THE EFFECT OF DIETARY ENERGY AND PROTEIN INTERACTION ON THE PHYSIOLOGICAL PLASTICITY OF THE SMALL INTESTINE OF THE YOUNG OSTRICH

by

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DECLARATION

I declare that the thesis, which I hereby submit for the degree PHILOSOPHIAE DOCTOR at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature:…… Date:……

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ABSTRACT

The study includes separate papers, which are all linked by their emphasis on the effect of different diets on the impact of the development of the small intestine of the young ostrich. This abstract is intended to provide readers with a broad overview of the outcomes of the study.

Part 1: Yolk utilisation and the development of the small intestines

Chapter 2 deals with the composition of egg yolk as it is absorbed by starved ostrich (*Struthio camelus* L.) chicks from one to seven days post-hatching and for ostrich (*Struthio camelus* L.) chicks from one to sixteen days post-hatching on a pre-starter broiler diet.

Chapter 3 provides information on the intestinal enzymes of ostrich (*Struthio camelus* L.) chicks from one to sixteen days post-hatching on a pre-starter broiler diet. The effect of growth on enzyme activity immediately post-hatching up to sixteen days of age was studied.

Chapter 4 details a histological and morphological study of the gastrointestinal tract of ostrich (*Struthio camelus* L.) chicks from two to sixteen days post-hatching on a pre-starter broiler diet. The effect of growth on histological and morphological changes in the various parts of the small intestines was examined.

Part 2: Influence on various pre-starter diets on growth and the development of the intestinal tract

Chapter 5 discussed a growth and digestibility study of ostrich (*Struthio camelus* L.) chicks on eight different pre-starter diets. The performance, growth and physiological development of the chicks on high and low variations of different nutritional components were examined.

Chapter 6 provides information on certain intestinal enzymes of ostrich (S*truthio camelus* L.*)* chicks on the eight different pre-starter diets.

Chapter 7 dealt with the histological and morphological changes of the gastrointestinal tract of ostrich (*Struthio camelus* L.) chicks as a result of high and low variations of different nutritional components in pre-starter diets.

Implications

The first part of this study revealed that although it appeared that yolk content was absorbed faster in starved ostrich chicks, intake of external feed had a positive influence on the absorption of fat from the yolk. The changes in the fatty acid composition of the yolk fat content that were observed may indicate that ostrich chicks have the ability to withdraw certain fatty acid components, especially unsaturated fatty acids. The digestive tract of ostrich chicks was compared with that of broiler chicks and differences observed in amylase and lipase activity between the birds in this trial and in poultry could be due to genetic differences between species. It could also be due to the genetic homogeneity of the poultry chicks that were used in the trials, whereas ostrich chicks have not been subjected to the same extent of advanced breeding improvement to date. Results on histological and morphological growth of the small intestines of the ostrich chick indicate increased absorption with an increase in age.

The second part of this study revealed that ostrich chicks performed differently on diets formulated with different nutritional components. It appears that a high fat and low sugar content in the diet is advantageous in ostrich pre-starter diets. Although there were a few mortalities during the trials, there is no clear indication as to why the chicks died, as the causes of mortalities were not investigated. Differences in composition of the eight pre-starter diets did not seem to have an influence on the protein content or enzyme activity of the small intestines of ostrich chicks. An anti-nutritional factor within certain feedstuffs may, however, have had an influence on enzyme activity. Similar results were obtained for the histological and morphological parameters which were measured in chicks fed the different diets. There was no single diet that promoted intestine development, although it seemed as if the small intestines of the chicks fed the high protein diet were under higher digestive stress than those of the chicks fed the other diets.

CONTENT Page

This thesis represents a compilation of manuscripts; each chapter is an individual entity and some repetition between chapters is therefore unavoidable.

Language and style in this thesis basically follows the style of the *South African Journal of Animal Science*. This thesis represents a compilation of manuscripts; each chapter is an individual entity and some repetition between chapters is therefore unavoidable.

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Chapter 1

General Introduction

Ostriches are flightless, primitive birds, classified as paleognathic ratites (Huxley, 1867). Ratites share the characteristic of not having a keel on their sternum that anchors wing muscles, therefore even if they did develop suitable wings, the ratites would never be able to fly. Other living birds from this group include the emu and cassowaries from Australia, the rhea from South America and the kiwi from New Zealand (Harshman *et al.*, 2008).

The natural conditions in large parts of South Africa are ideal for ostrich farming, as these birds prefer open, short-grass plains and semi-desert areas (Ullrey & Allen, 1996). About 75% of ostriches in South Africa are found in the Western and Southern Cape regions with its desert grasslands, Karoo shrubs and coastal fynbos, while 25% are found in the Eastern Cape region where there are semi-arid savannah areas, and the remainder in the Northern provinces (South African Ostrich Business Chamber, 2010).

Commercial ostrich farming was established in South Africa between 1857 and 1864 (Smit, 1963) and served mostly to supply the world market with feathers. This changed over the next century, with ostrich products expanding to include the hides and ostrich meat. In 1983 the South African ostrich industry produced 51 500 hides (58% of total income), 117 tons of feathers (25.9%) and 1 500 tons of meat (16.1%) (Swart, 1985). Today more than 65% of the world's ostriches are found in South Africa, and with 250 000 to 300 000 ostriches slaughtered per annum, this country supplies 90% of ostrich products world-wide (South African Ostrich Business Chamber, 2010). The ostrich industry contributes R1.2 billion per annum to the South African Economy. This will probably increase due to lifestyle trends that favour healthier living (ostrich meat contains almost no fat or cholesterol and is high in iron) and an increasing preference for luxury leather goods (South African Ostrich Business Chamber, 2010).

In 2001 Cloete *et al.* studied chick mortality in 2522 ostrich chicks which hatched during the 1999/2000 breeding season at the Klein Karoo Agricultural Centre near Oudtshoorn, South Africa. The authors reported a very high mortality rate, with 78.4% of these chicks dying before 90 days after hatching. Of these chicks 46.7% died before 28 days of age and 30.7% died between 28 and 90 days of age. The authors concluded that the high mortality rate of chicks up to 90 days post-hatching could have been due to an inability to adapt to the intensive rearing environments, hence resulting in stress. Low body weight at 28 days post-hatching was also a common factor in chick mortalities before 90 days of age (Cloete *et al.*, 2001). These results corresponded with other observations for South Africa regarding ostrich chick mortality. Smith *et al.* (1995a) reported 50% mortalities up to 3 months of age and Verwoerd *et al.* (1998) reported ostrich chick mortality to be 10-20% at one week post-hatching and 10-30% at 3 months of age. This high rate of ostrich chick mortality is unfortunately not limited to South Africa. More (1996) reported an average

of 37.1% of ostrich chicks dying at 4 months of age on farms in Queensland, Australia, while Deeming *et al.* (1993) reported the mortality of ostrich chicks under quarantine conditions in Britain, and at 3 months of age, to be 33.3 and 21.7%, respectively.

Verwoerd *et al.* (1998) attribute this high mortality to the so-called 'ostrich fading chick syndrome'. This phenomenon takes into account not only the infectious causes that can result in ostrich chick mortality (such as viruses, bacteria, fungal poisoning and parasite infestations), but also non-infectious causes (such as social stress, environmental stress, nutritional stress and immune incompetence) and their influence on the general well-being of the ostrich chick. They also suggest that ostrich chicks have an inherent incapacity to fully utilise feed nutrients during the first two weeks post-hatching and are thus dependant on the nutritional, as well as passive immunological factors of the yolk. The authors further remark that the large variation in management systems and nutritional regimes, as well as the wide variation in genetics, are limiting factors which prevent the ostrich industry from setting an industry standard for ostrich production. This differs from other production animals, such as cattle in feedlots, dairy cows, pigs or poultry where production standards are the norm. They do, however, conclude that a key factor in successful ostrich farming is the production of high quality ostrich chicks that are able to withstand most of the stressors which they have to face in the first 3 months of its life (Verwoerd *et al.*, 1998).

These problems during the early period of the ostrich's life lead to the following questions:

1) What does ostrich yolk consist of and how does the ostrich chick utilise the yolk?
2) Is the utilisation of the yolk influenced by fasting or feeding of the ostrich chicks?
3) Is the small intestine of the ostrich chick specially adapted to digest and absorb nutrients during the first two weeks post-hatching?
4) What contributes to the ability of the ostrich chick to be able to make the transition to utilise exogenous feed?
5) What does the perfect pre-starter diet consist of in order to enhance growth and produce a high quality ostrich chick?
6) Does the digestive tract of the ostrich chick have the ability to adapt to digesting certain diets, or should the diets be formulated and adjusted to what is physiologically already present?

In order to obtain answers to some of these questions, it is necessary to look at the following factors which play an important role in the growth of the ostrich chick post-hatching:

1. Development of the gastrointestinal tract of ostriches

The gastrointestinal tract (GIT) of ostriches differs from that of other birds, especially with regard to changes in age and diet. In the newly hatched chick the ratio of the small intestine to the colon is 1:1, at 3 months of age it is 1:1.5 and as the bird reaches maturity the ratio changes to 1:2 (Bezuidenhout, 1999). The

GIT of the post-hatching ostrich chick resembles that of a typical monogastric animal, but changes over the next 70 to 80 days to resemble that of a hindgut fermenter (Van der Walt *et al.*, 2003), which can digest both cellulose and hemicellulose (Swart, 1988). Ostriches have the ability to obtain between 12 and 76% of their energy from volatile fatty acids formed during the digestion of fibre-rich feeds in the large intestine (Swart, 1988).

The size of the gastrointestinal tract, especially of the large intestine, is further influenced by the amount of fibre in the diet. In a study by Baltmanis *et al.* (1997), a comparison was made between the GIT of ostriches fed either a high forage diet or a complete diet provided in the form of pellets. They found that the entire GIT of ostriches fed the high forage diet weighed more than that of ostriches on the complete diet. They ascribed this increase in content to a longer retention time of feed in the GIT which was needed to digest the plant material. The colon was significantly heavier in the ostriches fed high forage diets, while the caeca and ileum were significantly heavier in the ostriches fed on the complete diet provided as pellets (Baltmanis *et al.*, 1997). Viljoen *et al.* (2004) reported similar results in a study where diets with three energy and five protein levels were used to determine the effect of the different energy and protein concentrations on the development of the gastrointestinal tract of the ostrich. The authors found that as the dietary energy content increased, so the total length and weight of the GIT decreased. They attributed this to the higher fibre content of the lower energy diets. They also suggested that the physical characteristics and bulkiness of higher fibre diets, which have a longer retention time in the digestive tract, resulted in a larger GI tract.

2. Histology of the small intestine

The small intestine consists of three parts, the duodenum, jejunum and ileum. Hodges (1974) reported that these parts have the same essential structure throughout the whole length of the small intestine in the chicken and that the distinction between them is mainly due to the size and function of the different parts. The duodenum is defined as the part that leaves the proventriculus (gizzard), loops around the pancreas and joins the jejunum at approximately the point where the pancreatic and bile ducts enter the duodenum (Hodges, 1974; Bezuidenhout, 1986). The duodenum of the ostrich forms a secondary loop on the ascending or distal limb of the duodenal loop, which is unique to this species and is not present in any of the other ratite species (Cho *et al.*, 1984; Bezuidenhout, 1986). Hodges (1974) also reported that there is no distinct differentiation between the jejunum and ileum of the chicken, while Duerden (1912) made the same observation in ostriches. Bezuidenhout (1986) however, reported that the jejunum of the ostrich ended at the small vitelline diverticulum. The vitelline diverticulum is a small outgrowth found in avian species where the yolk stalk enters the jejunum (Esteban *et al.*, 1991). The ileum, which is the longest part of the small intestine, is connected to the jejunum and ends at the ileo-caecal junction (Bezuidenhout, 1986).

There are three distinct functions of the small intestine:

1) moving the chyme along the digestive tract;

2) mixing food with digestive secretions from cells lining the intestines and from accessory glands (pancreas and liver) thus assisting digestion, and

3) absorbing the digested end-products into the blood and lymph vessels (Ham & Leeson, 1961; Bloom & Fawcett, 1962).

This is similar for both mammals and birds. Digestive secretions originate from three general sites in mammals (Bloom & Fawcett, 1962): 1) The accessory glands (pancreas and liver, which deposit their secretions in the small intestines via ducts); 2) Brunner's glands situated in the sub-mucosa of the duodenum and 3) Crypts of Lieberkühn situated in the lamina propria. Birds do not have Brunner's glands, which may be the reason why the sub-mucosal layer in the small intestine of the chicken is almost non-existent (Hodges, 1974).

The general histological structure is organized similarly throughout the small intestine and one description applies to all three parts for both mammalian and avian animals (Ham & Leeson, 1961; Bloom & Fawcett, 1962; Hodges, 1974, Burkitt *et al.*, 1999). Externally there is a layer of epithelium forming part of the serosa. The *tunica muscularis* is situated inside the serosa and consists of a rather poorly developed outer longitudinal layer and a much thicker inner circular layer. The sub-mucosa is poorly developed in the small intestine of the chicken and its presence is only obvious where cell bodies of the sub-mucosal nerve plexus or an occasional large blood vessel increase the thickness of the layer. The *muscularis mucosae* is not well developed in the chicken and is similar in thickness to the outer longitudinal layer. Crypts of Lieberkühn stretch throughout most of the glandular layer between the villi and the *muscularis mucosae*. The lamina propria extends between these crypts and into the core of each villus (Hodges, 1974). The villi project into the lumen from the mucous membrane (Ham & Leeson, 1961) and contain a rich vascular and lymphatic network for the absorption of digestive products. The villi differ in number, size and shape according to the region in the small intestine. There is an overall decrease in depth of the mucous membrane from the duodenum to the ileum. The villi become shorter and broader and the depth of the crypts of Lieberkühn decrease considerably in the chicken (Hodges, 1974). Ham & Leeson (1961) and Bloom & Fawcett (1962) describe the villi of the duodenum to be broader than elsewhere, with a leaf-like appearance, while the villi of the jejujum are more tongue-shaped and those of the ileum resemble finger-like projections.

Bezuidenhout & Van Aswegen (1990) observed that the villi of the small intestine of the ostrich were long and branched compared to that of the chicken. They also reported that no Paneth cells were observed in the small intestines of the ostrich. This corresponds with reports that Paneth cells are also absent in the intestinal epithelium of chickens (Hodges, 1974).

3. Enzyme activity in the small intestine

The growth of poultry during the first week of life has been studied quite intensively (Mitchell & Smith, 1991; Dunnington & Siegel, 1995; Noy & Sklan, 1995; Iji *et al.*, 2001a & 2001b; Noy *et al.*, 2001),

as this particular period has become a large portion of the life span of meat-type poultry. Lilja already suggested in 1983 that the ultimate growth of poultry can be directly correlated to the development of especially the gastrointestinal and cardiovascular systems. Birds must be able to adapt to exogenous diets rapidly, in order to achieve their genetic growth potential (Dibner, 2000). Early growth rate may be influenced by various other factors (Dibner, 2000). Yolk sac residue supplies the energy which the bird needs during hatching. The composition of the yolk lipid is, however, more suited for the efficient development of cell membranes and of the central nervous system (Dibner, 2000). It would therefore be better to supply carbohydrates in the diet which provide the energy to the post-hatching chick. Yolk proteins are also essential to provide passive immunity rather than being a source of structural amino acids in chicks (Dibner, 2000), which also suggests that oral protein is important post-hatching. This may differ for ostriches, as many chicks dying at three weeks post-hatching have significant amounts of yolk. These mortalities may have been due to yolk sac infection (Deeming, 1996).

Levels of pancreatic and intestinal enzymes influence early growth rate. The increase in enzyme synthesis and secretion is necessary to keep up with increasing feed intake (Moran, 1985). Enzyme secretion does not seem to limit growth within the first week of growth for chicks (Moran, 1985), as oral nutrients are required for enzyme secretion, but thereafter the enzymes are necessary to break down the macromolecules for absorption (Dibner, 2000). The surface area of the gastrointestinal tract may be more likely to limit early growth than enzyme availability (Nitsan *et al.*, 1991). These authors reported that an increase in intestinal weight relative to body weight, as well as an increase in villi length and villi diameter, require oral nutrient intake. Nutrient digestibility and absorption varies for different nutrients and will thus have an influence on early growth. An increase in the digestibility of carbohydrates, proteins and fats in young growing chickens is reported during the first few weeks (Noy & Sklan, 1995). Low fat digestibility in chicks and poults (Sell *et al.*, 1986) may be associated with low bile salt synthesis rather than deficiencies in lipase enzymes (Escribano *et al.*, 1988).

The quality of the feed provided for chicks immediately post-hatching has an influence on early growth rate. The provision of the correct essential nutrients, the right protein:energy ratio and added fibre are necessary for the chick to best utilize the specific feed (Dibner, 2000; Cooper, 2004). Uni (2003) suggested that poultry chicks undergo rapid physical and functional development of the GIT during the first week post-hatching and that this gives the chicks the capacity to digest feed and assimilate nutrients. Sklan (2001) reported an increase of small intestine weight as a percentage of total body weight (0.02 - 0.08 %) during the first 8 days for chicks.

It is often noted that ostrich chick mortalities occur around 2-3 weeks post-hatching and suggest that it is due to chronic starvation as a result of environmental, social or nutritional stress to which the ostrich chicks are exposed in the first week post-hatching (Verwoerd *et al.*, 1997). Survivors are also often found to have a low growth rate, inefficient feed utilisation and reduced resistance to diseases (Uni, 2003).

Noy & Sklan (1999) suggested that pancreatic and brush border enzymes have to be available in sufficient quantities for effective digestion and absorption in chickens. However, they reported that quantitative determination of digestive enzymes in the intestines of poultry immediately post-hatching was not carried out at that time (Noy & Sklan, 1999). Iji *et al.* (2001b) conducted a study to determine the development and characteristics of certain intestinal enzymes (maltase, sucrase, aminopeptidase N (APN) and alkaline phosphatase (AP)) in broiler chicks on a commercial starter diet. They found that there was a general reduction in specific activities of intestinal enzymes as broiler chicks aged from 1 to 21 days post-hatching, but that the increased length and surface area of the gastrointestinal tract compensated for this reduction per unit of mucosal surface. The authors suggested that enzyme activities differ between different intestinal sites and that this may also have an influence on digestion (Iji *et al.*, 2001b). Specific enzyme activities (µ mole product / mg protein / min) at 1 vs. 21 days post-hatching for the various enzymes in the various regions were: duodenum (maltase = 2.44 vs. 2.21; sucrase = 1.66 vs. 0.64; APN = 1.57 vs. 0.94; AP = 2.06 vs. 1.27), jejunum (maltase = 2.53 vs. 2.15; sucrase = 1.81 vs. 0.77; APN = 1.58 vs. 0.97; AP = 1.84 vs. 1.19) and ileum (maltase = 2.69 vs. 2.17; sucrase = 1.85 vs. 0.77; APN = 1.65 vs. 0.97; AP = 1.36 vs. 0.58).

In contrast to literature found on digestive enzymes in mammals and poultry, limited information is available on levels of digestive enzymes in the intestines of ratites (Hartley *et al.*, 1987; Angel, 1996; Cilliers & Angel, 1999). Some work on the characterisation of pancreatic enzymes in ostriches has been done. Hartley *et al.* (1987) isolated and characterised trypsin from the pancreas of the ostrich and suggested that the trypsin performs the same role in intestinal digestion in ostriches as mammalian trypsin in mammals. Similarly Van der Westhuizen (1988) isolated and characterised pancreatic chymotrypsinogen and suggested that the same is true for ostrich chymotrypsiongen when compared to mammalian chymotrypsinogen. Sutherland (1990) isolated and characterised pro-elastase and elastase from the pancreas of the ostrich and suggested that these enzymes fulfil similar functions in the ostrich as in mammals, but may also play a key role in the ostrich's defence system against foreign micro-organisms in the diet. Oosthuizen *et al.* (1992) isolated and partially characterized α-amylase from the pancreas of the ostrich and concluded that this enzyme seems to show similar physical, chemical and biological properties to other known α-amylases, but with differences in primary structure. It showed an especially close resemblance to chicken α-amylase, and has an optimum activity for starch hydrolysis at pH 7.5 (Oosthuizen *et al.*, 1992). Smith (1993) and Bodley *et al.* (1995) both conducted research on ostrich pancreatic trypsinogen, while Smith (1993) also researched ostrich pancreatic trypsin. Both studies found ostrich trypsins and chymotrypsins to closely resemble those of other species, but with distinct differences. Ostrich trypsinogen is closely related to its serine protease counterparts and the authors concluded that it must therefore play an important role in collaborative digestion of protein.

Streicher (1984) isolated and characterised protease from the proventriculus of the ostrich, while Pletschke *et al.* (1995) activated and immunochemically characterised ostrich pepsinogens I and II from the

proventriculus of the ostrich. These two enzymes were found to be immunologically separate entities and that only two pepsinogens are present in the ostrich.

Roos *et al.* (1993) isolated and partially characterised alanine aminopeptidase from ostrich duodenal mucosa, while Naudé *et al.* (1993) did the same with enterokinase from ostrich duodenal mucosa. The amino acid composition of ostrich enterokinase was similar to that of mammalian enterokinase and the highest rate of bovine trypsinogen activation by ostrich enterokinase was at pH 5.2-5.7. Naudé *et al.* (1993) conducted a study on the characterisation of enterokinase from ostrich duodenal mucosa. Enterokinase plays a key role in activating pancreatic trypsinogen to trypsin. Trypsin, in turn, activates pancreatic chymotrypsinogen to chymotrypsin (Anderson *et al.*, 1977). Naudé *et al.* (1993) reported that ostrich enterokinase has a similar amino acid composition to its mammalian counterpart and that it showed the highest activation of bovine trypsinogen at pH 5.2 – 5.7.

Alkaline phosphatase is expressed in active and mature mucosal enterocytes and has therefore been used in enzyme studies as an enterocyte maturation marker (Wieser, 1973; Traber *et al.*, 1991). In a study conducted by Toyoda *et al.* (1985) the authors found that proteases from the pancreas caused the release of enterokinase and alkaline phosphatase from rat enterocytes and that chymotrypsin, specifically, causes the selective release of enterokinase over that of alkaline phosphatase.

One of the limited studies on the development of enzyme activity in ostrich chicks were conducted in 2003 by Iji *et al.* in a study on ostrich chicks at 3, 27, 41, 55 and 72 days of age. The authors suggested that the evaluation of enzyme activity be done at closer intervals, especially in early age, to get a clearer picture of enzyme development when the changes are rapid and possibly irreversible. These changes are usually observed within the first two weeks post-hatching (Uni *et al.*, 1998; Uni *et al.*, 1999).

4. Yolk utilisation

In the early 20th century, studies were conducted to determine the effect of feeding on yolk assimilation in chickens (Roberts, 1928; Heywang & Jull, 1930). The chicks were starved for about 48 to 72 hours post-hatching to allow the yolk to be completely absorbed and to prevent possible bowel problems which were thought to occur when yolk assimilation took place while exogenous food was fed simultaneously (Heywang & Jull, 1930). Since then, various studies have investigated the effect of feeding and starvation on yolk utilisation together with the development of the digestive tract and enzyme activity in poultry (Bierer & Eleazer, 1965; Chamblee *et al.*, 1992; Noy *et al.*, 1996; Noy & Sklan, 1998; Dibner, 2000; Smirnov *et al.*, 2003; Uni, 2003). It was concluded that yolk utilisation was more efficient in chicks that received feed and water after hatching and that starvation has a negative effect on the development of the digestive tract and digestive enzymes. Much less work has been done on yolk utilisation in ostrich chicks, especially with regard to the effect of feeding and starvation on yolk utilisation. Most of the work has been limited to the rate of yolk utilisation (Smit, 1963; Mushi *et al.*, 2004), and yolk sac infection and its significance with regard to the early mortality of ostrich chicks (Deeming, 1995; Deeming *et al.*, 1996).

5. Pre-starter diets and growth performance

Information about the nutritional requirements of ostriches, especially compared to that available for other production animals, is scarce (Ullrey & Allen, 1996). Research on ostrich nutrition has taken giant leaps since 1995 (Cilliers *et al.*, 1998; Brand *et al.*, 2000, Cooper, 2004) when ostrich diets were formulated based on both the energy value of the ingredients and the nutritional requirements of other poultry, such as chickens and turkeys. Ingredients based on nutritive values obtained with poultry were often used (Angel, 1996; Brand *et al.*, 2000). This led to ostrich nutrition being based on assumptions from other species (Ullrey & Allen, 1996). In a trial by Gandini *et al.* (1986) on ostrich chicks under intensive conditions, five of the ostrich chicks developed leg abnormalities. The researchers suggested that it may have been due to inadequate dietary calcium, but the study was not designed to address this problem. Ullrey & Allen (1996) also suggested that high protein, low fibre diets typically fed to young turkeys are likely to lead to leg problems if fed to ostriches. In the study by Gandini *et al.* (1986) no differences in production on diets varying between 16% and 20% protein were found, but birds on these diets performed better than birds on a diet containing 14% protein. Diets based on nutritive values of other species usually overestimated ostrich nutritional requirements and underestimated the ostrich's ability to effectively digest certain feedstuffs, especially those with high fibre content (Cilliers *et al.*, 1998; Brand *et al.*, 2000). This has been costly for the ostrich industry as 70-80% of total intensive production costs can be attributed to feeding costs (Brand *et al.*, 2000).

Cilliers *et al.* (1994) and Cilliers *et al.* (1995) were the first authors to report values for the true metabolisable energy (ME) in ostriches, while five years later Brand *et al.* (2000) compared the estimates of feed energy obtained from ostriches with estimates obtained from pigs, poultry and ruminants. Nine balanced diets differing in fibre content were fed to ostriches, pigs, poultry and ruminants. Ostriches had higher ME values than both pigs and ruminants on low, medium and high fibre diets, as well as on the medium and high fibre diets when compared with poultry. These authors concluded that ostriches had a better ability to utilise low quality raw material than the other two monogastric species and maintained higher ME values than ruminants on high fibre diets (Brand *et al.*, 2000)

The ostrich digestive tract undergoes dramatic changes in the first two to three months post-hatching (Van der Walt *et al.*, 2003). At hatching it resembles that of a typical monogastric animal, but changes to resemble that of a hindgut fermenter which can digest both cellulose and hemicellulose, feedstuff normally inaccessible to the typical monogastric animal. As the digestive tract of the ostrich is not yet fully developed at three months of age (Angel, 1993), Gandini *et al.* (1986) suggested that high-quality diets should be fed to young ostriches up to three month of age.

