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EFFECTS OF *OSTERTAGIA CIRCUMCINCTA* LARVAE AND ADULT PARASITES ON ABOMASAL AND INTESTINAL TISSUES IN SHEEP

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ABSTRACT

Ostertagia circumcincta parasites infect the abomasum of sheep causing damage to the abomasal tissues and significant production losses to the sheep farming industry. Ingested larvae enter the gastric glands and emerge as adults which live in the abomasal lumen. The effect of adult parasites on the abomasum has not been systematically investigated. In the present study, sheep raised to be free of helminth parasites were given either adult *O. circumcincta* parasites *via* an abomasal cannula or larvae *per os*.

Adult as well as larval *O. circumcincta* parasites stimulate hypergastrinaemia, a decreased abomasal pH and elevated serum pepsinogen concentrations. While the concentration of G cells did not change in the larval parasite infected sheep compared with the non-infected control sheep, the total number of G cells was increased due to an increase in mucosal thickness. There appeared to be fewer G cells present in the adult parasite infected sheep compared with the non-infected control sheep, which was most likely due to a depletion of their gastrin content due to overstimulation. The hypergastrinaemia observed during ostertagiasis is not due to a change in the ratio of G:D cells.

The lumen dwelling adult *O. circumcincta* affect the mucosa of the abomasum resulting in an apparent inflammatory reaction, demonstrated by the presence of eosinophils and neutrophils in the lamina propria. Mucous production and/or secretion is also affected, shown by the presence of large mucus-secreting cells in the mucosa.

The total wet weight of the abomasum/kg body weight is increased in sheep infected with *O. circumcincta*, with an increase in the total size of the abomasum. The larval parasites evoke a hyperplasia in both the antral and body mucosae with little change in cell size. In sheep infected with adult parasites, the thickness of the abomasal mucosa is increased in the body, but not the antrum. This increase is most likely due to hypertrophy.

Either the larval *O. circumcincta* or the hypergastrinaemia have trophic effects on the upper duodenum, with an increased mucosal thickness which did not occur more

distally. This did not occur in the adult parasite infected sheep.

The larval parasites or hypergastrinaemia provoked a hyperplasia in the jejunal mucosa. This did not occur in the adult infected sheep.

The larvae and adult parasites did not appear to exert a hypertrophic or hyperplastic effect on the ileum, caecum or colon.

These results indicate that adult *O. circumcincta* parasites have substantial effects on the ovine abomasum.

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LIST OF ABBREVIATIONS

Abbreviation		
@	=	at
A cell	=	glucagon containing cells
API	=	adult parasite infected
BSA	=	bovine serum albumen
°C	=	degrees Celcius
CCK	=	cholecystokinin
D cell	=	somatostatin containing cell
DAB	=	diaminobenzidine
DNA	=	deoxyribonucleic acid
EC cell	=	enterochromaffin cell
ECL cell	=	enterochromaffin-like cell
EGF	=	epidermal growth factor
ED	=	external diameter
e.p.g.	=	eggs per gramme
g	=	grammes
g	=	gravity
G cell	=	gastrin containing cell
GRP	=	gastrin releasing peptide
H. contortus	=	*Haemonchus contortus*
hr	=	hour
HCl	=	hydrochloric acid
ID	=	internal diameter
IGF	=	insulin growth factor
kg	=	kilogramme
L	=	litre
LPI	=	larval parasite infected
m	=	metres
min	=	minute
mm	=	millimetres
mm^2	=	square millimetres
mmol	=	millimoles
mol	=	moles
mRNA	=	messenger RNA
n	=	number
N	=	normality
Na_2CO_3	=	sodium carbonate
nm	=	nanometres
N.Z.	=	New Zealand
OD	=	optical density
O. circumcincta	=	*Ostertagia circumcincta*
/	=	per
%	=	percent
P	=	probability
PLP	=	phosphate-lysine-periodate
pmol	=	picamoles
RNA	=	ribonucleic acid

rRNA	=	ribosomal RNA
PBS	=	phosphate buffered saline
PLSD	=	probability of least significant difference
tRNA	=	transfer RNA
s.e.	=	standard error
SE	=	secretory-excretory
SOD	=	super oxide dismutase
µg	=	microgrammes
µm	=	micromoles
VFAs	=	volatile fatty acids

ANIMAL ETHICS

The protocols for the experiments described in this thesis have been approved by the Massey University Animal Ethics Committee.

1. LITERATURE REVIEW

1.1. The Ruminant Stomach

The ruminant stomach is divided into four compartments; the reticulum, rumen, omasum and abomasum (Figure 1.1.). The first three form the forestomach, which is the site of microbial fermentation of the complex carbohydrates which form the major part of the ruminant's diet. Approximately 75% of the daily maintenance energy requirement of ruminants is provided by the volatile fatty acids (VFA's) produced by microbial fermentation (Sellers and Stevens, 1966). Fermentation precedes the hydrolytic phase of digestion which occurs in the abomasum and intestines by the action of the animal's own digestive enzymes. The enormous storage capacity of the combined reticulum and rumen and near continuous passage of ingesta from the reticulum into the distal parts of the stomach maintains a constant movement of digesta into the abomasum (Dyce *et al.*, 1987). The mucosa lining the abomasum secretes acid and pepsinogen which begin the breakdown of microbial protein and helps sterilise the digesta before it reaches the more delicate absorptive surfaces of the small intestine. Microbes which enter the abomasum from the forestomach are an important source of protein for the ruminant (Hutton *et al*, 1971).

1.1.1. Rumen and Reticulum

Food entering the rumen and reticulum does so from the oesophagus which opens at the cardia of the reticulum, the most cranial division of the sheep stomach. The reticulum derives its name from the many short (2-8 mm high) mucosal folds called reticular ridges arranged in a hexagonal pattern, giving the mucosa a honeycomb-like appearance. The mucosal surface of the entire forestomach is lined with an aglandular stratified squamous epithelium.

The reticulum communicates with the cranial (anterior) sac of the rumen *via* the reticulo-rumen opening. Ingesta flows freely from one compartment to the other (Church, 1976; Dyce *et al.*, 1987). As the rumen and reticulum are incompletely

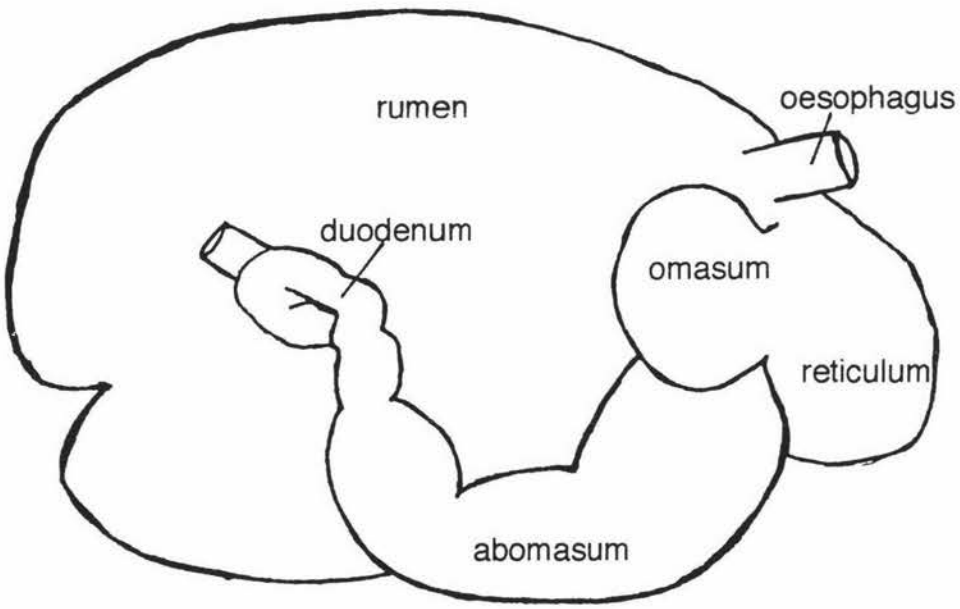

Figure 1.1. Diagrammatic view of the sheep's stomach from the right side.

separated, they are often collectively referred to as the reticulo-rumen.

The rumen is the largest compartment of the adult sheep stomach, containing up to 85% of the total stomach volume (Warner and Flatt, 1965). The inner surface area of the rumen is formed into numerous papillae which increase the surface area available for absorption of volatile fatty acids (see below).

Solid matter is retained in the rumen until it is reduced to a size suitable for passage through the reticulo-omasal orifice into the omasum (Kay, 1983). The reduction in size is due to microbial fermentation, contractions of the reticulum and rumen, and regurgitation and remastication of the ingesta during rumination (Kay, 1983).

There is a regular sequence of rumen contractions, which pass over the rumen and reticulum up to 3 times each minute in fed sheep (Reid, 1963). The backwards moving primary contraction, or "A sequence", commences with a biphasic contraction of the reticulum and then spreads to involve the rumen (Reid, 1963). It serves to mix the rumen contents with the ingesta already present in the rumen (Perruzzo de Naville *et al.*, 1987). The secondary contraction or "B sequence" does not involve the reticulum. It begins in the dorsal blind sac in a forward moving contraction passing over the rumen in a craniocaudal direction and is associated with eructation (Stevens and Sellers, 1959; Ruckebusch and Tomov, 1973; Perruzzo de Naville *et al.*, 1987). The cycle is regulated by the central nervous system, with the rate and strength of contraction adjusted according to the information supplied to the gastric centres in the medulla by receptors in the stomach wall. The receptors in the stomach wall are stimulated by distension, tactile stimulation and/or chemical stimuli (Cottrel and Gregory, 1991). The frequency and amplitude of secondary ruminal contractions are chiefly determined by the degree of ruminal distension, with chemical and tactile stimulation less important (Cottrel and Gregory, 1991).

During rumination the stomach movements are coordinated with those of the chest wall and oesophagus. Rumination begins with an additional reticular contraction, which floods the cardia with ingesta. The ingesta is then drawn into the oesophagus by an

inspiration with the upper airway closed and carried to the mouth by a peristaltic wave moving from the cardia to the pharynx. The regurgitated ingesta (cud) is then remasticated and further insalivated and divided, and then reswallowed. Along with aiding the mechanical breakdown of ingesta, rumination also assists the continuation of fermentation by breaking down the hard outer coating of the grass and grain, which allows the microbes access to the centre (Habel, 1975).

Fermentation of the microbes produces volatile fatty acids (VFAs) including acetic, butyric and propionic acids as well as ammonia, which are mostly absorbed from the forestomach (Kay, 1983; Dyce *et al.*, 1987). Some microbes pass from the forestomach into the abomasum where they are digested, providing a valuable source of protein and amino acids to the animal (Hutton *et al.*, 1971).

The gases produced during fermentation (mainly carbon dioxide and methane) are mostly discharged from the reticulo-rumen through the oesophagus during eructation (Dougherty, 1968). The B sequence of contraction clears the cardia of digesta allowing the gases to enter the oesophagus where they are moved towards the mouth by a very rapid reverse peristaltic contraction of the oesophagus. Some of the gases escape by mouth, but most are directed into the lungs.

1.1.2. Omasum

The interior of the ovine omasum is occupied by approximately one hundred crescentic laminae, arising from the sides and greater curvature and projecting towards the lesser curvature where there is a more open passage for digesta; the omasal canal. The laminae are classified into 1°, 2°, 3°, 4° or 5° laminae according to their structure; the 1° laminae extend the furthermost and the 5° the least from the greater curvature towards the omasal canal (McSweeney, 1988). They are covered by a mucous membrane from which numerous papillae arise. The omaso-abomasal orifice at the caudal end of the omasal canal is partially occluded by the abomasal folds (Dyce *et al.*, 1987).

The omasum contracts in a biphasic manner, with the first phase moving ingesta from the omasal canal into the recesses between the laminae. The second contraction involves the entire omasum and is thought to aid in squeezing fluid from the material between the folds. The percentage of water from the reticulo-rumen and omasum which is absorbed in the omasum is not clear, but has been estimated to be between 5 and 60% in different experiments (Engelhardt & Hauffe, 1975).

Approximately 50% of the VFAs that leave the reticulo-rumen *via* the reticulo-omasal orifice are absorbed from the omasum (Joyner *et al,* 1963). This estimation does not however take into consideration the VFAs which may be formed in the omasum due to continued fermentation. Overall it is thought that of the total VFAs produced in the forestomach of the sheep, nearly 85% are absorbed in the reticulo-rumen and 10% in the omasum (Joyner *et al,* 1963; Bost, 1970). Some ammonia, sodium and potassium is also absorbed in the omasum and chloride is secreted (Engelhardt & Hauffe, 1975).

A reduction in the size of food particles inside the omasum has been demonstrated (Bost, 1970) but the processes responsible have not been adequately described.

1.1.3. Abomasum

The abomasum is analogous in structure and function with the simpler form of stomach seen in many monogastric animals such as dogs and humans. In adult sheep, it comprises approximately 12% of the total stomach volume (Warner and Flatt, 1965).

1.1.3.1. Gross Anatomy

There are three regions in the abomasum characterised by gland type; the cardia, body and antrum (Figure 1.2.). The mucosa in the body region is characterised by large folds projecting into the lumen. These commence at the omaso-abomasal junction and, moving caudally, soon reach their maximum height before gradually decreasing in size towards the antrum. The folds increase the mucosal surface area approximately seven times (Hill, 1968). A few small irregular mucosal rugae are present in the antral region.

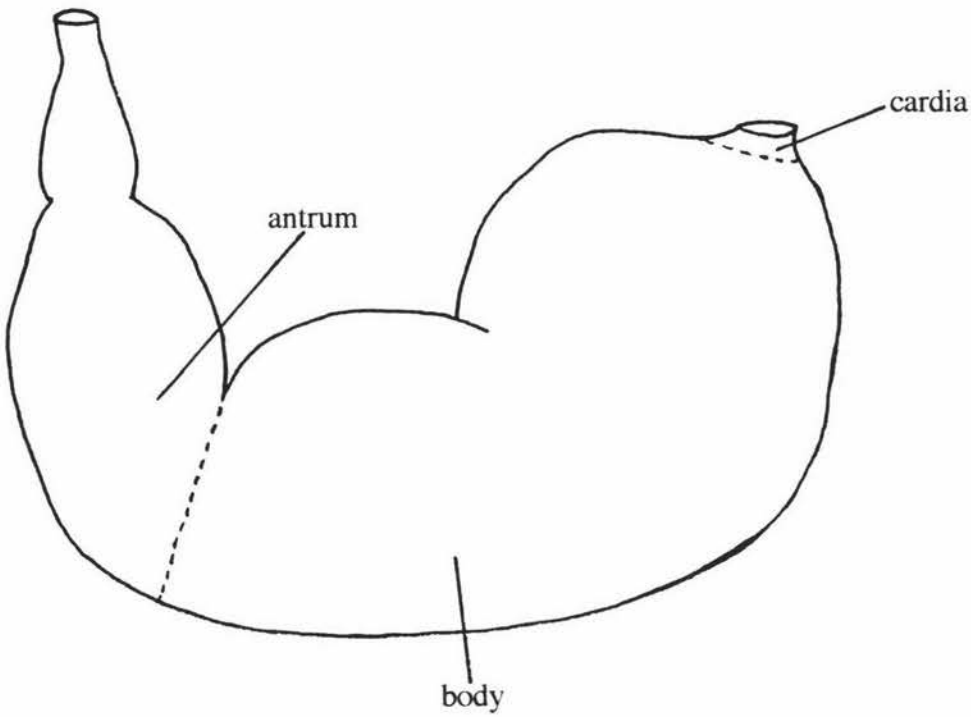

Figure 1.2. The 3 regions of the abomasum.

1.1.3.1.1. Blood Supply

The abomasum has a copious blood supply to support its secretory activities. It is derived from 4 branches of the coeliac artery. On the left side, the left gastric artery and left gastroepiploic artery run in the omental fat on the lesser and greater curvatures respectively, continuing to the pyloric sphincter. On the right side of the abomasum, the right gastroepiploic artery runs in the greater omentum and the right gastric artery in the lesser omentum. The right gastroepiploic artery anastomoses with the right gastric artery and the left gastroepiploic artery anastomoses with the left gastric artery.

The abomasal arteries are accompanied by the corresponding veins, which combine to form the gastric vein, which then joins the cranial mesenteric vein which enters the liver as the portal vein (Comline *et al.*, 1968).

1.1.3.1.2. Innervation

The abomasum is innervated by both branches of the autonomic nervous system. The following description is taken from Comline *et al.* (1968).

Parasympathetic innervation is from the ventral and dorsal vagi. The ventral vagus contributes to the reticular plexus which gives off branches to the cardia, reticulum, reticulo-omasal junction, atrium ventriculi and oesophageal groove. The long pyloric nerve arises from this plexus and passes in the lesser omentum to the duodenum. The main ventral trunk continues in the lesser omentum giving off branches to the omasum and parietal surfaces of the abomasum before terminating on the abomasal antrum.

The main trunk of the dorsal vagus nerve, after giving off branches to the reticular-omasal junction, atrium ventriculi and omasum, runs in the lesser omentum, innervating the visceral side of the abomasum before terminating near the pylorus. In addition, the dorsal vagus gives rise to the long abomasal nerve which innervates the parietal side of the greater curvature of the abomasum.

The sympathetic innervation of the abomasum is from the coeliac plexus, which receives fibres from the splanchnic nerves and dorsal vagus. Fibres leaving the plexus and

innervating the abomasum accompany branches of the coeliac artery. The sympathetic innervation is thought to be involved in vasomotor activities (Habel, 1975).

1.1.3.1.3. Microanatomy

The abomasum is composed of four layers of tissue, the *Tunica serosa*, *Tunica muscularis*, *Tunica submucosa* and *Tunica mucosa* (Figure 1.3.). The *Tunica mucosa* is composed of three layers, the *Lamina epithelialis*, *Lamina propria mucosae* and *Lamina muscularis mucosae*. There appear to be few systematic studies made of the microanatomy of the ovine abomasum. The following description is based on descriptions of the bovine by Murray (1970), Domeneghini and Castaldo (1981) and Rizzotti *et al.* (1980a and 1980b). The endocrine cells in the ovine body and antral glands of the ovine abomasum have been examined by Gurnsey (1985).

The abomasal mucosal surface is covered with multiple gastric pits (foveolae gastricae) which have gastric glands opening into their bases. The depth of these gastric pits varies depending on the region of the stomach; those in the pyloric gland area are deeper than those in other areas of the abomasum. The *Lamina propria mucosae* contains connective tissue with blood capillaries, nerve fibres, lymphatics, and some smooth muscle fibres. Gastric glands penetrate variable distances into the lamina propria. A distinct boundary between the mucosa and submucosa is created by the *Lamina muscularis mucosae* which contains 2-4 muscle layers orientated either longitudinally or circularly. Strands of the smooth muscle may extend from the *Lamina muscularis mucosae* between the gland cells in the lamina propria.

The cardiac gland area in the bovine comprises a thin band at the proximal end of the abomasum immediately distal to the omaso-abomasal junction. Cardiac glands are branched, and coiled. The glands are composed of parietal cells and some chief cells, along with mucous gland cells (Domeneghini and Castaldo, 1981). Numerous endocrine cells are also present in bovine cardiac glands, either as single cells or occasionally as clusters of 2 or 3 cells, in the lower one-third of the glands. The most numerous cell-type in the bovine cardiac glands is the enterochromaffin (EC) cell, which comprise

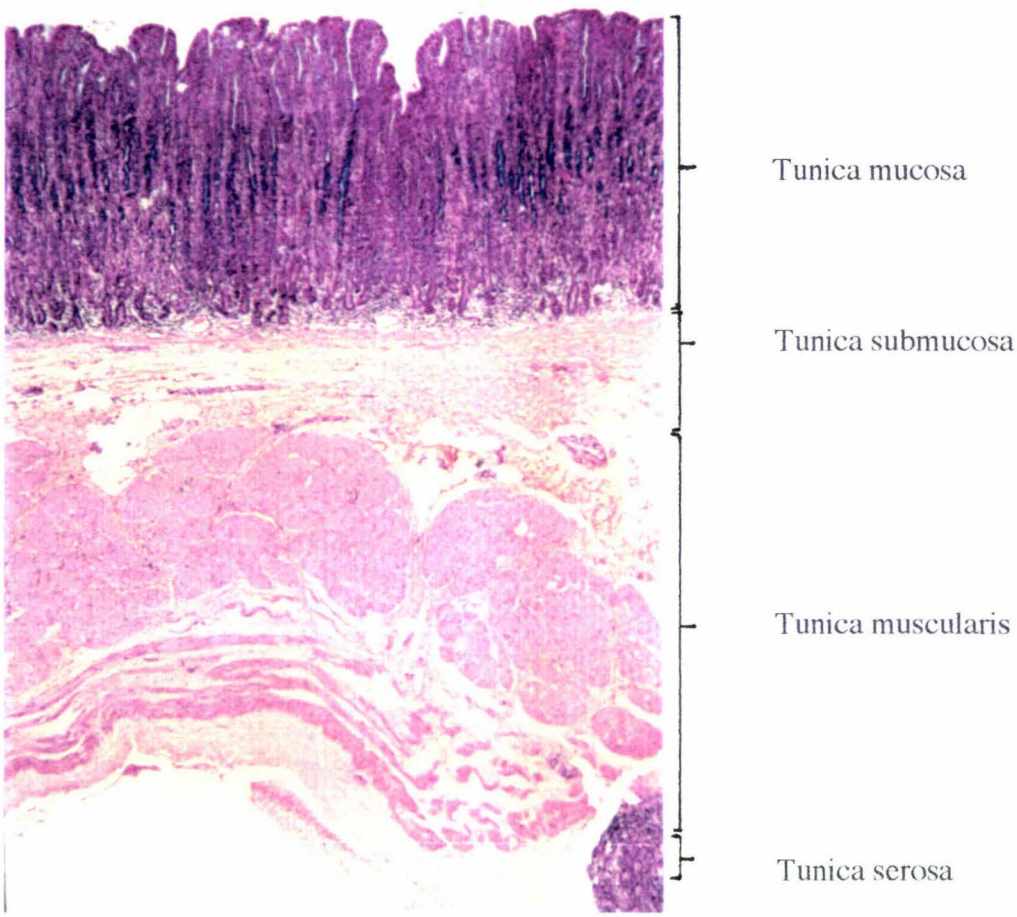

Figure 1.3. Tissue layers in the abomasal wall of the sheep (S.M. Hodgkinson, unpublished).

54.4% of the endocrine cells in this region. These contain serotonin (Domeneghini and Castaldo, 1981). Enterochromaffin-like (ECL) cells constitute 17.5% of the cardiac gland endocrine cells. In the mouse, these cells secrete histamine (Håkanson and Owman, 1967; 1969). In the cardiac glands 10.4% of the endocrine cells are "X" cells (Domeneghini and Castaldo, 1981) which have an, as yet, undefined function. Of the remaining endocrine cells, 4.5% are somatostatin producing D cells and 4.3% are "D_1" cells (Domeneghini and Castaldo, 1981) with unknown function. The remaining 8% of the endocrine cells have only a small portion of their cytoplasm visible on the section or very few equivocal granules making these cells unidentifiable (Domeneghini and Castaldo, 1981). The endocrine cells of the bovine cardiac glands are of the closed type, having no contact with the gland lumen (Domeneghini and Castaldo, 1981).

The glands in the mucosa of the abomasal body are called *gastric glands proper*. They are straight branched tubules opening into the base of pits. Cuboidal or low columnar mucus producing cells occurring singly or in clusters line the gland necks. Pepsinogen-secreting chief cells (*zymogenic cells*) are predominant in gastric glands. Parietal and mucous-producing cells are also present. The mucus produced is less viscous than that from the cardiac glands. This mucus provides protection to the mucosa against the proteolytic and hydrolytic activity of proteases and acid (Banks, 1986). Parietal cells secrete acid and intrinsic factor (McKay and McLeay, 1981). The latter is required for the absorption of vitamin B_{12}. Gurnsey (1985) reported that EC cells are uncommon in the ovine abomasum, but suggested this result may have been due to poor staining of their serotonin content. In monogastric species, EC cells are generally numerous in the body region of the stomach (Solcia *et al.*, 1975). A large part of the endocrine cell population of the abomasal body are ECL cells (Rizzotti *et al.*, 1980a). Glucagon-producing A cells are present in small numbers in the ovine cardiac, body and antral regions of the abomasum (Calingasan *et al.*, 1984). "Closed" type D cells are present in the ovine abomasal body (Gurnsey, 1985; Vergara-Esteras *et al.*, 1990). They lie directly on the basal lamina and have no contact with the gland lumen. The apical surface of "open cells" is exposed to the gastric lumen (Fujita and Kobayashi, 1973).

Gastric pits in the pyloric gland region are deeper than those in other areas of the

abomasum, but the pyloric glands themselves are short and simple or branched. Mucus-secreting cells are the most common cell type in the pyloric glands (Rizzotti *et al.*, 1980b). In the bovine, parietal and chief cells are present in small numbers. Endocrine gastrin secreting G cells are present in the ovine antrum, as well as both "open" and "closed" D cells. EC cells are rare in the ovine abomasal antrum (Gurnsey, 1985).

1.1.3.2. Abomasal secretions

Abomasal digesta is always highly acidic, despite the continual entry into the abomasum of near neutral reticulo-rumen content. This low pH is due to the secretion by the abomasum of a gastric juice containing up to 0.12 M HCl (Kay, 1983). The gastric juice is secreted continuously. Fluctuations in volume and acidity occur under different feeding conditions with an increase in acidity and volume following feeding, and a decrease in acidity and volume following fasting (Babkin, 1950; Hill, 1955; McLeay and Titchen, 1975). Hill (1965) estimated that the ovine abomasum produces 5-6 litres of gastric juices per 24 hours. Subsequently, McLeay (1971) estimated from studies using fundic pouches, that the volume of fundic secretion could approach 12-16 litres per 24 hours. McLeay cautioned against the use of pouches to determine the amount of gastric secretion produced, as acid normally enters the duodenum where it may inhibit gastric acid secretion in the sheep (Ash, 1961a).

A low abomasal pH is necessary for pepsin's proteolytic activity, with the optimum pH being 2.0 (Taylor, 1968). A mucus-rich secretion is provided by the antral mucosa, with a pH range of 7.5-9.0 (Harrison and Hill, 1962). This has been postulated to serve a protective function for the antral mucosa (McLeay and Titchen, 1975). The abomasum of milk fed lambs secretes rennin, which causes rapid clotting of the ingested milk (Grosskopf, 1959). Rennin has an optimum pH of 4.0 (Fish, 1956).

The continuous secretion of gastric juice appears to be maintained by a "phasic" activity of the various cell types; chief cells can be observed in all stages of activity, from repletion to exhaustion of pepsinogen granules (Hill, 1965).

The most important stimulus to secretion is the continuous passage of digesta into the abomasum (Hill 1965). During feeding increased motility of the forestomach causes an increase in the flow of reticulo-rumen content into and through the abomasum, stimulating the secretion of acidic gastric juice (Hill, 1965). Evidence for this has been provided by the observation that the consumption of a meal when the reticulo-rumen is empty does not evoke the same secretory response as when the reticulo-rumen is full; mucus is secreted, but little acid (Hill, 1955; 1960; Ash, 1961b). Also, Ash (1961a) showed that acid secretion by an abomasal pouch and the outflow of digesta from the abomasum increased during the first hour following feeding, peaking between the second and third hours, after which both parameters declined. Increasing the daily food intake increased the flow of digesta from the abomasum and the secretion of acid. Decreasing the food intake had the opposite effect.

Further evidence that the flow of digesta stimulates abomasal secretion is the increased gastric secretion when the flow of digesta through the abomasum is increased by stimulating motility of the gastrointestinal tract by insulin-induced hypoglycaemia. This response is greatly reduced if the forestomach is emptied prior to the injection of insulin (Hill, 1960). The frequency of reticulo-rumen contractions, measured using partial exteriorisations of the reticulum and rumen, increases within 30 seconds of sheep initiating feeding (McLeay and Titchen, 1970).

Both the composition of the digesta and abomasal distension are important in the stimulation of abomasal secretions. Distension of either the antral and/or body regions of the abomasum provokes a secretory response (Hill, 1955; 1960; Ash, 1961a). This is thought to be due, in part at least, to increased gastrin secretion (see Section 1.1.3.2.1.). In monogastric animals such as the dog, graded increases in intragastric pressure due to infusing isotonic saline into the stomach under barostatic control produces graded increases in acid secretion (Strunz and Grossman, 1978).

Rumen fluid stimulates secretion of gastric juice when introduced directly into the abomasum (Hill, 1955; Ash, 1960). Serum gastrin levels are also increased (Reynolds *et al.*, 1989; 1991). Volatile fatty acids may be important for this response. Acetic acid

when introduced into the abomasum, stimulates gastric acid secretion (Hill, 1960). If the rumen contents are titrated to pH 3.0 before infusion into the abomasum, gastrin and the abomasal pH remain close to control levels throughout the infusion (Reynolds *et al.*, 1991), indicating the importance of the raised pH for the response. The mechanisms by which the entrance of digesta into the abomasum elicits a secretory response are unclear. In monogastric animals such as humans, the entrance of digesta into the stomach stimulates a secretory response due to contact of the food with the gastric mucosa. Amino acids are potent stimulators of acid secretion. Some amino acids stimulate the secretion of acid more strongly than others. Phenylalanine is a potent stimulant, whereas glycine is no more effective than an equal volume of saline (Byrne *et al.*, 1977).

In monogastric animals, the sight, taste, smell, and actions of chewing and swallowing palatable food provoke gastric secretions. This is thought to be due to vagal stimulation of gastrin release from antral G cells as well as cholinergic stimulation of the chief and parietal cells of the body region of the stomach (Grossman, 1967; Giduck *et al*, 1987). In 1960, Hill concluded that no cephalic phase existed in the ruminant, as secretions from vagally innervated pouches of the body region of the abomasum did not increase in response to sham feeding in sheep with oesophagostomies or feeding when the reticulo-rumen was empty. However, McLeay and Titchen (1970) reported an increase in the secretion of acid and pepsin in sheep with vagally innervated abomasal pouches within 15-30 minutes of the sheep being teased with food or fed. These responses continued for up to 45 minutes after the cessation of teasing. The rapidity of the response suggested that it was due to direct vagal stimulation of the abomasum and not the result of the movement of digesta into the abomasum following stimulation of reticulo-ruminal contractions.

1.1.3.2.1. Gastrin

Gastrin is a peptide hormone which was first isolated from the antral mucosa of the pig in 1964 by Gregory and Tracey. Gastrin producing G cells have been identified by immunocytochemistry in the antral mucosa and the mucosa of the proximal duodenum in sheep (Bunnett and Harrison, 1979; Bunnett, 1984; Gurnsey, 1985). Very little gastrin is produced in other regions of the gastrointestinal tract (Reynolds *et al.*, 1984).

Gastrin can be extracted from the antral and duodenal mucosa and serum of sheep in two forms, G17 and G34 which are C terminally amidated peptides of 17 and 34 amino acids respectively (Simpson *et al.*, 1993). In the antrum, 95% is G17 and 5% G34 (Lichtenberger *et al.*, 1981). Small amounts of component 1 have also been isolated from the mucosa of the ovine antrum (Reynolds *et al.*, 1991). Serum gastrin in sheep contains gastrin in the form of G34; 14%, G17; 72%, and G14; 11% (Reynolds *et al.*, 1991).

Gastrin is known to have a trophic effect on the mucosa of the body region of the stomach, duodenum and jejunum (Johnson, *et al*, 1969; Chandler and Johnson, 1972; Willems *et al*, 1972; Johnson and Chandler, 1973; Casteleyn *et al*, 1977; Johnson, 1977; Blom and Erikoinen, 1984). The duodenal mucosa is more responsive to gastrin's trophic effects than is the mucosa of the gastric body (Johnson and Guthrie, 1974a). This may be due either to the relatively faster cell turnover rate in the duodenum or the duodenum may be more sensitive to the trophic actions of gastrin, or a combination of both. The trophic effect of gastrin on gastric and duodenal tissue is direct and independent of a second agent (Johnson and Guthrie, 1974a) such as histamine (Johnson *et al*, 1969; Willems *et al*, 1972; Johnson and Guthrie, 1974a). The size of the effect increases with increasing doses of pentagastrin (Johnson *et al*, 1969). Gastrin is thought to stimulate the progenitor cells (Blom and Erikoinen, 1984). A hypertrophy of pancreatic acinar cells is also stimulated by gastrin (Mayston and Barrowman, 1971; Majumdar and Goltermann, 1978). Gastrin has no trophic effect on the antrum and may in fact inhibit cell proliferation in the antral mucosa (Casteleyn *et al*, 1977). It also has no trophic actions on the oesophagus, or diaphragm (Johnson, 1977). In many of these studies, gastrin's trophic effects were determined by measuring the DNA concentration and the ratio of RNA:DNA and protein:DNA for the tissues (Section 5.1.).

Gastrin is a very potent stimulator of gastric acid secretion by parietal cells (Forte and Soll, 1989). It has been reported to also stimulate the secretion of water and electrolytes by the stomach, pancreas, liver and Brunners glands; inhibit absorption of water and electrolytes from the colon; stimulate the secretion of enzymes from the stomach and pancreas; stimulate the lower oesophageal (cardiac) sphincter and stomach muscle;

inhibit tone of the sphincter of Oddi; increase gastric mucosal blood flow; release histamine and induce histidine decarboxylase activity in the gastric mucosa of rats, and stimulate the incorporation of amino acids into protein in the gastric mucosa (Dockray and Gregory, 1989).

G cells have microvilli projecting into the gland lumen, allowing luminal stimuli to directly influence gastrin release (Dockray and Gregory, 1989). The base of the cell is in close proximity to blood capillaries in the lamina propria. Evidence that gastrin is released into the circulation by exocytosis at the base of the G cells has been obtained using radioimmunoassay and electron microscope techniques. Gastrin's release into the general circulation has been demonstrated by radioimmunoassay. Thus, mechanisms modulating gastrin's release may act at the lateral surface *via* paracrine or neural pathways, or due to luminal contents and at the base of the cell through circulatory mediators.

In monogastric animals such as the dog, decreasing the pH in the antral lumen results in a decrease in immunoreactive gastrin levels in the venous blood draining from the antrum (Becker *et al.*, 1973). This mechanism may also be present in the sheep as decreasing the pH of abomasal contents evokes a decline in abomasal acid secretion (Ash, 1961a; b). The infusion of rumen fluid into the abomasum stimulates the secretion of serum gastrin, but if the rumen contents are titrated to pH 3.0 before the infusion, the serum gastrin concentration and abomasal pH remain close to control levels throughout the infusion (Reynolds *et al.*, 1991). The threshold pH for the gastrin response according to McLeay and Titchen (1977) is 2.7 or less. They reported a decrease in the resting secretion of both acid and pepsin from pouches in the abomasal body when antral pouches were acidified to below this pH. They also reported that the increase in pepsin and acid secretion normally observed upon feeding was reduced or abolished when antral pouches were perfused with solutions of pH 1.3-2.0. They interpreted these effects to be due to a decrease in gastrin release.

Luminal protein may also exert an effect on gastrin release in sheep. Infusions of 10% soya protein suspension into the abomasum increase circulating gastrin levels, whereas

alpha- or beta-alanine or glycine infusions have no effect (van Bruchem, 1977).

Gastrin release in anaesthetised sheep is increased by electrical stimulation of both the dorsal and ventral vagal trunks (Bladen *et al.*, 1983). Gastrin release also occurs in response to insulin hypoglycaemia (Reynolds *et al.*, 1979b), which is an established method for stimulation of vagal efferent activity in conscious animals.

There is a cholinergic component to the gastrin response to feeding in sheep (Reynolds *et al.*, 1991). Intravenous injections of atropine sulphate (a muscarinic cholinergic agonist) at low doses to sheep on a restricted food intake enhanced the postprandial increase in circulating gastrin in the first hour after the commencement of feeding. High doses of atropine sulphate decreased the gastrin response during the first hour following the start of feeding in some animals but not others. The enhanced gastrin response observed with low atropine doses occurred despite lowered food intakes. This effect of atropine is similar to the effect in monogastric animals, where atropine blocks inhibitory pathways at low doses (Farooq and Walsh, 1975) and excitatory pathways at high doses (Nilsson *et al.*, 1972). In sheep atropine sulphate has very little effect on basal circulating levels of gastrin (Reynolds *et al.*, 1991).

1.1.3.2.2. Somatostatin

Paracrine agents are released from their cell of origin into the interstitial fluid surrounding the adjacent target cells, whose action they influence (for review, see Yamada, 1987). Somatostatin, which is believed to be a paracrine agent, is released from endocrine-like D cells in the upper third of the glands in the gastric mucosa of the rat (Saffouri *et al.*, 1980), as well as in the duodenum and pancreas and from extrinsic and intrinsic neurons in the submucosal and muscle layers throughout the gut (Yamada and Chiba, 1989). D cells in the antral and fundic regions have extensions which terminate close to nearby secretory cells, so are postulated to modulate both gastrin release and gastric secretion.

Somatostatin has an inhibitory effect on gastric secretion in the dog and has been postulated to be responsible for the inhibition of acid secretion in response to duodenal

acidification (Uvnas-Wallensten *et al.*, 1981). Intravenous infusions of somatostatin inhibit gastric secretion with no significant effects on gastrin release (Seal *et al.*, 1982). The concentration of somatostatin at the G cells when somatostatin is intravenously infused may not be sufficient to inhibit gastrin release; somatostatin works *via* a paracrine mechanism, so the concentration of somatostatin at the G cells would presumably be high under normal physiological conditions. Somatostatin infusion at high concentrations has been shown to result in gastrin inhibition and infusion of antisomatostatin antiserum enhances gastrin secretion in the rat (Saffouri *et al.*, 1979). Chiba *et al.* (1980) demonstrated that the decreased gastrin release is directly proportional to the increased somatostatin secretion from rat stomachs perfused with secretin, glucagon and vasoactive intestinal polypeptide.

Intravenous infusion of somatostatin in the sheep increases gastrin levels substantially, with the increases inversely proportional to the somatostatin dose; i.e. the lower doses of somatostatin (625 and 1250 pmol/kg/hr) were more effective in raising gastrin levels than the highest dose used (2500 pmol/kg/hr) (Reynolds *et al.*, 1991). These results were postulated to be due to the lower doses of somatostatin selectively inhibiting acid secretion, inducing an increase in abomasal pH with the resultant stimulation of gastrin secretion, whereas the highest dose inhibited both acid secretion and gastrin release partially.

There is not always an inverse relationship between gastrin and somatostatin secretion. Martindale *et al.* (1982) confirmed a reciprocal change in gastrin and somatostatin secretion from the isolated perfused stomach of the rat in response to cholinergic stimulation. This was also demonstrated by Saffouri *et al.* (1980). By contrast, beta-adrenergic agonists provoke the release of both somatostatin and gastrin (DuVal *et al.*, 1981). Gastrin and somatostatin secretion in the antrum have therefore been postulated to be linked by a dual paracrine feedback system, with one pathway mediated by cholinergic neurons which stimulate gastrin secretion by inhibiting somatostatin secretion and the other paracrine pathway activated by gastrin, stimulating somatostatin secretion and thereby diminishing the gastrin response (Figure 1.4., Schubert and Maklouf, 1992). Met-enkephalin has been demonstrated to inhibit the release of both gastrin and

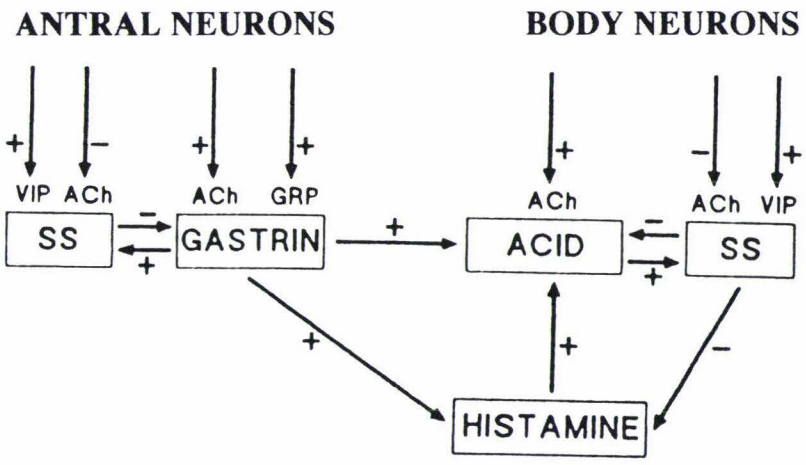

Figure 1.4. The control of gastric secretion by neural, hormonal and paracrine pathways in the gastric antrum and body. GRP, ACh and VIP are neurotransmitters. + stimulatory pathway, - inhibitory pathway. SS = somatostatin (adapted from Schubert and Makhlouf, 1992).

somatostatin (Chiba *et al.*, 1979). Thus, the control of gastrin release by somatostatin is not fully understood, nor are the mechanisms involved.

Increased gastrin release and an increase in gastric acidity both stimulate somatostatin release (Schubert and Maklouf, 1992). Somatostatin release is also increased by electrical stimulation of the vagus nerve, with GRP the postulated neurotransmitter (Holst *et al.*, 1987).

1.1.3.2.3. Other Secretogogues

Gastrin releasing peptide (GRP), the mammalian equivalent of the amphibian skin peptide bombesin is, as its name suggests, a strong stimulator of gastrin release (for review, see Walsh, 1989). The infusion of bombesin into the aorta of the rat provoked the release of both gastrin and somatostatin (Martindale *et al.*, 1982). The increase in somatostatin secretion stimulated by bombesin is abolished by the gastrin receptor antagonist L365,260; indicating that bombesin's effect on somatostatin secretion is mediated by gastrin. GRP is released by nerves in the enteric nervous system. Its distribution, determined by immunofluorescence staining using antisera to synthetic frog bombesin, is associated with nerve fibres in the mucosa and myenteric plexuses throughout the gut (Wathuta, 1986). In the ovine abomasum GRP containing neurons are present in the muscularis mucosa, lamina propria in the Meissner's plexus and in the muscularis externa (Wathuta, 1986).

Acetyl choline (ACh) is the neurotransmitter released by parasympathetic post-ganglionic nerves ending in the gastric mucosa. Exogenous ACh excites gastric acid and pepsinogen secretion in sheep (Hill, 1968; McLeay and Titchen, 1975). Endogenous Ach acts directly on parietal cells to stimulate acid secretion. Bethanechol, an ACh mimetic, potentiates the stimulatory effects of histamine (Hirschowitz and Hutchison, 1975) and gastrin (Hirschowitz and Hutchison, 1977) on gastric acid secretion and pepsinogen secretion in dogs. The effect is proportional to the dose of bethanechol and not affected by vagotomy. Thus ACh potentiates the excitatory effects of gastrin and histamine.

Histamine is a stimulator of acid secretion by the sheep's abomasum (Hill, 1968; McLeay and Titchen, 1975) presumably via histamine H_2-receptors. It appears to be essential that the histamine receptor is occupied for gastrin and ACh's full action; H_2 antagonists such as metiamide inhibit gastric secretion by histamine, gastrin and ACh in the dog (Grossman and Konturek, 1974). Histamine-induced acid secretion is accompanied by somatostatin secretion (Schubert and Maklouf, 1992). Gastric acid secretion is potentiated by histamine, gastrin and carbachol acting together on *in vitro* dog cells (Soll and Walsh, 1979). This potentiation of gastric acid secretion is reduced by metiamide.

The secretion of acid by abomasal pouches provoked by the infusion of a VFA and phosphate buffer solution into the abomasum of sheep is inhibited by exogenous adrenaline (Ash 1961b). In the dog, intravenous isoprenaline and noradrenaline inhibit pentagastrin stimulated gastric acid secretion (Curwain and Holton, 1972). Vasoconstriction of the gastric blood vessels resulting in reduced gastric gland blood flow is postulated to account for these effects (Davonport, 1977).

Prostaglandins E_1 and E_2 inhibit basal and pentagastrin stimulated acid secretion in humans (Robert, 1976), but the effects of these compounds in sheep have not been studied.

1.2. *Ostertagia circumcincta*

Ostertagia circumcincta is a nematode parasite in the trichostronglyae family. It infects the ovine abomasum where it develops in the gastric glands causing a parasitic gastritis. This parasite also has several systemic effects on sheep (see Section 1.2.3.).

