8	438.0 \pm 12.2	429.3 \pm 22.1	368.1 \pm 8.2

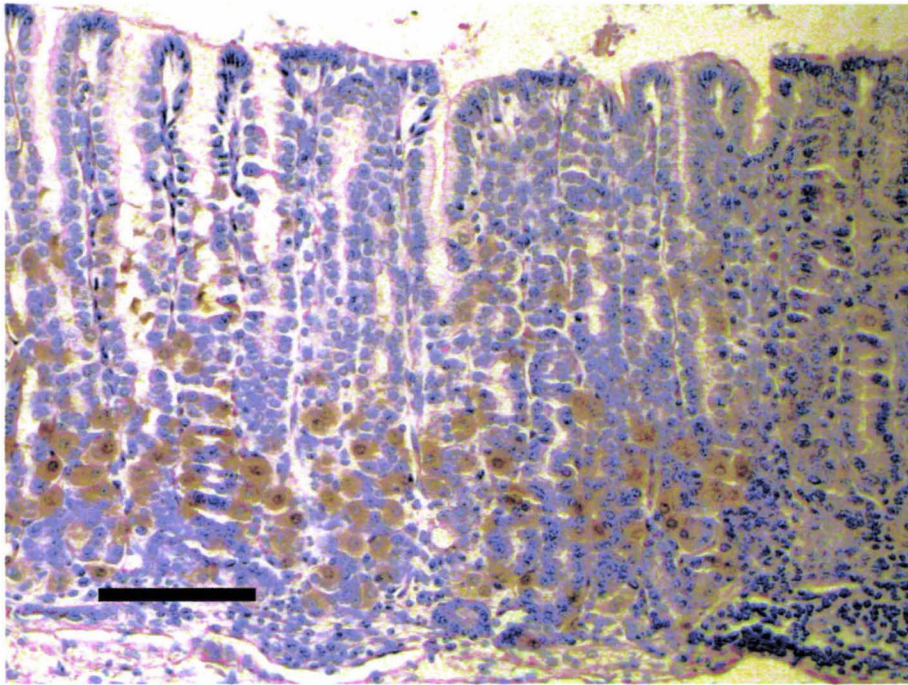


Figure 3.6. Fundic mucosa of a control (C) sheep, 28 days after infection with 50,000 L₃ *O. circumcincta*. TGF- α positive parietal cells are distributed mainly within the lower half of the mucosa. TGF- α immunohistochemistry, paraffin-embedded, 5 μ m section, Mayer's haematoxylin counterstain. Bar = 100 μ m

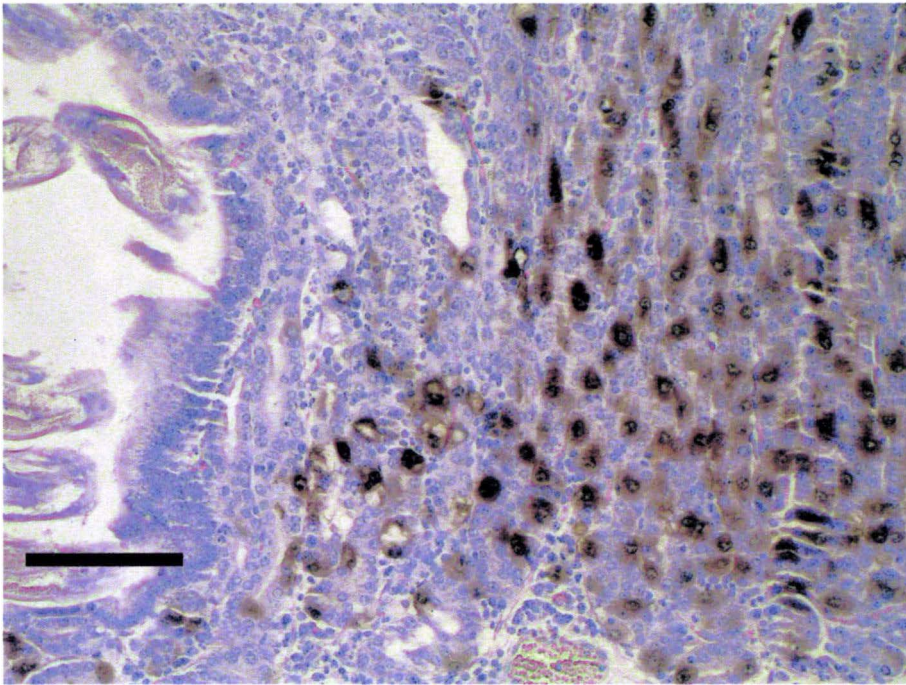


Figure 3.4. Fundic mucosa of a control (C) sheep, 8 days after infection with 50,000 L₃ *O. circumcincta*. A larval-infested gland can be seen on the left. Some parietal cells in adjacent glands are stretched and some contain vacuoles. TGF- α immunohistochemistry, paraffin-embedded, 5µm section, Mayer's haematoxylin counterstain. Bar = 100µm

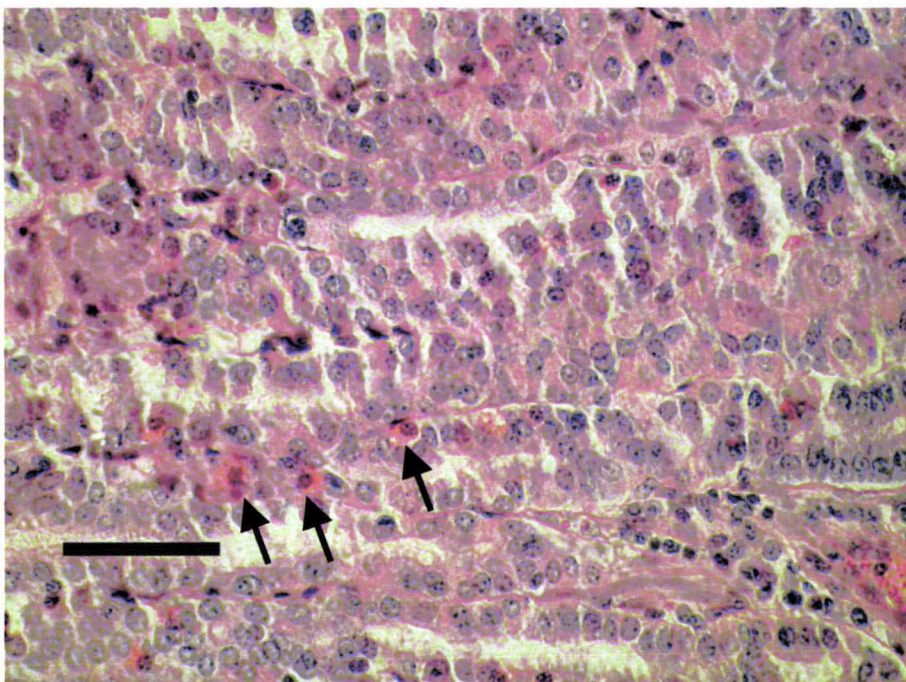


Figure 3.5. Fundic mucosa of a control (C) sheep, 28 days after infection with 50,000 L₃ *O. circumcincta*. Globule leucocytes (arrows) can be seen positioned between the epithelial cells. Paraffin-embedded, 5µm section, H&E. Bar = 100µm

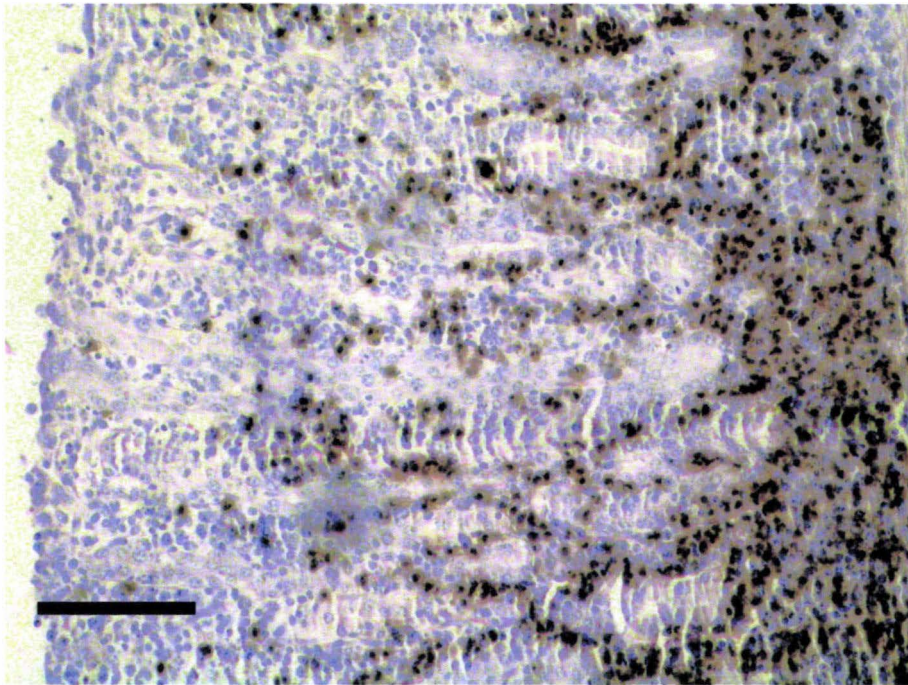


Figure 3.2. Fundic mucosa of a control (C) sheep, 8 days after infection with 50,000 L₃ *O. circumcincta*. The section was stained with a specific antibody for ovine eosinophils, which can be seen migrating in large numbers into the lamina propria. Paraffin-embedded, 5µm section, Mayer's haematoxylin counterstain. Bar = 100µm

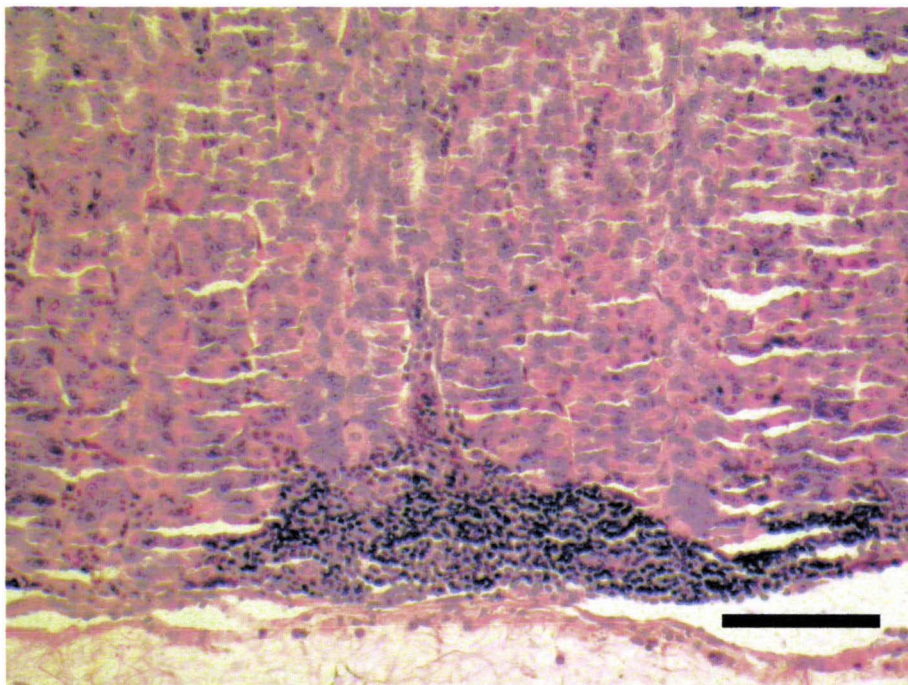


Figure 3.3. Fundic mucosa of a control (C) sheep, 8 days after infection with 50,000 L₃ *O. circumcincta*. An aggregation of lymphocytes can be seen in the base of the mucosa. Paraffin-embedded, 5µm section, H&E counterstain. Bar = 100µm

substantial numbers had migrated higher up into the mucosa forming longitudinal tracts (Figure 3.2). Lymphocytes were also present, mainly in the base of the mucosa, accumulating to dense conglomerates (Figure 3.3). Lymphocytes were less frequently encountered within the upper half of the mucosa.

In nodular areas, TGF- α positive parietal cells were primarily located in the lower half of the mucosa and, in the immediate vicinity of parasitised glands; parietal cells were often stretched and vacuolated (Figure 3.4).

3.3.1.2.2 Day 28

By Day 28, globule leucocytes were a common feature of both groups and were positioned between the epithelial cells (Figure 3.5). The number of eosinophils was markedly reduced when compared with Day 8, and eosinophils were mainly present in the sub-mucosa.

TGF- α positive parietal cells were primarily found in the lower half of the thickened mucosa and rarely within the extended pits (Figure 3.6). However, several TGF- α negative parietal cells were also detected.

3.3.2 Group LI

3.3.2.1 Abomasal Parameters

Mean values for abomasal pH, tissue eosinophils, TGF- α positive parietal cell counts and mucosal thicknesses are presented in Table 3.2 and the results for each individual animal in Figure 3.7. Mean serum gastrin concentrations in Sheep #50 and the mean for all animals surviving on each day are plotted in Figure 3.8. Abomasal pH was significantly higher on Days 10 p.i. ($p < 0.001$) and 15 p.i. ($p < 0.01$) in comparison with controls. Mucosal thickness was increased on Days 10 p.i. ($p < 0.05$), 15 p.i. ($p < 0.01$) and 20 p.i. ($p < 0.01$). A significant increase in the number of eosinophils within infected animal mucosae was detected on Days 10 p.i. ($p < 0.001$) and 15 p.i. ($p < 0.01$). Any difference between the TGF- α positive parietal cell counts of the various groups was only of borderline significance ($p = 0.06$).

The number of mucosal eosinophils was strongly correlated with abomasal pH ($r^2=0.73$, $p<0.0001$), but not with parietal cell counts. A negative, but weak, correlation existed between TGF- α counts and abomasal pH ($r^2=0.2$, $p<0.05$).

The results for the evaluation of TGF- α labelled sections after counterstaining with H&E are presented in Table 3.3. The number of parietal cells that appeared not to take up the antibody stain varied from less than 1% to 35%. TGF- α negative parietal cells were more frequently observed within control, Day 5, Day 20 and Day 30 sections, but not within Day 10 and Day 15 sections.

3.3.2.2 Light Microscopy

3.3.2.2.1 Controls

In control animals, TGF- α labelled parietal cells, were distributed along the pit and the gland (Figure 3.9). They were easily identified by their typical round to triangular shape, abundant cytoplasm and a round and dark-staining nucleus. Most of the parietal cells were present in the upper base or neck of the glands, with fewer cells in the base of the glands and in the pits (Figures 3.10 and 3.11). Parietal cells in the gland base often stained more intensely for TGF- α than parietal cells in the other locations.

There was marked variation in the numbers of TGF- α positive cells between control animals, although mucosal thickness varied little across the group. The small number of parietal cells that did not take up the TGF- α stain could be found anywhere along the fundic unit and appeared morphologically like other mature parietal cells.

The mucosal pits were no longer than a third of the total mucosal thickness and each pit was associated with one or more glands at its base. Surface mucous cells had a cuboidal to columnar shape, contained moderate amounts of mucus and many showed a weak reaction with the TGF- α antibody. All epithelial cells appeared normal and few cells of the inflammatory system were detected within the mucosa and only occasionally were a few seen within the sub-mucosa. In comparison, the mucosa on the tip of the abomasal folds often contained more inflammatory cells, mainly round cells and some eosinophils.

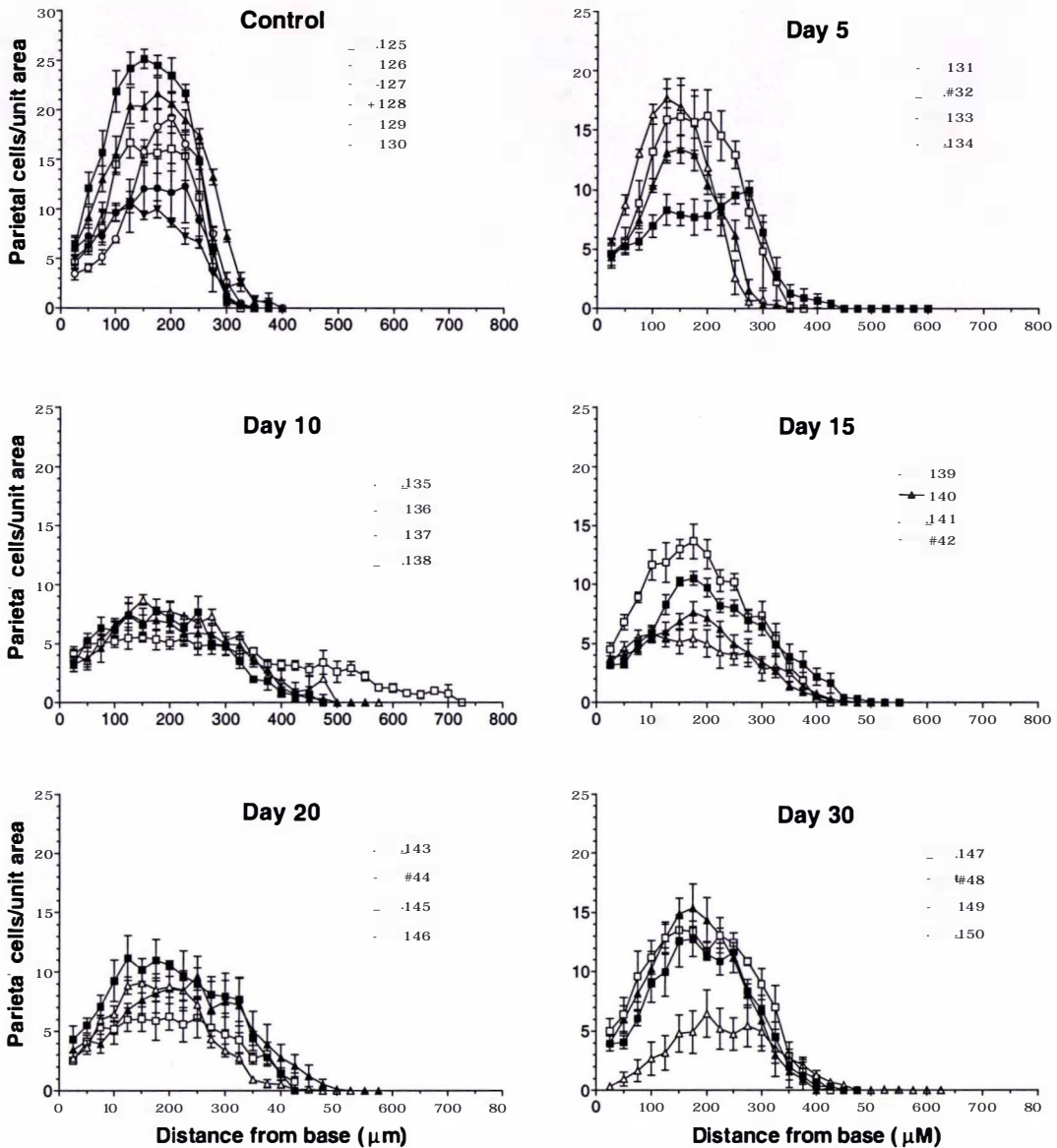


Figure 3.11. Numbers of TGF- α stained parietal cells (mean \pm SEM) per unit area in 258 μ m wide columns of fundic mucosa of individual sheep before and 5, 10, 15, 20 or 30 days after infection with 35,000 L₃ *O.circumcincta*.

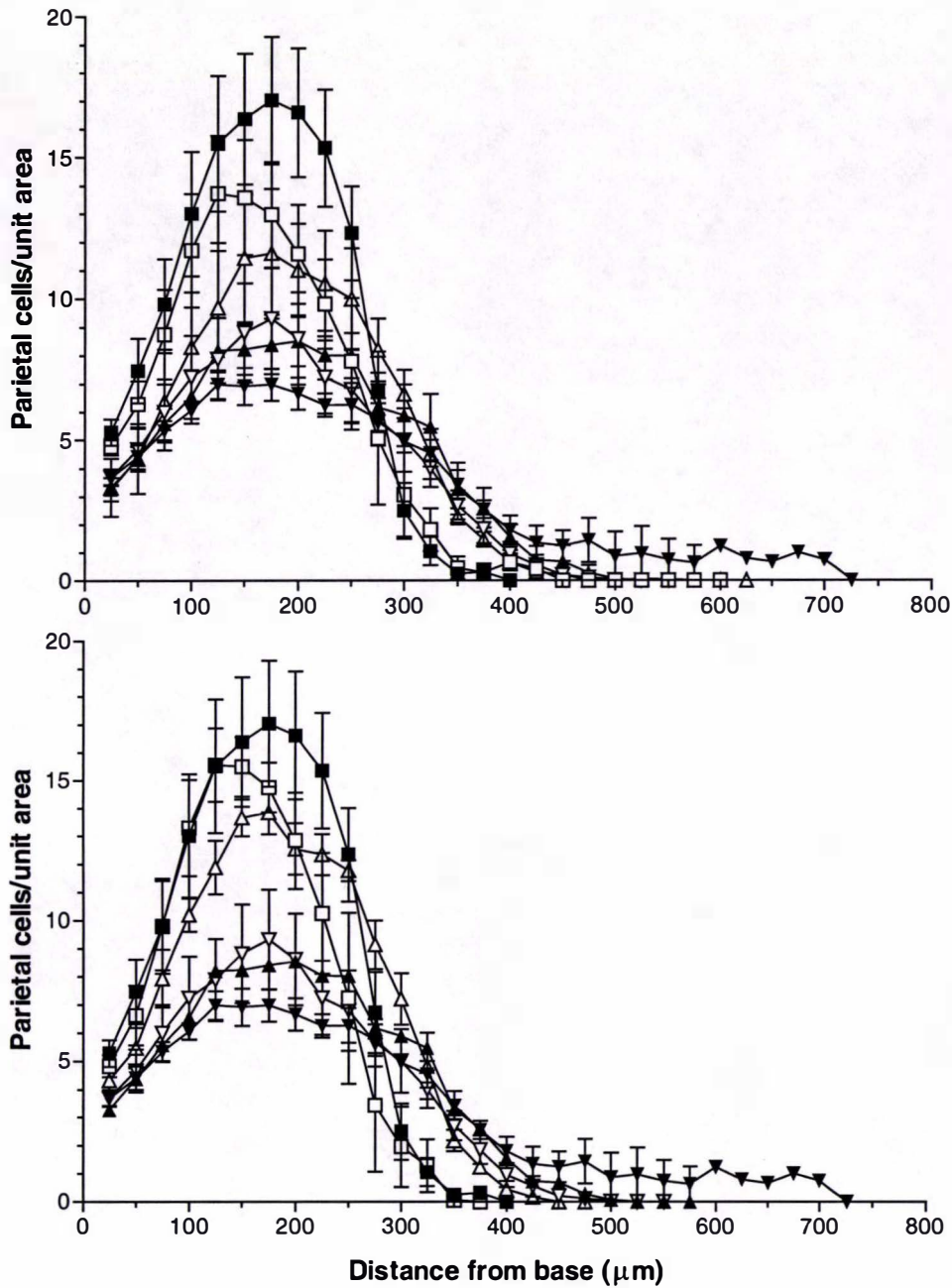


Figure 3.10. Numbers of TGF- α stained parietal cells (mean \pm SEM) per unit area in 258 μ m wide columns of fundic mucosa of groups of sheep before (control CT) and 5, 10, 15, 20 or 30 days after infection with 35,000 L₃ *O.circumcincta*. Top: all sheep included; Bottom: means with Sheep #31 excluded from the Day 5 group data and Sheep #50 excluded from the Day 30 group data. Symbols: CT: ■; Day 5: □; Day 10: ▼; Day 15: ▽; Day 20: ▲; Day 30: △.

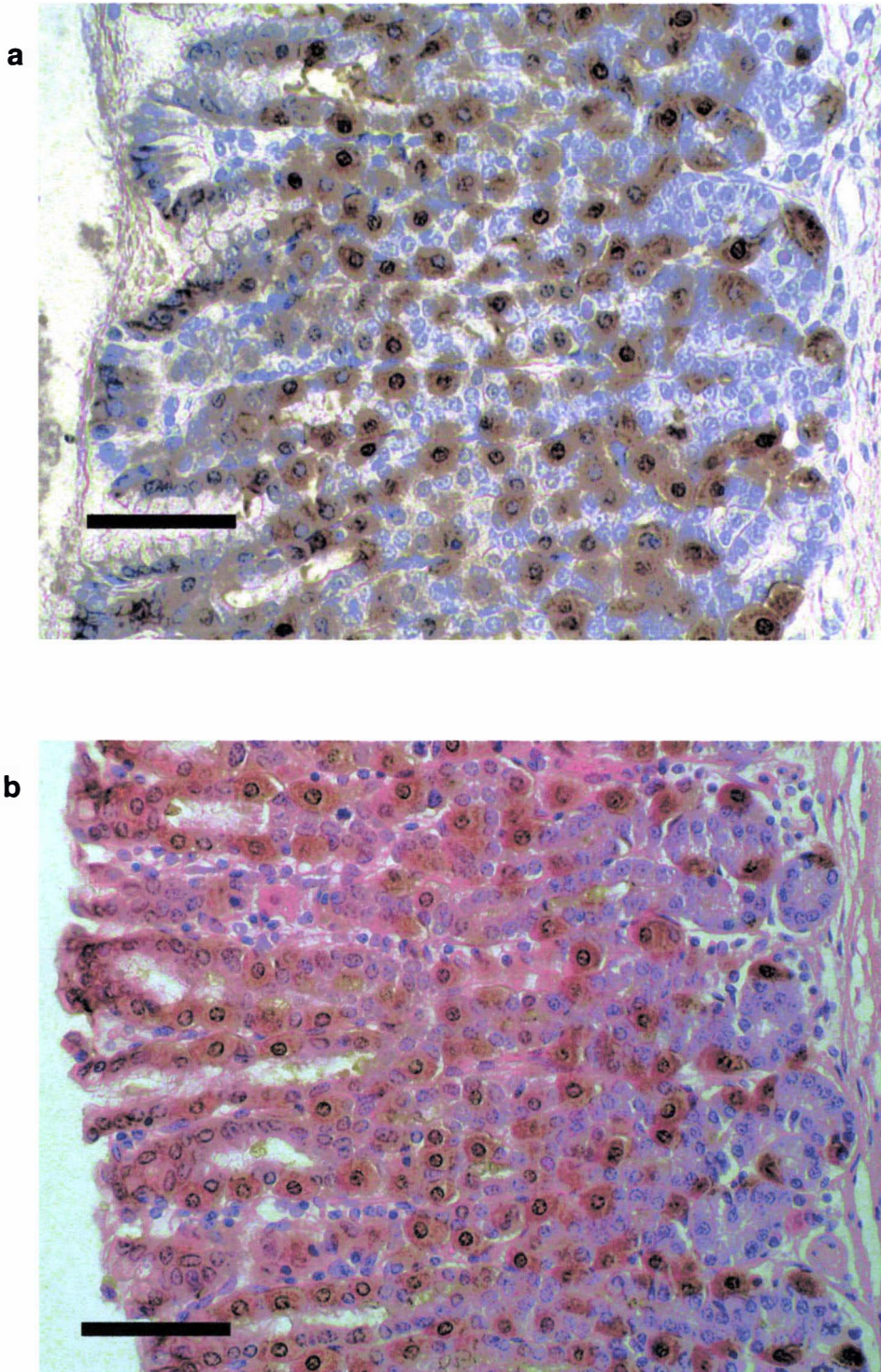


Figure 3.9a and b. Fundic mucosa of an uninfected control sheep from the larval infection experiment, showing the distribution of TGF- α positive parietal cells within the pits and glands. TGF- α immunohistochemistry, paraffin-embedded, 5 μ m section, counterstained with (a) Mayer's haematoxylin and (b) H&E. Bar = 50 μ m

The number of mucosal eosinophils was strongly correlated with abomasal pH ($r^2=0.73$, $p<0.0001$), but not with parietal cell counts. A negative, but weak, correlation existed between TGF- α counts and abomasal pH ($r^2=0.2$, $p<0.05$).

The results for the evaluation of TGF- α labelled sections after counterstaining with H&E are presented in Table 3.3. The number of parietal cells that appeared not to take up the antibody stain varied from less than 1% to 35%. TGF- α negative parietal cells were more frequently observed within control, Day 5, Day 20 and Day 30 sections, but not within Day 10 and Day 15 sections.

3.3.2.2 Light Microscopy

3.3.2.2.1 Controls

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There was marked variation in the numbers of TGF- α positive cells between control animals, although mucosal thickness varied little across the group. The small number of parietal cells that did not take up the TGF- α stain could be found anywhere along the fundic unit and appeared morphologically like other mature parietal cells.

The mucosal pits were no longer than a third of the total mucosal thickness and each pit was associated with one or more glands at its base. Surface mucous cells had a cuboidal to columnar shape, contained moderate amounts of mucus and many showed a weak reaction with the TGF- α antibody. All epithelial cells appeared normal and few cells of the inflammatory system were detected within the mucosa and only occasionally were a few seen within the submucosa. In comparison, the mucosa on the tip of the abomasal folds often contained more inflammatory cells, mainly round cells and some eosinophils.

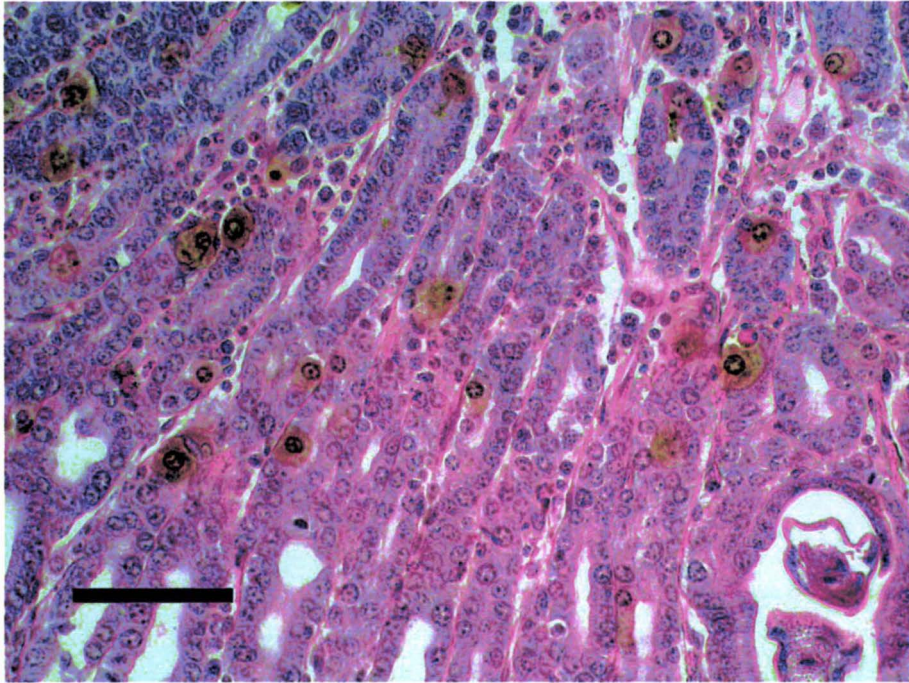


Figure 3.16. Fundic mucosa of sheep 31, 5 days after infection with 35,000 L₃ *O. circumcincta*. In the immediate vicinity of a parasitised gland (bottom right) there is a marked loss of labelled parietal cells. Some inflammatory cells are also present. TGF- α immunohistochemistry, paraffin-embedded, 5 μ m section, H&E counterstain. Bar = 50 μ m

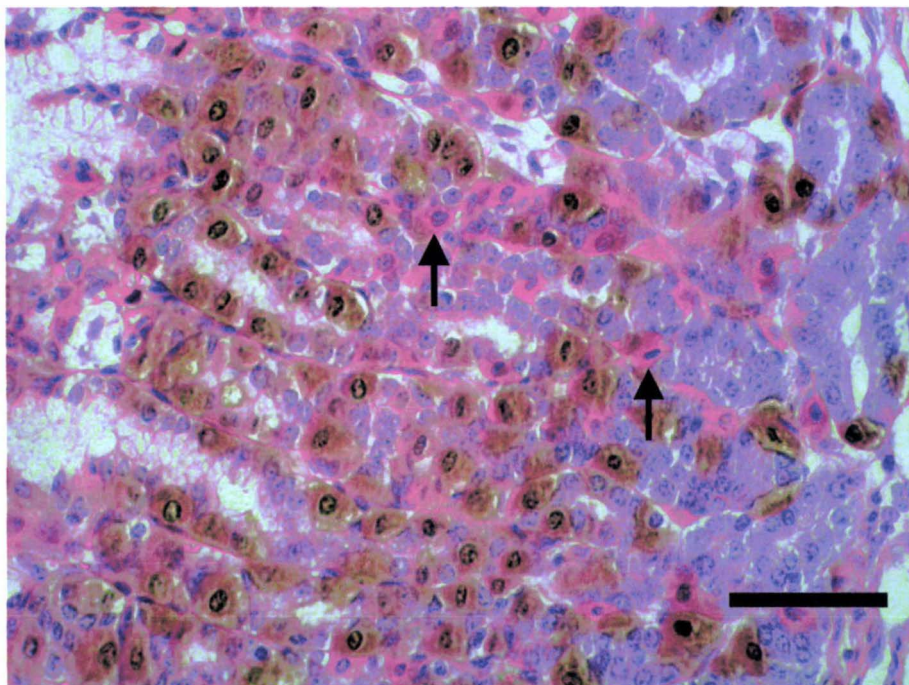


Figure 3.17. Fundic mucosa of a sheep, 5 days after infection with 35,000 L₃ *O. circumcincta*. Some parietal cells are TGF- α negative (arrows) and have abnormal morphology, having strongly eosinophilic cytoplasm and small, dense nuclei. TGF- α immunohistochemistry, paraffin-embedded, 5 μ m section, H&E counterstain. Bar = 50 μ m

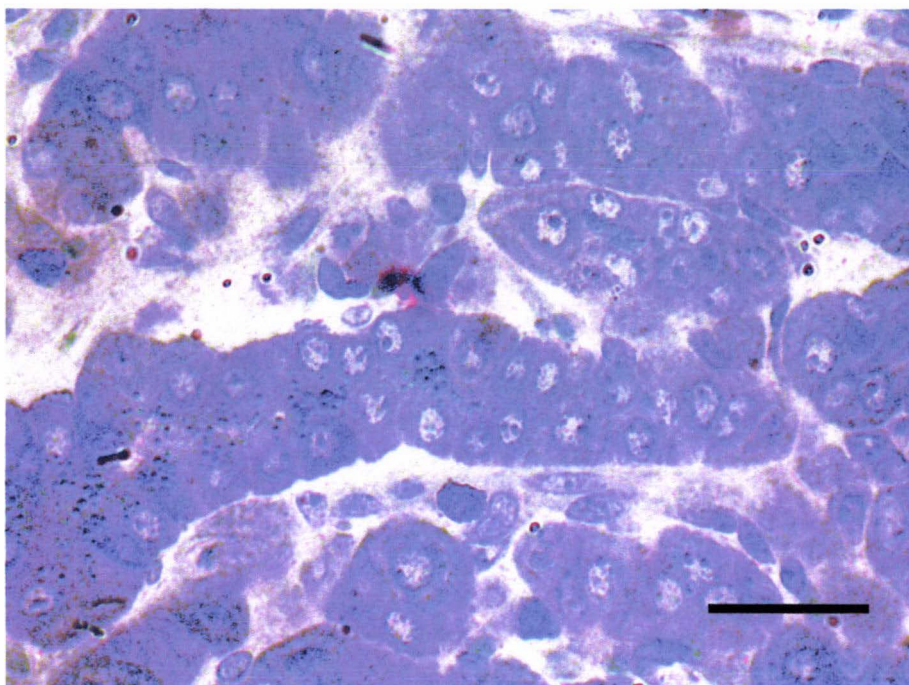


Figure 3.14. Fundic mucosa at mid-gland level of an uninfected control sheep from the larval infection experiment. At this level of the mucosa, triangular parietal cells with large nuclei and abundant cytoplasm are present alongside poorly differentiated cells and mucous neck cells. Paraffin-embedded, semithin section, Toluidine blue. Bar = 25 μ m