Various studies have been conducted to determine nutritional requirements for growing ostriches. Studies included research on the effect of dietary energy and protein levels on the performance (Brand *et al.*, 2002) and production (Brand *et al.*, 2000) of growing ostriches; dietary energy and protein levels on the production of male (Brand *et al.*, 2000) and female breeding ostriches (Brand *et al.*, 2003); as well as

research on determining energy, protein and amino acid requirements for growth and maintenance in ostriches (Cilliers *et al.*, 1998). Even though these studies have been conducted on growing ostriches, little scientific literature or specifications are available for energy and protein levels in pre-starter diets for ostrich chicks. The predicted energy level is suggested as 14.65 ME (Du Preez, 1991, Smith *et al.*, 1995b), while a crude protein level of 22.89% is indicated by Smith *et al.* (1995b). It therefore remains a constant challenge for nutritionists to fully comprehend the nutritional physiology and nutritional requirements of ostriches, especially 90 days post-hatching.

Hypotheses

The following hypotheses were posed:

1. The nutrients supplied from the yolk content of ostrich chicks need to be supplemented with external feed post-hatching.
2. In spite of similarities between the anatomy of the gastro intestinal tract of ostrich chicks and poultry during the first weeks post-hatching, the external nutrient requirements differ between these species from hatching.
3. Enzyme activity in the digestive tract of ostrich chicks changes during the first two weeks post-hatching to adapt from the digestion of yolk content to that of exogenous feed.
4. The histology of the small intestines develops over the first two weeks post-hatching to accommodate absorption of exogenous feed.
5. The composition of pre-starter diets has an influence on the growth of ostrich chicks.
6. The composition of diets has an influence on the development of enzyme activity. Enzyme activity will adjust according to the composition of the diet.
7. The composition of diets has an influence on the development of the digestive tract. Higher growth rate will correspond with an increased surface area of the gastro intestinal tract.

This study can be divided into two separate parts:
In order to address the hypotheses two separate trials were conducted during the first part to determine the changes in yolk content as it is utilised by starved ostrich chicks over the first seven days post-hatching, and then to evaluate the effect on ostrich chicks when fed a broiler pre-starter diet over the first 16 days post-hatching. While the yolk absorption of the ostrich chicks in these two trials were not directly compared, similarities and differences are discussed, as well as compared with similar work conducted on chickens and turkeys. Samples were taken from the small intestines of the fed ostrich chicks to determine the effect of growth on enzyme activity immediately post-hatching up to sixteen days of age, as well as to measure histological and morphological changes in the various parts of the small intestines.

In the second part of the study eight pre-starter diets were fed to ostrich chicks from hatching until they were slaughtered at 60 days of age. To further examine the hypotheses the performance, growth and physiological development of the chicks on high and low variations of different nutritional components were examined. The ostrich chicks were then slaughtered to determine the effect of the different diets on the activity of certain enzymes in the small intestines. Samples were also taken from the small intestines to measure possible histological and morphological differences in the various parts of the small intestines as a result of the different diets.

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Part 1

Yolk utilisation and the development of the small intestine of ostrich chicks

Chapter 2

The composition of egg yolk absorbed by starved ostrich (*Struthio camelus* L.) chicks from one to seven days post-hatching and for ostrich (*Struthio camelus* L.) chicks from one to sixteen days post-hatching on a pre-starter broiler diet

Introduction

In the early 20th century, studies were conducted to determine the effect of feeding on yolk assimilation in chickens (Roberts, 1928; Heywang & Jull, 1930). The chicks were starved for about 48 to 72 hours post-hatching to allow the yolk to be completely absorbed and prevent possible bowel problems which were thought to occur when yolk assimilation took place while exogenous food was fed simultaneously (Heywang & Jull, 1930). Since then, various studies investigated the effect of feeding and starvation on yolk utilisation and the development of the digestive tract and enzyme activity in poultry (Bierer & Eleazer, 1965; Chamblee *et al*., 1992; Noy *et al*., 1996; Noy & Sklan, 1998; Dipner, 2000; Asya *et al.*, 2003; Zehava, 2003). It was concluded that yolk utilisation was more efficient in chicks that received feed and water after hatching and that starvation has a negative effect on the development of the digestive tract and digestive enzymes. Much less work has been done on yolk utilisation in ostrich chicks (Bertram & Burger, 1981; Deeming, 1995; Mushi *et al*., 2004), especially with regard to the influence of feeding and starvation on the effect of yolk utilisation.

In this chapter, two separate trials were conducted. The first trial was to determine the chemical composition of the yolk of starved ostrich chicks, one to seven days post-hatching. The second trial was to determine the chemical composition of the yolk of ostrich chicks fed a broiler pre-starter diet which could conceivably affect the uptake of the yolk and stimulate growth and development of the digestive tract and digestive enzymes. The two trials are not compared, as there were too many variables in the methodology of the trials. The project had ethical approval from the Onderstepoort Animal Use and Care Committee (Protocol 36-5-623).

Materials and Methods

Animals

The first trial involved 35 ostrich chicks. Fertilized eggs were obtained from the Oudtshoorn Experimental farm of the Department of Agriculture Western Cape, South Africa and transported to the Faculty of Veterinary Science of the University of Pretoria, Onderstepoort, South Africa. Eggs were moved on day 36 of incubation. The eggs were packed by a professional ostrich egg transport company in suitable containers which eliminated excessive shaking and cooling down of the eggs during transport, but allowed

sufficient ventilation. Should the egg temperature fall below the critical temperature of 25°C, or excessive shaking occur, increased shell deaths would result. The hatchability of eggs decreases by 5-10% during the transportation process and the chicks in such cases normally need assistance with hatching, as they tend to get weaker, due to lower oxygen and higher carbon dioxide levels in the containers (Danie Terblanche, 2009, personal communication).

Eggs were hatched in a poultry incubator at 36°C with the humidity set at 24%. Eggs that did not hatch within the first two days of arrival at Onderstepoort were manually cracked to assist with hatching. Those chicks were slaughtered in the trail first, as they tended to be weaker than the others (Zanell Brand, 2003, personal communication). To make up for losses sustained, eight day-old chicks were obtained from a nearby ostrich farm to maintain a sufficient number of birds for the trial. Chicks were kept in a clean, disinfected room which was kept cool and dark. Noise and human contact were restricted to the minimum to limit stress. Chicks were provided with clean drinking water, but no food. Five chicks were slaughtered each day for seven consecutive days, starting from the day of hatching (day one). The mean hatching and slaughter weights of the starved chicks, as well as the yolk weight and pH, are presented in Table 1.

Table 1 Mean hatching and slaughter weights (± SE) of starved ostrich chicks as well as the yolk weight and pH of the yolk on 7 consecutive days after hatching (n=5 for each day)

Slaughter age (days)	Hatching weight (g)	Slaughter weight (g)	Yolk weight (g)	Yolk pH
1	832.6 ± 101.5^{a}	832.6 ± 101.5^{a}	230.3 ± 104.0^{ab}	7.34 ± 0.22^{a}
2	883.5 ± 81.6^{a}	838.4 ± 91.9^{a}	249.3 ± 74.9^{a}	7.18 ± 0.35^{ab}
3	835.8 ± 99.6^{a}	862.0 ± 148.1^{ab}	205.5 ± 39.9^{ab}	7.00 ± 0.28^{bc}
4	835.3 ± 101.0^{a}	782.0 ± 79.4^{a}	163.9 ± 76.5^{bc}	6.98 ± 0.18^{bc}
5	836.4 ± 84.4^{a}	749.2 ± 83.2^{ab}	109.4 ± 51.8^{cd}	6.81 ± 0.80^{c}
6	767.6 ± 86.6^{a}	656.8 ± 67.1^{b}	78.0 ± 12.0^{d}	6.46 ± 0.30^{d}
7	824.4 ± 57.3^{a}	661.4 ± 46.0^{b}	102.4 ± 7.3^{cd}	6.69 ± 0.30^{cd}

$^{a, b, c}$ Column means with different superscripts differ significantly at $P<0.05$

During the second trial, six chicks were slaughtered every second day (except on day 8 when 7 chicks were slaughtered) over a period of 16 days. The first group of chicks were thus two days old when sacrificed and a total of 49 chicks were slaughtered. The chicks were obtained from the same source as for the first trial and similarly handled and transported to Onderstepoort. The average weight of the day-old chicks was 839.3 g.

The trial continued for 16 days as it was found that yolk was still present in 14-day old chicks (Adriaan Olivier, 2005, personal communication). Chicks were kept under similar conditions as in the first trial, except for the provision of a poultry pre-starter diet (AFGRI Animal Feeds, South Africa). The

minimum specifications for the commercial pre-starter diet were: protein 24.5%, moisture 11.5%, energy 12 MJ/kg feed, fat 6.7%, crude fibre 3.6% and ash 6.2%. The values for the fed chicks are presented in Table 2.

Table 2 Mean hatching and slaughter weights (± SE) of fed ostrich chicks, as well as the yolk weight and pH on 16 days post-hatching

Slaughter age (days)	Number of chicks in group	Hatching weight (g)	Slaughter weight (g)	Yolk weight (g)	Yolk pH
2	6	869.6 ± 131.8^{a}	771.6 ± 118.2^{d}	229.2 ± 61.1^{a}	7.28 ± 0.16^{ab}
4	6	858.0 ± 65.0^{a}	786.6 ± 38.3^{d}	160.4 ± 29.6^{b}	7.03 ± 0.17^{bc}
6	6	925.0 ± 129.1^{a}	853.3 ± 123.4^{d}	135.0 ± 42.4^{b}	6.81 ± 0.16^{c}
8	7	827.4 ± 128.5^{a}	801.4 ± 166.8^{d}	75.2 ± 36.7^{c}	7.01 ± 0.19^{bc}
10	6	766.6 ± 136.0^{a}	901.6 ± 106.8^{cd}	68.6 ± 36.0^{c}	7.49 ± 0.40^{a}
12	6	881.0 ± 44.2^{a}	1040.0 ± 170.2^{bc}	51.6 ± 20.1^{cd}	7.04 ± 0.21^{bc}
14	6	828.8 ± 89.0^{a}	1191.6 ± 273.3^{ab}	18.4 ± 28.5^{de}	7.40 ± 0.25^{a}
16	6	830.5 ± 115.5^{a}	1341.6 ± 144.8^{a}	4.5 ± 4.0^{e}	-

$^{a, b, c}$ Column means with different superscripts differ significantly at $P<0.05$

Measurements and Calculations

In both trials, chicks were euthanized with CO_2 in a closed container. The yolks sacs were immediately removed from the carcasses, weighed and pH values determined (Thermo Orion pH meter, Orion Research, Inc.500 Cummings Centre, Beverly, MA 01915, USA). As much yolk as possible was taken from the yolk sac for proximate analysis (ash, dry matter, crude protein and ether extract analyses) and immediately frozen at -20°C.

Yolk samples from both trials were freeze-dried and analyzed for ash, dry matter (DM), crude protein (CP) content and fat content (measured as ether extract (EE)) by standard methods (AOAC, 1995).

Yolk samples from the first trial (the starved chicks) were further analyzed for amino acids, fatty acids and glucose composition. As the same tendencies were observed for crude protein and fat values in both trials, the analyses for amino acids, fatty acids and glucose composition were not repeated for the second trial due to insufficient funds and were assumed to be similar.

Amino acids were determined on freeze-dried samples by ion-exchange chromatography of the acid-hydrolysed protein. Samples were hydrolysed (AOAC, 1995) with 6M HCl in a sealed tube under N_2 for 22 hours in an oil bath at 110°C and then stored at -20°C. On the day the analyses were done, the samples were thawed to room temperature. Each sample was then mixed by vortex for 5-10 seconds and centrifuged at 15 000 g for 5 minutes in a Hermle bench centrifuge (HERMLE Labortechnik GmbH, Wehingen). The supernatant (25 µl) was placed in a glass hydrolysis tube and dried under vacuum for 1 hour.

The pH was adjusted to pH=7 by adding 20 µl methanol : water : triethylamine 2:2:1 and the samples were re-dried for 1 hour. Each sample was derivatised by adding 20 µl derivatising solution (methanol : water : triethylamine : phenylisothiocyanate (PITC) 7:1:1:1). The mixture was incubated at room temperature for 10 min. and then dried under vacuum for a minimum of 1 hour and a maximum of 3 hours until completely dry. The derivatised dried sample was dissolved in 400 µl of Picotag® sample diluent (Waters, Millford, MA, USA), filtered through a 0.45 uM filter and 16 µl of sample was subjected to HPLC using a standard method for PTC-amino acid chromatography.

Data was collected and analysed using Breeze software (Waters, USA). The percentage recovery of standards was determined by analysing standards of a known quantity. For each batch of samples at least two standards were dried and treated under the exact conditions as the samples.

Fatty acid methyl esters (FAME) were prepared according to the method of Morrison & Smith (1964). The FAME were analysed with a GLC: Varian Model 3300, equipped with flame ionisation detection and two 30m fused silica mega bore DB-225 columns of 0.53 mm internal diameter (J & W Scientific Folsom, CA). Gas flow rates were: hydrogen, 25 ml/min; air, 250 ml/min. and nitrogen (carrier gas), 5-8 ml/min. Temperature programming was linear at 4°C/min; initial temperature, 160°C; final temperature, 220°C held for 10 min.; injector temperature, 240°C and detector temperature, 250°C. The FAME were identified by comparison of the retention times to those of a standard FAME mixture (Nu-Chek-Prep Inc., Elysian, Minnesota) and the milligram fatty acid per gram tissue sample was calculated.

Glucose content was determined using the ACE™ Glucose Reagent (Reagent number NAE2-27) intended for the quantitative determination of glucose in serum using the ACE™ clinical chemistry system. In the ACE Glucose method, glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The resultant hydrogen peroxide reacts under catalysis of peroxidase with phenol and 4-aminophenazone to form a red-violet quinoneime dye as an indicator. The absorbance of the reaction is bi-chromatically measured at 505 nm/692 nm.

Statistics

The experimental design for the first trial entailed the random selection of five ostrich chicks every day from hatching up to seven days, with the subsequent measurement of the yolk content parameters. Amino acids and fatty acids were only analysed on days one, three, five and seven.

Linear regression functions were fitted to weight and yolk-variables over the period of seven days post-hatching to quantify changes over time.

Amino acid and fatty acid composition was subjected to one-way analysis of variance using SAS version 9.2 (SAS, 2000). Levene's test was performed to test for homogeneity of daily analysis of variance (Levene, 1960). The Shapiro-Wilk test was performed to test for non-normality (Shapiro & Wilk, 1965). Student's t-Least Significant Difference was calculated at the 5% confidence level to compare means for age (days) (Ott, 1998).

The experimental design for the second trial also entailed random selection of chicks with body weight and yolk observations made on six chicks every second day from hatching up to sixteen days. Linear regression functions were fitted on body weight and yolk-variables over the period of sixteen days post-hatching to quantify change over time.

Results and Discussion

Starved chicks trial

There was no difference in the hatching weight between the different chicks (P = 0.56) (Table 1). The average hatching weight was much lower than reported in other ostrich chick studies which ranged from 1.4-1.6 kg (Keffen & Jarvis, 1984; Mushi *et al*., 2004). There was a decrease (P<0.05) in slaughter weight from one to seven days post-hatching (Table 1). The chicks lost an average of 31.3 g body weight over the first seven days post-hatching (Figure 1). This was expected as the chicks did not receive any feed and utilised 44% of their yolk over the trial period.

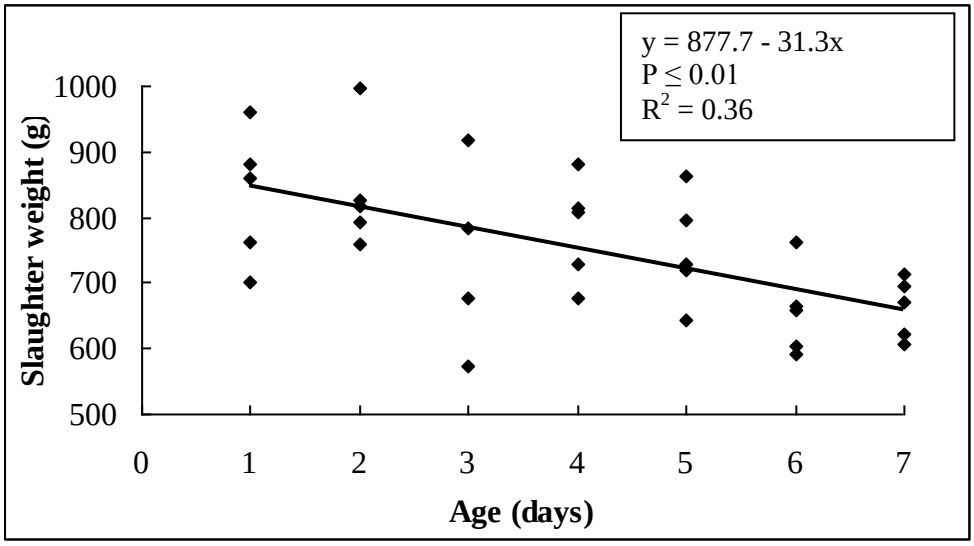

Figure 1 Slaughter weights of starved ostrich chicks from one to seven days post-hatching

Yolk weight decreased linearly (P<0.05) over the trial period. On day one the average yolk weight was 230.4 g and decreased daily by 28.9 g to reach an average weight of 102.5 g on day seven post-hatching (Figure 2). Yolk weight, as a percentage of body weight, was 28% for day-old and two day old post-hatch ostrich chicks and decreased to 12% of total body weight on day seven. Romanoff (1960) and Sklan & Noy (2000) reported yolk weight as 20% of body weight for chicks and Noy & Sklan (1998) reported 13-15% for poults. Only 44.4% of the ostrich chick yolk however, was assimilated over the seven day post-hatching period. Bierer & Eleazer (1965) reported that chickens deprived of feed for the first seven days post-hatching utilised 88.8% of their yolk.

The total dry matter content of the yolk indicated a significant ($P<0.05$) decline of 1.53% per day from one to seven days post-hatching. Due to the sample size, the regression was not very accurate ($R^2 = 0.22$) (Figure 3). This may, however, indicate a preference to withdraw moisture from the yolk together with the other nutrients.

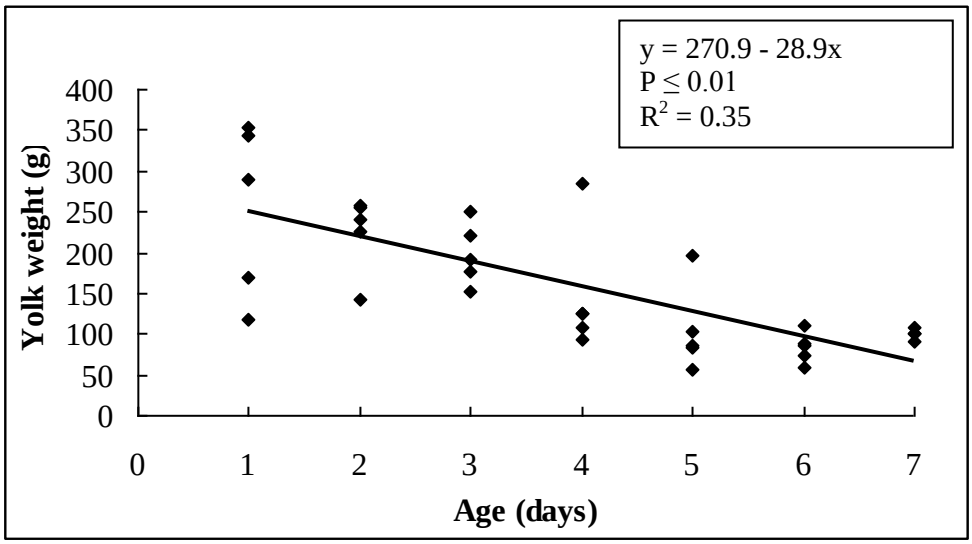

Figure 2 Yolk weights of starved ostrich chicks from one to seven days post-hatching

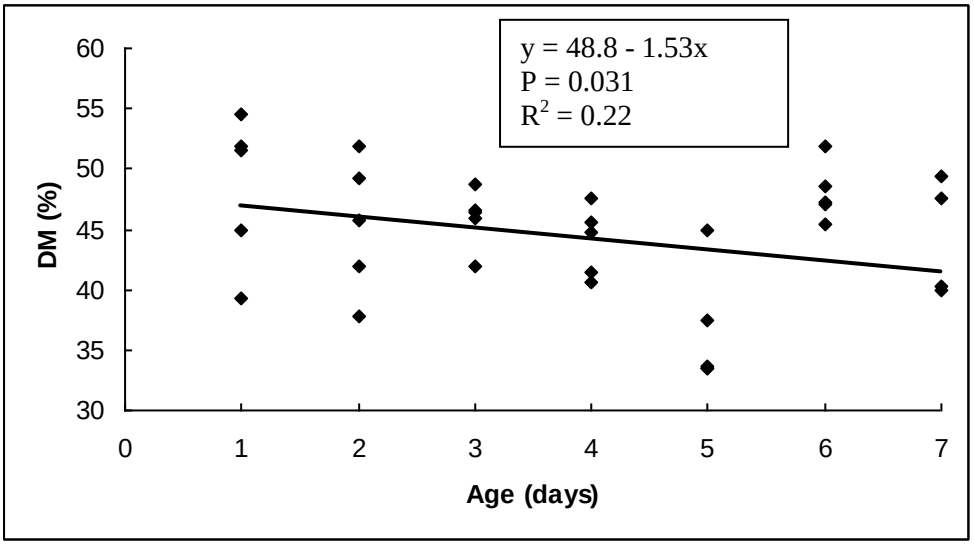

Figure 3 Dry matter content (%) of the yolk of starved ostrich chicks from one to seven days post-hatching

The average pH of the yolk of chicks slaughtered on day one post-hatching was 7.34. Yolk pH showed a linear decline ($P<0.05$) of 0.10 daily over the trial period, with an average pH of 6.69 on day seven (Figure 4).

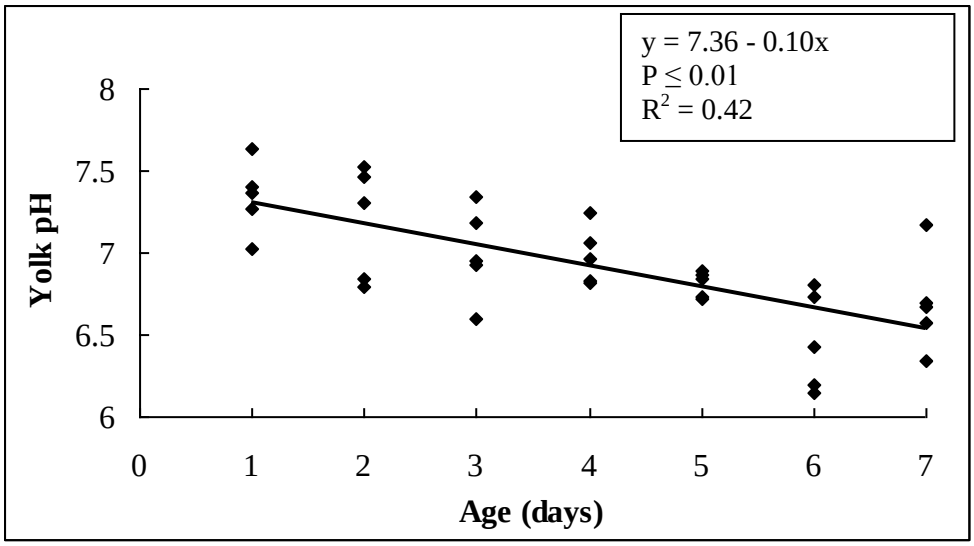

Figure 4 Yolk pH of starved ostrich chicks from one to seven days post-hatching

Crude protein as a percentage of yolk composition is depicted in Figure 5 and total crude protein content in grams is depicted in Figure 6. Fat content as a percentage of yolk composition is depicted in Figure 7, while total fat content in grams is depicted in Figure 8. Crude protein content decreased significantly ($P<0.05$) by 1.5% daily from day one (44.3%) to day seven (32.5%) post-hatching (Figure 5). This accounts for a daily decrease of 13.2 g in protein content (Figure 6). Fat content, on the other hand, increased significantly ($P<0.05$) by 1.77% daily from one (41.1%) to seven (48.8%) days post-hatching (Figure 7). Total yolk fat weight, however, decreased daily by 8.91 g from 94.9 g to 50.1 g. The increase in yolk fat percentage was therefore simply an increase in concentration of the component as a percentage of total yolk weight. Crude protein content decreased to 43.7% for two day old ostrich chicks, which was less than fasted poults that reflected a 49.8% yolk crude protein content two days post-hatch (Moran & Reinhart, 1980). Yolk fat content for ostrich chicks two days post-hatching (42.3%) was also less than that reported for poults (50.6%) two days post-hatching (Moran & Reinhart, 1980). Chicks reportedly showed a decrease in yolk protein and fat content after two days of fasting (Noy & Sklan, 1999).

Although no significant ($P<0.05$) difference in the glucose content over the entire trial period ($P = 0.36$) was observed, the glucose level in the yolk increased ($P<0.05$) from day three (8.48 mmol/l) to day seven post-hatching (11.8 mmol/l). The same conclusion can be made as for yolk fat percentage i.e. that the apparent increase in glucose content within the yolk was only due to an increase in concentration of glucose as a percentage of total yolk volume. This was probably due to the high protein removal from the yolk. Noy & Sklan (1998) reported that the absorption of glucose did not change with age in poults, but although the absorption of glucose is low in chicks close to hatch, it increased slightly over the first seven days post-hatch (Noy & Sklan, 2001; Sklan, 2003).