1.2.1. Lifecycle

Third stage *O. circumcincta* larvae are ingested along with foliage (Figure 1.5.). They exsheathe in the rumen and move into the abomasum where they undergo a second ecdysis and enter the gastric glands on the third and fourth days after ingestion

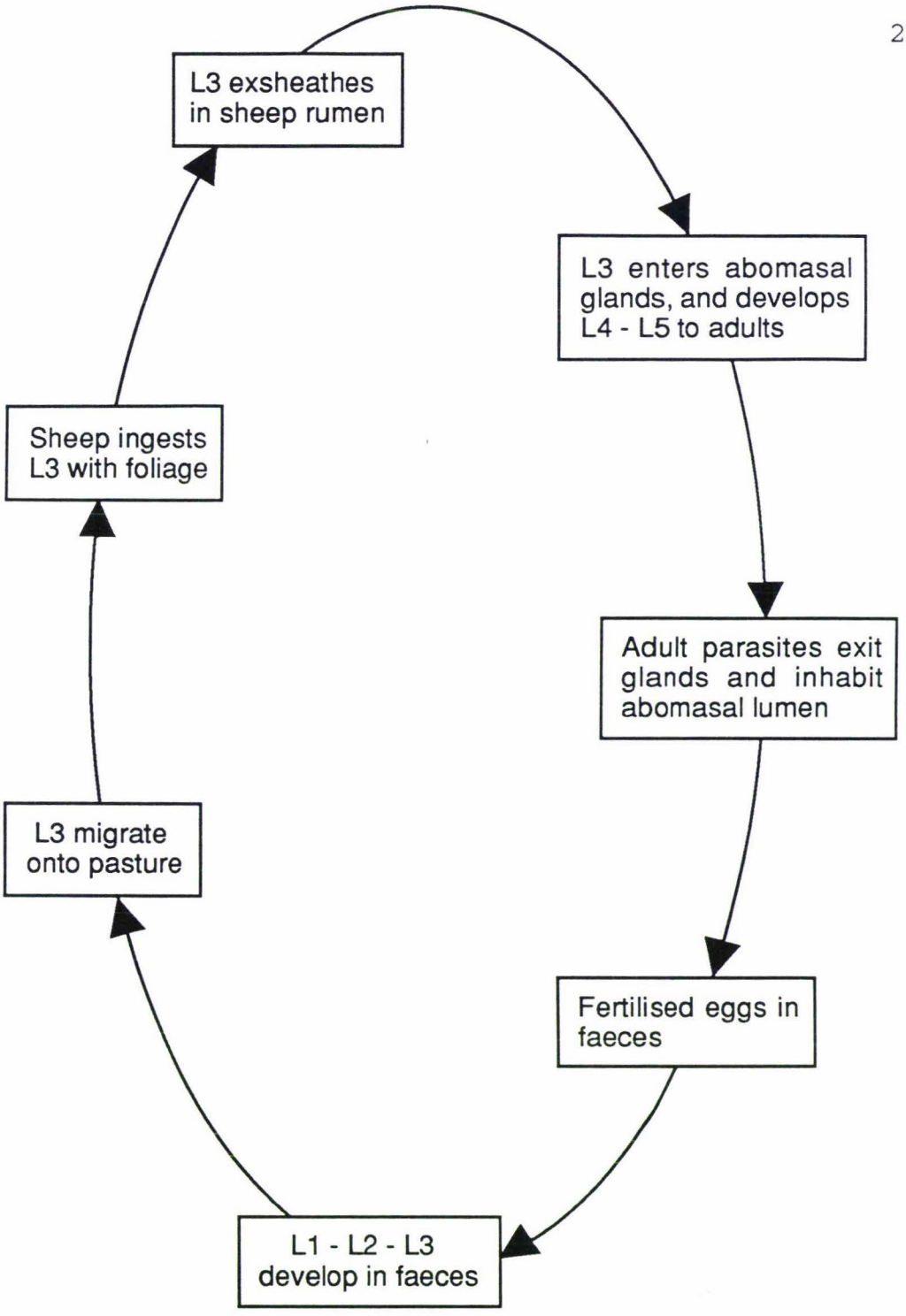

Figure 1.5. Lifecycle of the nematode parasite *Ostertagia circumcincta*. L1-L5 designate larval stages.

(Threlkeld, 1934; Sommerville, 1953). The majority of the larvae can be found singly or in pairs in small nodules in the pyloric region and on the valvulae terminales around the cardiac orifice, with the remainder scattered throughout the abomasal fundus (Sommerville, 1953).

Development may proceed in one of three ways. Most grow within the mucosa and emerge as young adults into the abomasal lumen. The larvae may exit from the mucosa after the third ecdysis and live on the surface. Some larvae do not develop but remain in the gastric pits and glands (Sommerville, 1953). Such larvae may remain in this inhibited state for up to three months before developing into adult parasites (Sommerville, 1954).

When the adult worms emerge from the glands, the glands are left distended and filled with cellular debris (Durham and Elliot, 1976). The average period of time required for the third stage larvae to develop into the adults is 16-28 days (Armour *et al.*, 1966).

The adult worms within the abomasum produce eggs, which are carried through the digestive tract along with the digesta and expelled in the faeces. The eggs hatch as first stage larvae (L_1). Under optimum conditions, larvae develop from the first to the third exsheathed infective stage (L_3) in seven days. It is this stage which is ingested with the foliage.

1.2.2. Local Effects

On the fourth day after sheep have been experimentally infected with a single dose of 100,000 larvae, larvae are observed within the gastric glands. Lesions are apparent on the mucosal surface, with the epithelial surface bulging outwards forming small nodules. The gastric glands containing the *O. circumcincta* larvae have a flattened epithelium where the larvae are in contact with the gland wall. There is a hyperplasia of surrounding glands (Armour *et al.*, 1966). Gastric glands adjacent to those containing the larvae are elongated and lined with cuboidal cells. Parietal cells in those glands lose normal shape and size. Neutrophil and eosinophil leucocytes migrate from local

capillaries of the lamina propria, moving between gland cells (Armour *et al.*, 1966).

On the eighth day after the infection, the gastric glands containing the larvae are distended and lined with relatively undifferentiated epithelia. They become progressively more distended as the parasites grow. The surrounding glands are elongated and also lined with undifferentiated epithelia with many mitotic figures. Neutrophils and eosinophils actively infiltrate the epithelium. Local areas of oedema due to the collection of protein-rich fluid occur in the submucosal connective tissue of the folds (Armour *et al.*, 1966; Durham and Elliot, 1976).

Larvae have completely emerged from some of the lesions by day twelve. In those lesions, the gastric glands are lined with a taller, clear, mucus secreting epithelia, with eosinophils and neutrophils in their lumen (Armour *et al.*, 1966). The mucosal lining is frequently replaced with mucus-secreting cells. In some cases the parasites provoke the formation of eosinophilic granulomata (Durham and Elliot, 1976b). A marked and severe hyperplasia of the surrounding gastric glands has been described (Armour *et al.*, 1966).

By day 16, there is a range of lesions. In the fundus, most of the mucosa is thickened with tall, uncoiled, undifferentiated glands. In the cardiac area, lesions are more pitted.

Inflammatory changes also occur, with congestion and cellular infiltration as described above. Mucosal and submucosal oedema also occur and are most marked in the fundus, especially at the margins of the folds (Durham and Elliot, 1976; Elliot and Durham, 1976).

The duodenal mucosa has been described to contain globule leucocytes and parasitic granulomata (Durham and Elliot, 1976).

The severity of these local effects is dependant on the larval dose. In sheep given 8,000 larvae *per os*, the gross abomasal lesions appear as little more than the formation of a few nodules and the mucosa returns to normal within 24 days post-infection (Durham

and Elliot, 1976). In sheep with a "medium" dose of larvae (64,000), the mucosa undergoes considerable recovery by 24 days post-infection and complete recovery 64 days after the infection (Durham and Elliot, 1976). A dose of 150,000 larvae induces a pronounced response to the larvae which continues for at least 45 days (McLeay *et al.*, 1973).

Trickle infecting, i.e. administering a small dose of larvae at regular intervals, maintains an experimental infection over a period of weeks and approximates the natural infection when the animals are on pasture. In a trickle infection, larvae are present in the mucosa in different stages, whereas after a single infection, the larvae are at the same stage of development except for inhibited larvae. Trickle infection allows the infection to be maintained for a greater length of time than a single infection.

1.2.3. Systemic Effects on Sheep

Sheep infected with *O. circumcincta* have a decreased food intake (McLeay *et al.*, 1973; Coop *et al.*, 1977; Sykes and Coop, 1977) and weight gain (Coop *et al.*, 1977; Sykes and Coop, 1977; Anderson *et al.*, 1988). The decrease in weight gain is not the direct result of anorexia, as pair fed controls grow at a greater rate than infected sheep (Coop *et al.*, 1977). This suggests a reduction in the efficiency of food utilisation. The magnitude of these effects are dose-related (Coop *et al.*, 1977).

Serum pepsinogen levels are elevated (McLeay *et al.*, 1973; Coops *et al.*, 1977; Reynolds *et al.*, 1979a; Anderson *et al.*, 1985). A slight hypoalbuminaemia has been reported to occur in some infected sheep (Coop *et al.*, 1977). In very heavy infections (300,000 larvae as a single dose), a decrease in packed cell volume and haemoglobin content has been observed, as well as a decrease in the plasma inorganic phosphate (Horak and Clark, 1964). There may also be a decrease in nitrogen absorption (Horak and Clark, 1964). The abomasal pH increases up to 5.9 in very heavy infections (McLeay *et al.*, 1973; Anderson *et al.*, 1974; 1976; 1981; 1985; Reynolds *et al.*, 1979a).

A hypergastrinaemia also occurs (Anderson *et al.*, 1975; 1981; 1985; 1988; Reynolds

et al., 1979a). The magnitude of the hypergastrinaemia reflects the severity of the infection, attaining a greater maximum in sheep receiving more larvae (Reynolds *et al.*, 1979). The cause of the hypergastrinaemia has not been established. The concentration of gastrin in the antral mucosa has been reported to increase, signalling an increase in production (Reynolds *et al.*, 1979a). This increase depends on either the age of the sheep involved or the dose of larvae; Anderson *et al.* (1988) reported no increase in the concentration of gastrin in the antral mucosa of 2-3 month old lambs trickle infected with 5,000 larvae 2x weekly for 7 weeks, whereas in 9-10 month old sheep trickle infected with 10,000 larvae twice weekly for 20 weeks the concentration of gastrin in the antral mucosa was significantly increased compared with that of non-infected control sheep. A decrease in duodenal gastrin content has also been reported (Reynolds *et al.*, 1979a).

With *O. circumcincta* infections, the abomasal weight and mucosal mass significantly increase (Coops *et al.*, 1977; Anderson *et al.*, 1988). It has been suggested, but not proven, that the trophic effects are caused by the hypergastrinaemia (Anderson *et al.*, 1988).

1.2.4. Prevalence and Control in New Zealand

O. circumcincta is widespread throughout New Zealand. "Subclinical" infections, where there are few or no obvious signs except a decreased weight gain, result in economically important production losses.

Anthelmintics are utilised to control ovine ostertagiasis. Strains of *O. circumcincta* which are resistant to anthelmintics are increasingly being reported in N.Z.. Resistance to benzimidazoles was first reported in N.Z. in a 1980/1981 study (Vlassoff and Kettle, 1985). A study of anthelmintic ineffectiveness in 1989-1990 confirmed the existence of resistant ovine parasite strains to both benzimidazoles and to a lesser extent levamisole/morantel and three suspected cases of ivermectin resistance (Bailey, 1991). Currently the number of farms with resistance to one or more anthelmintics in the

southern North Island of New Zealand is estimated to be between 28 and 45% (McKenna, 1991).

Because of this resistance, the continued use of the present anthelmintics does not appear to be a viable long term option for controlling ostertagiasis. Recently, concern has arisen regarding the reliance of farms on chemicals for parasite control, with the attendant risk of residues in animal products. It is desirable to decrease the use of chemicals on farms, in order to promote New Zealand's "clean green" image. The exact effects and mechanisms involved in the parasitic infection need to be elucidated in the hope of developing more effective means of control.

From the foregoing discussion, it is apparent that the production losses due to ostertagiasis and the way in which the disease will be controlled in the future are of major concern for the animal production industries in New Zealand. Before new strategies can be developed to reduce the impact of *O. circumcincta* parasites on these industries, a greater understanding is required of the pathophysiological changes occurring in the ovine host. It is apparent that the extent of the hyperplasia reported to occur during ostertagiasis needs to be elucidated, as well as whether this hyperplasia is a hyperplasia as opposed to hypertrophy. This will help to ascertain the mechanisms involved in the gastrointestinal response to *O. circumcincta*. Determining the frequency of the individual endocrine cells will help establish whether the hypergastrinaemia is due to a change in G cell numbers or the ratio of G to D cells. The effects of adult parasites as opposed to larvae has not been examined. Therefore, the following experiments were conducted:

1. A histological examination was carried out on abomasal tissue from sheep which had been infected with adult *O. circumcincta* and the changes present compared with those in sheep which had been infected with the larvae.

2. Multiple measurements of tissue layer thickness' were made for non-infected control sheep and sheep infected with larval or adult parasites to determine which areas of the gastrointestinal tract have a trophic response to ostertagiasis and which tissue layers are

increased in thickness. Morphometric analysis of both adult and larval infected sheep allowed the trophic effects due to the presence of the adults to be determined.

3. The tissue content of DNA, RNA and protein was determined in order to assess whether changes to wall thickness involved hypertrophy, hyperplasia or both.

4. The concentrations of the different endocrine cells in the abomasum were immunocytochemically examined to determine which endocrine cells were affected by this parasite and determine whether the hypergastrinaemia of ostertagiasis is due to a change in the number of G cells, the G:D cell ratio or the number of GRP containing neurons. The number of enteroglucagon-immunoreactive cells was also determined as enteroglucagon is proposed to be a trophic hormone.

To distinguish between the effects of adult and larvae *O. circumcincta* parasites, one group of sheep were infected with adult parasites (API) and one group with a single infection of larvae followed by a trickle infection of the larval parasites (LPI).

2. ORGAN WEIGHTS, SERUM GASTRIN AND PEPSINOGEN CONCENTRATIONS AND ABOMASAL pH IN SHEEP INFECTED WITH ADULT OR LARVAL *OSTERTAGIA CIRCUMCINCTA*

2.1. INTRODUCTION

O. circumcincta have several systemic effects on the sheep, as described in Section 1.2.3.. Briefly, the sheep have a decreased weight gain, not entirely due to the decrease in food intake. Serum pepsinogen levels are elevated. The abomasal pH increases to above 4 and up to 5.9 in very heavy infections and a hypergastrinaemia occurs. The magnitude of the hypergastrinaemia appears to reflect the severity of the infection, attaining a greater maximum in sheep receiving a greater number of larvae. The abomasal weight and mucosal mass significantly increase.

The local effects on the abomasum due to the presence of *O. circumcincta* larvae are described in Section 1.2.2.. Briefly, severe lesions occur on the abomasal surface, in the form of outward bulges of the abomasal mucosa forming "nodules". Gastric glands containing the larvae have a flattened epithelia, with what has been described as a hyperplasia of surrounding glands. Neutrophils and eosinophils migrate from local capillaries moving between gland cells. When the larvae have emerged from the glands, the glands remain filled with cellular debris, neutrophils and eosinophils and lined with mucus-secreting cells. In experimental infections the severity of these effects is proportional to the number of larvae given.

Whether the above changes are caused by the larvae or adult parasites has not been determined. The aim of this part of the study was to establish an infection with either adult or larvae *O. circumcincta* parasites and to determine the effects of the parasites on organ weight, serum gastrin and pepsinogen concentration and abomasal pH.

The serum gastrin and pepsinogen results were generously provided by D.E.B. Lawton.

2.2. METHODS

2.2.1. Preparation and Care of Experimental Animals

Fourteen Romney cross rams born at Flock House Agricultural Centre (AgResearch Grasslands, Crown Research Institute, Bulls) were raised to be free of Helminth parasites. They were removed from their mothers at birth and housed indoors on grating at Jennersmead Farm, Massey University. For the first two days, they were bottle fed sow colostrum then Anchor whole milk powder (Anchor Foods Ltd., Auckland, New Zealand). Lucerne nuts and chaffed red clover hay were available *ad libitum*. The lucerne used to prepare the nuts and chaff (see below) had been stored for 12 months or more in order to reduce the likelihood of the sheep becoming infected with parasites within the diet. Fresh water was available *ad libitum* in containers raised off the grating, to prevent faecal contamination.

Faecal floats performed when the lambs were five weeks of age showed the presence of small numbers of nematodirus in ten of the lambs. The maximum number in any sheep was 3 eggs per gram (e.p.g.) of faeces. At six weeks of age, all sheep were drenched with 160 mg levamisole HCl (Nilverne, Coopers Animal Health, N.Z.).

At 20 weeks of age, the sheep were transferred to individual metabolism crates in the Animal Physiology Unit, Massey University where they were kept for the remainder of the experiment. They were fed at 9:30 am daily with 200 g lucerne chaff and 800 g lucerne nuts consisting of 60% lucerne meal, 20% barley meal, 20% mill husk, 0.4 g/100 kg sodium molybdate and 4.4 g/kg sodium sulphate (anhydrous). The chaff and nuts were prepared by the staff of the Massey University Feed Centre. Fresh water was available *ad libitum*. The crates and floor of the animal room were hosed out once per day to remove the urine and faeces.

At 20.5 weeks of age, the sheep were weighed, and a cannula (see Figure 2.1.) was surgically inserted into the abomasum using full aseptic procedures while the sheep were anaesthetised. The cannulae were made in the laboratory from silastic tubing (ID 6.4

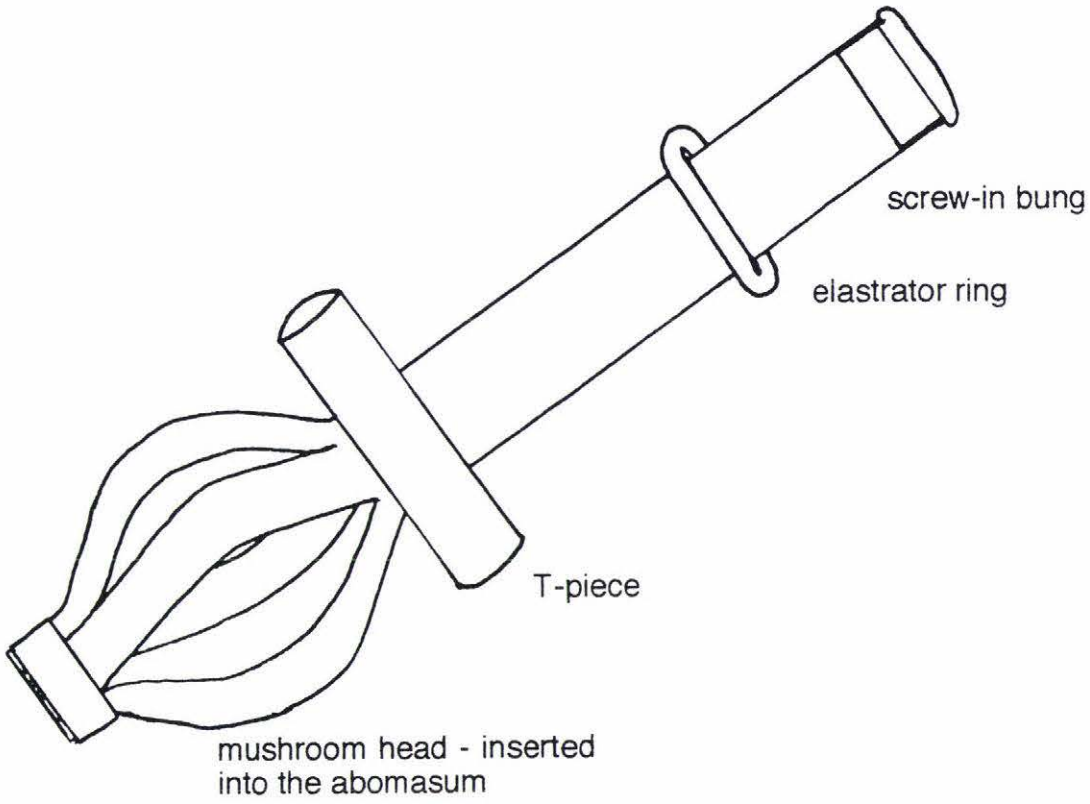

Figure 2.1. Diagram of the abomasal cannula.

mm, OD 7.7 mm, Medical Grade Silastic Tubing, Dow Corning, Michigan, U.S.A.). The end of the cannula to be inserted into the abomasum was formed into a mushroom shaped head and a T-piece (ID 9 mm, OD 12.5 mm, Medical Grade Silastic Tubing, Dow Corning) placed around the outside of the cannula 1-2 mm below the head. A screw-in bung was inserted into the external end of the cannula.

Anaesthesia was induced with Saffan (Pitman-Moore, N.Z.) at a dose of 3 mg/kg body weight injected into the jugular vein and maintained by inhalation of 3% halothane (Fluothane, Imperial Chemical Industries, England) in oxygen at a flow rate of 1-2 l/min. The sheep was then placed in left lateral recumbency and a right ventro-lateral paracostal incision made through the abdominal wall approximately 100 mm long and 40 mm caudal to the last rib. The cannula was inserted through the parietal surface of the abomasum at the junction of the pyloric antrum and body and held in place with a purse-string suture. The T-piece of the cannula was positioned within the abomasum and the free end of the cannula was exteriorised *via* a stab wound in the abdominal wall approximately 30 mm caudal to the laparotomy incision. The parietal surface of the abomasum near the cannula was then sutured to the peritoneum and the laparotomy incision closed. An elastrator ring (Allflex N.Z. Ltd., Palmerston North, New Zealand) was placed around the exteriorised part of the cannula to prevent it being pulled into the abdominal cavity.

Following surgery, the wound was dusted with antibiotic powder (Aureomycin, Cyanamid, N.Z.), and 1.25 g of porcaine penicillin and 1.25 g dihydrostreptomycin (Streptapen, Pitman-Moore N.Z.) given by intramuscular injection. The sheep were placed on their brisket in a pen with a slated floor to recover from the anaesthetic and then returned to their metabolism crates. The cannulae of two sheep were accidently pulled out several days after surgery when they became entangled in the wire mesh flooring of the metabolism crates. The cannulae of these two sheep were replaced with Silkolatex Casper Catheters (FG16, Rusch Ag, Germany). The cannulae in the other 12 sheep remained patent and in all sheep free from infection throughout the experiment.

Each sheep was assigned to one of three groups, according to pre-operative body weight

(Table 2.1). Two sheep with exceptionally low body weights (16 and 18 kg) were used as additional controls.

Table 2.1. The means and ranges for the body weights of the sheep in each of the 3 experimental groups.

Group	n	Body weight (kg) Mean	Range
Controls	4	23	20 - 25.5
Adult parasite infected (API)	4	21.4	20 - 24.5
Larval parasite infected (LPI)	4	22.4	19.5 - 25

2.2.2. Preparation of Larval *O. circumcincta* Parasites

The *O. circumcincta* larvae used in this experiment were obtained from a Romney cross ram experimentally infected with *Ostertagia* parasites. The ram was obtained from the Massey University farms and kept in a metabolism crate within the Department of Physiology and Anatomy, Massey University. It was orally drenched with fenbendazole (Panacure, Coopers Animal Health N.Z. Ltd., New Zealand) at a dose of 0.5 ml/kg body weight, ivermectin (Ivomec, Merck Sharpe and Dohme N.Z. Ltd) at a dose of 0.4 ml/kg body weight and rocobendazole and levamisole HCl (Leviben, Young's Animal Health N.Z. Ltd.) at a dose rate of 0.4 ml/kg body weight. Faecal floats performed one week after drenching showed the presence of no parasite eggs in the faeces. Ten days after drenching, the ram was infected with 50,000 larvae of a sheep-derived *O. circumcincta* strain maintained in the Department of Pathology and Public Health, Massey University. Prior to infection, 10 subsamples of the diluted larvae suspension were taken and the number of larvae per ml of solution counted using an Olympus CHS microscope (Olympus Optical Co. Ltd., Tokyo, Japan). The viability of the larvae was determined at the same time. Over 95% of the larvae were observed to move, indicating a viability

of over 95%.

After the infection, the faeces from the ram were sampled daily and when the number of eggs in the faeces was greater than 200 eggs per gram (e.p.g.), the faeces were collected continuously and treated as follows. The faeces from each 24 hour period were broken down by hand and mixed with vermiculite (Fine grade, Nuplex Industries Ltd., Auckland, New Zealand) and approximately 200 ml of distilled water to ensure that the faeces remained moist and aerated. The faeces were stored in a 27 °C room for 7 days in a plastic tray covered with a piece of glass to increase humidity. After the larvae had emerged from the faeces, they were recovered using the Baermann technique (Ministry of Agriculture, Fisheries and Food, 1986). Briefly, the faeces/vermiculite mixture was placed onto 2 layers of tissue paper (Snowtex, Caxton Products Pty. Ltd., Australia) in a glass funnel (250 mm diameter) with a short length of rubber tube attached to the outlet and clamped. The funnel was filled with distilled water and left overnight at room temperature. The larvae moved through the tissue paper and sunk to the bottom of the funnel. The clamp at the bottom of the funnel was then opened, and the bottom few mls of water which contained the larvae were collected and stored at 10 °C.

2.2.3. Preparation of Adult *O. circumcincta* Parasites

The adult parasites used to infect the sheep in the adult parasite infected (API) group were raised in 8 rams (4 months old) obtained from Massey University Farms and kept in a pen in the Department of Veterinary Clinical Sciences, Massey University. The rams were given anthelmintic as described in Section 2.2.. Faecal floats 10 days after drenching revealed the presence of no parasites. Fourteen days after drenching each sheep was infected *per os* with 100,000 sheep-derived *O. circumcincta* larvae obtained as described in Section 2.2.2.

After 28 days, the rams were euthanased by captive bolt and exsanguination.

Immediately following exsanguination the abomasum was opened and the abomasal contents collected from each sheep into buckets and pooled in a large glass flask. The mixture was allowed to settle for approximately 3 hours during which time the worms sunk to the bottom. The supernatant was decanted off and the remaining mixture divided into 5 approximately equal parts. Only adult parasites were present in the infection mix, as opposed to larvae. The donor sheep were euthanased 4 weeks after infection so that most of the larvae would have developed into adults except for any "inhibited" larvae which would remain in the mucosa. Also, multiple samples of the mixture were taken and examined under the microscope, confirming the presence of adult worms and no fourth stage larvae.

2.2.4. Treatment of the Larval Parasite Infected Group

The four sheep in the larval parasite infected (LPI) group were each infected with 150,000 larvae *per os*, obtained as described in Section 2.2.. The rumen was intubated *via* the oesophagus with a lubricated rubber hose attached to a syringe and the larvae injected into the rumen. Starting 21 days after the initial infection, the sheep were trickle infected with 10,000 larvae *per os* three times weekly for 5 weeks. The sheep were then euthanased with sodium pentobarbitone (Pentabarb 500, Chemstock Animal Health Ltd., N.Z.) injected into the jugular vein. Tissues were then collected from the sheep as described in Section 2.2.11..

2.2.5. Treatment of the Adult Parasite Infected Group

One part of the worm mixture produced in the manner described in Section 2.2.3. was infused into the abomasum of each of the 4 sheep in the API group using a lavage syringe connected to the abomasal cannulae. Each part was divided into 2 doses, each 280 ml, given two hours apart and flushed into the abomasum with a syringe full of warm tap water. The worms were administered in 2 doses to prevent the introduction of a large volume of fluid into the abomasum at one time. The remaining one fifth of the worm mixture was used to determine the number of worms each sheep obtained. Distilled water was added to the suspension up to a total volume of 500 ml. Five

subsamples of 25 ml were removed, and the total number of worms in each subsample counted after dilution. The adult parasites were administered to the sheep within 5 hours of their removal from the rams.

Eight days after receiving the adult parasites, the API sheep, plus the two outlying control sheep, were euthanased with sodium pentobarbitone (Pentabarb 500, Chemstock Animal Health Ltd., N.Z.) injected into the jugular vein. Tissues were then collected from the sheep as described in Section 2.2.11.

2.2.6. Treatment of the Non-infected Control Group

The control sheep were housed and fed in the same manner as the parasite infected sheep, as described in Section 2.2.1. When they were 20.5 weeks of age, a cannula was inserted into the abomasa in the same way as described in Section 2.2.1. The control sheep were euthanased on day 57 in the same way as the LPI sheep (Section 2.2.4.). Tissues were then collected in the manner described in Section 2.2.11.

Each control sheep was matched according to body weight with a larval infected sheep for pair feeding. However the food intake of the parasitised sheep did not decrease compared with either the preinfection levels or the control sheep.

2.2.7. Collection of Samples

Samples of peripheral blood (5 ml per sample) and abomasal contents were collected at least daily throughout the experiment starting 1 week before the infection. The blood samples were collected by venipuncture of the jugular vein. The abomasal contents were collected (2-5 ml per sample) from the abomasal cannula using a syringe. The cannula was flushed with warm tap water before each sample to remove any contents remaining in the cannula after the previous sample. The serum gastrin and pepsinogen concentrations and abomasal pH values were determined in the manner described in Sections 2.2.8., 2.2.9. and 2.2.10.. A sample of faeces was collected from the anus of

each sheep daily and the number of e.p.g was counted using the McMaster Method (Gordon and Whitlock, 1939).

2.2.8. Radioimmunoassay of Serum Gastrin

The blood samples were allowed to clot at room temperature, centrifuged at 250 *g* (Beckman GPR refrigerated centrifuge) and the serum frozen at -20 °C, for subsequent gastrin analysis. The serum was assayed for gastrin-like immunoreactivity using the procedure described by Hansky and Cain (1969), with the modification of using a second antibody (sheep antirabbit gamma-globulin) instead of dextran coated charcoal for the separation of bound from unbound fractions as described by Simpson *et al.* (1993). The antiserum used was Hansky's Ab74 (a kind gift from Dr J. Hansky). The antiserum has an equal affinity for the sulphated and nonsulphated forms of human, sheep and pig G34, G17 and G14 (see Section 1.1.3.2.1.). The antiserum has one one-thousandth the affinity for CCK compared to gastrin. Synthetic human NSG17 (Research Plus, Bayanne, N.J., U.S.A. Plus) was used to prepare labels and standards. Antisera Ab74 has been shown to react with synthetic ovine gastrin (for standard curve, see Anderson *et al.*, 1981).

2.2.9. Abomasal pH

The pH of the abomasal contents was measured using a combined glass electrode and pH meter (Radiometer PHM61, Copenhagen).

2.2.10. Serum Pepsinogen Determination

A test and a control tube containing 1.25 ml of 0.06 M HCl each had 0.25 ml of serum added. The control tube had 1 ml of 10% trichloroacetic acid (TCA) added immediately after the serum and the tube was mixed using a vortex mixer. After the test tube had been incubated in a 37 °C waterbath for 3 hours, 1 ml of TCA was added and the tube mixed.

Both the test and control tubes were centrifuged at 875 *g* for 15 minutes. From each tube, 1 ml of supernatant was added to 2 ml of Folin Ciocaltaeu's reagent (diluted 1:2). The absorbance of each tube was measured at 700 nm after 6-9 minutes to allow the colour to develop.

A standard curve using a tyrosine standard was used to determine the amount of tyrosine residues in the serum, which gives an indication of the amount of pepsinogen present. The equation used to calculate the tyrosine concentration was:

Tyrosine (μM) = $(OD_{test} - OD_{control}) / (OD_{total} - OD_{blank}) \times 0.1$

The result was expressed as mU tyrosine/ml of serum.

2.2.11. Postmortem Procedures

Following euthanasia, the entire gastrointestinal tract from where the oesophagus passes through the diaphragm to the point where the colon enters the pelvic inlet and including the pancreas was removed from the sheep and placed on a metal tray. Tissue samples for microscopic examination and morphometric measurements were taken from the sites described in Table 3.2., in the manner described in Section 3.2. Samples for biochemical analysis were collected from the sites listed in Table 4.2., in the manner described in Section 4.2.. Tissue samples for immunocytochemistry were removed from the sites listed and as described in Section 5.2.

The abomasum and intestines of all sheep were opened longitudinally and their contents collected. After completing the postmortems multiple samples of the intestinal and abomasal contents were examined for the presence of parasites using an Olympus CHS microscope (Olympus Optical Co. Ltd., Tokyo, Japan).

The omasum, abomasum, duodenum and pancreas were weighed separately using a Mettler AT200 balance (Watson Victor N.Z. Ltd.) and the reticulo-rumen, small intestine, caecum and colon weighed using a SD 1000T balance (Sauter, Germany). The organs were opened, and except for the abomasum and intestines, the contents were removed by washing with tap water. Excess water was removed by draining before the

organs were weighed.

The duodenum was defined as that part of the small intestine between the pylorus and the cranial flexure. The pancreas was dissected free from the surrounding mesentery. Associated lymph nodes were removed where possible and the duct system external to the pancreas detached before weighing. The small intestine, minus the duodenum, was defined as that part of the gut between the cranial flexure of the duodenum and the ileo-caecal junction. The terminal ileum was defined as the last 0.5 m of intestine proximal to the ileo-caecal junction. The jejunum and ileum weight refers to the intestines from the cranial flexure of the duodenum to 0.5 m proximal to the ileo-caecal junction. The colon was defined as the intestines from distal to the ileal-caecal junction to the point where the colon enters the pelvic inlet.

All samples for histological examination were collected before the organs were weighed or washed as washing the samples may have damaged the microstructure of the cells, whereas the samples for biochemical analysis were removed after the organs were weighed and washed to remove food particles and digestive secretions which could have affected the assay results.

2.2.12. Statistics

A two-tailed analysis of variance was performed on the results with the computer program StatView SE + Graphics version 1.03 (Abacus Concepts Inc., California, U.S.A.) using the Fisher probability of least significant difference (PLSD) coefficient to determine whether differences between the means were significant, and the degree of significance present. P values less than 0.05 were considered to be significant.

2.3. RESULTS

2.3.1. Confirmation of Infection

Infection of each sheep in the API and LPI groups was confirmed by the presence of

O. circumcincta eggs in the faeces and increases in abomasal pH, serum gastrin levels and serum pepsinogen levels and the presence of parasites in the abomasa at postmortem.

2.3.1.1. Faecal Egg Counts

O. circumcincta eggs were first present in the faeces of the LPI sheep 20 days after the initial infection (Figure 2.2.). Thereafter, eggs were always present in the faeces, with the number fluctuating between 50 and 1300 eggs per gram (e.p.g.).

In the API sheep, eggs were excreted in the faeces within 48 hours of the infection (Figure 2.2.). The mean number of e.p.g. increased until the fourth day after infection, reaching over 2000 e.p.g., then steadily decreased over the next 4 days to be 800 e.p.g. at day 8 when the sheep were euthanased.

2.3.1.2. Presence of Parasites in Abomasal and Intestinal Contents

Following euthanasia, the abomasal and intestinal contents were examined for the presence of parasites other than *O. circumcincta*. None were observed. Accurate counts of the number of *O. circumcincta* parasites was not possible due to the sampling regime; most of the parasites inhabit between the mucosal folds and would need to be scrapped out. Scraping the mucosa would have damaged the tissues and affected the morphometric measurements and histological appearance of the mucosa.

2.3.1.3. pH of Abomasal Contents

Before the infection began, there were no significant differences between the abomasal pH values for either group of parasitised sheep relative to control sheep.

In the LPI sheep, the mean pH of the abomasal contents (Table 2.2.) was significantly ($P<0.01$) greater than that for the control animals by the second week following the infection. The pH remained elevated during weeks 3 ($P<0.01$), 4 ($P<0.05$) and 5 ($P<0.01$). By weeks 6 and 7, the pH of the abomasal contents in the LPI sheep was not significantly different from that for the control sheep.

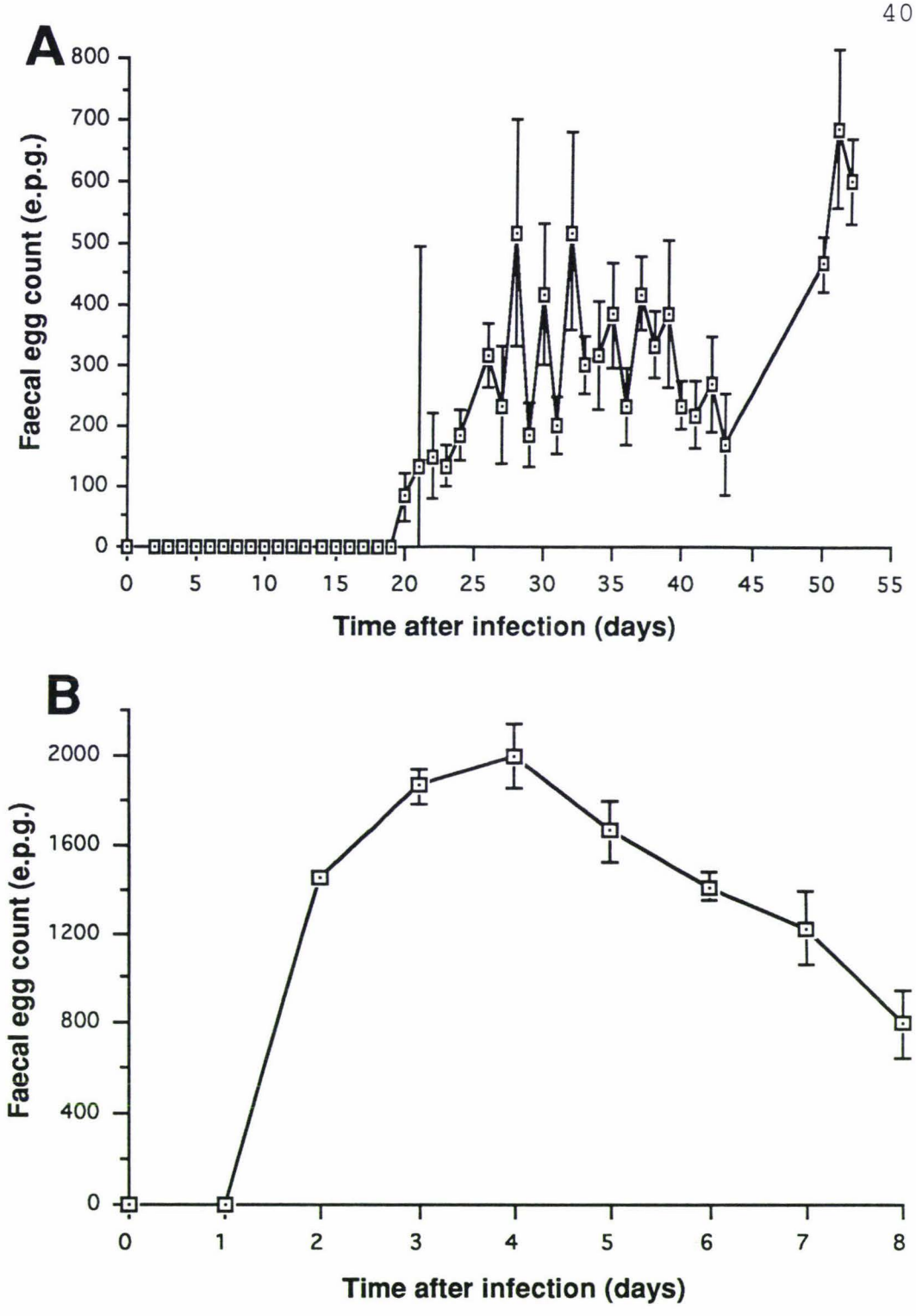

Figure 2.2. Mean number of *O. circumcincta* eggs per gramme (e.p.g.). A: larval parasite infected sheep, B: adult parasite infected sheep (mean ± s.e.). Day 0 = day of initial infection. Trickle infection of the LPI sheep began on day 21.

The mean pH of the abomasal contents of the API sheep (Table 2.2.) was significantly (*P<0.01*) increased compared to control animals in the first week after the infection and remained significantly elevated (*P<0.01*) throughout the experiment.

Table 2.2. Mean (± s.e.) weekly pH of the abomasal contents of the parasitised and control sheep throughout the experiment. Levels of significance are for the differences between the means for the infected and control groups.

Week after infection	Experimental Group		
	Control n=4	LPI n=3	API n=4
Pre-infection	2.86 ± 0.08	2.81 ± 0.04	2.80 ± 0.03
1	2.81 ± 0.11	3.00 ± 0.01	4.12 ± 0.31**
2	2.85 ± 0.05	4.70 ± 0.61**	4.68 ± 0.31**
3	2.91 ± 0.06	4.13 ± 0.24**	
4	2.90 ± 0.01	3.24 ± 0.13*	
5	2.79 ± 0.05	3.27 ± 0.05**	
6	2.80 ± 0.04	2.86 ± 0.13	
7	2.80 ± 0.11	3.04 ± 0.08	

* *P<0.05* ****P<0.01*

2.3.1.4. Serum Gastrin Concentrations

Serum gastrin concentrations for the LPI sheep were significantly (*P<0.01*) increased relative to control sheep within the first week after the start of the infection (Table 2.3.) and remained significantly (*P<0.01* in weeks 2 and 5, and *P<0.001* in weeks 3, 4, 6 and 7) greater than those for the control sheep throughout the remainder of the experiment.

Serum gastrin concentrations for the API sheep (Table 2.3.) were not significantly different than those for the control sheep before the experiment began. In both the first

and second weeks after infection, the serum gastrin concentrations were significantly (*P<0.01*) greater than those for the control sheep.

Table 2.3. Mean (± s.e.) weekly gastrin concentrations (pM) in the serum of the parasitised and control sheep before and during the experiment. The levels of significance are for the differences between the means for the infected and control groups.

Week after infection	Experimental group		
	Control n=4	LPI n=3	API n=4
Preinfection	42.6 ± 6.5	37.0 ± 1.0	31.8 ± 4.3
1	33.0 ± 4.3	85.5 ± 12.1**	195.1 ± 51.7**
2	32.2 ± 4.1	105.6 ± 18.3**	234.4 ± 54.2**
3	41.1 ± 3.3	99.6 ± 7.6***	
4	33.8 ± 1.7	101.9 ± 6.9***	
5	32.6 ± 2.5	93.3 ± 10.1**	
6	34.0 ± 3.2	112.2 ± 9.4***	
7	30.7 ± 2.7	84.7 ± 5.2***	

** *P<0.01* *** *P<0.001*

2.3.1.5. Serum Pepsinogen Concentrations

Serum pepsinogen concentrations in the LPI sheep were significantly (*P<0.01*) increased relative to preinfection values within the first week after the start of the infection (Table 2.4.) and remained significantly (*P<0.001* in weeks 2, 3 and 7, and *P<0.01* in weeks 4, 5 and 6) greater than the preinfection values throughout the remainder of the experiment.

In the API sheep, the serum pepsinogen concentration was significantly (*P<0.001*) increased compared with the preinfection values throughout the infection.

Table 2.4. Mean (± s.e.) weekly pepsinogen concentrations (milli-units tyrosine) in the serum of the parasitised sheep before and during the experiment. The levels of significance are for the differences between the means for the preinfection values and the values throughout the infection.

Week after infection	Experimental group	
	LPI n=3	API n=4
Preinfection	189.7 ± 42.4	201.6 ± 22.1
1	534.9 ± 129.8**	1043.0 ± 135.5***
2	1108.9 ± 366.1***	1239.1 ± 259.5***
3	1049.2 ± 437.6***	
4	882.5 ± 341.6**	
5	987.3 ± 288.8**	
6	939.8 ± 124.2**	
7	1244.3 ± 296.8***	

** *P<0.01* *** *P<0.001*

On day 43, one sheep in the LPI group died from causes unrelated to the experiment, reducing the number of sheep in this group to 3.

2.3.2. Body and Organ Weights

The API sheep weighed significantly (*P<0.01*) less than non-infected control sheep at the conclusion of the experiment (Figure 2.3.). This was most likely due to the differences between the ages of the API and control sheep. The mean body weights for the 2 additional control sheep at both the beginning and end of the experiment were significantly (*P<0.01*) less than the corresponding mean body weights for the other control sheep. Data from these two sheep for the organ weights and morphometric measurements were significantly (*P<0.01*) different from those from the other non-infected sheep and hence have not been included in the results for the control group.

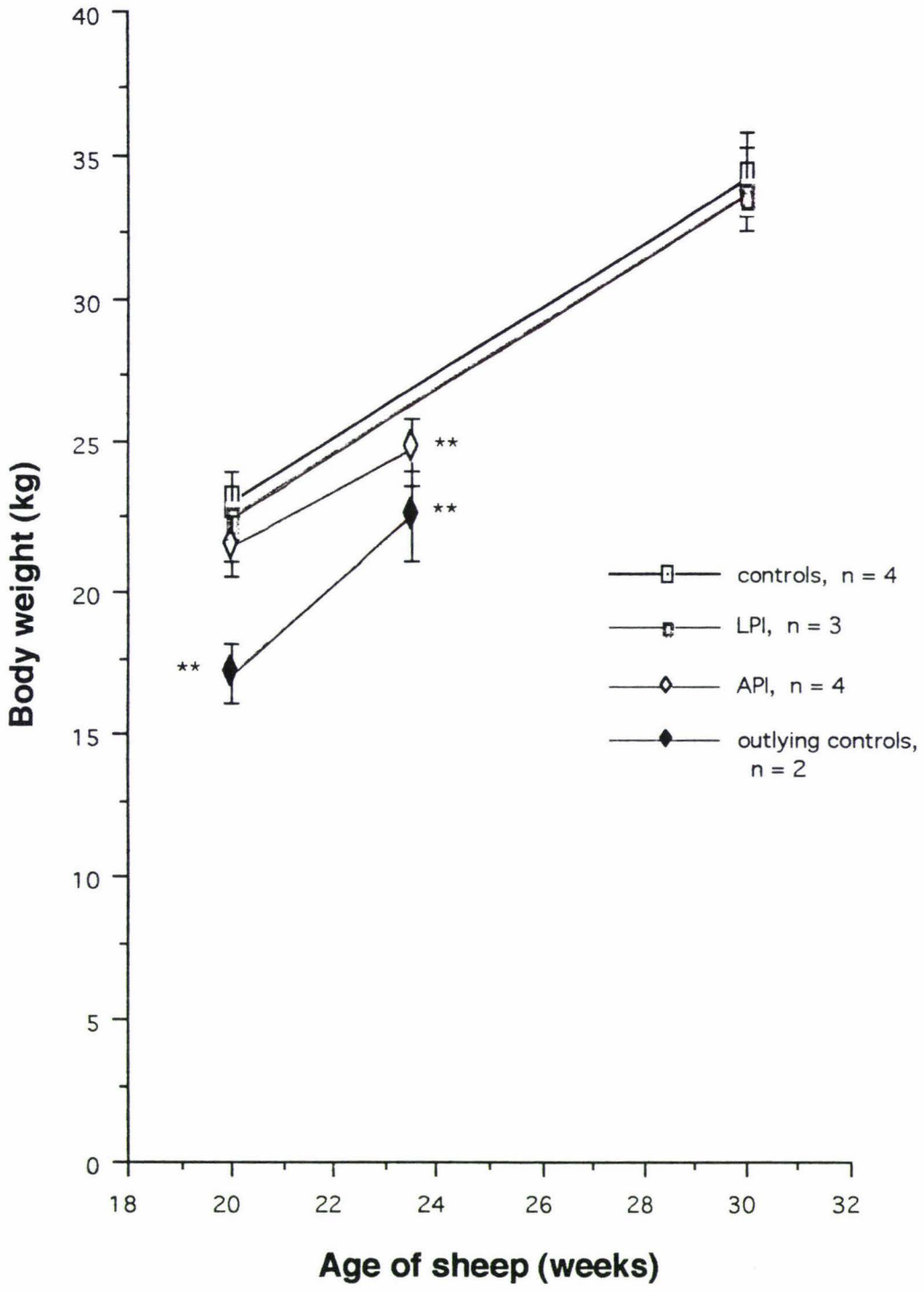

Figure 2.3. Body weight of sheep at the beginning and end of the experiment (mean ± s.e.). LPI = larval parasite infected sheep, API = adult parasite infected sheep.
** $P < 0.01$ relative to control group.