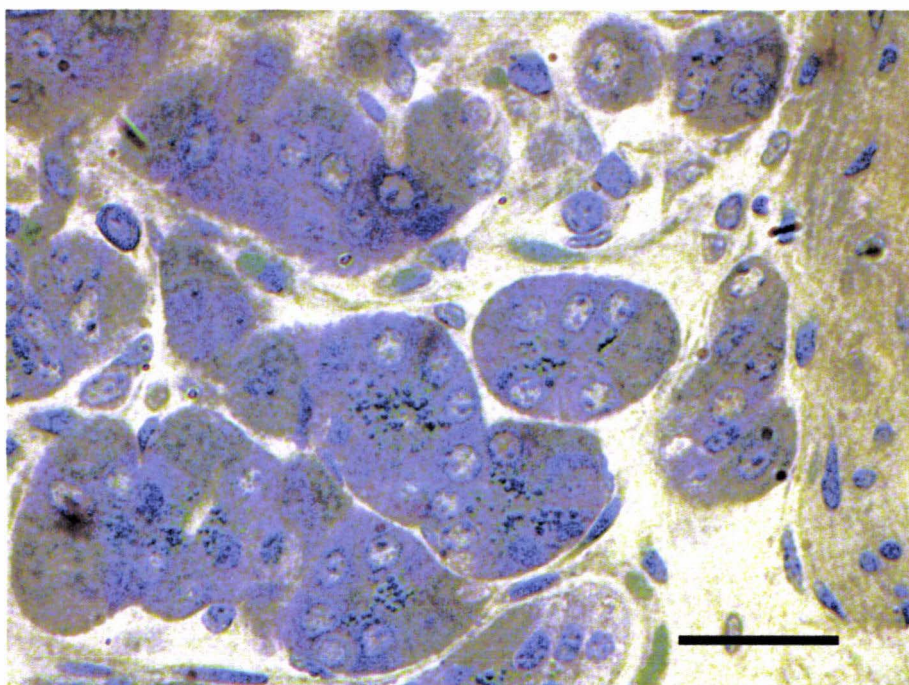


Figure 3.15. Fundic mucosa at the level of the gland base of an uninfected control sheep from the larval infection experiment. At this level of the mucosa, chief cells with their large apical granules are present alongside parietal and endocrine cells. Paraffin-embedded, semithin section, Toluidine blue. Bar = 25 μ m

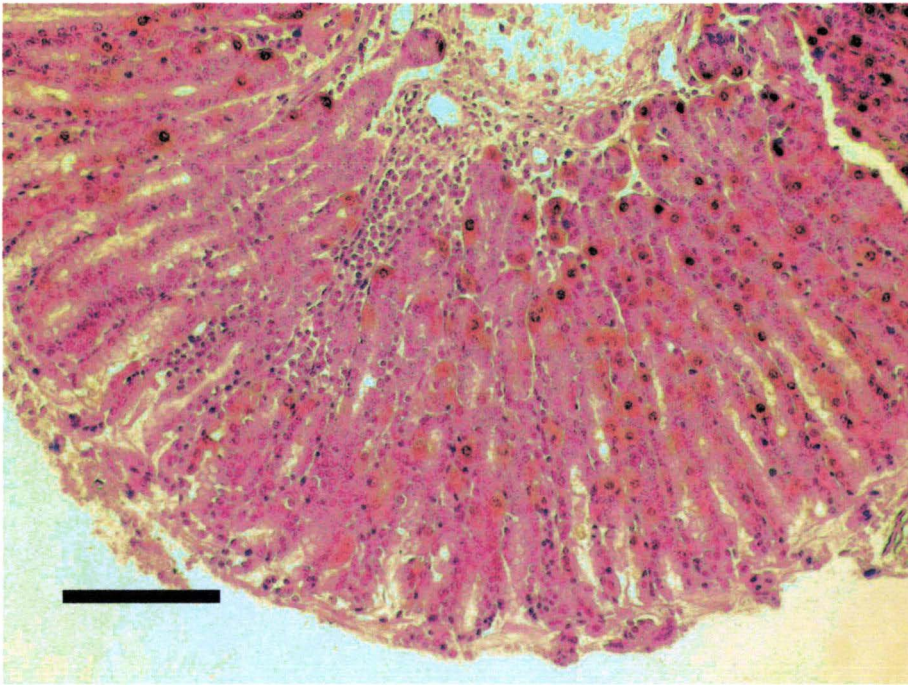


Figure 3.12. Fundic mucosa of an uninfected control sheep from the larval infection experiment, showing the distribution of TGF- α positive parietal cells within the pits and glands in the tip of the fold. TGF- α immunohistochemistry, paraffin-embedded, 5 μ m section, H&E counter-stain. Bar = 100 μ m

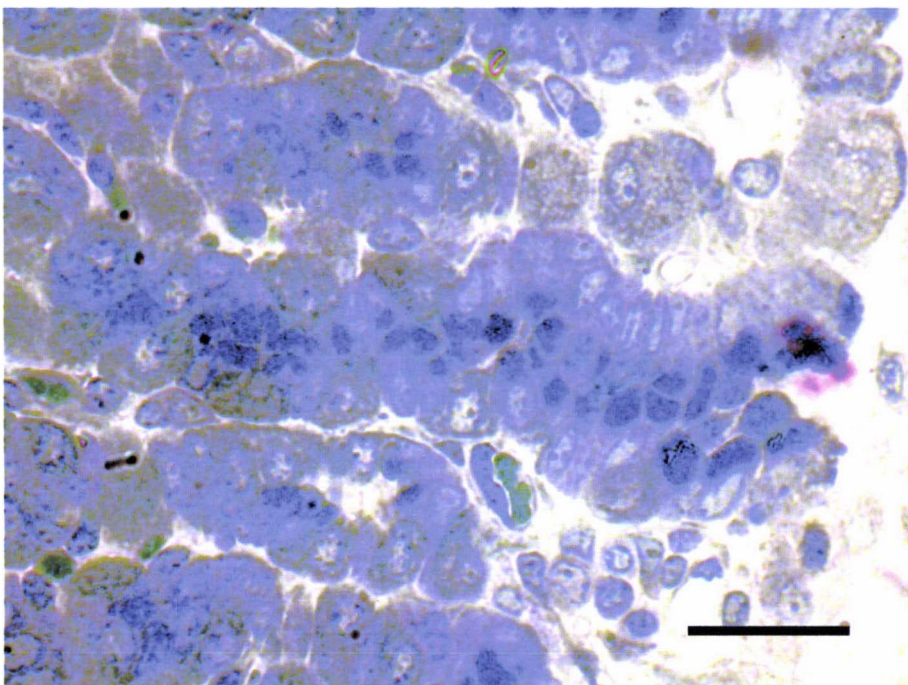


Figure 3.13. Fundic mucosa of an uninfected control sheep from the larval infection experiment. The pits are lined by surface mucous cells with abundant granules in their apical cytoplasm. Paraffin-embedded, semithin section, Toluidine blue. Bar = 25 μ m

At the tip of the folds there appeared to be fewer TGF- α positive parietal cells in glands and the pits seemed deeper (Figure 3.12).

In semithin sections stained with toluidine blue, mucous cells at the mucosal surface and in the pit contained abundant densely stained granules in the apical cytoplasm (Figure 3.13). The isthmus was characterised by the presence of poorly differentiated cells with large nuclei. Parietal cells appeared as large, often triangular-shaped cells, situated between mucous, mucous neck cells, chief cells and endocrine cells. Parietal cells were easily recognised by their large sized nuclei and generous cytoplasm (Figure 3.14). Endocrine and chief cells were found in the base and chief cells contained large dark round granules in their apical cytoplasm (Figure 3.15).

3.3.2.2.2 Day 5

Three out of the four sheep that had been killed on Day 5 p.i. had areas of moderate histopathological change, while Sheep #31 displayed more dramatic mucosal change (Figure 3.16).

In sections of all animals, normal healthy mucosa could be found beside localised affected areas, which were associated with the presence of larvae within glands (=nodules). Hyperplasia within nodules resulted in the presence of increased numbers of poorly differentiated cells and reductions in the numbers of mature cell types. In the close vicinity of parasitised glands mitotic figures were also a more frequent observation in the mid-gland region.

Within nodules, strongly stained, TGF- α positive parietal cells were mostly confined to the base and neck of glands (Figures 3.10 and 3.11), whilst many parietal cells in the upper gland did not stain or only had weakly stained nuclei. Counterstaining with H&E revealed that some TGF- α negative parietal cells had abnormal morphology, having strongly eosinophilic cytoplasm and very dense and small nuclei (Figure 3.17).

In non-nodular areas and in most of the animals, the surface mucous cells often contained a visibly increased amount of mucus in their apical cytoplasm. Infrequently a small number of inflammatory cells, mainly lymphocytes and

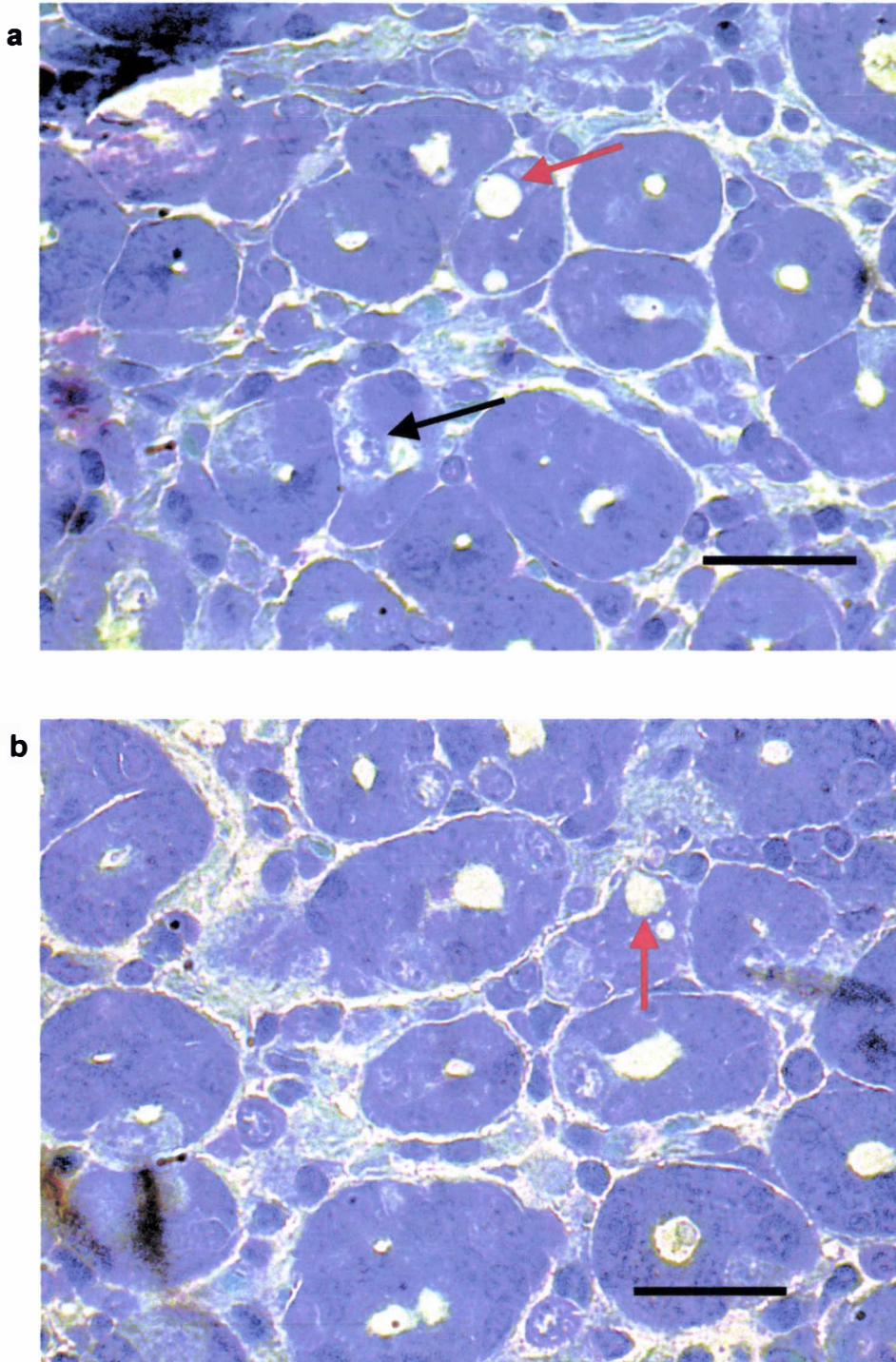


Figure 3.19a and b. Fundic mucosa of a sheep, 5 days after infection with 35,000 L₃ *O. circumcincta*. (a) Some parietal cells have pale, swollen nuclei and pale cytoplasm (black arrow) whilst other cells are vacuolated (red arrows). Paraffin-embedded, semithin section. Toluidine blue. Bar = 25µm

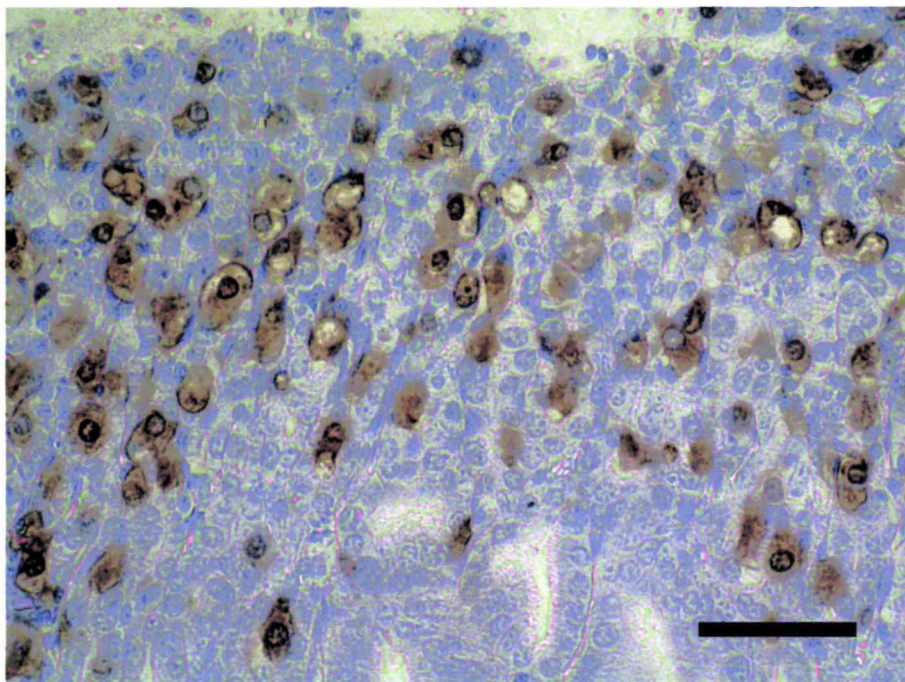


Figure 3.18. Fundic mucosa of a sheep, 5 days after infection with 35,000 L₃ *O. circumcincta*. Some TGF- α positive parietal cells also contain vacuoles. TGF- α immunohistochemistry, paraffin-embedded, 5 μ m section, Mayer's haematoxylin counterstain. Bar = 50 μ m

granulocytes, were observed in the submucosa and in the base of the abomasal mucosa, although not necessarily in the mucosa immediately surrounding a nodule. The majority of parietal cells in all sheep appeared normal, although a minority was affected by vacuolation. Affected parietal cells were recognised by the presence of quite large vacuoles, which marginalized the cytoplasm, did not take any stain and gave the cell a bulging appearance (Figure 3.18). In Sheep #31, the previously described changes and vacuolation of parietal cells were more generalised and pronounced, and mucosal thickness was also visibly increased compared to the other group members.

Vacuolated parietal cells were examined further in both semithin and EM sections. Affected parietal cells had one or more vacuoles (Figure 3.19). When several vacuoles were present in the one cell, the vacuoles usually did not communicate and were each lined by a thin membrane. In some cases the nuclei were flattened and squashed against the cell membrane. Other non-vacuolated, but still abnormal parietal cells had pale slightly swollen nuclei and a thinned cytoplasm within which were multiple paler areas. Otherwise, the majority of epithelial cells examined appeared normal.

3.3.2.2.3 Day 10

All four sheep euthanased ten days after infection demonstrated very similar histopathology within the sections investigated. Day 10 sheep appeared to have fewer TGF- α positive parietal cells, although there had been no significant difference compared to the controls or animals killed on other days. Overall, however, the TGF- α counts recorded for sheep sacrificed on Day 10 p.i. were amongst the lowest recorded in this study (Figures 3.7, 3.10 and 3.11).

Regions which showed a reasonably normal distribution of TGF- α positive parietal cells were still detectable in sections derived from these sheep, yet, most areas were affected by a generalised hyperplasia associated with an increasing length of wider pits. The division between pit and gland also became less distinct. Areas with generalised hyperplasia were easily recognised by the increased numbers of poorly differentiated cells with large nuclei and pale cytoplasm, amongst which were an increased number of mitotic

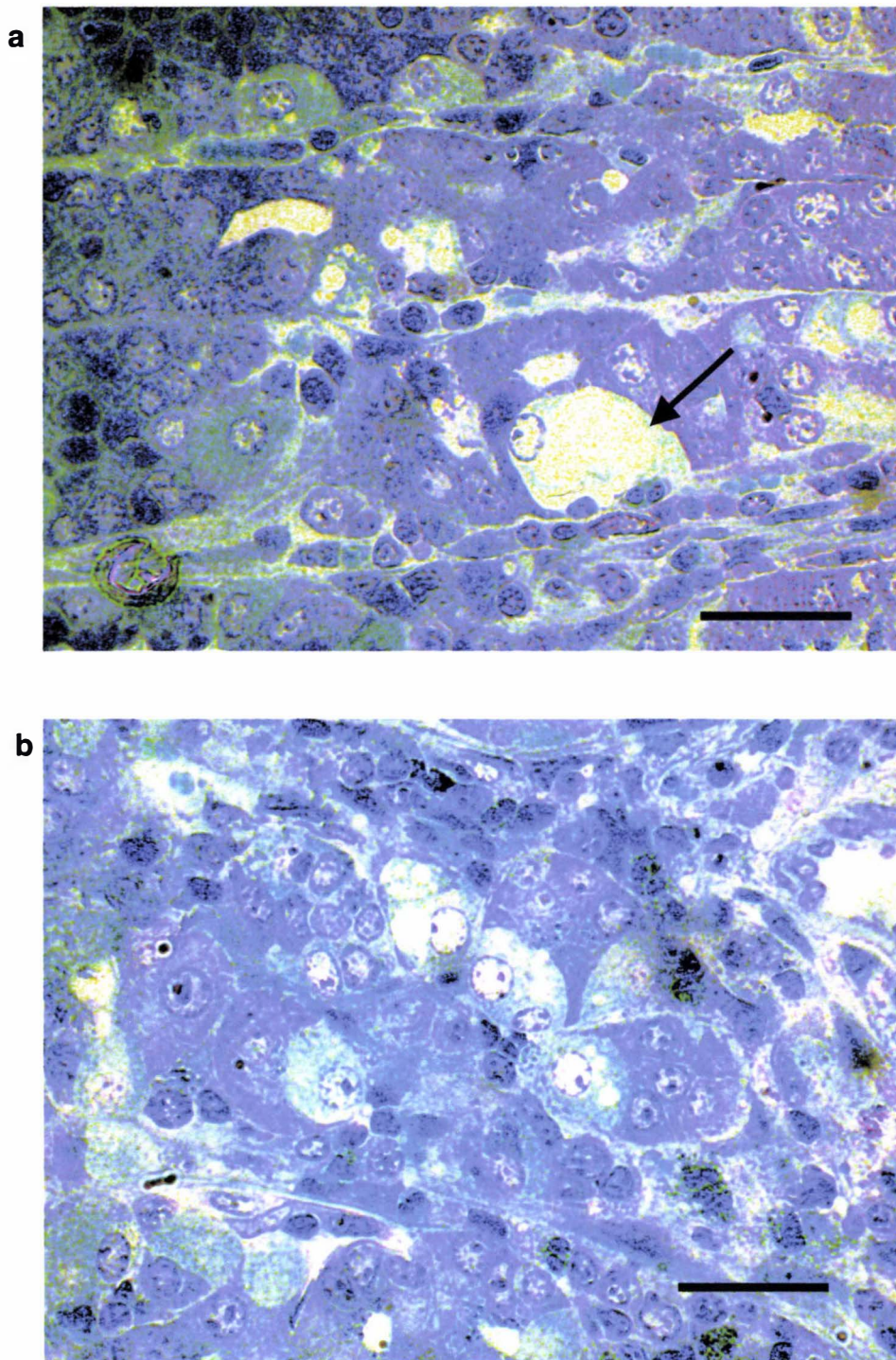


Figure 3.25a and b. Fundic mucosa of a sheep, 10 days after infection with 35,000 *O. circumcincta*. In both photomicrographs, vacuolation of parietal cells is obvious. In (a) the size of one affected parietal cell has been markedly increased by the presence of several vacuoles (arrow). Paraffin-embedded, semithin section. Toluidine blue. Bar = 25µm

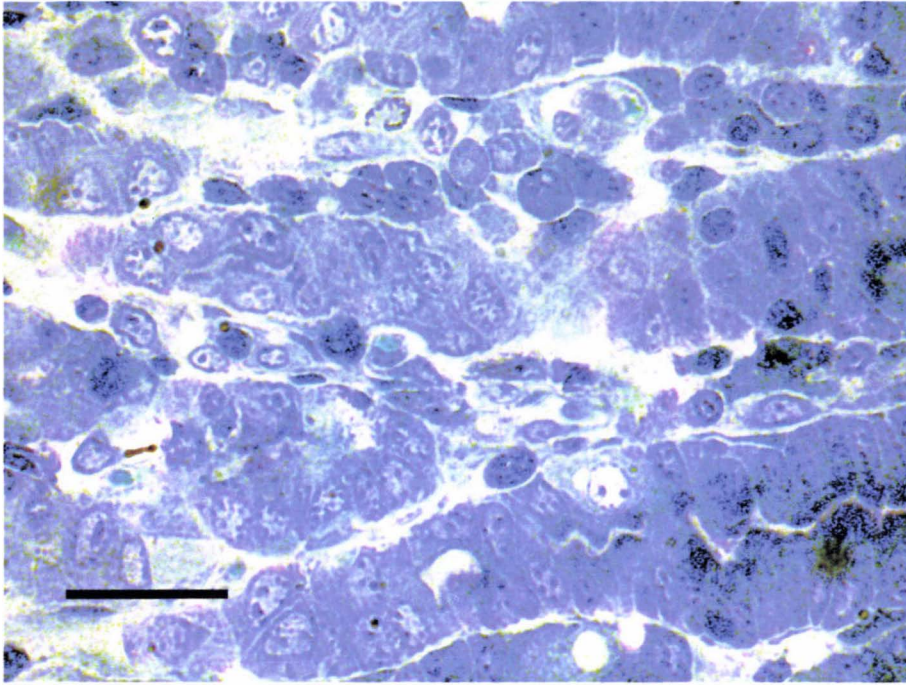


Figure 3.24. Fundic mucosa at mid-gland level of a sheep, 10 days after infection with 35,000 L₃ *O. circumcincta*. This level is now dominated by poorly differentiated cells with irregularly shaped, often large, nuclei. Paraffin-embedded, semithin section, Toluidine blue. Bar = 25 μ m

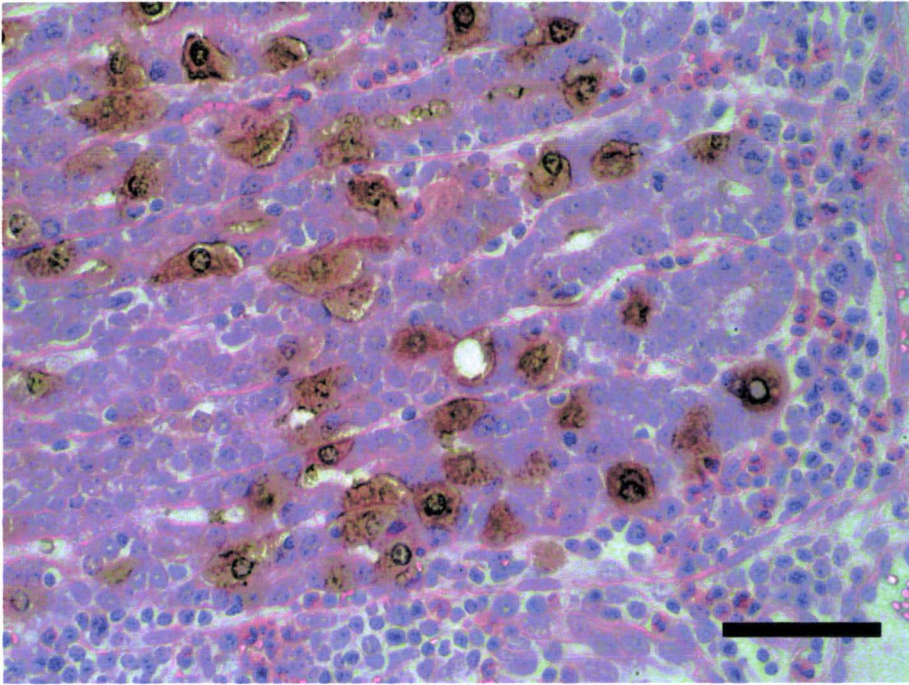


Figure 3.22. Fundic mucosa of a sheep, 10 days after infection with 35,000 L₃ *O. circumcincta*. Large numbers of eosinophils and lymphocytes can be seen migrating into the lamina propria. TGF- α immunohistochemistry, paraffin-embedded, 5 μ m section, H&E counterstain. Bar = 50 μ m

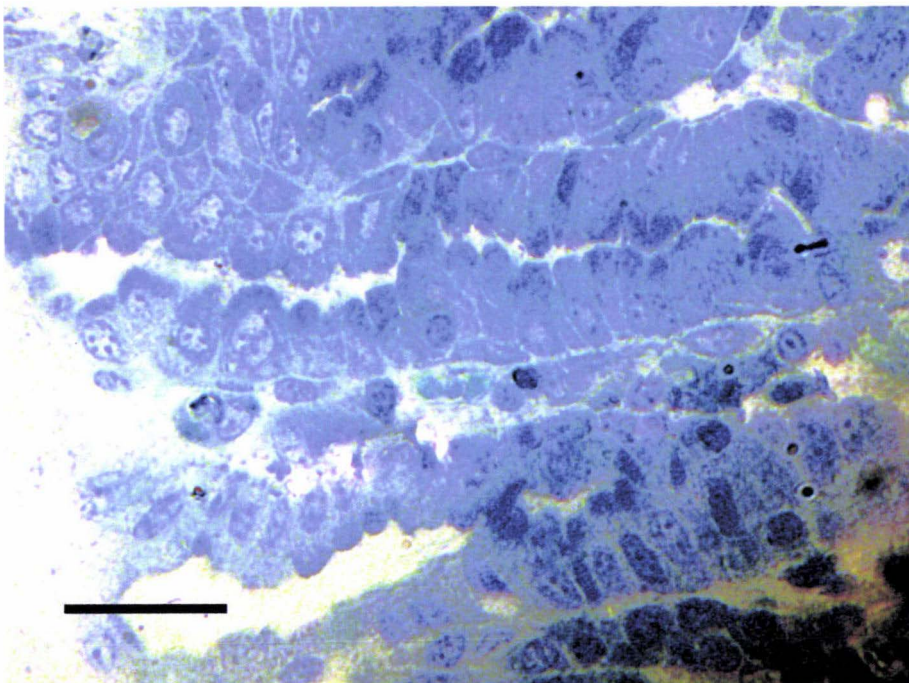


Figure 3.23. Fundic mucosa of a sheep, 10 days after infection with 35,000 L₃ *O. circumcincta*. Taller pits extend deeper into the tissue. Paraffin-embedded, semithin section, Toluidine blue. Bar = 25 μ m

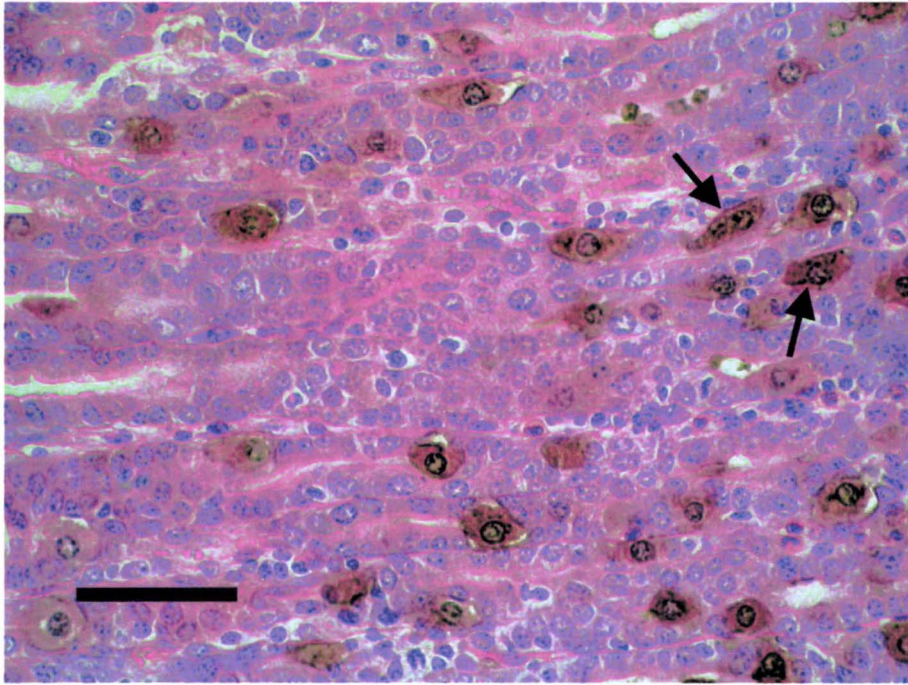


Figure 3.20. Fundic mucosa of a sheep, 10 days after infection with 35,000 L₃ *O. circumcincta*. In an area with reduced numbers of parietal cells, some of those remaining are abnormally stretched (arrow). TGF- α immunohistochemistry, paraffin-embedded, 5µm section, H&E counterstain. Bar = 50µm

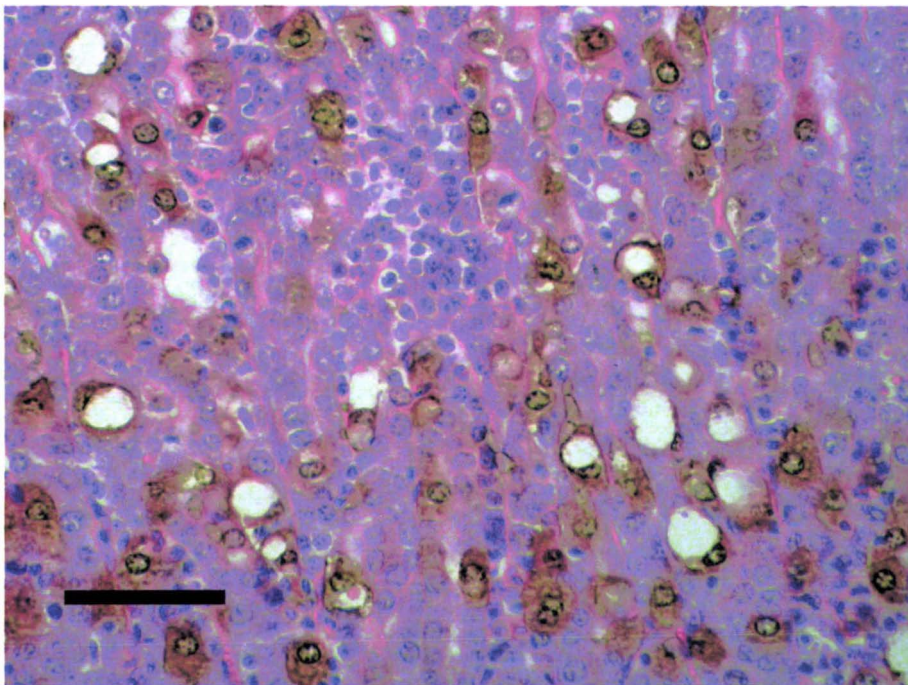


Figure 3.21. Fundic mucosa of a sheep, 10 days after infection with 35,000 L₃ *O. circumcincta*. Many parietal cells are now vacuolated. TGF- α immunohistochemistry, paraffin-embedded, 5µm section, H&E counterstain. Bar = 50µm

figures. Pit cells appeared to contain more mucus than pit cells of control animals.

In some focal areas, parietal cell number was clearly depleted. The remaining parietal cells were spread out within glands and some were abnormally shaped, appearing to have been stretched in line with the long axis of the glands (Figure 3.20). Vacuolated parietal cells were extremely common with the majority being located at base and mid-gland levels (Figure 3.21). The appearance of vacuolated parietal cells varied, although the vacuoles were in general larger than the vacuoles seen on Day 5. Vacuolated cells had either one big vacuole, which on occasions indented or even flattened the nucleus, or a number of vacuoles was evident, though it was not clear if they communicated with each other. Another change present in Day 10 tissues was the now prominent influx of inflammatory cells into the altered mucosae. The inflammatory cells were mainly lymphocytes and granulocytes, mostly eosinophils and were present in tracts within the submucosa and mucosa (Figure 3.22). Lymphocytes were sometimes located in large clusters in the submucosa and in the base of the mucosa.

The above changes were even more pronounced in fold-tip areas, which demonstrated long pits, marked loss of TGF- α positive parietal cells and a massive influx of inflammatory cells.

In semithin sections from the Day 10 animals, the pits were extended (Figure 3.23) and numerous poorly differentiated cells were clearly identifiable, the latter within the mid-gland level (Figure 3.24). Vacuolated parietal cells varied in terms of the number, shape and size of the vacuoles they contained (Figure 3.25). Most of the vacuoles were round or oval in shape and frequently were of similar size as the nucleus. Affected parietal cells sometimes also had a pale nucleus.