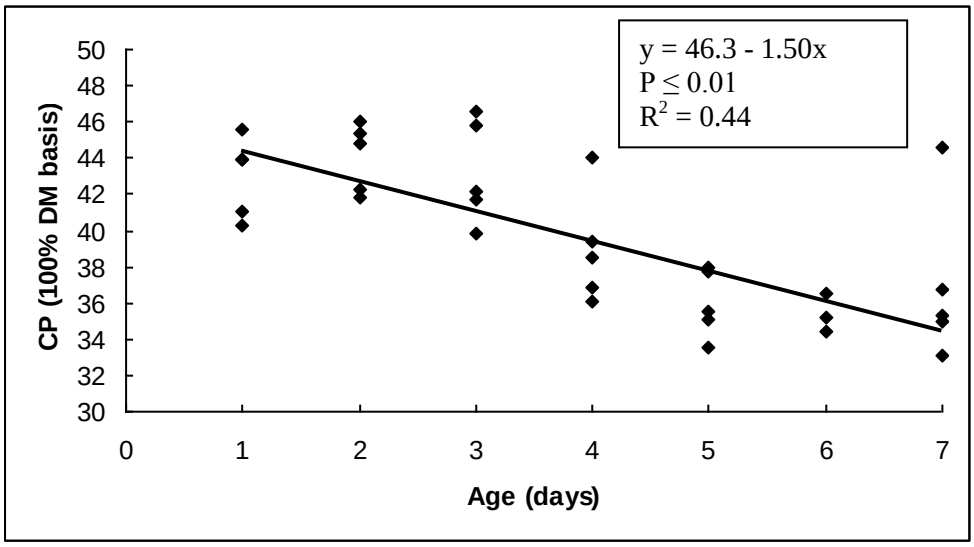

Figure 5 Crude protein content (%) of yolks from starved ostrich chicks from one to seven days post-hatching

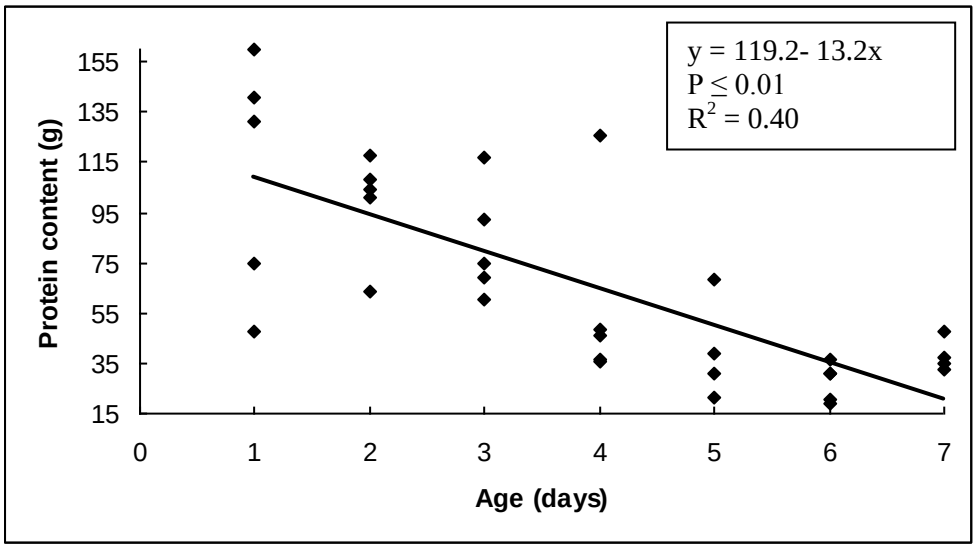

Figure 6 Crude protein content (g) of yolks from starved ostrich chicks from one to seven days post-hatching

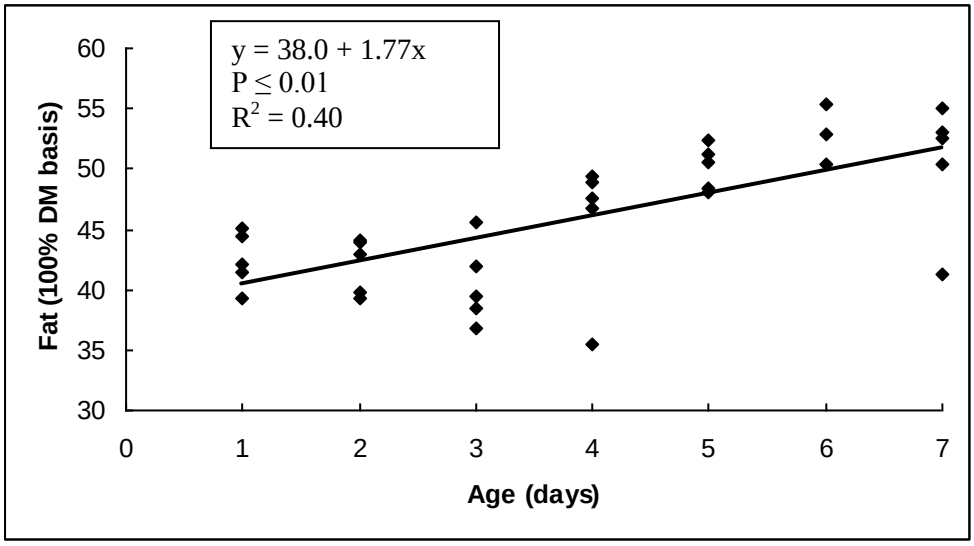

Figure 7 Fat content (%) of yolks from starved ostrich chicks from one to seven days post-hatching

There was no literature found which referred to the glucose content of the yolk in either fasted or fed birds. From this study it seemed that little glucose, or lipids, was utilised from the yolk as an energy source for the fasted ostrich chick. The starved ostrich chick would therefore have had to use yolk protein as an energy source, which could also contribute to the weight loss observed over the seven day trial period, as less protein was then available for growth.

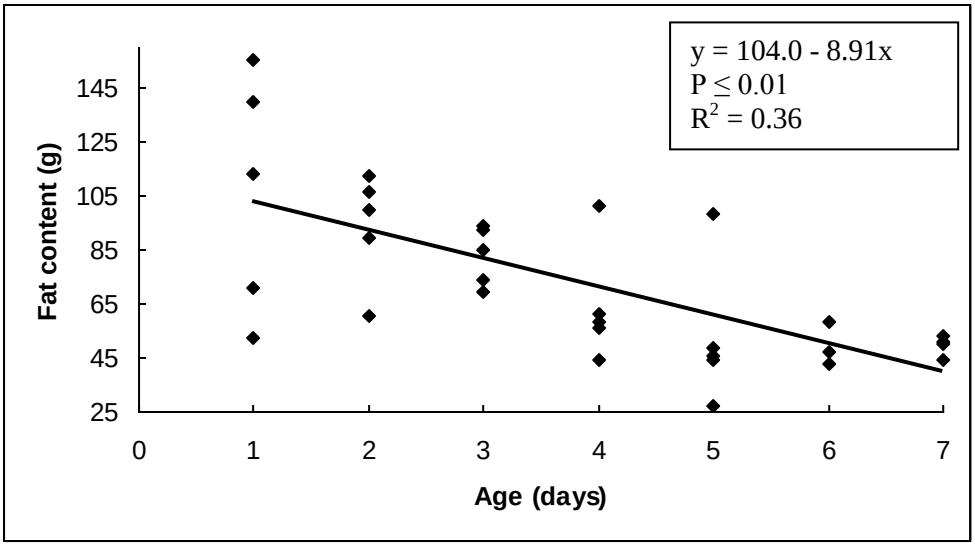

Figure 8 Fat content (g) of yolks from starved ostrich chicks from one to seven days post-hatching

Amino acid composition of yolk from starved ostrich chicks is listed in Table 3. From the eighteen amino acids analysed, only seven amino acids showed any significant ($P<0.05$) differences in percentage contribution to the total amount of protein in the yolk over the one to seven day post-hatching trial period. Glycine and threonine showed the same pattern over the seven day period, with an initial decline in content from day one to day three (although not significant ($P>0.05$)), followed by an increase ($P<0.05$) from day three to day five and declining again on day seven, but not significantly so. Methionine and serine showed the same tendencies, with first a slight increase from day one to day three, a significant ($P<0.05$) decline from day three to day five and a slight decrease from day five to day seven. Histidine declined ($P<0.05$) from day one to day five, while valine decreased ($P<0.05$) from day one to day five and then increased ($P<0.05$) again from day five to day seven. Norleucine increased ($P<0.05$) from day one to day three and then again from day three to day five, followed by a decrease ($P<0.05$) from day five to day seven post-hatching.

Table 3 Amino acid composition (% of total yolk protein content) of yolk from starved ostrich chicks from one to seven days post-hatching

Amino acid	1	3	5	7	LSD
Alanine	7.18 [a]	7.26 [a]	7.23 [a]	7.27 [a]	0.21
Arginine	3.24 [a]	3.15 [a]	3.21 [a]	3.09 [a]	0.22
Aspartate	8.54 [a]	8.49 [a]	8.64 [a]	8.53 [a]	0.16
Cysteine	1.80 [a]	1.81 [a]	1.84 [a]	1.84 [a]	0.19
Glutamate	11.85 [a]	11.70 [a]	11.65 [a]	11.98 [a]	0.34
Glycine	5.64 [ab]	5.63 [b]	5.78 [a]	5.75 [ab]	0.15
Histidine	1.84 [a]	1.78 [ab]	1.68 [c]	1.71 [bc]	0.08
Isoleucine	4.58 [a]	4.50 [a]	4.47 [a]	4.58 [a]	0.22
Leucine	9.50 [a]	9.53 [a]	9.52 [a]	9.69 [a]	0.24
Lysine	5.86 [a]	6.15 [a]	5.98 [a]	5.97 [a]	0.53
Methionine	2.90 [ab]	2.97 [a]	2.89 [b]	2.95 [ab]	0.08
Norleucine	0.29 [c]	0.36 [ab]	0.40 [a]	0.32 [bc]	0.07
Phenylalanine	3.78 [a]	3.87 [a]	3.88 [a]	3.89 [a]	0.15
Proline	4.94 [a]	4.91 [a]	4.96 [a]	4.99 [a]	0.13
Serine	11.99 [ab]	12.10 [a]	11.76 [b]	12.04 [ab]	0.29
Threonine	6.49 [bc]	6.46 [c]	6.69 [a]	6.62 [ab]	0.16
Tyrosine	3.51 [a]	3.53 [a]	3.61 [a]	3.58 [a]	0.12
Valine	6.09 [a]	5.81 [b]	5.85 [b]	6.00 [a]	0.11

[a, b, c] Row means with different superscripts differ significantly at P<0.05

Fatty acid composition of yolk is given in Table 4 and is expressed as percentage contribution to the total amount of fat in the yolk. Saturated fatty acid (SFA) content remained constant from day one to day five post-hatching and then increased significantly (P<0.05) from day five to day seven. The main contributors to this increase were palmitic acid (16:0), stearic acid (18:0), arachidic acid (20:0) and lignoceric acid (24:0). Results obtained in a study conducted in poults (Reidy *et al.*, 1998) indicated that stearic acid (18:0) also increased post-hatch from 10.4 - 11.3% 24 hours post-hatch. Mono-unsaturated fatty acids (MUFA) also remained constant over the first five days post-hatching, but decreased (P<0.05) from day five to day seven. This decrease was due to similar decreases (P<0.05) in oleic acid (18:1n9c) and nervonic acid (24:1). Reidy *et al.* (1998) however, reported an increase in oleic acid (18:1n9c) from 45.0 - 46.9%. There were no differences in the poly-unsaturated fatty acid (PUFA) content from one to seven days post-hatching, regardless of differences (P<0.05) in fatty acid content within the group, mostly from five to seven days post-hatching.

Table 4 Fatty acid composition (% of total yolk fat content) of yolk from starved ostrich chicks from one to seven days post-hatching

Fatty acids		1	3	5	7	LSD
Palmitic acid	16:0	32.77^{b}	33.33^{b}	35.20^{b}	58.62^{a}	3.53
Stearic acid	18:0	9.82^{b}	9.87^{b}	10.40^{b}	18.50^{a}	3.56
Arachidic acid	20:0	0.07^{b}	0.08^{b}	0.08^{b}	0.15^{a}	0.02
Heneicosanoic acid	21:0	0.02^{a}	0.03^{a}	0.02^{a}	0.03^{a}	0.02
Behenic acid	22:0	0.02^{b}	0.02^{b}	0.22^{a}	0.04^{b}	0.04
Lignoceric acid	24:0	0.18^{b}	0.27^{b}	0.27^{b}	0.40^{a}	0.11
	SFA [1)]	42.90^{b}	43.58^{b}	46.22^{b}	77.96^{a}	6.09
Palmitelaidic acid	16:1	7.43^{ab}	8.47^{ab}	6.39^{b}	8.98^{a}	2.46
Elaidic acid	18:1n9t	0.22^{b}	0.30^{a}	0.35^{a}	0.34^{a}	0.07
Oleic acid	18:1n9c	34.12^{a}	33.29^{a}	36.76^{a}	0.07^{b}	5.99
Gondoic acid	20:1	0.26^{b}	0.26^{b}	0.26^{b}	0.59^{a}	0.08
Erucic acid	22:1n9	0.03^{a}	0.04^{a}	0.03^{a}	0.05^{a}	0.03
Nervonic acid	24:1	0.13^{a}	0.09^{b}	0.10^{b}	0.10^{b}	0.04
	MUFA [2)]	42.20^{a}	42.44^{a}	43.87^{a}	10.77^{b}	6.73
Linolelaidic acid	18:2n6t	0.02^{a}	0.03^{a}	0.03^{a}	0.05^{a}	0.03
Linoleic acid	18:2n6c	6.02^{a}	5.86^{a}	1.99^{a}	0.09^{a}	9.67
Eicosadienoic acid	20:2	0.22^{a}	0.18^{ab}	0.15^{ab}	0.10^{b}	0.09
Brassic acid	22:2	0.05^{a}	0.04^{ab}	0.02^{b}	0.03^{ab}	0.02
y-Linolenic acid	18:3n6	0.22^{ab}	0.21^{ab}	0.19^{b}	0.30^{a}	0.09
a-Linolenic acid	18:3n3	3.05^{a}	2.25^{ab}	2.78^{a}	1.14^{b}	1.59
Dihomo-y-linolenic acid	20:3n6	0.12^{b}	0.15^{ab}	0.15^{ab}	0.20^{a}	0.07
Eicosatrienoic acid (ETE)	20:3n3	3.24^{a}	3.79^{a}	3.18^{a}	0.22^{b}	1.15
Arachidonic acid	20:4n6	0.02^{b}	0.05^{b}	0.02^{b}	5.19^{a}	0.22
Eicosapentaenoic acid (EPA)	20:5n3	0.15^{b}	0.11^{b}	0.18^{b}	0.30^{a}	0.07
Docosapentaenoic acid (DPA)	22:5n3	0.13^{b}	0.12^{b}	0.16^{b}	0.35^{a}	0.14
Docosahexaenoic acid	22:6n3	1.15^{ab}	1.02^{b}	1.06^{b}	1.63^{a}	0.50
	PUFA [3)]	14.90^{a}	13.98^{a}	9.92^{a}	10.27^{a}	8.54
	UFA [4)]	57.10^{a}	56.42^{a}	53.78^{a}	22.05^{b}	6.09
	SFA:UFA	0.76^{b}	0.78^{b}	0.87^{b}	3.65^{a}	0.54

[a, b, c] Row means with different superscripts differ significantly at P<0.05

[1)]Saturated fatty acids, [2)]Mono-unsaturated fatty acids, [3)]Poly-unsaturated fatty acids, [4)]Unsaturated fatty acids

The SFA:UFA ratio also remained constant over the first five days, but then increased (P<0.05) from day five to day seven. This is the result of the large increase in SFA content from day five (46.2) to day seven (77.9). Reidy *et al.* (1998) reported that poults and chicks also differ in fatty acid utilisation from the yolk and that poults prefer palmitoleic acid (16:1) over oleic acid (18:1n9c).

Fed chicks trial

There was an increase (P<0.05) in slaughter weight (Table 2) from one to sixteen days post-hatching (Figure 9).

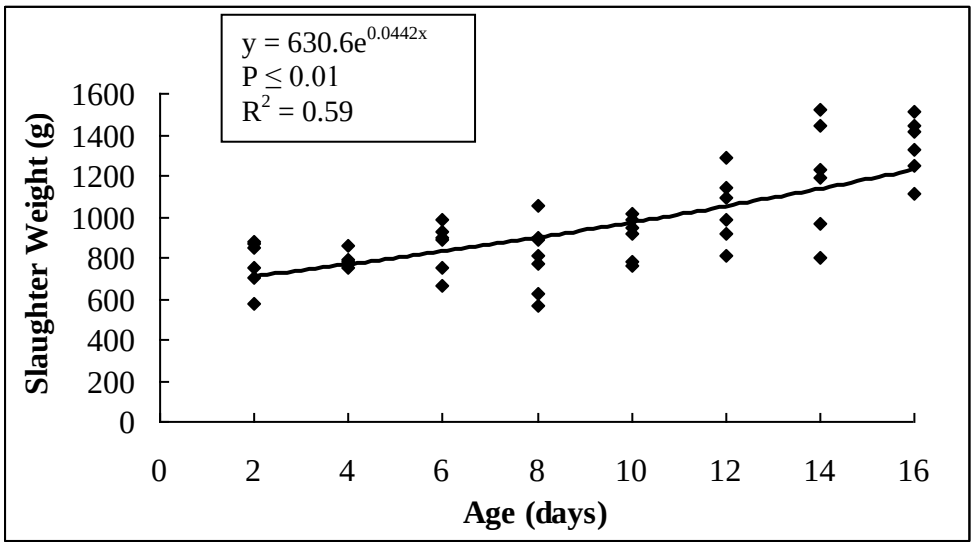

Figure 9 Slaughter weights of fed ostrich chicks from two to sixteen days post-hatching

Yolk weight decreased (P<0.05) by 16.3 g daily from day two (229.2 g) to day 16 (4.56 g) post-hatching (Figure 10). Chamblee *et al.* (1992) suggested that feed and water did not have an effect on body weight or yolk sac utilisation of broilers during the first day post-hatch. Yolk content for fed ostrich chicks in this trial was 26% of body weight, which was more than reported for poults (10.1%) (Moran & Reinhart, 1980) and broilers (16.7%) (Chamblee *et al.*, 1992). Several studies on newly hatched chicks reported that the yolk is absorbed in the first three days post-hatching (Heywang & Jull, 1930; Jull & Heywang, 1930; Heywang, 1940; Chamblee *et al.*, 1992), whereas this trial indicated that ostrich chicks only absorb 30% of the yolk over the first four days post-hatch, 67% after eight days post-hatch and only deplete the yolk reserve after 14 days post-hatch. This corresponded to work done by Mushi *et al.* (2004) that suggested yolk reserves were considered retained beyond 13 days post-hatch.

Crude protein as a percentage of yolk composition is depicted in Figure 11 and total crude protein content in grams is depicted in Figure 12, while fat content as a percentage of yolk composition is depicted in Figure 13 and total fat content in grams is depicted in Figure 14.

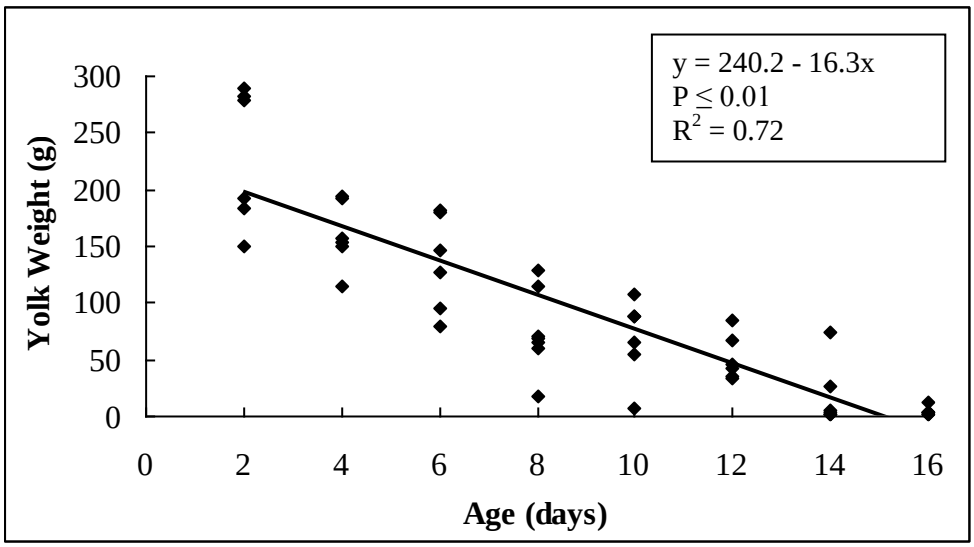

Figure 10 Yolk weights of fed ostrich chicks from two to sixteen days post-hatching

Both constituents showed the same tendencies reported for crude protein and fat content in the yolks of starved ostrich chicks. Crude protein content decreased ($P<0.05$) by 1.2% daily from day two (44.8%) to day 14 (28.7%) post-hatching (Figure 11), which amounts to a daily decrease of 6.84 g (Figure 12). This result corresponds with work reported by other authors. Noy & Sklan (1999) reported that yolk protein content decreased post-hatch for chicks and was depleted four days post-hatch. The same results were obtained for poults (Moran & Reinhart, 1980) who reported that the yolk protein content decreased from 51.3% one day post-hatch to 45.3% two days post-hatch.

Fat content of the yolk increased ($P<0.05$) by 1.39% daily from day two (40.8%) to day 14 (61%) post-hatch (Figure 13), although total yolk fat weight decreased by 6.61 g daily from 93.75 g at two days of age to 11.25 g at 14 days of age (Figure 14). There was too little yolk left to measure fat content at 16 days of age. The increase in yolk fat percentage could therefore be attributed to fat being slowly assimilated from the yolk content, hence increasing as a percentage of total yolk concentration. These results were in direct contrast to results obtained for chicks and poults. Moran & Reinhart (1980) reported that lipids were utilised faster than protein from the yolk of poults, while Romanoff & Romanoff (1967) made the same observation for chicks. Sklan & Noy (2000) reported that the mechanisms for the utilisation of lipids are present in the developing embryo, so that little change is necessary in the digestive tract of the post-hatch poult to be able to utilise lipid absorption. One would therefore expect that utilisation of lipids from the yolk would be an efficient mechanism to meet the energy requirement of the post-hatch ostrich chick. Several authors have suggested that the composition of the yolk only provides maintenance requirements for chicks and poults, whereas exogenous energy sources are utilised for growth (Romanoff, 1960; Thaxton & Parkhurst, 1976; Chamblee *et al.*, 1992; Sklan & Noy, 2000). Chamblee *et al.* (1992), however, suggested that yolk fat is necessary for early growth, as dietary fat is only effectively utilised 10 days after hatching.

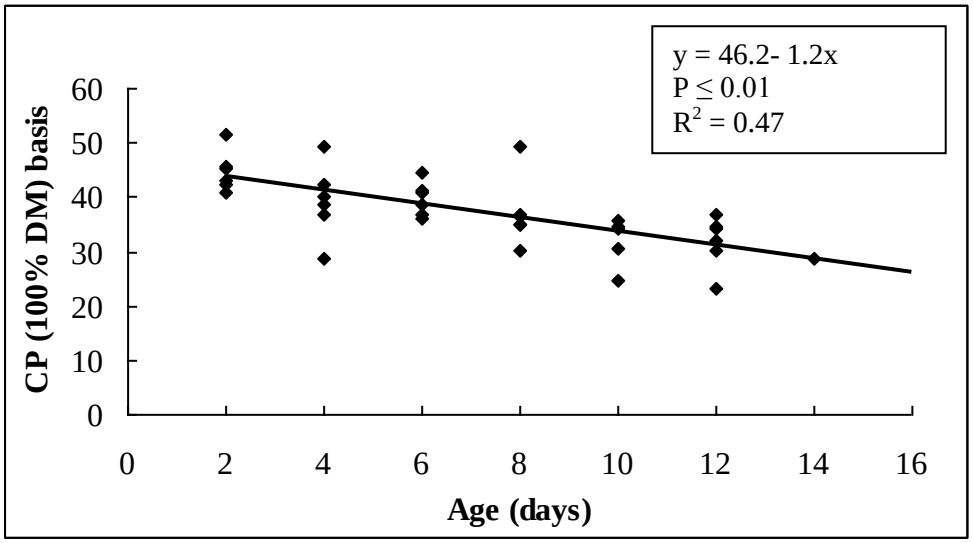

Figure 11 Crude protein content (100% DM basis) of yolks from fed ostrich chicks from two to sixteen days post-hatching

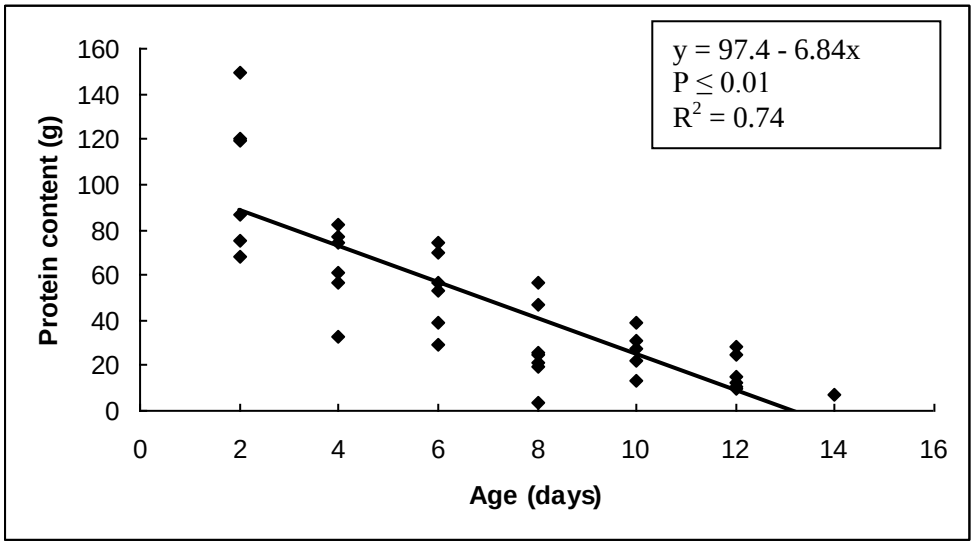

Figure 12 Protein content (g) of yolks from fed ostrich chicks from two to sixteen days post-hatching

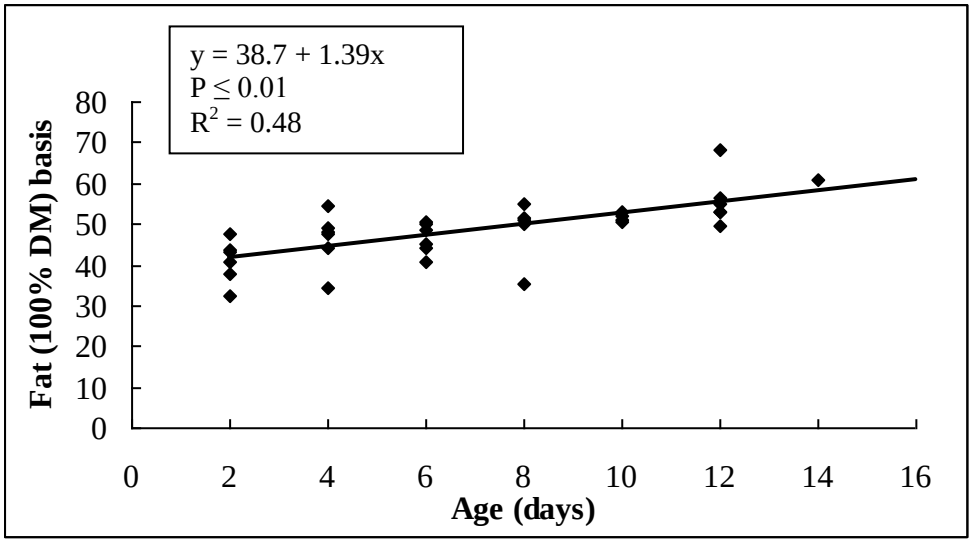

Figure 13 Fat content (100% DM basis) of yolks from fed ostrich chicks from two to sixteen days post-hatching

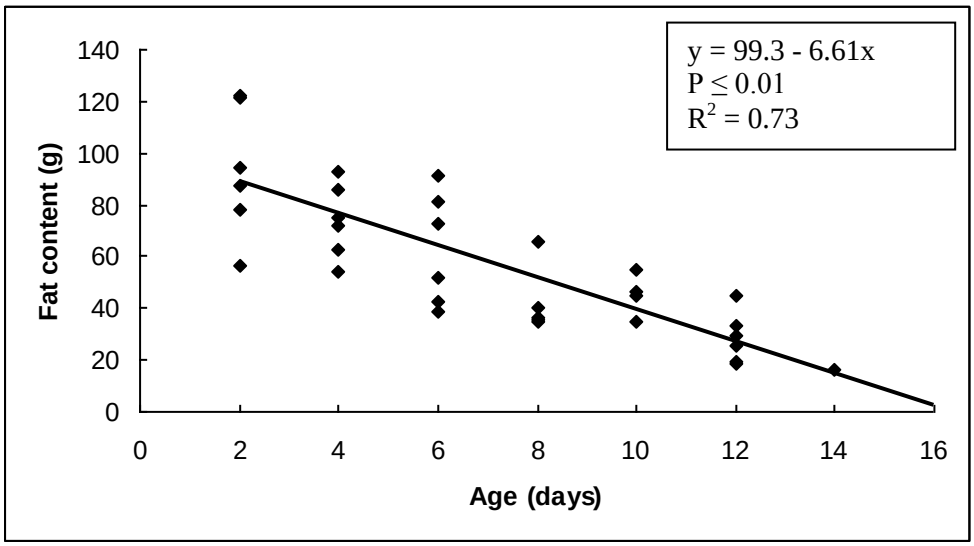

Figure 14 Fat content (g) of yolks from fed ostrich chicks from two to sixteen days post-hatching

Although statistically not comparable, the results of the two trials indicate that yolk weight decreased faster over the first seven days in the starved ostrich chicks than over the first eight days or over the entire trial period for the fed ostrich chicks (Table 5). The same tendency was observed for crude protein, which indicates that the decrease in yolk weight could be attributed to the absorption of protein from the yolk. Fat content, however, decreased faster over the first eight days from the yolk of the fed ostrich chicks than over the first seven days from the yolk of the starved ostrich chicks, although it was not the case over the entire 16 day trial period for the fed ostrich chicks (Table 5). This could indicate that external feed has a positive influence on the absorption of fat from the yolk content.