The results for the biochemical analyses and immunocytochemical staining from the 2 additional control sheep did not significantly differ from the data for the other control animals and have been included in the overall analysis of results.

The wet weight of the reticulo-rumen/kg body weight* for the API sheep was significantly ($P<0.01$) greater than that for the non-infected controls (Figure 2.4.). There were no significant differences between the reticulum weights between the LPI sheep and control sheep.

The abomasal wet weight/kg body weight for both the LPI and API sheep groups were approximately twice (both P values <0.0001) those for the non-infected controls (Table 2.5.). The increased abomasal weight per kg body weight for the LPI sheep was due to an increase in the abomasal weight ($P<0.001$) rather than a decrease in body weight compared to the control sheep. By contrast, the increase in the abomasal weight per kg body weight for the API sheep was mainly due to a lower ($P<0.01$) body weight rather than an increase in abomasal weight compared to control sheep. Abomasal weight increased by about 29 % in the API sheep compared to the control sheep but this difference was not significant.

The duodenal wet weight/kg body weight was significantly ($P<0.01$) greater in LPI sheep compared with non-infected controls. There was no difference in the weight of the duodenum between the API sheep and the control sheep.

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* Traditionally, wet wets of organs are expressed as per kg body weight (Johnson *et al.*, 1975; Fox *et al.*, 1993). A more accurate relationship is given by the following equations (Brody, 1945):

Cattle: stomach weight = 3.76 x (body weight)$^{0.34}$

intestinal weight = 0.94 x (body weight)$^{0.45}$

Rats: stomach and intestinal weight = 0.114 x (body weight)$^{1.26}$

Dogs: stomach and intestinal weight = 0.120 x (body weight)$^{0.64}$

Birds: stomach and intestinal weight = 0.0899 x (body weight)$^{0.985}$

There is no equivalent data for the sheep; therefore the organ wet weights were compared as organ wet weight/kg body weight.

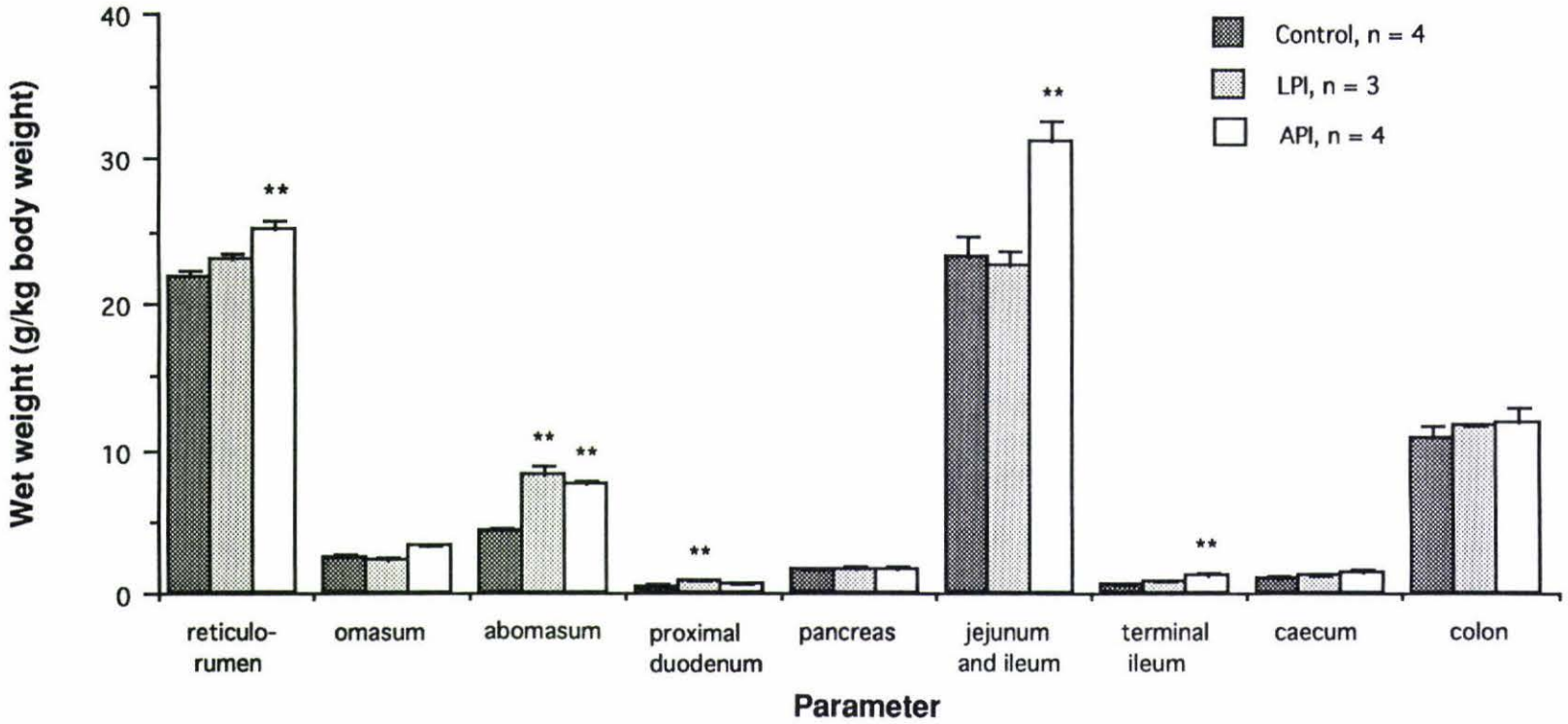


Figure 2.4. Wet weights/kg body weight (mean ± s.e.) for the sheep organs at postmortem. LPI = larval parasite infected sheep, API = adult parasite infected sheep.
** $P < 0.01$ compared to controls

Table 2.5. Ratio of abomasal weight to body weight of parasitised and non-parasitised sheep at the conclusion of the experiment (mean ± s.e.). LPI = larval parasite infected sheep, API = adult parasite infected sheep.

Group	Body weight (kg)	Abomasal weight (g)	Abomasal weight/ body weight ($x10^{-3}$)
Controls	34.4 ± 0.57	145.8 ± 10.8	4.25 ± 0.09
LPI	33.8 ± 0.70	282.7 ± 27.0***	8.33 ± 0.22 ****
API	24.8 ± 0.51**	188.3 ± 8.3	7.53 ± 0.12 ****

** *P<0.01* compared to control sheep
*** *P<0.001* compared to control sheep
**** *P<0.0001* compared to control sheep

The jejunum and ileum, and terminal ileum were all significantly (*P<0.01*) greater in the API sheep than the control sheep. There were no differences in the weight of the jejunum, ileum and terminal ileum in the LPI sheep compared to the control sheep.

The omasum, pancreas, caecum and colon wet weights/kg body weight for the infected sheep did not differ significantly from the corresponding weights for the non-infected control animals.

2.3.3. Gross Morphology of the Abomasum

The abomasa of the LPI sheep were noticeably larger than those in the control animals (Figure 2.5.) and the mucosal folds in the fundus and body appeared longer and thicker. The increase in size was more pronounced in the body region, than in the antrum. There were many small (2-3 mm in diameter) "nodules" present over most of the mucosal surface of the abomasa of the LPI animals (Figure 2.5.). There were no nodules present in the abomasal mucosa of the API or control sheep.

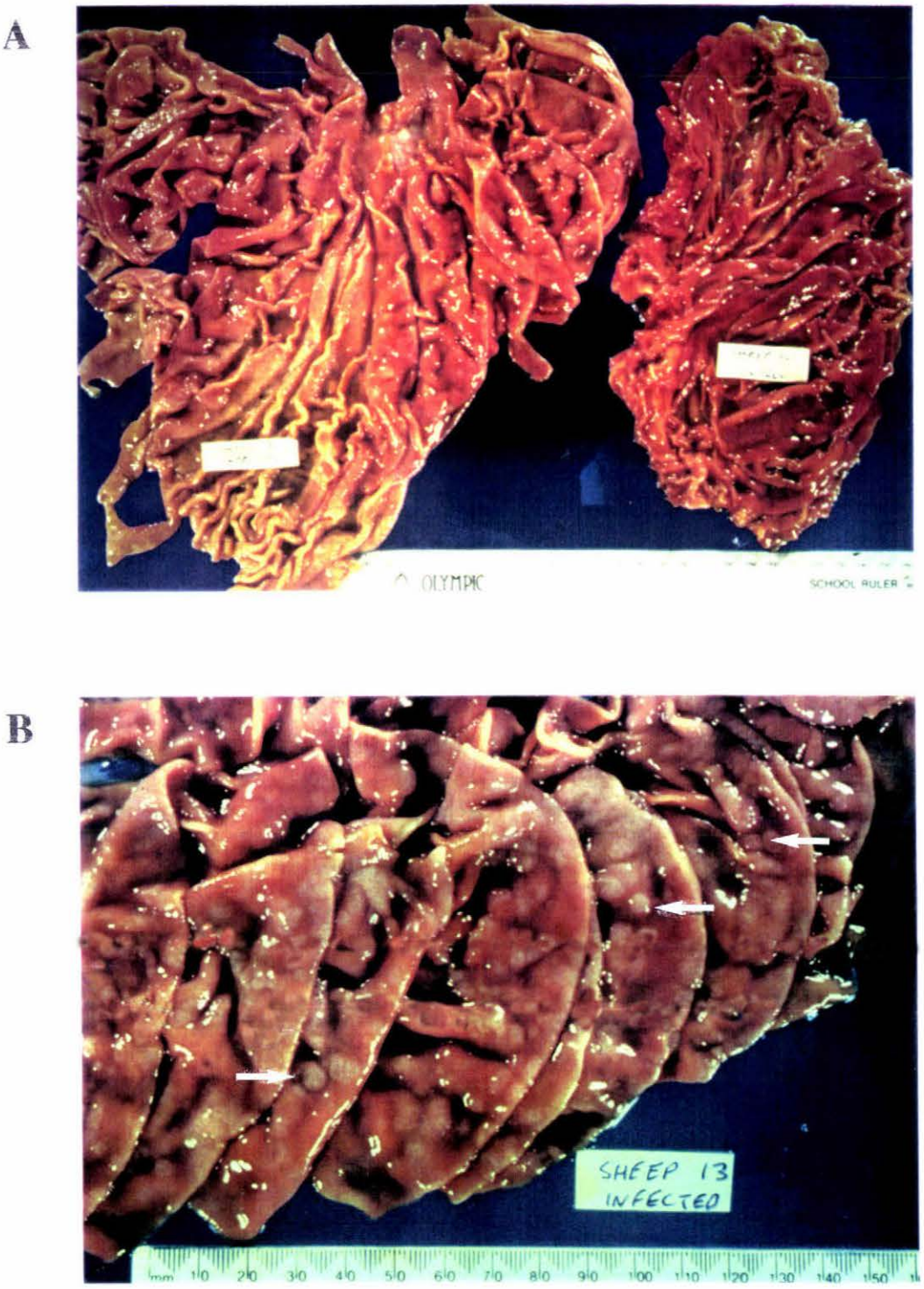


Figure 2.5. A: Abomasum of a larvae parasite infected sheep (left) and a non-infected control sheep (right). B: Mucosal folds with nodules (arrows) from the body region of a larvae infected sheep.

2.4. DISCUSSION

The infection of sheep with adult *O. circumcincta* resulted in a hypergastrinaemia and increased abomasal pH and serum pepsinogen concentration, showing that the adult parasites affect abomasal function. Increases also occurred in the wet weight/kg body weight of the reticulo-rumen, abomasum, jejunum and ileum, and terminal ileum compared with the non-infected contol sheep.

The wet weight for the reticulo-rumen of the API sheep but not the LPI sheep was significantly greater than that for the non-infected control sheep. As the parasites were introduced directly into the abomasum of the API sheep, it is unlikely that the increase in weight was due to a direct effect of the parasites on the reticulo-rumen. Instead the most likely cause of the increase in weight is the trophic effect of gastrin. Pentagastrin has been shown to increase DNA synthesis in ruminal epithelial cells *in vitro* in a dose-dependant manner and to increase the mitotic index of ruminal epithelial cells *in vivo* (Galfi *et al.*, 1991).

Alternatively, the increase in the weight of the reticulo-rumen of the API sheep may have been due to enteroglucagon secreted from glucagon-secreting (A) cells in the abomasum. The effects of abomasal parasites on enteroglucagon secretion are unknown. However, chronic (for 2 weeks) administration of glucagon stimulates mitosis of *in vitro* ruminal epithelial cells as well as mixed colonies of epithelial cells and connective tissue (Galfi *et al.*, 1991). The tissue and serum glucagon levels were not examined in this study. However, if the increased weight of the reticulo-rumen is due an increase in enteroglucagon secretion, this would be expected to have the same effects in the LPI sheep, resulting in an increased weight of the reticulo-rumen in the LPI sheep which did not occur.

Cortisol levels in the blood are elevated during stress in animals (Moberg, 1985). There is some, albeit equivocal, evidence that cortisol causes proliferation of ruminal epithelial cells *in vitro* (Galfi *et al.*, 1991). The effects of cortisol on cell proliferation of ruminal cells *in vivo* are not known, nor is there information on the effects of intestinal parasites

on circulating cortisol levels. If elevated cortisol levels were responsible for the increased reticulo-rumen weight per kg body weight, it is unclear why they did not provoke the same response in LPI sheep.

The abomasal weights for both groups of parasitised sheep were significantly greater than that for the controls. The entire abomasum of the infected sheep appeared larger (Figure 2.5.) than the abomasum of control sheep. The increase in abomasal size was very pronounced in the body region, with the antrum appearing a similar size in control and parasitised sheep. Gastrin has been demonstrated to have a trophic effect on the mucosa of the body but not the antral region of the stomach (see Section 1.1.3.2.1.). Hence a likely cause of the increase in abomasal size is the increased circulating levels of gastrin. In Chapter 3, the constituent layers of the abomasum will be examined to determine which tissue layers are increased in thickness in parasitised sheep compared with the control group.

The weight of the duodenum was significantly ($P<0.01$) greater for the LPI sheep than for the controls. There was no difference between the duodenal weights of the API and control sheep. This indicates that either the larval parasites are responsible for this increase, or the stimulus provoking this increase was not present for a sufficient length of time in the API sheep (euthanased 8 days following infection, compared with LPI sheep which were euthanased 55-57 days after infection). The most likely stimulus for this increase is gastrin which has been demonstrated to have a trophic effect on the duodenum (Johnson *et al.*, 1969; Chandler and Johnson, 1972; Johnson and Chandler, 1973; Johnson and Guthrie, 1974a; Casteleyn *et al.*, 1977). The 8 days that the parasites were present in the API sheep may not have been sufficient time for gastrin to exert a significant trophic effect on the duodenal mucosa. It is possible that the reticulo-rumen is more sensitive than the duodenum to the trophic effects of gastrin.

Secretin has been reported to inhibit the trophic effect of gastrin on the duodenum (Johnson and Guthrie, 1974b). However, it is unlikely that secretin affected the response of the duodenal tissues to gastrin in the present study.

The jejunum and ileum were significantly ($P<0.01$) heavier in the API sheep than in the controls. No change in this parameter was present in LPI sheep. This was unexpected. Any change in organ wet weight in API sheep was also expected to occur in LPI sheep which had a mixed population of both larvae and adult *O. circumcincta* parasites.

The reticulo-rumen, omasum, abomasum and small intestinal wet weights have been previously measured following *O. circumcincta* infection (Anderson *et al.*, 1988). That study showed a significant ($P<0.001$) increase in the abomasal wet weight of 2-3 month old sheep infected with 5,000 *O. circumcincta* larvae twice weekly for 7 weeks, as well as 9-10 month old sheep infected with 10,000 larvae 3x weekly for 20 weeks, compared to non-infected control sheep. The small intestine wet weight in Anderson *et al.*'s (1988) study was only significantly ($P<0.005$) increased in 9-10 months old sheep, not in the 2-3 months old lambs. No differences were observed in the reticulo-rumen or omasum. The present results correlate with those of Anderson *et al.* (1988). The sheep used in the present study were approximately 7 months of age at euthanasia; this age is between the ages of the 2 groups in the study by Anderson *et al.* (1988).

The presence of mucosal nodules with coiled larvae within them in sheep infected with *O. circumcincta* larvae have been described previously (Threlkeld, 1934; Sommerville, 1953; Armour *et al*, 1966; Durham and Elliot, 1976; Elliot and Durham, 1976). A small number of nodules have been reported to occur in non-infected abomasal pouches of 3 *O. circumcincta* infected sheep (McLeay *et al.*, 1973), suggesting that the nodules are not a direct effect of the parasites. Nodules were present on the abomasal wall of the LPI sheep, but not the API sheep. This implies that the nodules are in some way evoked by the larvae as opposed to adult *O. circumcincta*. The presence of nodules has been reported within 4 days of infection with the larvae (Armour *et al.*, 1966) so it is unlikely that the parasites were not present in the API sheep for a sufficient length of time to stimulate the formation of nodules.

Hypochlorhydria resulting in an increase in the pH of the abomasal contents has been previously reported to occur following infection with larval *O. circumcincta* (McLeay *et al.*, 1973; Anderson *et al*, 1974; 1976; 1981; Reynolds *et al.*, 1979).

Hypergastrinaemia has also been previously reported (Reynolds *et al.*, 1979; Anderson *et al.*, 1981; 1988). Hypergastrinaemia, an increase in the serum pepsinogen concentrations and an increased abomasal pH occurred in this study, confirming that the worms were exerting an effect on the animals. Faecal egg counts confirmed the presence of the parasites during the experiment. Elevations in abomasal pH, plasma pepsinogen and gastrin concentration have also been previously reported following infection of sheep with adult *Ostertagia* species (Anderson *et al.*, 1985). These also occurred in the present experiment.

The hypergastrinaemia of the API sheep was greater than that of LPI sheep. This indicates that the parasite burden in API sheep may have been greater than that of LPI sheep. The higher e.p.g. in the faecal egg counts of API sheep than LPI sheep indicate this also.

2.5. SUMMARY

The occurance of a parasitic gastritis in the sheep infected with adult *Ostertagia* parasites and those infected with *Ostertagia* larvae was confirmed by an increase in the abomasal pH, serum pepsinogen, hypergastrinaemia and the presence of *O. circumcincta* eggs in the faeces for both groups of parasite infected sheep.

There were significant increases in the wet weights of the abomasum and proximal duodenum of the larval parasite infected sheep and the reticulo-rumen, abomasum, jejunum and ileum and terminal ileum of the adult parasite infected sheep. The most likely candidate for the trophic factor stimulating these changes is gastrin, which was present in the serum in high concentrations in parasitised sheep.

3. CHANGES IN ABOMASAL AND INTESTINAL WALL THICKNESS DUE TO INFECTION WITH ADULT OR LARVAL *OSTERTAGIA CIRCUMCINCTA*

3.1. INTRODUCTION

Hyperplastic gastritis has been demonstrated to occur in ostertagiasis (Armour *et al*, 1966, see also Section 1.2.2). Briefly, the glands containing the larvae become increasingly distended as the larvae develop and a "severe" hyperplasia occurs in the gastric glands surrounding those containing the larvae (Armour *et al.*, 1966). Hyperplastic and metaplastic changes in gastric glands containing the *O. circumcincta* larvae have also been described by Durham and Elliot (1976). The pyloric mucosa thickens to twice its normal depth in very severe infections (e.g. after oral dosing with 2,000,000 infective larvae), due to what was postulated to be hyperplasia of the cells lining the gastric glands (Durham and Elliot, 1975).

Additional information on the extent of the hyperplastic changes has been provided by Anderson *et al.* (1988). They reported significant increases in the wet weight of the abomasum (180-250%) and small intestine (214%) following *O. circumcincta* infection of parasite-naive sheep. There was an increase of 253-293% in the wet weight of the abomasum in the body region, and of 168-194% in the antrum. The mucosal-to-abomasal weight ratio also increased by between 131 and 137%. These changes were more pronounced in sheep with a heavier worm burden.

The aim of the present study was to determine whether the increase in the weights of the abomasum and intestines reported in the previous Chapter to this thesis (see Chapter 2) are accompanied by increases in wall thickness and if so to determine which tissue layers are involved and to quantify the increases in tissue thickness. To these ends multiple measurements were made of thickness of the tissue layers in the infected and non-infected sheep. The tissues were also examined histologically for the presence of changes due to the parasitic infection.

The effects of both larval and adult *O. circumcincta* parasites on the tissues of the stomach and intestines were investigated by examining tissues from groups infected with either larval or adult parasites.

3.2. METHOD

The sheep used for the present experiment were those described in Chapter 2. They were raised to be free of Helminth parasites and infected with *O. circumcincta* larvae or adults as described in Sections 2.2.4 and 2.2.5. The sheep were euthanased in the manner described in Sections 2.2.4. and 2.2.5., and then treated as described in Section 2.2.11. Samples of the tissues for this study were then taken from the sites described in Table 3.1.. The samples were kept free of contaminants such as blood and other body fluids, and handled as little as possible. All samples were taken within half an hour after euthanasia.

Table 3.1. Regions of the digestive tract from which tissues were collected.

Region	Sample size	Sample site
abomasum	10 mm	body - 80 mm distal to the omasal-abomasal junction, measured along the greater curvature antrum - 10 mm proximal to the cranial edge of the Torus Pyloricus
duodenum	10 mm	upper - 30 mm distal to the pylorus lower - 60 mm distal to the pylorus
jejunum	20 mm	halfway between the cranial flexure of the duodenum and the jejuno-ileal junction
ileum	20 mm	0.5 m proximal to the ileocaecal junction
caecum	10 mm	apex of the caecum
colon	20 mm	1 m distal to the ileocaecal junction

Rings of tissue were taken from all of the sample sites with the exceptions of the abomasum and caecum. All tissues were fixed at room temperature for 24 hours in Bouin's fluid (Culling, 1985) comprising 14 parts saturated picric acid (laboratory grade, May and Becker Ltd., England), 5 parts formaldehyde in the form of a 37% w/v solution (Andrew Industries Ltd, New Zealand) and 1 part glacial acetic acid (analytical reagent, Rhone-Poulenc Chemicals Ltd., England). The tissues were placed on pieces of card before being placed in Bouin's fluid to reduce tissue folding due to muscular contraction during fixation. They were then stored in 70% ethanol. The tissues were impregnated with paraffin using an automatic tissue processor (SE400, Shandon Scientific Co. Ltd., England) and following the schedule described in Table 3.2..

Table 3.2. Paraffin embedding schedule

Process	Reagents	Time
Dehydration	absolute ethanol	4 changes x 1 hour 1 change x 2 hours
	chloroform	1 hour
Clearing	xylene	2 changes x 1 hour
Impregnation	paraffin wax @ 56°C	2 changes x 2 hours

The paraffin-impregnated tissues were then mounted in paraffin blocks and sections 6 μm thick cut with a rotary microtone (Leitz Wetzlar, Germany), and placed on P.V.A. coated slides. The slides were stained with haematoxylin and eosin and alcian blue (Culling, 1985, see Table 3.3.), mounted with DPX mountant (BDH Ltd Poole, England) and cover slipped. The sections were then studied using an Olympus CHS microscope (Olympus Optical Co. Ltd., Tokyo, Japan).

Measurements of wall thickness were made using the Sigma Scan program, version 3.9 (Jandel Scientific, U.S.A) and a graphic digitising tablet (Sketch Master, GTCO Corporation, Columbia, U.S.A.) attached to an Olympus CHS microscope (Olympus

Optical Co. Ltd., Tokyo, Japan). The instruments were calibrated with an Olympus objective micrometer (Olympus Optical Co. Ltd., Tokyo, Japan).

Table 3.3. Method for staining sections with haematoxylin and eosin and alcian blue

Process	Reagent	Time
Deparaffinising	xylene	2 changes x 5 minutes
	absolute ethanol	3-5 seconds
	70% ethanol	3-5 seconds
	tap water	3-5 seconds
Staining	alcian blue	10 minutes
	tap water	3-5 seconds
	Mayer's haemalin	10 minutes
	tap water	3-5 seconds
	Scott's tap water pH 9.5-10	2 minutes
	tap water	3-5 seconds
	1% aqueous eosin	2 minutes
	tap water	1-2 seconds
Differentiate and dehydrate		
	70% ethanol	3-5 seconds
	absolute ethanol	2 changes x 3-5 seconds
	xylene	2 changes x 3-5 seconds

Special care was taken to avoid bias when selecting the sites to be measured. Measurements were made along each of the fixed grid lines on the digitising pad rather than along lines subjectively selected during viewing of the tissue section. The grid lines were 0.2 mm apart on the tissue section when the 4x objective lens was used.

The total wall thickness and the *Tunica mucosa, Tunica submucosa* and *Tunica muscularis* were measured in each region of the gut (see Table 3.1.), as well as the mucosal pit depth and gland depth in the abomasum, and crypt depth, villus height and width in the duodenum, jejunum and ileum. Measurements of pit depth and gland depth in the abomasum and villus height, width and crypt depth in the small and large intestines were made using a 10x objective lens. All other measurements were made

using a 4x objective lens.

Five non-successive slides from each of 2 paraffin blocks from each area of the gut were examined and 10 measurements made of each parameter per slide, giving a total of 100 measurements per parameter per sheep. The mean of these 100 measurements was calculated for each sheep. A two-tailed analysis of variance was performed on the means with the computer program StatView SE + Graphics version 1.03 (Abacus Concepts Inc., California, U.S.A.) using the Fisher probability of least significant difference (PLSD) coefficient to determine whether the differences between the means for the different treatment groups were significant. The level of significance was determined when n was equal to the number of sheep in each group. A *P* value of 0.05 or less was considered significant.

The repeatability of the measurements was determined by making 10 measurements using the grid lines over the same approximate area of 1 slide at 10 different times. The results are shown in Table 3.4. and were within 5% of each other.

Table 3.4. Results for the test of the accuracy of the measurements

Measurement number	mean $\pm$ s.e.
1	1.052 $\pm$ 0.019
2	1.054 $\pm$ 0.015
3	1.062 $\pm$ 0.010
4	1.044 $\pm$ 0.021
5	1.058 $\pm$ 0.017
6	1.064 $\pm$ 0.014
7	1.064 $\pm$ 0.022
8	1.086 $\pm$ 0.013
9	1.087 $\pm$ 0.010
10	1.070 $\pm$ 0.016

3.3. RESULTS

3.3.1. Histology

In the API sheep, aggregates of large clear mucus secreting cells were present in the upper third of the mucosa (Figure 3.1.). Neutrophils were present in the lamina propria and within the lumen of some glands (Figure 3.2.) Other than these two differences, the mucosa of the API sheep appeared no different from the mucosa of the control sheep.

Larvae at various stages of development were present in the mucosa of the LPI sheep at euthanasia. The appearence and location of the larvae ranged from being coiled deep within the mucosa to being in the process of emergence from the glands (Figure 3.3.). The glands containing the coiled larvae were distended and lined with a flattened undifferentiated epithelium. Glandular leukocytes and many mitotic figures were present in the lamina propria close to the glands containing larvae (Figure 3.4.). Other glands were filled with cellular debris as well as eosinophils and neutrophils (Figure 3.2.). These were presumably glands from which worms had already emerged. An abundance of neutrophils, eosinophils and plasma cells were present in the lamina propria. Aggregates of large almost clear mucus secreting cells (identified from the slight blue staining with alcian blue) were present on the lumen side of the mucosa (Figure 3.5.). In some regions, cells had been sloughed off from the luminal surface, presumably by emerging worms.

3.3.2. Morphometry

Since the mean for the body weights of the API sheep at the conclusion of the experiment was significantly different from that for the control sheep (Figure 2.1.), the data was first examined to see whether or not a statistical relationship existed between the body weight and total wall thickness in each region of the gastrointestinal tract. This was done by plotting the logarithms for the total wall thickness in the non-infected control sheep against the logarithms for the body weights of the same sheep (Figure 3.6.). As the gradients of the lines from the different regions of the gastrointestinal tract were very different, some with a positive slope, and others with a negative slope, there

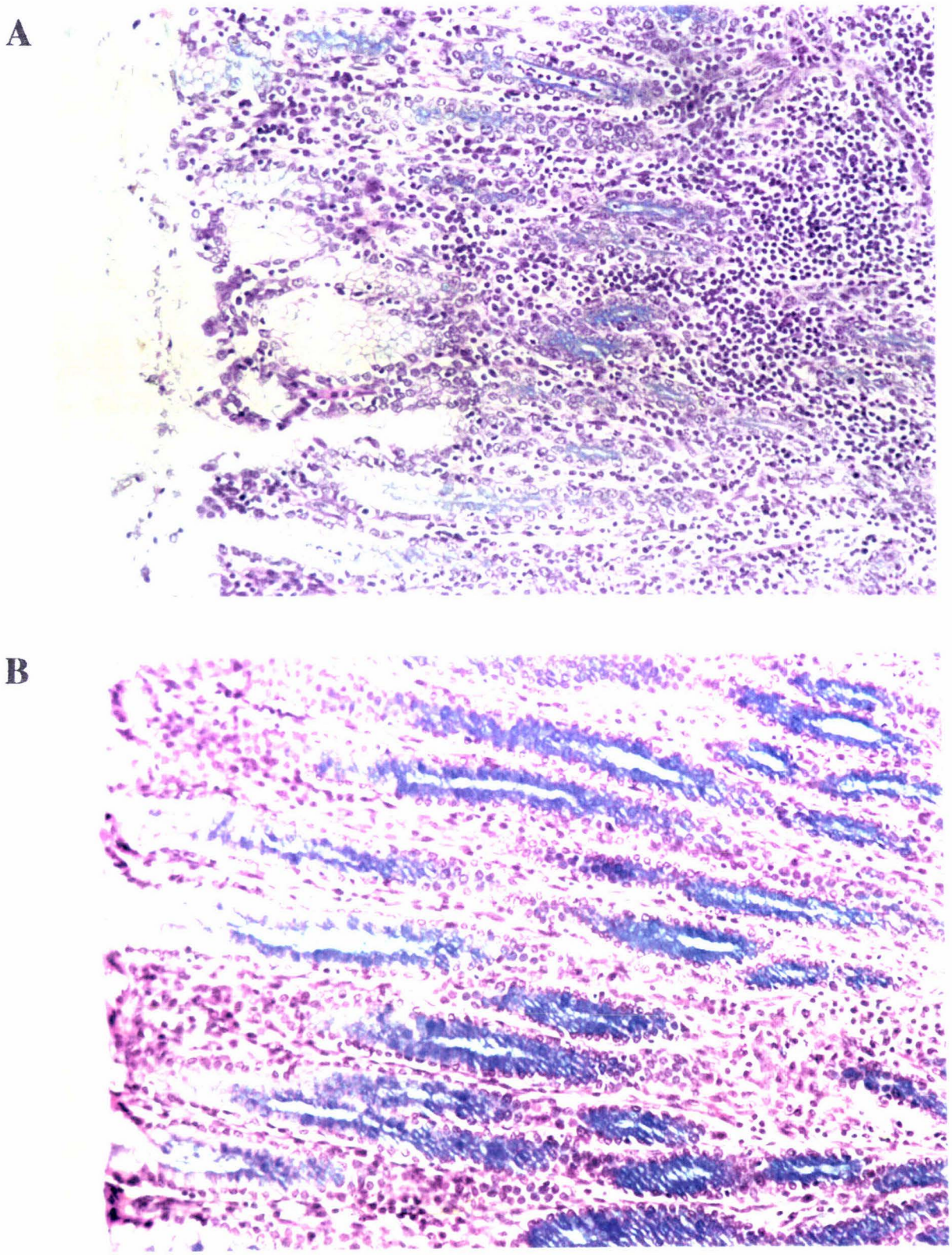


Figure 3.1. Mucous cells in the antral mucosa of an adult parasite infected sheep (A) and a non-infected control sheep (B). Note the intense blue staining of the control sheep that is not present in the adult parasite infected sheep. Magnification : x50. Stain: haematoxylin and eosin and alcian blue.

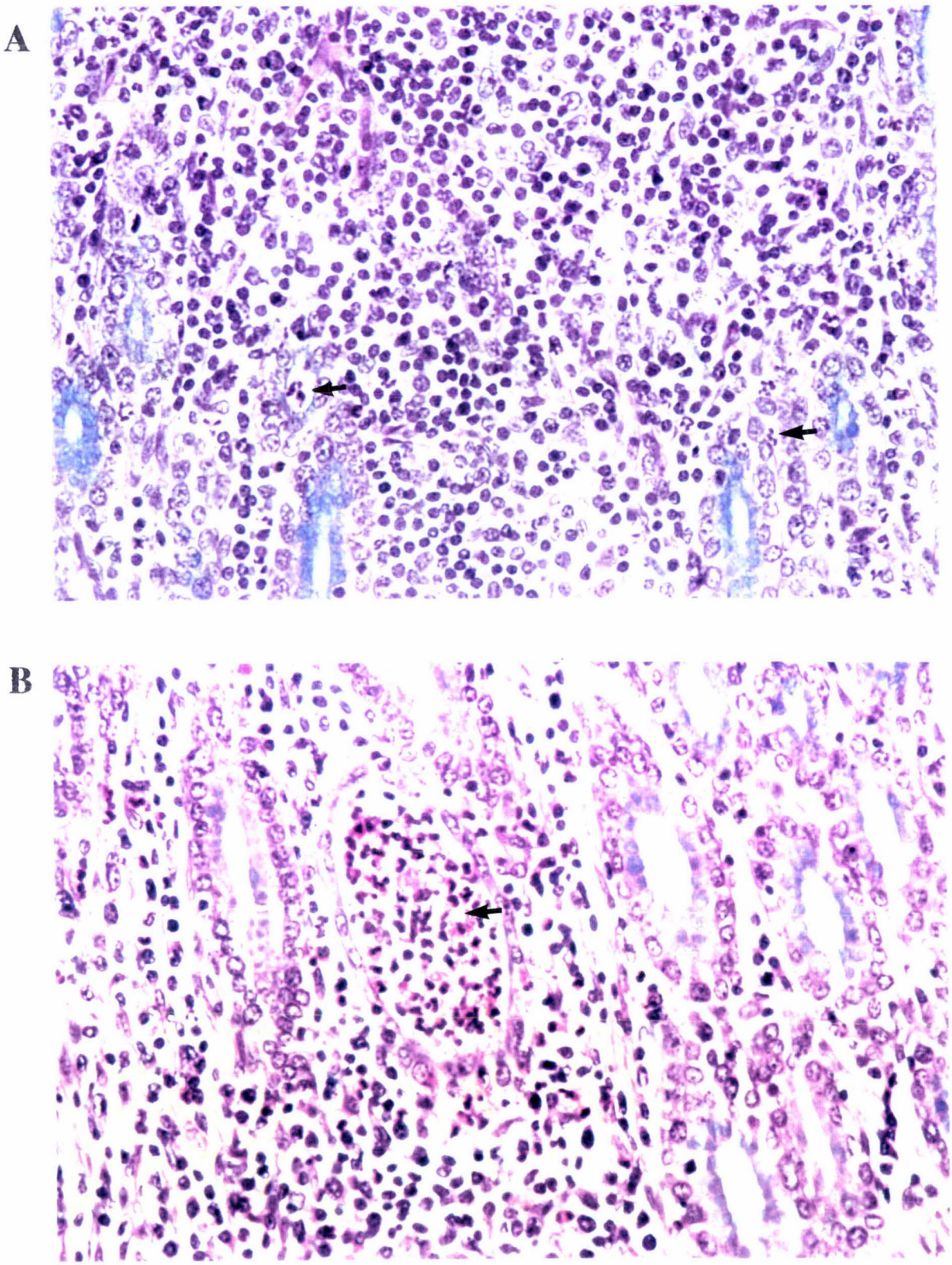
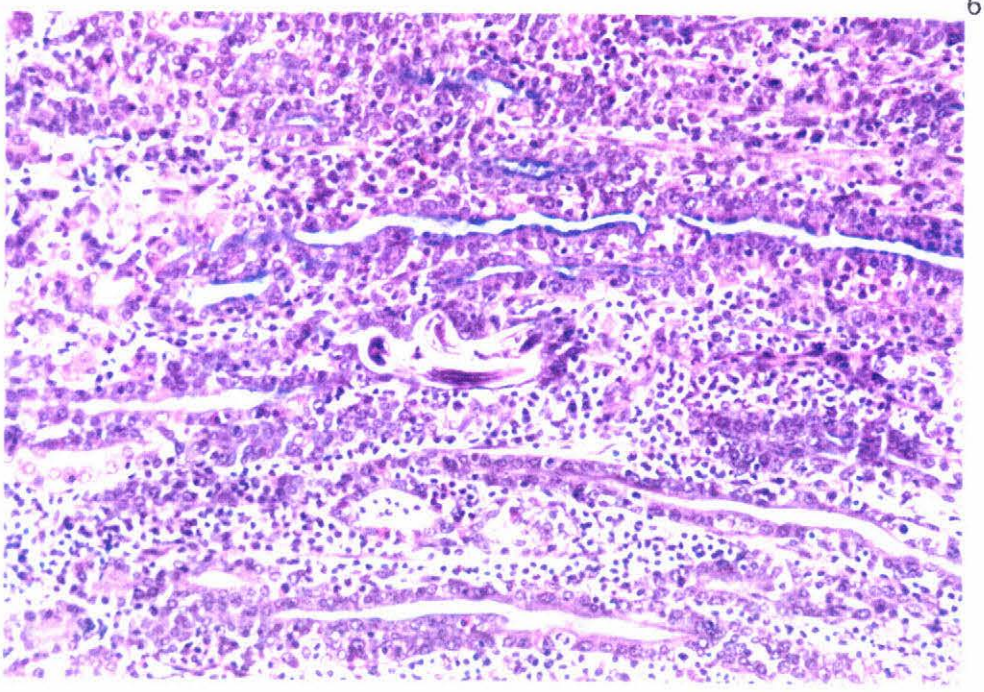


Figure 3.2. Neutrophils and eosinophils (arrows) in the antral mucosa of an adult parasite infected sheep (A) and a larvae parasite sheep (B). Magnification: x100. Stain: haematoxylin and eosin and alcian blue.

A



B

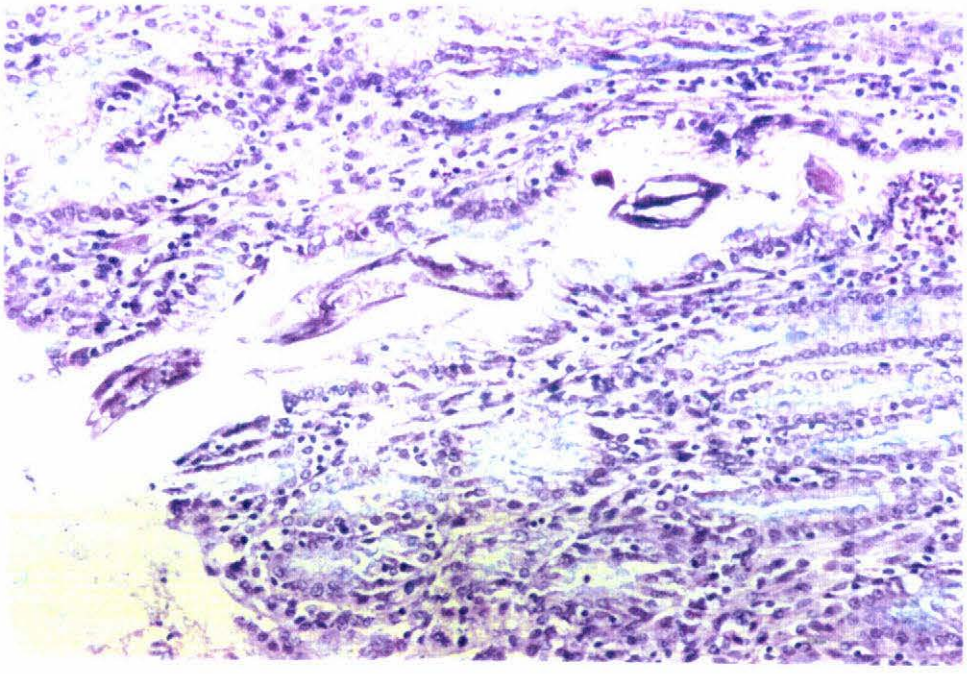


Figure 3.3. An *O. circumcincta* larvae coiled deep within the antral mucosa (A, magnification: x80) and an adult worm emerging from the antral mucosa (B, magnification: x50) of a larval parasite infected sheep. Stain: haematoxylin and eosin and alcian blue.

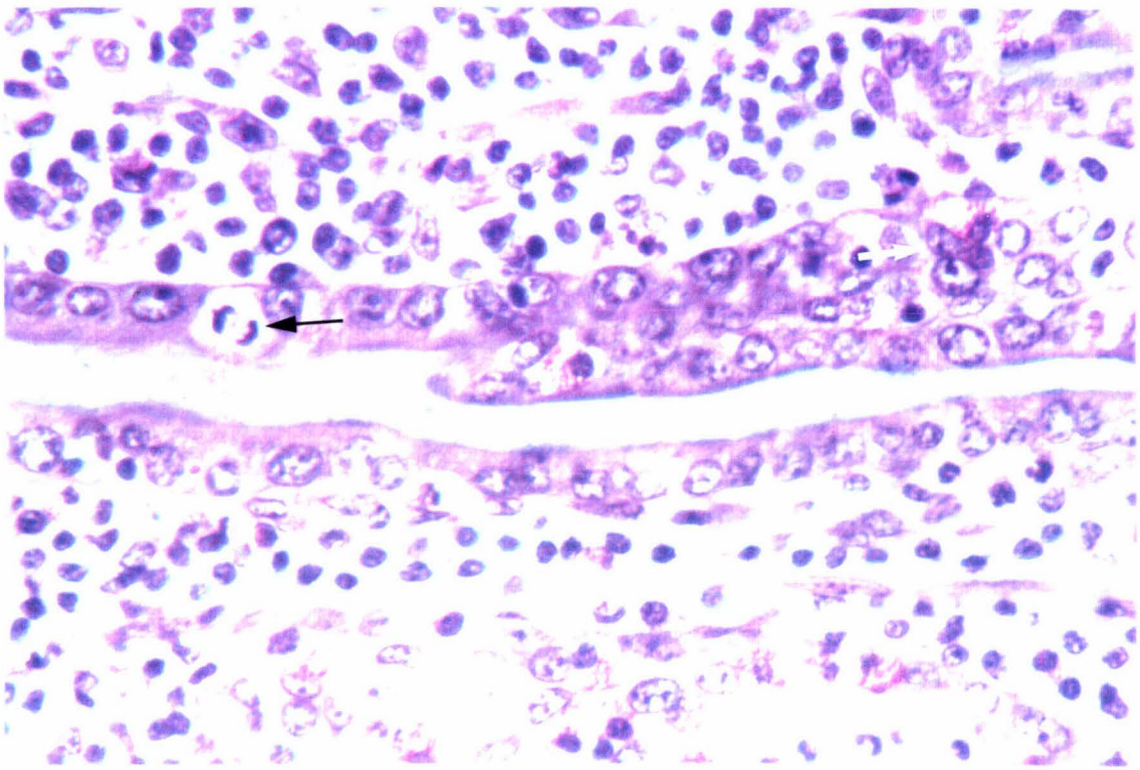


Figure 3.4. Globular leukocytes (white arrows) and mitotic figures (black arrows) in the antral mucosa of a larval parasite infected sheep. Magnification: x160. Stain: haematoxylin and eosin and alcian blue.