3.3.2.2.4 Day 15

Tissues from Day 15 animals also featured generalised mucosal hyperplasia and parietal cells were distributed mainly at mid-gland and base level (Figures

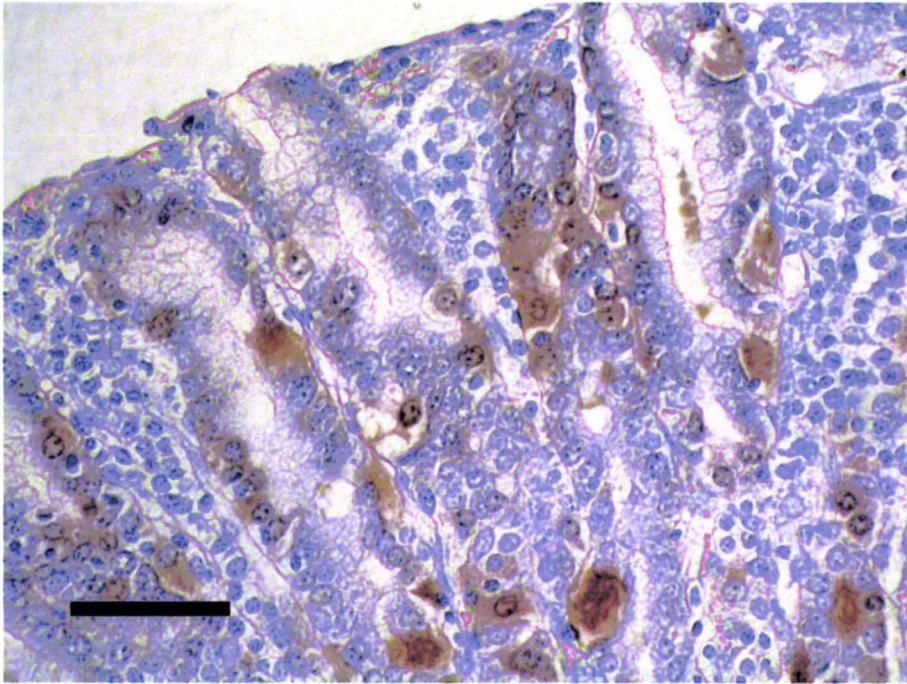


Figure 3.28. Fundic mucosa of a sheep, 15 days after infection with 35,000 L₃ *O. circumcincta*. Lymphocytes and plasma cells can be seen within the expanded lamina propria. TGF- α immunohistochemistry, paraffin-embedded, 5 μ m section, H&E counterstain. Bar = 50 μ m

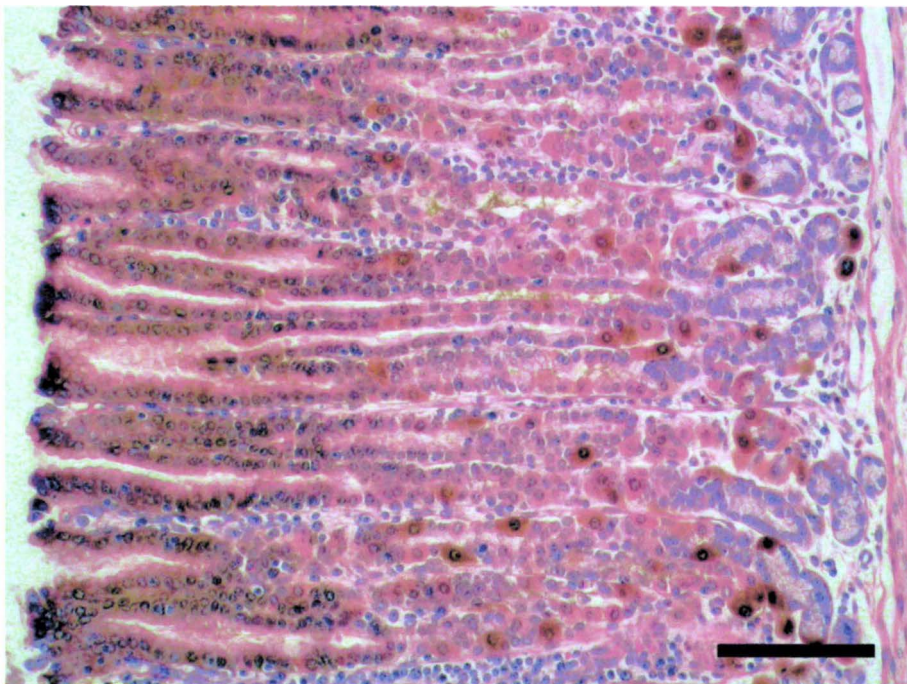


Figure 3.29. Fundic mucosa of a sheep, 20 days after infection with 35,000 L₃ *O. circumcincta*. The division between the pit and the gland has become indistinct, the pits appearing to extend deeper into the tissue. The number of TGF- α positive parietal cells appears reduced and confined to the basal mucosal half. Numerous non-staining parietal cells are present. TGF- α immunohistochemistry, paraffin-embedded, 5 μ m section, H&E counterstain. Bar = 100 μ m

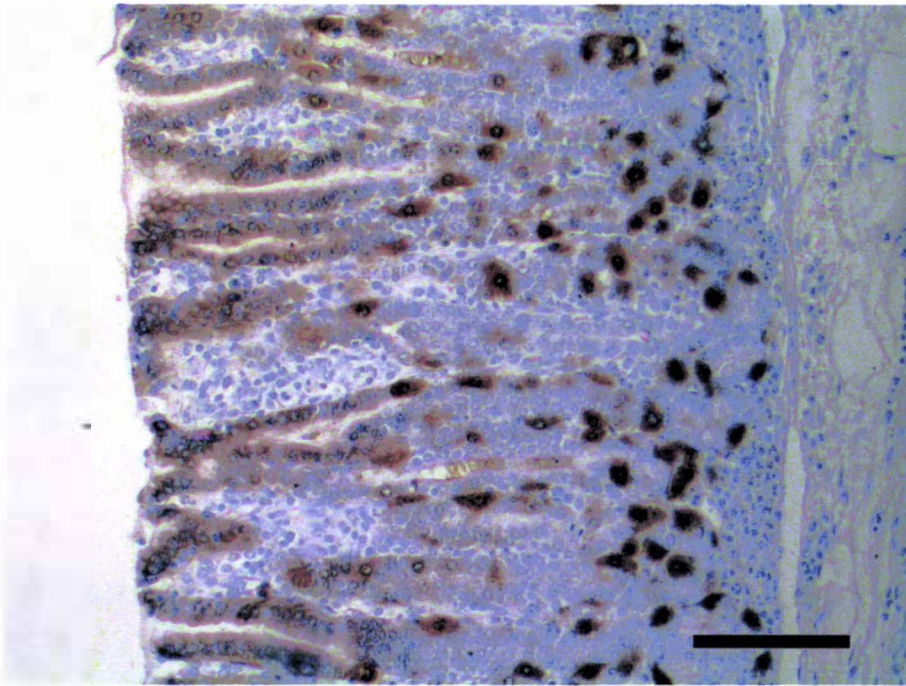


Figure 3.26. Fundic mucosa of a sheep, 15 days after infection with 35,000 L₃ *O. circumcincta*. TGF- α positive parietal cells are more numerous in the basal mucosal half. The mucous cells lining the pits are also positive. TGF- α immunohistochemistry, paraffin-embedded, 5 μ m section, Mayer's haematoxylin counterstain. Bar = 100 μ m

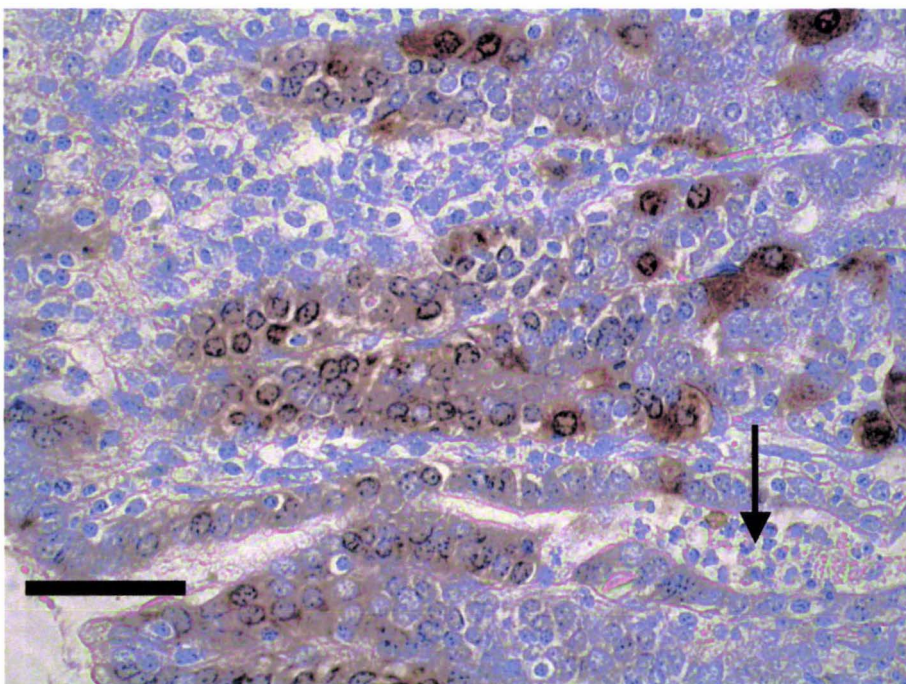


Figure 3.27. Fundic mucosa of a sheep, 15 days after infection with 35,000 L₃ *O. circumcincta*. Across the bottom of the photomicrograph lies a pit/gland with a dilated lumen containing cellular debris (arrow) that may include degenerate granulocytes. TGF- α immunohistochemistry, Mayer's haematoxylin counterstain. Bar = 50 μ m

3.10 and 3.11). As a result of the hyperplasia, the depth of the mucosa was still clearly increased.

Cells reacting positively with TGF- α antibodies were not only parietal cells, but also cells of the mucous cell type. In these cells, the cytoplasm, especially that around the nucleus seemed to take up the stain. In contrast, the majority of the parietal cells in the base stained equally strongly in both the cytoplasm and nucleus (Figure 3.26). By Day 15, fewer vacuolated parietal cells were present in comparison with Day 10.

The pits were elongated, reaching 1/2 or up to 2/3 of the way into the mucosa, and had wide-open lumina, some containing cellular debris and inflammatory cells (Figure 3.27). Mitotic figures were frequently spotted at the mid-gland level, although they could also be seen in the pit region.

In comparison with Day 10, a new feature of the inflammatory response was the presence of plasma cells, located within either the mucosa or the submucosa in a similar distribution as those of lymphocytes and granulocytes (Figure 3.28). These latter cells were often grouped together in a wedge-shaped aggregation.

3.3.2.2.5 Day 20

Generalised mucosal hyperplasia causing an increase in mucosal thickness was also a characteristic of tissues obtained from sheep euthanased 20 days after infection.

TGF- α positive parietal cells with both a strong staining cytoplasm and nucleus were mainly present in the gland base (Figures 3.10 and 3.11), though a number of pale-staining parietal cells were present in the neck and isthmus. Parietal cells, which did not stain for TGF- α , resembled mature cells and were mainly found in the mid-gland and upper base region. Vacuolated parietal cells were not found.

In some tissues there was now little distinction between the pits and the glands so that the pits, lined with TGF- α positive mucous cells, appeared to extend the full depth of the mucosa or at least 2/3 into it (Figure 3.29). A new feature was

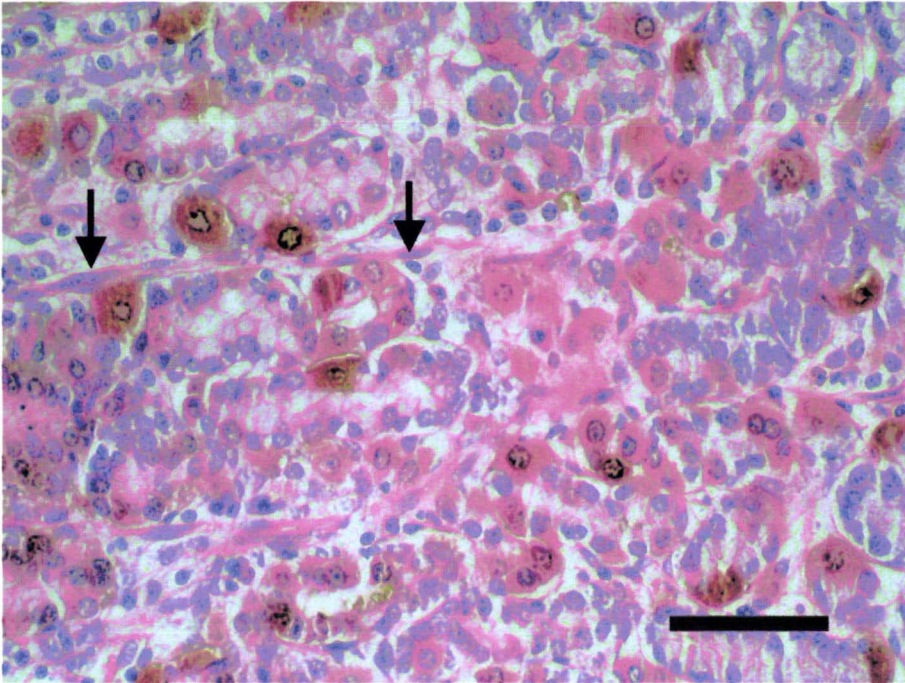


Figure 3.30. Fundic mucosa of a sheep, 20 days after infection with 35,000 L₃ *O. circumcincta*. Increased numbers of fibrocytes (arrows) can be seen between the glands. TGF- α immunohistochemistry, paraffin-embedded, 5µm section, H&E counterstain. Bar = 50µm

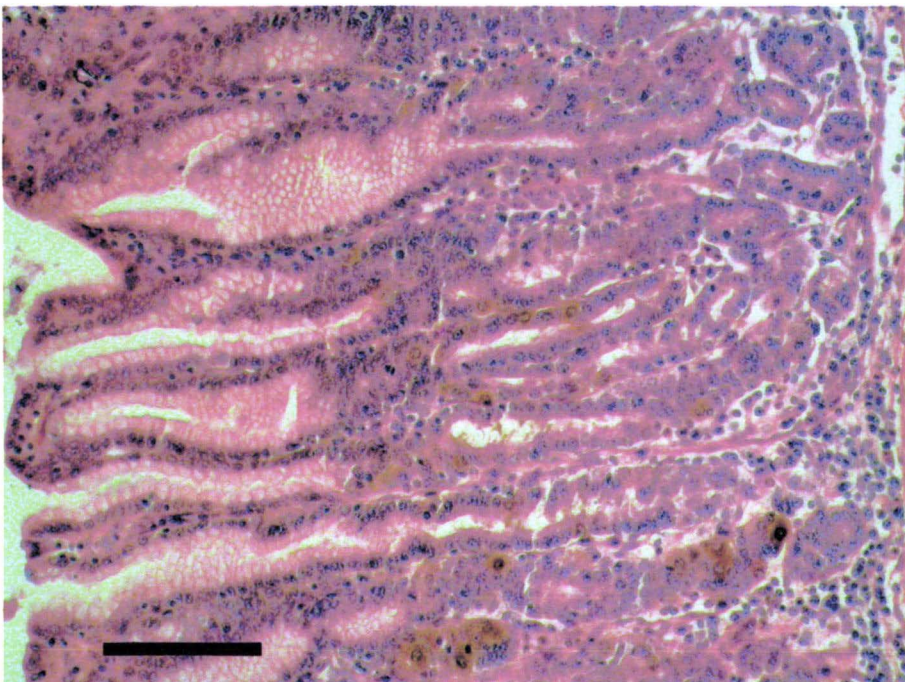


Figure 3.31. Fundic mucosa of sheep **50**, 30 days after infection with 35,000 L₃ *O. circumcincta*. There is marked hyperplasia of mucous cells in the outer mucosal half and a near complete depletion of parietal cells. TGF- α immunohistochemistry, paraffin-embedded, 5µm section, H&E counterstain. Bar = 100µm

the presence of tracts or bands consisting of stroma cells/fibrocytes lying between gland units (Figure 3.30). The inflammatory response consisted of plasma, round cells and granulocytes in the mucosa and submucosa.

3.3.2.2.6 Day 30

Fundic tissues from three sheep were similar in appearance, whereas tissues from Sheep #50 were markedly different, resembling more closely the tissue of an animal euthanised 10 or 15 days after inoculation. There were, however, no vacuolated parietal cells present in any section from any animal.

The distribution of strongly labelled TGF- α positive parietal cells within the sections of the three comparable sheep, was similar to that observed on Day 5 (Figures 3.10 and 3.11). In TGF- α stained sections counterstained with H&E derived from Sheep #47, parietal cells appeared like normal mature cells. In Sheep #50, however, the majority of parietal cells had a strongly eosinophilic cytoplasm, a very dense nucleus and appeared smaller than mature parietal cells. This sheep also had the lowest parietal cell count of all animals in this study.

Hyperplastic areas around formerly parasitised glands harboured the majority of granulocytes, round cells and plasma cells present in the mucosa and in some areas the submucosa still contained a substantial number of round cells and plasma cells. Globule leucocytes, lying between epithelial cells, were easily recognised by their characteristic eccentric nuclei and their abundant cytoplasm containing large eosinophilic granules. There were moderate numbers of mitotic figures in the isthmus, neck and pit regions in all sheep.

Diffuse staining of surface mucous cells for TGF- α could be observed in sections derived from two sheep, and fibrous bands were present in all animals.

Sheep #50 showed none of the previously listed features, but its TGF- α positive parietal cells were severely depleted and the pits/glands were wide open and lined by mucus-filled cells. The mucosa of this animal was also

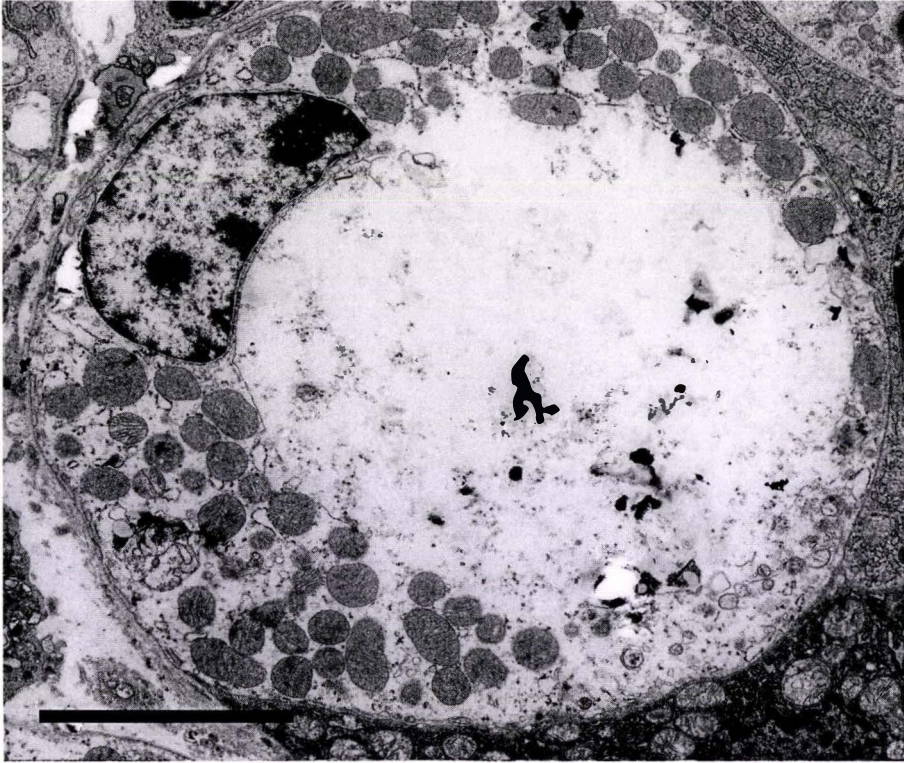


Figure 3.37. Parietal cell from a sheep, 10 days after infection with 35,000 L₃ *O. circumcincta*. The nucleus has been pushed to one side by the presence of a large vacuole, the lining membrane of which is indistinct. The cytoplasm is thinned and the mitochondria appear swollen. Epoxy-resin embedded, 70-90nm section, EM. Bar = 5 μ m

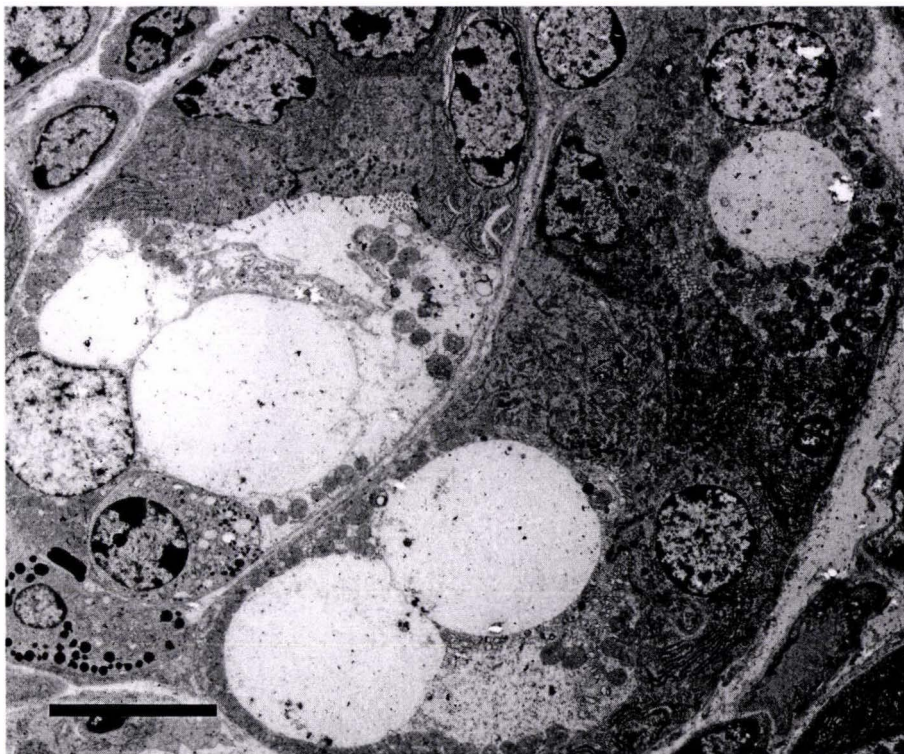


Figure 3.38. Parietal cells from a sheep, 10 days after infection with 35,000 L₃ *O. circumcincta*. Two parietal cells contain at least two large vacuoles separated by thin membranes. A third cell has one smaller vacuole (top right). Epoxy-resin embedded, 70-90nm section, EM. Bar = 5 μ m

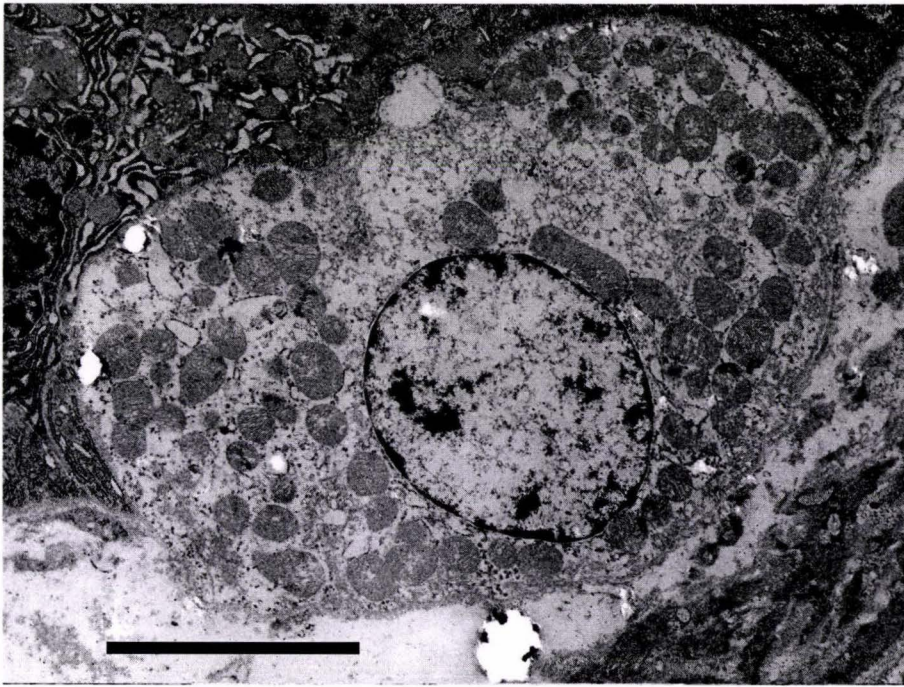


Figure 3.35. Parietal cell from a sheep, 10 days after infection with 35,000 L₃ *O. circumcincta*. This cell has a swollen pale nucleus and the cytoplasm is thinned. The mitochondria contain condensed cristae. Epoxy-resin embedded, 70-90nm section, EM. Bar = 5µm

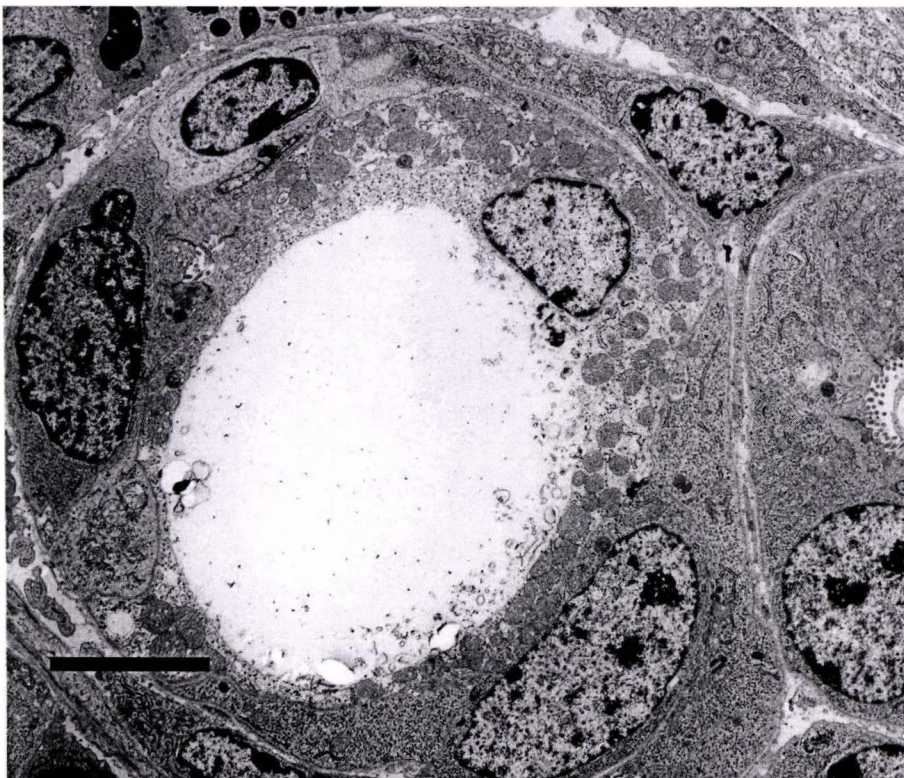


Figure 3.36. Parietal cell from a sheep, 10 days after infection with 35,000 L₃ *O. circumcincta*. The whole gland has been distorted by the presence of this vacuolated cell. The vacuole itself is larger than many of the surrounding cells. Epoxy-resin embedded, 70-90nm section, EM. Bar = 5µm

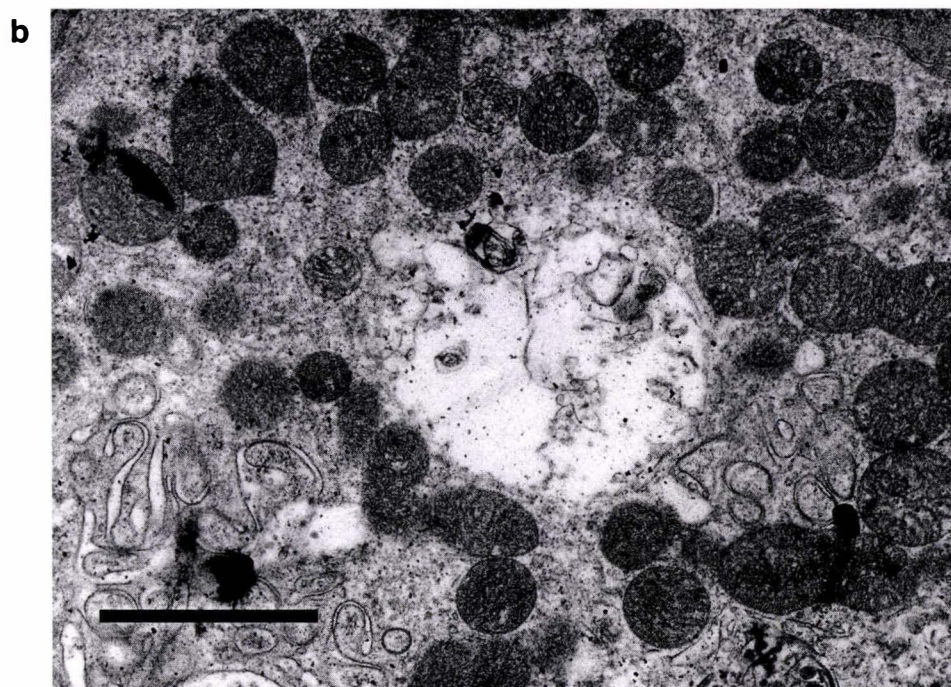
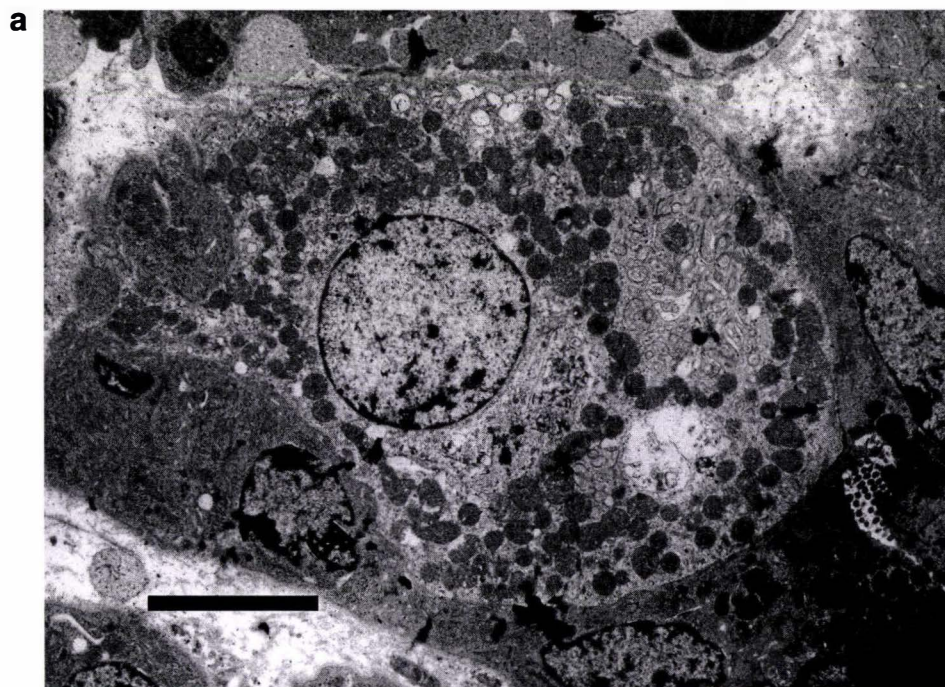


Figure 3.34a and b. Parietal cell from a sheep, 5 days after infection with 35,000 L₃ *O. circumcincta*. The nucleus appears slightly swollen and the cell contains what appears to be a small vacuole although it lacks a distinct membrane. Epoxy-resin embedded, 70-90nm section, EM.

(a). Bar = 5 μ m

(b) Bar = 2 μ m

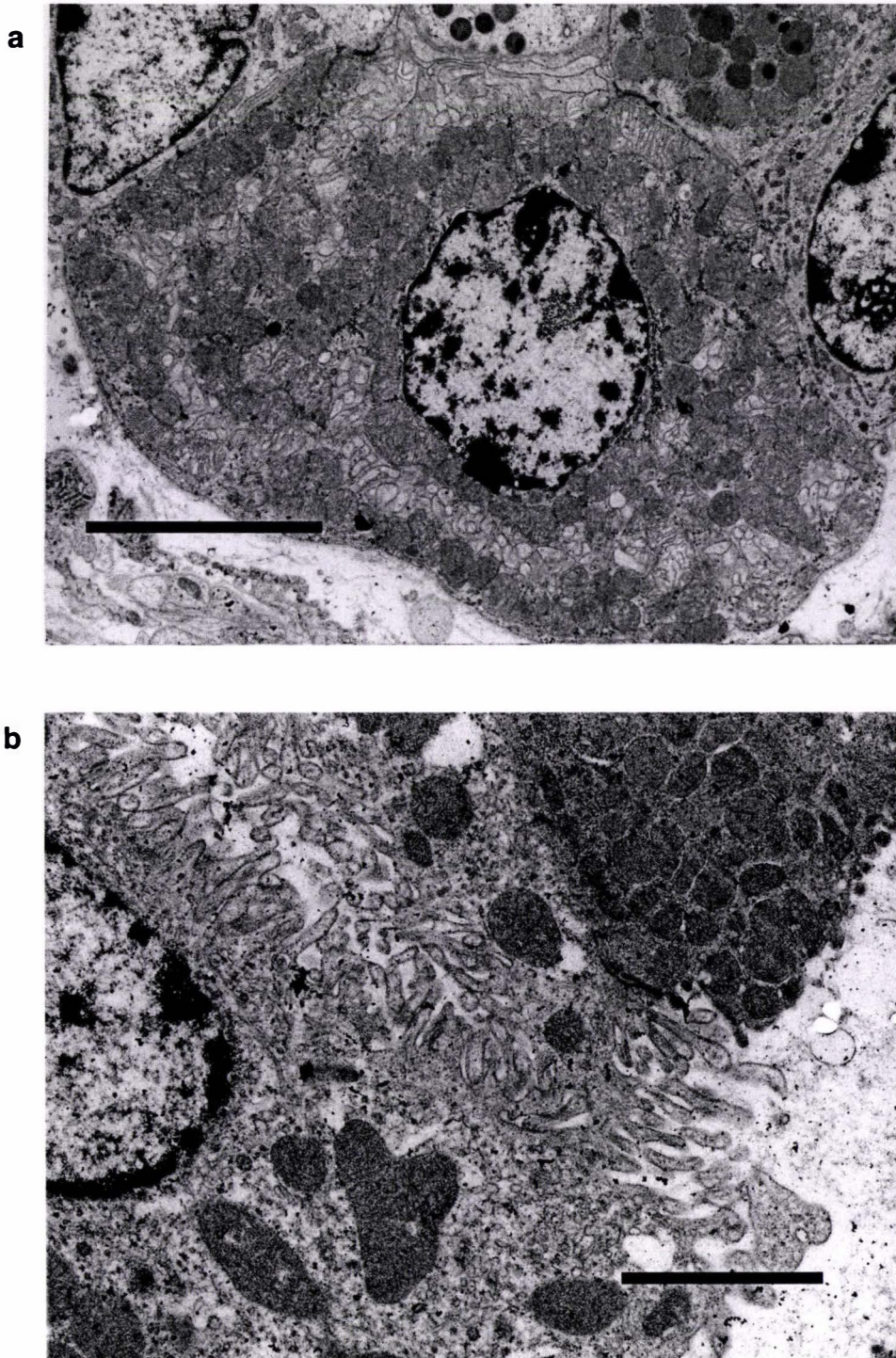


Figure 3.33a and b. Parietal cells from uninfected control sheep from the larval infection experiment, with what appear to be true secretory canaliculi consisting of numerous microvillar extensions of the plasmalemma. Epoxy-resin embedded, 70-90nm sections, EM.

(a) Bar = 5 μ m

(b) Bar = 2 μ m

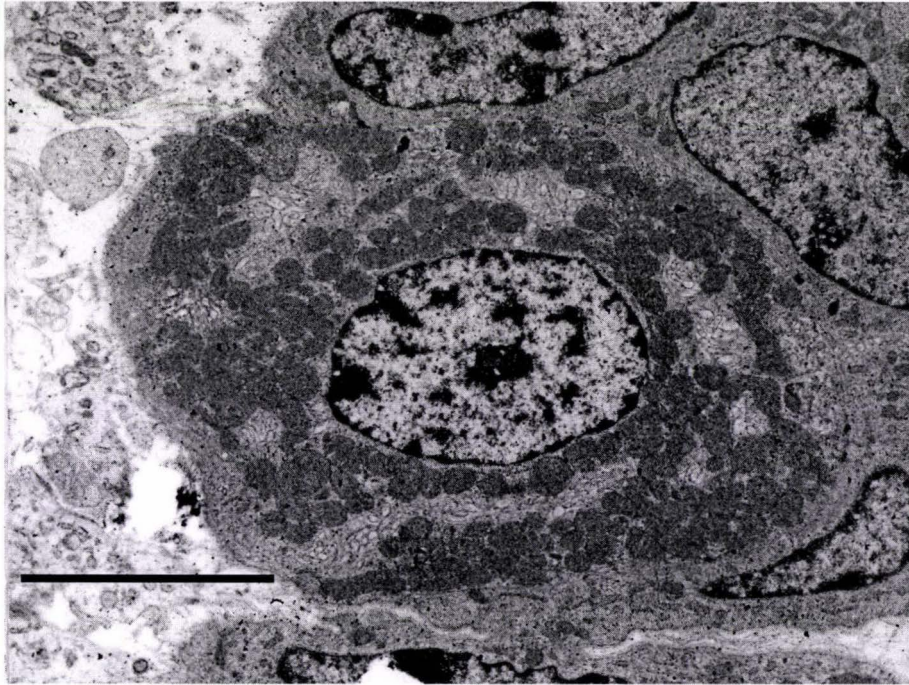


Figure 3.32. Parietal cell from an uninfected control sheep from the larval infection experiment. The cell has a large, central nucleus with obvious chromatin, abundant large mitochondria and numerous tubulovesicles. Epoxy-resin embedded, 70-90nm section, EM. Bar = 5 μ m

invaded by inflammatory cells, mainly granulocytes and plasma cells, and was markedly thickened (Figure 3.31).