Table 5 Regression statistics

	Starved 1-7 days	Fed 2-8 days	Fed 2-16 days
Slaughter weight (g)	y = 877.7 - 31.3x	$y = 767.4e^{0.01x}$	$y = 630.6e^{0.04x}$
Yolk weight (g)	y = 270.9 - 28.9x	y = 264.9 - 22.3x	y = 240.2 - 16.3x
Crude protein (%)	y = 46.3 - 1.50x	y = 45.7 - 1.08x	y = 46.2 - 1.20x
Crude protein content (g)	y = 119.2 - 13.2x	y = 120.9 - 11.7x	y = 97.4 - 6.84x
Fat content (%)	y = 38.0 + 1.77x	y = 39.8 + 1.16x	y = 38.7 + 1.39x
Fat content (g)	y = 104.0 - 8.91x	y = 115.3 - 10.1x	y = 99.3 - 6.61x

Conclusion

Thaxton & Parkhurst (1976) reported that adding carbohydrate and amino acids to the diet in the first two weeks post-hatching, is essential for the initiation of growth of chicks. This is especially important, as dietary fat is only utilised effectively 10 days post-hatch in broilers (Chamblee *et al.*, 1992). In both the trials conducted on ostrich chicks, protein was assimilated from the yolk sacs, while it appeared that fat was

absorbed at a much slower rate. The changes observed in fatty acid composition on day seven post-hatching may indicate that ostrich chicks have the ability to withdraw certain fatty acid components, especially mono unsaturated fatty acids from the yolk content. It would be interesting to see whether this apparent ability to withdraw certain fatty acids is repeated with exogenous feed over the first weeks post-hatching. Moran & Reinhart (1980) and Reidy *et al.* (1998) indicated that poults have the ability for selective withdrawal from the yolk, whereas poults and chicks display differences in metabolism of various lipid fractions. Even though this study indicated that yolk absorption is faster in starved than fed ostrich chicks, the author agrees with previous studies on chicks and poults which suggest that ostrich chicks should be fed immediately post-hatch. The author suggests that further studies should be performed with pre-starter diets that include added amino acids, especially histidine and valine, as well as mono-unsaturated fatty acids during the first few weeks post-hatching, due to the fact that a preference for these nutrients from the yolk content was observed in this trial.

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Chapter 3

Enzyme activity in the small intestine of ostrich (*Struthio camelus* L.) chicks from two to sixteen days post-hatching on a pre-starter broiler diet

Introduction

The growth of poultry during the first week of life has been studied quite intensively (Mitchell & Smith, 1991; Dunnington & Siegel, 1995; Noy & Sklan, 1995; Iji *et al*., 2001a & b; Noy *et al*., 2001), as this particular period represents a large proportion of the life span of meat-type poultry. Lilja (1983) suggested that the ultimate growth of poultry can be directly correlated to the development of specifically the gastrointestinal and cardiovascular systems. Birds must be able to adapt to exogenous diets rapidly, in order to achieve their genetic growth potential (Dibner, 2000). Early growth rate may be influenced by various factors, including yolk sac residue, levels of pancreatic and intestinal enzymes, surface area of the gastrointestinal tract, nutrient digestibility and absorption, and quality of feed (Dibner, 2000). Uni (2003) suggested that poultry chicks undergo rapid development, both physical and functional, during the first week post-hatching and that this development of the gastrointestinal tract gives the chicks the capacity to digest feed and assimilate nutrients.

It is often noted that ostrich chick mortalities occur around 2-3 weeks post-hatching, due to chronic starvation (Verwoerd *et al*., 1997). The authors ascribe this situation to environmental, social or nutritional stress to which the ostrich chicks are exposed in the first week post-hatching. Survivors are also often left with low growth rate, inefficient feed utilisation and reduced resistance to diseases (Uni, 2003).

Noy & Sklan (1999) suggested that pancreatic and brush border enzymes have to be available in sufficient quantities for effective digestion and absorption to occur in chickens. They reported, however, that quantitative determination of digestive enzymes in the intestines of poultry immediately post-hatching was not carried out at that time (Noy & Sklan, 1999). Iji *et al*. (2001) conducted a study to determine the development and characteristics of certain intestinal enzymes in broiler chicks on a commercial starter diet. They found that there was a general reduction in the specific activities of intestinal enzymes as broiler chicks aged, but that the increased length and surface area of the gastrointestinal tract compensate for this reduction per unit of mucosal surface. The authors suggested that enzyme activities differ between different intestinal sites and that this may also have an influence on overall digestion (Iji *et al*., 2001).

In 2003 Iji *et al*. conducted a study on ostrich chicks at intervals of 3, 27, 41, 55 and 72 days of age intervals to test the development of enzyme activity. Based on these findings, the authors suggested evaluation of enzyme activity at closer intervals, particularly during the early stages, to obtain a clearer

picture of enzyme development when changes are profound in other avian species. These changes are usually observed within the first two weeks post-hatching (Uni *et al*., 1998; Uni *et al*., 1999).

For this trial 49 ostrich chicks were slaughtered over a sixteen day period and the activity of certain enzymes was determined in the small intestine to establish the effect of growth on enzyme activity immediately post-hatching on a pre-starter broiler diet. The project had ethical approval from the Onderstepoort Animal Use and Care Committee (Protocol 36-5-623).

Materials and Methods

Animals

Forty nine chicks were slaughtered every second day for sixteen days, starting from two-day old chicks. Fifty nine ostrich chicks were originally obtained from the Oudtshoorn Experimental Farm of the Department of Agriculture Western Cape, South Africa, where they were hatched and weighed. The chicks were transported by air to the Faculty of Veterinary Science of the University of Pretoria, Onderstepoort, South Africa, for slaughtering. The average weight of the day-old chicks was 839.31 g.

Chicks were kept in a clean, disinfected room which was kept as cool and dark as possible. Noise and human contact were limited to the absolute minimum to restrict stress. Chicks were provided with clean drinking water and a poultry pre-starter diet. The minimum specifics for the commercial pre-starter diet were: CP = 24.5%; Moisture = 11.5%; Energy = 12 MJ/kg feed; Fat = 6.7%; Fibre = 3.6%; Ash = 6.2%. The average hatching and slaughter weights of the chicks are presented in Table 1.

Table 1 Mean hatching- and slaughter weights (± SE) of ostrich chicks

Slaughter age (days)	Number of chicks in group	Hatching weight (g)	Slaughter weight (g)
2	6	869.6 ± 131.8^{a}	771.6 ± 118.2^{d}
4	6	858.0 ± 65.0^{a}	786.6 ± 38.3^{d}
6	6	925.0 ± 129.1^{a}	853.3 ± 123.4^{d}
8	7	827.4 ± 128.5^{a}	801.4 ± 166.8^{d}
10	6	766.6 ± 136.0^{a}	901.6 ± 106.8^{cd}
12	6	881.0 ± 44.2^{a}	1040.0 ± 170.2^{bc}
14	6	828.8 ± 89.0^{a}	1191.6 ± 273.3^{ab}
16	6	830.5 ± 115.5^{a}	1341.6 ± 144.8^{a}

$^{a, b, c}$ Column means with different superscripts differ significantly at $P<0.05$

Collection of Material

Chicks were euthanized with CO_2 in a closed container. The digestive tracts were immediately removed from the carcasses after death. Each section of the digestive tract was identified. Samples of the wall of the small intestine were taken from the duodenum (ascending or distal limb of the duodenal loop, just

before the secondary distal loop); jejenum (before the vitelline diverticulum, where the yolk sac stalk entered the jejunum) and ileum (approximately 5 cm before the ileo-ceacal junction). The samples were approximately 2 cm in length and weighed between 0.11 and 2.01 g. These samples were used for enzyme analyses.

Tissue samples were rinsed with ice-cold saline (0.9% NaCl), using a syringe to flush out any intestinal content. The samples were then cut open lengthwise and placed on ice with the luminal surface facing away from the surface of the ice. An 18 gauge (18G) needle was attached to a syringe and the tissue surface was thoroughly rinsed, taking special care not to damage the mucosal layer of the wall. The tissue samples were wrapped in pre-marked pieces of aluminium foil and stored in liquid nitrogen, in a thermos flask. When all samples had been collected, the tissue samples were transferred to plastic bags and stored in a freezer at approximately minus 85°C.

Measurements and Calculations

Brush-border membrane vesicles were isolated from the intestinal tissues according to the method described by Shirazi-Beechey *et al.* (1991) with the following additions: A crushed ice-bed of about 5 cm thick was prepared in a flat container (24 cm by 30 cm). All instruments and buffer were kept on cold crushed ice. All the steps of the procedure were completed on the ice bed. Samples (eight per day) were taken from the freezer and placed on the ice-bed to prevent thawing. Each sample was quickly weighed, cut into small pieces, with a pair of sharp scissors while still frozen, and immediately transferred to pre-marked (for identification) conical plastic tubes and placed in the ice-bed in an upright position. Depending on the weight of the tissue sample, the appropriate amount of pre-cooled Buffer A (Table 2) was added to each tissue sample (< 1-1 g tissue weight, use 10 ml of Buffer A; >1-1.5 g use 20-25 ml). The tubes were sealed with parafilm to prevent any spillage. Each sample was vibromixed with a Vortex tube mixer (Heidolph REAX top) at maximum speed for 60 seconds and filtered through a 70 mm diameter Buchner funnel, where after each sample was homogenized individually for 30 seconds with an ULTRA-Turrax T25 homogenizer at 13 500 rpm. To prevent contamination, the homogenizer dispersing head was spun in clean distilled water and wiped dry after each sample. Immediately after mixing, the homogenate was divided (± 600 µl each), into three 1.5 ml Eppendorf tubes, recapped firmly and kept on the ice-bed. After preparation of all the samples, they were placed in a freezer at -85°C until they could be analysed for alkaline phosphatase and protein content.

The remaining homogenate was weighed (to the nearest 0.5 g) into centrifuge tubes. The weights of the samples to be placed opposite each other in the centrifuge were balanced by adding cold Buffer A (Table 2). This dilution was carefully noted. Where 10 ml of Buffer A (Table 2) was added at the start of the procedure (corresponding with a ± 1 g cut sample), 50 µl of $MgCl_2.7H_2O$, from a 2.5 M Stock Solution (50.825 g/100 ml) was added to each sample. Accordingly, 100 µl of $MgCl_2.7H_2O$ was added if the sample volume was between 20 ml and 25 ml. The exact amount added was taken into account during enzyme activity calculations. After the $MgCl_2.7H_2O$ was added, each sample was vibromixed briskly and allowed to

stand for 20 minutes on the ice-bed to aid subsequent sedimentation during centrifugation. Suspensions were centrifuged at high speed, using a Du Pont Refrigerated Sorvall Ultra-Centrifuge RC 6, Rotor SS-34, at 30 000 g (5 000 r.p.m.) and with the temperature set at 6°C for 15 minutes. The supernatant was then transferred to clean pre-marked tubes, discarding the pellets this time, and again centrifuged at 30 000 g (14 500 r.p.m.), at 6°C for 30 min. The supernatant was removed and the pellets re-suspended in cold Buffer B (Table 2). Homogenization was performed at this stage in the procedure, using a 18G needle. Samples were centrifuged for the third time at 30 000 g (14 500 r.p.m.) at 6°C for 45 minutes. The final pellets were re-suspended in 500 µl of cold Buffer C (Table 2) and passed through a 25G needle to obtain a homogenous suspension. The suspension was then divided and transferred to four pre-marked 1.5 ml Eppendorf tubes kept on ice. These homogenized tissue samples were also stored at -85°C for later analyses for chymotrypsin, trypsin, amylase and lipase activity.

Table 2 Chemical composition of Buffers A, B and C (Shirazi-Beechey *et al.*, 1991)

Buffers	A	B	C
Mannitol	100mM (18.22g/l)	100mM (18.22g/l)	300mM (13.665g/250ml)
	2mM	2mM	20mM
Hepes &	(0.477g Hepes/l)	(0.477g Hepes/l)	(1.192g Hepes/250 ml)
Trizma/HCl	(0.315g Trizma/HCl/l)	(0.315g Trizma/HCl/l)	(0.788g Trizma/HCl/250 ml)
$MgSO_4$	-	0.1mM (0.012g/l)	0.1mM (0.003g/250ml)
NaN_3	-	-	0.02% (0.05g/250 ml)
pH	7.1	7.4	7.4

Samples were analysed for alkaline phosphatase according to the method described by Forstner *et al.* (1968) and Holdsworth (1970). Protein was determined according to the method described by Bradford (1976). Chymotrypsin amidase and trypsin amidase were determined according to the method described by Servière-Zaragoza *et al.* (1997).

Amylase content was determined using the ACE™ Amylase Reagent (Reagent number AE2-5) intended for the quantitative determination of alpha (α) amylase activity in serum using the ACE™ clinical chemistry system. The method uses a modified p-nitrophenyl-maltoheptaoside as substrate. A multifunctional glucosidase cleaves the amylase reaction products and releases the p-nitrophenol. The terminal glucose of the substrate is chemically blocked preventing cleavage by the indicator enzyme. The rate of release of pNP is monitored at 408nm and is proportional to the α-amylase activity in the sample.

Lipase content was determined using the ACE™ Lipase Reagent (Reagents 1 and 2, Catalog number 11821792), intended for the quantitative determination of lipase activity in serum using the ACE™ clinical chemistry system. This chosen method uses a 1,2-O-dilauryl-rac-glycero-3-glutaric acid-(6-methylresorufin) ester as substrate. The chromogenic lipase substrate 1,2-O-dilauryl-rac-glycero-3-glutaric acid-(6-methylresorufin) ester is cleaved by the catalytic action of alkaline lipase solution to form 1,2-O-dilauryl-rac-

glycerol and an unstable intermediate glutaric acid-(6-methylresorufin) ester. This decomposes spontaneously in alkaline solution to form glutaric acid and methylresorufin. The colour intensity of the red dye formed is directly proportional to the lipase activity and was determined photometrically.

Enzyme activity can either be expressed as mole substrate hydrolysed/mg protein/min (Shirazi-Beechey *et al.*, 1991; Iji *et al.*, 2001) or as Units/mg protein (Pletschke *et al.*, 1995; Servière-Zaragoza *et al.*, 1997). The final enzyme activity was expressed as mmole product/min/mg protein.

Statistics

The data were analysed according to a standard eight (age) x three (region of small intestine) factorial analysis (Snedecor & Cochran, 1980) for protein of the brush border membrane and the enzymes alkaline phosphatase, trypsin and chymotrypsin. For the enzymes amylase and lipase a standard eight x two (region of small intestine) factorial analysis was done, as activity of these two enzymes was only tested in the duodenum and ileum. Repeated records from the same experimental units assessed for different parts of the small intestines were accounted for by adding the random effect of animal in the mixed model analysis (Harvey, 1990). Although the interaction between age and region of the small intestine was not significant ($P>0.05$) in all analyses, these interactions are provided to depict a clear picture of the results.

Results and Discussion

Protein

The protein content of the brush-border membrane (BBM) was higher ($P<0.05$) in the duodenum and jejunum than in the ileum, at two and four days of age. Thereafter there was no significant differences ($P<0.05$) between the protein content of the BBM of the different parts of the small intestine up until 16 days of age (Figure 1). Iji *et al.* (2001a) reported that the protein content in broiler chicks was the same in all the regions on the first day post-hatching, but that it was higher thereafter in the jejunum than in the other two regions. In a study on ostrich chicks Iji *et al.* (2003) observed that the protein content of the BBM of the jejunum and ileum was higher than that observed in the duodenum on three and 27 days of age. This was attributed to the growth of microvilli in these regions (Iji *et al.*, 2003). The protein reported of the BBM for this trial, was also much higher than the protein content reported by Iji *et al.* (2003). The protein content in the BBM of the duodenum on two days of age, for example, was 1.97 mg/g tissue, compared to 0.18 mg/g tissue reported by Iji *et al.* (2003) for three days of age. The ostrich chicks in the trial conducted by Iji *et al.* (2003) were fed a starter diet between 3 and 27 days of age, which was formulated with an ostrich feed database (CP = 17.94%; Energy = 16.2 MJ/kg feed; fat = 4.19%; fibre = 8.08%). The differences in the protein content could be due to nutritional differences within the diets of the two trials, different ages of chicks, different sites at which enzyme activities were measured and the way analyses were conducted.

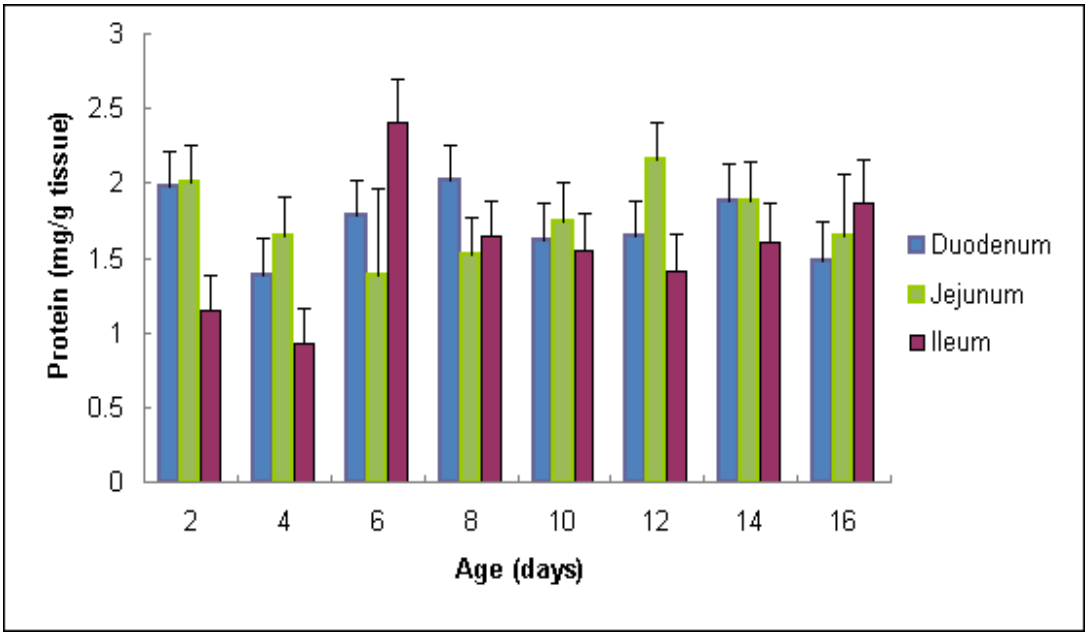

Figure 1 Protein content (± SE) in the different sections of the small intestine of ostrich chicks from two to 16 days post-hatching in ostrich chicks

In the following section the value for protein content is expressed as milligram per gram tissue (mg/g tissue). There was a decrease ($P<0.05$) in protein content of the duodenal BBM from two days (1.97 ± 0.24) to four days (1.39 ± 0.24), where after it increased ($P<0.05$) up to eight days (2.02 ± 0.22) of age. From eight days onwards, the protein content declined ($P<0.05$) up to 16 days (1.48 ± 0.26) (Figure 2), despite an increase on 14 days (1.88 ± 0.24) of age. This differs from broiler chicks where Iji *et al.* (2001a) reported that the protein content remained constant in the duodenum from hatch to 14 days of age.

In the jejunum the protein content of the BBM decreased from two (2.01 ± 0.24) to six days (1.39 ± 0.58), although not significant ($P<0.05$) due to the large variation between the samples at six days of age. There was an increase ($P<0.05$) in protein content from eight (1.53 ± 0.24) to twelve days (2.16 ± 0.24) (Figure 3) after which a gradual decline was observed. A sharp peak was observed ($P<0.05$) in the ileum BBM protein content at six (2.40 ± 0.29) days of age, but it remained constant thereafter (Figure 4). Similarly, Iji *et al.* (2001a) also reported a peak in both the jejunum and ileum in broiler chicks at 7 days of age, where after the protein content remained constant in both regions up to 21 days of age.

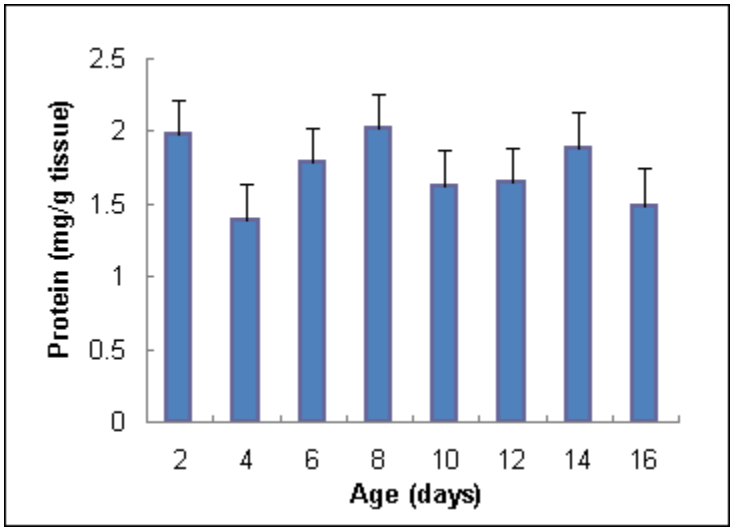

Figure 2 Protein content (± SE) in the brush border membrane of the duodenum

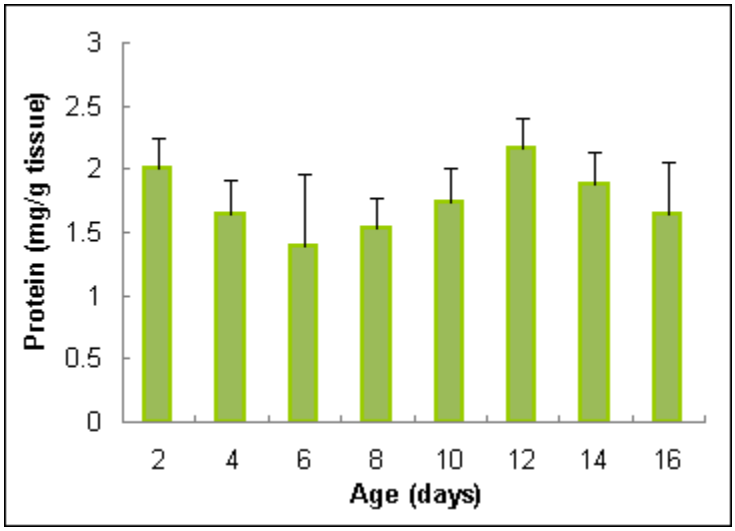

Figure 3 Protein content (± SE) in the brush border membrane of the jejunum

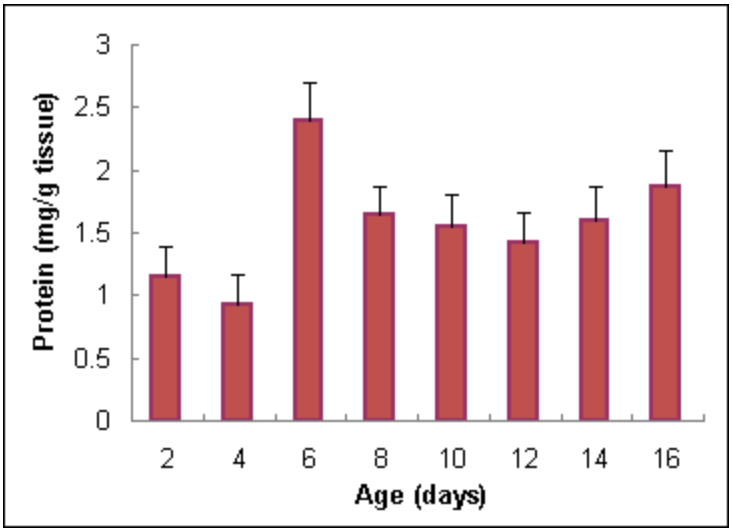

Figure 4 Protein content (± SE) in the brush border membrane of the ileum

Trypsin

The activity of trypsin was constant throughout the length of the small intestine up to twelve days, but at fourteen- and sixteen days of age, the trypsin activity decreased ($P<0.05$) from the duodenum to the ileum (Figure 5). Iji *et al.* (2003) could not detect trypsin in the pancreas of 3 day old ostrich chicks, but reported quite high trypsin activity at 27 days of age. Noy & Sklan (1995) also reported an increase of trypsin with age in the young chick.