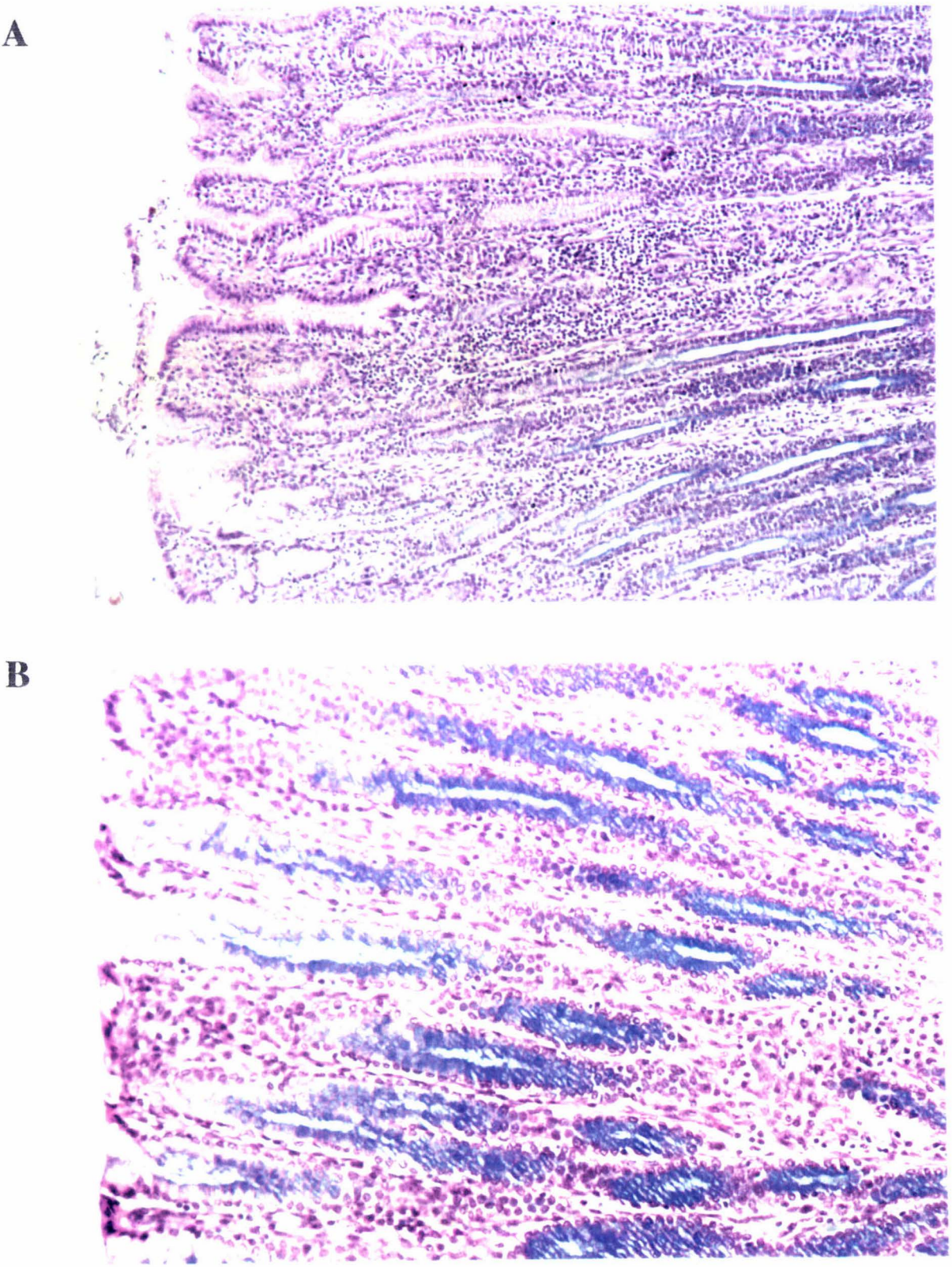


Figure 3.5. Mucous-producing cells (blue) in the antral mucosa of a larval parasite infected sheep (A) and a non-infected control sheep. Magnification: x50. Stain: haematoxylin and eosin and alcian blue.

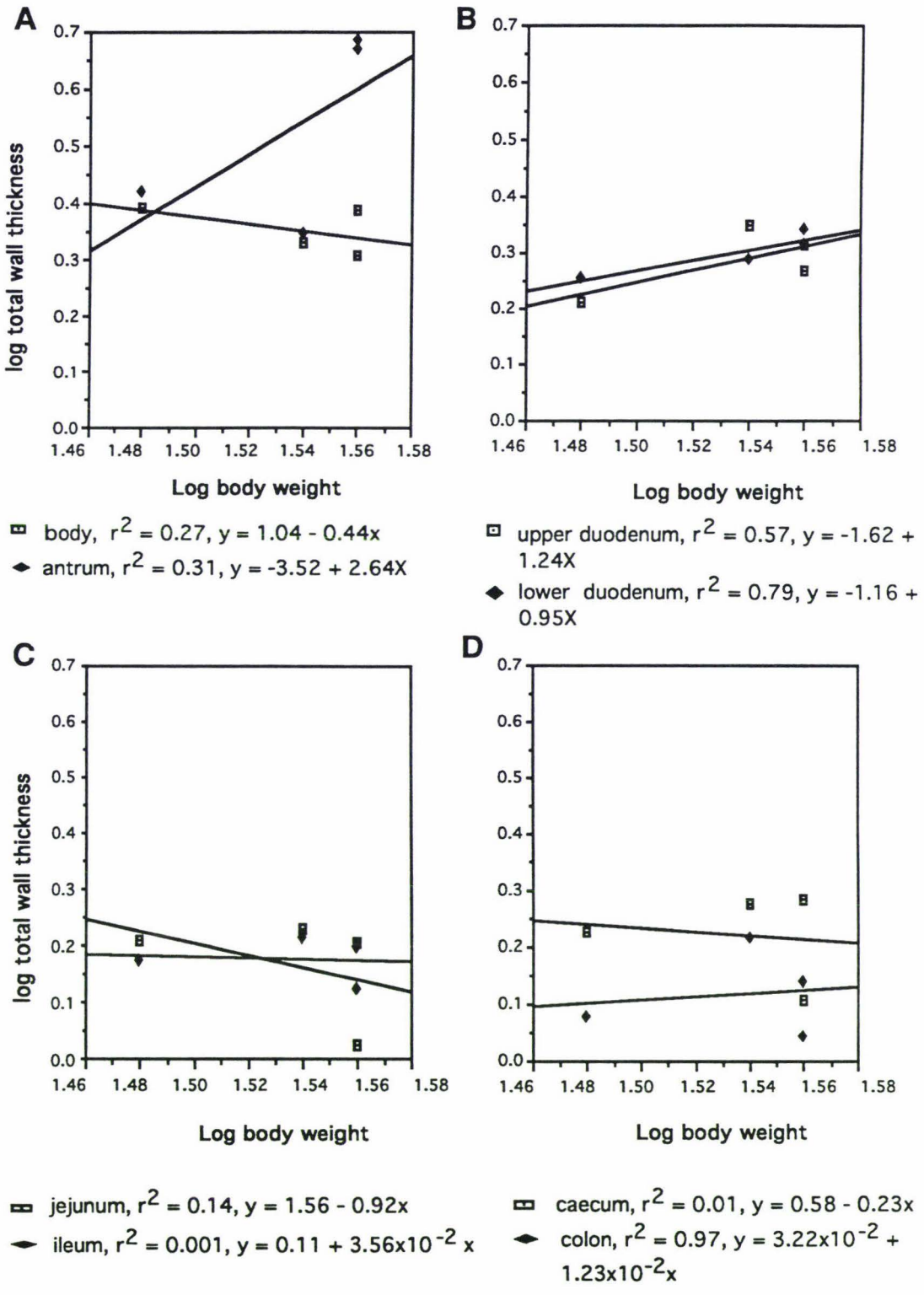


Figure 3.6. Logarithm of body weight vs. logarithm of total wall thickness in the control sheep. A. abomasum, B. duodenum, C. small intestine, D. large intestine

appeared to be no significant correlations between body weight and the thickness of the gastrointestinal tract. Therefore the data was examined without being corrected for differences in body weight.

In the body of the abomasum, (Figure 3.7.) the thickness of the mucosa was significantly increased in both the LPI ($P<0.01$) and the API ($P<0.05$) sheep compared to the control animals. The difference was more pronounced in the LPI sheep than in the API sheep. This increase in mucosal thickness of the LPI sheep comprised significant enlargements of both the gland ($P<0.01$) and pit ($P<0.05$) depths. In the API sheep, neither the pit depth nor gland depths were significantly increased. No significant differences were present in the total wall thickness or the thickness of the submucosa or muscularis in the body region of the abomasum of either group of parasitised sheep compared to the control sheep.

The antral mucosa (Figure 3.8.) in the LPI sheep was significantly ($P<0.01$) thicker than that in the control sheep. The gland depth of the LPI group was also significantly ($P<0.01$) increased. No significant differences were present in the antral pit depth, submucosa or muscularis of the LPI sheep compared to the control animals. There were no significant differences in the total wall thickness or the thickness of the mucosa, submucosa or muscularis between the API sheep and the control sheep.

The thickness of the total intestinal wall and the mucosa in the upper duodenum (Figure 3.9.) were significantly ($P<0.05$) increased in the LPI sheep compared to the control sheep. In the API sheep, there were no differences in the thickness of the tissue layers relative to controls in either the upper or lower duodenum (Figure 3.10.). In the LPI sheep, all duodenal parameters at both sites except the villus width at the first site, were slightly but insignificantly increased relative to controls. This trend was not present in the API sheep; in the upper duodenum the total wall thickness, submucosa and crypt depth were decreased and the muscularis and villus height was increased and in the lower duodenum the mucosa and crypt depth were decreased and the muscularis and villus height were increased compared with the same measurements for the control sheep.

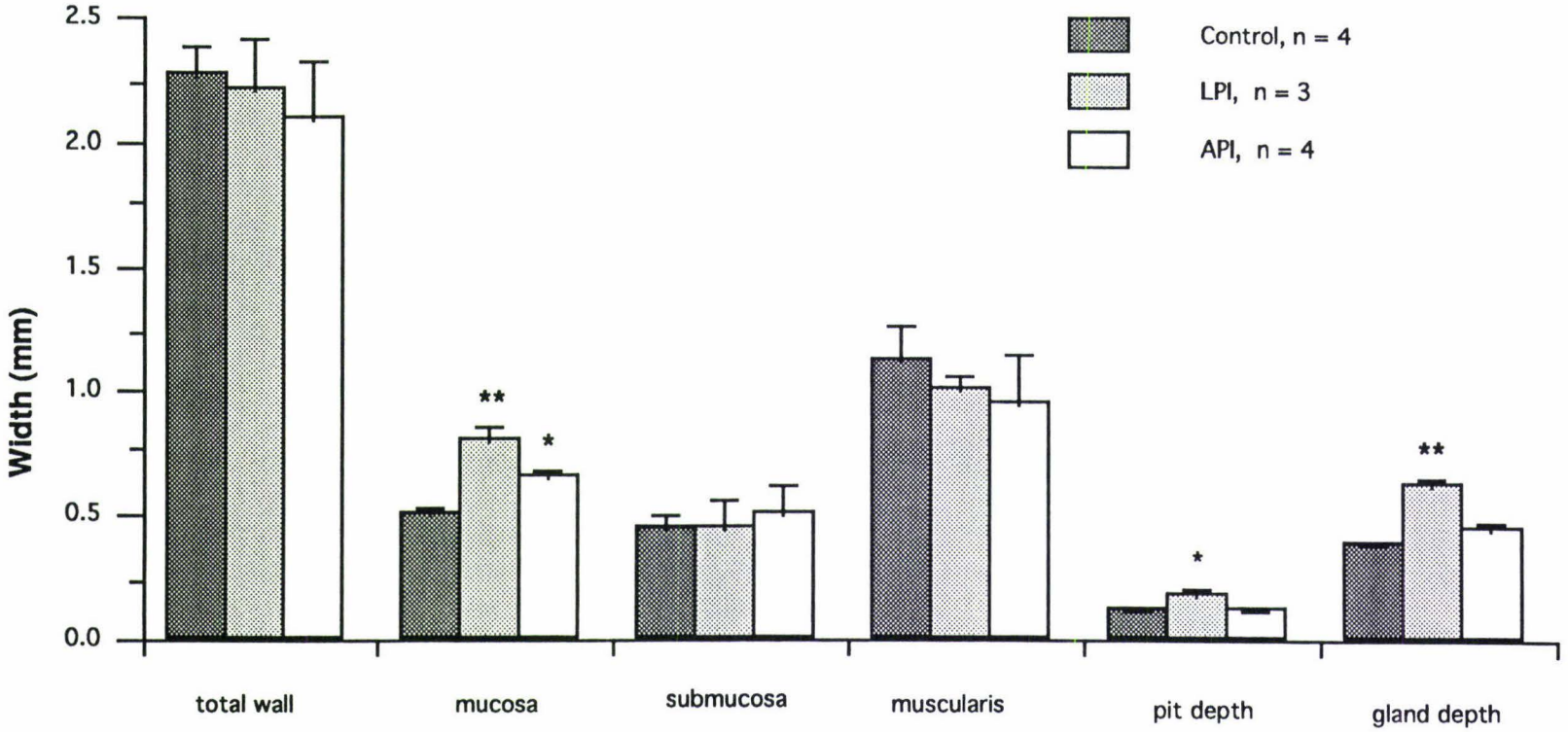


Figure 3.7. Tissue thickness, pit depth and gland depth in the abomasal body (mean ± s.e.). LPI = larval parasite infected sheep, API = adult parasite infected sheep.
* $P<0.05$ ** $P<0.01$

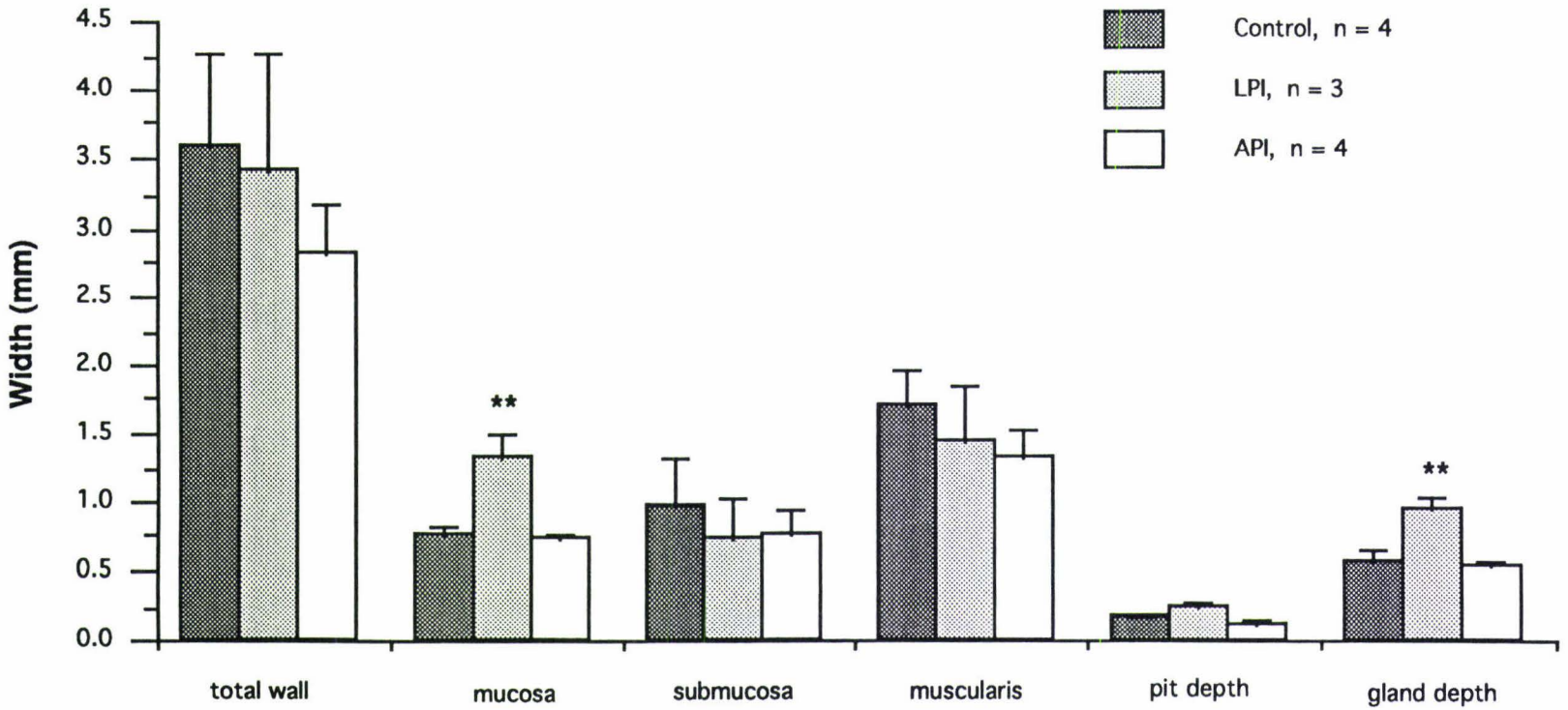


Figure 3.8. Tissue thickness, pit depth and gland depth in the abomasal antrum (mean ± s.e.). LPI = larval parasite infected sheep, API = adult parasite infected sheep. Note the different scale on this graph to the other graphs in this chapter.
** $P < 0.01$

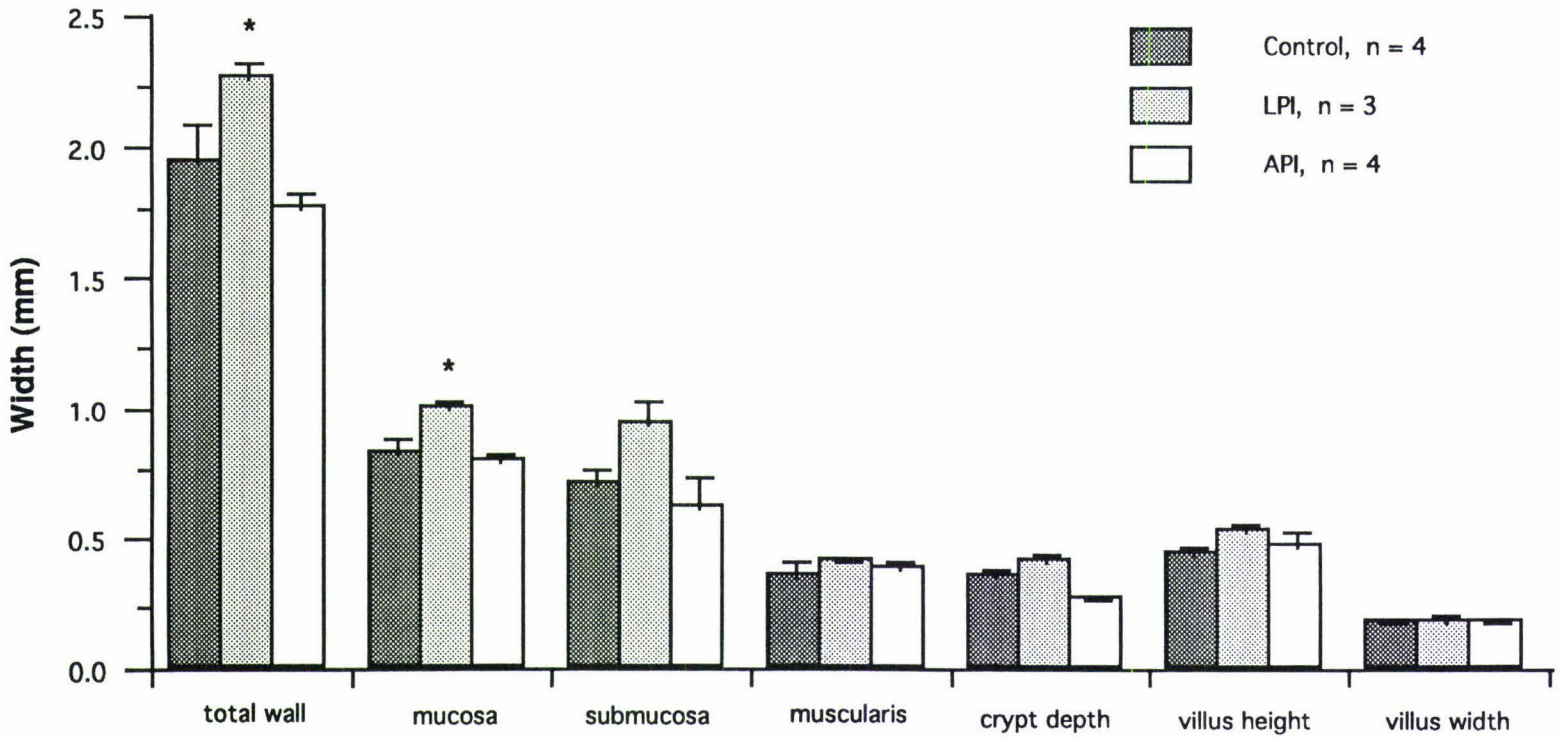


Figure 3.9. Tissue thickness, crypt depth, villus height and villus width in the upper duodenum (mean ± s.e.). LPI = larval parasite infected sheep, API = adult parasite infected sheep.
* $P<0.05$

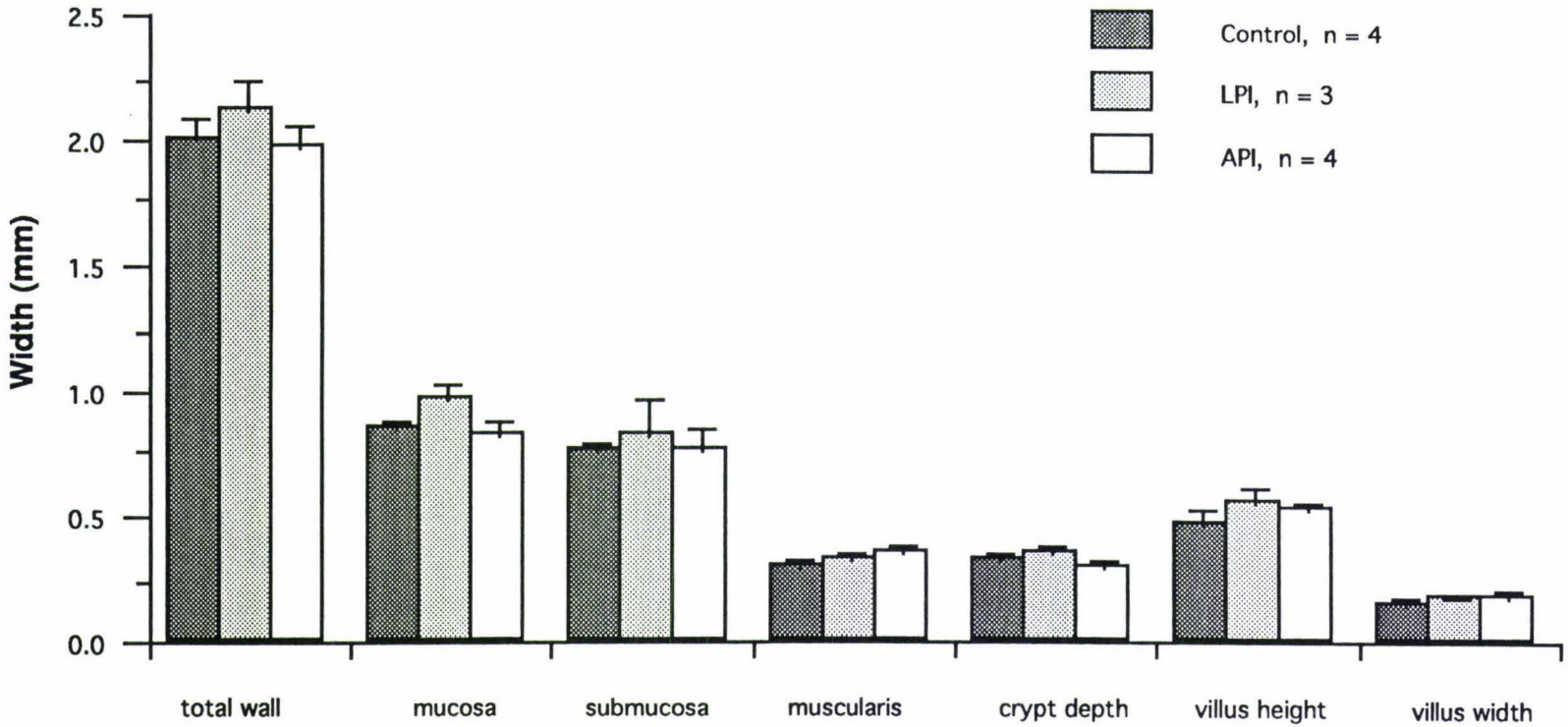


Figure 3.10. Tissue thickness, crypt depth, villus height and villus width in the lower duodenum (mean ± s.e.). LPI = larval parasite infected sheep, API = adult parasite infected sheep.

In the jejunum (Figure 3.11.), the villus width was significantly ($P<0.05$) increased in the LPI sheep compared to the non-infected control sheep. There were no other significant changes to the jejunum of the parasitised sheep compared to controls, however the means for all of the measurements made on the jejunum of the API sheep except for the villus width, were less than those for the control sheep.

There were no significant differences between the tissue measurements in the ileum (Figure 3.12.), caecum (Figure 3.13.) or colon (Figure 3.14.) of either the API or the LPI groups compared to the control group.

3.4. DISCUSSION

3.4.1. Histology

The histological changes to the abomasal tissues of the infected sheep included the following.

1) In the sheep infected with the adult parasites, there were aggregates of large clear mucous-secreting cells located on the luminal side of the abomasal mucosa. Neutrophils were present in the abomasal lamina propria and within the lumen of some abomasal glands.

2) At euthanasia, larvae at different stages of development were present in the abomasal mucosa of the LPI sheep. The glands containing the larvae were distended and most which did not contain larvae were filled with cellular debris, eosinophils and neutrophils.

3) In the LPI sheep, there were aggregates of large clear mucous-secreting cells in the upper quarter of the abomasal mucosa. In the lamina propria of the abomasum there were glandular leukocytes and mitotic figures present, as well as neutrophils, eosinophils and plasma cells.

The histological changes to the abomasum in response to infection with adult *O.*

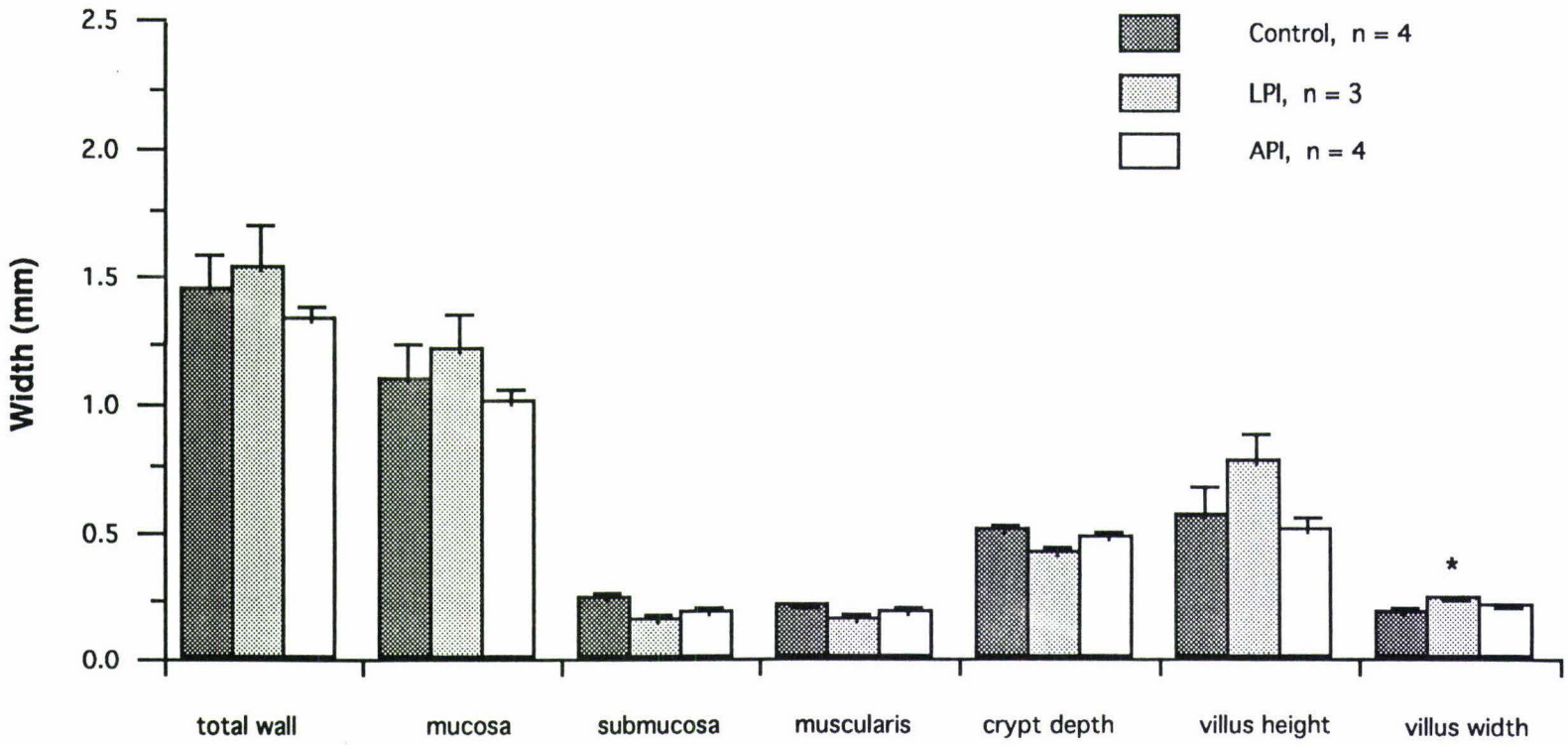


Figure 3.11. Tissue thickness, crypt depth, villus height and villus width in the jejunum (mean ± s.e.). LPI = larval parasite infected sheep, API = adult parasite infected sheep.
* $P<0.05$

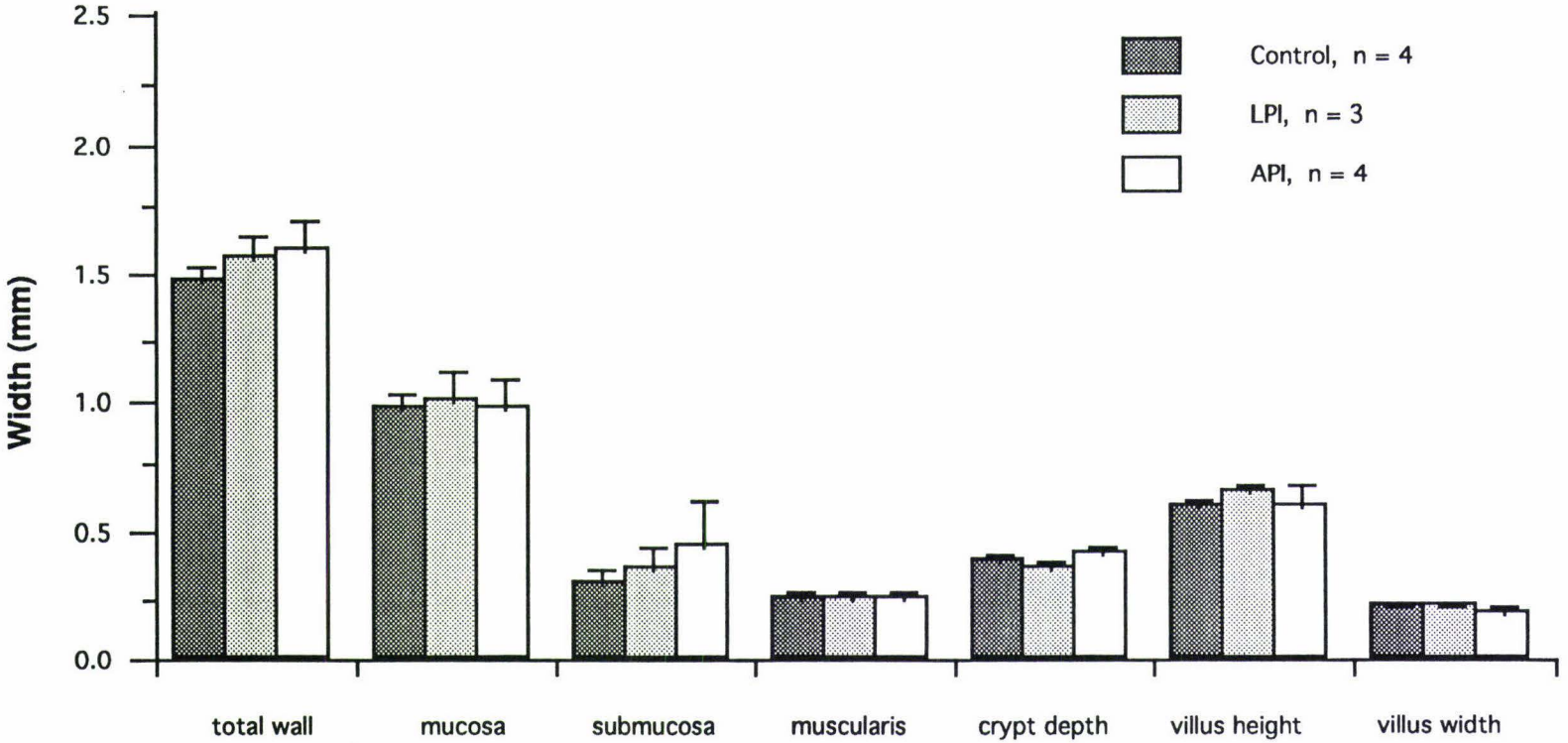


Figure 3.12. Tissue thickness, crypt depth, villus height and villus width in the ileum (mean ± s.e.). LPI = larval parasite infected sheep, API = adult parasite infected sheep.

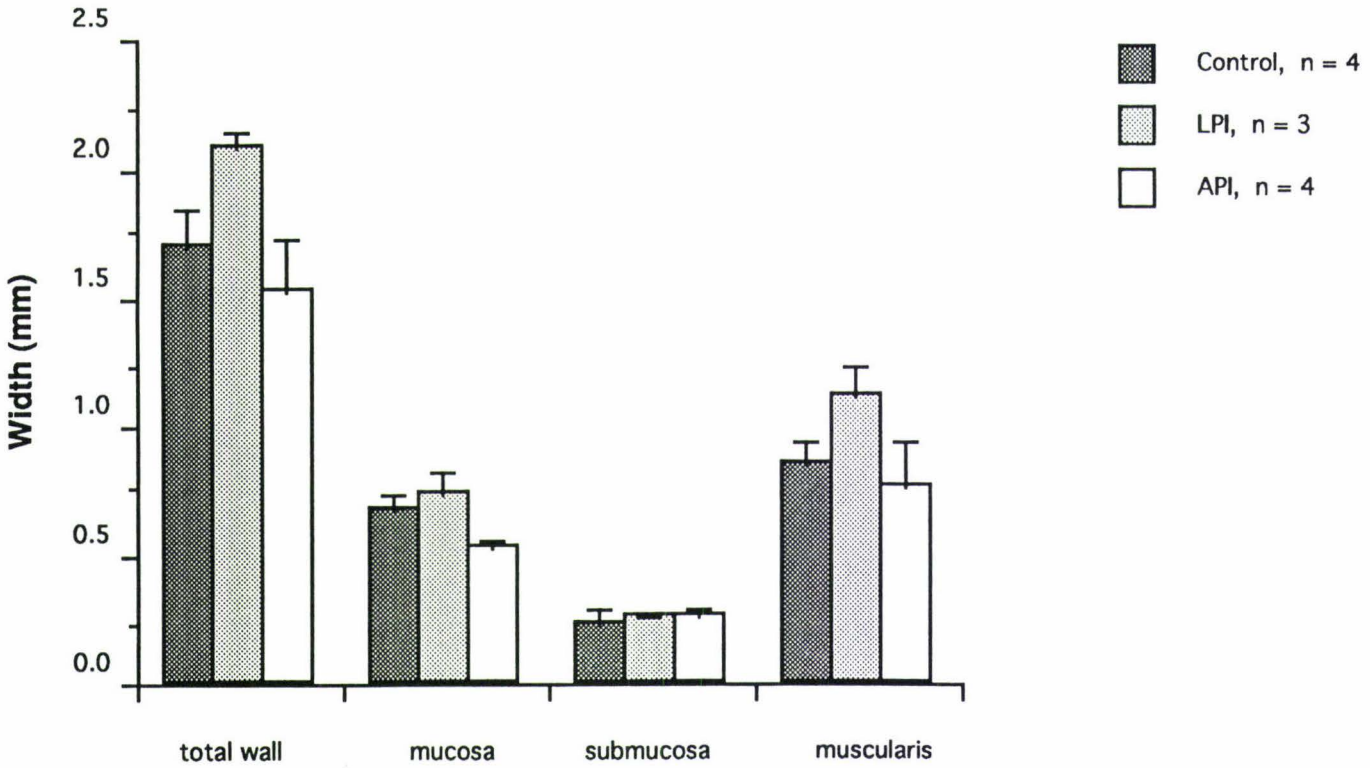


Figure 3.13. Tissue thickness in the caecum (mean ± s.e.). LPI = larval parasite infected sheep, API = adult parasite infected sheep.

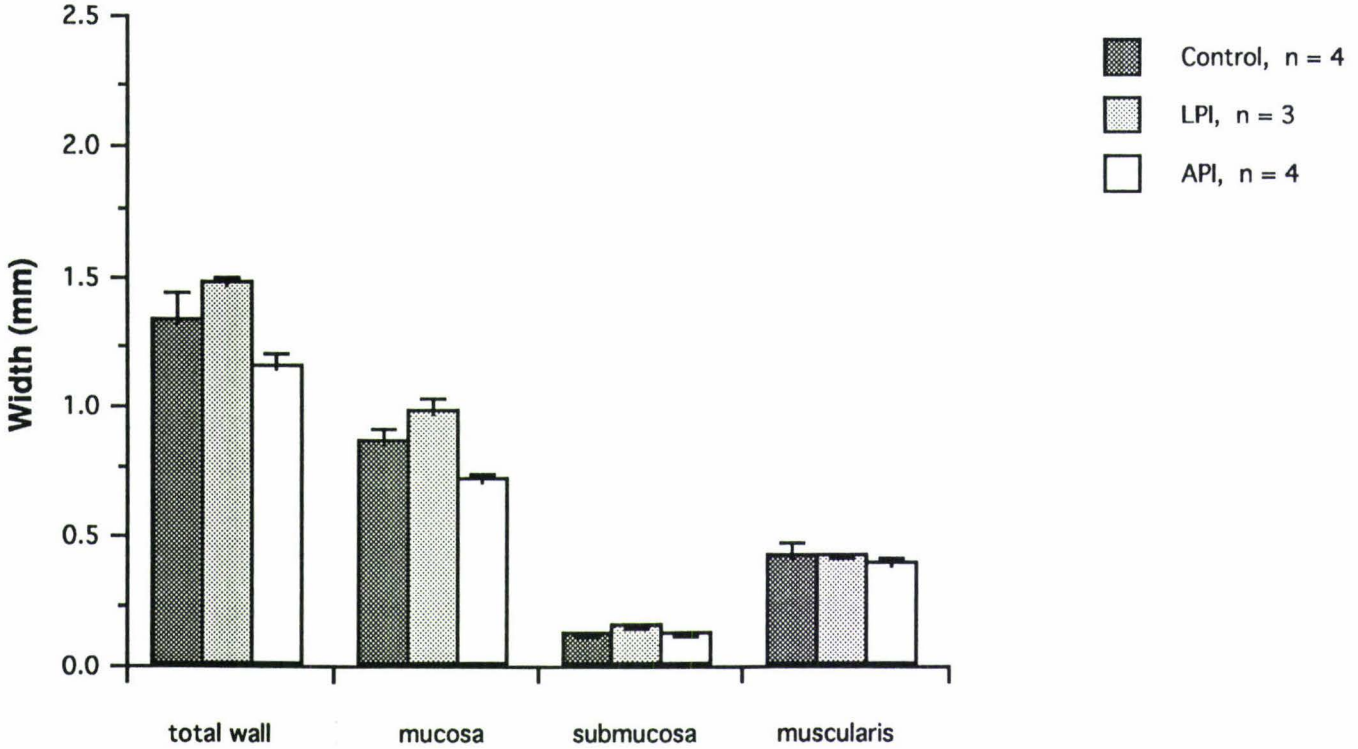


Figure 3.14. Tissue thickness in the colon (mean ± s.e.). LPI = larval parasite infected sheep, API = adult parasite infected sheep

circumcincta parasites have not been previously described. There appeared to be fewer changes present in the abomasal mucosa of the API sheep than in the LPI sheep. This is presumably because most of the damage done to the mucosa in the LPI sheep was due to the larvae entering the abomasal glands and their subsequent development in and emergence from the mucosa. *O. circumcincta* larvae "invade" individual gastric glands and the glands are reported to become progressively distended as the parasites grow (Armour *et al.*, 1966). The epithelial cells adjacent to the parasites are reported to develop a "dedifferentiated" appearance, with similar regressive changes in adjacent non-parasitised glands as the infection develops (Armour *et al.*, 1966; Durham and Elliot, 1976). These changes indicate that the larvae entering the glands and their subsequent development within the glands damage the abomasal mucosa. It is logical to conclude therefore that there will be less damage to the abomasal mucosa due to the presence of adult parasites than larvae.

Evidence of an inflammatory reaction was observed in the abomasal mucosa of the API sheep. This was in the form of the presence of neutrophils throughout the lamina propria, both within the lumens of some gastric glands and external to the glands. Adult *O. circumcincta* inhabit the abomasal lumen (Armour *et al.*, 1966) and do not appear to reenter the glands once they have emerged. Hence the inflammatory reaction must be elicited indirectly; e.g. due to secretory-excretory (SE) products produced by the adult worms. The immune response which is thought to be associated with gastrointestinal parasites is termed the immediate-type hypersensitivity reaction (Jarrett and Miller, 1982). The inflammatory mediators associated with this reaction include histamine, serotonin, slow reacting substance of anaphylaxis (SRS-A), prostaglandins and several granule associated enzymes (for review, see Miller, 1986). One or more of these mediators may be involved in this inflammatory reaction.

Large clear mucous secreting cells were observed at the lumen end of the gastric pits in the LPI sheep. These were mucus neck cells. They were clear most likely due to a lack of mucus. The parasites themselves or SE products released from the worms or other substances released from host tissues in response to the presence of the parasites may disrupt the mucus layer covering the abomasal lumen surface, overstimulating the

secretion from these cells, resulting in their depletion of mucus. Alternatively, the depletion of mucus may be due to an inhibitory effect of the parasites or their SE products on mucus production. The clear cells were all adjacent to the abomasal lumen. Other mucus-producing cells further down the mucosa did not appear to be devoid of mucus. Either the parasites only affected the mucus neck cells, or the parasites and their SE products had a direct effect on the production and/or secretion of mucus, thus only affecting the cells adjacent to the abomasal lumen. To determine whether the adult parasites and their SE products were altering the production of mucus within the cells, an assay which quantified a precursor molecule to mucus would need to be developed. If the concentration of this precursor molecule decreased in the abomasal mucosa of sheep infected with adult parasites compared with non-infected control sheep, this would demonstrate that the production of mucus in these sheep is compromised by the presence of adult *O. circumcincta* in the abomasum.

In the LPI sheep, the trickle infection of 10,000 larvae 3 times per week was initiated 3 weeks after the original infection and continued for 5 weeks, after which the sheep were euthanased. The trickle infection resulted in the presence at euthanasia, of larvae within the abomasal mucosa at different stages of development. Coiled larvae deep within the glands would have been administered to the sheep 4-8 days earlier unless the larvae were inhibited (Armour *et al.*, 1966). Larvae which were in the process of emerging from the mucosa would have been administered to the sheep 12-15 days before euthanasia (Armour *et al.*, 1966). The gastric glands which contained the coiled larvae were distended and the epithelium lining these glands was flattened. Similar observations have been reported previously by Armour *et al.* (1966), Durham and Elliot (1976) and Elliot and Durham (1976).

The lamina propria contained globular leukocytes which have been previously reported in association with *O. circumcincta* infections (Armour *et al.*, 1966; Durham and Elliot, 1976). The presence of many mitotic figures in the lamina propria indicates an increase in the rate of cell division in the LPI sheep. Neutrophils, eosinophils and plasma cells were present in the abomasal lamina propria of the LPI sheep. These would have migrated from local capillaries in the lamina propria (Armour *et al.*, 1966).

There were aggregates of large nearly clear mucus-secreting cells present on the lumen side of the mucosa in the LPI sheep. These cells were identified due to the slight blue staining of the mucus within them with the stain Alcian Blue. Gastric glands from which larvae have emerged have been reported to be lined with a tall, clear, mucous-secreting epithelium (Armour *et al.*, 1966). In the present study, the pits were lined with these cells as opposed to the glands. The reason for this is not apparent.

3.4.2. Morphometry

The morphometric measurements of the tissues of the abomasum and intestines revealed the following.

1) Infection of the abomasum of sheep with adult *O. circumcincta* parasites caused an increase in the mucosal thickness of the abomasal body; no significant increases occurred in the thickness of any of the tissues in other regions of the gut in the API sheep.

2) Infection with larval *O. circumcincta* caused an increase in the thickness of the mucosa of the abomasal body and antrum, increases in the depth of the gastric and antral glands and an increase in the depth of the gastric pits.

3) In the duodenum, there was an increase in the total wall thickness and mucosal thickness at the more proximal site following infection with the larvae.

In the abomasal body, increases in mucosal thickness occurred in both LPI and API sheep relative to controls indicating that the adult parasites themselves have a trophic effect on the mucosa of the abomasal body. Visual examination of the folds in the body region of the LPI sheep showed them to be thicker. The increased mucosal thickness is readily apparent in the photomicrographs (Figure 2.5.).