3.3.2.3 Electron Microscopy

3.3.2.3.1 Controls

Investigations of sections prepared for electron microscopy revealed structures typical of healthy parietal cells. Figure 3.32 displays a parietal cell with a large nucleus, abundant large mitochondria and numerous tubulovesicles forming an extensive smooth membrane system. True secretory canaliculi, characterised by numerous microvilli extending from the plasmalemma and wide dimensions, were present in only some parietal cells (Figures 3.33a and b).

3.3.2.3.2 Day 5

The parietal cell pictured in Figure 3.34 illustrates the type of changes present in some parietal cells on Day 5. The parietal cell shown had dilated secretory canaliculi and contained abundant mitochondria, however, the pale nucleus and a circular vacuole, a quarter of the size of the nucleus, are clearly visible. In this case, the vacuole appeared to be without a distinct plasmalemma and seemed to communicate with the cytoplasm.

3.3.2.3.3 Day 10

By Day 10 p.i., many parietal cells had a general pallor of both the nucleus and the cytosol. The nucleus in the parietal cell pictured (Figure 3.35) was not centrally placed, was enlarged and appeared swollen, whereas some of the abundant mitochondria contained fragmented cristae. In parietal cells with only one vacuole, the vacuole occupied nearly the whole cytoplasm and had pushed all other cell organelles aside. The cell size was often increased and bordering cells were therefore distorted (Figures 3.36 and 3.37). Some vacuolated parietal cells possessed a reduced number of mitochondria and a pale nucleus, whereas others had stronger staining nuclei and contained more mitochondria (Figure 3.36). There was, however, an obvious pallor within the cytosol of both types. In some instances, the membrane encircling the vacuole was partially ruptured or disintegrated allowing communication between the vacuole and the cytosol. Cell borders appeared to be intact and interdigitations and infoldings of the basolateral membranes of adjacent cells could be observed. Other

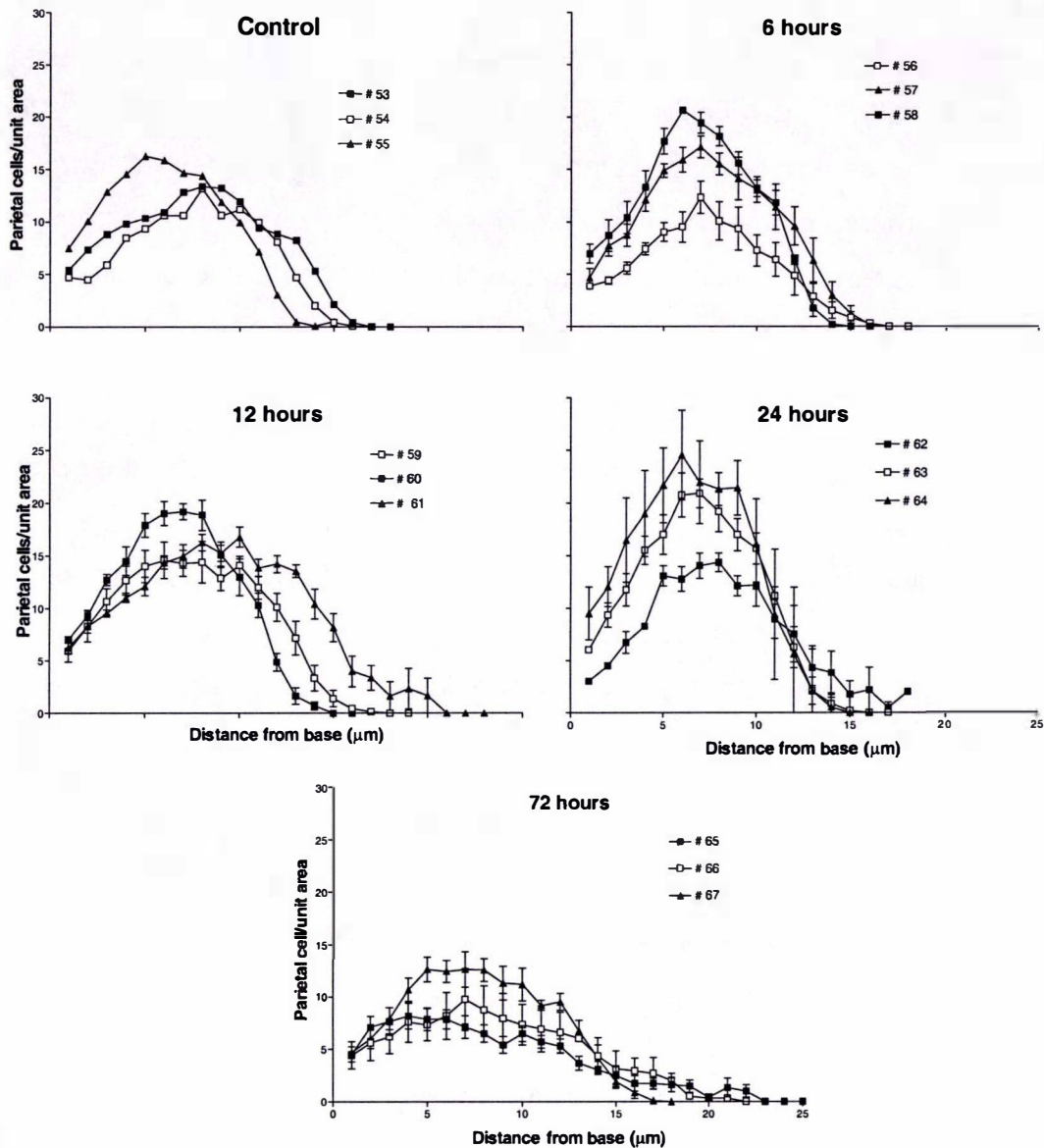


Figure 3.41. Numbers of TGF- α -positive parietal cells per unit area in a 258 μm wide column of fundic mucosa of individual sheep before (control, CT) or 6, 12, 24 or 72 hours after the transplantation of 10,000 adult *O. circumcincta*. Symbols for each sheep are shown on the Figure.

Table 3.4 Numbers of TGF- α -positive parietal cells (PC) and eosinophils in a 258 μ m wide column of fundic mucosa, abomasal pH at necropsy and mucosal thickness (μ m) (mean \pm SEM) in sheep before, (control, CT) or 6, 12, 24 or 72 hours after, the transplantation of 10,000 adult *O. circumcincta*.

Parameter	Group				
	CT	6 hours	12 hours	24 hours	72 hours
pH	2.76 \pm 0.12	2.98 \pm 0.40	2.69 \pm 0.24	3.36 \pm 0.51	4.14 \pm 0.70
TGF- α positive PC	129.6 \pm 7.1	137.0 \pm 21.0	170.8 \pm 12.4	165.8 \pm 21.6	111.1 \pm 11.7
Eosinophils	0.01 \pm 0.01	5.00 \pm 5.00	5.30 \pm 3.90	2.90 \pm 1.50	16.90 \pm 9.30
Mucosal thickness	385.7 \pm 26.9	386.3 \pm 11.7	397.7 \pm 33.9	349.9 \pm 5.1	442.8 \pm 18.2

Table 3.5. Percentage of total parietal cells which stained with Haematoxylin and Eosin, but not TGF- α (mean \pm SEM) from individual sheep sampled before, (control, CT) or 6, 12, 24 or 72 hours after, the transplantation of 10,000 adult *O. circumcincta*. The total parietal cell number was obtained from the total of TGF- α -positive and TGF- α -negative parietal cells.

Group	Sheep	Percentage TGF- α -negative parietal cells
Control	53	18.98 \pm 4.93
	55	15.16 \pm 2.02
6 hours	56	33.35 \pm 4.21
	58	13.52 \pm 5.80
12 hours	60	9.00 \pm 0.85
24 hours	62	6.35 \pm 1.76
72 hours	65	7.97 \pm 1.77
	66	0.43 \pm 0.13

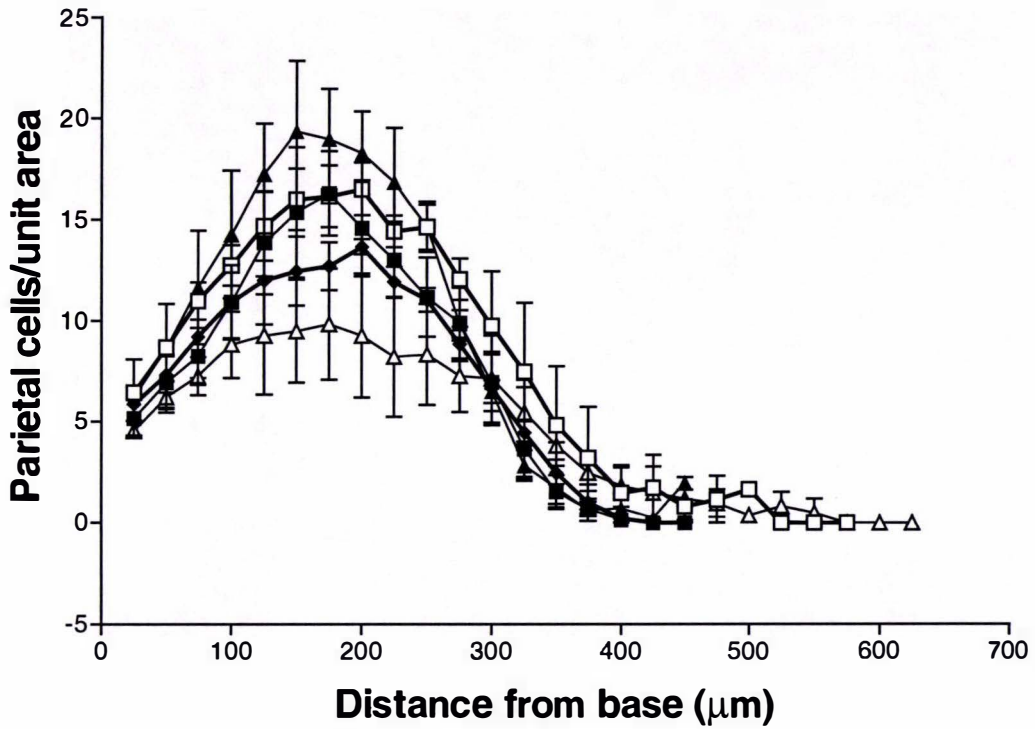


Figure 3.40. Numbers of TGF- α -positive parietal cells per unit area in a 258 μ m wide column of fundic mucosa (mean \pm SEM) in sheep before, (control) or 6, 12, 24 or 72 hours after, the transplantation of 10,000 adult *O. circumcincta*. Control: ◆ ; 6 hours: ■ ; 12 hours: □ ; 24 hours: ▲ ; 72 hours: △.

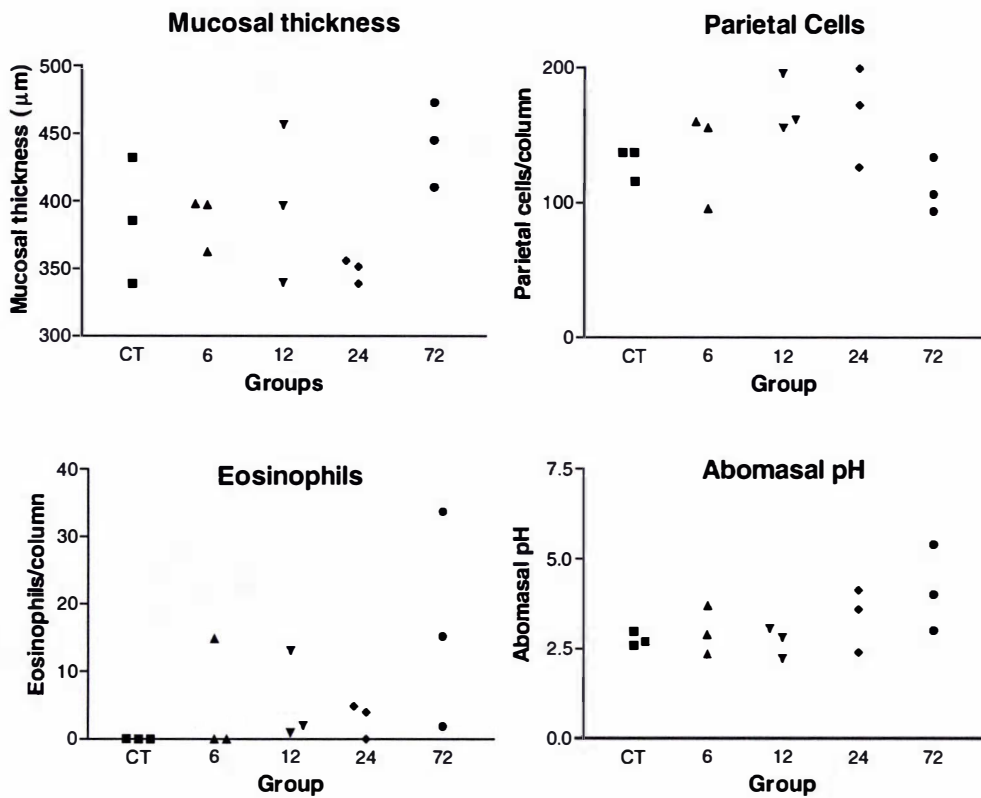


Figure 3.39. Abomasal pH at necropsy, mucosal thickness (μm) and numbers of TGF- α -positive parietal cells and eosinophils per 258 μm wide column of fundic mucosa of individual sheep before (control, CT) or 6, 12, 24 or 72 hours after the transplantation of 10,000 adult *O. circumcincta*.

parietal cells contained more than one vacuole and were abnormally shaped (Figure 3.38). When several vacuoles were present in the one cell, the vacuoles were confluent or separated by a thin membrane, were mostly of a round shape and together occupied nearly the whole cytosol, the nuclei were pale and indented or had disintegrated altogether.

3.3.3 Group AT

3.3.3.1 Abomasal Parameters

Table 3.4 and Figure 3.39 show the mean and individual values for tissue eosinophils, TGF- α positive parietal cell counts and mucosal thicknesses. Significant differences could not be detected for any parameter counted, when the various groups were compared.

There was a positive correlation detected between mucosal eosinophils and abomasal pH ($r^2=0.36$ with $p<0.05$) though no correlation was observed between mucosal eosinophils and parietal cell counts. Further, abomasal pH did not correlate with the number of TGF- α positive parietal cells.

The results for the evaluation of TGF- α labelled sections after counterstaining with H&E are presented in Table 3.5. Data varied considerably across groups.

3.3.3.2 Light Microscopy

Across members of all groups, mucosal changes in the tip area were more pronounced than in corresponding non-tip areas.

3.3.3.2.1 Controls

Histology was comparable to that described for control animals in Section 3.3.2.2.1.

3.3.3.2.2 6 hours

Sections derived from two sheep, which had been sacrificed 6 hours after adult transplant, had similar numbers of TGF- α positive parietal cells, as did the controls (Figures 3.40 and 3.41). In contrast, Sheep #56 had a lower number of TGF- α positive parietal cells, however, counterstaining with H&E revealed that 33.4% of the absolute total had not reacted with the TGF- α antibody. In

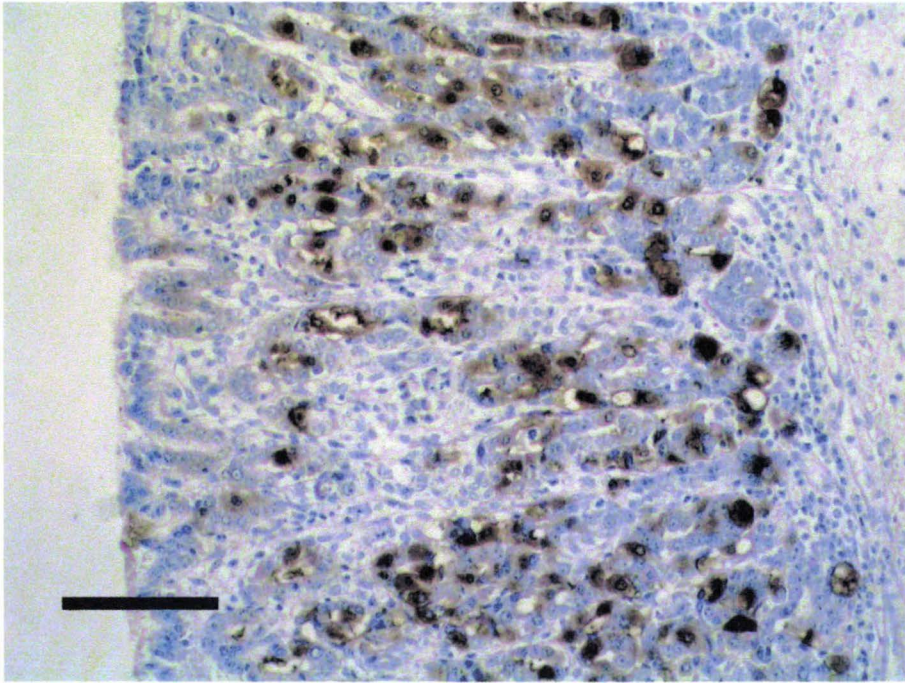


Figure 3.42. Fundic mucosa of a sheep, 12 h after the transplant of approximately 10,000 adult *O. circumcineta*. The number of parietal cells appears reduced and many cells are vacuolated. TGF- α immunohistochemistry, paraffin-embedded, 5 μ m section, Mayer's haematoxylin counterstain. Bar = 100 μ m

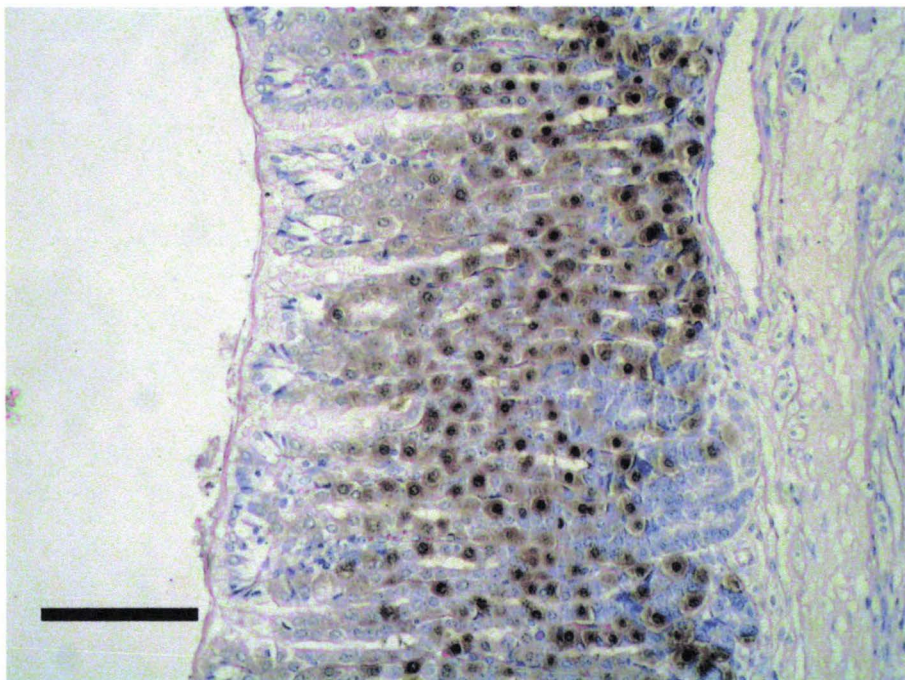


Figure 3.43. Fundic mucosa of a sheep, 24 h after the transplant of approximately 10,000 adult *O. circumcineta*. Neither an increase in mucosal thickness, nor a reduction of parietal cell numbers, is evident. TGF- α immunohistochemistry, paraffin-embedded, 5 μ m section, Mayer's haematoxylin counterstain. Bar = 100 μ m

comparison, only 13.5% of the parietal cells did not label with TGF- α in sections obtained from Sheep #58. TGF- α negative parietal cells were chiefly found in the upper pit. In all sheep, round cells and eosinophils were present mainly in the submucosa, though the number of cells present was not pronounced. All other cells appeared to be normal and no vacuolation of parietal cells was observed.

3.3.3.2.3 12 hours

In defined areas of the tissues of three animals, vacuolated parietal cells, similar to those described in Group LI sheep, were present, situated throughout all levels of fundic glands. The influx of inflammatory cells in the submucosa was moderate and consisted chiefly of round cells and granulocytes, namely eosinophils. Additionally, in Sheep #61, areas were detected, which were analogous in mucosal changes to those seen in larvae-infested glands and their surrounding tissues in Day 5 animals with nodule-like areas beside normal areas of the mucosa and with some vacuolated parietal cells being present (Figure 3.42). This animal also had an increased mucosal thickness.

3.3.3.2.4 24 hours

There were no measurable increases in mucosal thickness in comparison with control tissues and the number of antibody-labelled parietal cells was not reduced in comparison to controls (Figure 3.43). TGF- α positive parietal cells displayed different staining patterns throughout the whole mucosal depth, with parietal cells either having weak or strong staining nuclei and cytoplasm. There were also parietal cells with strong staining nuclei, but weakly staining cytoplasm. Pits were elongated, wider and contained mucous cells full of mucus granules in their apical cytoplasm, while glands seemed to be shorter. Granulocytes or lymphocytes were only seen in small numbers in the submucosa. Sheep #62 had an increased number of parietal cells displaying vacuoles.

3.3.3.2.5 72 hours

Overall changes in sections obtained from two sheep sacrificed had mucosal changes similar to those seen in sections derived from animals sacrificed 10 days after L₃ inoculation, with extensive loss of TGF- α positive parietal cells

and mucosal hyperplasia. Sheep #67, however, was histologically more like a 6 hour animal displaying no changes within the different epithelial cell types and only a subtle influx of inflammatory cells in the submucosa.

3.4 Discussion

The second experiment involving the HFW animals established that the HFW animals had fewer eosinophils in their abomasal tissues than did the controls. There was also a strong positive correlation between abomasal pH and mucosal eosinophils and a possible involvement of inflammation in the development of mucosal thickening was also indicated. The larval infection experiment showed an association between developing hypoacidity and eosinophil influx, while documenting an apparent reduction in parietal cells with obvious abnormalities among the remaining ones. Gradual recovery of the animals in the LI experiment towards Day 30 p.i. was accompanied by the regained ability to acidify abomasal contents and the disappearance of eosinophils. In the adult transplant experiment, major changes within the abomasal mucosa did not occur until a strong inflammatory response could be detected. These findings suggest therefore that the inflammatory response may play a significant role in the development of hypoacidity during parasitism. Yet, vacuolation of parietal cells on Day 5 in the LI experiment, when gastric pH was unchanged and inflammation minimal, suggested that, alternatively, the parasites may indeed have had some direct effect against the parietal cell population.

Important questions that stem from these findings are therefore: Do the histological findings elucidate the mechanism of resilience of HFW sheep? What is the mechanism underlying the development of hypoacidity in primary infections of the parasite-naïve host? Following on from the latter question are: Is hypoacidity caused by inhibition of parietal cells or their loss? What is the role of the inflammatory process and of the eosinophil in particular? Is there a direct effect of parasites on the parietal cell?

At this point, and before addressing the fate of parietal cells in the present experiments, it is worth considering the use of TGF- α immunohistochemistry as a marker for parietal cells. Both parietal cells and mucous cells lining the pits have been shown to stain when labelled with TGF- α antibodies (Thomas *et al.*, 1992). Both cell types also stained in the present experiments, although in many instances the staining intensity was greatest in the parietal cells. This allowed a rapid enumeration of parietal cells to be made. Some parietal cells, however, were observed that did not stain. The number of these non-staining cells was variable, but obviously their existence means that counts of TGF- α positive cells only would potentially underestimate the actual total. For this reason, counterstaining with H&E was done and the number of none-staining cells assessed for some animals. In the larval infection experiment, the comparison between the parietal cell counts of the control animals versus those killed 10 days after infection, was one of the most critical. For both groups of animals the numbers of non-staining cells were not large. Thus a comparison of stained cells was valid and to compare the parietal cell populations of various animals the counts of TGF- α positive cells were used.

3.4.1 Do the Histological Findings Elucidate the Mechanism of Resilience of HFW Sheep?

In Chapter 2, the pathophysiological changes occurring in HFW animals were reported and a possibly, immune-dependent mechanism of resilience for the HFW lambs was suggested. Peripheral eosinophilia was consistently lower in HFW animals and now a lower tissue eosinophilia has also been demonstrated.

3.4.1.1 Tissue Eosinophilia

High fleeceweight sheep, in spite of a similar worm burden, had a significantly less pronounced tissue eosinophilia on Day 8 p.i. in comparison with control sheep. Thus the HFW hosts either were able to actively suppress eosinophil recruitment into the mucosa early in infection or the production following appropriate stimuli of eosinophils by their bone marrows was reduced. The latter seems more likely, since blood eosinophils in HFW lambs stayed low throughout the whole experiment. There was limited evidence that IL-5 levels in

HFW animals were lower and this itself would be sufficient to explain the lower eosinophilia.

Inflammatory cells, like eosinophils, are known to produce and release a variety of substances, e.g. cytokines and leukotrienes, able to injure the integrity of the gastrointestinal mucosal layer (Rothenberg *et al.*, 2001). Similarly, a detrimental effect of mucosal eosinophils was suspected in the gastrointestinal mucosa, when ewes with the highest dag score also displayed an abundance of mucosal eosinophils (Larsen *et al.*, 1994).

Other cells of the immune/inflammatory response, e.g. lymphocytes and globule leucocytes were also prominent and certainly could play significant parts in the pathogenesis of parasitism, but given the known deleterious effects of eosinophils in diseases such as asthma (Hamelmann & Gelfand, 2001), the presence of large numbers of these cells in any disease is always going to be important.

The ability to suppress the eosinophilic inflammatory response supports the resilient status of the HFW lambs. If resilience is seen as the ability to maintain productivity while parasitised, then a reduced inflammatory response is desirable. As a consequence, HFW sheep would experience reduced losses and need to invest less in compensation and/or reconstruction and thus productivity would be less affected by parasitism.

3.4.1.2 Mucosal Thickness

It is likely that the inflammatory process is linked to the re-structuring of the mucosa. In the larval infection experiment, mucosal thickness and tissue eosinophilia were both maximal 10 days p.i. In the high fleeceweight experiment, the HFW lambs had thinner mucosae than did the controls, 8 days p.i. and this was potentially linked to more mild inflammatory changes. The fact that the HFW lambs retain a more normal gastrointestinal structure when parasitised provides additional support for their resilient status.

In humans, diagnosed with *H. pylori* associated enlarged fold gastritis, pro-inflammatory cytokines like IL-1 β , and HGF, appear to trigger increases in

mucosal thickness (Yasunaga *et al.*, 1996, 1997). The clinical picture of this latter gastropathy is very similar to that of abomasal parasitism and includes many of the same features of parasitism, namely, hypoacidity, mucosal hyperplasia via increased foveolar length as well as inflammatory infiltration (Murayama *et al.*, 1999).

Factors known to stimulate mucosal hyperplasia also include TGF- α , a known epithelial mitogen having an additional regulatory role in cell differentiation (reviewed by Salomon *et al.*, 1990), which is highly expressed in healing tissue (Konturek *et al.*, 1997). A possible source of TGF- α could have been mucous cells themselves, since a strong signal was found in some mucous cells, particularly later in infection (Figure 3.26). Hyperplasia was, however, present focally in Day 5 animals and was more generalized on Day 10 when immunohistochemical staining of the tissues rather suggested that the mucosal content of TGF- α had declined.

Another possible source of TGF- α is the eosinophil (Elovic *et al.*, 1990; Walz *et al.*, 1993), but in no instance did eosinophils stain for the growth factor. Nevertheless, a contribution of TGF- α can still be considered, since a low sensitivity of the antibody employed cannot be excluded. Moreover, mRNA levels in tissues were not measured, which could give an indication of the production of TGF- α .

Hypergastrinaemia is consistently present during abomasal parasitism and different forms of gastrin have been shown to enhance cell proliferation (reviewed by Dockray *et al.*, 2001). A possible role for gastrin as a trophic agent was perhaps demonstrated in Sheep #50, one of the Day 30 animals. This animal had extremely high serum gastrin concentrations that occurred far later in the experiment than was seen in other animals. This sheep also had a severely thickened mucosa (Figure 3.31) in comparison with that in the other sheep of this group, although it also had markedly fewer parietal cells than other members of the group.

It may be impossible to exclude TGF- α or gastrin as acting as trophic agents, and perhaps there is a combined effect of both. Transgenic mice over-

expressing amidated gastrin, which display similar mucosal changes to those reported here, also show an increase in TGF- α production (Wang *et al.*, 2000).

As mentioned earlier, the closest parallel to abomasal parasitism is enlarged fold gastritis in humans, due to *H. pylori* infection in the fundus. This type of gastritis also features gastric inflammation accompanied by reduced acid secretion and hypergastrinaemia is secondary to this. In addition it has also been demonstrated that as the mucosal levels of cytokines such as IL-1 β increase, the mucosal content of TGF- α decreases (Russo *et al.*, 1998).

This leads to other work which shows that simply losing the parietal cell is sufficient stimulus for hyperplasia to occur (Li *et al.*, 1996), most likely due to the lower mucosal levels of TGF- α that result from parietal cell loss thus confirming that interference with the parietal cell plays a pivotal role in parasitic infections and other forms of gastritis.

3.4.2 What is the Mechanism underlying the Development of Hypoacidity in Primary Infections of the Parasite-naïve Host?

To answer this question, the fate of the parietal cells themselves will first be considered then the ability of the parasites themselves to directly affect the parietal cells and the potential role of the inflammatory response will be considered.

3.4.2.1 Is Hypoacidity Caused by Inhibition of Parietal Cells or their Loss?

Studies have shown that parietal cells may be lost during parasitic infection, (Scott *et al.*, 1998a; 2000), if not completely, whilst other studies have shown that parietal cell function may be inhibited (Stringfellow & Madden, 1979; Hertzberg *et al.*, 2000). The loss of parietal cells within nodules caused by developing larvae is well known. Of greater concern is the usually larger area of tissue outside the nodules. The question exists as to whether these cells are also lost or whether they are inhibited by infection.

In both high fleeceweight experiments, patterns of gastric secretion in the intact animals showed that there was some ability to lower the pH of the abomasum

in response to feeding, suggesting that there was at least some degree of inhibition present. When the tissues collected on Day 8 p.i. were examined, parietal cells were present in good number in both groups, even though hypoacidity was present. This suggested therefore that the parietal cells that were present were inhibited.

In contrast, although it could not clearly be demonstrated statistically, there was evidence that parietal cells had been lost in both the larval infection and adult transplant experiments. Animals killed 10 days after infection with larvae had some of the lowest overall parietal cell counts and the cells that were present were distributed through an increased mass of tissue at a lower density. There were, however, obvious areas in which parietal cell loss had occurred that were adjacent to areas in which the numbers appeared more normal. These depleted areas were not associated with any increase in mucosal depth so it is not likely that these areas represented nodules associated with larval development. Similar depleted areas were also observed in animals in the adult transplant experiment that clearly could not have resulted from the presence of larvae.

Similar areas were not observed in control animals, although parietal cell counts did vary widely in this group and large variation in the control group was one possible reason why lower counts in the infected animals failed to be significant statistically.

Diminution of parietal cells may happen in two stages. Parietal cell loss beginning in confined areas in the immediate vicinity of parasite-infested glands and later, when parasite stages will be present in the gastric lumen, a generalised mucosal hyperplasia accompanied by a more marked loss of parietal cells occurs. Some parietal cells may remain, but many may be abnormal, having vacuoles and ultrastructural features suggesting that the cells are dying.

There is therefore evidence for both inhibition and loss of parietal cells occurring and it is entirely possible that the continued inhibition of parietal cells leads eventually to their deaths. The fact that not all cells are lost provides at

least one avenue for the resumption of secretion once the parasites have been removed. However, no studies so far have looked closely at the numbers of pre-parietal cells and their precursors present/left following abomasal parasitism. This cell type may have been indicated by the presence of relatively mature looking parietal cells that did not label with the TGF- α antibody at 20 and 30 days p.i. and in some animals receiving an adult transplant. Thus, it is possible that pre-parietal cells survive parasitic infection and are able to re-introduce a physiological pH following their maturation. Since the number of functioning parietal cells necessary to maintain a physiological pH is not known, even the naturally small number of pre-parietal cells present could be enough.

The two stages of parietal cell loss might be taken as evidence for two different mechanisms. Generalised parietal cell loss associated with the presence of worm stages in the abomasal lumen, was generally accompanied by marked inflammation suggesting that the inflammatory response may have caused the rise in abomasal pH. In contrast, the earlier parietal cell loss within nodules often occurred with minimal associated inflammatory changes, providing evidence perhaps for a direct effect of the parasites on the parietal cell.

3.4.2.2 What is the Role of the inflammatory Process and of the Eosinophil in particular?

Tissue eosinophilia has been reported to be part of a rejection mechanism activated by tissue penetrating larvae (Balic *et al.*, 2000), however, control lambs harboured a worm burden similar to that of HFW lambs, despite having a higher number of tissue eosinophils 8 days p.i. One reason for the ineffectiveness of eosinophils against gastrointestinal parasites in the previously parasite-naïve host could be that at this stage the cells are not fully equipped to target the parasites (Meeusen & Balic, 2000). If not beneficial to the host by preventing larval establishment, the presence of eosinophils may rather be detrimental to the host and may even be beneficial for the parasite.

Numerous mediators have been identified that interfere with acid secretion, TGF- α being one of them. The growth factor is able to inhibit acid secretion *in*

vitro and *in vivo* (Guglietta *et al.*, 1994). Yet, as stated earlier, TGF- α was not detected immunohistochemically in eosinophils. Cytokines with a documented ability to suppress acid secretion include IL1- β and TNF- α , and IL-1 β is produced by granulocytes (reviewed by Cassatella, 1994). Levels of IL-1 β have been shown to correlate with hypoacidity in enlarged fold gastritis (Yasunaga *et al.*, 1997) and both IL1- β and TNF- α are potent inhibitors of acid secretion *in vitro* (Beales & Calam, 1998).

Many other pro-inflammatory agents have been shown to originate in a broad range of inflammatory cell types. Nitric oxide is a highly reactive and toxic oxidant, produced by activated macrophages (Stuehr & Nathan, 1989) as well as lymphocytes (Liew, 1995) and could easily interfere with parietal cell survival. Nitric oxides are suspected to contribute to neoplastic mucosal changes in humans following bacterial, viral and parasitic infection (reviewed by Ohshima & Bartsch, 1994) and recently have been discovered as a co-factor in *H. pylori* induced apoptosis of rat parietal cells (Neu *et al.*, 2002). Eosinophils are also known to produce many mediators that are potentially toxic to mammalian tissues - mediators such as Major Basic Protein (Rothenberg *et al.*, 2001).