For the rest of this section the values for enzyme activity are expressed as millimole product per minute per milligram protein (mmole product/min/mg protein).

There was no difference in the activity of trypsin in the duodenum over the first eight days, but the activity increased ($P<0.05$) from ten (3.22 ± 0.82) to sixteen days (6.36 ± 0.90) of age (Figure 6). Although there was a significant ($P<0.05$) increase in trypsin activity in the jejunum from ten (1.49 ± 0.90) to twelve (3.94 ± 0.82) days of age, there was no change in trypsin activity in the jejunum between two (2.61 ± 0.82) and sixteen days (2.27 ± 1.43) of age (Figure 7). The activity of trypsin was constant in the ileum, regardless of the two peaks ($P<0.05$) on two (4.98 ± 0.82) and twelve days (4.35 ± 0.82) of age (Figure 8). These values could be correlated with that of other authors who also reported an increase in trypsin activity for poultry with an increase in body weight over the first two weeks post-hatching (Noy & Sklan, 1999; Sklan & Noy, 2000; Noy *et al.*, 2001; Noy & Sklan, 2001).

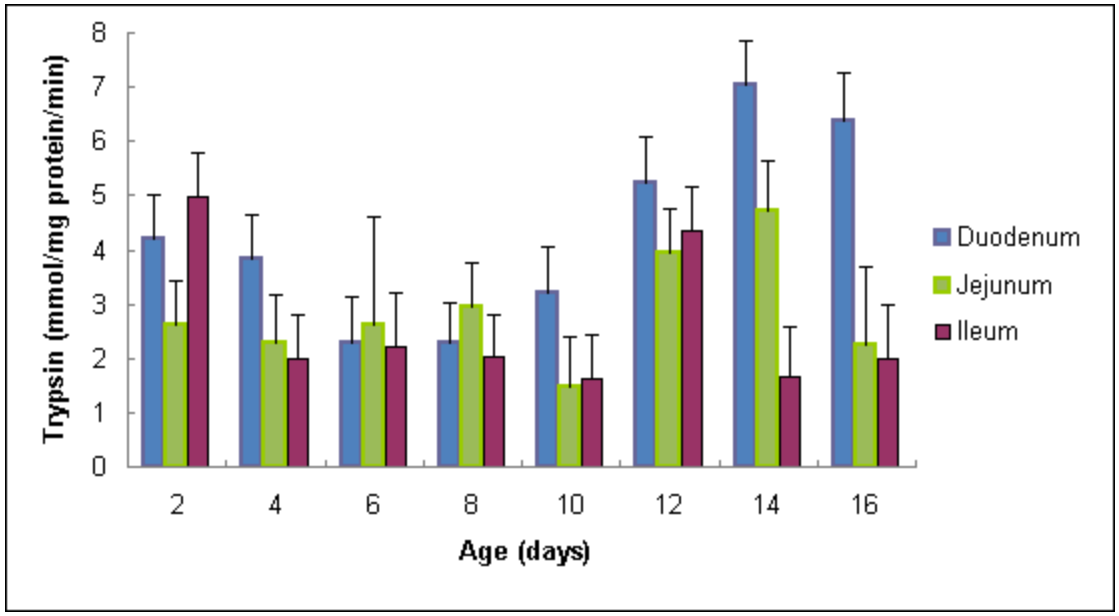

Figure 5 Trypsin activity (± SE) in the small intestines from 2 to 16 days post-hatching in ostrich chicks

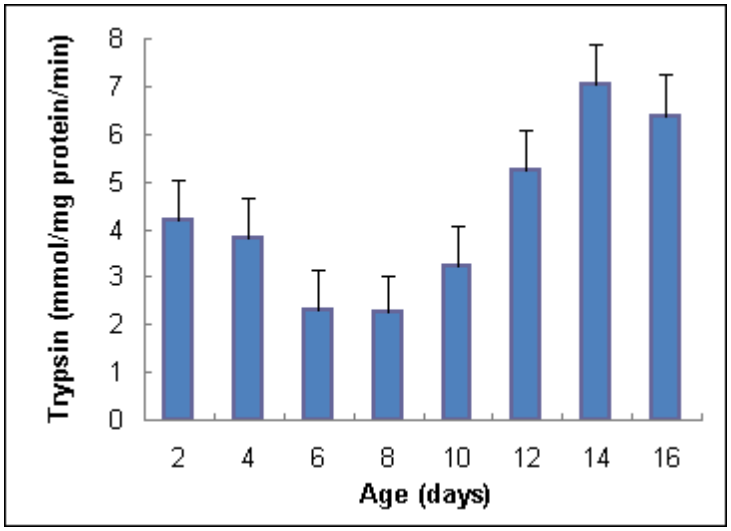

Figure 6 Trypsin activity (± SE) in the duodenum

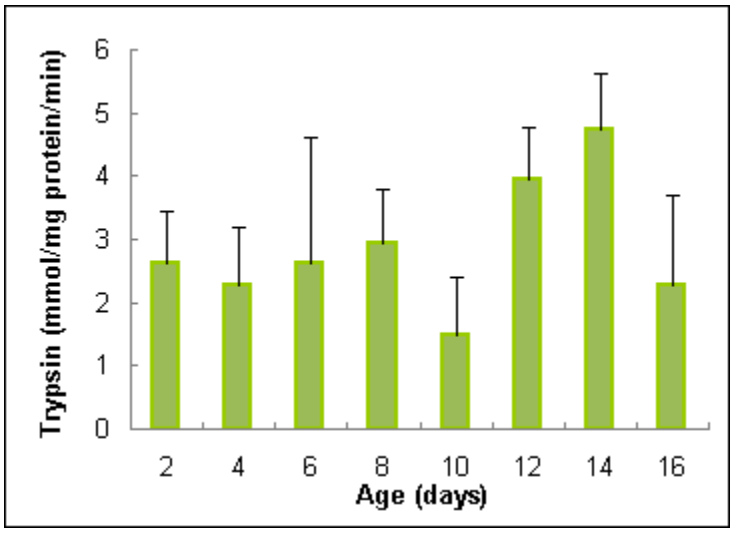

Figure 7 Trypsin activity (± SE) in the jejunum

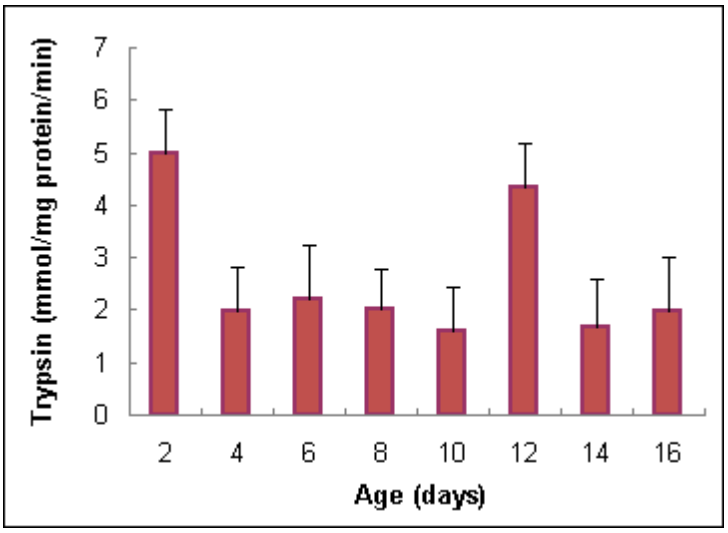

Figure 8 Trypsin activity (± SE) in the ileum

Chymotrypsin

The activity of chymotrypsin was constant throughout the small intestine, regardless of region, except for an increase in activity ($P<0.05$) in the ileum at four days of age (Figure 9).

Chymotrypsin was constant in the duodenum (Figure 10) and ileum (Figure 12) except for a peak ($P<0.05$) in activity at four days (41.85 ± 3.98 and 63.80 ± 3.98) of age. In the jejunum (Figure 11) the activity was constant over the first eight days and then declined ($P<0.05$) from eight (30.51 ± 3.97) days to ten (17.50 ± 4.35) days of age and remained constant for the next eight days. Iji *et al.* (2003) made a similar observation for chymotrypsin in a trial on ostrich chicks, where chymotrypsin activity was high at 3 days post-hatching, but declined up to 27 days of age.

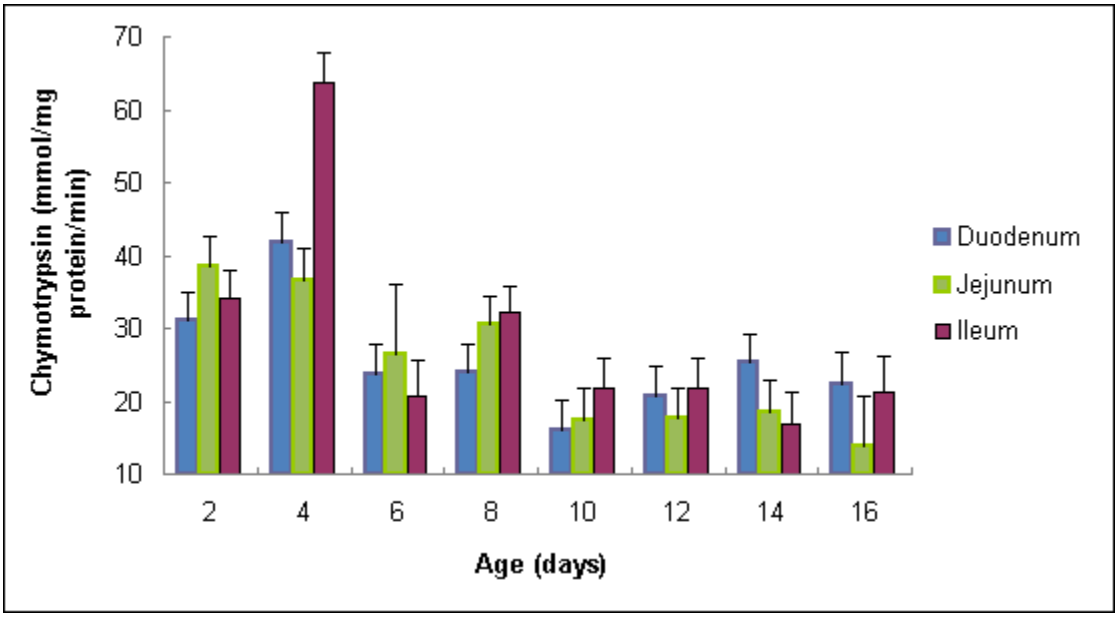

Figure 9 Chymotrypsin activity (± SE) in the small intestines from two to 16 days post-hatch in ostrich chicks

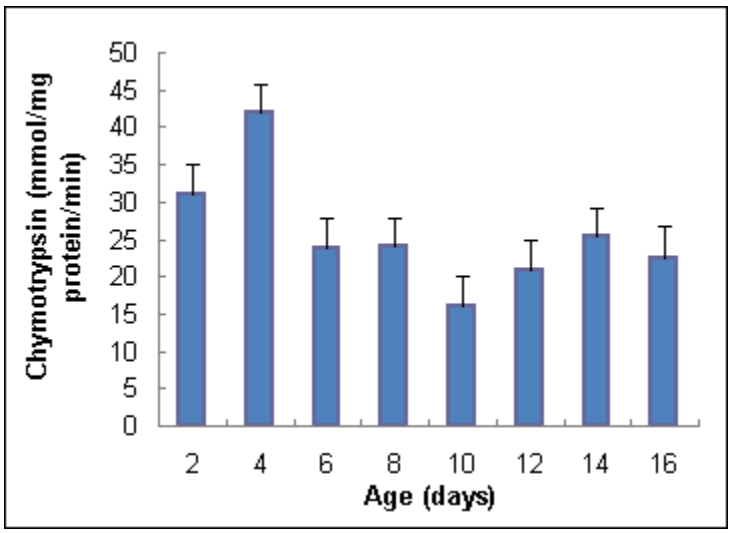

Figure 10 Chymotrypsin activity (± SE) in the duodenum

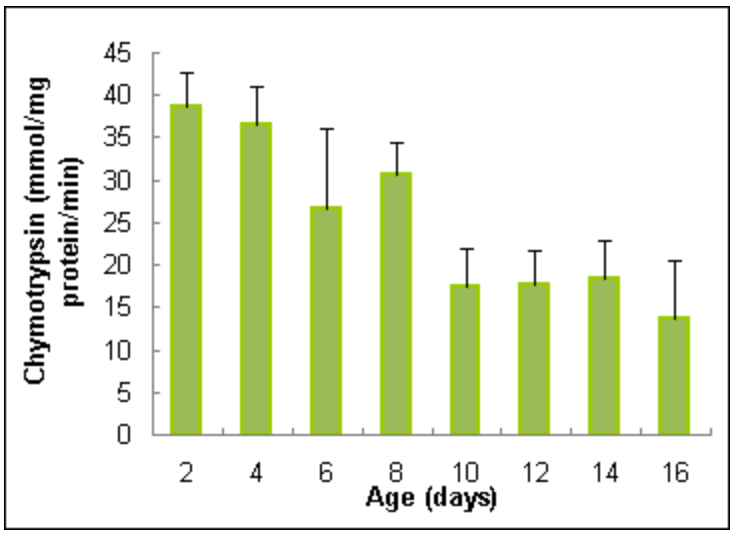

Figure 11 Chymotrypsin activity (± SE) in the jejunum

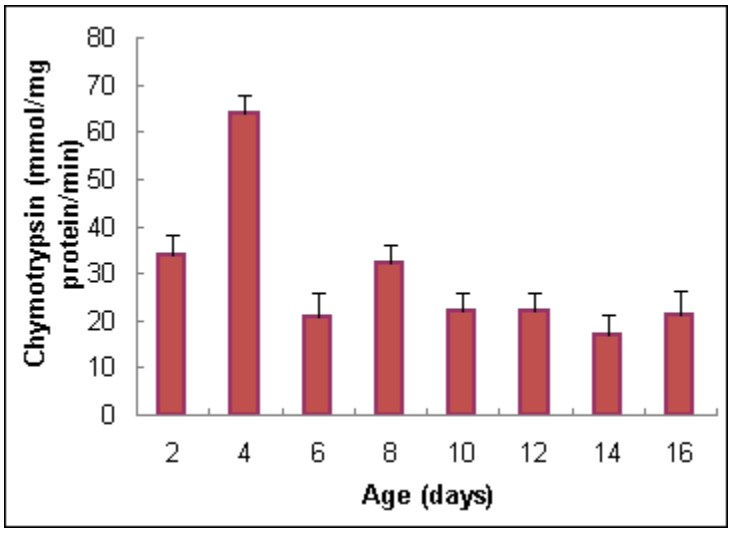

Figure 12 Chymotrypsin activity (± SE) in the ileum

Alkaline Phosphatase

Alkaline Phosphatase (AP) activity showed a tendency to decline along the length of the small intestine, although it was only significant ($P<0.05$) at two, 12 and 16 days of age (Figure 13). Iji *et al.* (2003) reported that AP activity was similar in the duodenum and jejunum of ostrich chicks at 3 days post-hatching, but that it declined, though not significantly ($P>0.05$) to the ileum. They also indicated that total AP activity declined from 3 days to 27 days of age and that the activity was higher in the ileum than in the other two regions on 27 days of age, though not significantly ($P>0.05$). In broiler chicks AP was observed to be higher in the jejunum than in the other two regions (Iji *et al.*, 2001b). Uni *et al.* (2003) reported little change in AP content with age in the duodenum and jejunum of broiler chicks. In a study by Uni *et al.* (1999) the authors found the differences between AP activities in intestinal regions to be more pronounced in poults than in broiler chicks. Iji *et al.* (2003) suggested that the development of the gastrointestinal tract occurs more slowly in the ostrich than poultry. The authors claimed that this can be expected, as the

production cycle of the ostrich is much longer than in poultry. A decrease in the AP activity may therefore be an indication that the development of mucosal enzyme activity in the ostrich is slower than in poultry, resulting in slower growth during the first days post-hatching (Uni *et al.*, 1999).

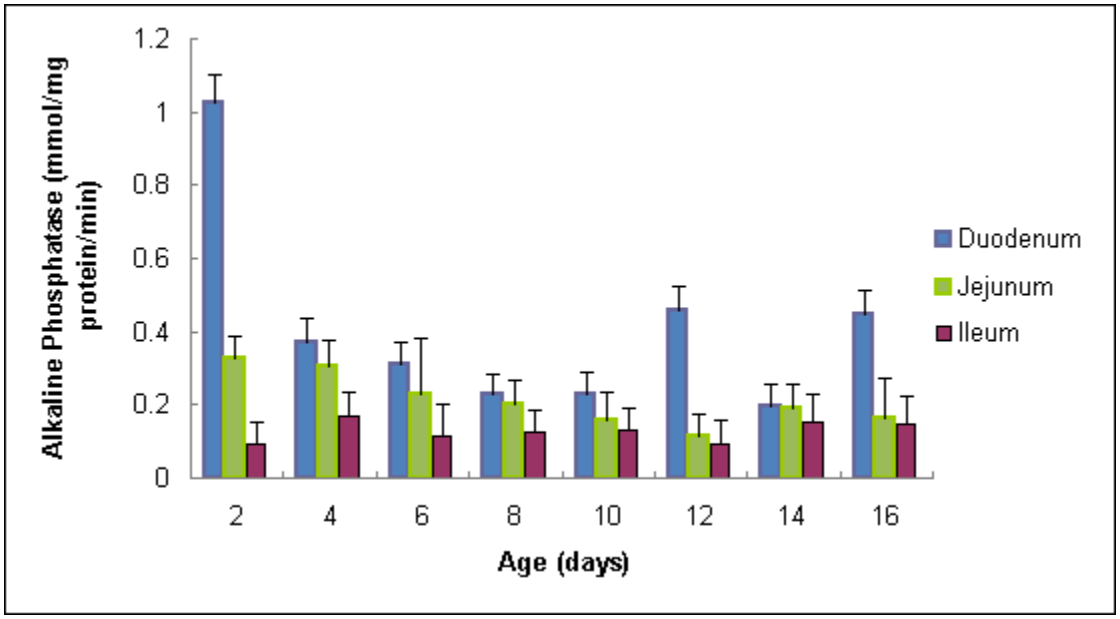

Figure 13 Alkaline Phosphatase activity (± SE) in the small intestines from two to 16 days post-hatch in ostrich chicks

In the duodenum, AP activity decreased ($P<0.05$) from two days (1.03 ± 0.08) to four days (0.37 ± 0.06) of age and remained constant over the rest of the sixteen day observation period (Figure 14). The AP activity decreased ($P<0.05$) from two days (0.33 ± 0.06) to twelve days (0.12 ± 0.06) in the jejunum, after which it remained constant (Figure 15). There was no difference ($P<0.05$) in the activity of AP over the 16 days observation period in the ileum (Figure 16). Similar results were observed in broiler chicks where AP activity declined in the duodenum and ileum from one to 14 days post-hatching, but declined in the jejunum only up to 7 days post-hatching and then increased up to 14 days of age (Iji *et al.*, 2001b).

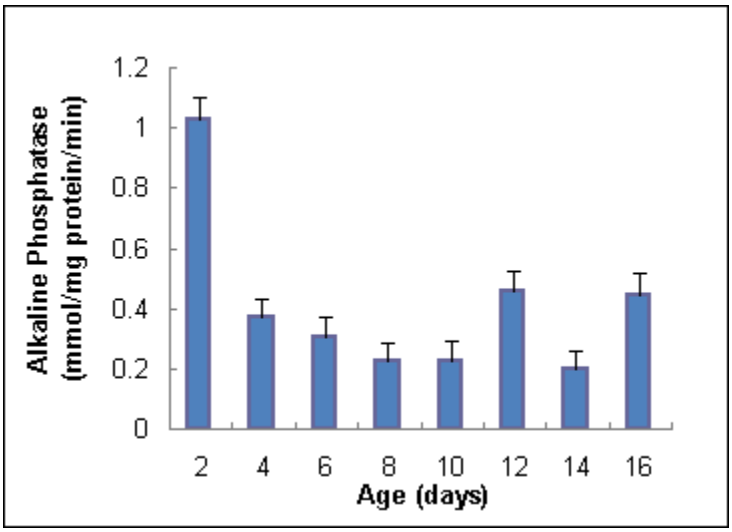

Figure 14 Alkaline Phosphatase activity (± SE) in the duodenum

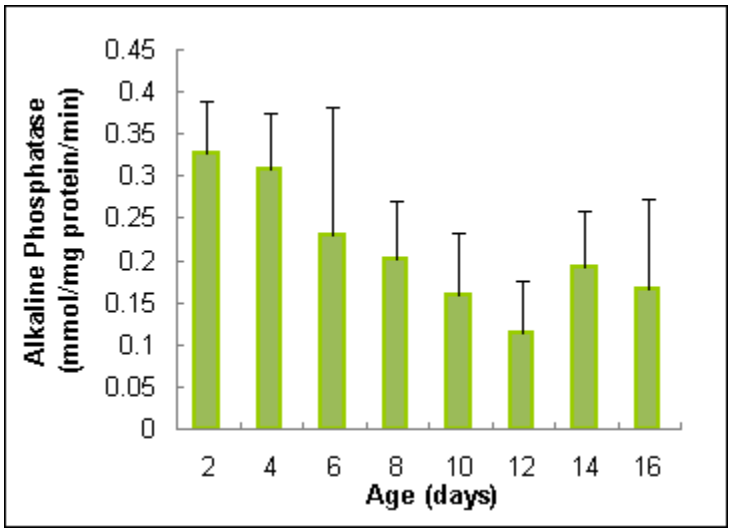

Figure 15 Alkaline Phosphatase activity (± SE) in the jejunum

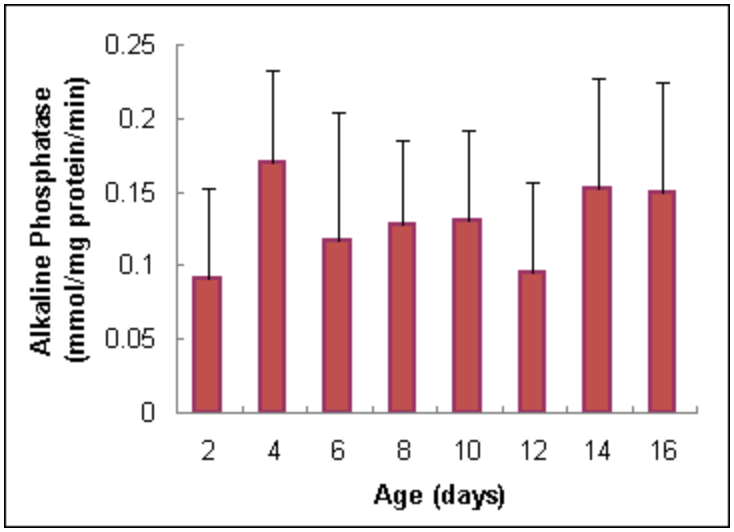

Figure 16 Alkaline Phosphatase activity (± SE) in the ileum

Amylase

Amylase activity remained constant from the duodenum to the ileum throughout the sixteen day observation period (Figure 17). The graphs (Figures 17-19) are presented on the natural log scale, as data was transformed to ensure a normal distribution and equal variances during the treatment period. Values in the text are back transformed to geometric means for ease of interpretation. The activity of amylase showed no differences from two days to 14 days of age in the duodenum (range from 70.83 ± 25.28 to 54.38 ± 24.01; Figure 18), but declined ($P<0.05$) at 16 days (20.19 ± 8.914). The activity of amylase in the ileum declined ($P<0.05$) from two days (188.03 ± 73.80) to six days (28.38 ± 14.61) of age and subsequently remained constant up to 16 days (range from 28.38 ± 14.61 to 38.87 ± 15.26; Figure 19) of age. This corresponds with results from Iji *et al.* (2003) observed for ostrich chicks from 3 to 27 days of age, although the authors reported that the amylase activity tended to be lower in ostrich chicks than in poultry. These results differ from results reported for broiler chicks in the literature. Several authors reported that amylase activity in broiler chicks increases with age and body weight, especially over the first couple of days post-hatching (Noy & Sklan, 1999; Sklan & Noy, 2000; Noy *et al.*, 2001; Noy & Sklan, 2001). Iji *et al.* (2003) attributed the lower activity of amylase compared to that of poultry to the low concentration of soluble sugars in the diet of the ostrich.