In the LPI group, the parasites were present for 55-57 days, during which time the larvae would have developed into adult parasites; *O. circumcincta* larvae develop into adult parasites over a period of 16-28 days unless inhibited (Armour *et al.*, 1966). The

presence of adult parasites in the abomasum was confirmed at post mortem by visual inspection of the abomasal contents and by the presence of eggs in the faeces of the sheep in both of the infected groups (Figure 2.4.). In the LPI sheep, the presence of the developing larvae in the mucosa may have contributed to the increase in body mucosal thickness, as the increase in mucosal thickness was more pronounced in LPI sheep than API sheep. Alternatively, the greater increase in the thickness of the mucosa in LPI sheep compared with API sheep may have been due to the greater length of time that the parasites were present in the abomasum of the LPI sheep (55-57 days compared to 8 days in the API sheep). The hypergastrinaemia which acompanies ostertagiasis occurred in the LPI sheep for a longer time than in the API sheep (Table 2.4), thus any trophic effects of gastrin (see Section 1.1.3.2.1.) would be expected to be more pronounced in the LPI sheep than in the API sheep.

Although there was an increase in the thickness of the mucosa of the abomasal body in both the LPI and API sheep, there was no significant increase in the total wall thickness of either group. This was due to an insignificant decrease in the thickness of the muscularis layer. The total size of the abomasum appeared to increase in the LPI sheep compared with the controls (Figure 2.5.). The trophic factor inducing this increase in total abomasal size is most likely gastrin. There is no evidence that gastrin has a trophic effect on abomasal muscle tissue as it does on connective tissue and glandular tissue (Johnson, 1989). Therefore, the muscle layer may not increase in size in proportional to the other tissue layers such as the mucosa and submucosa.

In the body mucosa of the LPI sheep, there was a significant increase in both the gland depth and the pit depth, whereas in the antrum, there was a significant increase in the depth of the glands only. Gastrin has been previously shown to stimulate growth of the body mucosa (Johnson *et al.*, 1969; Chandler and Johnson, 1972; Stanley *et al.*, 1972; Johnson and Guthrie, 1974a; Castelyn *et al.*, 1977; Johnson, 1977; Majumdar and Goltermann, 1978). The present results indicate that the effect of gastrin on the mucosa may include increases in both the gland depth and pit depth of the mucosa. Gastrin has been demonstrated not to have a trophic effect on the mucosa of the antrum (Casteleyn *et al.*, 1977; Johnson, 1977). It is possible that the larval parasites and/or their

secretory/excretory products have a trophic effect on the mucosa, independant of gastrin. As the larvae inhabit the glands of both the antral and body regions of the abomasum, the glands in both these regions would show a trophic response. Therefore, in the body region, the increased thickness of the glands may be due to both the trophic effects of gastrin and the parasites directly, whereas in the antrum, the increased gland depth may be only due to the direct trophic effects of the parasites. A greater increase in mucosal weight in the body than in the antrum was reported by Anderson *et al.* (1988), who measured the weight of the mucosa following ostertagiasis in sheep.

A direct trophic effect of the larvae would also explain why the mucosal thickness in the body region increased to a greater extent in the LPI sheep than in the API sheep; no larvae were present in the API sheep. In the API group, no significant differences were present in abomasal gland or pit depths relative to the control sheep.

From these results it appears that the increase in the weight of the abomasum reported in the previous Chapter was largely due to an increase in the thickness of the mucosa which increased 98-174% relative to that in the control animals, as shown in Table 3.5. There also appeared to be an increase in the total area of the abomasum, as shown in Figure 2.5. which would have contributed to the increase in abomasal weight. The increased abomasal weight may also have been partly due to oedema which occurs as part of the inflammatory response. The occurrence of both an inflammatory response (Armour *et al.*, 1966; Durham and Elliot, 1975) and oedema (Durham and Elliot, 1976; Elliot and Durham, 1976) have been previously reported in association with *O. circumcincta* infections. Indications of an inflammatory response such as the infiltration of neutrophils and eosinophils into the lamina propria were present in this study (Section 3.3.1.). Processing tissues into paraffin wax with the attendant shrinkage of tissues as well as dehydration with the alcohol may have removed signs of oedema. This would result in an underestimation of the thickness of the mucosa. Any increase in mucosal thickness due to hypertrophy and hyperplasia would however be unaffected by the tissue processing.

Table 3.5. Percentage increase in abomasal wet weight and mucosal thickness in the adult and larval infected sheep relative to the control sheep.

Group	% increase in abomasal wet weight	% increase in thickness of the body mucosa	% increase in thickness of the antral mucosa
adult parasite infected	180%	129%	98%
larval parasite infected	197%	157%	174%

In the upper duodenum, the total wall and mucosal thickness increased in the LPI sheep relative to the controls. In both duodenal sites, a trend was present in the LPI sheep, with an increase in all parameters compared to the control sheep with the exception of the villus width at the upper site, which was the same in the LPI sheep as in the controls. This trend correlates with the heavier wet weight of the LPI duodenum, compared to the controls (Figure 2.4.) at the conclusion of the experiment. There was no increase in the thickness of any tissue layers in the lower duodenal site. The reason that there was an increase in the total wall thickness and mucosa in the upper duodenum in the LPI sheep but no increase in these parameters in the lower duodenum is not known. There is no evidence indicating a difference between the trophic effects of gastrin on the upper and lower duodenum. In this context, it is interesting to note that the gastrin content of the duodenum in sheep decreases from the pylorus to the hepatic flexure (Simpson *et al.*, 1993). Whether this decrease distally in the gastrin content of the duodenum influences the impact that ostertagiasis has on the tissues of the duodenum remains to be determined.

The thickness of the duodenal tissue layers in the API sheep were not significantly affected by the parasitism. This difference in results between the API and LPI sheep may have been due to either the greater length of time that the parasites were present in the LPI sheep compared with the API sheep allowing more time for the trophic

effects due to the hypergastrinaemia to occur or the trophic effects may have been due to the presence of the developing parasites as discussed above.

The width but not the height of the villi in the jejunum of the LPI sheep was significantly greater than in the control sheep. In the following Chapter, results for the concentrations of DNA, RNA and protein in the jejunal mucosa will be presented to determine whether this increased villus width is due to the presence of more cells (hyperplasia), or larger cells (hypertrophy).

Neither the adult not the larval parasites had a significant effect on the thickness of the tissue layers in the ileum, jejunum, caecum or colon. The wet weights of the ileum and jejunum and terminal ileum were significantly increased in API sheep relative to controls. This suggests that the small intestine may have increased in length in these animals as opposed to an increase in wall thickness.

3.5. SUMMARY

Both larval and adult *O. circumcincta* parasites exert a trophic effect on the abomasal body mucosa. The larvae affect both the glands and gastric pits in the mucosa. Larvae also have a trophic effect on the antral mucosa, with an increase in gland depth, with no significant effects on pit depth.

In the duodenum, adult *O. circumcincta* have no significant effects on tissue layer thickness. Larvae increase mucosal and total wall thickness at proximal sites and may have a slight trophic effect on other tissue layers.

O. circumcincta parasites do not affect the thickness of the ileum or jejunum tissue layers, or the caecum or colon.

In the following Chapter, the results from assaying the mucosa of the regions used in this study for RNA, DNA and protein will be presented to determine whether the increases in thickness measured in this study were due to hypertrophy or hyperplasia.

4. HYPERTROPHIC AND HYPERPLASTIC CHANGES IN THE GASTROINTESTINAL MUCOSA OF SHEEP INFECTED WITH ADULT OR LARVAL *OSTERTAGIA CIRCUMCINCTA*

4.1. INTRODUCTION

Growth of an organ may involve hyperplasia, i.e. an increase in the number of its component cells, or hypertrophy, i.e. an increase in the size of existing cells, or a combination of both of these. The terms hyperplasia and hypertrophy were originally used to refer to abnormal growth, but both are now commonly used to describe "normal" growth. The DNA content of cells with the same ploidy (number of chromosome copies) is constant in each animal species (Boivin *et al.*, 1948). This enables a tissue's DNA content to be used as an index of the number of cells in that tissue. An increase in the DNA content of an organ represents hyperplastic growth. Dividing the RNA and protein content of a tissue by the DNA content gives an index of the amount of RNA and protein per cell. An increase in either of these indices is a measure of hypertrophic growth (Winick *et al.*, 1972). The concentration, as opposed to content of DNA, RNA and protein within a tissue can also be used to determine the protein:DNA and RNA:DNA ratios which give an index of the amount of RNA and protein per cell respectively.

There is rapid turnover of enterocytes in the mucosa of the gastrointestinal tract (for review see Johnson, 1989). Proliferation and growth of the gastrointestinal mucosa are balanced by cell loss from the mucosal surface through exfoliation, maintaining the cell population in a steady state. An increase in cell division or decreased rate of exfoliation may result in hyperplasia.

During cell division, there are certain "restriction points" at which influences which are external to the process of cell division are required for cell division to continue. The ability of eukaryotic cells to cross these "restriction points" and complete cell division is modulated by both positive and negative influences (Korc, 1991). These may include environmental factors (e.g. cell density and ambient temperature), nutritional factors (e.g.

the availability of amino acids and trace elements), intracellular regulatory mechanisms (Korc, 1991) and growth factors and mitogens (Sato *et al.*, 1991). Examples of growth factors (for review see Korc, 1991) are insulin, insulin-like growth factors (IGF) I and II, relaxin, epidermal growth factor (EGF), inhibin A and B, bombesin, cholecystokinin (CCK), gastrin, basic and acidic fibroblast growth factors, enteroglucagon and neurotensin. Growth factors may exert trophic effects on 1 or 2 cell types (e.g. gastrin, CCK and bombesin) or they may exert trophic effects on many different cell types (e.g. IGF-I). Growth factors reach their target cells as neurotransmitters, as paracrine agents affecting nearby cells or as hormones affecting distant target cells (Woll and Rozengurt, 1989; Rozengurt *et al*, 1990).

The peptide bombesin and its mammalian analogue gastrin releasing peptide (GRP) have been shown to be potent mitogens for Swiss 3T3 cells in the absence of other growth promoting factors (Rozengurt *et al*, 1990), indicating that the bombesins have direct trophic effects on cells. Chronic administration (1 week) of bombesin has been shown to induce gastrin cell hyperplasia in the rat (Lehy *et al.*, 1983). The mitogenic actions of the bombesins are potentiated by insulin which increases the maximal response to bombesin and reduces the bombesin concentration required for the half maximal effect (Woll and Rozengurt, 1989).

Cell proliferation in cell culture systems is stimulated by EGF (Hollenburg and Cuatrecasas, 1975). Intestinal DNA synthesis and mitosis are also stimulated by EGF in the intact rat when EGF is administered intravenously. This effect is most pronounced in the colon (Goodlad *et al.*, 1987). In humans, EGF has also been demonstrated to inhibit gastric acid secretion (Carpenter, 1985; Burgess, 1989).

Gastrin has a trophic effect on the mucosa of the body region of the stomach (Johnson *et al.*, 1969; Chandler and Johnson, 1972; Stanley *et al.*, 1972; Johnson and Guthrie, 1974a; Castelyn *et al.*, 1977; Johnson, 1977; Majumdar and Goltermann, 1978), the duodenum (Johnson *et al.*, 1969; Chandler and Johnson, 1972; Johnson and Guthrie, 1974a; Casteleyn *et al.*, 1977; Johnson, 1977), the jejunum (Casteleyn *et al.*, 1977), the ileum (Johnson and Guthrie, 1974a) the colon (Johnson, 1977) and the pancreas

(Mayston and Barrowman, 1971; Majumdar and Golterman, 1978) in the rat. Gastrin has no trophic effect on the oesophagus (Johnson, 1977), the antrum (Casteleyn *et al.*, 1977; Johnson, 1977), the liver (Johnson and Guthrie, 1974a) or the diaphragm (Johnson, 1977) in the rat.

The gastritis resulting from ostertagiasis has been described in many of the publications on this subject as involving a hyperplastic reaction. Armour *et al* (1966) described a severe hyperplasia of the gastric glands surrounding those which contained developing *O. circumcincta* larvae. Histological evidence for hyperplastic changes in the abomasal epithelial and glandular cells of parasitised sheep have been reported by Durham and Elliot (1976), however, the changes described were not proven to be hyperplastic, as opposed to hypertrophic. This distinction can be made by quantifying the protein, DNA, and RNA concentrations of infected mucosal tissues, calculating the RNA:DNA and protein:DNA ratios, and comparing these and the DNA content to those of non-infected control animals. The data presented in Chapter 2 showed increases in the wet weight/kg body weight of the abomasum and intestines during *O. circumcincta* infection. In Chapter 3, increases in the mucosal wall thickness in the abomasal body of both the larval and adult parasite infected sheep, the antrum of the larval parasite infected sheep and the upper duodenum of the larval parasite infected sheep were reported. The aim of the present study was to determine whether these increases in mucosal thickness are due to either hyperplastic or hypertrophic growth or a combination of the two.

4.2. METHODS

4.2.1. Sample Collection

The sheep used for the present study were those described in Chapter 2. They were raised to be free of Helminth parasites and infected with either larvae or adult *O. circumcincta* parasites as described in Sections 2.2.4. and 2.2.5. The sheep were euthanased and the gastrointestinal tract dissected as described in Section 2.2.11. Samples of the mucosa from the sites described in Table 4.1. were used in this study. Care was taken during the collection of the samples to keep them free of contaminants.

All samples were taken within half an hour of euthanasia. The mucosa was removed from the submucosa by blunt dissection or by scrapping with the back of a scalpel blade. The mucosal samples were then cut up finely with a scalpel blade, placed in Eppendorf tubes (Labserv, Biolab Scientific, N.Z.) and snap-frozen in liquid nitrogen. The tissues were stored frozen at -70 °C until assayed.

Table 4.1. Sites from which mucosa was collected for DNA, RNA and protein assays.

Sample	Site of sample
Abomasal body	90 to 120 mm distal to omasal-abomasal junction
Abomasal antrum	immediately proximal to the torus pyloricus
Duodenum	from the pylorus to the cranial flexure minus the tissue collected for morphometric measurement (Table 3.1.)
Pancreas	right lobe
Jejunum	halfway between the cranial flexure of the duodenum and the jejuno-ileal junction
Ileum	480 mm proximal to the ileo-caecal junction
Caecum	10-20 mm proximal to the apex
Colon	1 m distal to the ileo-caecal junction

4.2.2. Methods of Biochemical analysis

Duplicate subsamples of mucosa weighing between 100 and 105 mg (Mettler AT200 scales, Watson Victor, NZ) were used for the DNA, RNA and protein assays. Each subsample was homogenised in 5 ml of cold saline with an ultrasonic disintegrator (Soniprep 150, MSE Scientific Instruments, Sussex, England) and an exponential probe. To determine the optimum sonication amplitude, samples of mucosa from the antrum of one sheep were assayed for DNA and RNA after they had been sonicated at an

amplitude of 2-20 microns for intervals of 1 and 2 minutes as shown in Table 4.2.. An amplitude of 10 microns for 1 minute was found to give the maximum amounts of DNA and RNA following analysis (Table 4.2.), indicating that the cells were broken up sufficiently to release all of the cellular RNA and DNA, but the DNA and RNA sugars which are detected by this assay were not fragmented. The amplitude of 10 microns for 1 minute was therefore used for the experimental tissue.

Table 4.2. The effects of sonication amplitude and time on mucosal DNA and RNA concentrations.

Amplitude (μm)	Time (min)	Tissue concentration (μg/g) DNA	RNA
2	1	0.16	28
2	2	0.19	17
4	1	0.36	37
4	2	0.30	41
6	1	0.22	34
6	2	0.18	28
8	1	0.45	23
8	2	0.43	40
10	**1**	**0.61**	**44**
10	2	0.26	24
12	1	0.54	34
12	2	0.44	35
14	1	0.37	35
14	2	0.53	34
16	1	0.51	33
16	2	0.52	36
18	1	0.35	36
18	2	0.51	30
20	1	0.34	43
20	2	0.50	25

4.2.2.1. Protein Determination

The protein concentration of the homogenates produced as described in Section 4.2.2. was determined using the method of Lowry *et al* (1951). The standard used was bovine serum albumen (BSA) (Sigma, Louis, USA) dissolved in sterile 0.9% saline at a concentration of 500 µg/ml. This stock solution was further diluted with sterile 0.9% saline to produce standard solutions with BSA concentrations of 0, 10, 20, 30, 40, 50, 60 and 70 µg/0.2 ml. The 500 µg/ml stock solution of BSA was stored at 4 °C. It was discarded after 1 week and a fresh solution made.

Duplicate 0.2 ml aliquots of the homogenates or standards were mixed with 2 ml of a reagent containing 2% Na_2CO_3 (May & Baker Ltd., Dagenham, England), and 0.01% $CuSO_4$ (Scientific Supplies, Auckland, NZ) with 0.02% sodium-potassium-tartrate (Univar Analytical Reagents, Ajax Chemicals, N.S.W., Australia) added in 12 mm x 75 mm polystyrene disposable test tubes (Greiner GmbH, Germany). The samples were mixed with a vortex mixer (Chiltern MT19 Auto Vortex Mixer) at medium speed and left to stand at room temperature for 10 minutes. Folin's reagent diluted 1:1 with distilled water was then added (0.2 ml) and each sample mixed with the vortex mixer. After standing at room temperature for a further 30 minutes, the absorbance was read at 500 nm (Ultraspec III UV/visible Spectrophotometer, Pharmacia LKB, Cambridge, England) against a blank containing 0.9% saline, which was treated in the same manner as the standards and unknowns.

4.2.2.2. RNA and DNA extraction

The methods for the extraction of RNA and DNA were those described by Johnson and Chandler (1973). Duplicate 1 ml samples of the tissue homogenates (see Section 4.2.2.) were mixed using a vortex mixer at medium speed with 1 ml of 0.9% saline and 1 ml of 0.6 N perchloric acid (BDH Ltd., Poole, England) in 12 mm x 75 mm polystyrene disposable test tubes (Greiner GmbH, Germany). The samples were placed on ice for 10 minutes, then centrifuged at 500 *g* for 10 minutes (Beckman GPR centrifuge) and the supernatant discarded. The precipitates were then washed twice with 2 ml of 0.2 N perchloric acid (BDH Ltd., Poole, England) and centrifuged (Beckman GPR centrifuge) at 500 *g* for 10 minutes between each washing. All reagents were cooled to 4 °C before

being used and the samples centrifuged at 4 °C.

The pellets were resuspended in 2 ml of 0.3 N potassium hydroxide (BDH Analar, Poole, England) using a vortex mixer. The tubes were then incubated at 37 °C in a waterbath for 1 hour to dissolve the RNA. One ml of perchloric acid (BDH Ltd., Poole, England) diluted to a concentration of 1.2 N with double distilled water was then added and mixed using the vortex mixer. The tubes were left to stand on ice for 10 minutes to precipitate the DNA and protein and then centrifuged (Beckman GPR centrifuge) at 500 *g* for 15 minutes at 4 °C. The supernatant was collected into clean labelled test-tubes. The pellets were washed with 2 ml of 0.2 N perchloric acid (BDH Ltd., Poole, England), recentrifuged at 500 *g* for 15 minutes at 4 °C (Beckman GPR centrifuge) and the supernatants were pooled with the supernatants previously collected. This fraction contained the RNA.

The pellets containing the DNA were dissolved in 4 ml of 1.0 N perchloric acid (BDH Ltd., Poole, England) by heating in a boiling water bath at 100 °C for 10 minutes. This procedure caused the protein in the suspension to precipitate. The precipitated protein was removed by centrifugation at 500 *g* for 20 minutes (Beckman GPR centrifuge). The supernatant contained the DNA.

4.2.2.3. RNA Quantification

The absorbance of the RNA in the supernatant produced as described in section 4.2.2.2. was measured at the wavelengths 232 and 260 nm using a spectrophotometer (see section 4.2.2.1.) using 1 N perchloric acid as a blank. The equation by Fleck and Begg (1954) was used to determine the concentration of RNA.

$$C_{RNA} = (3.40 \times OD_{260} - 1.44 \times OD_{232})/0.068$$

4.2.2.4. DNA Assay

The fraction which contained the DNA (see section 4.2.2.2.) was assayed using the diphenalamine method reported by Burton (1956), as modified by Giles and Myers (1965). Herring sperm DNA (Boehringer Mannheim, West Germany) in 1 N perchloric acid (BDH Ltd., Poole, England) was used as a standard. A 0.5 mg/ml stock solution

of the DNA standard was made by dissolving 7.5 mg of Herring sperm DNA in 15 ml of 1 N perchloric acid (BDH Ltd., Poole, England) in a boiling water bath for 15 minutes. Evaporative losses were made up by adding 1 N perchloric acid to make up the 15 ml volume. The solution was frozen at -18 °C in aliquots of 1.5 ml in 12 mm x 75 mm polystyrene disposable test tubes (Greiner GmbH, Germany). The stock solution was thawed immediately before use. Triplicates of standards at DNA concentrations of 0, 5, 10, 20, 30 and 40 mg/ml were prepared in 1 N perchloric acid and assayed as described below.

Each sample (1 ml) and standard (1 ml) was mixed in 12 mm x 75 mm polystyrene disposable test tubes (Greiner GmbH, Germany) with 1 ml of 4% diphenalamine (Sigma, St Louise, USA) in glacial acetic acid (Rhone Poulenc Laboratory Products, Bristol, United Kingdom) using a vortex mixer (Chiltern MT19 Auto Vortex Mixer) at maximum speed. Aqueous 0.8 mg/ml acetaldehyde (BDH Ltd., Poole, England) (0.1 ml) was added and the tubes were mixed with the vortex mixer at maximum speed. Following incubation for 16-20 hours in a waterbath at 30 °C, the absorbances of the samples and standards were measured at 595 nm using a spectrophotometer (see section 4.2.2.1) against a 1 N perchloric acid blank treated as described above.

A total of four duplicates were assayed for each tissue sample. Duplicates which differed from each other by more than 10% were reassayed.

The inter- and intra-assay variations (Table 4.3.) were determined by assaying 4 duplicate samples of the same tissue in each assay. The variation was calculated by dividing the square root of the mean squares by the mean value.

4.2.3. Cell Counts

The number of cells/mm^2 of mucosa in the abomasum, duodenum, ileum and jejunum were counted as follows. Slides were prepared of the tissue samples collected from the regions described in Table 3.1., in the manner described in Section 3.2. and stained with haematoxylin and eosin and alcian blue as described in Table 3.3.. Two non-sequential sections from the same paraffin block were examined for each region of each sheep.

Stained nuclei were counted using a 40x objective lens, in an area 0.14 x 0.14 mm (Leitz Periplan GF10^{x} eyepiece, Ernst Leitz, Wetzler, Germany with counting graticule), measured with an Olympus objective micrometer (Olympus Optical Co. Ltd., Tokyo, Japan). The number of nuclei in five randomly selected areas was counted on each slide, giving a total area of 0.196 mm^2 counted per tissue region. The number of cells with nuclei in this area was then converted to the number of cells per mm^2 by dividing the number of stained nuclei by 0.196.

Table 4.3. Inter- and intra-assay variations for the DNA, RNA and protein assays.

Assay	Inter-assay variation (%)	Intra-assay variation (%)
DNA	4.3	6.6
RNA	15.2	5.9
protein	14.6	8.1

4.2.4. Statistics

The significance of the differences between the means was determined using a two-tailed analysis of variance and the Fisher probability of least significant difference (PLSD) coefficient. All analyses were performed using the computer program StatView SE + Graphics version 1.03 (Abacus Concepts Inc., California, U.S.A.) A *P* value of 0.05 or less was considered significant.

4.3. RESULTS

The concentration of RNA in the mucosa of the abomasal body of the larval parasite infected (LPI) sheep was significantly ($P<0.05$) less than that for the control sheep (Figure 4.1.). This resulted in a significantly ($P<0.05$) lower RNA:DNA ratio indicating that the amount of RNA per cell in the mucosa of the abomasal body of the LPI sheep had decreased compared to the control sheep. There was no corresponding decrease in

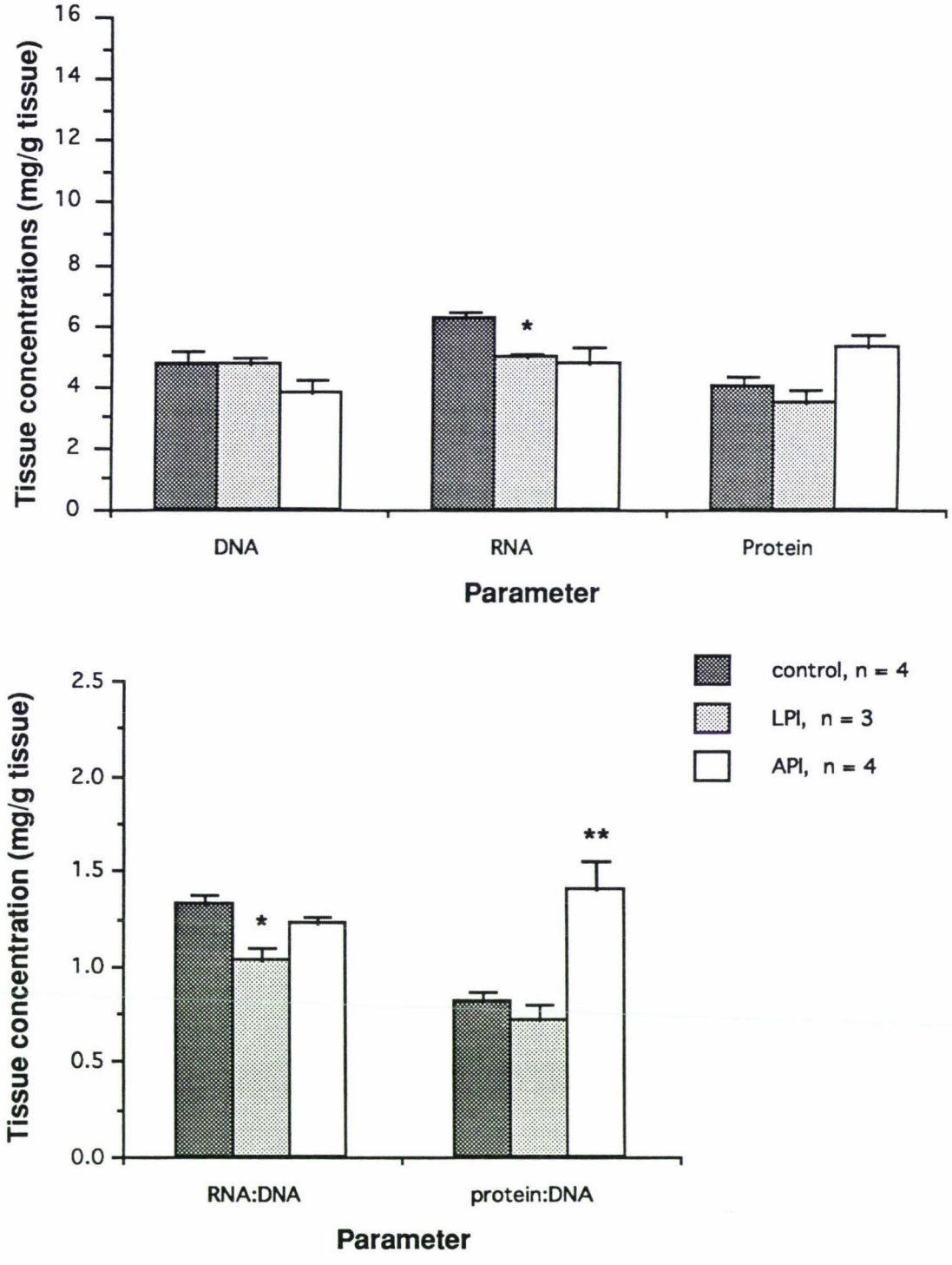


Figure 4.1. Mucosal DNA, RNA and protein concentrations and RNA:DNA and protein:DNA ratios in the body region of the abomasum (mean ± s.e.). LPI = larval parasite infected sheep, API = adult parasite infected sheep.
* $P < 0.05$ ** $P < 0.01$

the amount of protein per cell (protein:DNA ratio). The DNA concentration in the mucosa of the abomasal body of the LPI sheep did not differ significantly from that of control sheep. In the adult parasite infected (API) sheep, the protein:DNA ratio in the mucosa of the abomasal body (Figure 4.1.) was significantly ($P<0.01$) increased compared to non-infected controls. This increase in the protein:DNA ratio appeared to be due to a combination of a non-significant decrease in the mucosal DNA concentration and a non-significant increase in the mucosal protein concentration.

Infection with larvae or adult *O. circumcincta* parasites had no significant effects on the antral concentrations of RNA, protein or DNA, or the antral RNA:DNA and protein:DNA ratios (Figure 4.2.).

The duodenal concentration of RNA was significantly lower in both the LPI ($P<0.01$) and the API ($P<0.05$) groups compared to the control animals (Figure 4.3.). These differences were not reflected by significant reductions in the RNA:DNA ratios. No other significant differences were present in the duodenal mucosa of either the LPI or the API sheep, compared to the control sheep.

The larvae and adult parasites had no significant effects on the pancreatic RNA, protein and DNA concentrations or the RNA:DNA and protein:DNA ratios compared to the control sheep (Figure 4.4.).

The concentration of DNA in the jejunal mucosa was significantly ($P<0.01$) greater in the LPI sheep than in the control animals (Figure 4.5.). There was a corresponding significant ($P<0.01$) decrease in the RNA:DNA ratio of the LPI sheep compared to the non-infected controls. The jejunal protein concentration was also significantly ($P<0.05$) greater in the LPI sheep than in the controls, but no significant difference in the protein:DNA ratio was observed. There were no significant differences in the jejunum of the API sheep compared to the non-infected control group.

The RNA, DNA and protein concentrations and the RNA:DNA and protein:DNA ratios for the ileum (Figure 4.6.) and caecum (Figure 4.7.) of the 2 infected groups were not

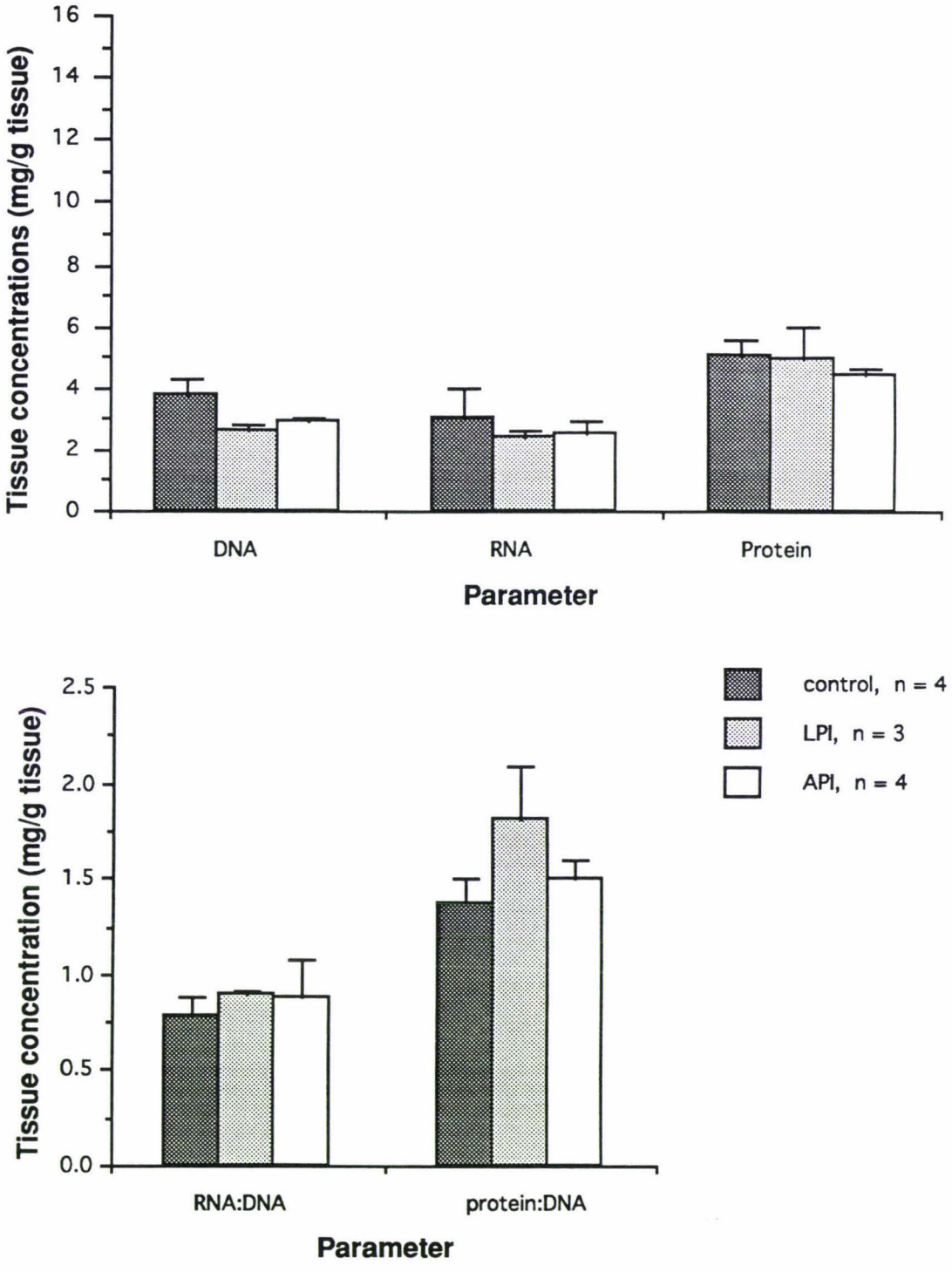


Figure 4.2. Mucosal DNA, RNA and protein concentrations and RNA: DNA and protein: DNA ratios in the antral region of the abomasum (mean ± s.e.). LPI = larval parasite infected sheep, API = adult parasite infected sheep

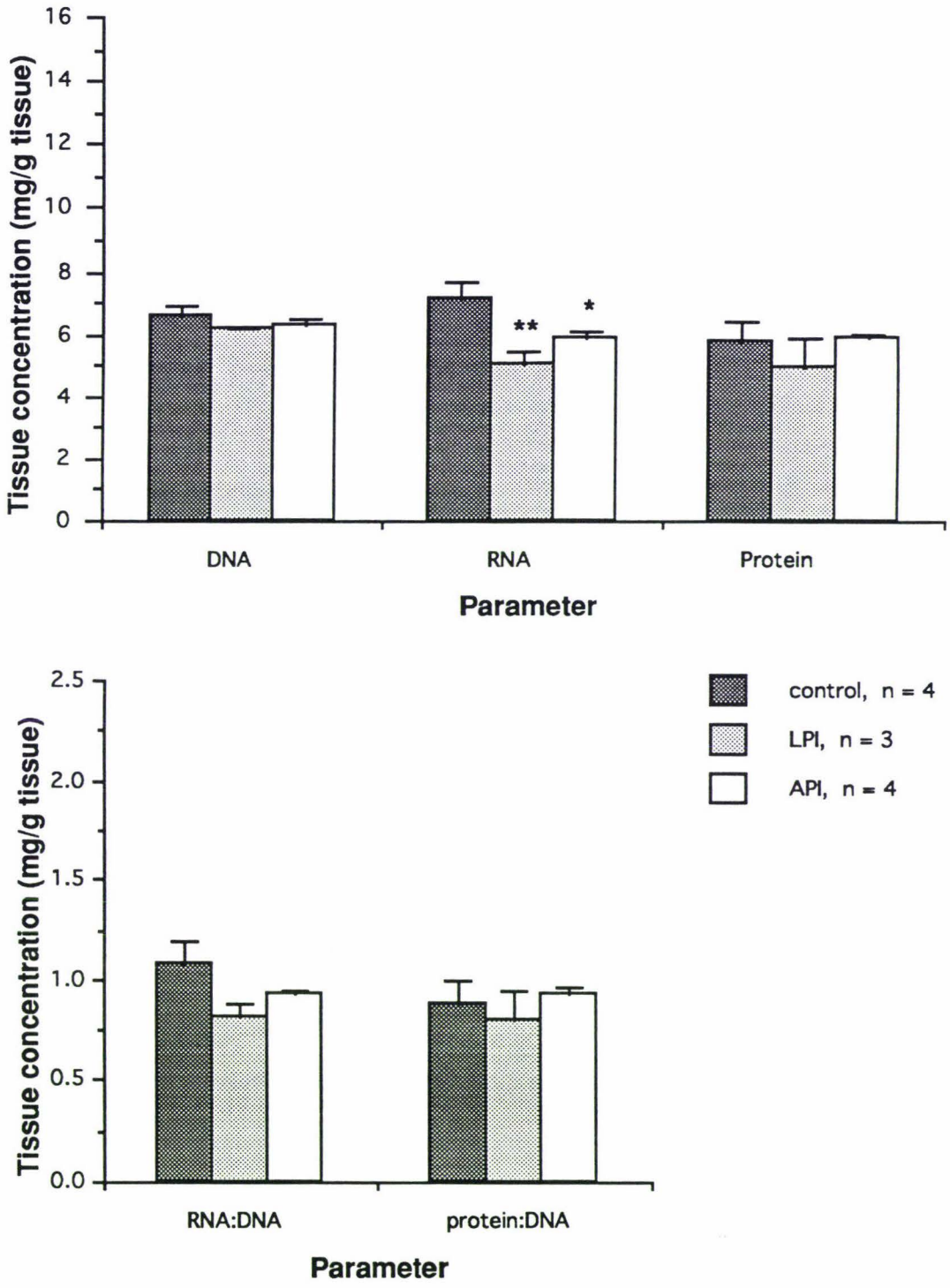


Figure 4.3. Mucosal DNA, RNA and protein concentrations and RNA: DNA and protein: DNA ratios in the duodenum, (mean $\pm$ s.e.). LPI = larvae parasite infected sheep, API = adult parasite infected sheep.
* $P < 0.05$ ** $P < 0.01$

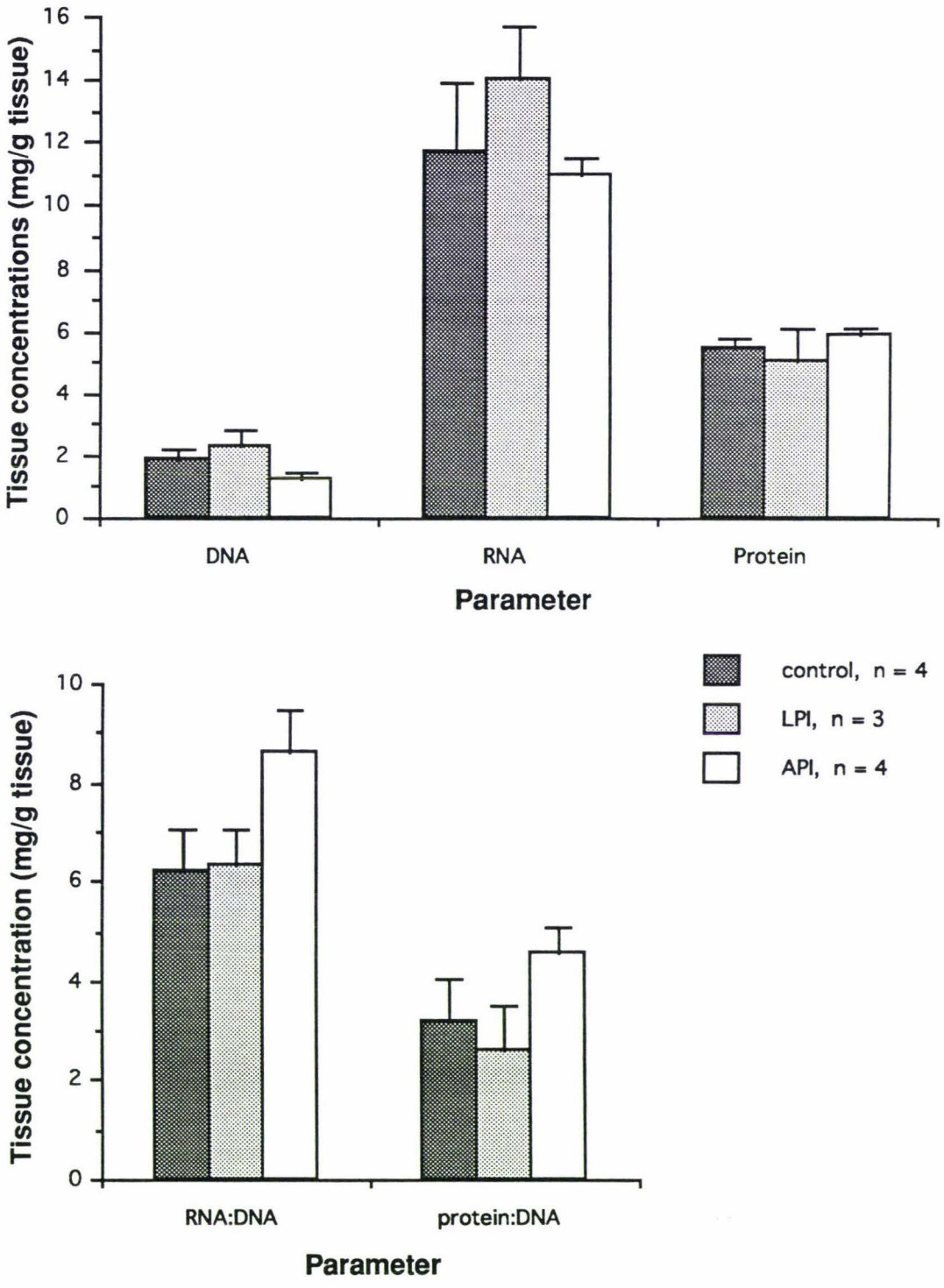


Figure 4.4. DNA, RNA and protein concentrations and RNA: DNA and protein: DNA ratios in pancreatic tissue, (mean ± s.e.). Note the different scale in the second graph to other graphs in this chapter. LPI = larval parasite infected sheep, API = adult parasite infected sheep.

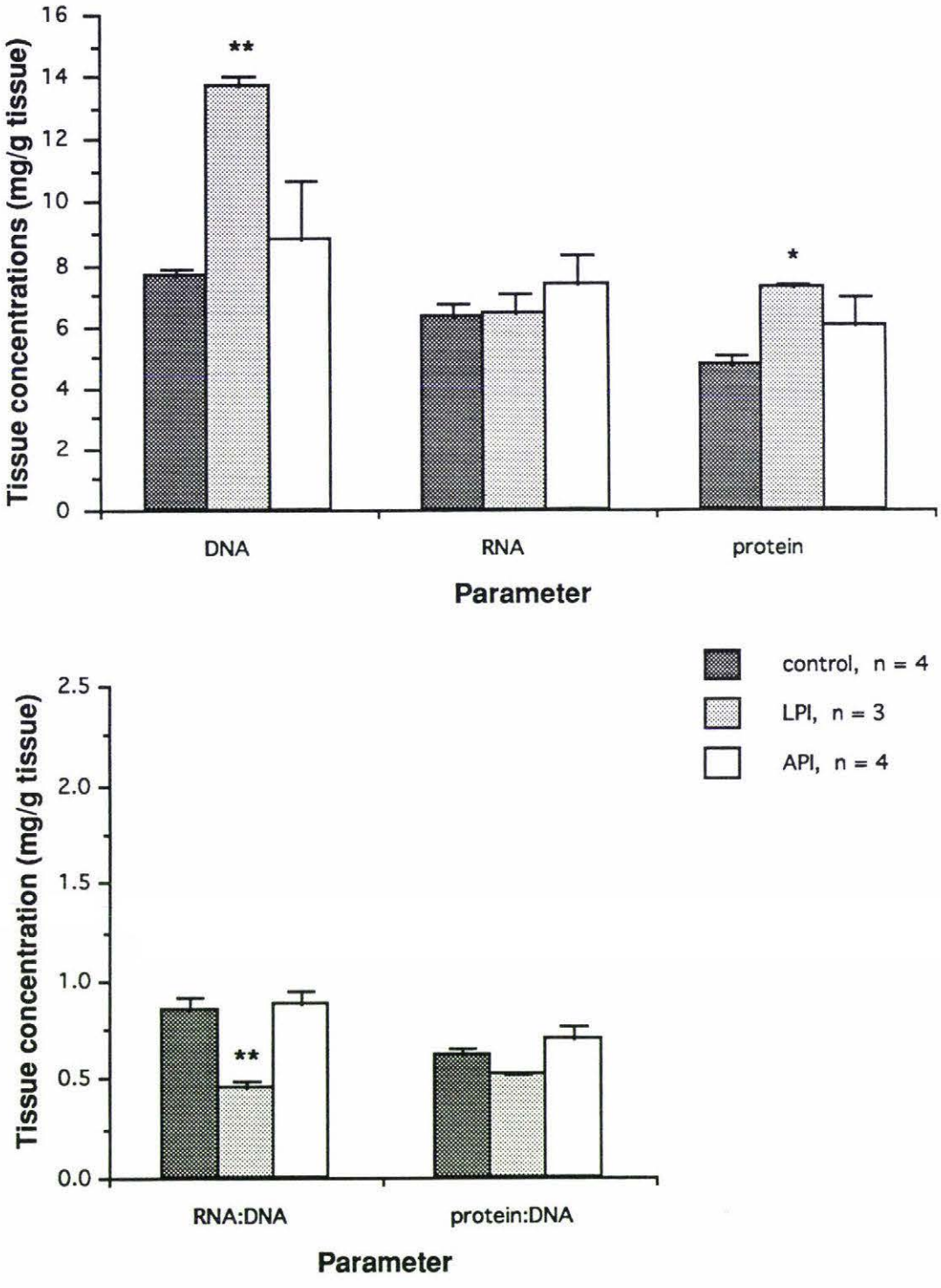


Figure 4.5. Mucosal DNA, RNA and protein concentrations and RNA: DNA and protein: DNA ratios in the jejunum, (mean ± s.e.). LPI = larval parasite infected sheep, API = adult parasite infected sheep.
* $P < 0.05$ ** $P < 0.01$

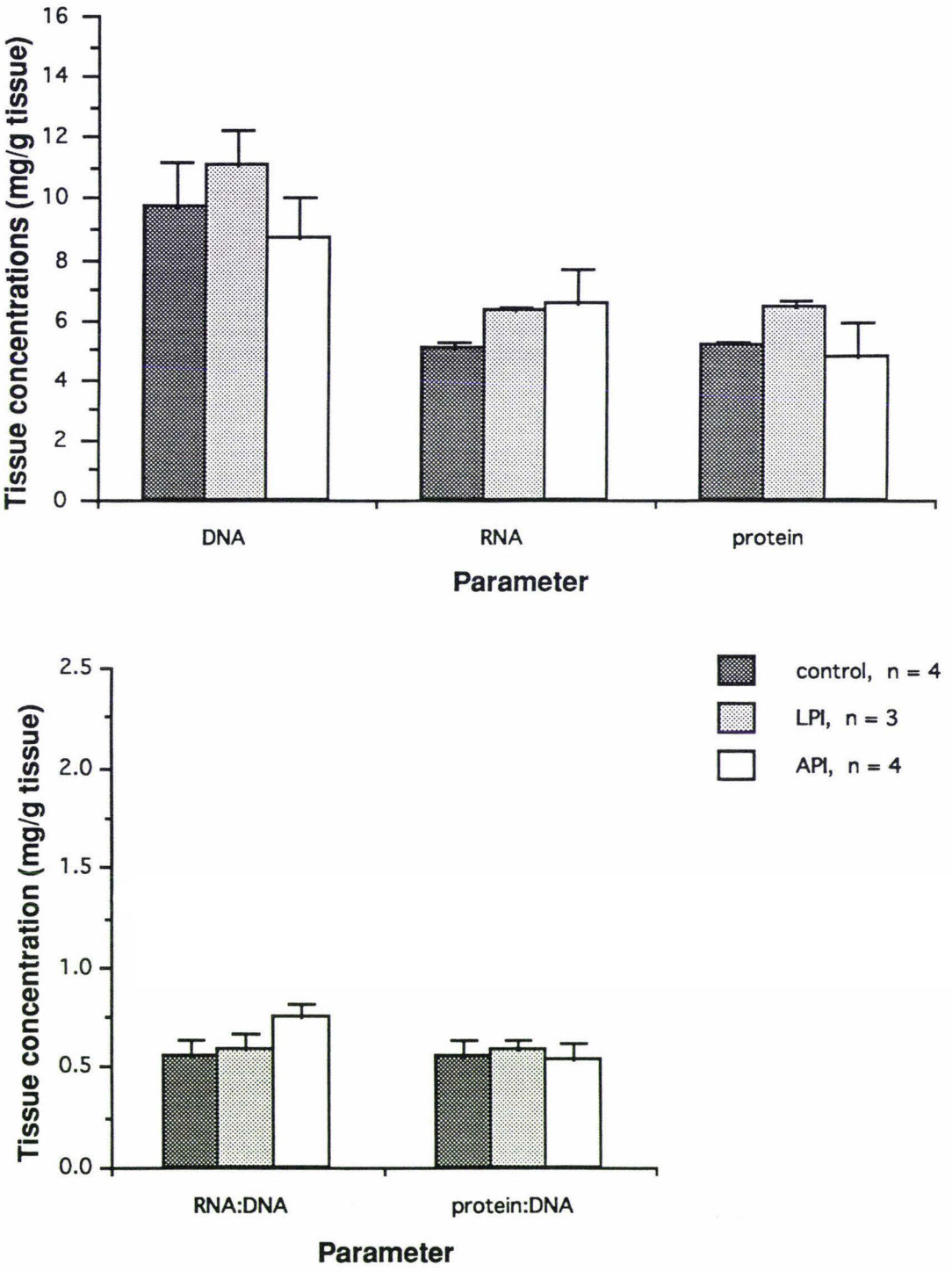


Figure 4.6. Mucosal DNA, RNA and protein concentrations and RNA: DNA and protein: DNA ratios in the ileum, (mean ± s.e.). LPI = larval parasite infected sheep, API = adult parasite infected sheep.