On the other hand, an inflammatory response secondary to hypoacidity is also possible. Studies on laboratory animals in which acid secretion is chemically inhibited have revealed an increased inflammatory response within the gastric mucosa (Lamberts *et al.*, 1993).

In summary, the inflammatory response may easily affect parietal cell function, by inhibiting the cells or accelerating their loss, but other parasite-derived factors able to influence both parameters at the same time could also be involved in the response to nematodes. An eosinophil chemotactic factor has been isolated in *O. ostertagi* (Klesius *et al.*, 1985, 1986), while other studies offer *in vivo* (Hertzberg *et al.*, 2000, 2001) and *in vitro* (Merkelbach *et al.*, 2002) evidence for the ability of parasites to inhibit acid-secretion.

3.4.2.3 Is there a Direct Effect of Parasites on the Parietal Cell?

Since adult *O. circumcincta* live on top or within the mucus layer covering the gastric mucosa it is unlikely that their effects result from their physical presence and activity. Far more likely is the involvement of chemical stimuli such as ES, which are released by the worms into their immediate environment. A variety of enzymes (Ogilvie *et al.*, 1973; Karanu *et al.*, 1993; Gamble & Mansfield, 1996) as well as ammonia (Barrett, 1981; Merkelbach *et al.*, 2002) has been identified among ES derived from *Ostertagia* and other parasites. Ammonia especially can affect mucosal integrity (Murakami *et al.*, 1993) and interfere with parietal cell viability (Hagen *et al.*, 1997) as well as function (Triebling *et al.*, 1991; Ricci *et al.*, 1993). Additionally, ammonia causes vacuolation in epithelial cells (Cover *et al.*, 1991; Hagen *et al.*, 1997), a phenomenon frequently observed in parietal cells in the present experiments. Vacuolated parietal cells have also been described previously in biopsies taken from sheep infected with *O. circumcincta* (Scott *et al.*, 2000) and in sections obtained from calves infected with *T. axei* (Ross *et al.*, 1971). In the LI experiment, some of the vacuolated parietal cells carried signs of necrosis - pale swollen nuclei and fragmented mitochondrial cristae (Figure 3.28a). Parietal cells with a similar appearance have also been detected in other studies (Murray *et al.*, 1970; Scott *et al.*, 2000) In contrast, TGF- α -negative parietal cells on Day 5 displayed apoptotic features such as condensed nuclei. Sommerville (1956) reported parietal cells of identical appearance following a nematode infection. Vacuolation may well be a significant feature of parietal cell degeneration, but affected parietal cells may recover and resume secretion of acid as has been seen in rabbit parietal cells inhibited with omeprazole (Karam & Forte, 1994).

In two other studies (Ross, *et al.*, 1971; Scott *et al.*, 2000) a significant feature of the pathology of parasitism has been the presence of dilated canaliculi, whereas the vacuoles documented here show more similarities to those seen in parietal cells derived from omeprazole-treated rabbits (Karam & Forte, 1994) or homozygous null mutant mice of the Na⁺/H⁺ exchanger (NHE2) gene (Schultheis *et al.*, 1998). It is not clear what exactly causes vacuolation following H⁺/K⁺ATPase inhibition. An accelerated degeneration due to long-

term inhibition has been suggested (Karam & Forte, 1994) and loss of NHE2 may disturb cell fluid homeostasis (Schultheis *et al.*, 1998).

There is more than one potential mechanism for how parasites cause vacuolation of acid secreting cells. Exposure of epithelial cells to *H. pylori vacA* results in similar cytoplasmic vacuoles, the appearance of which can be augmented by the addition of ammonia (Cover *et al.*, 1991). Thus, the possibility should be considered that abomasal nematode parasites operate in a comparable manner to *H. pylori* by secreting a vacuolating factor, which may or may not be augmented by ammonia.

Chapter 4

Early changes within the abomasal mucosa following infection with *Ostertagia circumcincta*

4.1 Introduction

Results documented in the previous chapters not only confirmed a pronounced mucosal eosinophilia, but also revealed a prominent drop in parietal cell counts at the same time. A strong correlation between abomasal pH and tissue eosinophil counts proposed a possible negative relationship between the inflammatory cells and the acid producing cell.

Only a few studies have so far targeted the earliest changes arising when parasite-naïve sheep are inoculated with nematodes (Charleston, 1965; Ritchie *et al.*, 1966; Murray *et al.*, 1970). Most agree that eosinophils make an early appearance after infection. Yet, only a small number of studies concentrated specifically on effects on parietal cells during very early changes in abomasal parasitism (Murray *et al.*, 1970). Of particular interest is the speed at which parietal cells are lost, from both the gland actually invaded by the parasite and glands in the immediately adjacent mucosa.

Thus, the following experiment was created to enable investigations into very early changes of tissue eosinophil population and parietal cell numbers following larvae inoculation of a parasite-naïve sheep.

4.2 Materials and Methods

4.2.1 Animal

A 3 month-old male Romney cross lamb, which had been raised parasite-naïve, was infected with 25,000 *O. circumcincta* L₃ and euthanased 30h later with an overdose of pentobarbitone (Pentobarb 500[®], NZVet, Auckland, NZ).

At necropsy, the abdomen was opened and the abomasum was tied off at both ends and removed. The abomasum was opened along the greater curvature, the contents discarded and the mucosa washed with 0.9% normal saline. Several fundic mucosal folds were removed and placed in Bouin's fluid and the remaining abomasal tissue was then discarded.

4.2.2 Histology

4.2.2.1 Tissue Preparation and Staining

Tissues were fixed, embedded in paraffin and 5 μ m thick sections cut as described in Section 3.2.5.1. The sections were stained with H&E and periodic acid Schiff (PAS) to visualise neutral mucin.

4.2.2.2 Immunohistochemistry

Dewaxed and hydrated 5 μ m thick sections were pre-treated with 0.05% Saponin (Sigma-Aldrich, St. Louis, USA), washed with PBS and exposed to 0.5% BSA (Fraktion V, Boehringer Mannheim GmbH, Germany). The full methods for the immunohistochemical staining of sections for TGF- α and eosinophils are described in Section 3.2.5.3.

4.2.2.3 Eosinophil and TGF- α positive Parietal Cell Counts

All cell-types were counted at 40x magnification using a 1cm² eyepiece graticule. Cells were counted in columns of mucosa (locations) 258 μ m wide, from the muscularis mucosa to the luminal surface.

In larval-infested sections, two locations immediately adjacent to each side of the larvae, were counted. In sections which did not contain any larvae, two locations immediately adjacent to each other were counted and served as controls. Results were calculated separately for infested and non-infested tissue in both tip and fold (non-tip) areas.

4.2.2.4 Light Microscopy

Histopathological assessment of sections from the 30h-sheep was made by comparison of infested tip or fold areas with non-infested comparable sections and also with sections from uninfected parasite-naïve control animals (reported in Section 3.3.2.2.1).

4.2.3 Statistical Analyses

All data are presented as mean \pm SEM ($\mu \pm$ SEM).

Statistical calculations were performed only on counts derived from sections of the 30h-sheep. Data were examined for normality using Kolmogorov-Smirnov test. Means were compared using two-way ANOVA with Boniferroni's post hoc test.

4.3 Results

4.3.1 L₃ in Folds

4.3.1.1 Uninfected Control Animals

Control tissue from an uninfected animal stained for TGF- α is shown in Figure 3.9 and was normal in appearance. Pits were generally not longer than a third of the total mucosal thickness and each pit was associated with one or more glands at its base. Surface mucous cells were cuboidal to columnar in shape and contained moderate amounts of mucous. Parietal cells, identified by positive staining for TGF- α , were distributed along the whole length of the glands. The neck and the upper base of each gland unit harboured the majority of the parietal cells, whilst parietal cells at the base of glands stained the most intensely. Surface mucous cells were labelled by TGF- α antibodies within the uninfected mucosa, but did not stain as strongly as did the parietal cells. All epithelial cells appeared normal and no inflammatory cells were detected within the mucosa. Similar observations were made in non-infested areas of the 30h-sheep.

Table 4.1. Numbers of TGF- α stained parietal cells and eosinophils (mean \pm SEM) per 258 μ m wide column of fundic tissue in infested and non-infested areas of the main portion and in the tip of the fundic folds of a sheep killed 30 hours after infection with 25,000 L₃ *O. circumcincta*. The number in parentheses indicates the number of observations made.

	Non-infested fold	Infested fold	Non-infested tip	Infested tip
TGF- α	69.08 \pm 2.11 (12)	62.50 \pm 3.78 (16)	55.67 \pm 2.93 (6)	69.25 \pm 4.59 (4)
Eosinophils	6.08 \pm 1.03 (12)	6.19 \pm 1.44 (16)	21.83 \pm 2.09 (6)	79.00 \pm 13.59 (4)

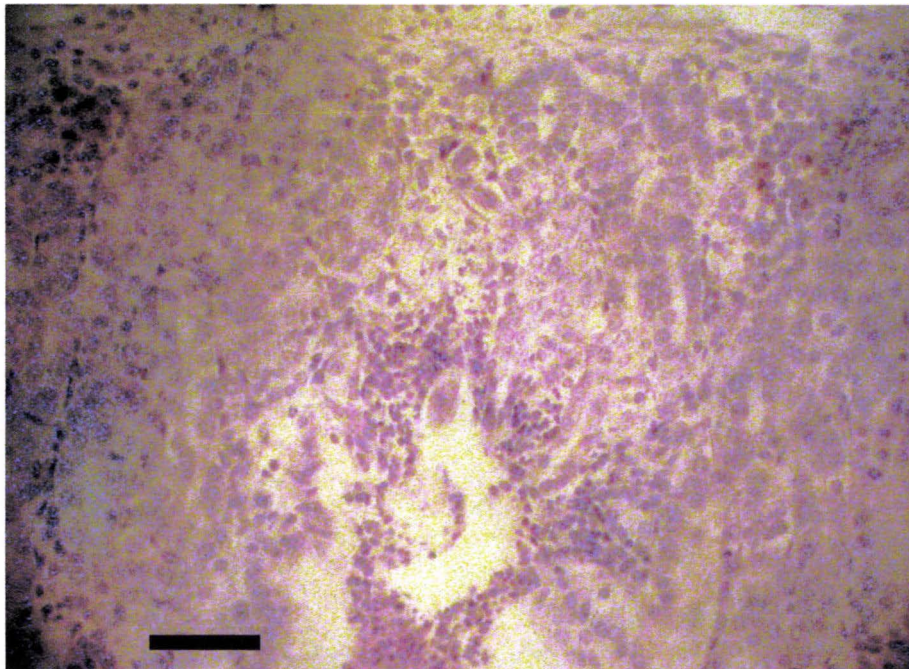


Figure 4.7 Fundic mucosa from a lamb, 30h after infection with 25,000 L₃ *O. circumcincta*. This is the same lesion as seen in Figure 4.6. Numbers of eosinophils are present in the mucosa and sub-mucosa. Eosinophil immunohistochemistry, paraffin-embedded, 5µm section, Mayer's haematoxylin counterstain. Bar = 50µm.

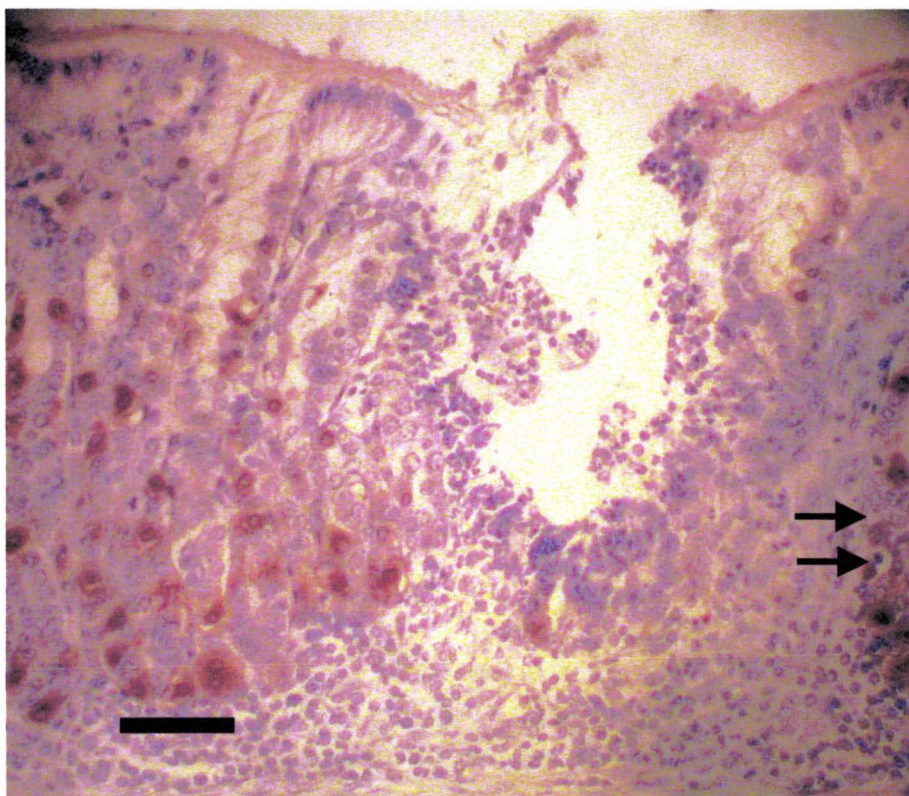
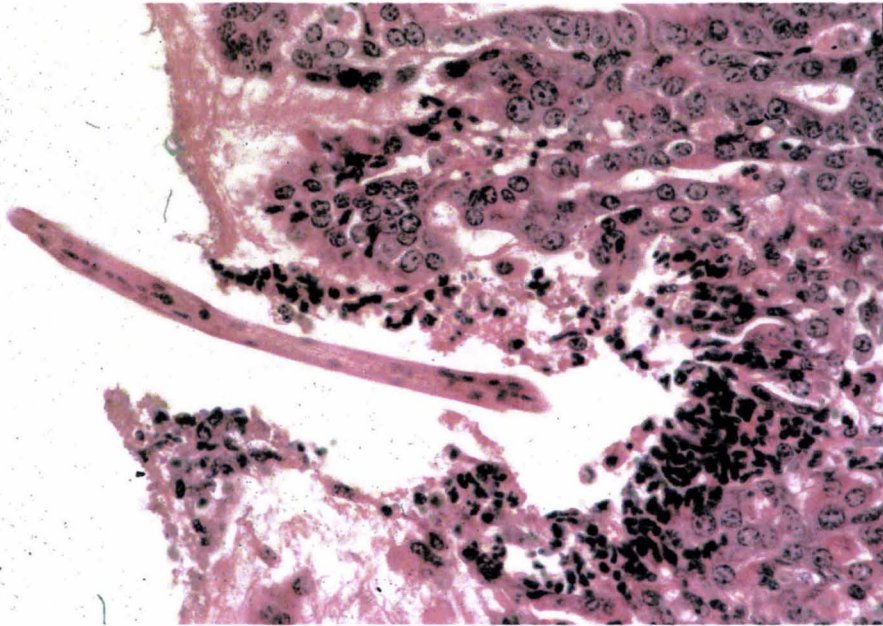


Figure 4.8 Fundic mucosa from a lamb, 30h after infection with 25,000 L₃ *O. circumcincta*. This is the same lesion as seen in Figure 4.6. Parietal cells have been depleted from areas immediately adjacent to the defect. A few parietal cells contain vacuoles (arrows). TGF- α immunohistochemistry, paraffin-embedded, 5µm section, Mayer's haematoxylin counterstain. Bar = 50µm.

a



b

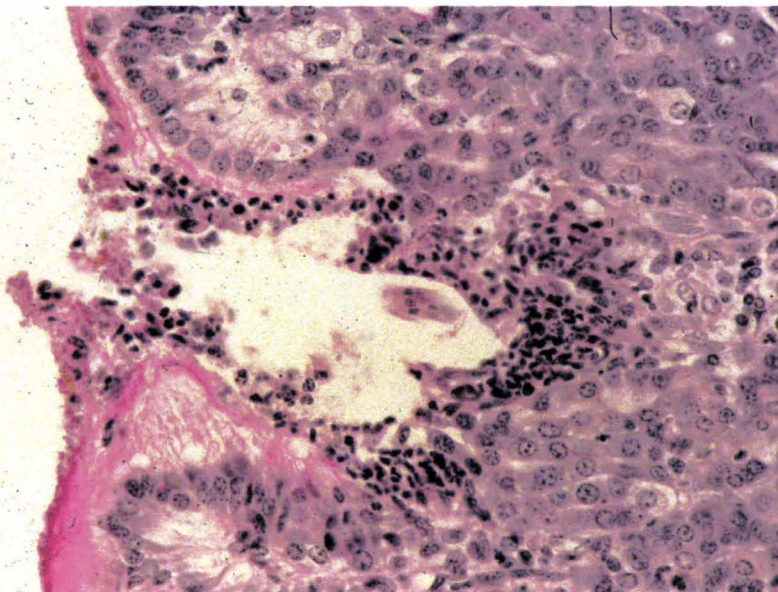


Figure 4.6a and b. Fundic mucosa from a lamb, 30h after infection with 25,000 L₃ *O. circumcincta*. Penetration of this L₃ into the mucosa appears to have resulted in localised tissue loss. The irregularly shaped defect contains the worm, cell debris and there are numerous lymphocytes at its margin..Paraffin-embedded, 5µm section, counterstained with H&E (a) or PAS (b)

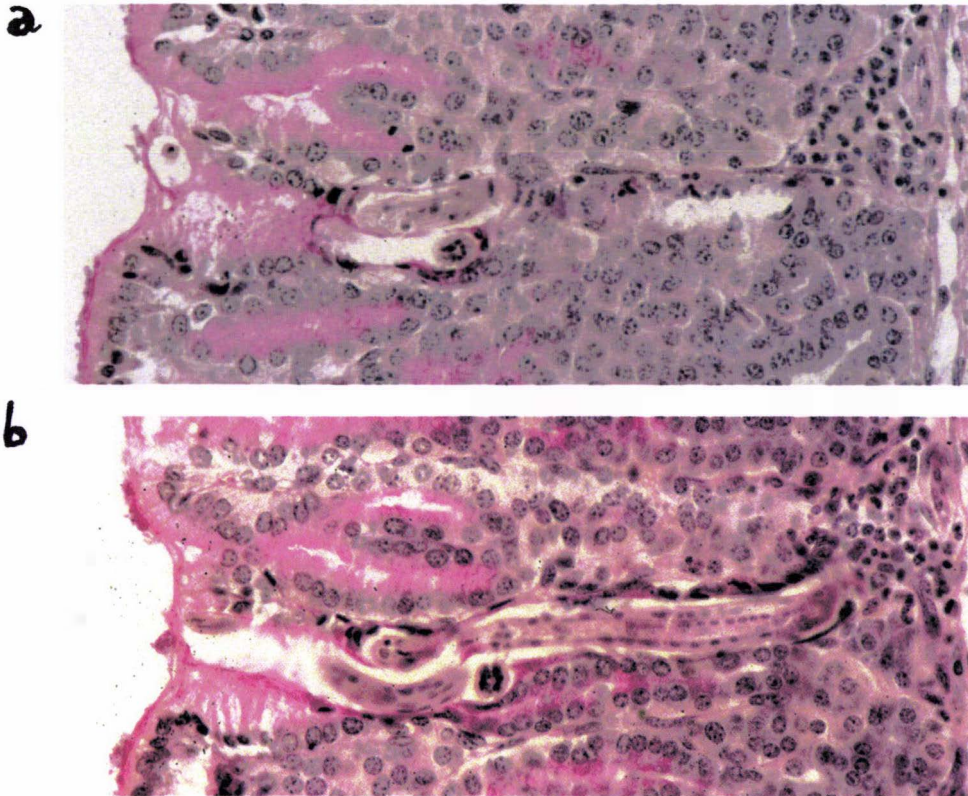


Figure 4.4 Fundic mucosa from a lamb, 30h after infection with 25,000 L₃ *O. circumcincta*. L3 have now penetrated down into the tissue. The epithelial cells of the affected pits/glands appear stretched and flattened and mature secretory cells are no longer identifiable. Paraffin-embedded, 5µm sections, PAS (a), H&E (b)

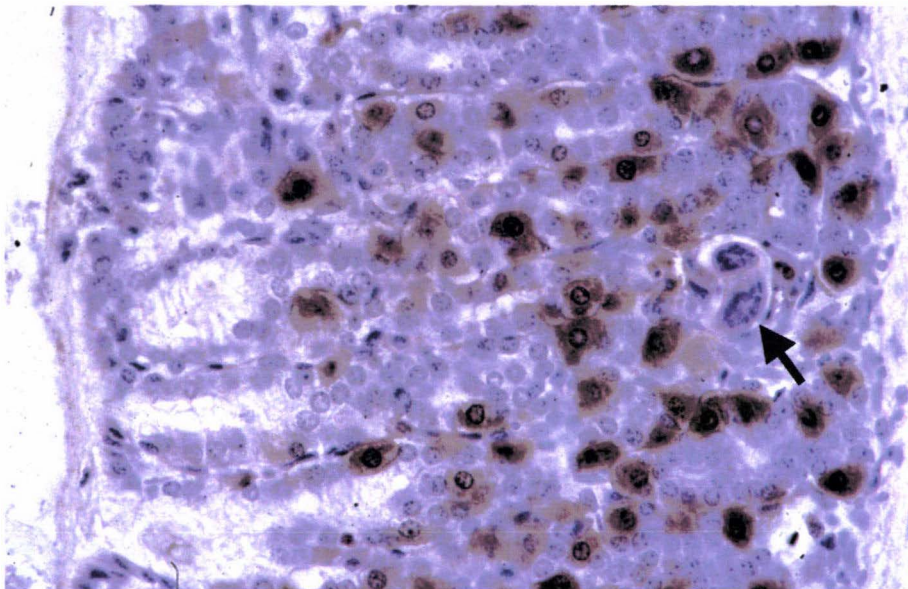


Figure 4.5 Fundic mucosa from a lamb, 30h after infection with 25,000 L₃ *O. circumcincta*. Parietal cells close to a parasitised gland appear normal and their number appears unaffected by the presence of the larva (arrow). TGF-α immunohistochemistry, paraffin-embedded, 5µm section, Mayer's haematoxylin counterstain.

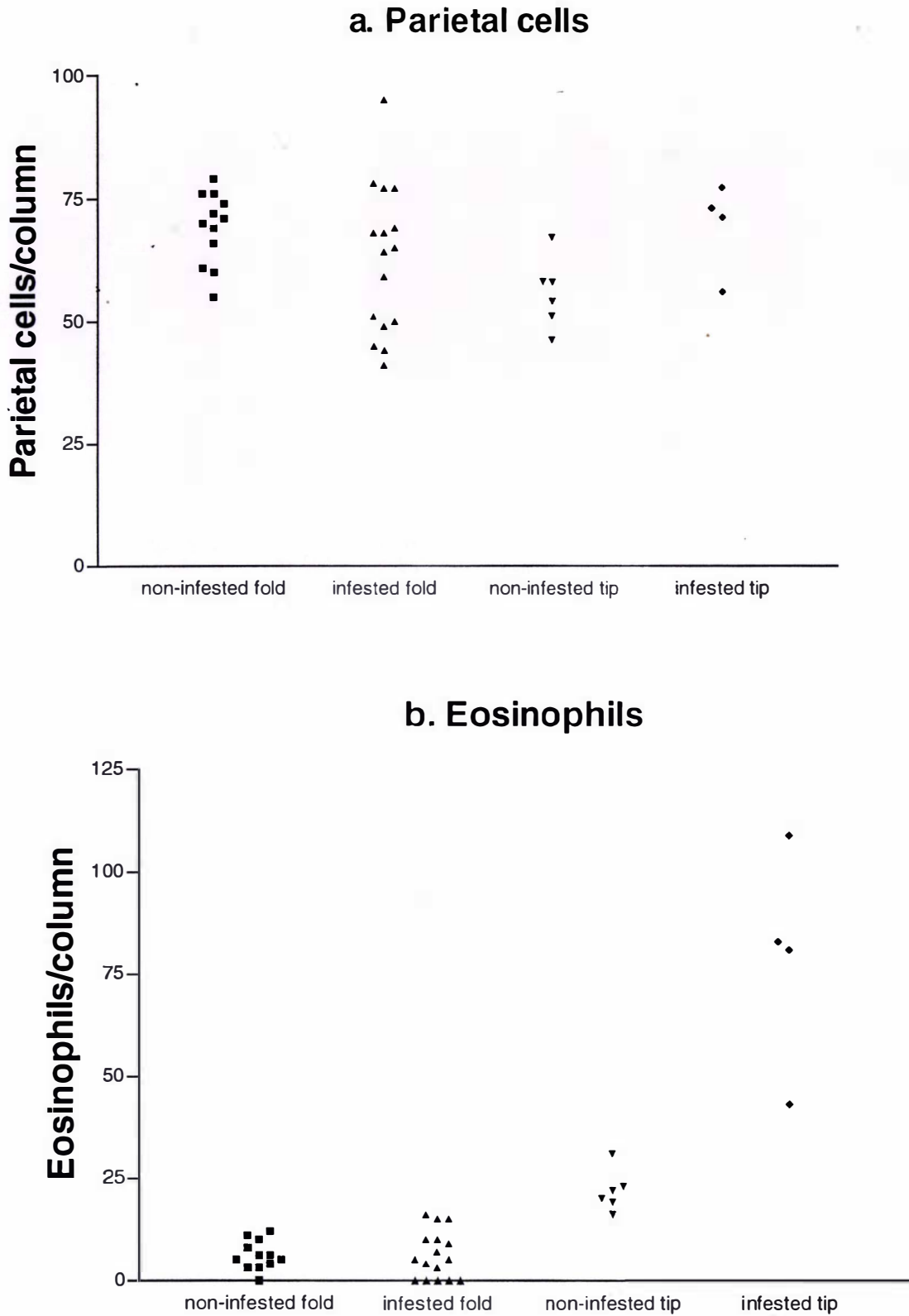


Figure 4.3 Number of parietal cells staining positively for TGF- α (a) or eosinophils (b) in 258 μ m wide columns of infested and non-infested areas mucosa in the main portion and in the tips of the fundic folds of a sheep infected with 25,000 L3 *O circumcincta*.

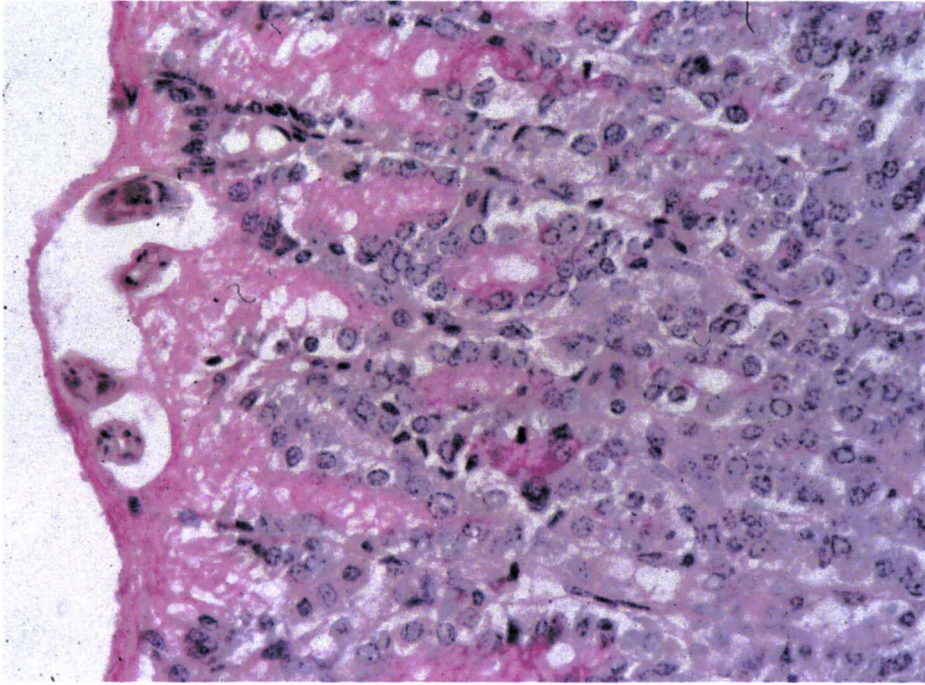


Figure 4.1 Fundic mucosa from a lamb, 30h after infection with 25,000 L₃ *O. circumcincta*. On the left of the picture, an L₃ can be seen on the mucosal surface, covered by a layer of mucus. Paraffin-embedded, 5µm section, PAS.

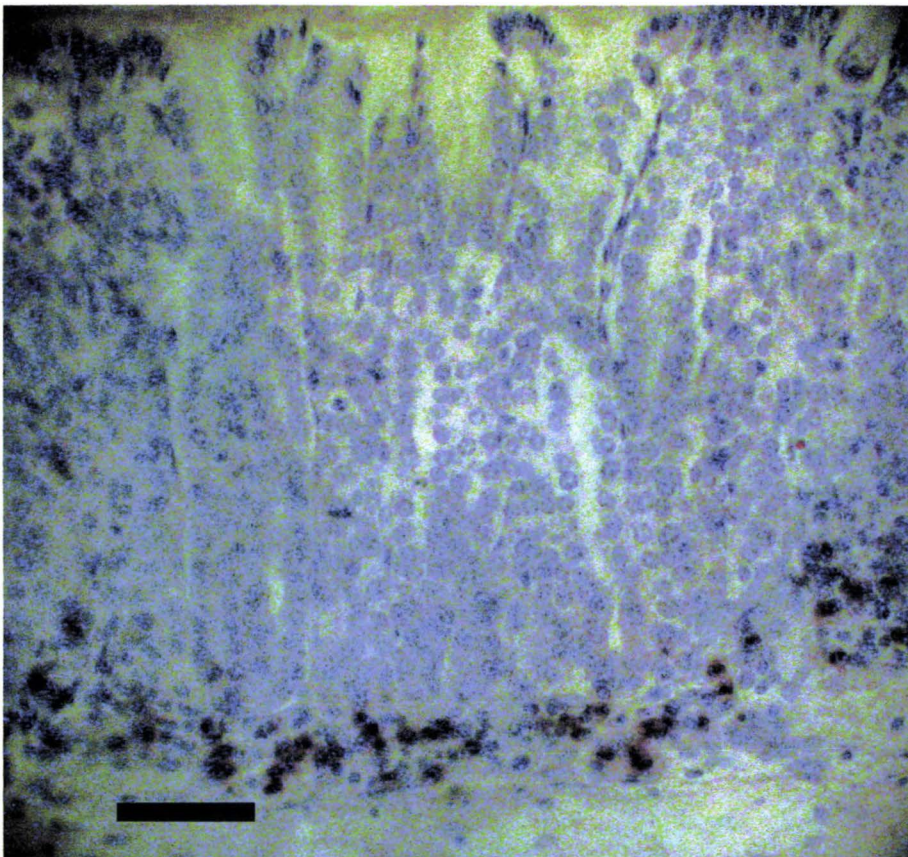


Figure 4.2 Fundic mucosa from a lamb, 30h after infection with 25,000 L₃ *O. circumcincta*. Large numbers of eosinophils have migrated into the lamina propria, but are mainly to be found at the base of the mucosa and have not migrated towards the L₃ that can be seen in the top right. Eosinophil immunohistochemistry, paraffin-embedded, 5µm section, Mayer's haematoxylin counterstain. Bar = 50µm

4.3.1.2 Non-infested Fold Areas

The non-infested fold areas of the 30h-sheep were identical in appearance to the equivalent areas of the control animals described above.

4.3.1.3 L₃ at the Surface

L₃ located on the surface of the abomasal mucosa were covered by PAS-positive mucus and were not associated with an inflammatory response of any kind. Epithelial cells were unaffected by the proximity of the larvae and looked healthy (Figure 4.1).

4.3.1.4 L₃ in the Pits and the Glands

Inflammatory cells, mainly eosinophils, had migrated into the tissues, but appeared to be poorly targeted at the larvae (Figure 4.2). There were, however, no differences between eosinophil counts in non-infested areas and infested areas (Table 4.1). Figure 4.3b shows that the presence of infective larval stages had no influence on the appearance of eosinophils in the immediate vicinity of the infested glands. Larvae in pits, upper glands or glands were clearly surrounded by poorly differentiated, but PAS-positive cells, which in some sections appeared flattened and slightly stretched (Figure 4.4). Parietal cells were no longer recognisable within the parasitised gland, but cells in the immediately adjacent mucosa showed no signs of changes.

In infested fold areas, parietal cell numbers were slightly reduced when compared to the numbers present in non-infected areas, though not significantly (Figure 4.3a), nor were they correlated with eosinophil counts. The morphology of the parietal cells did not appear abnormal and the staining intensity of antibody-labelled cells appeared normal (Figure 4.5).

Invasion of at least one pit/gland was associated with more severe changes (Figure 4.6). The affected gland was wide open, irregularly shaped and filled with cell debris. Mucus present stained strongly with PAS and inflammatory cells of lymphocytic character had migrated into the mucosa, while a small number of eosinophils were visible on the border of sub-mucosa and mucosa (Figure 4.7). TGF- α labelled parietal cells were missing within the close vicinity

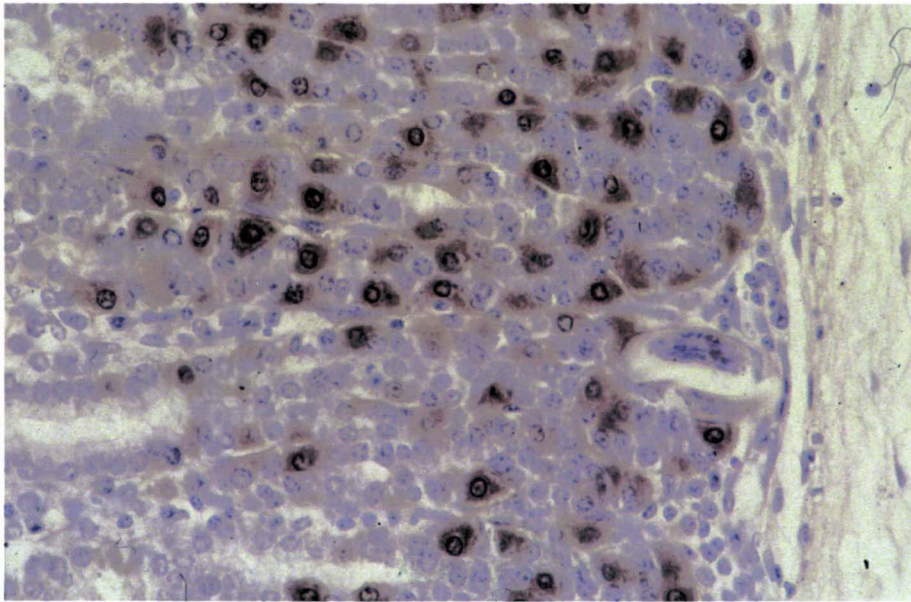


Figure 4.9 Fundic mucosa from a lamb, 30h after infection with 25,000 L₃ *O. circumcincta*. A L₃ has penetrated down to the base of the gland. The epithelial cells of this gland surrounding the larva are clearly abnormal. There are no changes in adjacent glands. TGF- α immunohistochemistry, paraffin-embedded, 5 μ m section, Mayer's haematoxylin counterstain.

of the affected area, while the surrounding tissue appeared to be denser (Figure 4.8). A few parietal cells surrounding the parasitised gland showed signs of vacuolation.

4.3.1.5 Gland Base

L₃ located in the base of the glands were encapsulated by distorted epithelial cells and caused no visible damage to cells in adjacent glands. There appeared to be no changes in the numbers of TGF- α positive parietal cells in immediately adjacent glands. No eosinophils or other inflammatory cells were usually present (Figure 4.9).