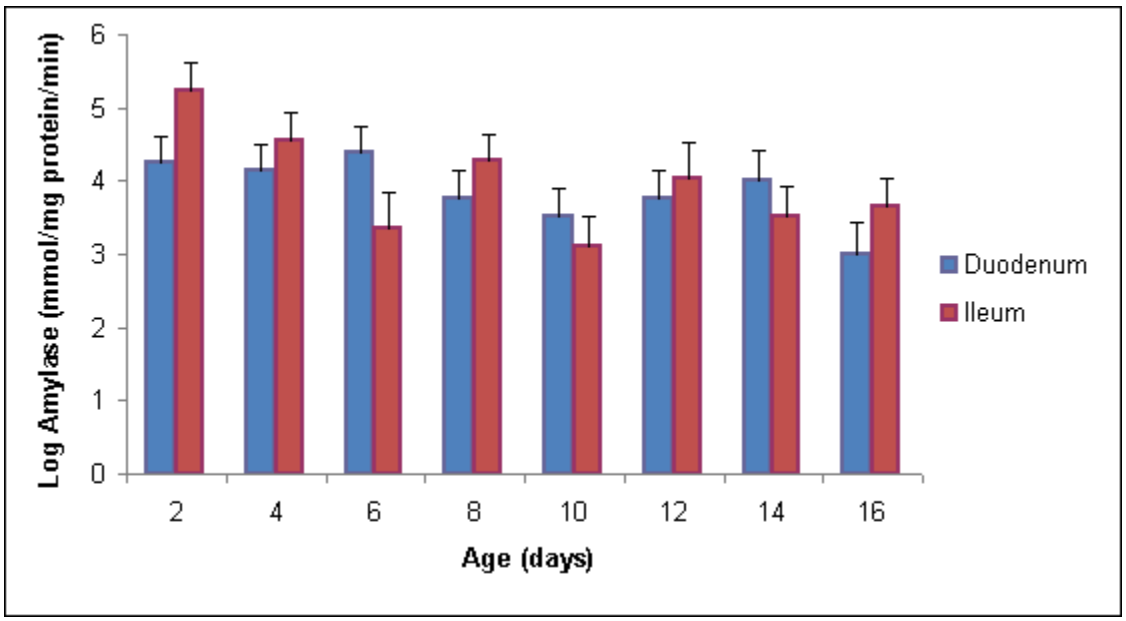

Figure 17 Amylase activity (± SE) in the small intestines from 2 to 16 days post-hatch in ostrich chicks

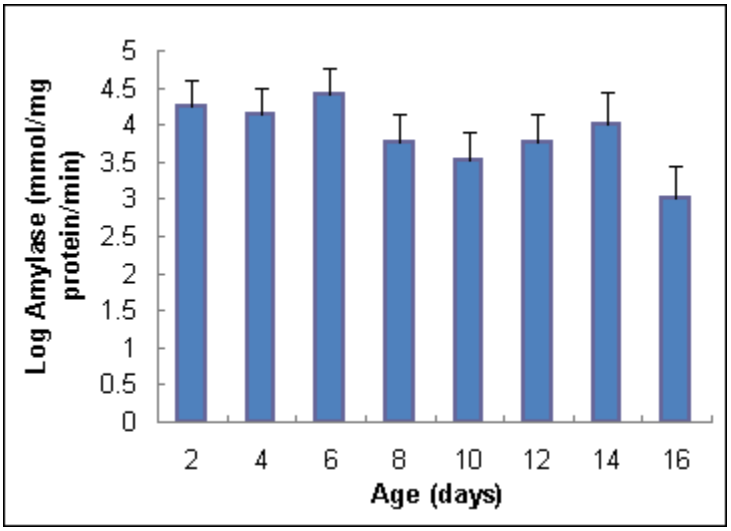

Figure 18 Amylase activity (± SE) in the duodenum

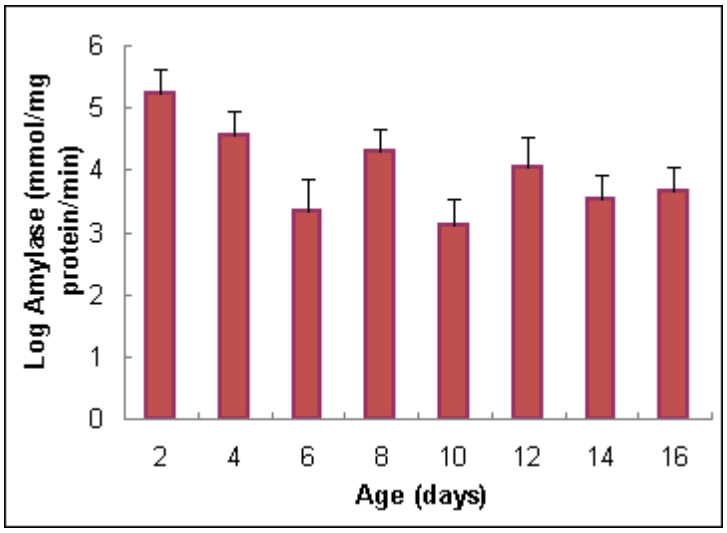

Figure 19 Amylase activity (± SE) in the ileum

Lipase

The age of the chicks had an effect on lipase activity in the duodenum and ileum in most of the observations (Figure 20). The graphs (Figures 20-22) are presented on the natural log scale, as data was transformed to ensure a normal distribution and equal variances during the treatment period. Values in the text are back transformed to geometric means for ease of interpretation. Lipase activity increased ($P<0.05$) from the duodenum to the ileum on two (42.25 ± 13.35 to 105.38 ± 37.37; Figure 20) and four (42.25 ± 13.35 to 79.36 ± 22.82; Figure 20) days of age, remained constant on six and eight days of age, but declined ($P<0.05$) from the duodenum to the ileum from 10 (40.70 ± 11.71 to 17.30 ± 5.46; Figure 20) to 16 (45.34 ± 14.32 to 12.43 ± 4.41; Figure 20) days of age. Lipase activity on the yolk sac membrane of turkey embryos was reported to be present on the seventh day of incubation (Escribano *et al.*, 1988). The authors reported that this lipase activity increased until hatch, but declined after four days post-hatching. They also reported

that pancreatic lipase activity increased up to 16 days post-hatching. This was also reported for lipase activity in broiler chicks (Noy & Sklan, 1999; Sklan & Noy, 2000; Noy *et al.*, 2001; Noy & Sklan, 2001). Lipase activity was also observed in broiler chicks before external feed intake and increased after feed had been consumed, but was less marked and occurred at a later age than the changes in trypsin and amylase activity (Noy & Sklan, 1995; Noy & Sklan, 1998). Furthermore, yolk of chickens is depleted within the first three days post-hatching (Heywang & Jull, 1930; Chamblee *et al.*, 1992), while yolk reserves for ostriches were considered to be retained beyond 13 days post-hatching (Mushi *et al.*, 2004). Yolk was depleted in this trial only after 14 days post-hatching (as described by the author in Chapter 2, page 27). One could therefore argue that ostriches might also have lipase secretion from the yolk membrane, which could cause the lipase activity to be higher in the ileum than in the duodenum. As the yolk reserve is depleted and the pancreatic lipase activity increases, one would expect to find an increase in lipase activity in the duodenum compared to the ileum, as was observed in this study. Iji *et al.* (2003) reported a decline in lipase activity in ostrich chicks from three days to 72 days of age, although the decline between three to 27 days of age was not significant ($P>0.05$).

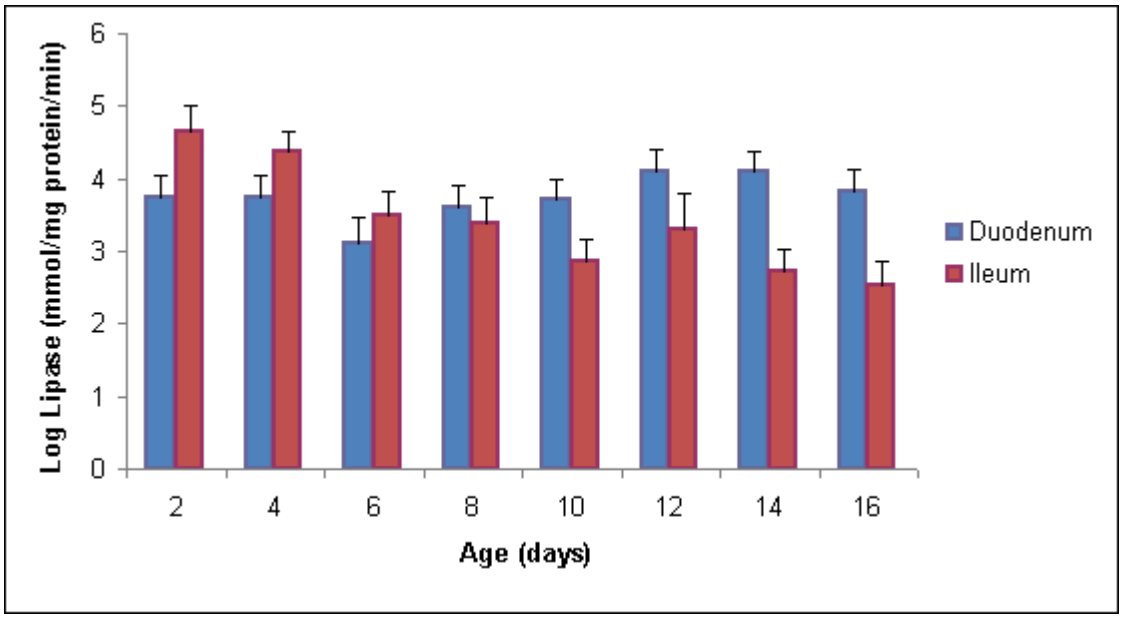

Figure 20 Lipase activity (± SE) in the small intestines from 2 to 16 days post-hatch in ostrich chicks

In the duodenum (Figure 21) lipase activity remained constant over all sixteen days, except for a drop ($P<0.05$) in activity on day six (22.43 ± 7.954). The activity of lipase in the ileum declined ($P<0.05$) from four (79.36 ± 22.82) to six (32.81 ± 11.63) days of age, remained constant up to 12 days (27.43 ± 14.03), where after it declined again to 16 days (12.43 ± 4.40) of age (Figure 22). The differences observed in amylase and lipase activity between the birds in this trial and poultry could be due to genetic differences between species, or because with poultry the animals used in the trials are genetically much more homogenous than ostrich chicks, which have not been subjected to the same extent of advanced breeding

progress than poultry up date. However, the choice of methods used for determining enzyme activity could also play a role in the differences observed.

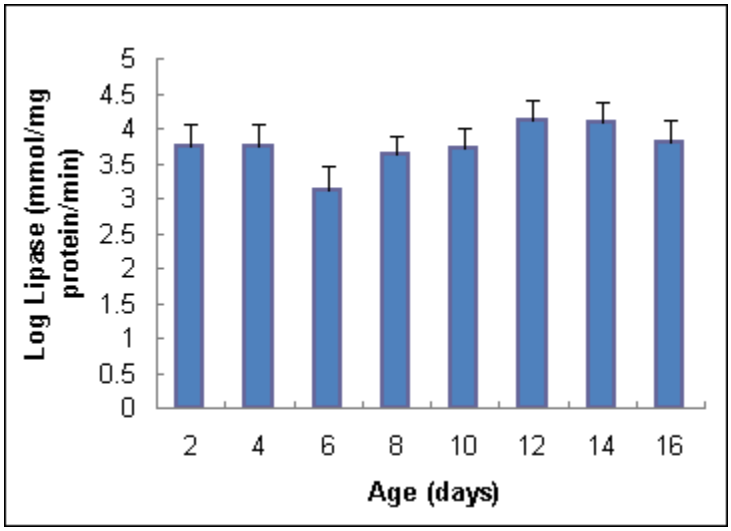

Figure 21 Lipase activity (± SE) in the duodenum

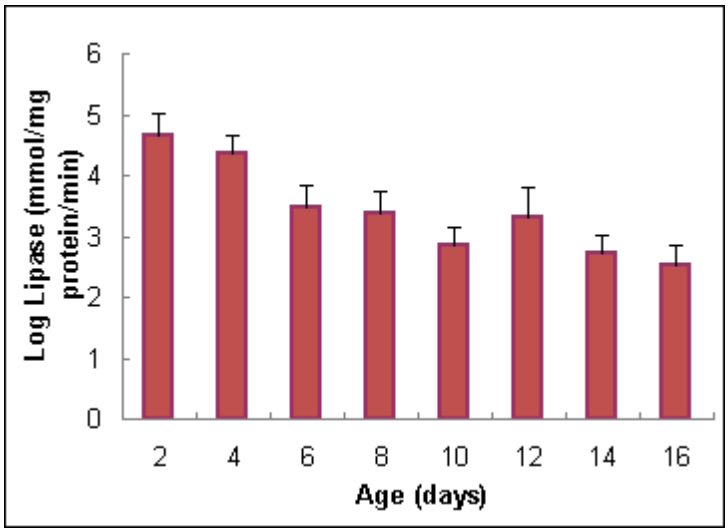

Figure 22 Lipase activity (± SE) in the ileum

Conclusion

Iji *et al.* (2003) observed that the pattern of enzymatic development for ostrich chicks was in many aspects similar to that in broiler chickens. In their study, however, chicks were slaughtered at intervals of between 14 and 17 days from 27 to 72 days of age, but with 24 days between the first slaughter on day 3 post-hatching and 27 days of age. The chicks were also fed a starter diet between 3 and 27 days of age, which was formulated with an ostrich feed database (CP = 17.94%; Energy = 16.2 MJ/kg feed; fat = 4.19%; fibre = 8.08%). These authors measured amylase, lipase, trypsin and chymotrypsin activity in pancreatic preparations.

In the current study the changes in enzymatic development were determined within the first two weeks post-hatching, with two day intervals. The chicks in this study were fed a commercial poultry pre-starter diet

(CP = 24.5%; Energy = 12 MJ/kg feed; fat = 6.7%; fibre = 3.6%). All the enzyme activities were measured in brush border membrane vesicles. Iji *et al.* (2003) reported higher protein content in the duodenum than in the other parts of the small intestine, which decreased from three to 27 days post-hatching, when it was reported to be higher in the jejunum and ileum than in the duodenum. No differences were found for protein content from two to 16 days post-hatching in this trial. In this trial trypsin activity increased from day 2 to 16 days post-hatching. Iji *et al.* (2003) could not detect any trypsin in three day old ostrich chicks, but reported relatively high trypsin activity on 27 days post-hatching. Alkaline phosphatase activity was higher in the duodenum than in the ileum in this trial and the activity remained the same in the duodenum and ileum from two to 16 days post-hatching. Iji *et al.* (2003) reported a decline in alkaline phosphatase activity from three to 27 days of age, as well as a higher activity in the ileum than the other two regions of the small intestine on 27 days of age. The differences observed between this trial and the results obtained by Iji *et al.* (2003) could be due to nutritional differences within the diets of the two trials, as well as the age differences of chicks and different sites at which enzyme activities were measured.

Amylase activity corresponded with results from Iji *et al.* (2003) observed for ostrich chicks from 3 to 27 days of age, but differed from results reported for broiler chicks. Amylase activity in broiler chicks increases with age and body weight, especially over the first couple of days post-hatching. Iji *et al.* (2003) attributed the lower activity of amylase compared to that of poultry to the low concentration of soluble sugars in the diet of the ostrich.

Lipase activity measured in ostrich chicks showed the same tendencies as lipase activity reported in poultry (Escribano *et al.*, 1988; Noy & Sklan, 1995; Noy & Sklan, 2001), although the time period during which activity changes occurred was much shorter for poultry than for ostrich chicks. This again, can be attributed to the longer production cycle of ostriches compared to poultry.

As ostrich production is advancing in the same direction as poultry production, more studies should follow to fully comprehend the development and functioning of the digestive tract of this ratite species. This will enable the nutritionist to formulate ostrich diets that will not only benefit initial growth of the ostrich chick, but also result in a stronger chick which will have higher resistance to external stresses that may cause the high mortalities which presently adversely affect ostrich production.

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Chapter 4

A histological and morphometric study of the small intestine of ostrich (*Struthio camelus* L.) chicks from two to sixteen days post-hatching on a pre-starter broiler diet

Introduction

The small intestine of birds consists of three parts, the duodenum, jejunum and ileum. It has been reported that this part of the digestive tract is similar in structure throughout its entire length (Hodges, 1974) and that the various regions cannot be distinguished grossly or histologically (McLelland, 1979). However, Nickel *et al.* (1977) state that differentiation of the various components can be made on the basis of morphological characteristics. The duodenum leaves the gizzard (ventriculus) and forms a characteristic "U"-shaped loop with descending (proximal) and ascending (distal) limbs (Hodges, 1974; Nickel *et al.*, 1977; McLelland, 1979; Bezuidenhout, 1986). The pancreas lies within the confines of the duodenal loop (Duerden, 1912; Hodges, 1974; Nickel *et al.*, 1977; Bezuidenhout, 1986). In the ostrich the ascending limb forms a secondary loop which is unique to this species and is not present in any other ratite (Cho *et al.*, 1984; Bezuidenhout, 1986). The jejunum is considered to begin where the ascending limb of the duodenum crosses the abdominal cavity from left to right caudal to the cranial mesenteric artery (Nickel *et al.*, 1977; McLelland, 1979). This point is described as being at the level of the seventh vertebral rib in the ostrich (Bezuidenhout, 1986, 1999). However, the junction between the duodenum and jejunum has alternatively been described, in the chicken, as the point where the pancreatic and bile ducts enter the intestine (Calhoun, 1954; Hodges, 1974). Although some authors fail to recognize a distinct demarcation between the jejunum and ileum (Calhoun, 1954; Hodges, 1974), the transition has been described as the point of origin of the vitelline (Meckel's) diverticulum (McLelland, 1979), a situation also described for the ostrich (Bezuidenhout, 1986, 1999). The ileum, which is the longest part of the small intestine in the ostrich, ends at the ileo-caecal junction (Bezuidenhout, 1986).

The general histological structure of the various parts of the avian small intestine is reported to be similar (Hodges, 1974). The lumen is lined by a mucosa which is thrown into numerous villi that vary in shape, number and size and which consist of a simple columnar epithelium supported by an extension of the underlying lamina propria that forms the core of the villus. Between the *muscularis mucosae* and the base of the villi lie the crypts of Lieberkühn that open into the lumen in between the villi. The final component of the mucosa is the *muscularis mucosae*, a thin but obvious layer of smooth muscle that extends fibres into the core of each villus. The submucosa is poorly developed in the small intestine of the chicken and its presence is only obvious where cell bodies of the submucosal nerve plexus or an occasional large blood vessel increase the thickness of the layer. The *tunica muscularis* consists of a poorly developed outer longitudinal

layer and a much thicker inner circular layer of smooth muscle. The muscular tunic is covered externally by a serosa supported by a loose connective tissue sub-serosa (Calhoun, 1954; Hodges, 1974; McLelland, 1979).

The main functions of the vertebrate small intestine are to: (1) mix and propel the chyme forward along the digestive tract through smooth muscle contraction; (2) continue the process of digestion through the action of enzymes released by the intestinal mucosa and accessory glands (pancreas and liver); (3) absorb the digested constituents into the blood and lymph vessels and (4) lubricate and protect the wall of the intestine from the action of the acidic chyme and digestive enzymes through the production of mucus (Seeley *et al.*, 1995). In birds, as in mammals, the intestine displays a variety of specializations aimed at increasing the surface area available for absorption including "lengthening of the small intestine, development of the complex surface relief of folds and villi, and the presence of microvilli on the absorptive epithelial cells." (McLelland, 1979).

The early development of the gastrointestinal tract has been linked to growth in poultry (Lilja, 1983). Early growth is dependent on various factors, including the surface area of the gastrointestinal tract available for digestion. Dibner (2000) and Uni (2003) have suggested that the rapid physical and functional development of poultry chicks during the first week post-hatch is partly due to the concomitant development of the gastrointestinal tract, which gives chicks the capacity to digest feed and assimilate nutrients.

The importance of early development of the gastrointestinal tract in birds has been widely recognized and a number of studies have examined the histological and morphological development of the small intestines in poultry, mainly in broiler chicks and turkey poults (Uni *et al.*, 1995; Uni *et al.*, 1996; Fan *et al.*, 1997; Uni *et al.*, 1998; Applegate *et al.*, 1999; Uni *et al.*, 1999; Noy *et al.*, 2001; Sklan, 2001; Iji *et al.*, 2001; Wu *et al.*, 2004; De Verdal *et al.*, 2010). Although some information is available on the histological features of the small intestine in ostriches (Bezuidenhout & Van Aswegen, 1990; Illanes *et al.*, 2006), very little is known about the morphological changes that take place in this species during the immediate post-hatch period. The only study to have addressed this topic is that of Wang & Peng (2008) who investigated the morphological development of the small intestine on days 1, 45, 90 and 334. In this study the histological and morphological development of the small intestine of the ostrich, from two to 16 days post-hatch, is examined to provide a better understanding of the ability of the small intestine to digest and absorb nutrients during this critical development phase.

Materials and Methods

Animals

Fifty nine newly hatched ostrich chicks were obtained from the Oudtshoorn Experimental Farm of the Department of Agriculture, Western Cape Province, South Africa. The chicks were weighed and then transported by air to the Department of Anatomy and Physiology at the Faculty of Veterinary Science, University of Pretoria, South Africa. Groups of chicks (a total of eight groups - see Table 1) were euthanized every second day for sixteen days, starting from day two. The average weight of the day old chicks was

839.31 g. A total of 49 chicks were used in the trial due to the death of some of the birds during the trial period.

Chicks were reared in a clean, disinfected room which was kept as cool and dark as possible. Noise and human contact were limited to the absolute minimum to restrict stress. Chicks were provided with clean drinking water and a poultry pre-starter diet. The minimum specifications for the commercial pre-starter diet were: protein 24.5%, moisture 11.5%, energy 12 MJ/kg, fat 6.7%, fibre 3.6% and ash 6.2%. The hatch and slaughter (CO_2 euthanasia) weights of the chicks are presented in Table 1.

Table 1 Mean hatching- and slaughter weights (± SE) of ostrich chicks

Slaughter age (days)	Number of chicks in group	Hatching weight (g)	Slaughter weight (g)
2	6	869.6 ± 131.8^{a}	771.6 ± 118.2^{d}
4	6	858.0 ± 65.0^{a}	786.6 ± 38.3^{d}
6	6	925.0 ± 129.1^{a}	853.3 ± 123.4^{d}
8	7	827.4 ± 128.5^{a}	801.4 ± 166.8^{d}
10	6	766.6 ± 136.0^{a}	901.6 ± 106.8^{cd}
12	6	881.0 ± 44.2^{a}	1040.0 ± 170.2^{bc}
14	6	828.8 ± 89.0^{a}	1191.6 ± 273.3^{ab}
16	6	830.5 ± 115.5^{a}	1341.6 ± 144.8^{a}

$^{a, b, c}$ Column means with different superscripts differ significantly at $P<0.05$

Collection and preparation of samples

Chicks were euthanized with CO_2 in a closed chamber and the gastro-intestinal tract immediately removed from the carcasses. The gastro-intestinal tract was spread open for identification of the different regions of the small intestine as described by Bezuidenhout (1986, 1999). Tissue samples representing transverse sections of the duodenum, jejunum and ileum were taken from the specific parts of the intestinal tract outlined in Table 2.

Tissue samples were thoroughly rinsed with saline (0.9%NaCl) to remove adhering intestinal contents and mucus and immersion-fixed in 10% neutral buffered-formalin for a minimum period of 48 hours. The samples were dehydrated through 70, 80, 96, and 2X 100 % ethanol and further processed through 50:50 ethanol : xylol, 2X 100 % xylol and 2X paraffin wax (60–120 min per step) using a Shandon Excelsior Automatic Tissue Processor (Shandon, Pittsburgh, PA, USA). Tissue samples were then imbedded manually into paraffin wax in plastic moulds. Sections were cut at 4–6 µm, stained with haematoxylin and eosin (H&E), then viewed and micrographed using an Olympus CX-31light microscope equipped with a digital camera.

Table 2 Regions of the small intestine sampled for histological and morphometric analyses

Region	Specific point of sample collection
Duodenum	Samples taken from the ascending limb of the duodenal loop, immediately proximal to the secondary loop of the ascending limb.
Jejunum	Samples taken approximately 5cm proximal to the point where the vitelline duct (Meckel's diverticulum) enters the small intestine.
Ileum	Samples taken approximately 5 cm proximal to the ileo-caecal junction.

Morphometric analysis

The following parameters were determined for each region of the small intestine in each of the eight groups of chicks by light microscopy of the H&E-stained tissue sections: Villus length (VL) and width (diameter) (VD); crypt depth (CD) (thickness of the glandular layer essentially representing that part of the lamina propria adjacent to the *muscularis mucosae* housing the crypts of Lieberkühn); thickness of the *muscularis mucosae* (MM); and thickness of the inner circular (ICL) and outer longitudinal (OLL) layers of the *tunica muscularis*. The measurements were made using an Olympus CX-31 light microscope equipped with the AnalySIS™ (Soft Imaging System, 1999) software package. The specific tissue layers/structures measured are indicated in Figure 1. Ten measurements were obtained for each layer in each of the chicks in each group. Similarly, the length and width of ten villi were determined for each chick. Villus width was determined by averaging three measurements taken near the base, at the middle and towards the tip of the villus. The data were analysed according to a standard one-way analysis of variance with the age of the chick at slaughter as the only fixed effect (Snedecor & Cochran, 1980). Separate analyses were performed for the duodenum, jejunum and ileum. The random effect of animal was included in the analysis to account for the covariance between measurements arising from the fact that ten measurements for each layer, as well as ten villi, were measured on each slide. The V:C ratio (the ratio of villus length to crypt depth) was determined for each segment of the small intestine from days 2 – 16.