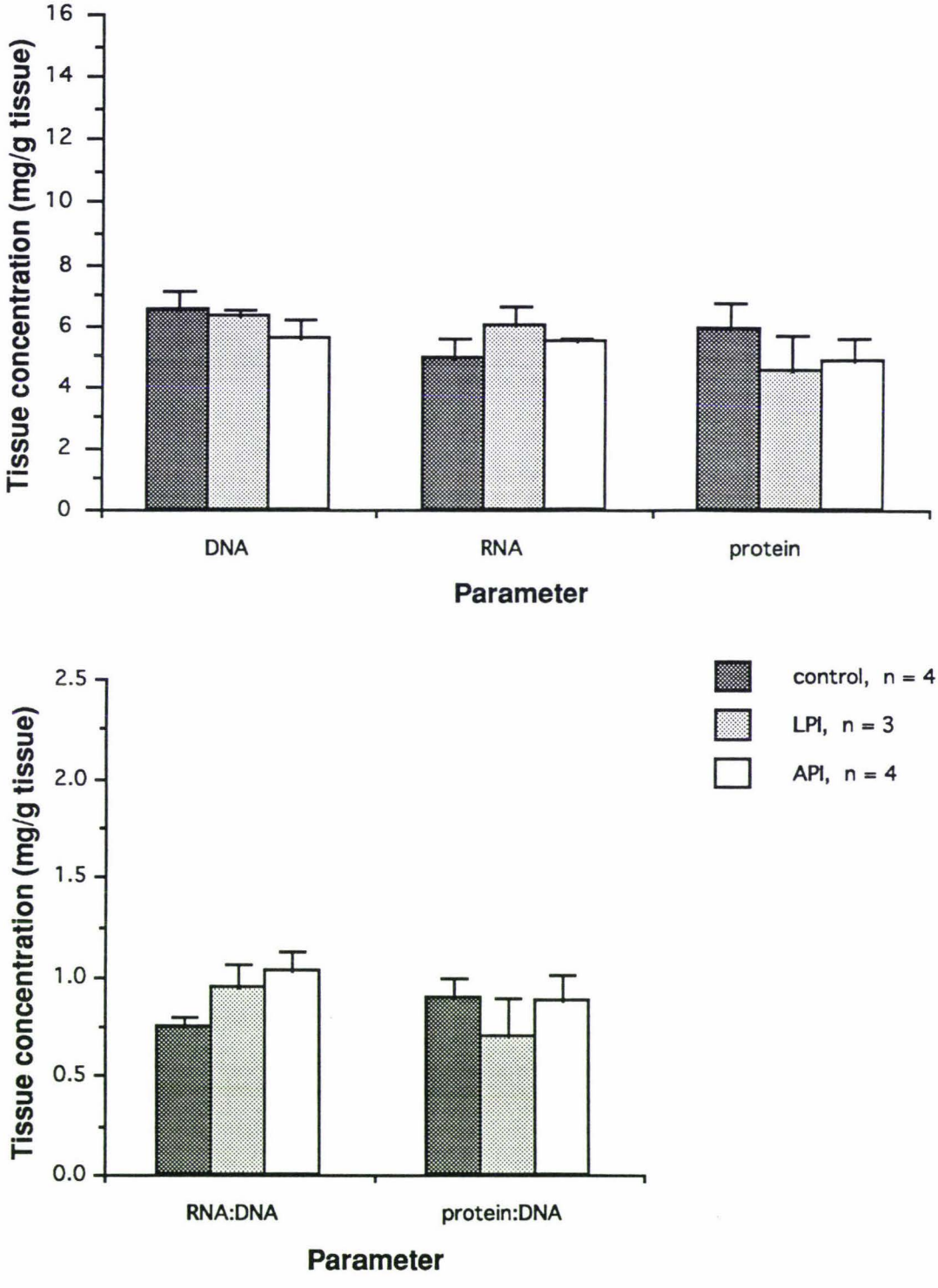


Figure 4.7. Mucosal DNA, RNA and protein concentrations and RNA: DNA and protein: DNA ratios in the caecum, (mean ± s.e.). LPI = larval parasite infected sheep, API = adult parasite infected sheep.

significantly different from those for the control group.

The concentration of RNA in the colon (Figure 4.8.) of the API sheep was significantly ($P<0.05$) greater than that for the control animals, but there was no change in the RNA:DNA ratio. No other significant differences were observed in the colonic mucosa of either the LPI or the API sheep relative to the control sheep.

The number of cells/mm^2 in the abomasal body was significantly ($P<0.01$) greater in the LPI sheep, but not the API sheep, compared to the controls (Figure 4.9.). Conversely, in the antrum of the API sheep, but not the LPI sheep, the number of cells/mm^2 was significantly ($P<0.01$) greater than that in the control animals. The number of cells/mm^2 in the jejunum was significantly ($P<0.01$) greater in LPI sheep than in the control sheep. There were no significant differences between the number of cells in the duodenum or ileum of either of the infected groups and the non-infected control animals (Figure 4.9.). The standard errors for the number of cells in the proximal duodenum of the LPI sheep and in the ileum of API sheep were very large, which may have obscured any differences between infected and control animals in these regions.

4.4. DISCUSSION

The effects of adult and larval *O. circumcincta* infections on mucosal RNA, DNA and protein levels have not been previously reported. The present study has shown that:

1) The mucosal concentration of RNA and the RNA:DNA ratio, i.e. the amount of RNA per cell, in the body region of the abomasum were both significantly decreased in sheep infected with *O. circumcincta* larvae, but not adult parasites. These changes in the larvae infected sheep were accompanied by an increase in the number of cells/mm^2 of mucosa.

2) The adult *O. circumcincta* parasites, but not the larvae, caused a significant increase in the protein:DNA ratio in the mucosa of the body region of the abomasum, but had no significant effect on cell numbers.

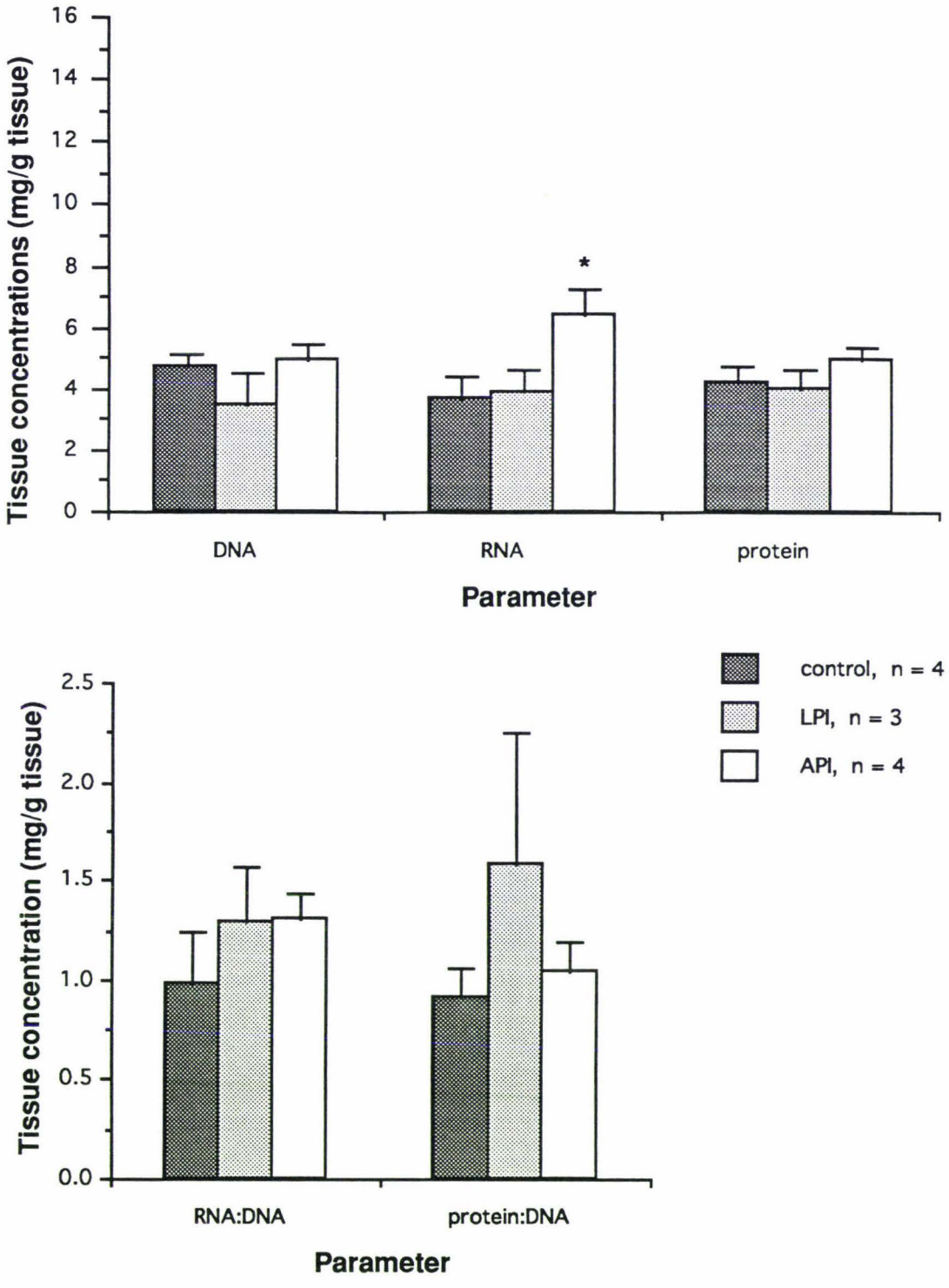


Figure 4.8. Mucosal DNA, RNA and protein concentration and RNA: DNA and protein: DNA ratios in the colon, (mean ± s.e.). LPI = larval parasite infected sheep, API = adult parasite infected sheep.
* $P < 0.05$

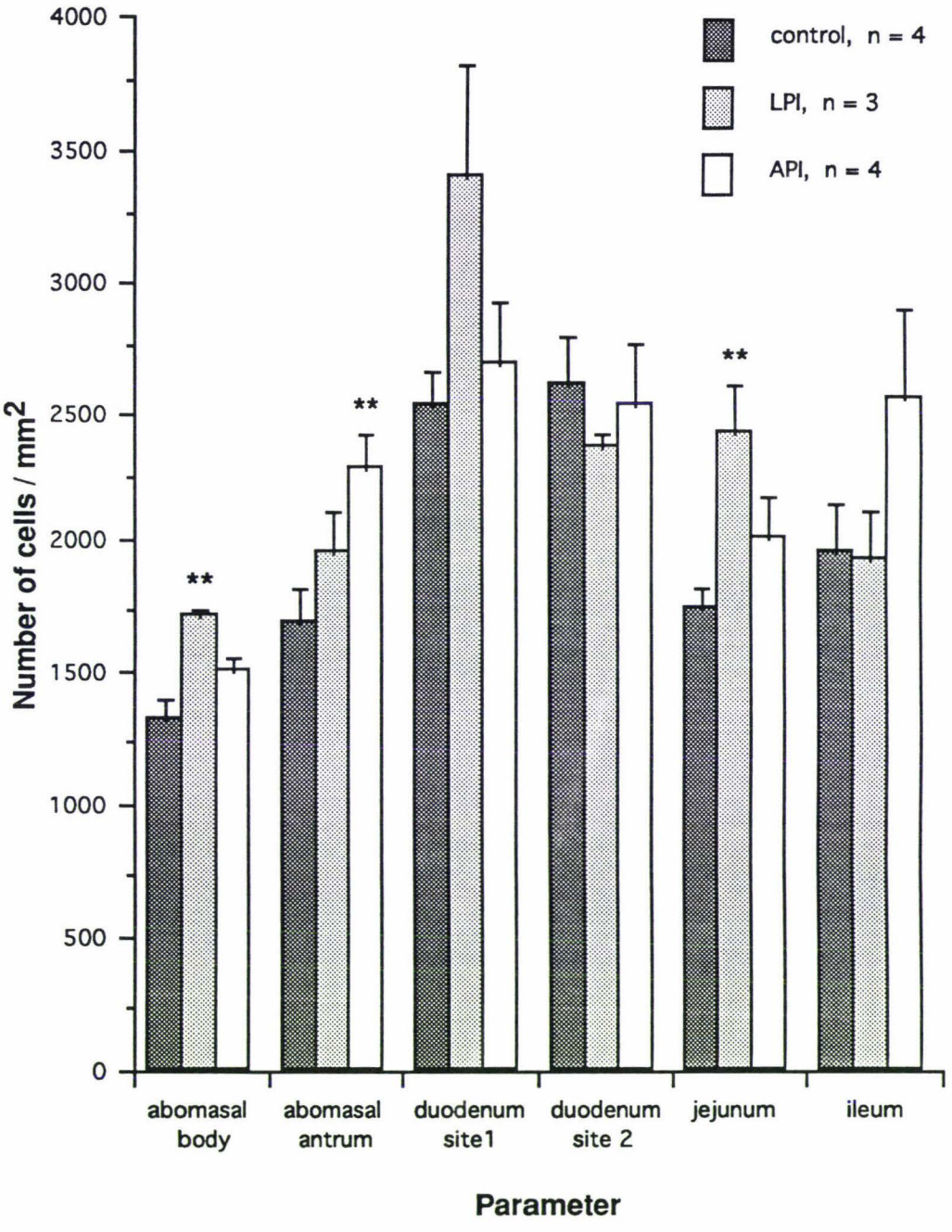


Figure 4.9. Number of cells/mm^2 in the mucosa of the abomasum, duodenum and intestines (mean ± s.e.). LPI = larval parasite infected sheep, API = adult infected sheep.
** $P < 0.01$

3) The adult parasites, but not the larvae, caused a significant increase in the number of mucosal cells/mm^2 in the abomasal antrum but neither the larvae nor the adult parasites affected the antral RNA, DNA and protein levels.

4) In the duodenum of both the larvae and adult infected groups, the RNA concentration was significantly lower than it was in the control animals. There were no significant changes in cell numbers within this region of the gut.

5) In the jejunum there were significantly higher concentrations of DNA and protein in the larvae infected sheep than in the controls. These changes were accompanied by an increase in the number of cells/mm^2 of mucosa in the jejunum. The jejunal RNA:DNA ratio decreased in the larvae infected sheep.

6) Neither the larvae nor the adult parasites affected mucosal RNA, DNA and protein levels or cell numbers within the ileum.

7) Caecal RNA, DNA and protein levels were not affected by the parasites.

8) In the colon, the adult parasites caused a significant increase in the mucosal concentration of RNA but neither the larvae nor the adult parasites affected the DNA or protein concentrations or the RNA:DNA and protein:DNA ratios.

The decreases in cellular RNA seen in the abomasum of the LPI group and the duodenum of both the API and LPI groups could have involved one or more of the three different types of RNA found within cells, all of which are involved in protein synthesis. These are messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA). During the process of transcription, mRNA forms using the cell's DNA as a template. The sequence of DNA nucleotide bases code for the eventual protein structure (for reviews, see Monier, 1972; Niederman, 1976; Polya, 1977). The mRNA then moves into the cytoplasm of the cell, and attaches to rRNA (Adams, 1977). A specific amino acid is attached to each tRNA, with the amino acid determined by the codon sequence

on the tRNA (Moldave, 1972; Zachau, 1972). The tRNAs then bind to the rRNA and peptide bonds form between the amino acids, forming the protein chain (for reviews, see Moldave, 1972; Niederman, 1976). Several copies of the protein are manufactured on each mRNA strand (Bloemendal, 1972; Adams, 1977). As the decreases in the concentration of RNA were not accompanied by decreases in the protein content of the mucosal cells (i.e. the protein:DNA ratio) the most likely type of RNA to be affected by the parasites is mRNA; a decrease in either rRNA or tRNA would be expected to cause a concomitant decrease in the cellular protein. There was no decrease in the protein concentration nor the protein:DNA ratio, possibly because several rRNAs can attach to a single mRNA strand (forming a polysome), producing many protein copies (Adams, 1977) from one mRNA strand. With fewer mRNAs present, more rRNAs would attach to each mRNA, forming more protein copies per mRNA strand.

An alternative cause for the decrease in the mucosal concentrations of RNA with no apparent change in the cellular protein content is the presence in the mucosa of protein from other sources such as serum albumen and plasma proteins. A hypoalbumenaemia is reported to occur during ostertagiasis (Coop *et al.*, 1977). Albumen may leak from the capillaries into the lamina propria. The results from the protein assay are the total protein within the mucosa. Therefore, there may have been a decrease in the amount of protein within each mucosal cell in the abomasal body of LPI sheep, which was not observed due to the presence of albumen and plasma proteins.

The decrease in RNA concentration in the body and duodenum of the LPI sheep may be due to a decrease in the general cell metabolism. Indicators of a decrease in cell metabolism are present in ostertagiasis. For example, parietal cells with the appearence of metabolically suppressed cells have been reported (McLeay *et al.*, 1973). McLeay *et al.* (1973) suggested that this inhibition of parietal cells was caused by the adult worms rather than the developing larvae due to the timing after the infection of the effects. The abomasal pH in the parasitised sheep increased (Table 2.4.), showing a decreased acid secretion. In the API sheep, there was a decrease in the duodenal concentration of RNA compared with the control sheep, but not in the abomasal body RNA concentration. There may have been a decrease in the general cell metabolism in

the API sheep, which was more marked in the duodenum than in the body, resulting in no significant changes in the RNA concentration of the abomasal body. A significant change in RNA concentration may have occurred in the abomasal body of the LPI sheep, but not the API sheep due to the greater length of time that the adults were present in the LPI sheep.

There were a greater number of cells/mm^2 in the abomasal body of the LPI sheep compared to the non-infected control sheep indicating that either hyperplasia had occurred or the cell size had decreased. Interestingly this increase in the concentration of cells was not accompanied by a significant increase in the mucosal DNA concentration. Why this should be so is not obvious. However, it is likely that the cell counts provide a more accurate estimate of cell concentration than does the assay of DNA. In the DNA assays, the inter-assay variation was 4.3%, and the intra-assay variation was 6.6%. The increased number of cells/mm^2 in the abomasal body of the LPI sheep may have been due to the neutrophils and eosinophils which had infiltrated the lamina propria. The decrease in the amount of RNA per cell in the LPI sheep compared to the control animals indicate that there was no hypertrophy occurring.

The increase in the protein:DNA ratio in the mucosa of the abomasal body in the API sheep was due to a non-significant increase in the mucosal protein concentration and a non-significant decrease in the mucosal DNA concentration. This increased protein:DNA ratio indicates that the increased mucosal thickness observed in this region and reported in Chapter 3 (Figure 3.1.) is due, in part at least, to hypertrophy, i.e. an increase in cell size. The increase in the protein content of the cells was not accompanied by an increase in the RNA content (RNA:DNA ratio), another indicator of hypertrophy. There are 2 possible explanations for this. Firstly, more protein copies may have been produced off each mRNA molecule (see above) and secondly, there may have been protein in the mucosa from other sources. Hypoalbumenaemia is reported to occur during infections with *O. circumcincta* larvae (Coop *et al.*, 1977). Serum albumen and plasma proteins may have leaked from capillaries into the lamina propria resulting in an increased protein concentration. The reason that there was an increased protein:DNA ratio in the abomasal body of the API sheep, but not the LPI sheep may

have been an increase in the albumen and plasma protein content of the API sheep mucosa. The API sheep appeared to have a larger parasite burden than the LPI sheep, as shown by the greater faecal egg counts (Figure 2.2.) and hypergastrinaemia (Table 2.4.) in the API sheep. Therefore, more albumen and plasma proteins may have leaked from the plasma into the lamina propria in the API sheep than in the LPI sheep, resulting in an increase in the protein:DNA ratio in the API sheep which did not occur in the LPI sheep. Also, as explained above, there may have been a decrease in the cellular protein content of the mucosa of the LPI sheep which was not detected due to the presence of albumen and plasma proteins.

If the increase in the protein content of the body mucosa is due to the presence of albumen and plasma proteins then no hypertrophy occurred. The number of cells/mm^2 would be expected to decrease if a hypertrophy occurred due to the increase in the size of the cells. Under normal circumstances there should be increases in the RNA and protein content of the cells and a decrease in the number of cells per unit area to confirm the presence of a hypertrophic reaction.

Gastrin is known to stimulate both hyperplasia (Stanley *et al.*, 1972; Johnson and Guthrie, 1974a; Castelyn *et al.*, 1977; Johnson, 1977) and hypertrophy (Johnson *et al.*, 1969; Chandler and Johnson, 1972; Johnson, 1977; Majumdar and Goltermann, 1978) in the body region of the rat stomach, and hyperplasia in the dog (Willems *et al.*, 1972) (see also Section 1.1.3.2.1.). A hypergastrinaemia occurred in the infected sheep (Table 2.4.) and may have stimulated growth of the mucosa of the abomasal body. Other factors such as an increase in the secretion and/or activity of other growth factors such as insulin, insulin-like growth factors (IGF) I and II, relaxin, epidermal growth factor (EGF), inhibin A and B, bombesin, cholecystokinin (CCK), gastrin, basic and acidic fibroblast growth factor, enteroglucagon and neurotensin as discussed in Section 4.1. may also be involved. Parasites produce and release secretory-excretory (SE) products within the host. Some of the actions of the SE products released by *O. circumcincta* have been demonstrated by McKellar *et al* (1990) such as stimulation of pepsinogen secretion from isolated preparations of dispersed gastric glands from the ovine and bovine abomasal mucosa. The decrease in the secretory activity of the parietal cells

observed by McLeay *et al.* (1973) may have been due to SE products from *O. circumcincta*. The oxygen scavenging enzyme, super oxide dismutase (SOD) has been detected in high levels in the L_3 form of *O. circumcincta* (Knox and Jones, 1992) and is released by the parasites inside the hosts (for review see Behnke *et al.*, 1992). SOD is thought to protect the parasites from free oxygen radical release during the host's response (Callahan *et al.*, 1988). The effects of SOD on the host tissues have not been investigated. The trophic effects of SE products on the abomasal mucosa have not been studied.

The antral concentrations of RNA, DNA and protein and the ratios of RNA:DNA and protein:DNA for both the API and LPI groups and the number of mucosal cells/mm^2 in the LPI group were the same as in the control group. These observations indicate that the increased mucosal thickness observed in the LPI sheep (Figure 3.4.) was not due to hyperplasia or hypertrophy alone, but may have been due to a balanced combination of both. Studies of the trophic effects of gastrin on the antral mucosa have produced conflicting results. The intravenous administration of purified porcine gastrin or pentagastrin has been reported to inhibit growth of the antral mucosa in starved rats (Casteleyn *et al*, 1977). By contrast, a study using fed guinea pigs showed gastrin to have a trophic effect on the antrum (Muller *et al*, 1980). Whether the discrepancy between these results is due to species differences or because the work by Muller *et al.* (1980) was carried out on fed animals as opposed to starved animals is unknown. Starvation of rats for 8 hours reduces fundic mucosal DNA synthesis by 63% and RNA synthesis by 81% (Muller *et al.*, 1980). In the present study, the sheep were not starved, so the decrease in RNA concentration in the abomasal body and duodenum could not be accounted for by this. The trophic effects of the parasites in the abomasal body of LPI sheep may be due to the increased circulating levels of gastrin, however as explained above gastrin is not generally considered to have trophic effects on the antral mucosa.

No differences in the antral wall thickness was observed in the API sheep compared to the control animals (Figure 3.4). In the present study, no changes in the DNA, protein or RNA concentrations were observed in the API sheep. This indicates that adult *O.*

circumcincta do not stimulate hyperplasia or hypertrophy of the antral region. The reason that there was a greater number of cells/mm^2 in API sheep may be the presence of neutrophils in the lamina propria.

The concentration of RNA in the duodenum decreased in both larval and adult infected sheep compared to control animals. This was accompanied by an insignificant decrease in DNA concentration resulting in no significant change to the RNA:DNA ratio. This is an interesting result in view of work carried out by Johnson and Guthrie (1974a) showing gastrin to stimulate growth in the duodenum at a higher rate than in the stomach.

Infection with either *O. circumcincta* larvae or adult parasites did not affect pancreatic growth. Gastrin has been reported to exert a trophic effect on the pancreatic acinar cells in rats (Mayston and Barrowman, 1971; Majumdar and Goltermann, 1979) hence when planning this experiment, it was thought that pancreatic growth may be affected by the hypergastrinaemia associated with ostertagiasis. In the rat, CCK has a trophic effect on the pancreas (Barrowman and Mayston, 1973; Mainz *et al.*, 1973; Brants and Morriset, 1976; Peterson *et al.*, 1978). The trophic effects of gastrin on the pancreas are thought to be due to the structural similarities between gastrin and cholecystokinin (CCK) as both peptides have the same 5 C-terminal amino acid sequence. Gastrin has been shown to bind to CCK receptors (for review see Rehfeld, 1989). The trophic effect of gastrin on the pancreas is much less than the effect of CCK (Walsh, 1981). The effects of gastrin on growth of the ovine pancreas have not been investigated. The weight of the pancreas did not increase in either group of infected sheep compared with the control sheep (Figure 2.3.). The present results indicate that pancreatic growth is not stimulated by the hypergastrinaemia of ostertagiasis in sheep.

The concentration of DNA and the number of cells/mm^2 in the jejunal mucosa significantly increased in the sheep infected with larval parasites compared with the control animals, indicating hyperplasia of the jejunal mucosa or a decrease in mucosal cell size. The increase in DNA concentration with the unchanged RNA concentration resulted in a decrease in the RNA:DNA ratio. The protein content of the jejunal

mucosal cells was also increased compared to controls, resulting in the amount of protein per cell (protein:DNA ratio) remaining at the same level in the LPI sheep as in the non-infected sheep. In Chapter 3, an increase in the jejunal villus width of the LPI sheep was reported. Therefore, there must have been an increase in the total number of cells in the jejunal villi. The number of cells on the villus is dependant on cell production in the crypts and cell loss (Klein and McKenzie, 1983). There must be either an increase in cell production or a decrease in cell loss in the jejunal villi of the LPI sheep, resulting in an increased villus width. Exfoliation of surface cells by food particles accounts for most of the cell loss (Creamer *et al.*, 1961). As there were no differences in the food intakes between the different sheep groups, it is unlikely that there was a difference in exfoliative cell loss between the groups. Therefore, there must have been an increase in cell production or a decreased villus transit time. The villus transit time is the time between cell production in the crypt and the movement of these cells up the villus (Rijke, 1980). This possible hyperplasia was most likely stimulated by the hypergastrinaemia. The difference between the results for the LPI and the API sheep, i.e. a decrease in the jejunal RNA concentration and increase in the DNA concentration in the LPI sheep, but not in the API sheep, may have been due to the greater length of time that the hypergastrinaemia was present in the LPI sheep compared to the API sheep (55-57 days compared to 8 days respectively). Gastrin has been shown to increase the proliferative activity in the jejunum of the rat within 76 hours of its administration (Casteleyn *et al.*, 1977). The proliferative activity was measured by calculating the percentage of cells labelled after the administration of tritiated thymidine and also the mitotic indices for each tissue. No similar data is available for the sheep. Because of the relative insensitivity of the methods used in the present study to detect cell division/hyperplasia it would presumably take a longer time for the increased rate of cell proliferation to become detectable.

The present results suggest that infection with *O. circumcincta* under the present experimental conditions does not cause either a hyperplasia or hypertrophy of the ileal and caecal mucosae. In the colonic mucosa of the API sheep there was a small but significant increase in the RNA concentration, but no corresponding significant increase in the average cell content of RNA (RNA:DNA ratio). The cause of the increase in

RNA concentration is not known, but may reflect a change in the general metabolism of the enterocytes in this region of the gut. This remains to be determined.

Assuming that the increases in wall thickness of the abomasal body region and jejunum reported in Chapter 3 were due to the trophic effects of the increased circulating gastrin levels, it is unclear why gastrin did not also stimulate growth of the mucosa of the duodenum and ileum. Any changes may not have been large enough to be detected by these protein, RNA and DNA assays. Alternatively secretin has been reported to inhibit the trophic effects of gastrin on the stomach (Stanley *et al*, 1972), duodenum (Johnson and Guthrie, 1974b) and colon (Johnson and Guthrie, 1974b). Whether or not secretin affected the trophic effects of gastrin on the duodenum and ileum in the present study is not known. The effects of ostertagiasis on secretin release have not been examined.

4.5. SUMMARY

The presence of adult *O. circumcincta* worms in the abomasum may stimulate hypertrophy of the body mucosa, while the developing larvae stimulate hyperplasia of the body mucosa. The high serum gastrin levels are the most likely growth factor responsible for these trophic reactions.

No changes in the DNA concentration or RNA:DNA or protein:DNA ratios were present in the antrum of either group of parasitised sheep relative to controls. Any increase in the thickness of the antral mucosa must therefore be due to a combination of both hyperplasia and hypertrophy.

Adult and larval *O. circumcincta* exerted no apparent trophic effects on the duodenal or ileal mucosae or pancreatic tissue. The developing larvae appeared to stimulate hyperplasia of the jejunal mucosa.

The mitogen or mitogens responsible for the trophic effects reported here are not known. Gastrin is the most likely stimulator of the trophic effects, but if so, did not appear to exert a trophic effect in some areas where it has been previously shown to have trophic

effects.

In the following chapter, the numbers of the different endocrine cells in the abomasum will be studied in order to further determine any functional effects of the hyperplasia and hypertrophy observed in these regions.

5. ENDOCRINE CELLS IN THE ABOMASUM OF CONTROL SHEEP AND SHEEP INFECTED WITH LARVAE OR ADULT *OSTERTAGIA CIRCUMCINCTA*

5.1. INTRODUCTION

The distribution of biologically active peptides including hormones, paracrine agents and neurotransmitters within the tissues of the gastrointestinal tract can be studied immunocytochemically by applying antibodies raised against a specific peptide to sections of tissue from the gut and detecting where binding between antibody and antigen has occurred. This technique has been used to determine the frequency and distribution of numerous endocrine and endocrine-like cells and peptidergic neurons within the gastrointestinal tract of a number of species (Larsson, 1988). Several peptides which have been studied in this way include the following.

5.1.1. Chromogranin A

Antibodies to chromogranin A bind to the secretory granules of endocrine and endocrine-like cells in the stomach and intestines of the guinea pig, dog, ox, cat, mouse, pig (Rindi *et al.*, 1986), rat (Cohn *et al.*, 1984; Rindi *et al.*, 1986) and human (O'Conner *et al.*, 1983; Facer *et al.*, 1985; Rindi *et al.*, 1986; Hearn, 1987) and have been used to determine the total number of endocrine cells in the gut. There have been no reports on the use of chromogranin antibodies to identify endocrine cells in the gastrointestinal tract of the sheep.

Chromogranin is a high molecular weight protein, originally extracted from granules of cells of the adrenal medulla (Smith and Winkler, 1967). Three chromogranin proteins have been identified; chromogranin A, chromogranin B (also called secretogranin I) and secretogranin II. The physiological functions of chromogranins are unknown but *in vitro* work on human and bovine chromogranins indicate that they may have intracellular roles in the packaging and/or processing of prohormones and neuropeptides (Seidah *et al.*, 1987). Chromogranin A is thought to be the precursor of pancreastatin (Eiden, 1987; Huttner and Benedum, 1987; Konecki *et al.*, 1987; Iacangelo *et al.*, 1988), a peptide

which partially inhibits insulin-induced glucose release from the *in vitro* pancreas (Tatemoto *et al.*, 1986). Whether this also occurs *in vivo* has not been studied.

5.1.2. Enteroglucagon

A hyperglycaemic-glycogenolytic factor with the same chemical and biological properties as pancreatic glucagon was first extracted from the fundic mucosa of the dog and rabbit in 1948 (Sutherland and deDuve, 1948). Fundic mucosal cells reacting with antibodies to pancreatic glucagon were described in 1971 (Polak *et al.*, 1971). This glucagon-like substance is termed enteroglucagon or gut glucagon. A similar substance to glucagon is glicentin, which is the proglucagon molecule, a biosynthetic precursor of glucagon (Moody, 1980; Moody *et al.*, 1981). There are many gastric mucosal cells containing glucagon-like-immunoreactivity (A cells) in the dog and cat, but they are either absent or scarce in other mammals such as the pig and human (Polak, 1989). The frequency of A cells has been immunocytochemically studied in the ovine gastrointestinal tract using antisera to enteroglucagon. Enteroglucagon-immunoreactive cells were present in small numbers in the cardiac (0.12 cells/mm^2), body (0.12 cells/mm^2) and antral (2.08 $\pm$ 0.56 cells/mm^2) regions of the abomasum (Calingasan *et al.*, 1984).

Enteroglucagon has been demonstrated to have a trophic effect on the small intestine in the rat. An increase in serum enteroglucagon in response to refeeding following fasting has been correlated with an increased rate of crypt cell production in the distal small intestine (Goodlad *et al.*, 1987). There is an association between an increase in the concentration of enteroglucagon in the plasma and conditions with stimulated intestinal growth such as the presence of a renal tumour which produced enteroglucagon (Gleeson *et al.*, 1971; Bloom, 1972), intestinal bypasses (Sarson *et al.*, 1981) and intestinal resections (Sagor *et al.*, 1982). Glucagon has also been shown to stimulate the growth of human intestinal epithelial cells *in vitro* (Simopoulos *et al.*, 1989). Enteroglucagon has not been shown to stimulate mucosal growth in the stomach.

The intravenous infusion of glucagon in the calf decreases the frequency of antral action

potentials and gastric emptying (McLeay and Bell, 1981). It also reduces gastric abomasal acid secretion accompanied by an elevation in blood glucose concentration (McLeay and Bell, 1981). The physiological significance of these effects of glucagon has not been determined.

5.1.3. Gastrin

The structure, secretion and actions of gastrin have been discussed in Section 1.1.3.2.1.. An apparent over-secretion of gastrin, or hypergastrinaemia, occurs during infection with *O. circumcincta* (Anderson *et al.*, 1975; 1981; 1985; 1988; Reynolds *et al.*, 1979a). The cause of the hypergastrinaemia has not been established. The concentration of gastrin in the antral mucosa has been reported to increase, indicating an increase in the production of gastrin (Reynolds *et al.*, 1979a).

The possibility that the hypergastrinaemia of ostertagiasis is associated with a change in the concentration of G cells has been investigated using calves infected with *Ostertagia ostertagi* (Fox *et al.*, 1993). Tissue samples were taken from the pylorus 10, 19 and 28 days after the infection and sections from these samples were immunocytochemically stained for gastrin-immunoreactivity. The number of immunoreactive G cells in the pyloric mucosa was significantly lower in the infected animals compared with the non-infected control cattle. The staining intensity of the G cells in the parasitised cattle was less than that in the control tissues, indicating that the apparent decrease in G cell number in the parasitised animals was due to the release of previously stored gastrin into the circulation rather than the presence of fewer G cells.

5.1.4. Somatostatin

Somatostatin is a paracrine agent which, among other things, inhibits the release of gastrin from G cells (see Section 1.1.3.2.1.). A change in the number of somatostatin-releasing D cells, resulting in a change in the ratio of G:D cells in the abomasal antrum theoretically could affect the amount of gastrin released from G cells. The G:D cell ratio was examined in the present study, as a change in this ratio could affect gastrin

secretion leading to a hypergastrinaemia.

5.1.5. Gastrin Releasing Peptide

Gastrin releasing peptide (GRP), the mammalian equivalent of the amphibian skin peptide bombesin is, as its name suggests, a strong stimulator of gastrin release (for review, see Section 1.1.3.2.1. and Walsh, 1989). GRP-containing neurons are closely associated with antral G cells and are thought to be involved in the physiological regulation of gastrin release. There is no information on the effect of ostertagiasis on the frequency and distribution of GRP-containing neurons in the abomasal mucosa of sheep.

The aim of the present study was to determine whether or not larval and adult *O. circumcincta* parasites alter the numbers of chromogranin containing endocrine cells, G cells, D cells, A cells and GRP neurons in the abomasum of the sheep. The results are discussed with reference to factors contributing to the hypergastrinaemia seen during ostertagiasis in ruminants.

5.2. METHOD

The sheep used for the present experiment were those described in Chapter 2. They were raised to be free of Helminth parasites, infected with *O. circumcincta* larvae and adults and euthanased as described in Sections 2.2.4. and 2.2.5. The sheep were then treated as described in Section 2.2.11. Two 10 mm wide samples of whole tissue (mucosa and muscularis externa) were removed from the abomasal body at a site 80 mm distal to the omasal-abomasal junction, measured along the greater curvature, and from the abomasal antrum at a site 10 mm proximal to the antral edge of the Torus Pyloricus. All samples were kept free of contaminants such as blood and other body fluids, and handled as little as possible. They were taken within 15 minutes of euthanasia.

The 2 tissue samples from both regions in every sheep were fixed separately in either Bouin's fluid or phosphate-lysine-periodate (PLP).

5.2.1. Bouin's Fluid-Fixed Tissues

The tissues were placed on pieces of card to reduce folding of the tissue due to muscular contraction and immersed in Bouin's fluid (Culling, 1985) at room temperature for 24 hours. They were then stored in 70 % ethanol until they were embedded in paraffin. The tissues were dehydrated, cleared and impregnated with paraffin wax (melting point 56-58 °C) using an automatic tissue processor (SE400, Shandon Scientific Co. Ltd., England) following the schedule shown in Table 3.2.. The paraffin wax processed tissues were then embedded using an embedding console (Tissue Tek II, Miles Scientific). Sections 6 μm thick were cut with a rotary microtome (Leitz Wetzlar, Germany), floated on warm water and mounted on P.V.A. coated slides.

5.2.2. PLP-Fixed Tissues

Bouin's fluid has been used extensively to fix tissues of the ovine and bovine abomasa for immunocytochemical staining with antisera to gastrin, somatostatin and glucagon (Calingasan *et al.*, 1984; Kitamura *et al.*, 1985; Weyrauch *et al.*, 1989) and to chromogranin in cattle (Nolan *et al.*, 1985). In the present study, the abomasal tissues fixed in Bouin's fluid had very few cells which stained for chromogranin. To determine if this was due to an effect of the Bouin's fluid, antral tissue from a sheep in the control group was fixed in phosphate-lysine-periodate fixative (PLP) and stained immunocytochemically for chromogranin. The fixative PLP forms long cross-links between the structural proteins, allowing space for the penetration of large antibody molecules. The long-cross links also cause less microstructural damage than the shorter cross-links between the structural proteins formed during fixation in Bouin's fluid (Kiernan, 1990). The major advantage of using Bouin's fluid is that fixation in Bouin's fluid requires fewer manipulations to the tissues than does fixation in PLP.

The tissues were immersed in PLP fixative at 4°C for 2-4 hours. The PLP fixative

consisted of 0.05 M phosphate buffer pH 7.4 containing 15.7 g/l L-lysine monohydrochloride (BDH Laboratory Supplies, Poole, England), 22.9 g/l paraformaldehyde (May & Becker, England) and 2.4 g/l sodium periodate (AnalaR, BDH Chemicals Ltd., Poole, England). The phosphate buffer comprised 75 mM disodium hydrogen orthophosphate (Analytical grade, Univar, Ajax Chemicals, Australia) and 25 mM sodium dihydrogen orthophosphate (AnalaR, BDH Chemicals Ltd., Poole, England) in distilled water, with the pH adjusted to pH 7.4 with sodium hydroxide (AnalaR, BDH Chemicals Ltd., Poole, England). The tissues were then cryoprotected with 0.05 M phosphate buffer pH 7.4 with 7% sucrose (Prolabo, Paris, France) for 4 hours, and then stored until use at 4°C in 0.05 M phosphate buffer pH 7.4 with 14% sucrose. The tissues were mounted onto the cryostat stubb (Jung Frigocut 2800E, Leica Instruments, Germany) using OCT imbedding compound (Miles Inc., Elkhart, U.S.A.) and rapidly frozen. Sections 5 μm thick were cut from the tissues and mounted onto P.V.A. coated slides.

5.2.3. Immunocytochemistry

The antisera listed in Table 5.1. were used to separately identify chromogranin A containing cells, G cells, D cells, A cells and GRP containing neurons.

The enteroglucagon antisera used had 100% cross-reactivity between gut and pancreatic glucagon. The antisera for GRP, somatostatin and chromogranin were all reported by the manufacturing companies to have negligible cross reactivity with any other peptides. The gastrin antisera had 50% cross reactivity with cholecystokinin. Because there is no cholecystokinin present in the stomach (Rehfeld, 1989) this cross-reactivity did not affect the identification and counting of antral G cells.

Table 5.1. Antisera dilutions and incubation times.

Antisera	Source	Dilution	Incubation
Chromogranin A	rabbit Ig to human Dako Corp., California	1/1000	4°C overnight
Gastrin	rabbit Ig to human Dako Corp., California	1/2000	4°C overnight
Somatostatin	rabbit Ig to human Amersham Int., England	1/2000	4°C overnight
GRP	rabbit Ig to human Dako Corp., California	1/1000	4°C overnight
Enteroglucagon	rabbit Ig to pig Amersham Int., England	1/500	1 hour room temperature

The *Bouin's fluid-fixed sections* were deparaffinised by immersing the slides in 2 changes of xylene (5 minutes/wash). This was followed by rehydration by sequentially submerging the slides in 2 washes (3-5 seconds/wash) of absolute ethanol, 70 % ethanol (3-5 seconds) and tap water. The slides were then rinsed in 0.01 M phosphate buffered saline (PBS) pH 7.4 made by dissolving 4.54 g of anhydrous Na_2HPO_4 (Analytical grade, Univar, Ajax Chemicals, Australia), 1.09 g of KH_2PO_4 (May and Becker Ltd., Dagenham, England) and 36 g of NaCl (AnalaR, BDH Chemicals Ltd., Poole, England) in 4 L of distilled water. The slides were drained of excess PBS and blotted with a lint-free paper towel without allowing the sections to dry. The slides were next placed on glass rods in a humidified chamber comprising a polypropylene tray lined with paper towels moistened with tap water and covered by a perspex sheet. The sections on the slides were covered with 1% bovine serum albumin (BSA) (Sigma, St Louis, U.S.A.) in PBS for 5 minutes to block nonspecific binding. The excess BSA was drained off and the slides were blotted with a lint-free paper towel without allowing the sections to dry. The slides were then incubated for the appropriate length of time in a primary polyclonal antisera (Table 5.1.) diluted with 1% BSA in PBS, in the humidified

chamber.