4.3.2 L₃ in Tips of Folds

Parietal cell counts in the non-infested tips were significantly lower than non-infested, non-tip areas ($p < 0.05$), but there was no reduction in numbers compared to infested-tips (Table 4.1). In each location, there were no correlations between eosinophil counts and counts of TGF- α parietal cells.

4.3.2.1 Controls

The tip-area of abomasal mucosa from uninfected sheep (See Section 3.3.2.2.1 and Figure 3.12) contained more inflammatory cells, mainly round cells and eosinophils, in the submucosa when compared to non-tip areas of the same animal. Inflammatory cells were also present within the mucosa. Pits appeared slightly deeper when compared to pits in non-tip areas and were lined with columnar mucous cells, which contained a moderate amount of apical mucins. There appeared to be fewer TGF- α positive parietal cells in the base of glands and in mid-gland levels and the pits themselves occasionally lacked parietal cells altogether. All other epithelial cells, however, appeared normal.

4.3.2.2 Infected 30-h Animal

Tissues from non-infested tip areas of the 30h-sheep were similar in appearance to the tips of control animal tissue, although the inflammatory response was slightly more prominent. Non-infested tips in the 30h-sheep contained significantly more eosinophils than non-infested fold ($p < 0.05$) of the

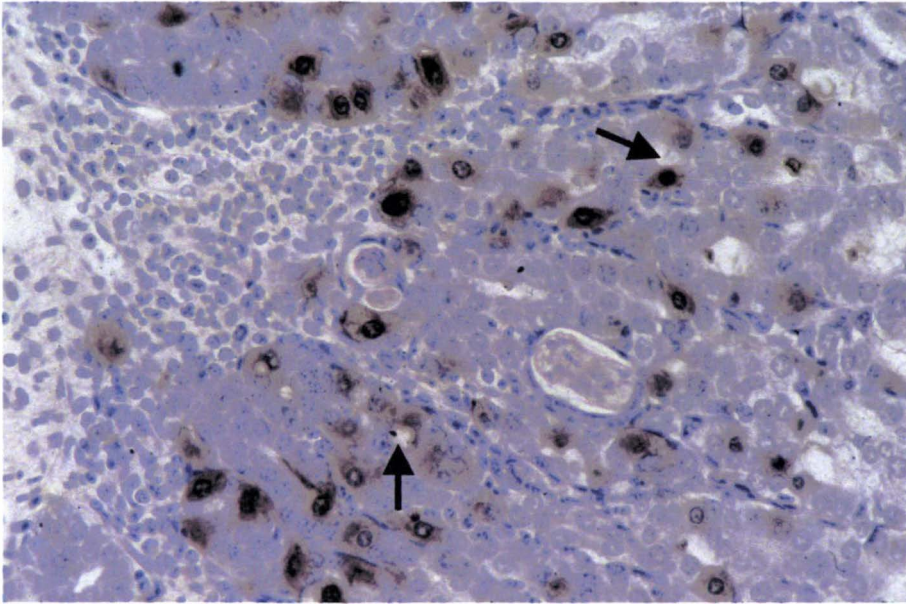
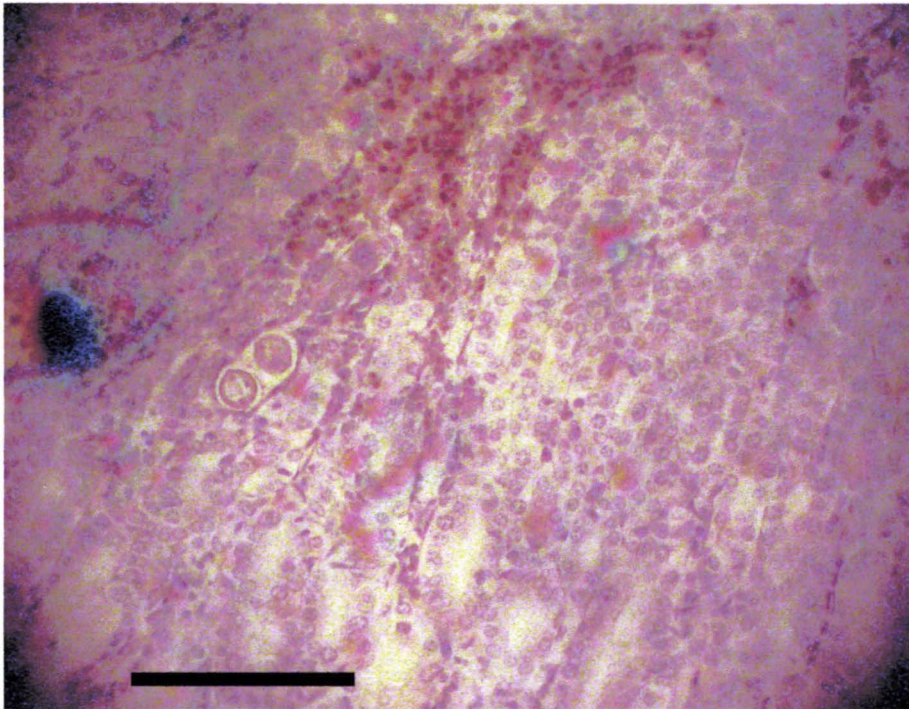


Figure 4.11 Fundic mucosa from a lamb, 30h after infection with 25,000 L₃ *O. circumcincta*. A larva has penetrated into the tissue in the tip of a fold and some parietal cells in neighbouring glands are vacuolated (arrows). TGF- α immunohistochemistry, paraffin-embedded, 5 μ m thick section, Mayer's haematoxylin counterstain.

a



b

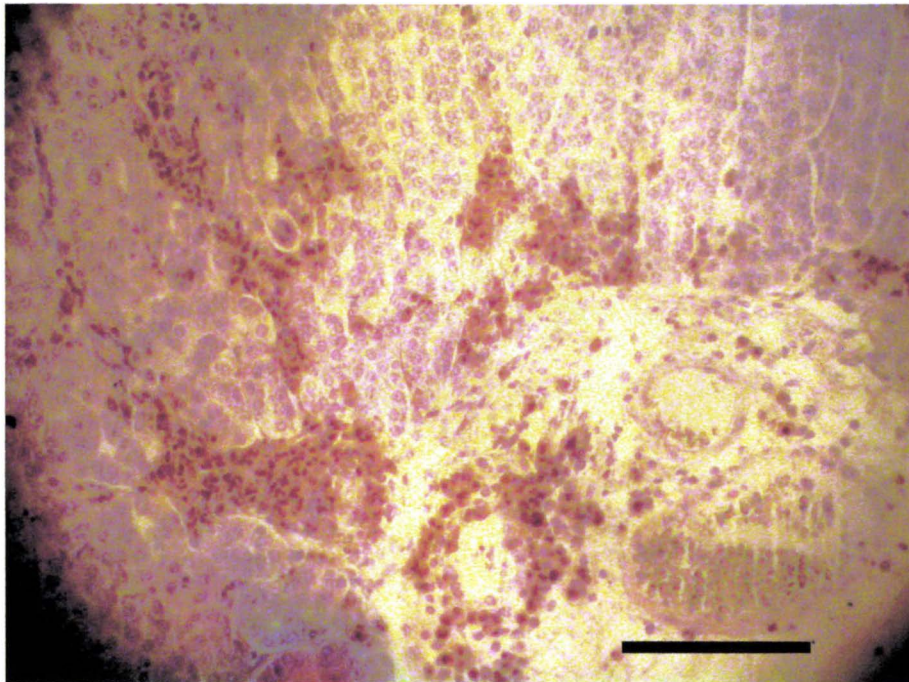


Figure 4.10 a and b Fundic mucosa from a lamb, 30h after infection with 25,000 L₃ *O. circumcincta*. Larvae have penetrated into the tips of the folds and have attracted large numbers of eosinophils into the mucosa and sub-mucosa. Eosinophil immunohistochemistry, paraffin-embedded, 5µm sections, Mayer's haematoxylin counterstain. Bar = 100µm

same animal. There was a significantly lower number of TGF- α positive parietal cells present within non-infested tips than within non-infested fold areas ($p < 0.05$) and indicated a naturally lower presence of parietal cells in the tips of folds (Table 4.1 and Figure 4.3a).

The numbers of inflammatory cells, mainly eosinophils and lymphocytes, in the submucosa and mucosa of larvae-containing tips were extremely high. Sections stained with an eosinophil antibody, revealed an apparently targeted migration of eosinophils into the tip areas when larvae were present. Eosinophils were recruited in very high numbers and could be seen surrounding the gland containing the larvae, though none had migrated into the gland itself (Figure 4.10). Significantly more eosinophils were present in infested-tips compared to non-infested tips ($p < 0.001$) (Figure 4.3b). Lymphocytes were also observed within the mucosa in the close vicinity of larvae-infested glands. In general, parietal cells appeared normal, although some vacuolated parietal cells were observed in close proximity to larvae-inhabited glands (Figure 4.11). There was no statistical difference between counts of TGF- α positive parietal cells in infested or non-infested tips, though Figure 4.3a indicates an increase, most likely caused by the small sample size. Further, there was no correlation between eosinophils and TGF- α positive parietal cells.

4.4 Discussion

The present experiment was designed to follow the parasites at a fairly early stage of a primary infection and more closely explore the timing of changes in eosinophils and parietal cell populations.

One of the most surprising results was the fact that L₃ invading glands located in mucosal tips caused a strong inflammatory response, while L₃ making their way into glands located in the main body of the folds only created a light inflammatory response. Further, mucosal tissue surrounding larvae buried deeply within folds generally showed minimal signs of damage. A naturally greater presence of granulocytes and lymphocytes within the submucosa and

mucosa of the tips, as observed in control tissues, may readily explain why the presence of L₃ created a more pronounced migration of eosinophil and lymphocytes into these areas. Such a readily available pool of cells was not observed in the main body of the folds and this might explain why the inflammatory response here was weaker.

In a previous study, granulocytes and mononuclear cells had infiltrated between the cells of occupied glands (Ritchie *et al.*, 1966) as early as two days after infection of previously parasite-naïve calves with *O. ostertagi*. Infective larvae therefore do seem to be able to cause an immediate inflammatory response in the parasite-naïve host in some circumstances, but in general an inflammatory response is slow to develop and may still be minimal even 5 days after infection (see Chapter 3).

Recently, Balic *et al.* (2000) reported a directed migration of eosinophils towards sites of larval penetration 24 hours after immunised sheep had been challenged with *H. contortus*. This is similar to what occurred in the present experiment in the tips of folds, however, eosinophils did not penetrate the layer of undifferentiated cells encapsulating the larvae, whereas in the immunised hosts, degranulating eosinophils were found in close proximity to damaged or dead larvae (Rainbird *et al.*, 1998). Thus eosinophils can migrate towards penetrating larvae even in the parasite-naïve host, though their role might be different from that in the immunised host.

Third-stage larvae were found on the surface of the mucosa and deep within gland lumina 30 hours after inoculation. What is not known is the time taken by larvae to move into the tissue, but given the short distance involved the process is likely to be quick. One intended aim of the present experiment was to follow the fate of parietal cells present in the glands invaded by the parasite. These clearly are the first parietal cells to disappear during infection, but how and when is not known. Unfortunately, the present experiment has not been able to provide an answer as to how, but no larvae were seen in a gland that still possessed recognisable parietal cells, so the speed at which the cells are lost is clearly rapid.

Overall, the number of parietal cells was not markedly reduced by the presence of larvae and this is in keeping with the hypothesis that parietal cell loss is primarily associated with later parasite stages. An increase of parietal cells in mucosa surrounding infested glands in the tips was recorded, but this was most likely due to the small number of sections investigated.

In the previous chapter, the possibility of a direct effect of L₃ on the parietal cell, mediated by ES products, was discussed. Vacuolation of parietal cells also occurred in the present experiment, within the tissue immediately adjacent to areas penetrated by larvae (Figures 4.8 and 4.11), but in contrast to previous observations of vacuolated parietal cells within 5 day old nodules, those in this experiment were more conspicuously associated with a greater number of inflammatory cells. Thus, a contribution of the host's immune response cannot be ruled out. Together with the results discussed in Chapter 3, investigations into the existence of a possible vacuolation factor excreted/secreted by abomasal parasites are therefore strongly supported.

Chapter 5

***In vitro* vacuolating activity of nematode excretory/secretory products**

5.1 Introduction

Vacuolated parietal cells have been seen in the close vicinity of glands infected by developing larvae, as well as more generally in the abomasal mucosal once the parasites have emerged or after adult worm transplantation (Chapter 3). In each case, the nematodes were not in direct contact with the vacuolated parietal cells, suggesting it may be mediated indirectly, perhaps through a chemical released by the parasites.

Parasites release a variety of agents in their ES, including chemotaxins, metabolic end-products, enzymes, immunomodulators and growth factors (reviewed in Section 1.3.2.4). Enzymes identified in ES of a range of gastrointestinal helminths include acetylcholinesterase (Lee & Hodsden, 1963; Ogilvie *et al.*, 1973; Knox & Jones, 1990; Griffiths & Pritchard, 1994; Huby *et al.*, 1999a), cysteine, aspartic and metalloproteases (Karanu *et al.*, 1993), elastase (Knox & Jones, 1990), N-acetyl- β -D-glucosaminidase and an acid phosphohydrolase (Gamble & Mansfield, 1996). Some of these could have effects on parietal cells.

ES components which have been implicated in abomasal pathophysiology are more likely candidates to mediate parietal cell vacuolation. These include a parietal cell inhibitor (Merkelbach *et al.*, 2002), an inhibitor of the ECL cell (Hertzberg *et al.*, 1999a) and a stimulant of pepsinogen release from previously exposed tissue (Scott & McKellar, 1998). ES also contains ammonia (Merkelbach *et al.*, 2002), which has been shown to cause vacuolation in epithelial cells (Hagen *et al.*, 1997).

Vacuolation of epithelial cells is a feature in humans suffering from chronic gastritis caused by *H. pylori* (Fiocca *et al.*, 1987) and is caused by vacuolating factor A (*vacA*), a toxin able to induce apoptosis in gastric epithelial cells *in vitro* (Cover *et al.*, 2003). *VacA* is a protein cytotoxin whose sequence can be found in nearly all *H. pylori* strains (Cover *et al.*, 1994), though there is a considerable variation in toxin activity within strains. One of its main biological activities is the induction of cytoplasmic vacuoles, which originate from late endosomal/lysosomal compartments (Papini *et al.*, 1993). *VacA* has been studied in a variety of cultured epithelial cell lines, particularly HeLa cells (Cover *et al.*, 1991; Harris *et al.*, 1996) using the Neutral Red (NR) uptake assay. The development of these vacuoles *in vitro* is dependent on the presence of extracellular weak bases like ammonia (Cover *et al.*, 1991), which could be provided *in vivo* by *H. pylori* urease (Megraud *et al.*, 1992).

The similarity in appearance and development of vacuolation between parietal cells in parasitised abomasal mucosa and that of other epithelial cells exposed to *vacA* raises the question whether parasites may release a vacuolation factor similar to *vacA* in the many components of ES. An additional reason for believing that abomasal parasites and a gastric bacterium, which can colonise the stomach for decades, might produce a related chemical is the marked resemblance between gastropathies evoked by *H. pylori* and abomasal nematodes.

Experiments based on methods used to study *vacA* were designed to seek a similar parasite vacuolation factor and possibly to characterise it. In addition, ammonia was tested as a candidate vacuolating factor in ES, in concentration similar to those present in the ES derived from adult *H. contortus* with which Merkelbach *et al.* (2002) inhibited histamine-stimulated AP-uptake in dispersed rabbit glands.

Table 5.1. Components of the three media MEM (10x), RPMI-1640 and EBSS. RPMI-1640 contained in addition Glutathione (reduced) 0.001g/l, L-Asparagine (anhydrous) 0.05g/l, L-Aspartic acid 0.02g/l, L-Glutamic acid 0.02g/l, L-Glutamine 0.3g/l, Glycine 0.01g/l, Hydroxy-L-Proline 0.02g/l, D-Biotin (traces), p-Amino Benzoic Acid 0.001g/l and Vitamin B12 (traces).

Components (g/l)	MEM (10x)	RPMI-1640	EBSS (E3261)
Inorganic Salts			
CaCl ₂	2.00 (anhydrous)		0.27 (2H ₂ O)
KCl	4.00	0.40	0.40
MgSO ₄	0.98	0.05 (anhydrous)	0.1 (anhydrous)
NaCl	68.0	6.00	6.80
NaHCO ₃		2.00	2.20
NaH ₂ PO ₄	1.40 (H ₂ O)		0.12 (anhydrous)
Na ₂ HPO ₄		0.80	
Other components			
D-Glucose	10.00		1.00
Phenol Red	0.10	0.005	
Amino acids			
L-Arginine	0.21 (HCl)	0.20	
L-Cystine	0.29 (2Na)	0.07 (2HCl)	
L-Histidine	0.21(HCl-H ₂ O)	0.02	
L-Isoleucine	0.13	0.05	
L-Leucine	0.13	0.05	
L-Lysine HCl	0.18	0.04	
L-Methionine	0.15	0.02	
L-Phenylalanine	0.17	0.02	
L-Threonine	0.12	0.02	
L-Tryptophane	0.20	0.01	
L-Tyrosine 2Na 2H ₂ O	0.26	0.03	
L-Valine	0.12	0.02	
Vitamins			
D-Ca Pantothenate	0.48	traces	
Choline Chloride	0.14	0.003	
Folic Acid	0.44	0.001	
myo-Inositol	0.18	0.04	
Niacinamide	0.12	0.001	
Pyridoxal HCl	0.20	0.001	
Riboflavin	0.38	traces	
Thiamine HCl	0.34	0.001	

5.2 Materials and Methods

5.2.1 *In vitro* Effects of ES on Cultured Epithelial Cells

5.2.1.1 General Experimental Design

Adult worms and L₃ stages of *O. circumcincta* and *H. contortus* were placed in RPMI 1640 or EBSS, both pH 7.3, and incubated at 37°C in a atmosphere containing 5% CO₂ and 95% humidified air. Incubations were terminated after different times, which varied from 24h to 168h, and ES was harvested by centrifugation and passed through a filter with a pore size of 0.45µm and/or 0.22µm. ES was then concentrated using dialysis tubing or ultrafiltration membranes with molecular weight cut-off (MWCO) of 1kDa or 10kDa and stored at -20°C or 4°C until use.

HeLa cells, grown for two days after passaging, were seeded in 96-well plates and were allowed to attach for 24h. HeLa cells were then exposed to serial dilutions of ES for 24h. Effects of ES on HeLa cells were measured spectrophotometrically to assay Neutral Red (NR) uptake into cells.

5.2.1.2 Cell Culture

HeLa cells, which had been 13x passaged since establishment (p13), were obtained from the Institute of Veterinary, Animal and Biomedical Sciences (Virology), Massey University, Palmerston North, NZ. All relevant cell culture techniques are described in Appendix 3.

5.2.1.3 Preparation of ES

Adult worms or L₃ stages of *O. circumcincta* and *H. contortus* were obtained, counted and exsheathed (L₃) as described in Appendix 1. The incubation media RPMI 1640 (R1383) and EBSS (E3261) (both Sigma Chemicals Co, St.Louis, USA) were made up as instructed by the manufacturer and 0.01g/l gentamicin and 0.25mg/l fungizone[®] (both Gibco BRL, Life Technologies Inc., USA) were added. The compositions are shown in Table 5.1.

Immediately before use, the pH of incubation medium was readjusted to pH 7.3 and the medium sterilised by filtration through a sterile 0.22µm syringe-filter

(Pall, Ann Arbor, USA). Parasites were incubated in either 50ml sterile RPMI 1640 or EBSS, in a sterile, non-pyrogenic 75cm² cell culture flasks with a ventilation cap fitted with a 0.2µm filter (Coming Inc., Coming, USA).

The flask was left at 37°C in an atmosphere containing 5%CO₂/95% humidified air (Queue CO₂ incubator, Parkersburg, USA) for time periods between 24h and 168h. Incubations were terminated by centrifugation for 5 min at 200g. The supernatant (designated ES) was poured into an autoclaved 50ml tube while carefully avoiding the parasite pellet on the bottom. ES was then filtered into another autoclaved 50ml tube using either 0.45µm and/or 0.22µm syringe filters and stored at -20°C or 4°C until further use. The remaining parasites were either discarded or resuspended in 50ml sterile incubation medium and poured into the previously used cell culture flask for further incubation under previously described conditions.

ES was then either fractionated and concentrated against 40% PEG (BDH Chemicals Ltd., Poole, England) using dialysis tubing with a MWCO of 1kDa (D7884, Sigma Chemicals Co., St. Louis, USA) at 4°C for 12h to 72h or by centrifugation at 3000g at 4°C using centrifugal filter units with a MWCO of 10kDa (Centricon[®] Plus-20, Amicon Millipore, Bedford, USA). Further into the project a pressurised (25kPa) stirred cell unit (Amicon Millipore, Bedford, USA) was employed. This device was fitted with semi-permeable membranes with MWCO of 10kDa (YM10, regenerated cellulose, Amicon Millipore, Bedford, USA) and operated with N₂ gas on a magnetic stirrer placed in a 4°C room. The resulting fractions of ES were transferred into autoclaved 1.5ml microcentrifuge tubes and kept at -20°C or 4°C until use. All methods used to fractionate and concentrate were operated as instructed by the manufacturer.

In order to establish a negative control, RPMI was prepared, incubated for 48h, harvested and filtered as described for ES and is referred to as RPMI-inc. It was fractionated and concentrated using a pressurised stirred cell unit.

5.2.1.4 Controls

NH₄Cl in CEM in a concentration series of 50mM, 25mM, 12.5mM, 6.25mM and 3.125mM served as positive controls, while CEM represented null controls.

Positive and null controls were prepared freshly and run within each performed assay. Negative controls such as RPMI diluted in CEM, RPMI-inc diluted in CEM as well as a 10K fraction of RPMI-inc diluted in CEM were used to assess a possible effect of media mixing.

5.2.1.5 Neutral Red (NR) Uptake Assay

The method, based on the method of Cover *et al.* (1996), and the reagents used are described in Appendix 3. Briefly, HeLa cells, suspended in CEM, were seeded at a concentration of 3000/well and allowed to attach and grow for 24h. CEM was then removed and test media (controls, ES samples or their dilutions) were added in a volume of 100 μ l per well. Each test medium was assayed in triplicate. Microtiter plates were placed back in the incubator and incubation was terminated after 24h.

The supernatant in the wells was discarded and 100 μ l of a freshly prepared 0.05% neutral red (NR)-CEM solution was added to each well. The plate was allowed to incubate at 37°C for 4min before the NR-CEM solution was withdrawn and cells washed twice with 150 μ l of 0.9% NaCl per well. To extract NR from HeLa cells, 100 μ l of acidified alcohol was pipetted into each well and the OD measured at 550nm.

5.2.1.6 Ammonia and Protein Determination in ES

Ammonia was estimated by an assay based on the reaction of ammonia with hypochlorite and phenol to produce indophenol. The quantity of ammonia was then measured spectrophotometrically at 635nm (Bolleter *et al.*, 1961). Protein concentrations were determined using the method of Bradford (1976). Briefly, the dye Coomassie Brilliant Blue G-250 binds to the protein and causes a shift in absorption maximum of the dye from 465nm to 595nm. Both methods are described in Appendix 3.

5.2.1.7 Data Presentation

All reported means are expressed as mean \pm SEM. Optical density (OD) is expressed as % deviation of the OD of the null control, which was CEM.

Calculation of a concentration-dependent response: a formula was derived to compare the effects on NR uptake of the different ES preparations, which varied in incubation time, parasite density, the extent to which ES was concentrated and the dilution of the ES. The final concentration (C_{final}) was a function of all of these:

$$C_{\text{final}} \propto \frac{\text{nematode density (ml}^{-1}\text{)}}{\text{dilution factor}} \times \frac{\text{incubation volume (l)}}{\text{final volume (l)}} \times \text{incubation time (h)}$$

$$= \text{equivalent nematode density} \times \text{incubation time (h ml}^{-1}\text{)}.$$

All results are interpreted on the presumption that NR uptake is zero in the absence of any ES.

5.2.2 Probing Worm Genomic DNA for a VacA-like Gene

5.2.2.1 DNA Extraction

Approximately 200 μ l of adult *O. circumcincta* (about 500) in a 1.5ml microcentrifuge tube were digested for 3h in 10mM Tris-HCl (TE) buffer (Sigma Chemical Co., St. Louis, USA) containing 0.5% sodium dodecyl sulfate (SDS) (Qbiogene[®], BDH Chemicals Ltd, Poole, England) and 0.1mg/ml proteinase K (Qiagen, Germantown, USA). A volume of 0.5ml of 50/50 phenol/chloroform (phenol: equilibrated with TE buffer and containing 0.1% hydroxyquinoline and 2% β -mercaptoethanol; chloroform: 24:1 v/v of chloroform and isoamyl alcohol) suspension (both Sigma Chemical Co., USA) was added to the digest and gently mixed. This was followed by a 2min centrifugation at 1600g at room temperature. The resulting upper aqueous phase was transferred to a new tube and the phenol/chloroform extraction step was repeated. The upper aqueous phase was then extracted with an equal volume of chloroform to remove all traces of phenol. Sodium acetate was added to give a final concentration of 0.3M and two equal volumes of cold 100% ethanol were added to the aqueous phase to precipitate DNA. The mixture was kept at -20°C for 30min and then centrifuged at 1200g at 4°C. The supernatant was discarded, cold 70% ethanol added and the tube centrifuged at 1200g at for 5min at 4°C. The supernatant was discarded and the DNA pellet re-suspended in 0.5ml TE-buffer.

RNA was removed from the DNA by incubation at 37°C for 1h with 40µg/ml RNAse A (Roche Boehringer Mannheim, Germany) in a final concentration of 40µg/ml, after which phenol/chloroform extraction and ethanol precipitation were repeated. The final DNA-pellet was resuspended in 100µl TE-buffer and stored at -70°C until use.

5.2.2.2 Restriction Digestion of Parasite Genomic DNA

Worm genomic DNA was digested overnight at 37°C with 10 units of restriction enzyme EcoRI or HindIII (both Invitrogen Life Technologies Inc., USA).

5.2.2.3 VacA Probe

The *H. pylori* vacA probe was amplified by polymerase chain reaction (PCR) using the primers vacA 1 (5' to 3': GCT TCT CTT ACC ACC AAT GC) and vacA 2 (5' to 3': TGT CAG GGT TGT TCA CCA TG) (both Gibco BRL Custom Primers, Life Technologies Inc., USA). Template *H. pylori* DNA was the kind gift of Dr P. O'Toole, Institute of Molecular Biosciences, Massey University.

The PCR reaction contained 5ng/µl of each primer and 0.1ng/µl template DNA, 0.3mM dNTP, 2.5units Taq, 0.25mM MgCl₂, PCR buffer and was made up with H₂O to a final volume of 50µl. The protocol for the PCR cycle (35x) was as follows:

strand denaturation at 94°C for 30s,

primer annealing at 58°C for 30s and

primer extension at 72°C for 60s.

5.2.2.4 Southern Blotting

5.2.2.4.1 Gel

A 0.7% agarose gel in 1x Tris acetate EDTA (TAE) buffer containing 50µg ethidium bromide was poured and lanes were loaded with 5µg of undigested, 5µg of EcoRI digested DNA, 5µg of HindIII digested genomic worm DNA. VacA was loaded in concentrations of 0.1ng, 1ng and 10ng. The gel was run overnight at 25V.

5.2.2.4.2 Southern Transfer

The gel was washed twice in denaturing solution (0.5M NaOH, 1.5M NaCl) for 15min, briefly rinsed with neutralising solution (1M Tris pH8, 1.5M NaCl) and then soaked twice in neutralising solution for 30min. The gel was then blotted overnight onto a nylon membrane (Biodyne plus[®], Gelman Sciences, Ann Arbor, USA) using 20x SSPE buffer as blotting solution. The next morning the nylon membrane (blot) was rinsed twice with 2x SSPE buffer, dried at 80°C for 2h and stored in a dry environment at room temperature until use.

5.2.2.4.3 Probing the Blot

The vacA DNA probe was prepared using a commercial kit (Ready to go kit[®], Pharmacia Biotech) and the radioactive label [$\alpha^{32}\text{P}$]dCTP (3000Ci/mmol) (Amersham Life Science, Little Chalfont, UK). 50ng of vacA DNA were labelled according to the manufacturer's protocol.

The blot was rehydrated in 6x SSPE buffer and transferred into a glass hybridisation tube. The tube was filled with 30ml of prehybridisation buffer (30ml 20x SSPE, 1ml boiled 10mg/ml salmon sperm DNA, 10ml 50x Denhardt's solution, 5ml 10% SDA made up to 100ml with H₂O) and incubated at 65°C for 2h.

The prehybridisation solution was then poured out and replaced by 20ml hybridisation solution (prehybridisation solution without Denhardt's solution) plus the denatured radioactive probe. The tube was then incubated at 65°C overnight.

The hybridisation solution was discarded, the blot removed and briefly rinsed with posthybridisation wash solution (0.2x SSPE, 0.5%SDS). The blot was transferred into a container, washing solution added and the container placed in 65°C shaking waterbath for 30min. This step was repeated once. The blot was removed, blotted dry using 3MM filter paper (Whatmans, Maidstone, England), wrapped in Saran[®] and exposed to a radiographic film (Kodak, USA) for 3 days.

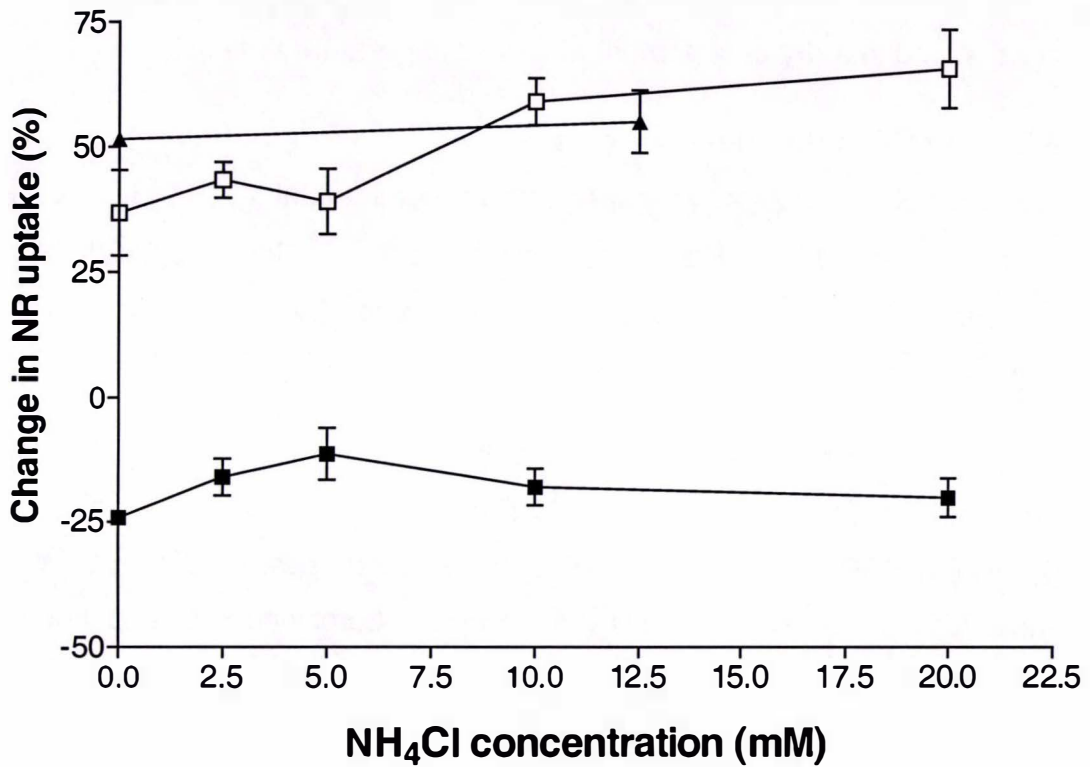


Figure 5.3. The effect of exposure for 24 hours to adult *O. circumcincta* excretory/secretory products, incubates OAD1 (2000h ml⁻¹) and OAD 2A (375 h ml⁻¹ and 187.5h ml⁻¹) at different concentrations in the presence of up to 20mM NH₄Cl on Neutral Red (NR) uptake by HeLa cells (mean ± SEM), relative to that in Complete Essential Medium. The letter “a” identifies incubates as the first incubate of the same batch of parasites. Symbols: ■ OAD2A 187.5; □: OAD2A 375; ▲: OAD1 2000.

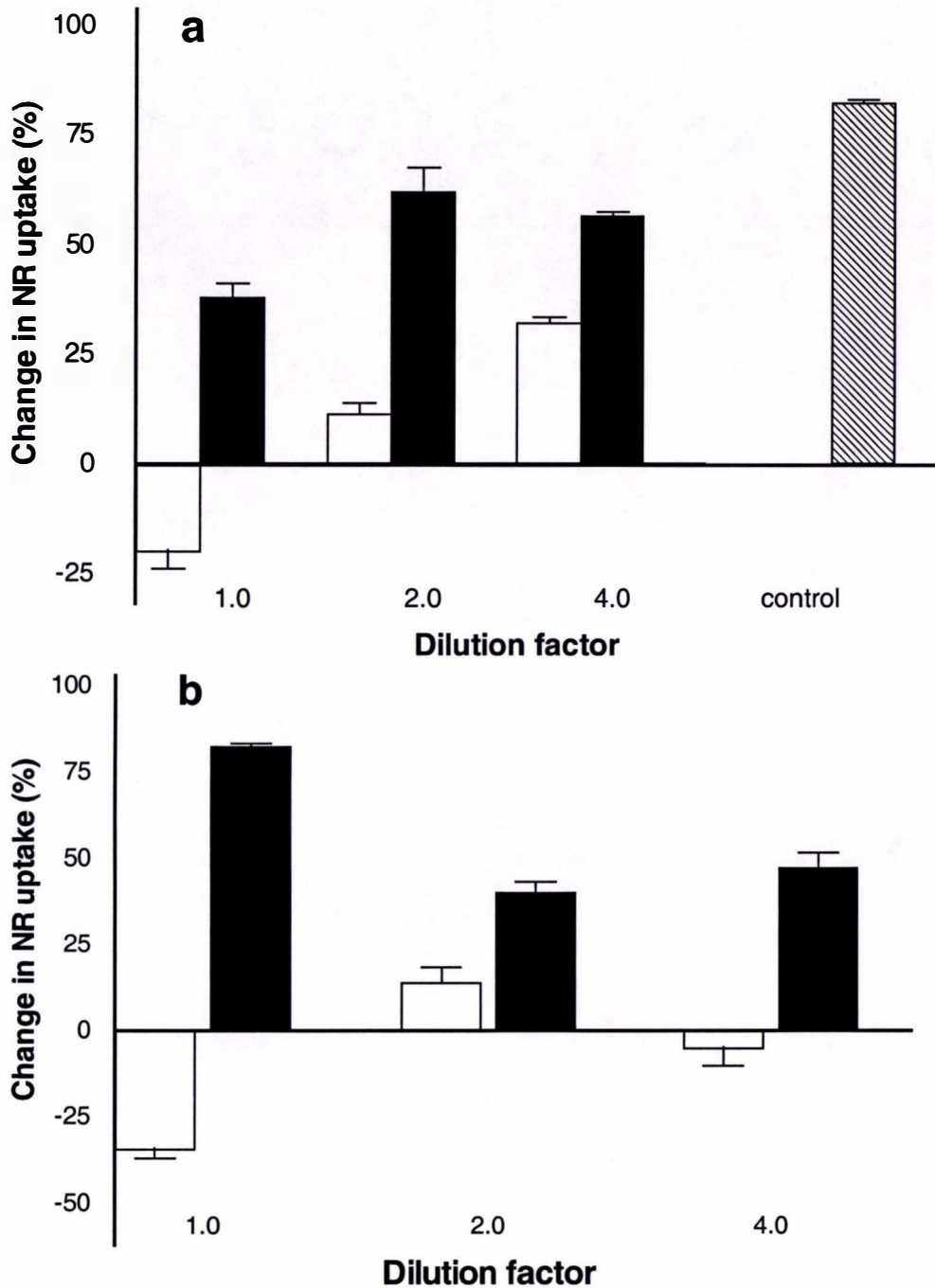


Figure 5.2. The effect of exposure for 24 hours to *H. contortus* L₃ excretory/secretory products (a) HL1c or (b) HL1e, with or without added 6.25 mM NH₄Cl, on Neutral Red (NR) uptake by HeLa cells (mean ± SEM), relative to that in Complete Essential Medium. Open bars show the responses to ES without NH₄Cl, closed bars the responses to ES with 6.25mM NH₄Cl and the hatched bar the response to 6.25mM NH₄Cl alone. The letters “c” and “e” identify incubates as the third or fifth, respectively, incubate for the same batch of parasites.