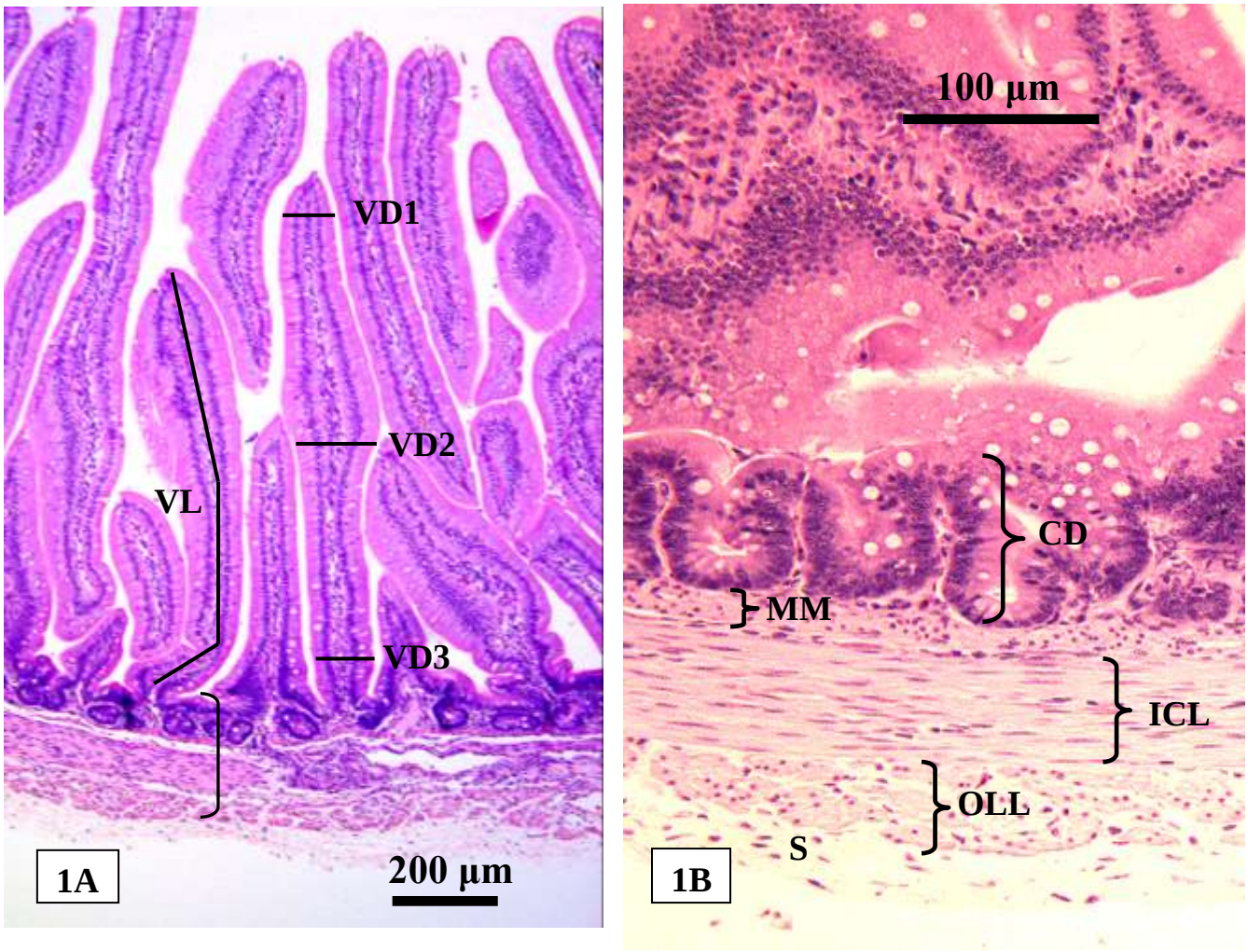


Figure 1A A cross section through the ileum of a 14 day old ostrich chick illustrating the linear measurements taken of villus length (VL) and diameter (VD1 – 3). The layers bracketed at the base of the villi are enlarged in Figure 1B. **B** An enlargement of the base of the villi and the muscle layers of the duodenum in a 12 day old chick indicating the measurements taken of crypt depth (CD), the thickness of the *muscularis mucosae* (MM), the inner circular layer (ICL) and outer longitudinal layer (OLL) of the *tunica muscularis* and the submucosa (S)

Results

Histological structure of the small intestine

Duodenum

Due to the similarities in basic structure observed between the three segments of the small intestine, particularly between the duodenum and jejunum, and between younger and older chicks, references to figures in the following section are not restricted exclusively to morphological features of the duodenum or to a specific age group.

The mucosa of the duodenum in two day old chicks formed numerous villi that extended into the lumen (Figure 2). Two types of villi were observed, namely, long, thin, finger-like villi, and appreciably shorter dagger or tongue-shaped villi. Both types often alternated with each other (Figures 2 and 7). The villi were generally tightly packed (Figure 2), although there were localized areas where they were more loosely

arranged. Each villus consisted of a core of relatively loose, but cell-rich, connective tissue (part of the underlying lamina propria) lined by a simple columnar epithelium. The epithelial (chief) cells displayed a round, vesicular nucleus situated in the basal third of the cell and a wide zone of apical cytoplasm (Figures 6, 8, 11). Higher magnification revealed a conspicuous brush border on the free surface of the chief cells (Figure 12). Typical pale-staining goblet cells were scattered at regular intervals between the chief cells and exhibited a narrow basal portion containing the nucleus and a swollen apical part containing secretory material (Figures 3, 6, 8, 11). Numerous mitotic figures were seen towards the base of the villi (and in the forming intestinal crypts), but entero-endocrine cells were not discernable. The lamina propria at the base of the villi formed a narrow layer that contained occasional shallow, simple tubular glands, the crypts of Lieberkühn (Figure 3), as well as blood vessels. Although rudimentary, the crypts showed varying degrees of development in individual birds. There were also indications in the two day old chicks that more than one crypt opened between adjacent villi. The blood vessels in the lamina propria, together with strands of smooth muscle emanating from the *muscularis mucosae*, extended throughout the length of the villus. Numerous thin-walled vessels (arterioles, venules and capillaries) were particularly conspicuous towards the free end of the villi (Figure 12). No lymphatic vessels were observed in the core of the villi or in the main part of the lamina propria housing the crypts of Lieberkühn. The final component of the mucosa, the *muscularis mucosae,* formed a thin band of longitudinally oriented smooth muscle fibres immediately beneath the lamina propria (Figures 2 and 6).

A sub-mucosa was virtually non-existent and could only be identified due to the presence of occasional blood vessels. In some sections there was a tendency for the tissue layers to separate at the sub-mucosa (Figure 3). The thick inner circular layer (ICL) of the *tunica muscularis* displayed longitudinally oriented smooth muscle fibres (Figures 3, 6, 9, 11). These fibres formed a continuous layer, broken only by occasional large blood vessels passing from the vascular plexus located between the inner circular and outer longitudinal layers (OLL) of the *tunica muscularis* to the lamina propria, where they formed a plexus of smaller vessels at the base of the villi. Although thin in comparison to the ICL, the OLL layer was noticeably thicker, although of similar orientation, to that of the *muscularis mucosae*. This layer was composed of clearly demarcated muscular bundles and often appeared as thick as the ICL (Figures 3 and 6). Large nerve (non-medullated) (Figure 15) and vascular plexuses were positioned between the ICL and OLL. In some instances large blood vessels and elements of the myenteric plexus separated the muscle bundles of the OLL and occupied part of the subserosa. The subserosa varied in thickness and was composed of a layer of loose, almost mucus-like, connective tissue covered externally by a serosa (Figures 3, 6, 9, 18). The subserosa displayed conspicuous vascular collections consisting of a prominent artery and vein and attendant lymphatic vessels and nerves (Figure 17).

The morphological characteristics of the duodenum remained remarkably similar from day two until day 16. The most noticeable difference was an increase in the length of the villi (Figure 4). This change was already evident on day four and in some instances was accompanied by folding of the villi to accommodate them within the lumen of the duodenum (see morphometric data below) (Figure 5). A limit degree of

branching was observed. There also appeared to be a general increase in the number and depth of the crypts (Figures 4, 5, 6). The latter phenomenon was also obvious at day 4 and was accompanied by an increase in the thickness of the lamina propria. As the number of crypts increased it was obvious that more than one crypt opened between neighbouring villi (Figure 6). Mitotic figures indicative of replicating enterocytes was a prominent feature of the crypt lining at all stages during the trial period. Although individual lymphocytes were seen traversing the epithelial layer (Figure 8) and in the lamina propria, no lymphoid tissue was observed in the material studied. However, the number of scattered lymphocytes appeared to increase in the older birds. The OLL of the *tunica muscularis* also appeared visibly thicker in some samples from day 12 to day 16 chicks. The muscular layers of all segments of the small intestine also appeared to form a relatively smaller proportion of the total thickness of the intestine in the older chicks (compare for example Figures 2 and 4 (Duodenum), and Figures 13 and 16 (Ileum)).

Jejunum

The histological features of the jejunum were similar in most respects to those of the duodenum (Figures 7, 8, 9). As in the duodenum, a dramatic increase in villus length was observed from day four onwards. A greater number of goblet cells appeared to be present (Figures 7 and 8). Although the ICL of the *tunica muscularis* appeared continuous, it was less compact than that seen in the duodenum and in some specimens was split into concentric rings by thin intervening layers of connective tissue. Similar to those found in the duodenum, the crypts of Lieberkühn were weakly developed at day two (Figure 9), but increased in number and size from day four onwards (Figure 10). Lymphatic vessels were not obvious in the villi but structures resembling lymph vessels lay between the two layers of the *tunica muscularis*. No aggregations of lymphoid tissue were observed.

Ileum

The ileum displayed structural features generally similar to those of the duodenum and jejunum. However, a number of important differences were noted. The villi were visibly shorter (see morphometric data below) than those of the other components of the small intestine and presented as stubby, finger-like projections, although club-shaped forms were also identified. Short and long villi were again apparent (Figures 13 and 16). The ICL of the *tunica muscularis* was broken into numerous concentric layers and the muscle bundles of the OLL were separated by obvious tracts of connective tissue (Figures 13, 14, 17, 18).

Duodenum Day 2

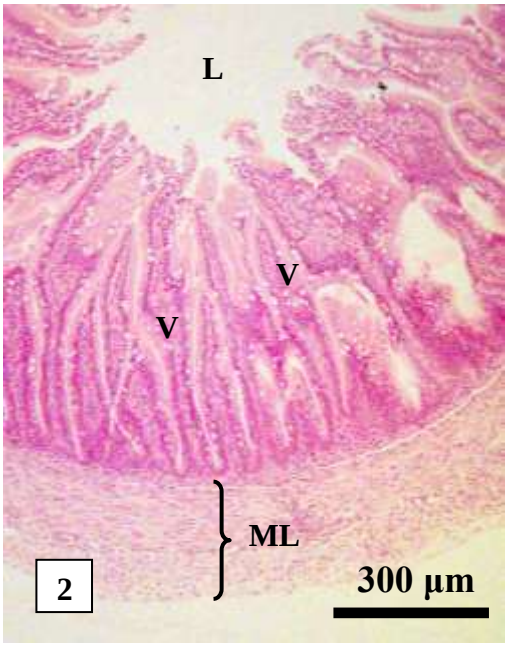
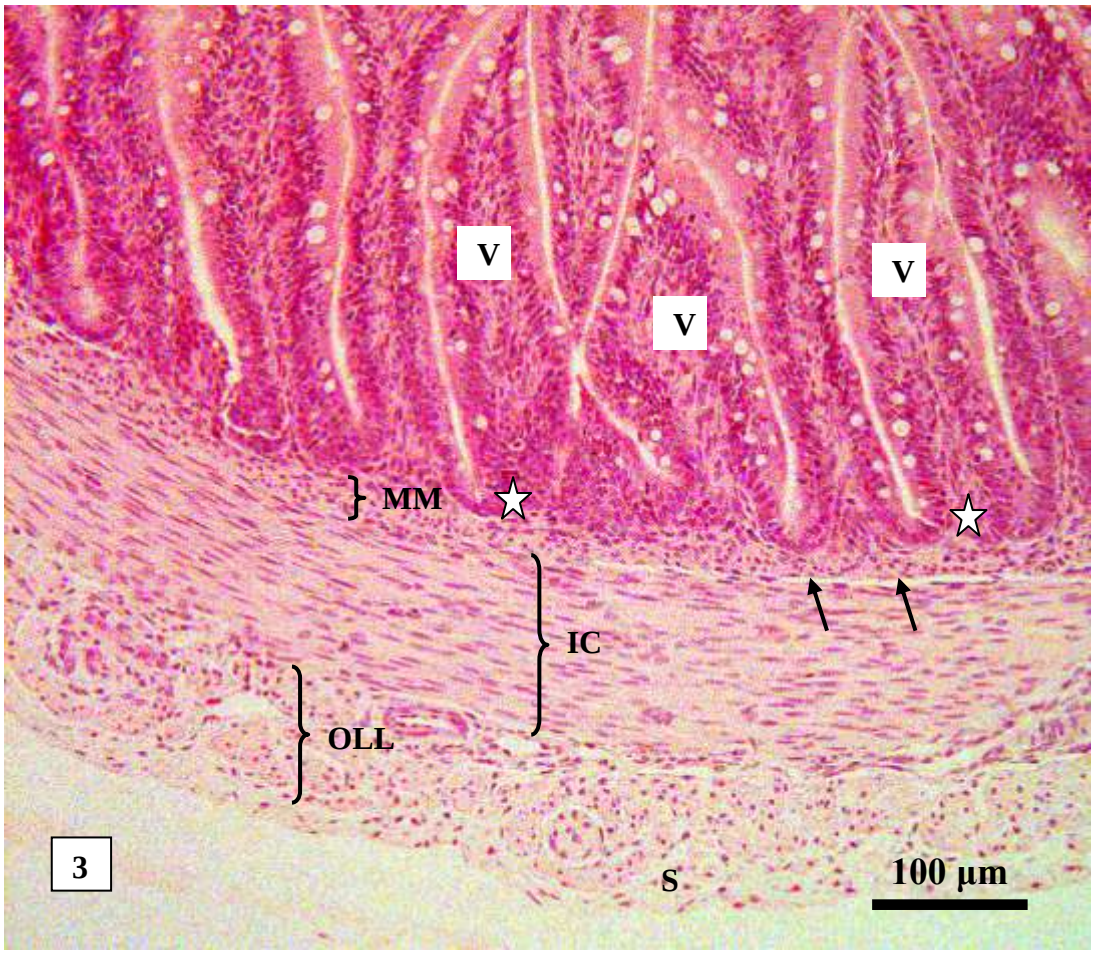


Figure 2 Transverse section of the duodenum of a two day old chick showing the tightly packed villi (V) extending into the lumen (L). Note the relatively thick muscle layer (ML) surrounding the mucosa

Figure 3 Enlargement of the muscle layers and base of the villi illustrated in Figure 1. Three short villi (V) are shown. The *muscularis mucosae* (MM) is separated from the inner (ICL) and outer (OLL) layers of the *tunica muscularis* by an extremely thin submucosa represented on the micrograph as a gap (arrows) between the two muscle layers. The crypts of Lieberkühn are poorly developed (stars) and numerous goblet cells are present. Subserosa (S)



Legend to Figures

Figure 4 Transverse section through the duodenum of a 12 day old chick. The finger-like villi (V) appear partially collapsed. Note the prominent crypts (stars) at the base of the villi and the relatively narrow muscular layer (ML) (compare with Figure 1)

Figure 5 A section similar to that shown in Figure 4, but from a 14 day old chick. The villi show extensive folding and are compactly arranged. The lamina propria at the base of the villi houses numerous crypts (stars)

Figure 6 Enlargement of the muscle layers and base of the villi illustrated in Figure 4. Three crypts are seen (arrows) opening between adjacent villi (V). *Muscularis mucosae* (MM), inner circular (ICL) and outer longitudinal (OLL) layers of the *tunica muscularis*, submucosa (S). Connective tissue from the lamina propria forms the core of each villus. Note the simple columnar epithelium and scattered goblet cells lining the villi

Figure 7 Transverse section of the jejunum of a two day old chick showing the tightly packed alternating long (V1) and short (V2) villi extending into the lumen (L). The crypts of Lieberkühn (C) are exaggerated due to the plane of section

Figure 8 The tips of the villi illustrated in Figure 7 showing the lining of simple columnar epithelial cells interspersed with scattered goblet cells. A few isolated lymphocytes are visible within the epithelium (arrows). Core of connective tissue from the lamina propria (Ct)

Figure 9 Enlargement of the muscle layers and base of the jejunal villi. Note the morphological similarities to that of the duodenum shown in Figure 2. Villi (V), *muscularis mucosae* (MM), inner (ICL) and outer (OLL) layers of the *tunica muscularis*, subserosa (S). The crypts of Lieberkühn are poorly developed

Figure 10 Overview of the components of the jejunum in a 14 day old chick. Notice the development of the glandular layer (crypts) (C) in comparison to that shown in Figure 9 and the similarity between this layer and that of the Day 14 duodenum illustrated in Fig. 4. Villi (V), Lumen (L), muscular layers (ML)

Figure 11 Higher magnification of the alternating short and long villi (V) of the jejunum demonstrating the simple columnar epithelium with goblet cells. *Muscularis mucosae* (MM), inner circular layer of the *tunica muscularis* (ICL)

Figure 12 High magnification of the epithelial lining of the jejunum. The simple columnar lining cells display a distinct brush border of microvilli (arrows) and occasional goblet cells (G) are present. The connective tissue core (Ct) of each villus is richly supplied with capillaries (white arrows)

Figure 13 Transverse section of the ileum demonstrating the short, loosely arranged villi (V) found in this segment of the small intestine. The muscular layers (ML) are well developed

Figure 14 Enlargement of the rectangle in Figure 13 showing the inner (ICL) and outer (OLL) layers of the *tunica muscularis* and the extension (white arrow) of the lamina propria and *muscularis mucosae* (MM) into the core of the villi (V). The crypts of Lieberkühn (stars) are poorly developed

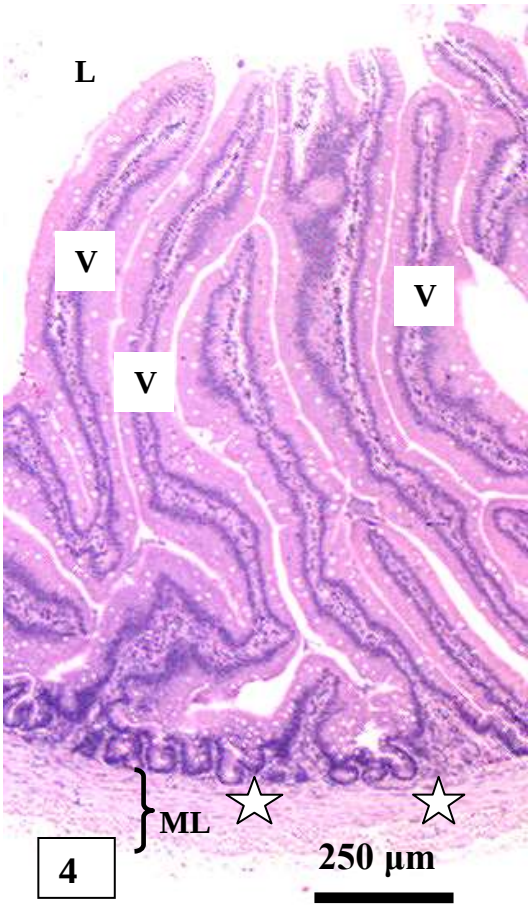
Figure 15 High magnification of part of the myenteric plexus (Mp) situated between the inner (ICL) and outer (OLL) layers of the *tunica muscularis*. The plexus contains neurons and supporting elements

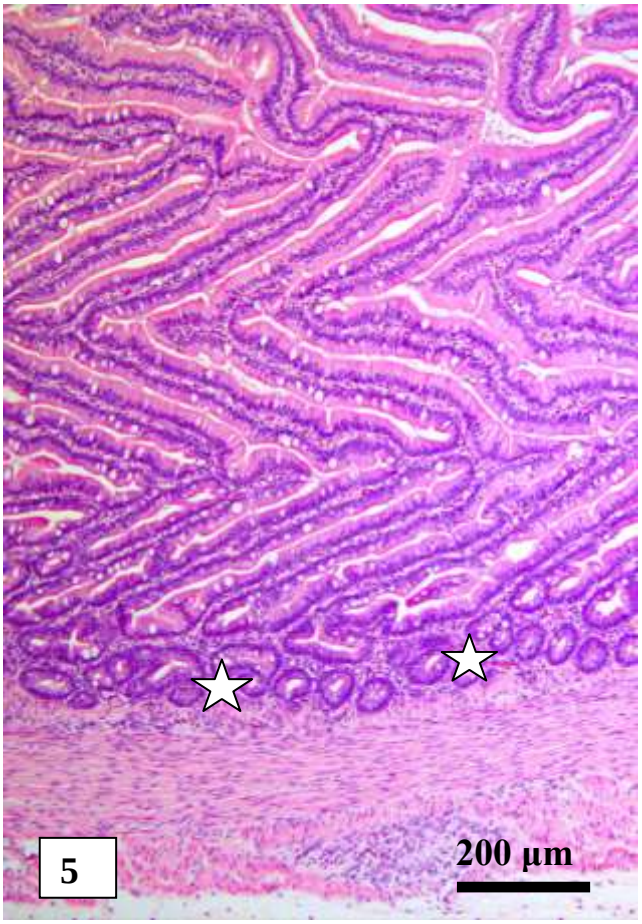
Figure 16 Overview of the components of the ileum in a 14 day old chick. The glandular layer (crypts) (stars) is well developed in comparison to that in the two day old chicks, but the muscular layers (ML) appear relatively thinner. Villi (V), Lumen (L)

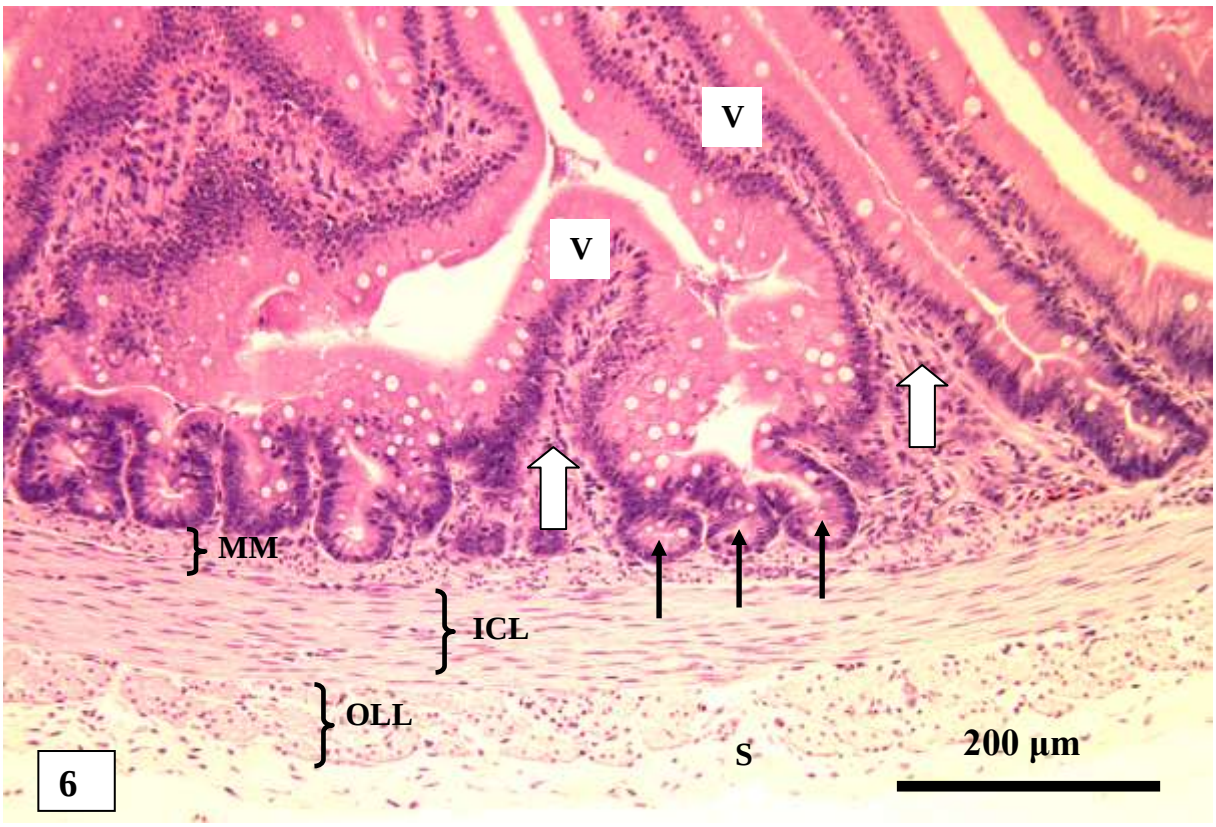
Figure 17 Details of the muscle layers of the ileum. The *muscularis mucosae* is relatively thin (stars), while both the inner circular (ICL) and outer longitudinal (OLL) layers of the *tunica muscularis* are discontinuous. Elements of the myenteric plexus (Mp) are visible. A large vascular plexus in the subserosa (S) reveals arteries (A) veins (Ve) and lymphatic vessels (arrows). Villi (V), Crypts of Lieberkühn (C)

Figure 18 High magnification of the base of the villi and muscular layers of the ileum. Note the rudimentary submucosa (arrows) and the broken nature of the inner (ICL) and outer (OLL) layers of the *tunica muscularis*. Villi (V), Crypts of Lieberkühn (C), *Muscularis mucosae* (MM), Subserosa (S)