The *PLP-fixed sections* from the antrum of 1 of the control sheep were immersed in distilled water for one minute to remove the medium used to attach the tissue to the cryostat stubb and some of the sucrose remaining on the tissue after the fixation process. The sections were then immersed in 3% hydrogen peroxide (Analytical grade, Ajax Chemicals, N.S.W., Australia) in absolute methanol (BDH AnalaR, Poole, England) for 30 minutes to block endogenous peroxidase and partially extract lipids which may hinder antibody binding. This was followed by a gentle wash in distilled water and then a 1 minute wash in PBS. The slides were then covered with 1 % BSA in PBS for 5 minutes in the humidified chamber to block non-specific binding. Chromogranin A antisera was then added in the same manner as for Bouin's fluid-fixed sections.

From this stage, the Bouin's fluid-fixed tissues and PLP-fixed tissues were treated in the same manner, as described below.

A positive control slide was included in each batch of 15 to 20 slides. The control slide contained a section of tissue known to stain with the primary antibody and was stained in the same manner as the experimental sections. The tissues used for positive controls were antral tissue for gastrin and somatostatin and GRP antisera and pancreatic tissue for enteroglucagon. The positive control sections were stained to ensure that an absence of staining on a test section was due to an absence of the appropriate cells and not to the inability of the primary antisera to bind with the antigen. A negative control was also included. This was an additional experimental section stained as described below except that the primary antibody was omitted and the tissue section remained covered with 1% BSA in PBS. The negative control slides were then treated in the same way as the other sections and used to assess nonspecific staining.

Following incubation with the primary antibody, the slides were washed 3 times (1 minute/wash) in fresh PBS. They were then incubated for 30 minutes at room temperature in the humidified chamber with anti-rabbit biotinylated IgG (Amersham Int., England) at a dilution of 1/200 in 1% BSA in PBS. This compound binds to the

primary antisera.

After 3 additional 1 minute washes in PBS, the slides were incubated for 10 minutes at room temperature in the humidified chamber with streptavidin biotinylated horse radish peroxidase pre-formed complex (Amersham Int., England), diluted 1/200 with 1% BSA in PBS. This binds to the IgG biotin conjugate. It has many peroxidase sites, amplifying the reaction. This incubation was followed by three more 1 minute washes in PBS.

The peroxidase sites on the biotin-streptavidin-peroxidase complex were then identified with a freshly made diaminobenzidine (DAB) solution with heavy metal intensification. The DAB solution comprised 10 ml of PBS solution containing 4 mg of 3,3'-diaminobenzidine tetra-hydrochloride dihydrate (Alderich, U.S.A), 10 µl of 30% w/v hydrogen peroxide (Analytical reagent, Ajax Chemicals, N.S.W., Australia) and 1 drop of nickel cobalt comprising of 1 % cobaltous chloride (BDH AnalaR, Poole England) and 1 % nickel chloride (Analytical grade, May and Becker Ltd., Dagenham, England) in distilled water. The DAB solution used to stain the slides incubated with the GRP primary antiserum did not have the nickel cobalt added. The DAB solution was applied for approximately 3 minutes at room temperature. The slides were then immersed in PBS for 1 minute. This was followed by counterstaining with eosin for 30 seconds, and dehydration with a graded series of ethanol changes (70% ethanol followed by 2 changes of absolute ethanol). The slides were then cleared in 2 changes of xylene and mounted in DPX (BDH Ltd., Poole, England).

When the sections were viewed under the microscope, cells containing the particular antigen being detected were identified by a black brown colour in their cytoplasm (secretory granules), except for sections stained for GRP containing neurons. In the sections where GRP was the primary antisera the neurons were a golden brown colour. This was due to the lack of nickel cobalt in the DAB solution and made the neurons more easily identifiable.

5.2.4. Cell counting

Two Bouin's fluid-fixed sections from different parts of the same paraffin block were stained with each antisera in each region of each sheep. The number of stained cells were counted on each slide in a randomly selected area 0.28 x 0.29 mm (Leitz Periplan GF10^{x} eyepiece, Ernst Leitz, Wetzler, Germany with counting graticule), measured using an Olympus objective micrometer (Olympus Optical Co. Ltd., Tokyo, Japan) and a 40x objective lens. Five of these areas were counted on each slide, giving a total area counted of 0.812 mm^2 per sheep per region (antrum and body). The number of stained cells/mm^2 was then calculated by dividing the total number of cells counted by 0.812.

Serial 5 μm thick sections of the body region of the control sheep were stained for chromogranin or somatostatin and compared with each other, to determine whether chromogranin detects D cells in the ovine abomasum.

5.3. RESULTS

5.3.1. Chromogranin A

In the control animals, the number of antral cells which stained for chromogranin A (5.5 $\pm$ 3.9) was less than the total number of D (66.3 $\pm$ 5.1) and G (158.4 $\pm$ 4.0) cells.

In the mucosa of both the abomasal body and antrum, most chromogranin A-immunoreactive (CI) cells were located in the lower half of the glands, with fewer CI cells present in the upper half of the glands (Figure 5.1.). Very few CI cells were present in the abomasal pits. There were no obvious differences in the distribution of CI cells within the mucosa between non-infected control sheep and the API or LPI sheep.

The cytoplasm as opposed to the nuclei of the CI cells were stained (Figure 5.2.). The shape of the cells varied from approximately circular to elongated. Some of the cells

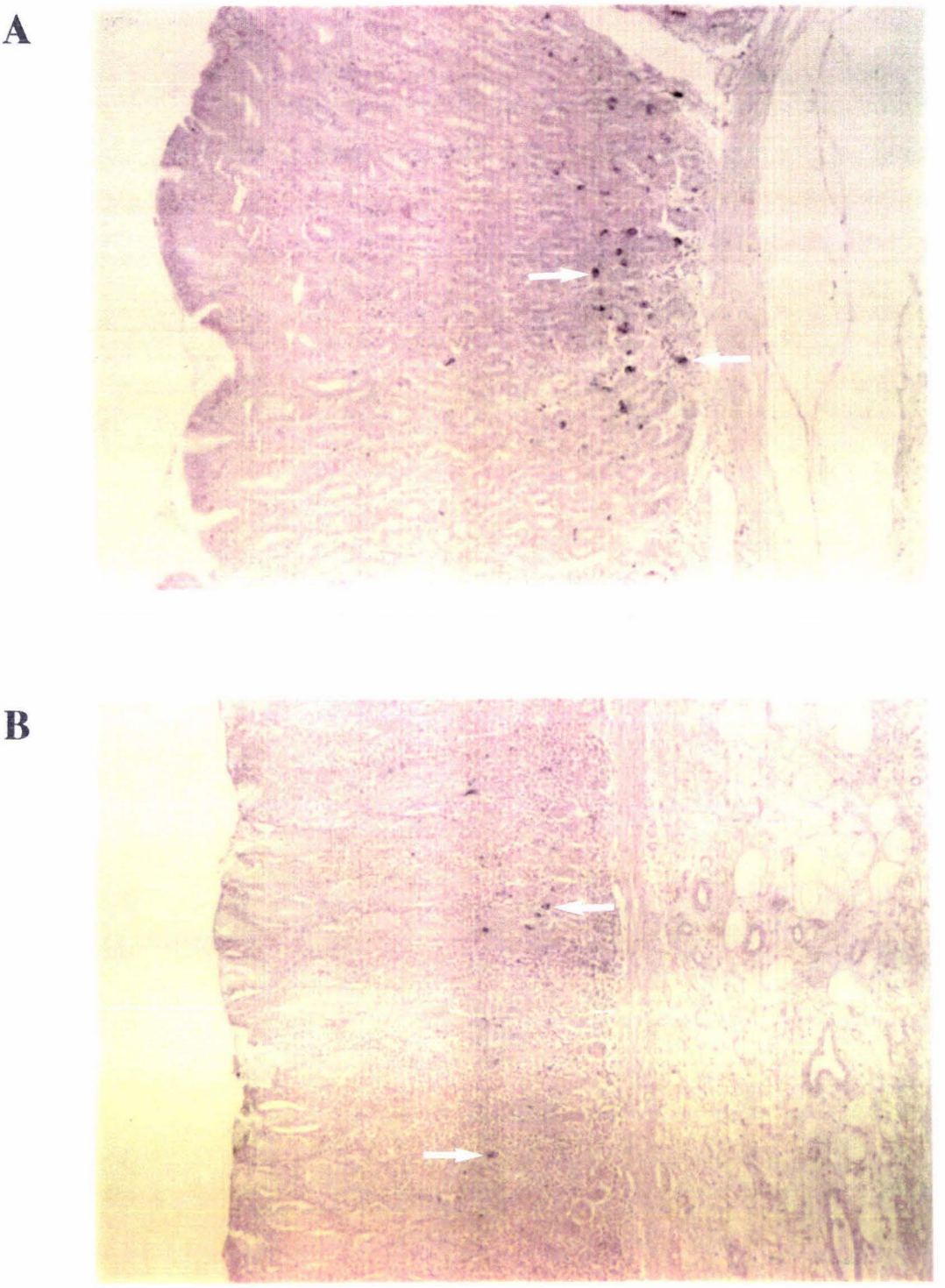


Figure 5.1. Distribution of chromogranin-immunoreactive cells (arrows) in the abomasal mucosa of a non-infected control sheep. A: body region, B: antral region. Magnification: x25. Stain: eosin.

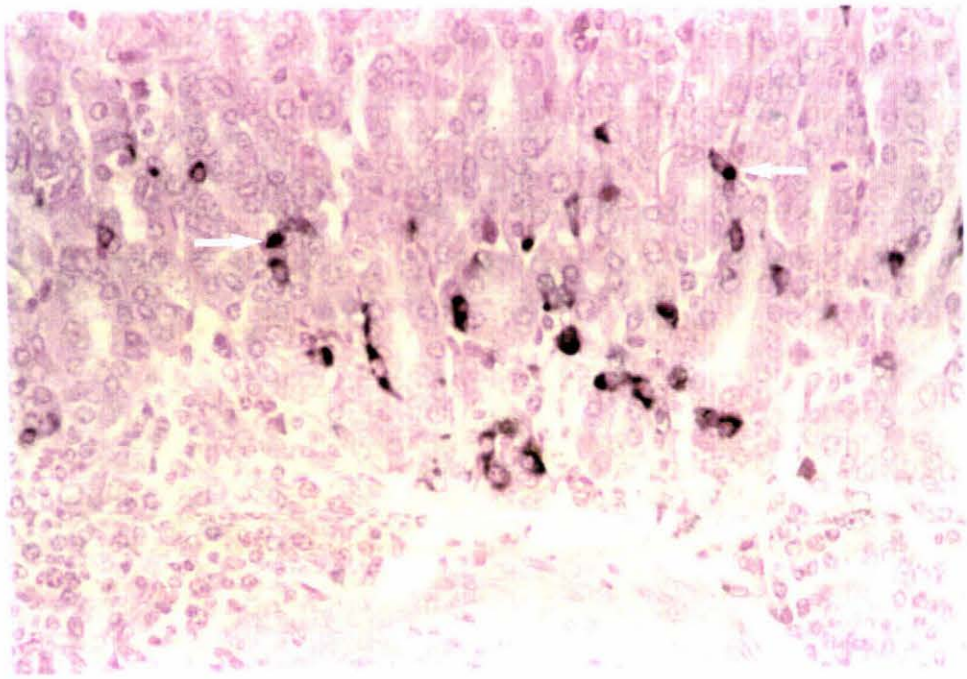


Figure 5.2. Chromogranin-immunoreactive cells (arrows) in the mucosa of the abomasal body of a non-infected control sheep. Magnification: x100. Stain: eosin.

had long cytoplasmic projections (Figure 5.2.). The intensity of the staining varied between cells within the same animals. There appeared to be no variation in the staining intensity between animals in the different experimental groups.

In both the body and antrum, there were no significant differences in the number of CI cells between control sheep and either of the two parasite infected groups (Figures 5.3., 5.4.). There was a large variation in the number of CI cells in the mucosa of the body region between individual animals, causing high standard errors of the mean. More CI cells were present in the body (72.3 $\pm$ 9.2 cells per mm^2) than in the antral (5.5 $\pm$ 3.9 cells per mm^2) mucosa of the control sheep.

The D cells did not appear to stain for chromogranin A. This was obvious from a comparison of serial sections of the abomasal body stained for chromogranin A or somatostatin (D cells) (Figure 5.5.).

There were no significant differences in the number of CI cells in the control antral tissue fixed in PLP fixative compared to those fixed in Bouin's fluid and immunocytochemically stained for chromogranin A. The tissues fixed in PLP fixative had 7 CI cells per mm^2 compared to an average of 5.5 $\pm$ 3.9 cells per mm^2 in control tissues fixed in Bouin's fluid.

5.3.2. G cells

There were no gastrin-immunoreactive (G) cells visible in the abomasal body of any of the sheep.

In the abomasal antrum of all of the sheep, G cells were distributed throughout the mucosa. Most cells were located in the central third of the glands; very few cells were present near the base of the glands (Figure 5.6.). The cells were pyramidal in shape and some had a cellular projection (Figure 5.6.).

The antral G cells in the control and LPI sheep were generally intensely stained

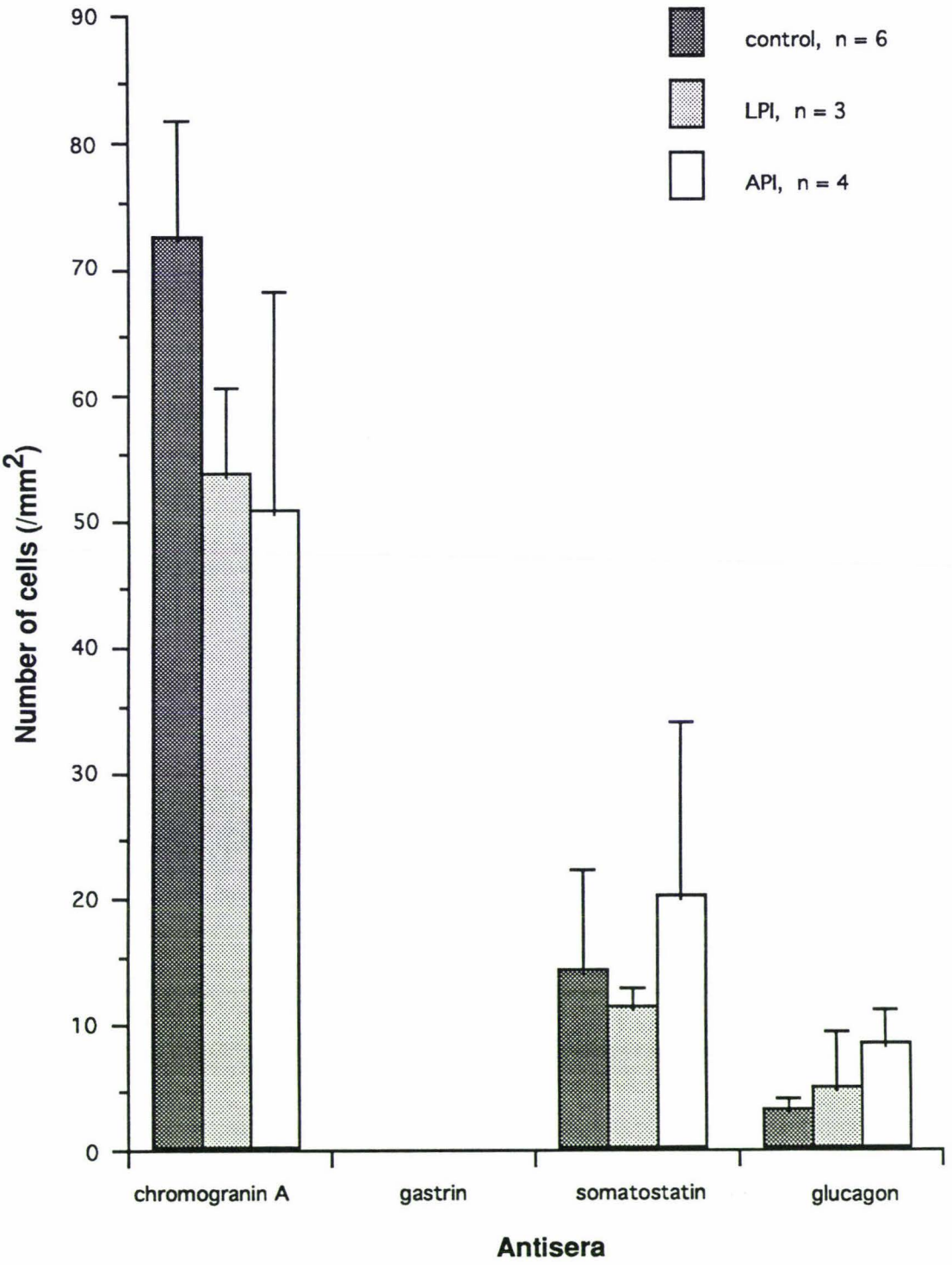


Figure 5.3. Number of cells in the abomasal body immunocytochemically stained with antisera to chromogranin, gastrin, somatastatin and glucagon (mean ± s.e.). LPI = larval parasite infected sheep, API = adult parasite infected sheep.

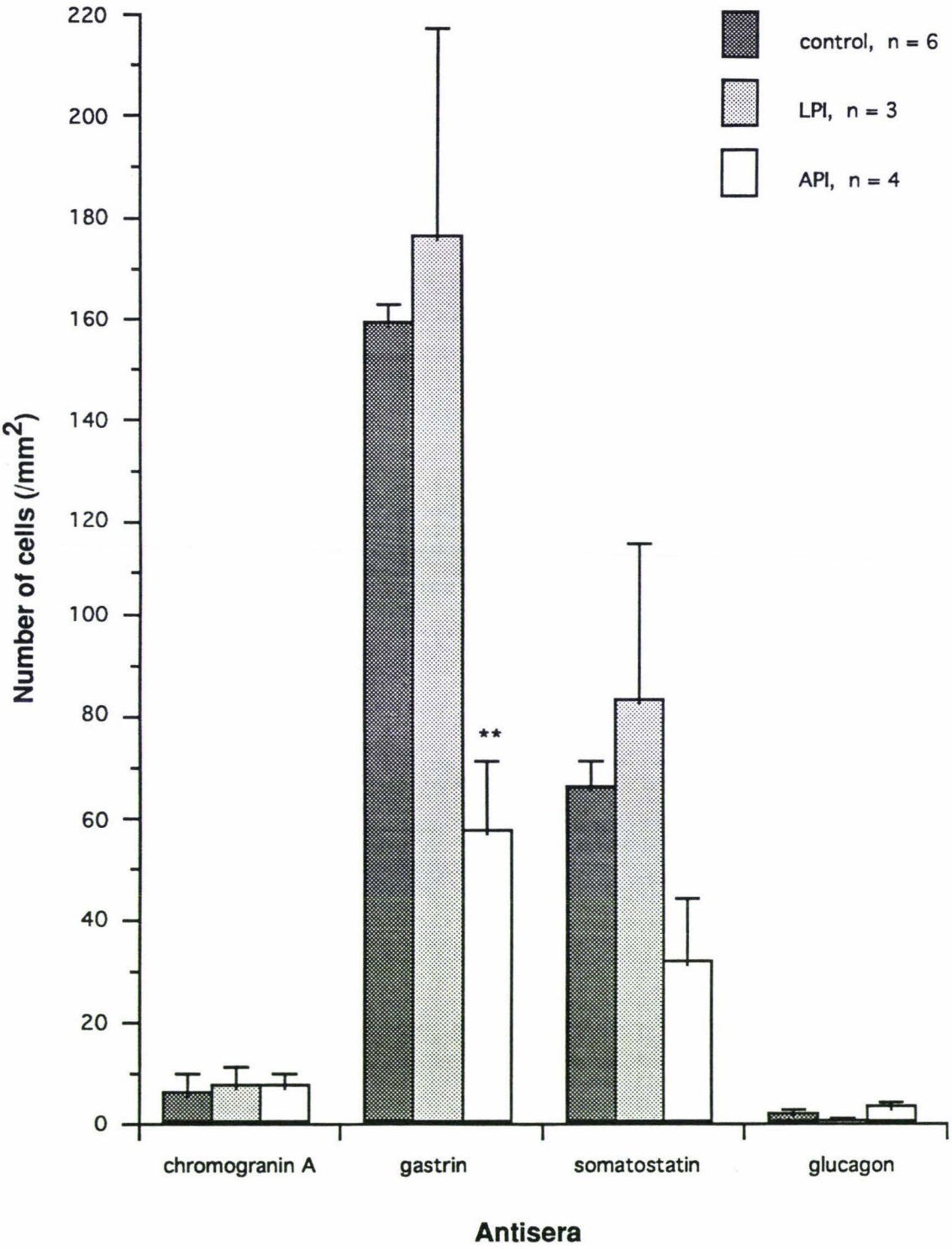
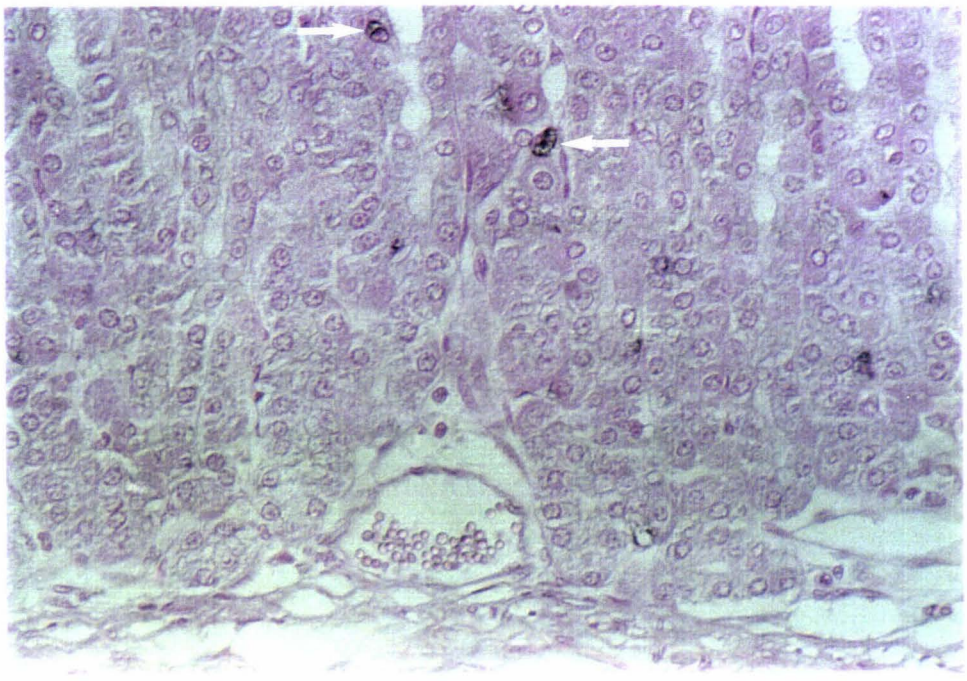


Figure 5.4. Number of cells in the abomasal antrum immunocytochemically stained with antisera to chromogranin A, gastrin, somatostatin, and glucagon (mean ± s.e.). LPI = larval parasite infected sheep, API = adult parasite infected sheep.
** $P < 0.01$

A



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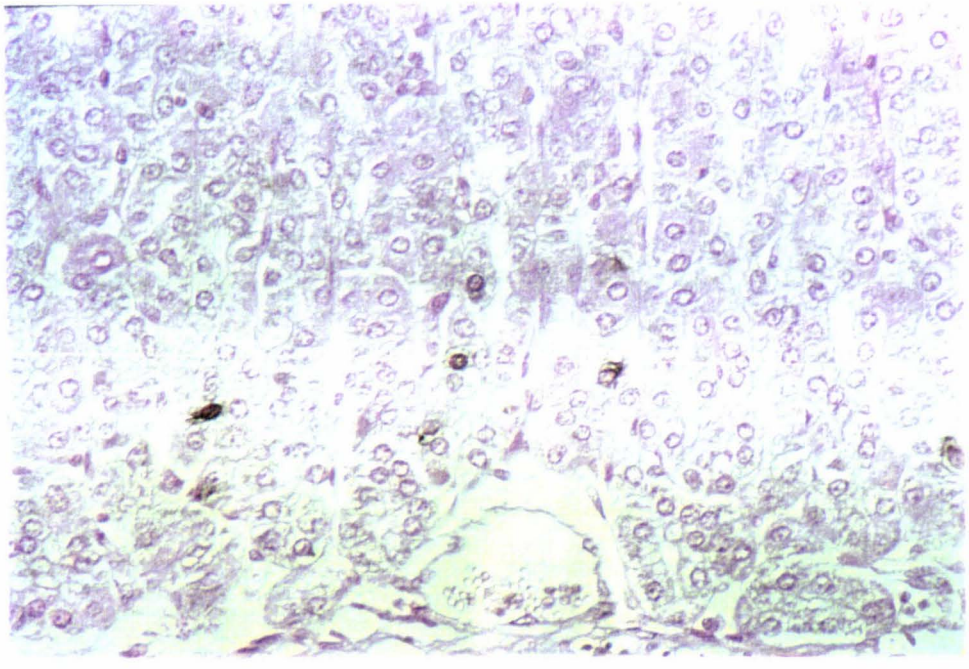


Figure 5.5. Serial sections of the abomasal body of a non-infected control sheep. A: chromogranin-immunoreactive cells (arrows), B: somatostatin-immunoreactive cells (arrows). Magnification: x100. Stain: eosin, D.I.C.

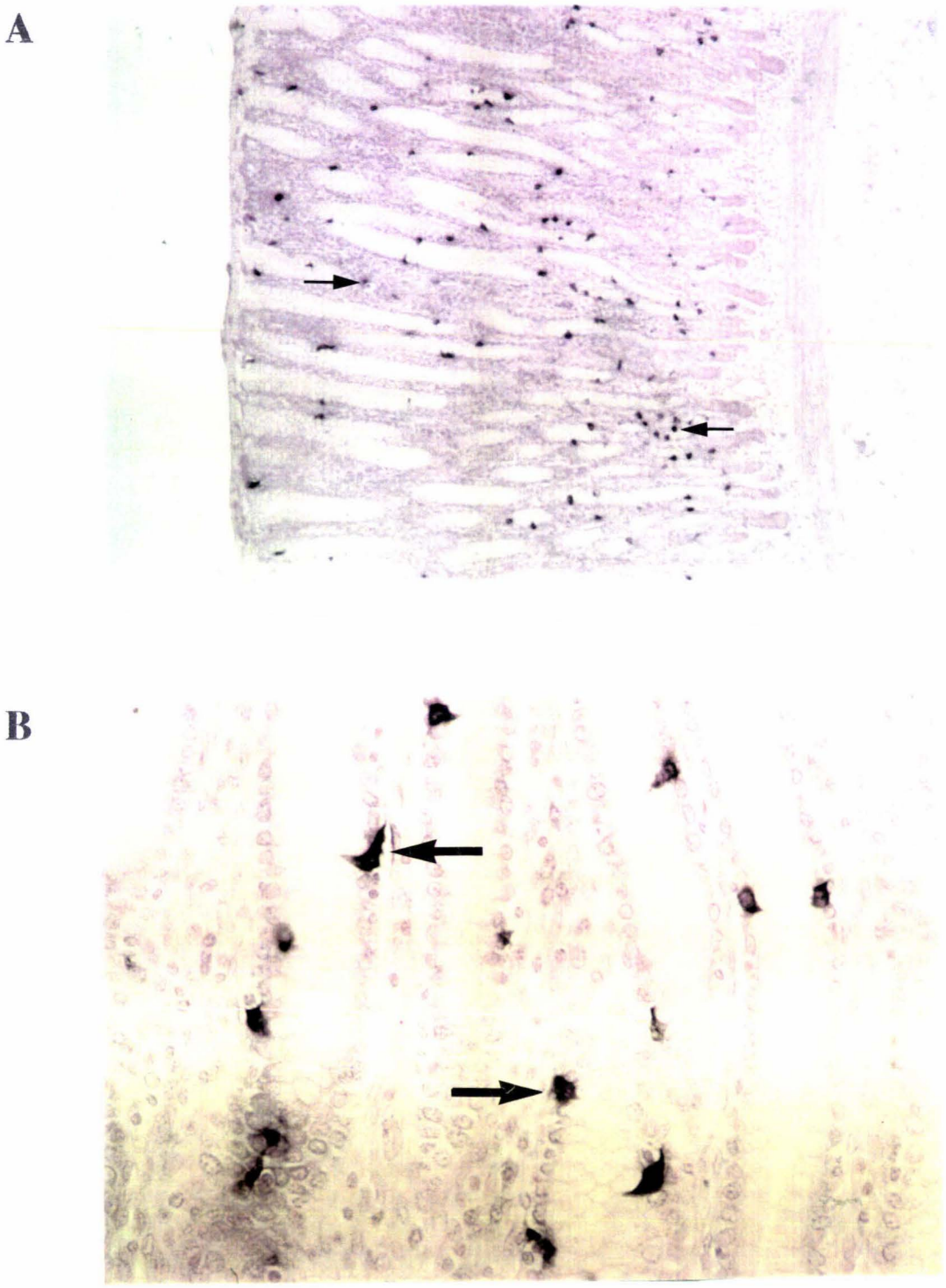


Figure 5.6. Gastrin-immunoreactive cells (arrows) in the antral mucosa of a control sheep. A: Magnification: x25. B: Magnification: x100. Stain: eosin.

throughout the cytoplasm. By contrast, although some of the G cells in the adult parasite infected (API) group were intensely stained, many were stained with a very low intensity and others were barely visible (Figure 5.7.).

There were significantly (*P<0.01*) fewer antral G cells stained in the API sheep than in the control sheep. There was no significant difference between the number of antral G cells in the LPI sheep and the control sheep (Figure 5.4.).

5.3.3. D cells

Somatostatin-immunoreactive (D) cells were distributed throughout the mucosa but were more concentrated in the lower half than in the upper half of the glands (Figure 5.8.). The D cells were circular or oval in shape. Some had cytoplasmic projections from the circular shape. Staining was confined to the cytoplasm of the cells; the nuclei were not stained (Figure 5.8.). In most cells, the staining was more intense in the cytoplasm at one side of the nucleus (Figure 5.8.).

In the control and LPI groups of sheep, the staining intensity appeared equal in all of the cells, whereas in the API sheep, some cells were intensely stained while others were less intensely stained (Figure 5.9.).

There were no significant differences between the number of D cells for either group of parasitised sheep relative to the control group in both the antrum and body (Figures 5.3. and 5.4.). There was a large inter-animal variation in the number of D cells/mm^2 of mucosa, especially in the LPI and API sheep. This large variation contributed to the high standard errors for the means of each group.

There was a significantly (*P<0.01*) higher density of D cells in the antral region (66.3 $\pm$ 5.1) than in the body region (14.3 $\pm$ 8.0) of the abomasum in the control animals. This difference was not significant in the parasitised animals due to the large inter-animal variations.

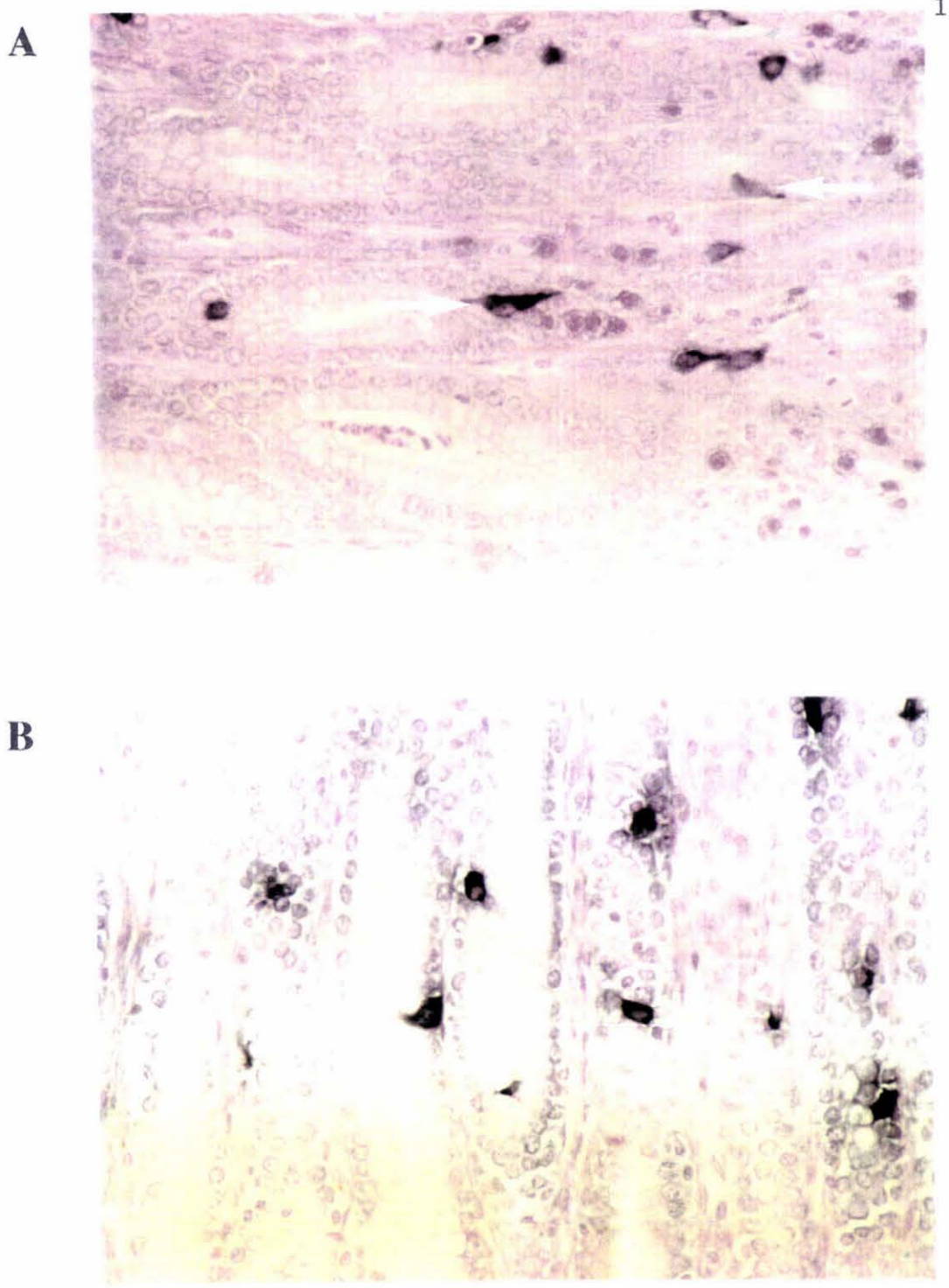


Figure 5.7. Gastrin-immunoreactive cells (arrows) in the antral mucosa of the abomasum. A: adult parasite infected sheep, B: non-infected control sheep. Magnification: x100. Stain: eosin.

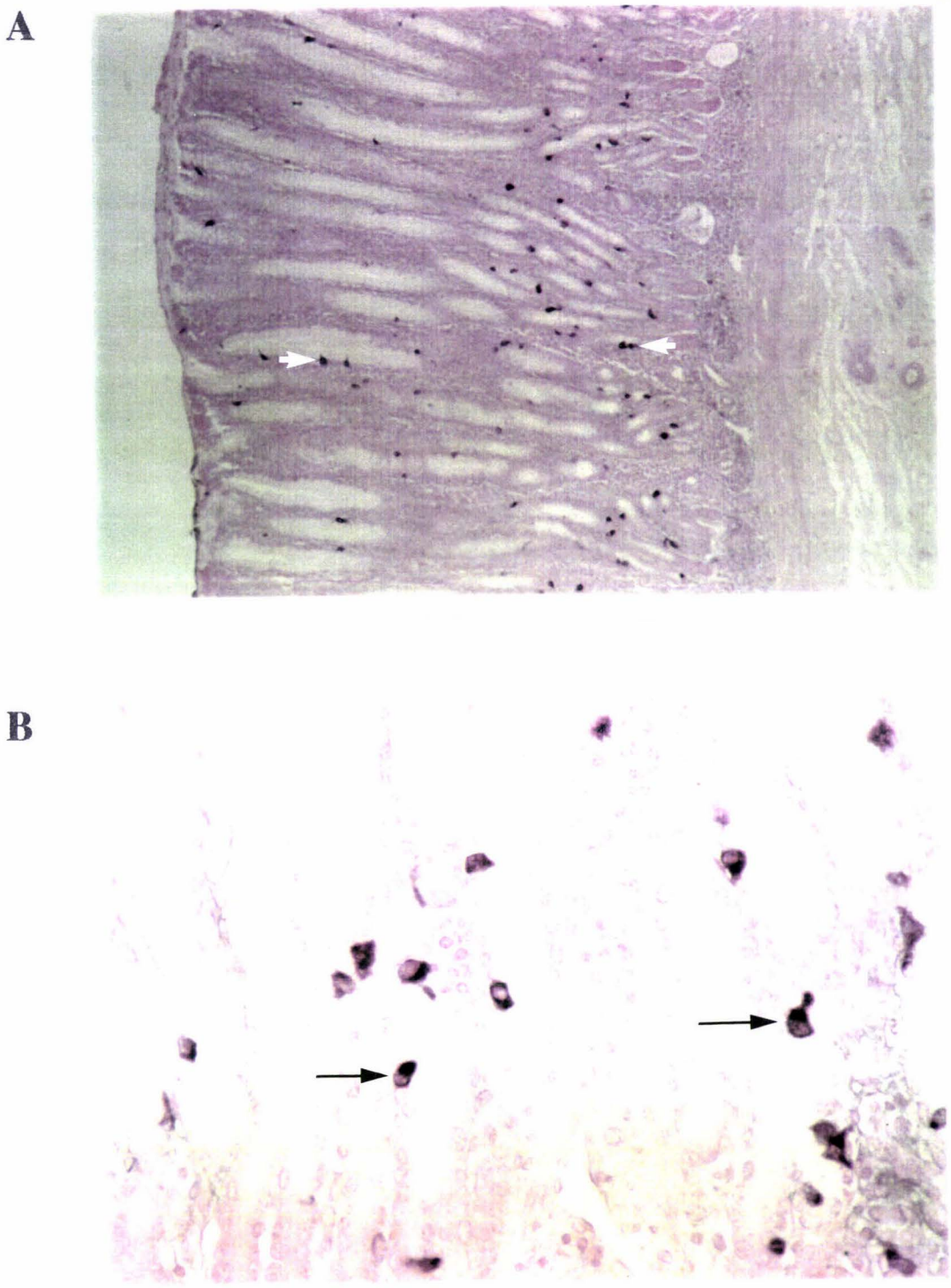


Figure 5.8. Somatostatin-immunoreactive cells (arrows) in the mucosa of the abomasal antrum of a non-infected control sheep. A: Magnification: x25. B: Magnification: x100. Stain: eosin.

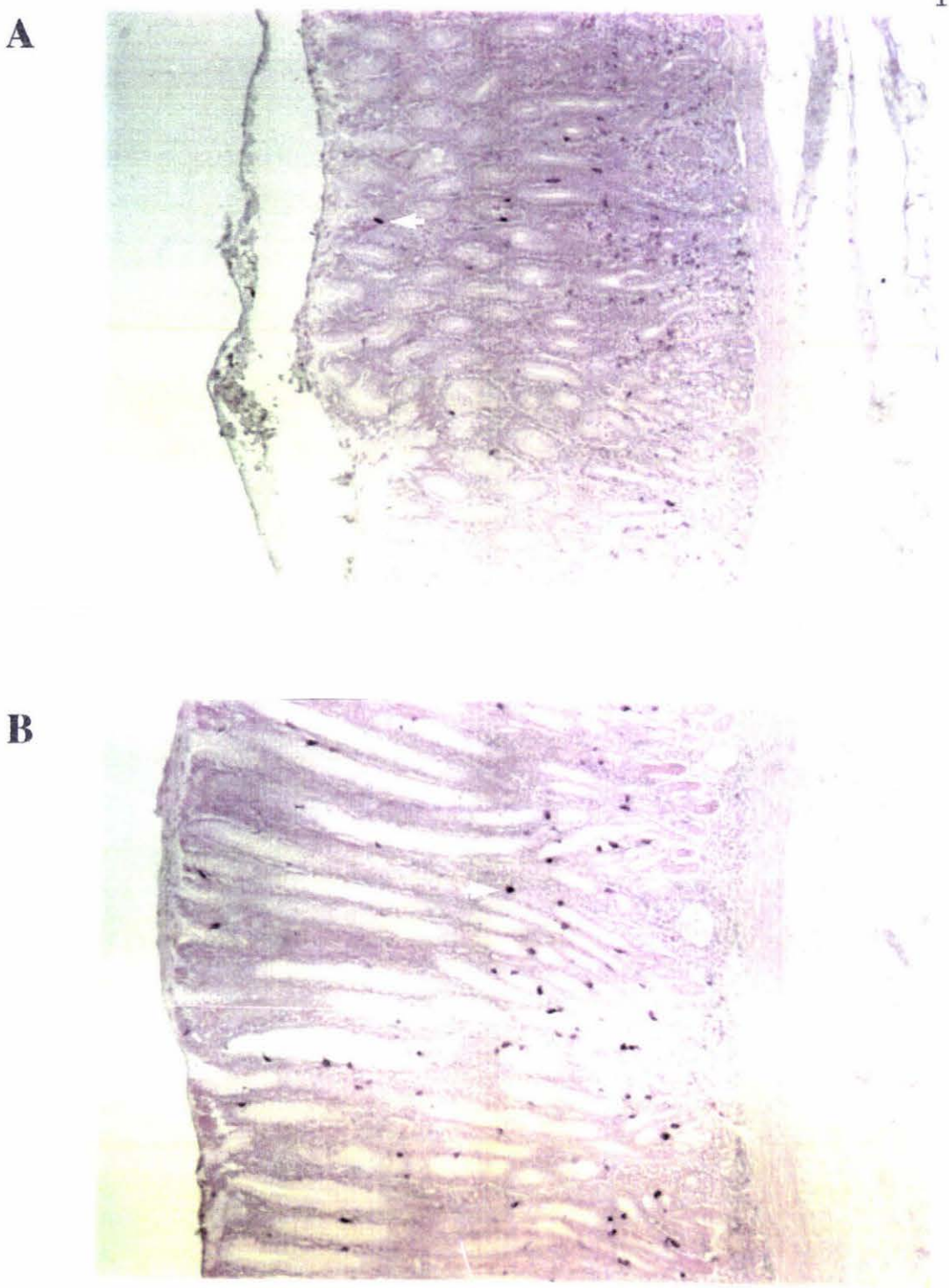


Figure 5.9. Somatostatin-immunoreactive cells (arrows) in the mucosa of the abomasal antrum. A: adult parasite infected sheep. B: control sheep. Magnification: x25. Stain: eosin.

There was a strong correlation between the number of D and G cells in all 3 experimental groups when combined (*r=0.90; P<0.001*) (Figure 5.10.).

5.3.4. A Cells

There were few enteroglucagon-immunoreactive (A) cells in the abomasum of all 3 groups of sheep. The A cells were mainly located in the middle of the glands in both the body and antral regions. Most cells were oval in shape although some had cytoplasmic projections (Figure 5.11.).

The staining was in the cytoplasm, with a greater staining intensity on one side of the nucleus than the other side (Figure 5.11.). The nuclei were unstained. Unlike the G cells, the A cells in all 3 groups appeared to be stained with equal intensity

There were no significant differences between the number of EI stained cells per mm^2 in either of the parasite infected groups and the control group (Figures 5.3., 5.4.).

5.4.5. GRP Neurons

The axons and dendrites of the GRP containing neurons in both the body and antrum of the abomasum were very small and difficult to see due to their size and low staining intensity. They were observed as small rings and were stained a golden colour (Figure 5.12.). These GRP immunoreactive nerve fibres were located within the lamina propria, muscularis mucosa, submucosa and tunica muscularis (Figures 5.12. and 5.13.). There were very few stained nerve fibres present in the sections; the density was too low to make accurate comparisons of their number between the parasite infected sheep and the controls.