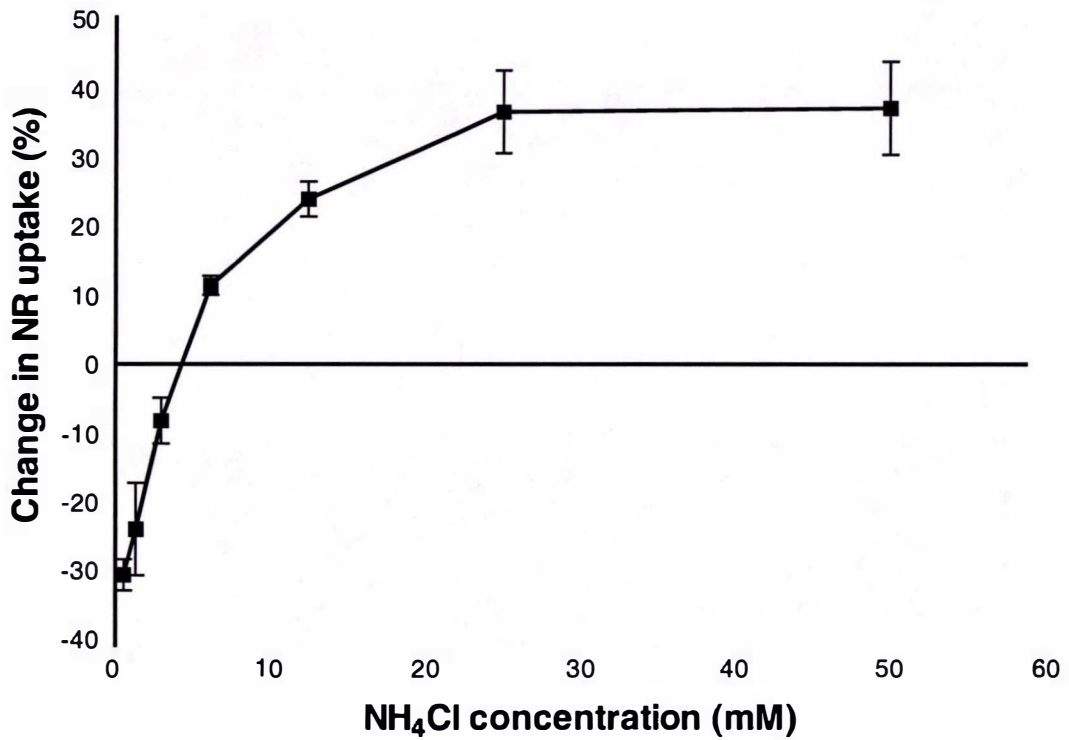


Figure 5.1. Effect of exposure to a series of ammonium chloride concentrations (3.125mM, 6.25mM, 12.5mM, 25mM and 50mM) for 24 hours on neutral red (NR) uptake by HeLa cells. NR uptake is expressed relative to that in Complete Essential Medium.

5.3 Results

5.3.1 Effects of Ammonia and ES on NR uptake by HeLa Cells

5.3.1.1 Ammonia Concentrations in ES

Ammonia concentrations were measured in ES prepared by incubating 5000L₃/ml in RPMI for 24h, 48h, 96h and 144h and in addition in both RPMI and RPMI-inc. ES derived from a 48h incubation of *O. circumcincta* L₃ did not differ in ammonia concentration (2.7μM) from a 96h-incubation of *O. circumcincta* L₃. *H. contortus* L₃ ES, when incubated for 144h, contained 29μM NH₃/NH₄⁺. Unincubated RPMI or RPMI incubated for 48h served as negative controls, and each contained 2.5μM NH₃/NH₄⁺. *O. circumcincta* L₃ ES incubated for 24h did not have an ammonia concentration above the 2.4μM measured in RPMI alone.

5.3.1.2 NR Uptake by HeLa Cells Incubated with NH₄Cl

The dose-response curve for NR uptake by HeLa cells exposed to different NH₄Cl concentrations represent the positive controls (Figure 5.1). At concentrations of 6.25mM and higher, NH₄Cl increased NR uptake and approached saturation at a concentration of 25mM. Based on this result it was decided to supplement ES with NH₄Cl in concentrations 6.25mM or higher. This concentration was chosen to overcome the inhibitory effect on HeLa cells by NH₄Cl concentrations below 6.25mM.

5.3.1.3 NR Uptake by HeLa cells Incubated with ES and NH₄Cl

The presence of different dilutions of ES derived from *H. contortus* L₃ did not interfere with the ability of NH₄Cl to increase NR uptake (Figures 5.2a and 5.2b), though exposure of HeLa cells to 6.25mM NH₄Cl clearly enhanced NR uptake.

Figure 5.3 illustrates the effect of incubating cells with NH₄Cl in the presence of adult *O. circumcincta* ES. Exposure of HeLa cells to a series of NH₄Cl concentrations in the presence of *O. circumcincta* adult ES showed little effect on NR uptake.

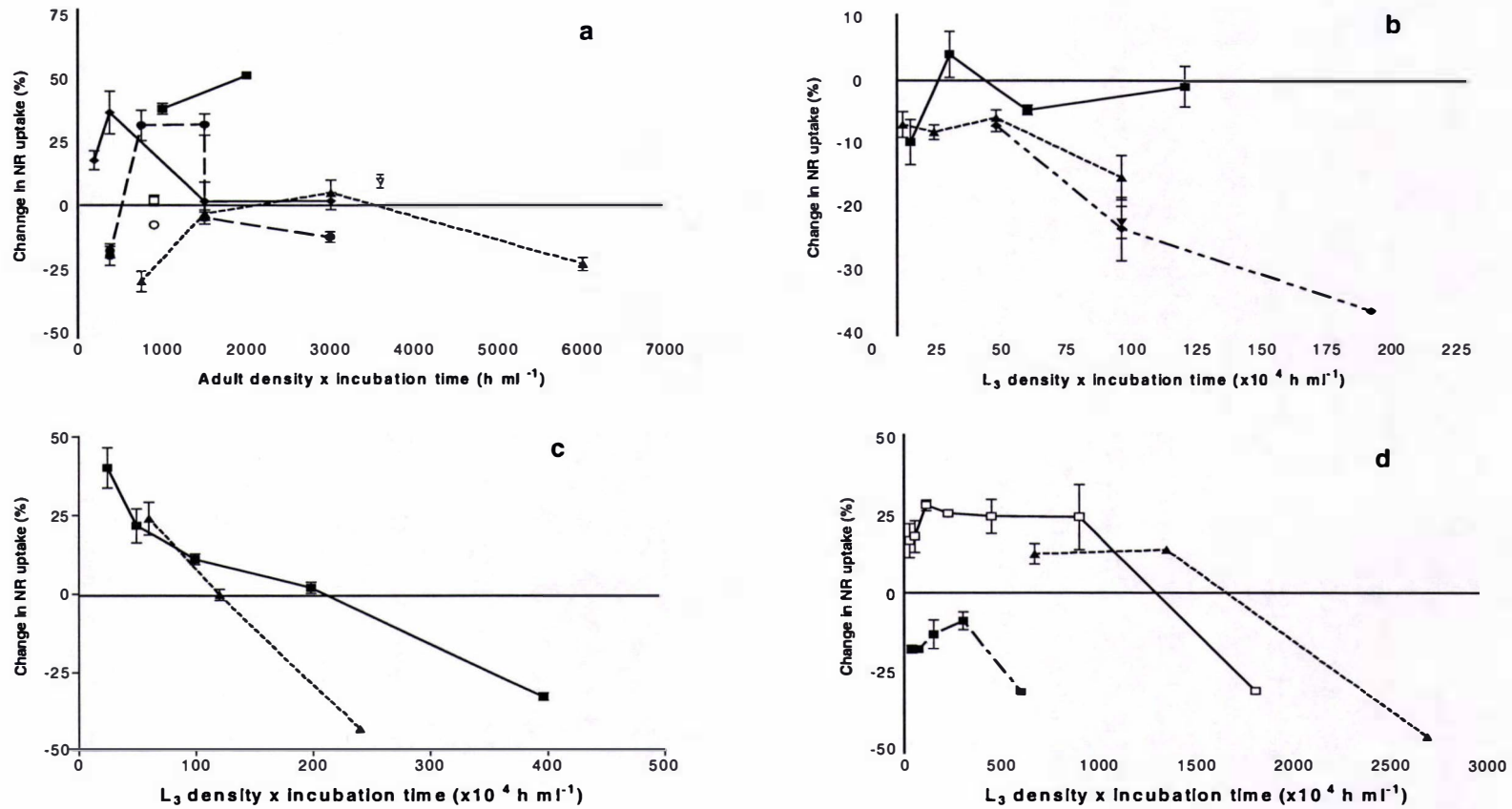


Figure 5.4. The effect of excretory/secretory products of adult *Ostertagia circumcincta* (a), *Ostertagia circumcincta* L₃, which had been incubated for 24h (b) and *Haemonchus contortus* L₃, which had been incubated for 24h, except HL4 (144h) and HL5 (168h) (c and d) on Neutral red uptake by HeLa cells after exposure for 24h. Concentration has been standardised by multiplying the density of larvae by incubation time. The letters “a, b, c, d” identify incubates as the first, second, third or fourth, respectively, incubation of the same batch of parasites. Symbols. (a): ■: OAD1; ◆: OAD2a; ●: OAD2b; ▲: OAD3c; □: OAD4a; ○: OAD4b; ▽: OAD4c; (b): ■: OL1b; ◆: OL2a; ▲: OL2b; ▼: OL2d; (c): ▲: HL2c; ■: HL1c; (d): ■: HL3; □: HL4; ▲: HL5.

5.3.1.4 NR Uptake by HeLa cells incubated with ES

The final concentration (C_{final}) of the different ES preparations was calculated according to the formula:

$$C_{final}^{\infty} = \frac{\text{nematode density (ml}^{-1}\text{)}}{\text{dilution factor}} \times \frac{\text{incubation volume (l)}}{\text{final volume (l)}} \times \text{incubation time (h)}$$

$$= \text{equivalent nematode density} \times \text{incubation time (h ml}^{-1}\text{)}.$$

All results are interpreted on the presumption that NR uptake is zero in the absence of any ES.

Exposure of HeLa cells to adult ES of *O. circumcincta* origin revealed a dependency on nematode density or incubation time. A higher density or longer incubation time enhanced NR uptake compared with lower C_{final} , though an inhibitory effect was observed with further increases of either parameter. Moreover, there was some evidence that the effect on NR uptake by ES changed with the overall time for which the adult nematodes had been incubated. Adult worms incubated for a third consecutive time (OAD3c) did not increase NR uptake in comparison with adult ES derived from a first or a second consecutive incubation (Figure 5.4a).

ES based on *O. circumcincta* L₃ and *H. contortus* L₃ incubations gave similar data, again reflecting a dependency of NR uptake on equivalent nematode density x incubation time. To compare these data with data obtained in adult experiments, the large difference in density x incubation time between the larvae and adult experiments had to be taken into account: L₃, 100-fold higher concentrated than adults, were not able to increase NR uptake, whereas L₃, 1000-fold or higher concentrated compared to adult ES caused NR uptake similar to it. Furthermore, the variability in the extent of the effect on NR uptake within the L₃ ES experiments has also to be considered: the potency of ES derived from a third consecutive 24h incubation was similar to that of ES derived from a long-term incubation, with both of *H. contortus* origin. A third consecutive incubation of *O. circumcincta* L₃ did not raise NR uptake, while an *H. contortus* L₃ ES at a similar concentration did raise NR uptake (Figure 5.4).

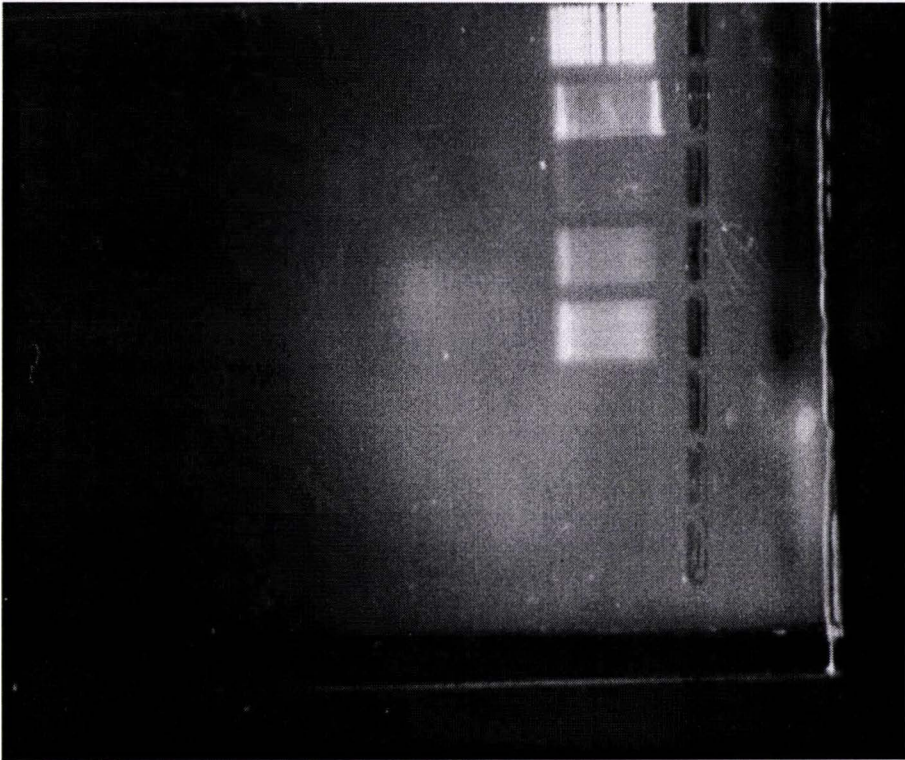


Figure 5.8. Genomic *O. circumcincta* DNA run on a 0.7% agarose gel. From top to bottom: DNA ladder, EcoRI digested DNA with rest RNA, empty and EcoRI digested DNA free from RNA, HindIII digested DNA free from RNA.

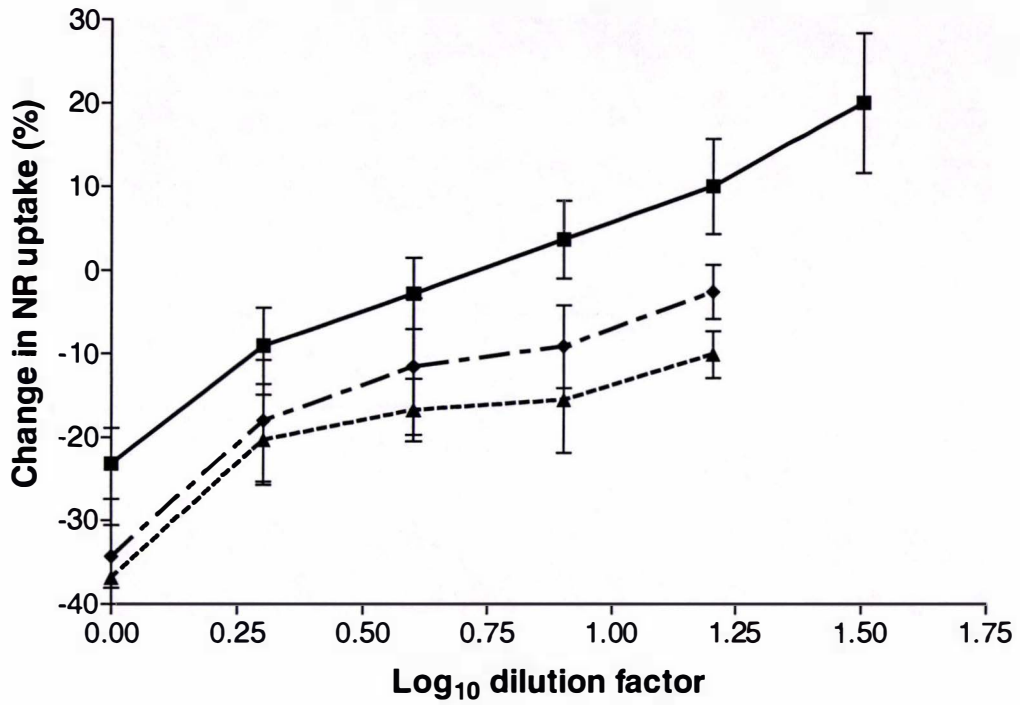


Figure 5.7. Effect on Neutral Red (NR) uptake by HeLa cells of exposure to CEM, combinations of CEM and RPMI, CEM and RPMI-inc and a 10kDa fraction of RPMI-inc and CEM, for 24 hours. NR uptake is expressed relative to uptake of NR in CEM. Symbols: RPMI and CEM: ■; RPMI-inc and CEM: ▲; RPMI-inc >10kDa and CEM: ◆.

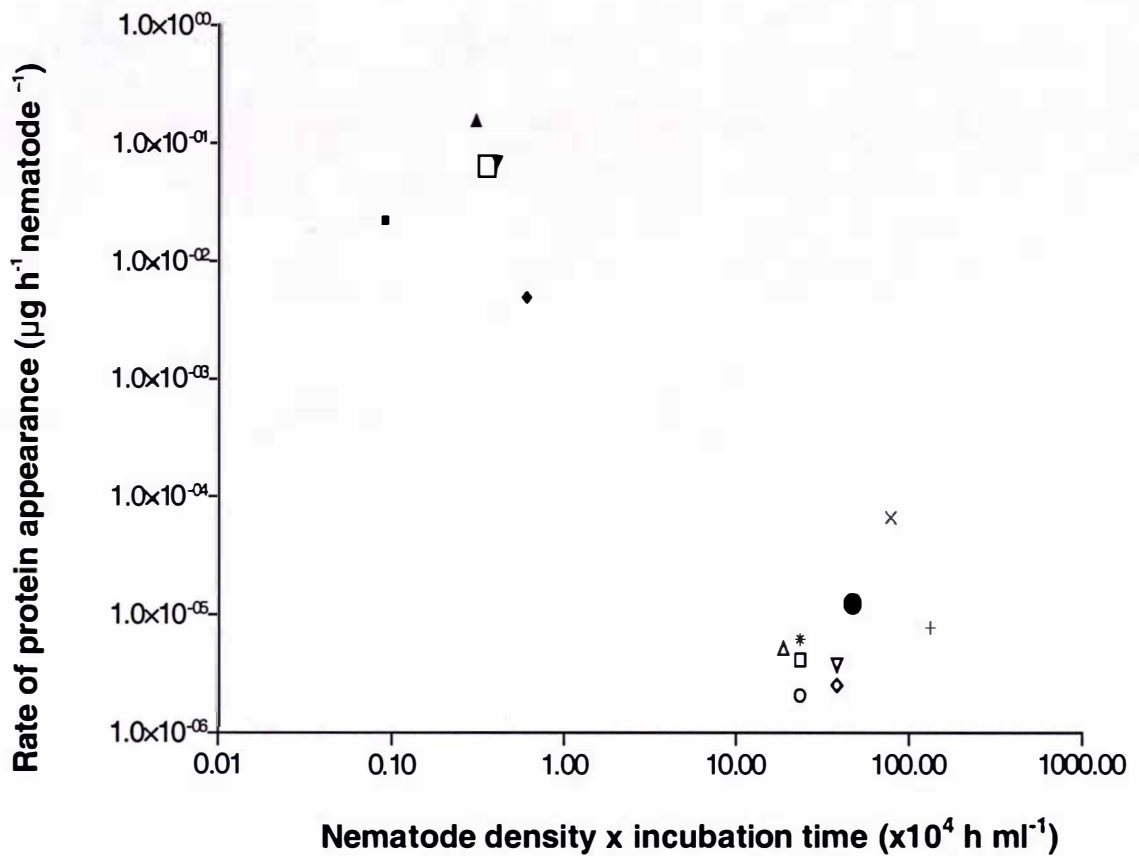


Figure 5.6. Rate of protein appearance in incubations with varying concentrations of *H. contortus* L₃ or *O. circumcincta* L₃ or adult worms. Concentrations have been standardised by multiplying the density of larvae by incubation time. The letters a, b, c and e identify incubates as the first, second, third and fifth incubation of the same batch of parasites. Symbols: OAD1: ▼; OAD2b: ▲; OAD3c: ■; OAD4a: ◆; HL2c: *; OL1b: □; OL2b: △; HL3c: ◆; HL3e: ◇; HL4: ○; HL5: ×; HL6: +; Adult average: □; Larval average: ●.

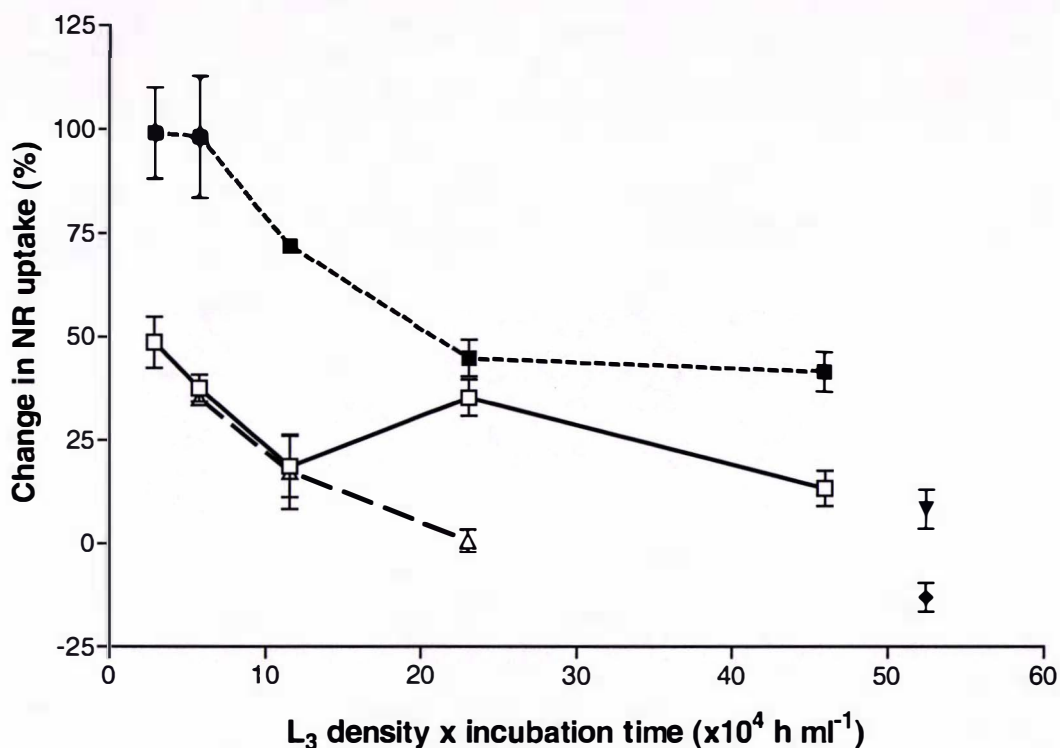


Figure 5.5. The effect of exposure for 24 hours to L₃ *O. circumcincta* excretory/secretory products which had been stored under different conditions on Neutral Red (NR) uptake by HeLa cells (mean \pm SEM), relative to that in Complete Essential Medium. The concentration has been standardised by multiplying the density of L₃ by incubation time: OL3aFi₁: 4d at 4°C; OL3aFi₂: 23d at 4°C; OL3aFe: 3d at 4°C, then 1d at -20°C; OL3c: not stored; OL3cFi/Fe: 1d at 4°C, then 29d at -20°C. The letter a and c identify incubates as the first and third, respectively, incubation of the same batch of parasites. Symbols: ■:OL3aFi₁; □:OL3aFi₂; △:OL3aFe; ▼:OL3c; ◆: OL3cFi/Fe.

5.3.1.5 Effect of Storage on ES Potency

Data shown in Figure 5.5 confirm previously reported results (Section 5.3.1.4), but also imply an influence of storage conditions on the potency of L₃ ES.

L₃ ES derived from *O. circumcincta* incubations (OL3a), stored for 4d, lost around 50% of its potency, when the storage condition had been switched from 4°C to -20°C for the last 24h. The same ES showed a similar loss of its potency after long-term storage at 4°C. Similarly, fresh ES was more potent than ES stored long-term at -20°C, though the loss was smaller compared to long-term storage at 4°C.

5.3.1.6 Protein Concentrations in ES

Protein concentrations appeared to depend on both density and time, though concentrations were in general low. L₃ in a 1000-fold higher concentration than adults produced a 1000-fold lower amount of protein (Figure 5.6).

5.3.1.7 Effect of Media on NR Uptake

While undertaking this study an unexpected effect of mixed media on NR uptake became apparent. Therefore controls (see Section 5.2.1.4) were tested in an identical manner to ES to rule out any false positive reactions among ES results. Figure 5.7 clearly shows that small volumes of RPMI mixed with CEM enhanced NR uptake, whereas it was inhibitory when added in higher concentrations. There is some minor offset between treatments of RPMI, but this might reflect the individual performance of the different cells.

There are changes in amino acid, vitamin and glucose concentrations due to mixing of media, but also important changes in certain ion concentrations. Ion changes are more evident when RPMI, not EBSS, is combined with CEM (Table 5.1).

5.3.2 Probing Genomic Worm DNA for a VacA-like Gene

The *vacA* probe used here did not show the presence of homologous sequences to *H. pylori vacA* in the *O. circumcincta* genome. The gel pictured in Figure 5.8 shows the DNA extracted from adult *O. circumcincta* run on a

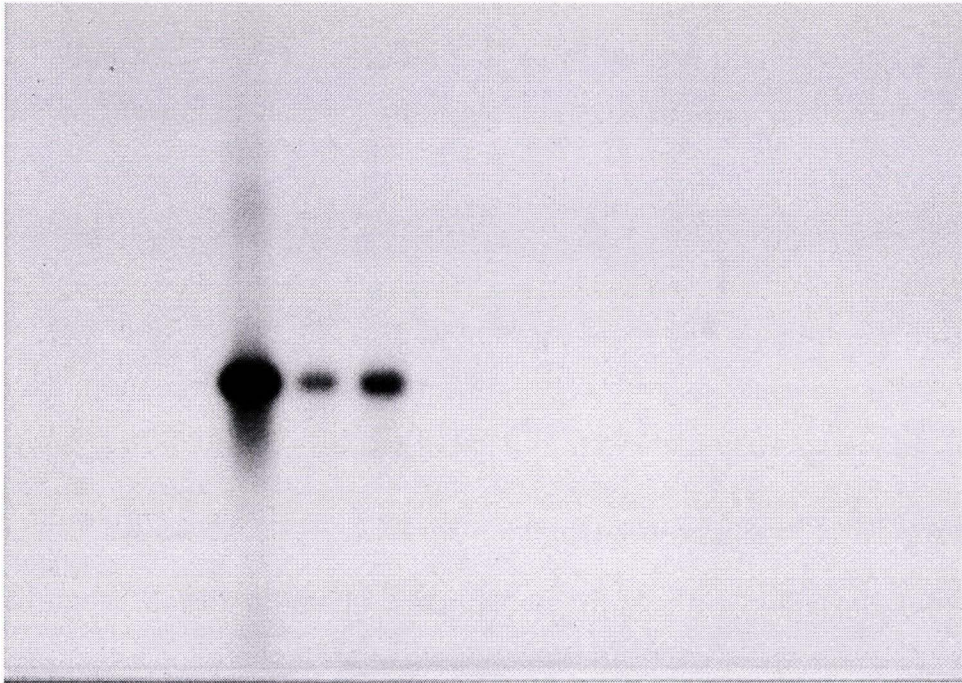


Fig. 5.9. Radiographic film after exposure to a radioactively probed Southern blot of genomic *O. circumcincta* DNA for 3 days. From left to right: 5 μ g of undigested, 5 μ g of EcoRI digested DNA, 5 μ g of HindIII digested genomic worm DNA; 10ng, 1ng and 10ng of vacA PCR product.

0.7% agarose gel. Only DNA preparations free from RNA were run together with the *vacA* PCR products on the overnight gel for the Southern blot. The radiographic film to which the blot had been exposed to for 3 days did not show any signal for the lanes containing worm DNA of different concentrations. Yet, all three concentrations of the PCR product resulted in three clearly distinct signals (Figure 5.9).

5.4 Discussion

Experiments were undertaken to investigate the existence and the character of a potential vacuolation factor secreted/excreted by *O. circumcincta* and *H. contortus*. The close similarity of histopathological changes observed in atrophic gastritis caused by *H. pylori* in humans and abomasal parasitism in sheep was a further incentive to look at a molecular level for a possible expression of *vacA* within *O. circumcincta*.

5.4.1 Effect of Mixing Media

In these experiments, there was a previously unreported effect of mixing media when using the NR uptake assay. In order to follow the method employed by Cover *et al.* (1991), RPMI, which was used to incubate worms, was mixed with CEM. The authors of this paper had experienced no effect on NR uptake when broth culture was mixed with complete essential medium (CEM). In the present study, there were unexpected influences of mixing media which made the development of control curves necessary in order to exclude any likely false positive results (Figure 5.7).

There could be a variety of reasons for this effect. The RPMI used here contained L-glutamine, which degrades in solution within days (Schneider *et al.*, 1996) and creates ammonia. As reported in Section 5.3.1.1, maximal amounts of ammonia measured in RPMI were $2.4\mu\text{M}$. As previously discussed, this is believed to be too low to affect NR uptake. Other possible factors include ion changes, especially those in Ca^{2+} and PO_4 following mixing of both media, which could possibly affect the buffer capacity. This may lead to under- or over-estimation of the effects of ES.

5.4.2 Is the Vacuolating Factor Ammonia?

Ammonia has been shown to be a component of the ES of nematode parasites (Merkelbach *et al.*, 2002), to induce vacuoles in epithelial cells (Hagen *et al.*, 1997) as well as to inhibit acid secretion secretion *in vitro* in concentrations of 1mM to 10mM (Hagen *et al.*, 2000; Merkelbach *et al.*, 2002). Therefore, first steps taken were to look into the possibility of ammonia as a potential vacuolation factor for parietal cells.

Ammonia also plays an important role in vacuolation caused by *H. pylori vacA*. Urease produced by *H. pylori* increases ammonia levels (Megraud *et al.*, 1992), which in turn augments the vacuolating activity of *H. pylori* derived *vacA* (Cover *et al.*, 1991). The mechanism by which ammonia causes vacuolation is based on the fact that ammonia is membrane permeable, while ammonium is not. Ammonia enters acidic cell organelles such as lysosomes/endosome, if intraorganelle pH (pH_{in}) is lower than the cytoplasmic pH (pH_{out}). Within cell organelles, ammonia is protonated to ammonium and trapped, whereas water is osmotically driven into the organelle, which then swells up forming a vacuole.

Vacuolation in the context of *H. pylori* vacuolation is commonly assessed by NR uptake into affected cells, though originally it was developed as a cell viability test. NR uptake as a quantitative measure for ammonia and *H. pylori* dependent vacuolation of lysosomes/endosomes is a well-established assay (Cover *et al.*, 1991). It works on a similar basis as ammonia, since unprotonated NR is preferably taken up into cellular lysosomes/endosomes, if their interior is acidic relative to the cytosol ($pH_{in} < pH_{out}$). Lysosomes/endosomes swollen through ammonia uptake have a greater volume and a relatively low pH_{in} , which causes a greater influx of NR.

Since ammonia causes reliably vacuolation ammonium chloride (NH_4Cl), as a source of ammonia, was employed as positive control throughout the whole experiment. Despite a very consistent performance on HeLa cells over time (Figure 5.1a), which verifies the method applied, explanation is needed.

The negative uptake in HeLa cells when exposed to concentrations of NH_4Cl ($[NH_4Cl]$) below 6.25mM was not surprising, since many cell lines are known to

be less viable and inhibited in their growth during exposure to low concentrations of ammonium/ammonia around 4 to 7mM (Schneider *et al.*, 1996). It is also possible that cell death, prior to the appearance of vacuolation induced by ammonia, occurred (Hagen *et al.*, 1997). However, application of $[\text{NH}_4\text{Cl}] > 6.25\text{mM}$ made enough NH_3 available to overcome losses based on susceptible cells by vacuolating the more resistant cells. Yet, even at pH 7.4, membrane permeable ammonia is present excluding the necessity to change pH_{out} to an alkaline level in order to overcome inhibition of low ammonium/ammonia concentrations.

In order to eliminate ammonia originating from ES products as a likely vacuolation factor, ammonia levels of several parasite incubations (Section 5.3.1.1) were determined. The highest concentration observed derived from a 168h incubation of *H. contortus* L₃ and reached $29\mu\text{M}$. When compared with Figure 5.1, it seems doubtful that such a concentration had any vacuolating effect. Together with earlier reported studies, when much higher levels were effective (Cover *et al.*, 1991), ammonia of parasite origin appears to be a unlikely source of the vacuolation seen here in HeLa cells.

However, larval ES products did not interfere with the ability of NH_4Cl to increase NR uptake, though an identical $[\text{NH}_4\text{Cl}]$ was slightly influenced in its activity by differently diluted ES products. Inhibition of NR uptake or loss/death of HeLa cells could have been responsible, as it was observed in cells incubated in the presence of ES products alone (Figure 5.1b). On the other hand, increasing amounts of NH_4Cl added to adult ES had hardly any effect on NR uptake. There are two hypotheses to explain how adult ES could have influenced NR uptake: inhibition or the death of cells. Inhibition implies that adult ES is capable of blocking the pathway of vacuole generation by ammonia, thus adult ES also occupies acidic cell organelles, changing organelle pH_{in} to alkaline levels and therefore preventing an active entry of ammonia. Since the ammonia can only enter cell organelles against a gradient with the help of the vacuolar ATPase pump (Papini *et al.*, 1993), which transports weak bases, adult ES must inhibit this pump as well. An alternative hypothesis would be for component(s) of adult ES to enter cell organelles driven by vacuolar ATPase(s)

and to exhaust ATP. Interestingly, experiments on epithelial cells exposed to *H. pylori* cytotoxin discovered that vacA made use of the vacuolar ATPase, when the addition of bafilomycin A, a specific vacuolar ATPase inhibitor, prevented the creation of vacuoles (Papini *et al.*, 1993).

5.4.3 Characteristics of ES Products

The discovery of an obvious difference between adult and L₃ ES, with adult ES being more potent than L₃ ES, confirmed data obtained in the ammonia experiments. Besides, in the light of histological observations discussed in Chapter 3, adult worms appear to be more pathogenic, though vacuoles had mainly been observed in the presence of late L₄/immature adults (IA). Yet, following adult transfections vacuolation of parietal cells could be observed, though in smaller numbers. Thus it is possible vacuolation *in vivo* appears later or ES of L₄/IA origin does not differ in its composition from that of adult ES. Still, parietal cells around glands harbouring L₃ showed signs of vacuolation, which is in contrast to the weaker effect monitored *in vitro*. A gastric gland represents a very confined space with a calculated volume of 700pl for the average gastric gland (Kurbel *et al.*, 2001), therefore L₃ ES might be more powerful *in vitro*. After all, conditions and media used do not provide a natural environment for the parasites, which most likely interfered with their ability to secrete/excrete. Furthermore, ES products of one species might not have only one effect, as shown by a study by Huby *et al.* (1995). After incubation of adult worms in a concentration of 200/ml for 24h in HEPES buffer, *O. circumcincta* ES was able to enhance the growth of HT29-D4 cells following incubation for 72 hours. Thus, different incubation conditions most likely lead to a great variety of components within the ES, which additionally could adversely affect results.