5.4.6. Neutrophils

Neutrophils were present in the mucosa of both groups of parasitised sheep and stained with the immunocytochemical reaction as the endogenous peroxidase was not blocked

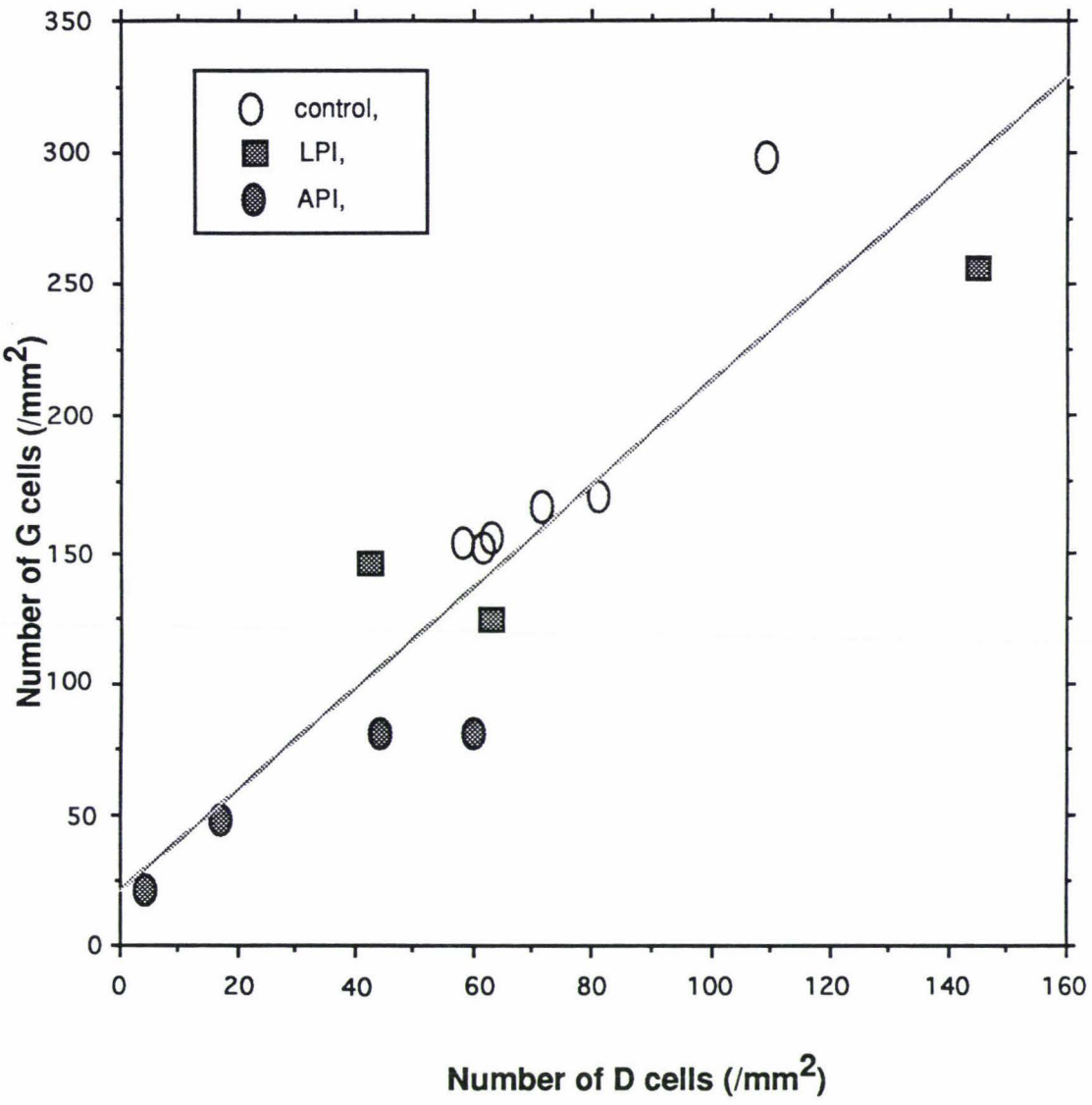


Figure 5.10. Relationship between the number of D cells and the number of G cells immunocytochemically stained in the abomasal antrum. LPI = larvae parasite infected sheep, API = adult parasite infected sheep.
$y = 1.918x + 21.7$, $r = 0.90$, $P<0.001$

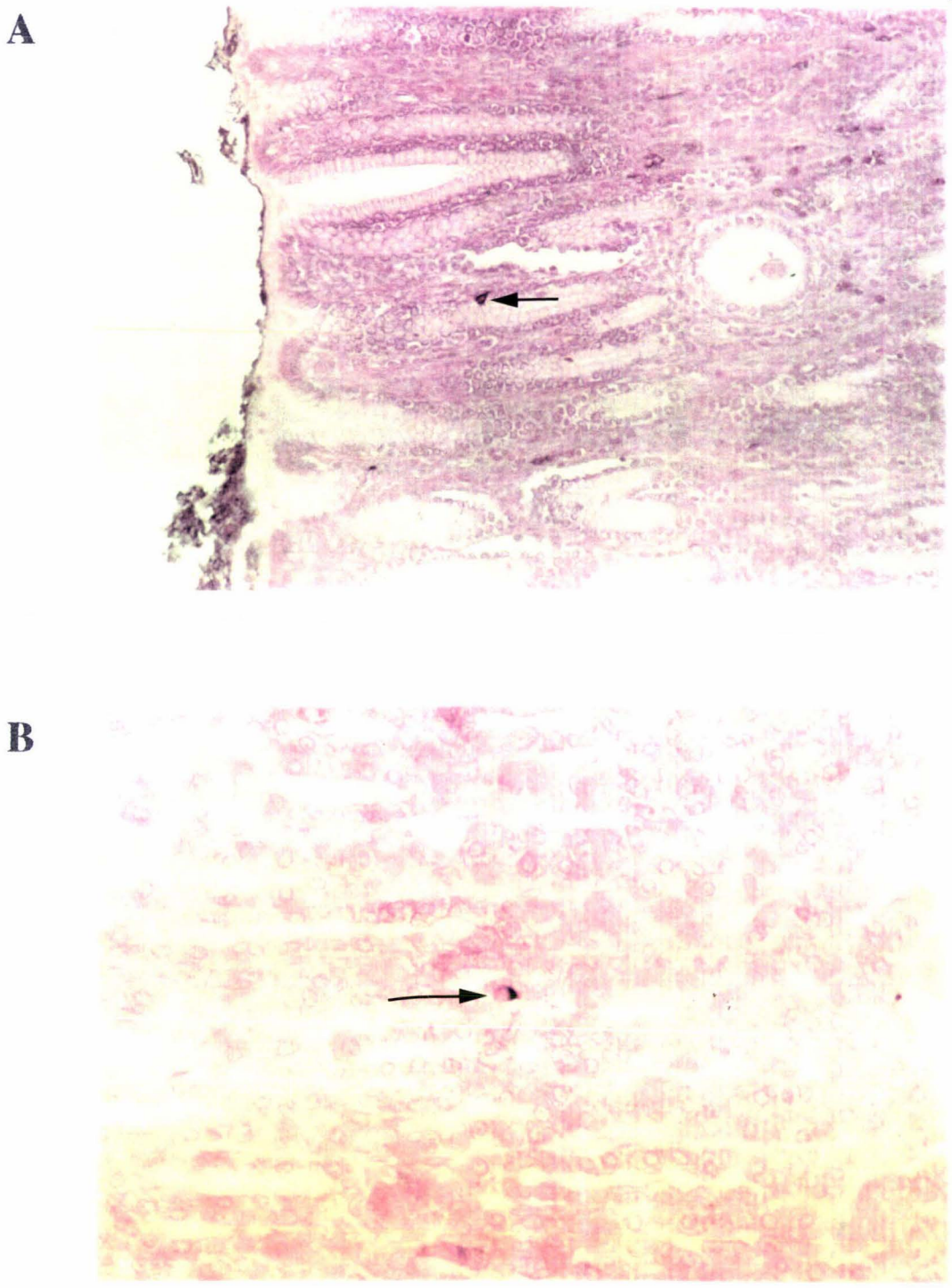


Figure 5.11. Enteroglucagon-immunoreactive stained cells in the abomasal mucosa. A: antrum, magnification: x25. B: body, magnification: x100. Stain: eosin.

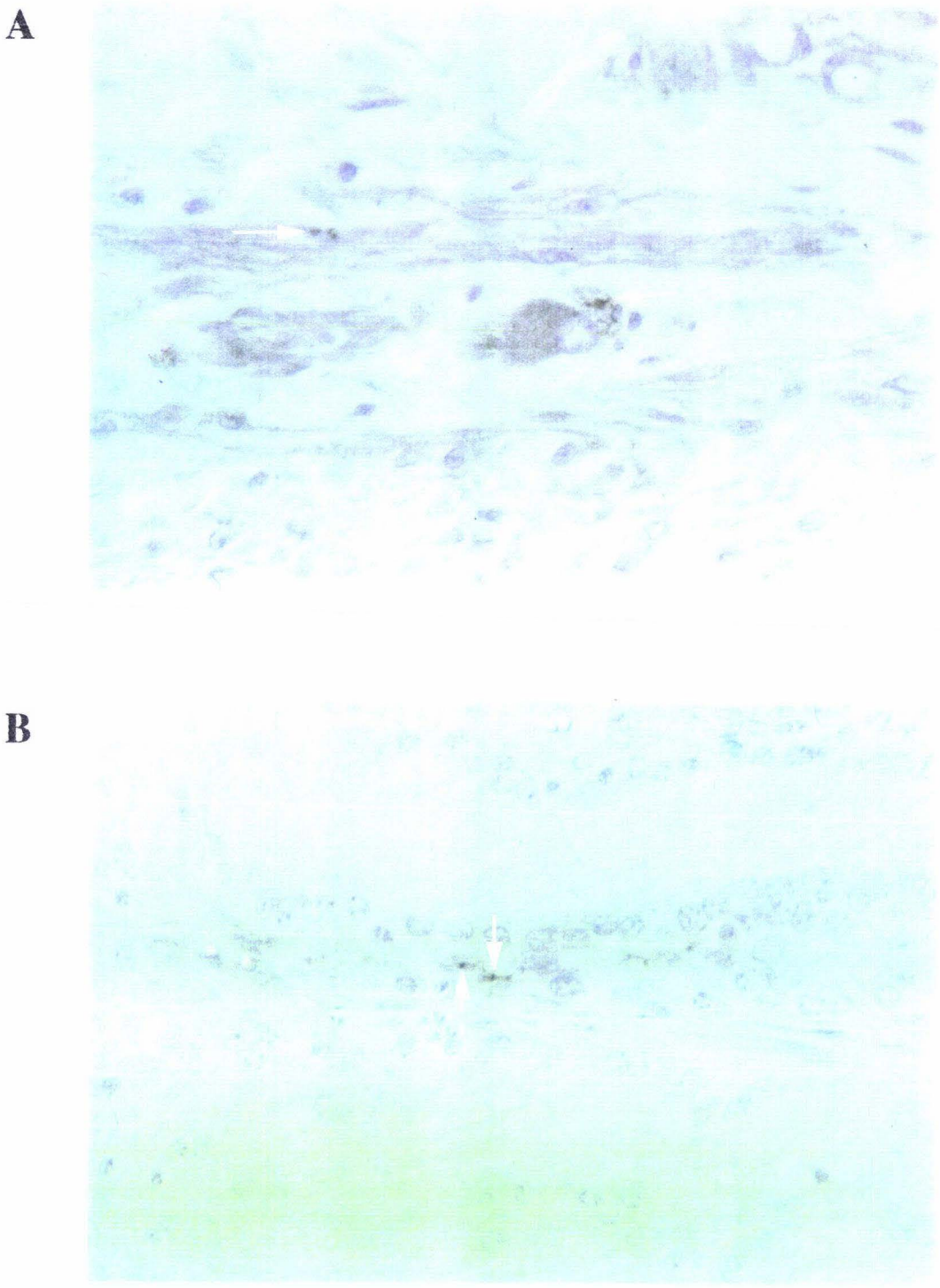
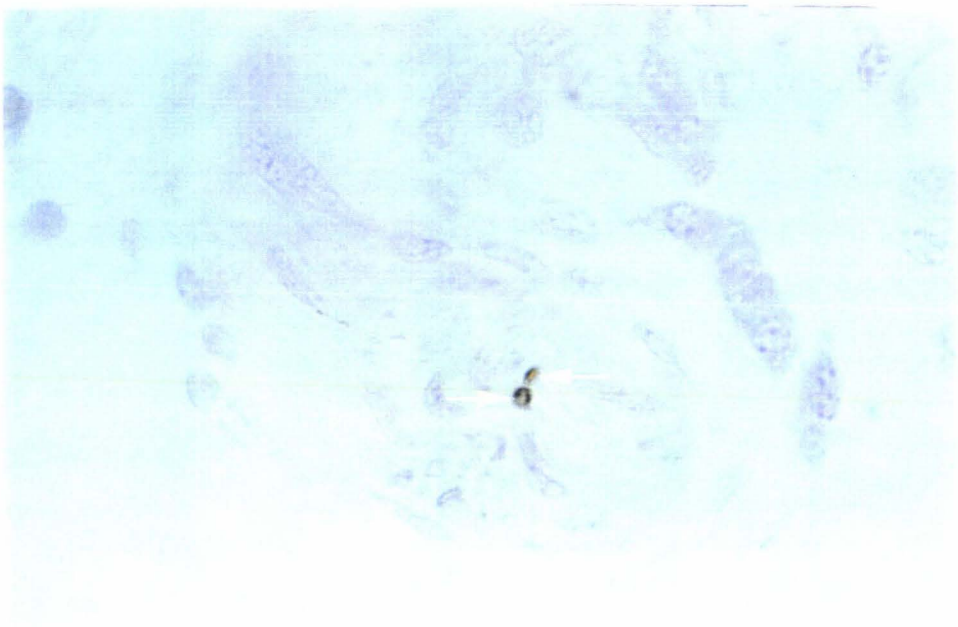


Figure 5.12. GRP-immunoreactive neurons (arrows) in the lamina propria of the antrum of a non-infected control sheep. Magnification: x160. Stain: eosin, D.I.C.

A



B

Figure 5.13. GRP-immunoreactive neurons (arrows) in the antrum of a non-infected control sheep. A: submucosa. B: tunica muscularis. Magnification: x160. Stain: eosin, D.I.C.

prior to staining. They were easily distinguished from endocrine cells due to differences in morphology (Figure 5.14.) and were not included in the endocrine cell counts.

5.4. DISCUSSION

5.4.1. Chromogranin A

Chromogranin A antisera reacts with the chromaffin located within the secretory granules of endocrine cells. It was used in this study to give an indication of the concentration of endocrine cells in the abomasum of sheep infected with either adult or larvae *O. circumcincta*, and compare the results with the non-infected control sheep. The results show that:

1) There were no significant differences in the number of chromogranin-immunoreactive cells in the antrum or body of either group of parasitised sheep relative to non-infected control sheep.

2) There were many more chromogranin-immunoreactive cells per unit area of mucosa in the body region of the abomasum than in the antrum.

3) The cytoplasm of the cells was stained more intensely on one side.

In the antral region, a greater density of cells stained for gastrin or somatostatin than for chromogranin, indicating that not all D or G cells react with antisera to chromogranin A. The protein chromogranin A is contained within chromaffin granules in the cytoplasm of the cell (Bloschko *et al.*, 1967; Fischer-Colbrie *et al.*, 1985). Gurnsey (1985) stained ovine abomasal sections using the chromaffin method, and found it to be unreliable, with a very poor staining intensity. The reason for this is not known. There may be some difference in the structure of chromaffin in the ovine abomasum compared to other species. Comparison of the amino acid sequences of human and bovine chromogranin A revealed that the amino acid sequence of the middle portion of the 429

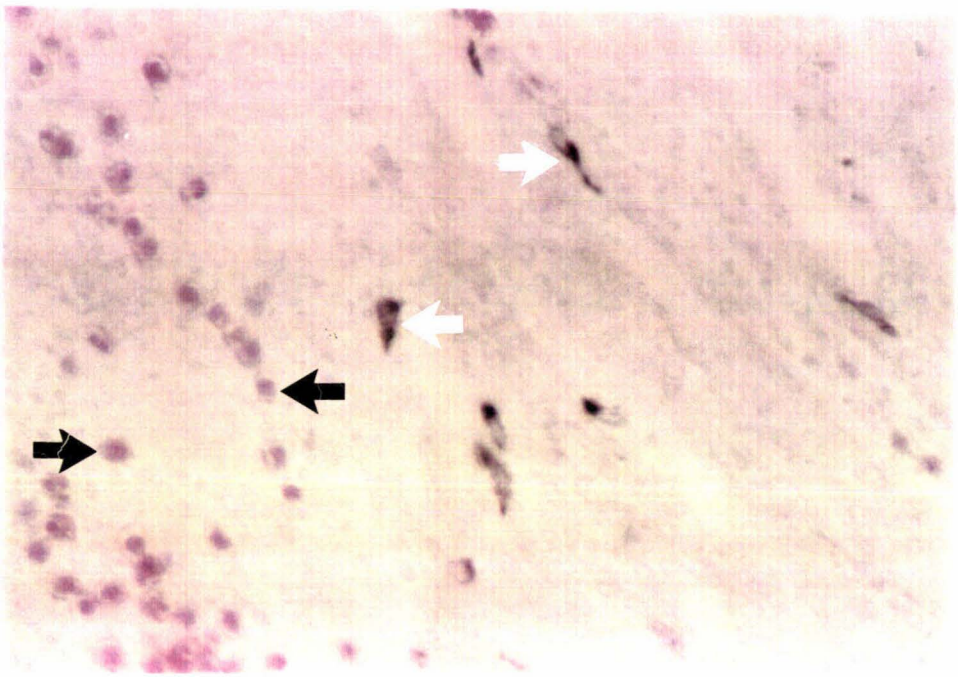


Figure 5.14. Neutrophils (black arrows) and endocrine cells (white arrows) in the antral mucosa of the abomasum of a larval parasite infected sheep. Magnification: x100. Stain: eosin.

residue long proteins differs between the 2 species by 36% (Konecki *et al.*, 1987). The structure of ovine chromogranin A has not been determined.

To test whether or not the poor staining obtained in the present study was due to the Bouin's fluid, antral tissue from a control sheep was fixed in PLP which is less damaging to the microstructure of the tissue (see Section 5.2.2.), and cryostat sections were cut and immunocytochemically stained for chromogranin. The PLP-fixed sections did not contain a greater number of stained cells than did the Bouin's-fixed tissues from the control sheep, indicating that fixation in Bouin's fluid did not adversely affect the staining reaction compared to PLP.

There have been no previous reports of which cell types in the ovine abomasum react with antibodies to chromogranin A. Comparison of serial sections immunocytochemically stained for chromogranin or one of several secretory products including gastrin and somatostatin has shown that the cell types that stain for chromogranin vary between species (Table 5.2.). These inter-species differences are most likely due to differences in the structure of chromogranin A (Konecki *et al.*, 1987). Facer *et al.* (1985) reported that in the human gastrointestinal tract D cells also bind antibodies against chromogranin A.

In the present study comparison of serial sections of the mucosa from the abomasal body which were stained for chromogranin A or somatostatin, showed that ovine D cells are not chromogranin-immunoreactive. This observation needs to be confirmed using the "elution technique" whereby a section is stained for one antibody and photographed, the staining reaction is "eluted" and the section restained with the second antibody and rephotographed. The 2 images are then compared. This allows the same cells to be examined following staining with more than one antisera (Larsson, 1988).

Table 5.2. Cells which stain for chromogranin A in various species, determined by immunocytochemical staining of serial sections (Rindi *et al.*, 1986).

Species	Cell type			
	Pancreatic glucagon	G cell	ECL cell	Serotonin producing EC cell
ox	-	+	+	+
guinea pig	+	+	+	+
dog	+	+	-	+
cat	-	-	-	+
rat	-	+	+	+
pig	+	-	-	+
mouse	+	-	+	+
human	+	+	+	+

+ stained, - did not stain

Based on the present evidence it appears that chromogranin A is a poor indicator of the number of endocrine cells in the sheep abomasum.

5.4.2. G cells

The concentration of G cells in the abomasum was examined to determine whether the hypergastrinaemia of ostertagiasis is accompanied by a change in the frequency of G cells. The following observations were made:

1) Significantly fewer G cells were stained in the antrum of the API sheep compared to the non-infected control sheep.

2) In the API sheep, many of the G cells were poorly stained and some were barely

visible, whereas in the control animals, all of the G cells appeared to be stained with the same high intensity. A few of the G cells in the API sheep stained with a high intensity.

3) Many of the G cells in the abomasal antrum had cytoplasmic projections. They occurred in both the control and parasitised animals.

4) There were no gastrin-immunoreactive cells visible in the body of the abomasum.

There were fewer antral G cells/mm^2 of mucosa in the API sheep compared with the non-infected control sheep. A decrease in G cell numbers has been observed in cattle 28 days after infection with *Ostertagia ostertagi* larvae (Fox *et al.*, 1993) however, this was thought to be due to a reduction in staining intensity rather than a decrease in the number of G cells. A reduced staining intensity may also have contributed to the low G cell count recorded for the API sheep in the present study. In the control sheep, all G cells appeared to have been stained with equal intensity, whereas in the API sheep, some cells were stained with a low intensity, whereas others were barely visible. It is assumed that the barely visible cells had a depleted gastrin content. Håkanson *et al.* (1982) removed the acid producing part of the stomach in rats (fundectomy), which in turn caused a hypergastrinaemia. The number of antral G cells in the fundectomised rats was increased 6-10 weeks later relative to non-fundectomised control rats and there were fewer cytoplasmic granules per G cell in the fundectomised rats. In the present study a hypergastrinaemia was present in the API sheep (Table 2.4.) indicating that some G cells may have been depleted of stored gastrin due to hyperstimulation and therefore were stained weakly or not at all with gastrin antisera. There may actually have been an increase in the number of G cells in the API sheep which was not observed due to a decrease in the number of gastrin granules in the G cells resulting in some G cells not being stained.

Omeprazole, an inhibitor of gastric acid secretion, has been shown to provoke a hypergastrinaemia in calves (Fox *et al.*, 1989) and rats (Allen *et al.*, 1986; Blom, 1986; Larsson *et al.*, 1986; Tielemans *et al.*, 1989; Axelson *et al.*, 1990; Dockray *et al.*, 1991).

After 14 days of omeprazole treatment in rats there is a twofold increase in the amount of gastrin stored in the antrum and a tenfold increase in the amount of messenger RNA (Dockray *et al.*, 1991). In non-omeprazole treated rats, about 0.08% of the stored gastrin was released per minute, compared with approximately 0.4% in the omeprazole-treated rats. The increased gastrin synthesis was attributed to the increased messenger RNA levels with most of the increased gastrin production being directly secreted as opposed to being stored. With more gastrin stored and then released in association with hypergastrinaemia, the amount of gastrin remaining stored within the G cells would decrease, which appeared to occur in the present study.

Comparison of the number of G cells with the extent of the hypergastrinaemia (Table 5.3.) in the individual sheep supports the hypothesis that not all of the G cells in the API sheep were observed due to the depletion of the gastrin stores. Analysis of the data showed that the correlation coefficient of the data was -0.632 and the regression coefficient was -0.95 ± 0.388, with $P< 0.037$. The sheep with the higher average serum gastrin concentrations had a lower concentration of G cells than did those with the lower average serum gastrin concentrations.

There was no significant difference between the number of G cells per area in the LPI sheep compared with non-infected control sheep. There are 2 possible explanations for this observation. The LPI sheep had a lower hypergastrinaemia than did the API sheep (Table 5.3.). The faecal egg counts were also lower in the LPI sheep (Figure 2.4.) suggesting that there were fewer adult parasites present in the LPI sheep than in the API sheep, hence the stimulus for gastrin release may have been greater in the API sheep. Therefore, sufficient stored gastrin may have been present in the G cells of the LPI sheep to enable them to be easily seen. The hypergastrinaemia of ostertagiasis is stimulated by both the larval and adult parasites. It was first detectable in the LPI sheep during the first week following the infection when no adult parasites would have been present. Another explanation is that as LPI sheep had their parasite burden for a much longer period of time than the API sheep (55 days compared with 8 days), gastrin synthesis may have increased in the LPI sheep. This could not be confirmed in the present experiment.

Table 5.3. The concentration of G cells in the antral mucosa (cells/mm^2) at the completion of the experiment and the average serum gastrin concentration (pM) during the experiment. API = adult parasite infected sheep, LPI = larval parasite infected sheep.

Group	Sheep number	Concentration of G cells	Average serum gastrin
controls	1	170	27.4
	2	154	41.8
	3	153	39.9
	4	155	32.7
API	1	80	283.2
	2	81	140.6
	3	21	319.8
	4	48	96.7
LPI	1	124	99.1
	2	146	93.5
	3	257	105.3

The cellular projections from the G cells appeared to extend either towards the lamina propria or into the gland lumens. These projections may be the means whereby the G cell maintains contact with its environment, enabling other substances to modulate the secretion of gastrin. The immunocytochemical staining revealed the presence of gastrin within these projections; gastrin may also be secreted from these projections, which may end near capillaries in the same way as D cells (Larsson *et al.*, 1979).

In conclusion, the hypergastrinaemia observed in ostertagiasis does not appear to be associated with an increase in the concentration of G cells in the ovine abomasum.

5.4.3. D Cells

Somatostatin inhibits the release of gastrin from G cells *via* a paracrine pathway (see Section 1.1.3.2.1.). Hence a change in the number of D cells, resulting in a change in the ratio of G:D cells in the antrum, could theoretically affect the amount of gastrin

released from the G cells. The results from the present study show that:

1) More somatostatin immunoreactive cells per unit area were present in the antral region than in the body region of the control sheep.

2) There were no significant differences in the number of D cells in the body or antrum of either group of parasitised sheep compared with the non-infected control sheep.

3) There was a strong correlation between the number of G cells and the number of D cells in the antrum of all 3 groups of sheep.

The presence of more D cells in the antrum than in the body region of the abomasum is in agreement with the results obtained by Gurnsey (1985) and has been previously reported by Vergara-Esteras *et al* (1990), who studied the effects of the parasite *Haemonchus contortus* on D cell density in the ovine abomasum. In the latter study, 3 sheep were infected with *H. contortus* larvae. After 5 weeks, the sheep received an anthelmintic, and the sheep were euthanased 14-15 days later. The number of D cells increased in 2 out of 3 sheep compared with non-infected control sheep. Vergara-Esteras *et al.* suggested that the increase in D cell number was due to an "overshoot in a rapidly recovering hyperplastic mucosa of the post-parasitised abomasum" as opposed to being a consequence of the parasitism *per se*. It would be interesting to test whether an increase in D cell number occurs in *O. circumcincta* infected sheep after treatment with an anthelmintic. This may contribute to the decrease in serum gastrin levels back to control levels after the parasites have been expelled.

In the present study, the concentration of D cells in the antrum of the control sheep (66 $\pm$ 5 cells/mm^2) was less than that (115 $\pm$ 16 cells/mm^2) in the study by Vergara-Esteras *et al.* (1990). This is most likely due to the difference in the thickness of the tissue sections; in the present study, the tissue sections were 6 μm thick, while Vergara-Esteras *et al.* (1990) used sections 10 μm thick. The other abomasal region studied in the present experiment was the body, whereas the "fundus" was examined by Vergara-Esteras *et al.* (1990). The exact location of the fundic sampling site was not described

by Vergasa-Esteras *et al.* (1990) but may correspond to the region designated the body in the present study. Vergara-Esteras *et al.* (1990) found approximately twice the number of D cells in the "fundus" as was found in the body in the present study, which may also be due to the thickness of the tissue sections (see above).

In the present study there was a significant correlation between the numbers of G and D cells in the antrum when all sheep were combined with approximately 1 G cell to 1.9 D cells. This ratio was similar in parasitised and control sheep. In the bovine, this ratio has been shown to be 1:1.2 (Weyrauch *et al.*, 1989).

As discussed above, there was a significant reduction in the concentration of antral G cells in the API sheep, possibly due to a reduction in the intensity of staining and hence the detection of G cells. The G:D cell ratio in the API sheep did however remain approximately the same as in the non-infected control sheep due to a non-significant reduction in the number of antral D cells in the API sheep (see Figure 5.4.). Therefore, some D cells may not have stained with the somatostatin antisera. This may have been due to depletion of the amount of somatostatin stored within the D cells, suggesting that the adult parasites excessively stimulated the release of somatostatin. Somatostatin inhibits gastric acid secretion *via* a paracrine pathway (Schubert and Maklouf, 1992). If somatostatin release is stimulated by the adult parasites, this may at least partially explain the decrease in gastric acid secretion resulting in the increased abomasal pH. The effects of *Ostertagia* parasites on somatostatin secretion could be investigated by measuring circulating levels of somatostatin and its concentration in samples of interstitial fluid collected from abomasal tissues using microdialysis probes (Ungeratedt, 1991). In children with hypergastrinaemia due to antral gastrin cell hyperfunction, the concentration of antral G cells is significantly increased and the concentration of antral D cells decreased relative to controls, resulting in a doubling of the G:D cell ratio (Rindi *et al.*, 1994). A decrease in the G:D cell ratio has been reported in humans with hypergastrinaemia due to Zollinger-Ellison syndrome, due to D cell hyperplasia (Arnold *et al.*, 1982; 1991). Therefore, there does not appear to be a mechanism controlling the G:D cell ratio.

In conclusion, the hypergastrinaemia observed in ostertagiasis does not appear to be associated with a change in the G:D cell ratio.

5.4.4. GRP Neurons

In the present study, the distribution and density of GRP containing neurons in the abomasal antrum were examined as these neurons stimulate G cells directly to release gastrin, as well as indirectly by inhibiting somatostatin release from antral D cells (Schubert and Makhlouf, 1992). The following observations were made:

1) Due to the low intensity of staining, there were too few GRP containing nerve fibres visible in the tissue sections to allow their density to be accurately determined.

2) Nerve fibres which stained with GRP antisera were observed in the lamina propria, muscularis mucosa, submucosa and muscularis externa.

Although the presence of GRP immunoreactive nerve fibres in the ovine abomasal muscularis externa, lamina propria and muscularis mucosa has previously been reported (Wathuta, 1986), their presence in the submucosa has not.

The effects of larval and adult *O. circumcincta* parasites on the number of nerve fibres containing GRP was not determined in the present study due to the very low staining intensity. This may have been due to weak binding of the antibody to the antigen because of species differences in the structure of the antigen-binding site (the GRP antisera was raised in rabbits against human GRP) allowing the antibody to be easily knocked off during subsequent washes of the tissues. The structure of human GRP has not been compared with the structure of ovine GRP. Alternatively, this may be due to the arrangement of the antigen within the nerves; if there was a low concentration of antigen, this will result in a low staining intensity (M.J. Birtles, pers. comm.). Better results could possibly be obtained by using an antisera raised against ovine GRP.

5.4.5. A Cells

Enteroglucagon secretion is increased in some disease states and thought to have a trophic effect on the gastrointestinal mucosa (see Section 5.1.2.), so may have been at least partly responsible for the increase in mucosal thickness observed in Chapter 3. The following observations were made in the present study:

1) Few enteroglucagon immunoreactive cells were present in both the antrum and body regions of the abomasum.

2) There were no significant differences in the number of enteroglucagon immunoreactive cells stained in the abomasal antrum or body of the parasitised sheep compared with the non-infected control sheep.

The number of A cells stained in the antrum in the present study are similar to the results obtained by Calingasan *et al.* (1984), who reported the presence of 2.08 $\pm$ 0.56 cells/mm^2 in the antrum, 0.12 cells/mm^2 in the body region and 0.12 cells/mm^2 in the cardiac region of the sheep abomasum. In this study there was a greater concentration of enteroglucagon-immunoreactive cells in the body than was reported by Calingasan *et al.*, (1984). This was most likely due to the thickness of the sections; 2-4 μm thick sections were used in the study by Calingasan *et al.* (1984) compared to 6 μm thick sections in the present study.

The number of A cells in the control sheep was similar in the body and antrum, with 2.0 $\pm$ 0.6 cells/mm^2 in the antrum and 3.0 $\pm$ 1.0 cells/mm^2 in the body. The low concentration of A cells and the high standard errors due to the between animal variation may have resulted in any difference between parasitised sheep and controls in the number of A cells remaining insignificant.

Although there appeared to be no difference in the concentration of A cells between the parasitised and the control sheep, more enteroglucagon may have been secreted from the A cells in the parasitised sheep resulting in an increased plasma concentration of

enteroglucagon. The effects of *O. circumcincta* on circulating enteroglucagon levels have not been reported.

5.5. SUMMARY

The present results show that chromogranin A is a poor indicator of the total number of endocrine cells in the antral mucosa of the sheep. Chromogranin A does not appear to stain ovine D cells.

Although the adult parasite infected sheep had fewer gastrin-immunoreactive cells than the control sheep, this is probably due to the depletion of gastrin granules in the G cells concomitant with the hyperstimulation resulting in the hypergastrinaemia, as opposed to the presence of fewer G cells. No differences were present in the number of G cells in larval parasite infected sheep compared to non-infected controls.

There was a high correlation in the G:D cell ratio in the antrum of both the control and the parasitised sheep. As there was no difference in the G:D cell ratio in the adult parasitised sheep which had fewer stained G cells, compared to the control sheep, some D cells may not have stained, possibly due to depletion of intracellular somatostatin granules. The large standard errors present in the results of staining for D cells may have masked any significant differences present.

Nerve fibres containing GRP immunoreactivity were observed in the abomasal muscularis externa, lamina propria, muscularis mucosa and submucosa.

Few enteroglucagon-immunoreactive cells were stained in the ovine abomasum in both control and parasitised sheep.

6. GENERAL DISCUSSION

The larvae of *Ostertagia* parasites are generally considered to affect abomasal function and decrease productivity to a greater extent than the adults (Coop *et al.*, 1977). This concept has arisen largely because the larvae physically invade the mucosa of the abomasum altering both its appearance (Armour *et al.*, 1966) and its secretory activities (McLeay *et al.*, 1973; Anderson *et al.*, 1976). The adult parasites do not invade the mucosa, instead they live in the lumen of the abomasum, albeit in close association with the mucosa (Threlkeld, 1934). Because the adults are considered "lumen dwellers" scant attention has been given in the past to their affect on the abomasal mucosa and its secretions. The present experiments have shown that adult *Ostertagia* parasites, when introduced directly into the abomasum of previously helminth-free sheep, have pronounced affects on both the histological appearance and the secretory activities of the abomasal mucosa (Table 6.1.).

Several of the affects of the adult parasites observed in the present study closely resemble those seen in sheep which have been given *O. circumcincta* larvae *per os* under experimental conditions (Armour *et al.*, 1966; McLeay *et al.*, 1973; Durham and Elliot, 1976; Anderson *et al.*, 1988) or have ingested the larvae while at pasture (Coop *et al.*, 1985). How the adult parasites bring about these changes to the abomasum are not known. One or both of two possible mechanisms may be involved. Firstly, adult helminth parasites are known to secrete/excrete compounds collectively called SE products (Behnke *et al.*, 1992). Little is known of the biological actions of SE products (Behnke *et al.*, 1992). It is possible they have direct trophic affects on the abomasal mucosa and cause increases in abomasal weight and mucosal thickness and hypertrophy such as those seen in the present study. Secondly, the same SE products may stimulate gastrin release which, because of its trophic actions (Johnson *et al.*, 1969; Chandler and Johnson, 1972; Johnson, 1977; Majumdar and Goltermann, 1978), causes hypertrophic growth of the abomasal mucosa.

Table 6.1. Comparison of the changes occurring to the gastrointestinal tissues of sheep infected with adult (API sheep) or larval (LPI sheep) *O. circumcincta* parasites.

Effect	adult	larval
Serum gastrin concentration	+	+
Abomasal pH	+	+
Serum pepsinogen concentration	+	+
Presence of nodules on abomasal mucosa	NC	+
Organ Weights		
Reticulo-rumen weight/kg body weight	+	NC
Abomasal weight/kg body weight	+	+
Duodenal weight/kg body weight	NC	+
Jejunum and ileum weight/kg body weight	+	NC
Terminal ileum weight/kg body weight	+	NC
Morphometric Measurements		
Thickness of mucosa of the abomasal body	+	+
Pit and gland depth in the mucosa of the abomasal body	NC	+
Thickness of the mucosa of the abomasal antrum	NC	+
Thickness of the mucosa of the upper duodenum	NC	+
Cell Counts (mucosal)		
Abomasal body	NC	+
Abomasal antrum	+	NC
Jejunum	NC	+
Hypertrophic or Hyperplastic Increases (mucosal)		
Abomasal body	t	p
Abomasal antrum	NC	p & t
Jejunum	NC	p
Endocrine Cell Numbers		
Concentration of antral G cells	-	NC

NC: no change, +: increased -: decreased p: hyperplasia t: hypertrophy

The sheep infected with *O. circumcincta* larvae, as did those infected with the adults, exhibited increases in abomasal weight and the thickness of the abomasal mucosa. The evidence presented in Chapter 4 indicates that the increased mucosal thickness in the larvae infected sheep involved hyperplasia, but not hypertrophy as occurred in the adult

infected sheep. Why one group of infected sheep but not the other should have hyperplasia is not known. The difference may be due to differences between the mechanisms where by larvae and adult parasites affect host tissues. It would appear that the trophic effects of both the larvae and the adults are not mediated solely by the increased circulating levels of gastrin; if gastrin was the sole mediator then its effect on mucosal growth should have been the same for both groups of animals. This does not however take into account the different lengths of time circulating gastrin levels were elevated in the 2 groups (see Chapter 2). The LPI sheep had their parasite burden for a much greater length of time than did the API sheep (55 days compared with 8 days), with the hypergastrinaemia also present for a greater period of time (Table 2.4.).

The role of gastrin in hypertrophic growth of the abomasum during ostertagiasis could be investigated by intravenously infusing exogenous gastrin into sheep for several weeks to maintain circulating gastrin at levels seen during ostertagiasis. Omeprazole inhibits gastric acid secretion and provokes a hypergastrinaemia in the calf (Fox *et al.*, 1989) and rat (Allen *et al.*, 1986; Blom, 1986; Larsson *et al.*, 1986; Tielemans *et al.*, 1989; Axelson *et al.*, 1990; Dockray *et al.*, 1991). Omeprazole treatment increases ECL cell density in rats, with this increase directly correlated with serum gastrin levels (Larsson *et al.*, 1986). An examination of general growth responses in the digestive tract following 16 weeks of omeprazole administration to rats revealed growth of the mucosa of the gastric body, but not the antrum, small intestine, large intestine and pancreas (Håkanson *et al.*, 1986).

In the rat, gastrin has been shown to increase the proliferative activity in the fundic, duodenal and jejunal mucosa after administration (Casteleyn *et al.*, 1977). The proliferative activity was measured by calculating the percentage of cells labelled after the administration of tritiated thymidine and also the mitotic indices for each tissue. Significant changes in these 2 parameters were observed 16 hours after gastrin administration. Hyperplasia most likely did occur in these regions in the sheep infected with adult parasites, however, because of the relative insensitivity of the methods used in the present study, the proliferation was not identified. Eight days may not have been a sufficient length of time whereas 55-57 days would be expected to be sufficient time

for the trophic effects to become detectable.

The reason for the difference in the times that the infections were maintained in the different groups was that the abomasal pH in the API sheep began to decrease towards pre-infection levels 8 days after the initiation of the infection. Therefore, this group of sheep was euthanased before the tissues started to recover. In the LPI sheep, the larvae would still have been within the mucosa at an immature stage 8 days after the infection (Threlkeld, 1934). The infection in the API sheep may be able to be maintained for a greater length of time by repeatably dosing the sheep with adult worms *via* the abomasal cannula every 2-3 days. Such an experiment would however have a high ethical cost; in the present experiment, 8 sheep were required to provide sufficient adult parasites to dose 4 sheep once. Whether this alternative method would result in the abomasal pH remaining elevated depends on the reason why the abomasal pH decreased in the present experiment. It may have been due to the sheep becoming refractory to the adult worms, suggesting that the presence of larvae is required in the abomasum for the effects on the abomasum to continue. However, this needs further investigation. There is no direct evidence that sheep become refractory to internal parasites, but there is evidence that sheep can tolerate substantial numbers of parasites in the abomasum without apparent effects (Watson and Baker, 1986). Alternatively, there may have been a loss of adult worms from the abomasum, in which case trickle infecting the sheep with adult parasites would result in the continuation of the infection. The faecal egg counts (Figure 2.4.) in the API sheep decreased over the last 4 days of the infection from 2,000 e.p.g. to 800 e.p.g., indicating that the number of adult parasites in the abomasum decreased during this time.

If the infection with adult parasites could not be maintained for a longer time, a second group of control sheep which were maintained for the same period of time as the API sheep should be added to the experiment. This would eliminate any differences due to the different ages of the sheep.

The difference between the effects of larvae and adult parasites is further emphasised by the trophic response of the antral mucosa to the larvae but not the adults. *Ostertagia*

larvae invade the antral glands as well as those in the body of the abomasum where they develop and emerge as young adults causing distension of the glands and histological changes to the surrounding tissues (Armour *et al.*, 1966). The results of the present study indicate that the changes to the antral mucosa during ostertagiasis are largely due to the effects of the larvae. How these changes are brought about is not known but may involve direct effects of the larvae or the actions of SE products produced by the larvae. Gastrin is unlikely to be involved as there is considerable evidence indicating that gastrin does not have a trophic affect on the antral mucosa (Casteleyn *et al.*, 1977).

In these experiments, there were large variations between the animals in each group, resulting in large standard errors in the data. The inclusion of additional sheep in each experimental group would have reduced the inter-animal variation. The lowest possible number of sheep was used in this experiment (4 in each experimental group) due to the high ethical cost of the experiment. Alternatively, biopsy samples could be taken from the abomasum through an abomasal cannula at least daily throughout infections with adult and larvae *O. circumcincta*. Morphometric measurements, biochemical assays and immunocytochemistry could be carried out on these samples in the same manner as in the present experiment, eliminating inter-animal variation. The development of the infection could be examined in this manner.

These experiments have also allowed a greater understanding of the mechanisms controlling the hypergastrinaemia present in ostertagiasis. The hypergastrinaemia is not due to an increase in the number of G cells in the antral mucosa. Therefore the hypergastrinaemia must be caused by an increase in the secretion of gastrin from the G cells and/or a decrease in the breakdown of gastrin in the blood. The half-life of circulating G17 is between 4 and 10 minutes in the human (Boniface *et al.*, 1976; Walsh *et al.*, 1976; Pauwels *et al.*, 1985), approximately 2 minutes in the cat and 3-5 minutes in the dog (Walsh *et al.*, 1974; Boniface *et al.*, 1976). Gastrin is metabolised at several different sites. In the dog, 20-30% of gastrin is removed in a single passage by the major vascular beds (Strunz, 1978). The kidney extracts 30% of the gastrin presented to it with less than 2% of the extracted gastrin excreted in the urine of the dog (Davidson *et al.*, 1973).

Whether or not the hypergastrinaemia is due to a decrease in the breakdown of gastrin in infected sheep could be determined by intravenously injecting labelled gastrin into infected and non-infected sheep and determining the amount of gastrin remaining in the circulation at predetermined times after the injection. The results from the 2 groups of sheep could be compared to determine whether the clearance of gastrin from the circulation changes during infection with *O. circumcincta*.

Adult parasites may stimulate gastrin secretion either due to the release of SE products which provoke gastrin release, or by causing reactions in the host tissues which result in the hypergastrinaemia.

An inflammatory reaction occurs in both adult and larval *O. circumcincta* infected sheep, as demonstrated by the presence of eosinophils and neutrophils within the mucosa. The administration of anti-inflammatory agents throughout the infection would allow the effects of the inflammatory reaction to be determined. The effects of anti-inflammatory agents on the tissues of parasitised animals has not been reported. Inflammation is thought to aid in the expulsion of parasites from the host's gut, with a combination of mediators involved (Moqbel and MacDonald, 1990). Aspirin, which inhibits prostaglandin synthesis, inhibits expulsion of *Nippostrongylus brasiliensis* by rats (Dineen *et al.*, 1974) but does not effect expulsion of *Trichostrongylus colubriformis* by guinea pigs (Rothwell *et al.*, 1977). The assays for RNA, DNA and protein would be more accurate without the presence of neutrophils and eosinophils in the mucosa as eosinophils and neutrophils are not generally present in non-infected sheep, but contain RNA, DNA and protein.

In both the adult and larval infected sheep, the mucus-secreting cells appeared to be devoid of mucous, as they were almost clear after staining with alcian blue. Further examination of the effects of the parasites on mucus production would allow a greater understanding of the local effects of *O. circumcincta*. Samples could be taken by biopsy as described above and fixed in the manner described by Kerss *et al.* (1982). The effects of the parasites on the mucous throughout the experiment could then be examined.

6.2. CONCLUSIONS

Experiments described in this thesis have shown that:

1) Adult as well as larval *O. circumcincta* parasites stimulate hypergastrinaemia, a decreased abomasal pH and elevated serum pepsinogen concentrations.

2) The lumen dwelling adult *O. circumcincta* affect the mucosa of the abomasum resulting in an apparent inflammatory reaction, demonstrated by the presence of eosinophils and neutrophils in the lamina propria. Mucous production and/or secretion is also affected, shown by the presence of large mucus-secreting cells in the mucosa.

3) The total wet weight of the abomasum/kg body weight is increased in sheep infected with *O. circumcincta*, with an increase in the total size of the abomasum. The larval parasites evoke a hyperplasia in both the antral and body mucosae with little change in cell size. In sheep infected with adult parasites, the thickness of the abomasal mucosa is increased in the body, but not the antrum. This increase is most likely due to hypertrophy.

4) Either the larval *O. circumcincta* or the hypergastrinaemia have trophic effects on the upper duodenum, with an increased mucosal thickness which did not occur more distally. This did not occur in the adult parasite infected sheep.

5) The larval parasites or hypergastrinaemia provoked a hyperplasia in the jejunal mucosa. This did not occur in the adult infected sheep.

6) The larvae and adult parasites did not appear to exert a hypertrophic or hyperplastic effect on the ileum, caecum or colon.

7) While the concentration of G cells did not change in the larval parasite infected sheep compared with the non-infected control sheep, the total number of G cells was increased due to an increase in mucosal thickness. There appeared to be fewer G cells present in

the adult parasite infected sheep compared with the non-infected control sheep, which was most likely due to a depletion of their gastrin content due to overstimulation.

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