A distinctive dependency on time and density on the outcome of adult as well as L₃ ES was noted, possibly a sign that parasites needed time to adjust their metabolism to culture conditions. A similar phenomenon has been reported in respect of *H. contortus* L₃, which were shown to secrete significant amounts of protein not unless incubated for 72h or 96h (Gamble & Mansfield, 1996). In this study a secreting activity was tied to the development of L₃ stage to the

feeding L₄ stage. Yet, in the present experiment vacuolating activity appeared to be independent of the developmental stage, though the strength did vary between L₃ and adult stages. However, protein concentrations in the present experiment did depend on time and density, too, although protein concentrations determined were much lower than those recorded by Gamble & Mansfield (1996). In comparison, actively vacuolating concentrations of the protein *vacA* of *H. pylori* were many times higher than those recovered here (Cover *et al.*, 1991; Harris *et al.*, 1996). However, a protein-like character for the vacuolating ES factor could not be excluded, because ES proteins could display high potency. Whereby great sensitivity to temperature and length of storage characterised by great loss of potency argued, together with previously discussed arguments, against ammonia as a possible candidate. Ammonia would be expected to stay in solution for longer periods of time than recorded here. Nevertheless, a supportive role for ammonia cannot be ruled out.

Adult *H. contortus* ES caused higher NR uptake than adult *O. circumcincta* ES. The reasons behind this phenomenon are unknown. On a speculative basis, *H. contortus* are known to penetrate the gastric epithelium in order to feed on blood. It is possible that ES products also contain more powerful compounds which cause vacuolation of parietal cells in order to ensure hypoacidity.

Chapter 6

General discussion

The objective of the work contained in this thesis was to study the interaction between the parasite and the host mucosa both *in vivo* and *in vitro*, in order to learn more about the mechanisms underlying abomasal hypoacidity in the parasitised host. Important aspects of this interaction are the fate of the parietal cells themselves and the relative importances of host inflammatory responses and ES in causing gastric hypoacidity. One key question is whether the parasites directly target parietal cells or whether the inflammatory response that inevitably accompanies their presence promotes the pathophysiological changes.

Four different approaches have been employed to better elucidate the mechanisms of infection: an *in vivo* model utilising sheep of different genetic backgrounds; histological studies of tissues derived from randomly bred sheep, as well as the selected sheep; *in vitro* investigations of the effects of ES on cultured epithelial cells and finally probing the parasite genome for a gene sequence homologous to a known bacterial vacuolating agent.

The *in vivo* model, comparing the responses of sheep of different genetic backgrounds to infection with abomasal parasites, investigated the relationship between breeding, inflammation and abomasal acid secretion. HFW sheep, which had been selected for higher greasy fleece weight, and their controls, had already been shown to differ in FEC following exposure to nematodes and could therefore be considered resilient. Prolonged inbreeding had also resulted in differences in a variety of biochemical parameters, which might themselves influence the pathophysiological consequences of abomasal parasitism. By carrying out the infections in indoor experiments the impact of some of the many factors that cannot adequately be controlled in field experiments, was reduced, allowing the influence of genetics to be more readily distinguished.

The results left little doubt that the HFW sheep show characteristics typical of resilience. The difference between these animals and the non-selected controls was most likely due to a less pronounced inflammatory/immune response. The delayed increase in abomasal pH at 6.5 months-of-age, but not at 4.5 months-of-age, would be consistent with an underlying immunological basis for the physiological effects of the parasites if the immune system in lambs is more fully mature around the age of 6 months, and thus the expression of resilience in physiological terms (abomasal secretion) is more likely to develop in animals with a more developed immune response.

Work with the HFW animals (and larvae- or adult-infected outbred sheep) also implied an inhibitory or cytotoxic effect of inflammation on parietal cells.

Delayed larval development, or extension of the time larvae reside within the glands, were both possible features of infection in the HFW animals. However, delayed development was not supported by the finding of eggs in the faeces of HFW lambs at the normal time, i.e. the pre-patent period was unaffected. Alterations in the lifecycle of the parasites are of particular interest in the light of the results in Chapter 3, in which the presence of larvae and adult stages in the gastric lumen, as opposed to worm stages confined to glands, invariably coincided with the beginning of abomasal dysfunction. In contrast, L₃ administered as a challenge infection seemed to have a more immediate inhibitory effect on parietal cells.

Examination of tissues derived from randomly bred sheep, which had been sacrificed on selected days following larval inoculation or adult transfer, allowed a detailed study of the development and time course of hypoacidity. In addition, the relationship between inflammation, the parasite and the parietal cell was again closely scrutinised. Findings demonstrated a loss of parietal cells, with the lowest number of TGF- α positive parietal cells occurring in sheep sacrificed 10 days after infection and coinciding with the peak of both hypoacidity and tissue eosinophilia, as well as the expected presence of late larval and adult stages within the gastric lumen. Later in the infection, resolution of the inflammation was accompanied by greater abomasal acidity and a recovery in the numbers of TGF- α -positive parietal cells. In summary,

the increase and decrease of abomasal pH was very much associated with the increase and decrease in mucosal eosinophils, while the time course of the disappearance and re-appearance of TGF- α positive parietal cells roughly matched both changes in pH and inflammation.

Although these experiments did not allow for a definitive answer concerning the mechanism responsible for the diminution in parietal cells, similarities to other gastropathies (Yanusaga *et al*, 1997; Murayama *et al.*, 1999) suggest that inflammation may be the most significant driving force behind the changes observed during abomasal parasitism.

A feature which was repeatedly observed across many experiments was the presence of vacuolated parietal cells. In the larval infection experiment, vacuolated parietal cells were detected by Day 5 p.i. and became more pronounced by Day 10. The discovery of vacuolated parietal cells in Experiment 2 and in the LI and AT experiments, as well as in the 30 hour study, suggests the existence of a vacuolating factor of parasite origin, since evidence of vacuolation being caused by inflammation has not so far been provided.

In vitro experiments on HeLa cells were intended to identify and characterise the putative vacuolating factor released by nematodes. Vacuolating activity in ES was confirmed, though its potency varied considerably between incubations and depended on parasite stage as well as on the incubation density of those stages and the length of the incubation. The experiments excluded ammonia as a sole cause, since ammonia concentrations in parasite incubates never rose above 29 μ M, a concentration considered too low to cause vacuolation. Yet, a supportive role of ammonia remains possible. Given the features of vacuolation in the parietal cells, and the HeLa cell findings, a similarity of effect to *H. pylori vacA* did not seem unlikely.

In order to see if any similarities existed on a molecular level between *H. pylori vacA* and the parasite-derived vacuolating factor, the nematode genome was probed. No corresponding genetic sequence was detected, but using the current approach, any homology of genes cannot be excluded, as only two out of many possible *vacA* primers had been chosen.

Future work:

Further experiments involving pasture-raised HFW sheep and their controls similar to those reported in this thesis would be desirable. Of key interest is testing the hypothesis of reduced inflammatory changes as a mechanism of resilience to the chronic infections that occur in animals at pasture and also examining the pathology of infection in the intestines as well as the abomasum. It would be highly desirable to repeat the cytokine assays on larger numbers of animals and also to extend the analysis to include cytokines such as IL-1 β . Unfortunately, the poor breeding record of the HFW flock may not allow many more studies to be conducted.

To better explore the participation and contribution of inflammation to parietal cell changes, studies using immunosuppressed animals offer one possible alternative. The use of agents that block eosinophil recruitment or production would be highly desirable.

It is more difficult to recommend appropriate future studies of the vacuolating factor. Because of the many difficulties encountered, a complete change of test system may be necessary. It may, for instance, be desirable to utilise a visual assessment of vacuole formation rather than rely on indirect evidence as furnished in the NR uptake studies. In addition, finding the ideal incubation conditions for parasites will be essential. To explore the similarity of the parasite-derived factor to *H. pylori vacA*, ES could be tested in the presence of bafilomycin, which is an important inhibitor of ammonia and vacA derived vacuolation.

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Appendix 1: Parasitology

1.1 Larval Culture

Infective L3 were regularly passaged through parasite-free sheep to maintain cultures of pure strains of *H. contortus* and *O. circumcincta*. Existing worm burdens were removed from donor sheep by drenching with a double dose of Leviben (8mg/kg ricobendazole + 15mg/kg levamisole; Young's Animal Health Ltd., N.Z.) and ivermectin (Ivomec, Merial, Auckland, NZ; 7ml). Dexamethazone (Dex 5, Virbac NZ, 0.15mg/kg) was injected i.m. twice weekly. After faecal floats were negative, the sheep were infected intraruminally via stomach tube with L₃.

Faeces collected in faecal bags were mixed with vermiculite, moistened and incubated in a 22-24°C room for 10days for *H. contortus* and 12 days for *O. circumcincta*. The mixture was moistened when necessary and turned over in the trays twice during this time. L₃ were recovered by a Baermann technique. The faeces mixture was placed in sieves lined with tissue paper in bowls of RO water and left for 24h. The water containing the larvae was then placed in a funnel lined with a single layer of tissue for a further 24h. *H. contortus* were stored at 10°C, and *O. circumcincta* at 4°C, in RO water.

To exsheathe L₃, they were incubated in 0.2% sodium hypochlorite at 37°C for 15 min, filtered, washed and resuspended in the incubation medium.

1.2 Faecal Egg Counts

The method was the modified McMaster method of Stafford *et al.* (1994). 2g of faeces were passed through a small sieve into 30ml of saturated NaCl solution. Aliquots of the resulting suspension were transferred with a Pasteur pipette into

the chambers of a McMaster slide. The suspension was continuously stirred while aliquots were being removed to assure an even distribution of eggs. After 2 min, eggs were counted under a microscope. Each egg represented 50 e.p.g.

1.3 Total Worm Counts

Half the abomasum was placed in a beaker containing 300ml of deionised water, 2g of pepsin (BDH Laboratory Supplies, Poole, England) and 10ml of concentrated HCl and maintained at 37°C in a water bath for 2h. The digestion liquid was then washed through a 53 μm mesh and 10% kept for counting worms. All worms were identified as L₃, L₄, immature adults (L₅) and mature female and male worms and recorded separately. The total number of eggs *in utero* were counted in each of 50 female worms.

1.4 Recovery of Adult Worms

Ligated abomasa were removed from donor animals immediately after death, opened and the contents collected. The luminal surface of the mucosa was washed with warm 0.9% NaCl. The washings were mixed with the abomasal contents and the worms were allowed to settle in a large measuring cylinder in a waterbath at 37°C. The volume in the cylinder was reduced by aspirating fluid at the surface fluid from which worms had sedimented.

A solution of 3% agar (Bacto Agar, DIFCO Laboratories, USA) was heated in a microwave oven and allowed to cool. When it had cooled to 40-50°C, it was rapidly mixed with twice its volume of worm mixture (final concentration 1% agar) and immediately poured into trays to set. Saline (0.9%) warmed to 37°C was carefully poured over the agar blocks until they were completely covered. The agar blocks were placed in large trays of saline at 37°C. The worms migrated out of the agar blocks into the saline and formed clumps. About 90% of those worms that migrated from the agar had done so in the first two hours. Clumps of worms were picked out of the saline for incubation to generate ES.

Appendix 2: Assays

2.1 Ammonia Assay

Assay solutions:

0.1mM NH₄Cl (positive control)

Milli Q H₂O (negative control)

Reagent A (kept at 4°C) 0.5M phenol and 0.4mM sodium nitroferricyanide

Reagent B (to be kept at 4°C) 0.67M NaOH and 0.45mM sodium hypochlorite in Milli Q H₂O

Method:

Set up in duplicate in 5ml plastic test tubes:

Negative control	0.5ml MQ H ₂ O
Positive control	0.5ml 0.1mM NH ₄ Cl
Standard solutions	1mM to 0.001mM NH ₄ Cl in Milli Q H ₂ O
Sample	0.5ml sample

To each tube, add 100μl of reagent A and briefly vortex the tubes.

Then add 100μl of reagent B and again briefly vortex the tubes.

Place the tubes on a 50°C heating block for at least 30min.

Remove the tubes and cool to room temperature.

Read the absorbance at 635nm against the negative control.

Construct a standard curve to obtain ammonia concentrations.

2.2 Protein Microassay

Set up in duplicate in 5ml plastic test tubes:

Negative control	0.2ml RPMI 1640
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Standard solutions	5 μ g to 100 μ g BSA in RPMI 1640
Sample	0.2ml sample

Add 0.8ml of Bradford reagent (Sigma Chemicals, St. Louis, USA) to each tube, mix and leave for 10min.

Read the absorbance at 595nm against the negative control.

Construct a standard curve to obtain protein concentrations.

2.3 Pepsinogen Assay

The method is a modification of the method of Berghen *et al.* (1987).

Assay solutions:

Assay buffer Two volumes of stock solution A containing 0.1M glycine (Sigma Chemicals Co, St.Louis, MO, USA) and 0.1M NaCl (BDH Laboratory Supplies, Poole, UK) were mixed with one volume of a stock solution B containing 0.27N HCl (BDH Laboratory Supplies, Poole, UK)

Substrate solution Slowly dissolve BSA (Fraktion V, Boehringer Mannheim GmBH, Germany) in a concentration of 2g/100ml in assay buffer at room temperature 1h before use. Immediately before use, filter through glass wool (BDH Laboratory Supplies, Poole, England).

Tyrosine standards 0.1, 0.2 and 0.3 μ mol/ml tyrosine in distilled water, prepared from a stock solution (0.01M L-tyrosine in 0.1N HCl) and stored in 1.5ml aliquots at -20°C.

Method:

Set up in microcentrifuge tubes:

Negative control	0.2ml distilled water (one sample after each 16 samples)
Serum	duplicate 0.2ml serum samples

To each tube add 0.5ml of substrate solution and briefly vortex.

Add 0.3ml of the serum-substrate solution to 0.5ml of trichloroacetic acid (4g/100ml) (Sigma Chemicals Co, St.Louis, USA) in a microcentrifuge tube and centrifuge at 10,000 r.p.m. for 5min.

Place 0.2 ml of the resulting supernatant in a 5 ml disposable plastic tube and keep a 4°C overnight.

Incubate the tube with the remaining 0.3ml in a 37°C water bath for 24h.

Tyrosine assay:

After the 24h incubation, 0.2ml of incubated samples, non-incubated samples and each of the tyrosine standards are assayed similarly.

To each tube add 2ml of a 0.25N NaOH (BDH Laboratory Supplies, Poole, England)

Then add 0.3ml of a diluted Folin & Ciocalteu's reagent (1:3 v/v) (Sigma Chemical Co, St. Louis, USA)

Leave the tubes at room temperature for 30min for the colour to develop, then read the absorbance at 680nm against the negative controls.

Calculate pepsinogen activity in I.U ($\mu\text{mol tyrosine released/l/min}$):

$$\text{I.U} = (\text{OD}_{\text{incubated sample}} - \text{OD}_{\text{non-incubated sample}}) \times F \times 11.11$$

$$\text{Where } F = \left\{ \frac{0.1}{(\text{OD}_{\text{tyr } 0.1\mu\text{Mml}^{-1}})} + \frac{0.2}{(\text{OD}_{\text{tyr } 0.2\mu\text{M ml}^{-1}})} + \frac{0.3}{(\text{OD}_{\text{tyr } 0.3\mu\text{M ml}^{-1}})} \right\}$$

2.4 Gastrin Radioimmunoassays

2.4.1 Primary Ab 74

The method is that of Simpson *et al.* (1993), a modification of the method of Hansky & Cain (1969).

Assay Buffer: 0.02M Veronal buffer pH 8.6 containing per litre:

4.12g Na barbiturate

0.744 g barbitone

5g BSA (Boehringer Mannheim, Fraktion V)

100mg thiomerosal (ethylmercuri-thiosalicyclic acid, sodium salt; Acros organics, NJ, USA)

10mg neomycin (neomycin sulphate; Sigma, St. Louis, USA)

Tracer: Synthetic non-sulphated human G17 (Research Plus, Bayanne N.J. U.S.A.) was labelled with I^{125} using the chloramine T method. The label was purified on a Sephadex G10 column followed by separation on a Diethylaminoethyl cellulose column with a NaCl gradient from 0-1M. The tracer for the assay contained 1200-1600 c.p.m. per 500 μ l.

Antiserum: Ab74 (the kind gift of Dr J. Hansky, Monash University, Melbourne, Australia) which binds equally with human, porcine and ovine sulphated and non-sulphated G14, G17 and G34 was used in a final dilution of 1:100,000 with 1:400 normal rabbit serum (NRS). The assay solution contained 1:250 NRS and 1:40000 Ab.

Standards: Synthetic non-sulphated human G17 was made up in assay buffer in concentrations of 0, 2, 5, 10, 20, 50, 100 and 200pM.

Second antibody: Serum from sheep immunized with rabbit gamma globulin was used as the precipitating antibody. This was standardised against Donkey anti-rabbit gamma globulin (IDS, UK).

Method:

Assays were conducted in triplicate. Assay tubes contained:

Total counts	500 μ l tracer
Non Specific Binding	100 μ l buffer, 400 μ l NRS without Ab, 500 μ l tracer
Standards	100 μ l standard solution, 400 μ l Ab, 500 μ l tracer
Samples	100 μ l sample, 400 μ l Ab, 500 μ l tracer

Incubate all tubes for 2 days at 4°C.

Then add 200 μ l second Ab to all tubes except the total counts and incubate for a further 3 days at 4°C

Centrifuge at 2000g for 30min, discard the supernatant and count the pellet for 5min in a gamma counter.

Gastrin concentrations (pM) are determined from the standard curve.

2.4.2 Primary Antibody 1296

The method is that of Ciccotosto & Shulkes (1992).

Assay Buffer: 0.02M Veronal buffer pH 8.7 containing per litre:

4.12 g Na barbiturate

0.744 g barbitone

0.01% sodium azide (Sigma Chemicals Co., St.Louis, USA)

2% charcoal stripped plasma

Tracer: Synthetic non-sulphated human G-17 (Research Plus, Bayonne N.J. U.S.A.) was labelled with I^{125} using the chloramine T method. The label was purified on a Sephadex G10 column followed by separation on a Diethylaminoethyl cellulose column with a NaCl gradient from 0 to 1 M. The tracer for the assay contained 1100 – 2000 c.p.m. per 500 μ l.

Antiserum: Ab 1296 (the kind gift of Dr J.H. Walsh, CURE, UCLA, Los Angeles, USA) which binds equally with human and ovine sulphated and non-sulphated G14, G17 and G34 was used in a final dilution of 1:40.000.

Standards: Synthetic non-sulphated human G-17 was made up in assay buffer in concentrations of 0, 2, 5 10, 20, 50, 100 and 200 pM.

Charcoal: 2.5% charcoal (activated charcoal; Sigma Chemicals Co., St. Louis, USA) in 0.02M Veronal buffer with 1% BSA.

Method:

Assays were conducted in triplicate. Assay tubes contained:

Blank	900 μ l assay buffer, 100 μ l tracer
Reference	800 μ l assay buffer, 100 μ l Ab, 100 μ l tracer.
Standards	800 μ l standard solution, 100 μ l Ab, 100 μ l tracer
Samples	800 μ l sample, 100 μ l Ab, 100 μ l tracer

Incubate all tubes for 2 days at 4°C.

Separate free and bound gastrin by adding to each tube 1ml charcoal solution in a 4°C room and vortexing.

Immediately, centrifuge at 800g for 30min at 4°C, discard the supernatant and count the pellet for 5 min in a gamma counter.

Gastrin concentrations (pM) are determined from the standard curve.

2.5 Serum Antibody Titres

Assays were carried out with the assistance of Mr. Richard Green in the laboratories of AgResearch Ltd, Wallaceville, NZ. Immunoglobulin isotypes and a total titre of immunoglobulins specific for ES antigens of either exsheathed L₃ or adult *O. circumcincta* worms were determined in serum samples using an ELISA method similar to that of Douch *et al.* (1994).

2.5.1 Antigen Preparation

Infective L₃ were exsheathed in sodium hypochlorite solution (0.025% available chlorine) and washed extensively with deionised water on an 8.0 μ m cellulose nitrate filter (Millipore, Bedford, USA) immediately before use. Adult *O. circumcincta* were obtained from donor sheep. The exsheathed L₃ or adult worms were then incubated in PBS at 37°C. The PBS was recovered by centrifugation and the resulting supernatant containing ES products was concentrated over a molecular filter with a 10K-cut off size (Amicon PM10 membrane, Amicon Corp., USA) using a stirred cell unit. The concentrate was divided into aliquots and frozen at -20°C until use.

2.5.2 ELISA Method

ELISA plates were coated with ES antigen containing 200ng protein, determined on the basis of absorbance at 230/260nm (Kalb & Benlohr, 1977), in 100 μ l of coating buffer. Plates were then incubated at 37°C for 2h and afterwards washed 3 times in deionised distilled water containing a 0.1% Tween 20 detergent washing solution. The plates were blotted with "Blotto" [10 mM phosphate buffer at pH 7.2 containing Tween 20 and 5% bovine skim milk powder (purchased at a local retail outlet)] and washed 6 times in the same washing solution.

Sheep serum was diluted 1/2000 with ELISA buffer [10mM phosphate buffered (0.15M) saline (PBS), pH 7.2 containing 0.5% Tween 20]. 100 μ l of diluted serum was added to ELISA plate wells in triplicate and incubated at 37°C for

1h. After incubation plates were washed 6 times in the above mentioned washing solution. Rabbit immunoglobulins to sheep immunoglobulins conjugated with horse radish peroxidase (Dako P163, Dakopatts A.S., Glostrup, Denmark) diluted 1/1000 with ELISA buffer was added to each well (100 μ l) and incubated at 37°C for 2h. A further washing step, identical to the washing steps before, followed. 100 μ l of *o*-phenylene diamine di-HCl (OPD, at 0.5mg/ml) and H₂O₂ (30%, 1 μ l/ml) in 100mM citric acid/Na₂HPO₄ buffer was added to each well and plates were incubated at room temperature for 10min. This reaction was stopped by addition of 50 μ l of 1M H₂SO₄ solution. The resulting colour from this reaction was measured in an ELISA plate reader (Biotech EL312 or Dynatech MR5000 at 490/630nm). Results, expressed as absorbance units, were means of triplicate assays.

2.5.2 Immunoglobulin Isotypes

Immunoglobulin isotypes were determined using an ELISA and monoclonal antibodies against sheep IgG1, IgG2, IgA and IgM (the kind gift of Dr. Ken Beh, CSIRO, McMaster Laboratory, Australia) (Beh, 1987, 1988).

Plates were coated with ES antigen, blotted and test serum diluted 1/20 (IgA, IgG2), 1/2000 and 1/100 (IgG1, IgM) with ELISA buffer was added as before described. After incubation plates were washed 6 times in washing solution made up as previously described. Monoclonal antibody against specific immunoglobulin isotype, diluted in ELISA buffer, was added (100 μ l per well) and incubated at 37°C for a further 1h. Another washing step (6 times) in washing solution followed.

Horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako P260, Dakopatts, A.S., Glostrup, Denmark) were diluted 1/1000 in ELISA buffer and 100 μ l added to each well and incubated at 37°C for 1h. The plates were then washed 6 times in the washing solution. The enzyme reaction was carried out and colour developed and measured as previously described.

2.6 Abomasal Lymph Node Cytokine Profiles

These analyses were conducted by Dr A.Pernthaner in the laboratories of AgResearch Ltd., Wallaceville, NZ.

2.6.1 Cell culture

Mesenteric lymph nodes were removed and stored in sterile normal saline. Single cell suspensions of each lymph node were prepared by slicing comparable portions of each node and teasing cells from the pulp using a pair of forceps. Cells obtained from lymph nodes were washed twice in RPMI 1640 (Gibco BRL, Life Technologies Inc., USA) and adjusted to concentration of 1×10^6 cells/ml in RPMI-1640 plus L-glutamine (Invitrogen, Auckland, NZ), supplemented with 0.2% sodium bicarbonate (BDH Laboratory Supplies, Poole, UK), 50 mM mercaptoethanol, 100 U/ml penicillin, 100 μ g/ml streptomycin (all Sigma Chemicals Co, St.Louis, USA,), and 5% FCS (Invitrogen, Auckland, NZ). Viability was always higher than 95% as determined by trypan blue dye exclusion.

Approximately 2×10^6 lymphocytes were cultured per well in the presence of 10 μ g/ml *O. circumcincta* larval excretory/secretory antigen (OcL3ESag), or with 5 μ g/ml concavalin A (Con A, Sigma Chemicals Co, St.Louis, USA). OcL3ESag was prepared as described as in 2.5.1. Cultures were incubated for 96 hours.

2.6.2 RNA extraction and reverse transcription

Total RNA was isolated from cells using TRI REAGENT[®]LS (Molecular Research Center, Inc., Cincinnati, USA) that combines phenol and guanidine thiocyanate to facilitate cell lysis and inhibition of RNAase activity according to the manufacturer's protocol. The lysate was separated into aqueous and organic phases by bromochloropropane addition and centrifugation. RNA was precipitated from the aqueous phase by the addition of isopropanol and the RNA pellet was washed in ethanol and solubilized. Total RNA yield was calculated based on the spectrophotometric measurement of absorption at

260/280nm. First-strand cDNA synthesis was performed using SuperScript™II RNase H⁻ reverse transcriptase (Invitrogen, Auckland, NZ) and poly(A) oligo(dT)₂₅ primer to reverse transcribe up to 5 μg of total RNA according to the manufacturer's protocol, and adjusted to an initial concentration of 20 ng/ml RNA. cDNA was stored at -20 °C until used for real time PCR.

2.6.3 Primer design

Primers were designed by Dr A.Pernthaner and can be viewed after publication in a scientific journal (manuscript in preparation for submission to *The Journal of Immunological Methods*).

2.6.4 Real time PCR and quantification of gene expression

Cytokine gene expression was detected by real time PCR using a GeneAmp™ 5700 sequence detection system in combination with sequence detector software (Applied Biosystems, Scoresby, AUS). All reactions were assembled in 25 μl in duplicates in optical 96-well reaction plates using AmpErase™ uracil N-glycosylase as a carry over prevention, SYBR®Green PCR master mix (Applied Biosystems, Scoresby, AUS) containing SYBR®Green 1 dye, AmpliTaq Gold™ and dNTPs with dUTP in optimised buffer components, 0.2 μM of each gene specific primer and 1 μl of cDNA template. For each gene of interest and on each plate no template and positive controls were included. 40 cycles of amplification with denaturation at 95 °C for 15 sec followed by annealing and extension at 60 °C for 1 min were performed after an initial incubation at 50 °C for 2 min followed by incubation at 95 °C for 10 min. For each sample a melting curve was generated after completion of amplification and analysed in comparison to the positive and negative controls.

The comparative C_T method was employed for relative quantification where the amount of target is normalized to an endogenous reference (housekeeping gene) (User Bulletin #2: ABI PRISM 7700 Sequence Detection System; Applied Biosystems, Scoresby, AUS). The threshold cycle (C_T) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold, and ΔC_T represents the difference in threshold cycles for the target and house keeping gene. A validation experiment that demonstrated the approximately

equal amplification efficiency for the target and housekeeping gene was conducted prior commencement of the experiment.

Appendix 3: Cell Culture

3.1 Culture Conditions

All chemicals and equipment were either purchased sterile or sterilised by autoclaving or passing through a sterile 0.2 μ m filter (Acrocip, Gelman Sciences, Ann Arbor, MI, USA). Cells were handled in a laminar flowhood using sterile technique and all solutions were pre-warmed in a 37°C-waterbath.

HeLa cells were grown in non-pyrogenic 75cm² cell culture flasks (with either a ventilation cap fitted with a 0.2 μ m filter [Corning Inc., Corning, NY 14831, USA] or with a ventilated cap [Nalge Nunc International, Rochester, NY, USA]) in a CO₂-incubator (Contherm, Lower Hutt, NZ) in an atmosphere containing 5% CO₂ and 95% humidified air at 37°C.

3.2 Culture Medium

The cell culture medium was complete essential medium (CEM) consisting of minimum essential medium based on Eagle's Medium without L-glutamine (MEM 10x) diluted in MilliQ-water and supplemented with fetal bovine serum (FBS) (Invitrogen, Auckland, NZ), a Penicillin-Streptomycin-Neomycin mixture (Penicillin-Streptomycin-Neomycin (PSN) antibiotic mixture 100x), non-essential amino acids (MEM Non-essential Amino Acids solution 100x, all Gibco BRL, Life Technologies Inc., USA) and 0.027M NaHCO₃ (BDH Laboratory Supplies, Poole, UK).

MilliQ-water in 75ml aliquots in 250ml glass bottles was autoclaved in batches of four twice weekly and stored at room temperature. MEM, non-essential amino acids solution and 0.9M NaHCO₃ were stored protected from light at 4°C, while FBS on arrival was heat-inactivated for 30 min in a waterbath at 56°C and stored in 10ml aliquots at -20°C. PNS was kept in 1ml aliquots at -20°C. FBS- and PNS-aliquots were thawed as required in a 37°C waterbath.

CEM was made up as required in 100ml volumes and kept at 4°C for a maximum of 7 days. 10ml MEM was added to 75ml MilliQ-water and supplemented with 10ml heat-inactivated FBS, 3ml 0.9M NaHCO₃ (final concentration in 100ml CEM 0.027M), 1ml PSN mixture and 1ml non-essential amino acids solution. The pH of freshly made up CEM was 7.2 - 7.4 and was re-adjusted with 1M HCl, if necessary. Phenol red, commercially added in MEM, acted as a pH-indicator with colour changes from orange-red (pH 7.2 to 7.4) to yellow-orange (pH below optimum of 7.2).

3.3 Passage of HeLa Cells

HeLa cells were independently passaged by trypsinisation as a stock and a working lot. In general, cells of the stock were a maximum of 10 passages behind cells of the working lot. The stock was passaged when the colour of the culture medium changed into a yellow-orange, a sign of consumption of nutrients of a culture medium. Generally, the stock was passaged every 5-6 days, while the working lot was passaged every 48h to ensure young and reactive cells for ES testing. The working lot was continuously passaged until stock and working lot differed by 10 passages. The working lot was then discarded and a new one started using cells of the stock.

All cells were passaged using an antibiotic-trypsin-versene solution (ATV) containing 0.5g/l trypsin (Sigma Chemicals Co, St.Louis, USA), 0.2g/l EDTA (disodium salt), 8g/l NaCl, 0.4g/l KCl, 0.58g/l NaHCO₃ (all BDH Chemicals Ltd., Poole, England), 1g/l dextrose (Riedel-de-Haën, Seelze, Germany) as well as 10ml PSN mixture was sterilised by filtration and kept in 10ml aliquots at -20°C until needed. A PBS solution pH 7.3 consisting of 8g/l NaCl, 0.2g/l KCl, 1.15g/l Na₂HPO₄ and 0.2g/l KH₂PO₄ (all BDH Chemicals Ltd., Poole, UK) was made up in a volume of 500ml and, if necessary, the pH was adjusted with 1M HCl. PBS was stored at 4°C and kept for a maximum of 4 weeks. The ATV-solution and PBS were warmed in a 37°C waterbath prior to use.

A flask was taken out of the incubator and placed under an inverted microscope (Zeiss, Germany) to check cells for confluence and abnormalities.

The flask was then placed in the laminar flowhood and the medium was removed and discarded. PBS in a volume of 10ml was added to the cells and to guarantee an efficient washing step the flask was held in a horizontal position and gently rocked for about 10sec. The PBS was discarded and the washing process repeated. Depending on cell density, 2 - 4ml of ATV were pipetted into the flask and the flask was placed in the incubator for 2min. Gentle tapping of the flask detached the cells visible to the naked eye. CEM was added to the ATV-cell suspension in at least the double amount of ATV previously added, to neutralise the effect of ATV. The suspension was carefully pipetted up and down to break up any existing cell lumps. It was microscopically certified that the cell suspension mainly consisted of single cells. Stock cells were split in a ratio of 1/20 while cells of the working lot were split in a ratio of 1/5. The cell suspension to be kept was either transferred in a new flask or retained in the old flask, whereas the remainder of the cell suspension was discarded. The volume in the flask was then made up to 25ml with CEM and the flask returned to the incubator.

3.4 Seeding 96-well Microtiter Plates

Cells of the working lot were grown for 48h before seeding onto 96-well microtiter plates (Coming Inc., Corning, USA). Generally, the cultures had not reached confluence when trypsinised. Numbers of viable cells were calculated using the trypan blue method. An aliquot of 1ml of the neutralised CEM-ATV-cell suspension was taken and transferred into a microcentrifuge tube, while the flask was put back into the incubator. 100 μ l of cell suspension was gently mixed with 400 μ l of 0.4% trypan blue solution in another microcentrifuge tube using a plastic Pasteur pipette. A Neubauer haemocytometer was filled with the cell-trypan blue suspension and all viable cells (= unstained cells) in each of the five large squares in both chambers were counted. The average was determined and the concentration of viable cells/ml was calculated using following formula (Burlison *et al.*, 1992):

$$\text{Viable cells/ ml} = (\text{viable cell count} / 5) \times (1 / \text{dilution}) \times 10^4$$

To calculate the dilution necessary to obtain a desired volume of cells at a specific concentration of cells/ml, the following formula (Burleson *et al.*, 1992) was used:

$$V_1 \times C_1 = V_2 \times C_2$$

where

V_1 is the volume in ml of the original cell suspension (to be calculated). This is the volume of the original cell suspension that will have to be diluted.

C_1 is the concentration of cells/ml of the original cell suspension

V_2 is the volume in ml of diluted cell suspension desired

C_2 is the concentration of cells/ml desired

So that $V_1 + \text{diluent} = V_2$ at the desired concentration.

Since cells were seeded in a density of 3×10^3 /well and $100\mu\text{l}$ of cell suspension was transferred to each well, a final concentration of 3×10^4 cells/ml was required:

$$V_1 \times C_1 = V_2 \times 3 \times 10^4 \text{ cells/ml}$$

Before seeding onto the plate, the original cell suspension was checked for any existing cell lumps and, if necessary, treated as before. It was then diluted with CEM as calculated, seeded in the wells of a 96 well microtiter plate and incubated for another 24h. The rest of the original cell suspension was passaged as previously described.

3.5 Neutral Red (NR) Uptake Assay

Reagents:

Neutral red solution Neutral Red (Sigma Chemical Co, St. Louis, USA) was made up as a 0.5% NR-stock solution in 0.9% NaCl and was kept at 4°C.

Acidified alcohol 63% ethanol, 23% distilled H₂O and 10% conc HCl (all Sigma Chemical Co, St. Louis,USA)

Method:

The method is based on the method of Cover *et al.* (1996). Test media were assayed in triplicate

HeLa cells, suspended in CEM, were seeded at a concentration of 3000/well and allowed to attach and grow for 24h. CEM was then removed and 100 μ l/well of test media (controls, ES samples or their dilutions) were added. The microtiter plates were replaced in the incubator for 24h.

The supernatant in the wells was discarded and 100 μ l of a freshly prepared 0.05% neutral red (NR)-CEM solution was added to each well. The plate was allowed to incubate at 37°C for 4min before the NR-CEM solution was withdrawn and cells washed twice with 150 μ l of 0.9% NaCl per well. To extract NR from HeLa cells, 100 μ l of acidified alcohol was pipetted into each well and the OD measured at 550nm.