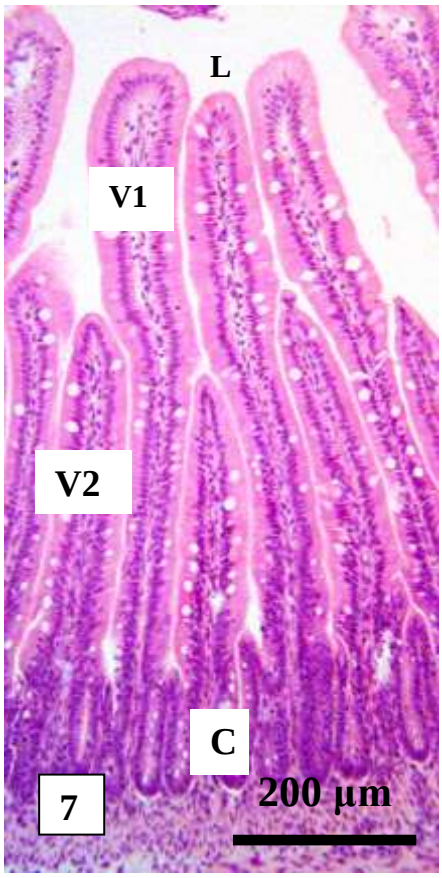
Duodenum Day 12 to 14

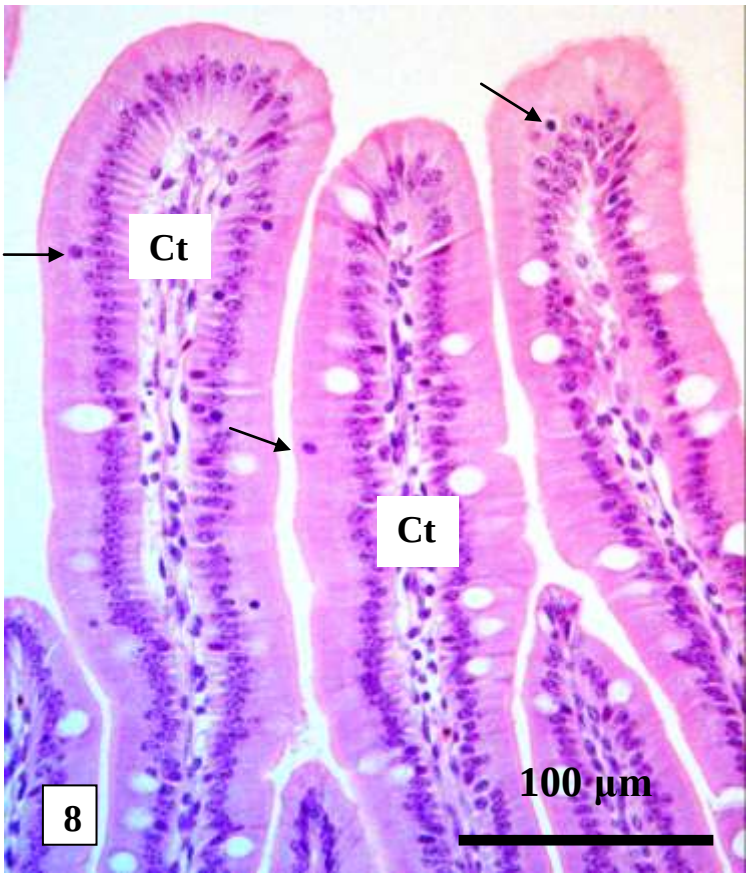


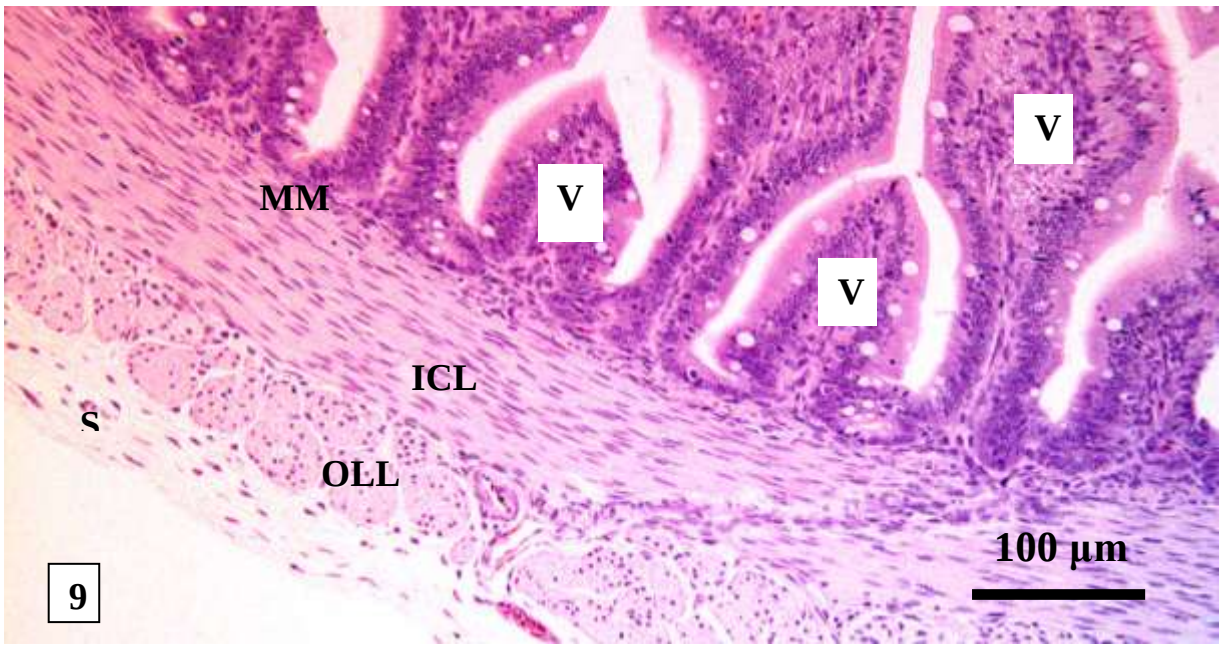




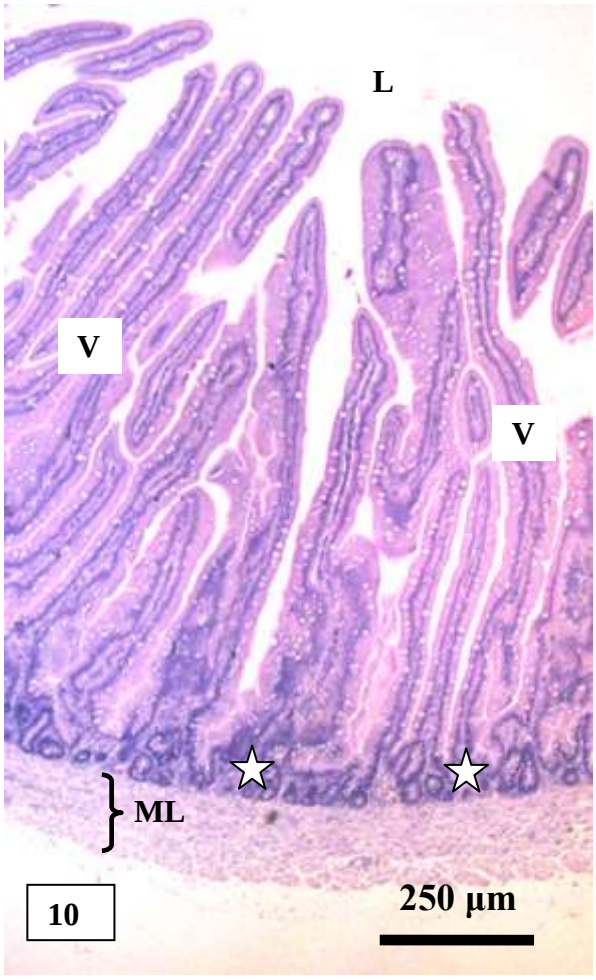
Jejunum Day 2

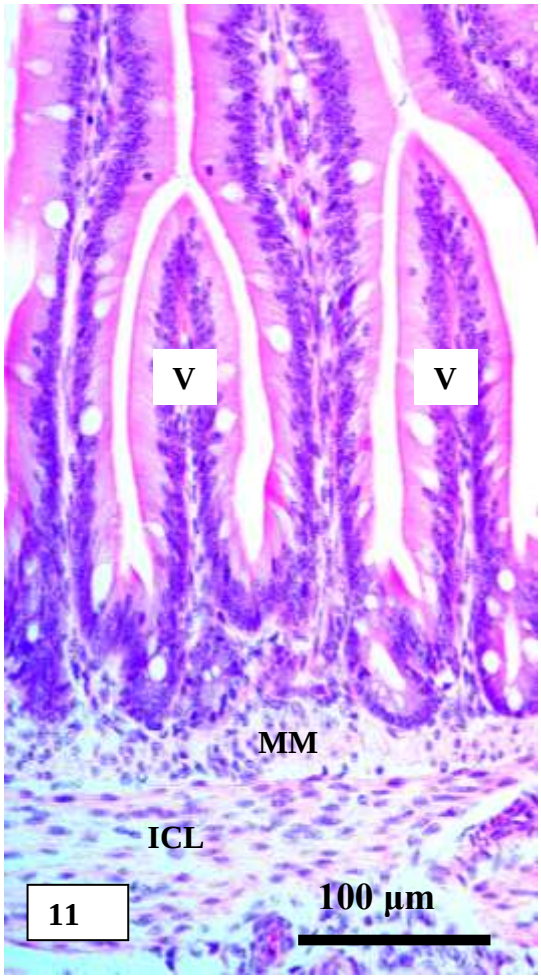


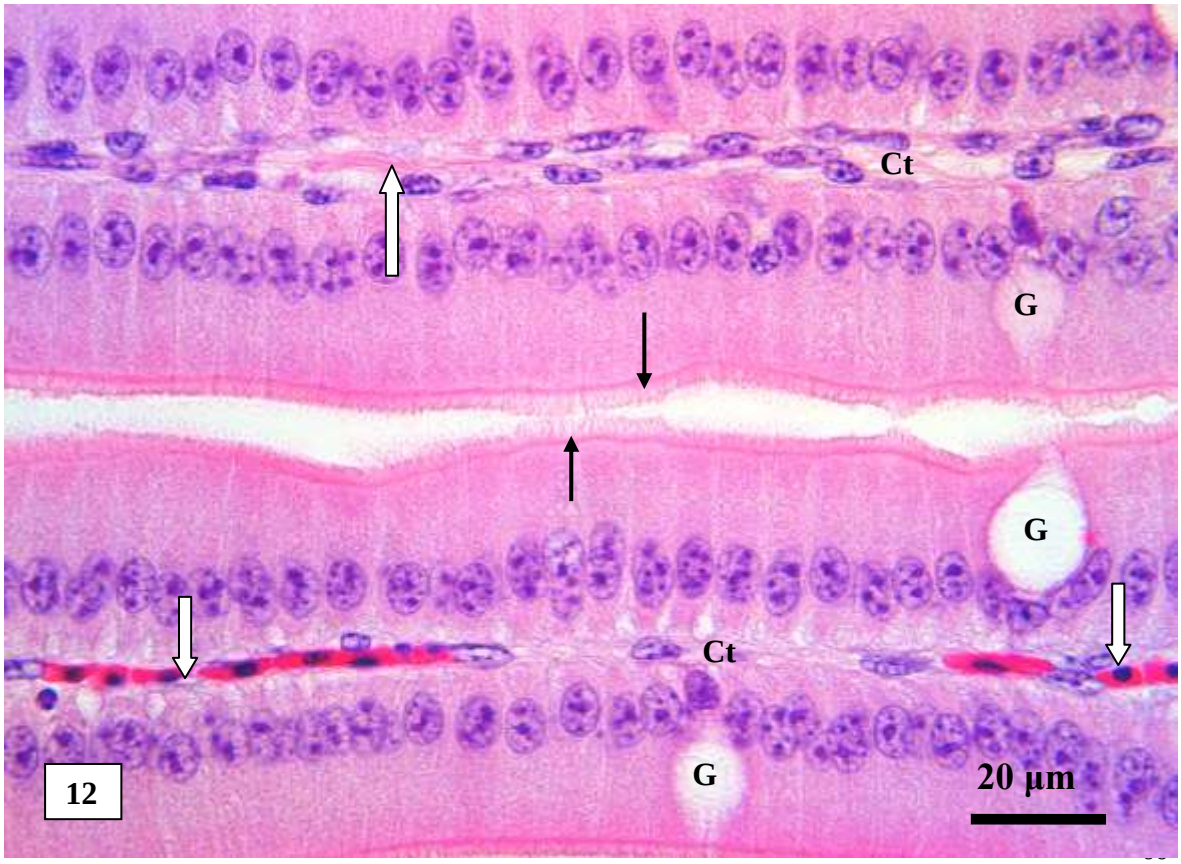




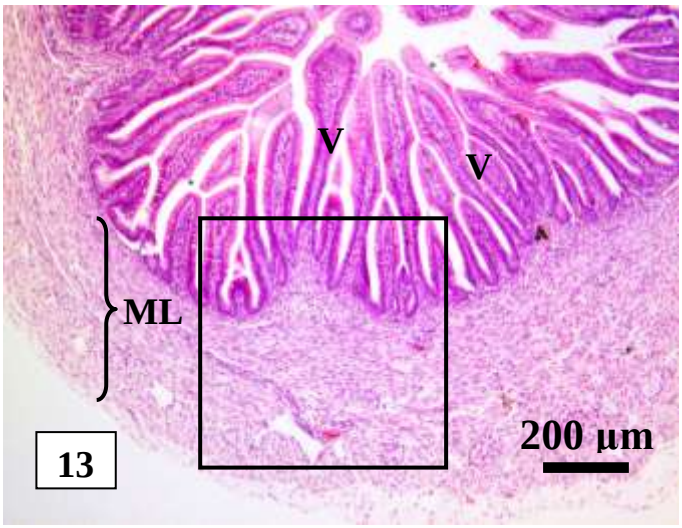
Jejunum Day 14

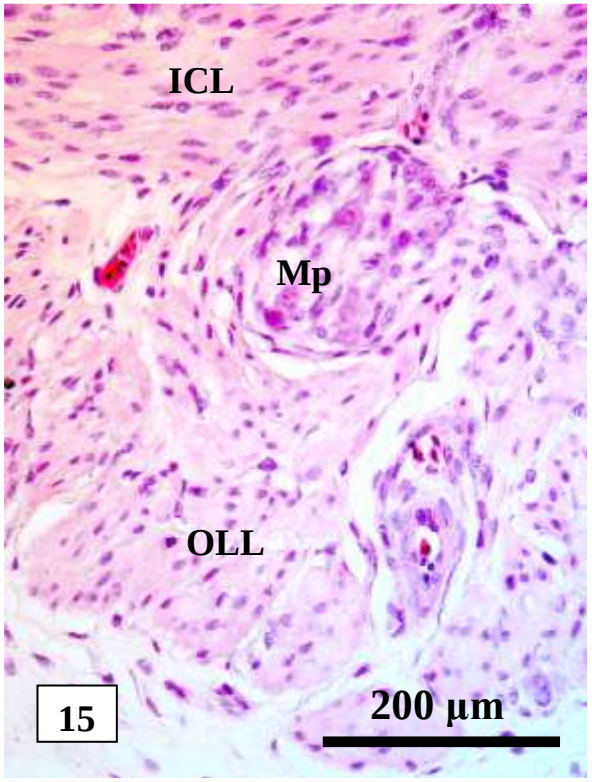


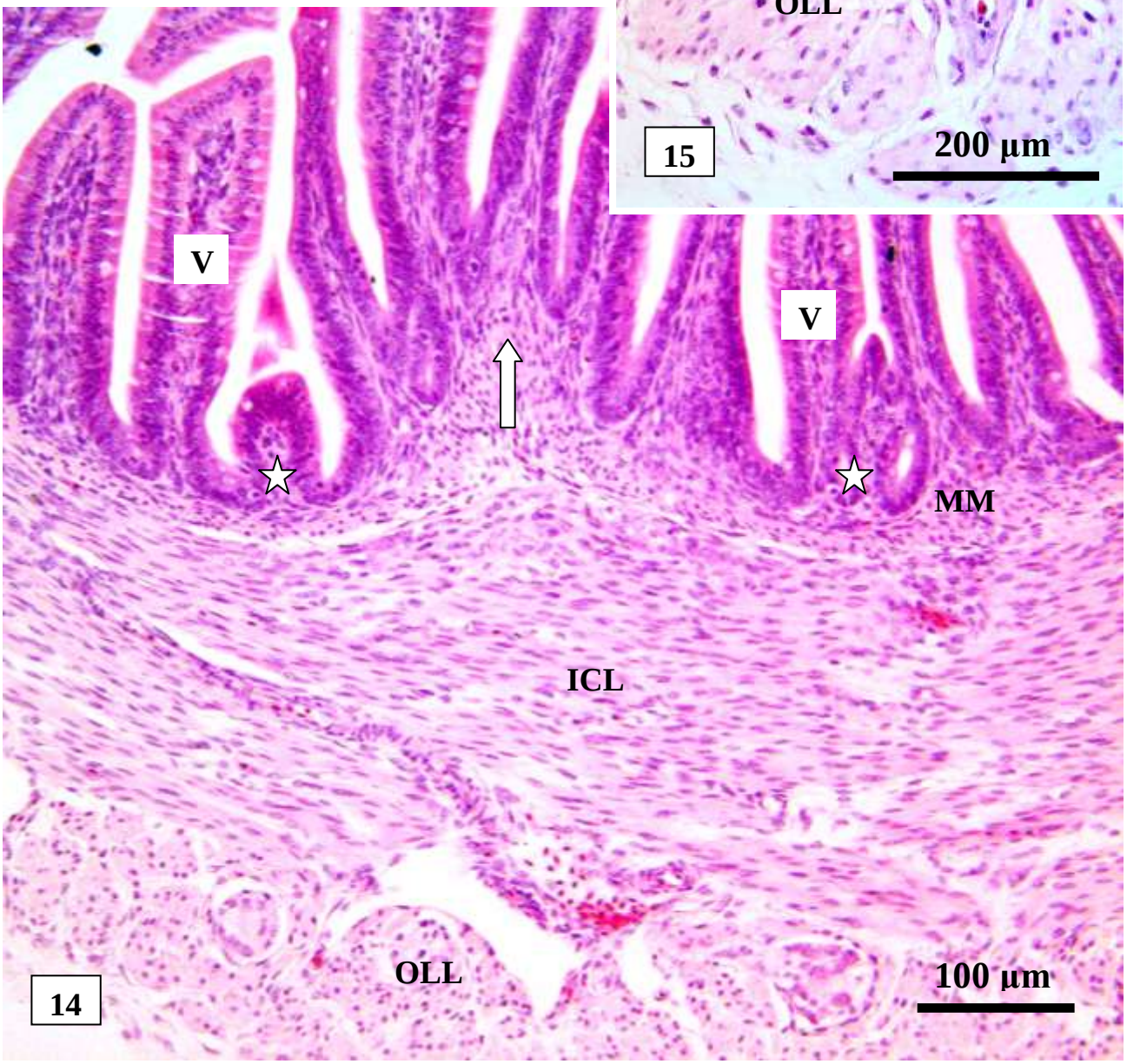




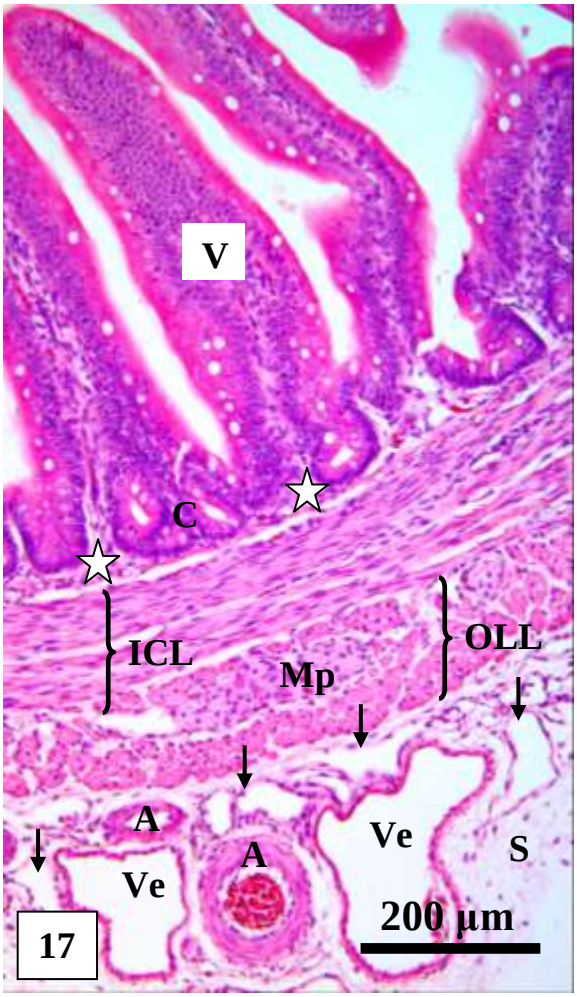
Ileum Day 2

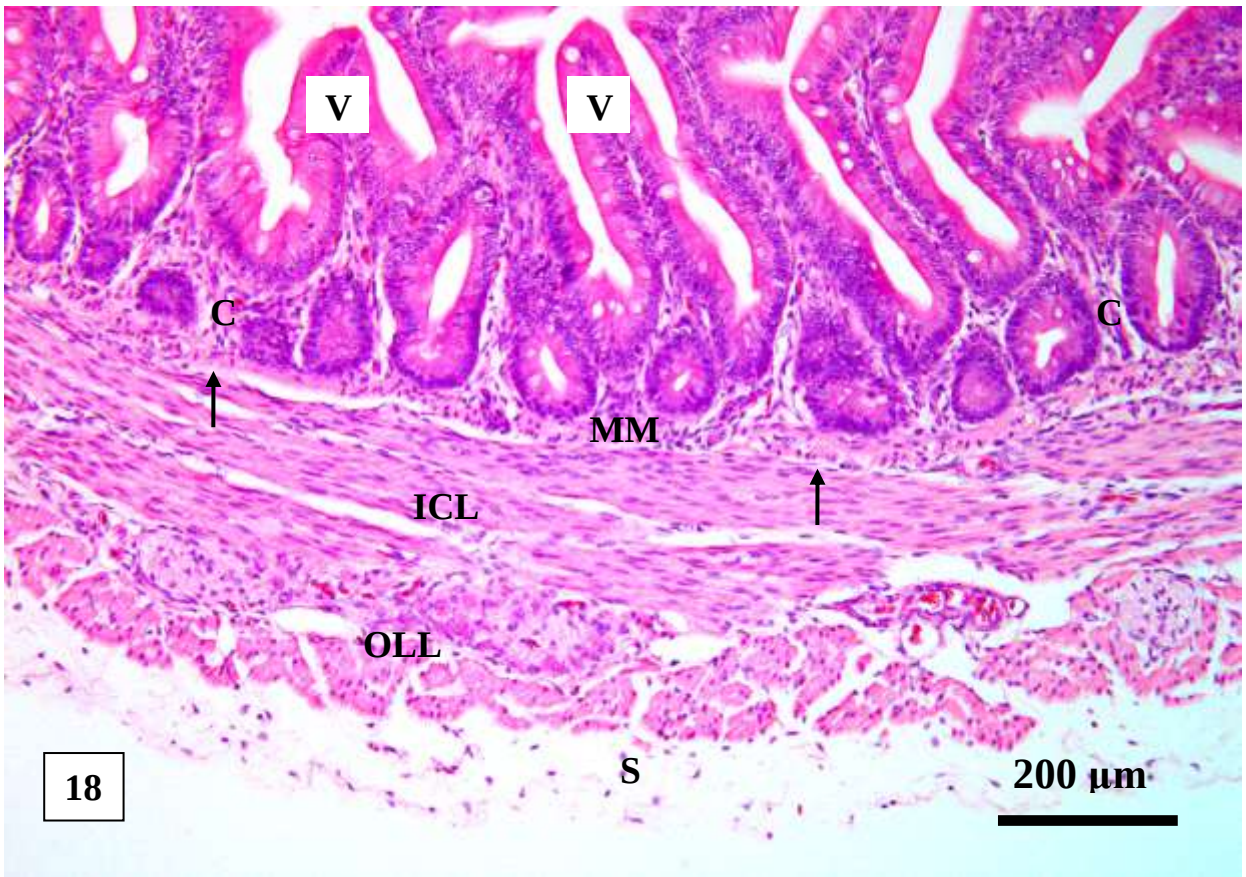






Ileum Day 14





Morphometric analysis

Duodenum

The dimensional changes that occurred in the various tissue layers and structures (villi) measured in the duodenum over the 16 day period are reflected in Figures 19 and 20. Standard errors are not indicated on Figure 20 as their inclusion makes the graph difficult to interpret. The duodenal villi showed a sharp increase in length (VL) over the trial period, with significant ($P<0.05$) increases being noted between days four and six and between days eight and 10. Villus length continued to increase until day 16, but was not statistically significant (Figure 19). The villi also demonstrated a progressive increase in diameter (VD) (based on the measurement of villus width) from day two to day 16, with significant increases ($P<0.05$) occurring between days four and six and again between days ten and 12 (Figure 20).

Crypt depth (CD) (essentially a glandular layer representing the part of the lamina propria adjacent to the *muscularis mucosae* housing the crypts of Lieberkühn) showed a significant ($P<0.05$) peak on day six, but dropped significantly ($P<0.05$) on day eight, only to increase more gradually, although significantly ($P<0.05$), from day eight to day 16 (Figure 20). The width of the *muscularis mucosae* (MM) remained relatively constant throughout the trail period (Figure 20). The inner circular layer (ICL) of the *tunica muscularis* demonstrated a gradual decrease in thickness, but only significantly ($P<0.05$) from day ten onwards (Figure 20). In contrast, the outer longitudinal layer (OLL) increased slightly in thickness from day two to day eight, but not significantly ($P<0.05$). However, a significant ($P<0.05$) decrease in the width of this layer was noted between day eight and day ten, thereafter remaining constant, with fluctuations, until the end of the trial period (Figure 20).

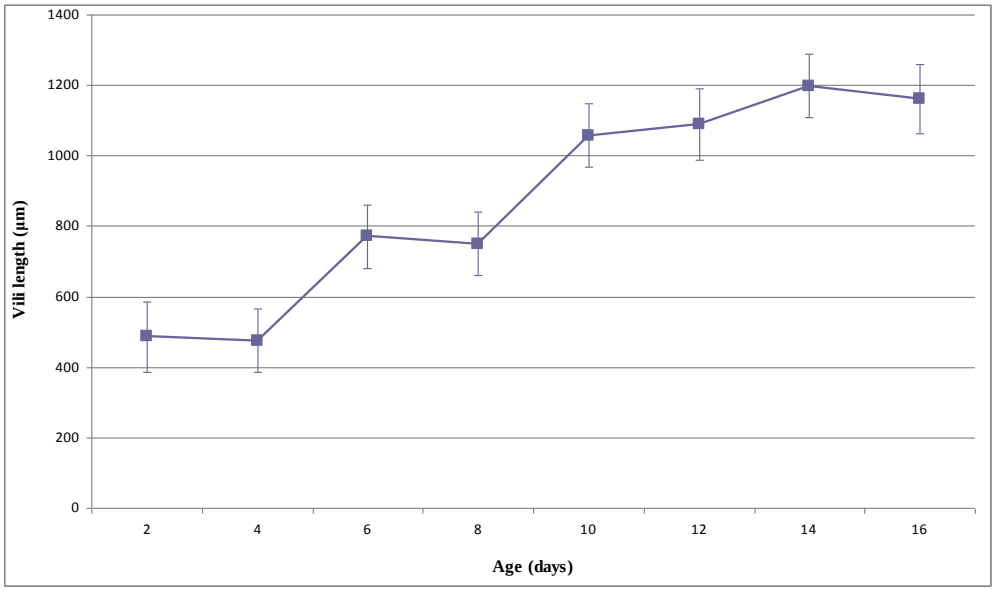


Figure 19 Trends of the villi length (± SE) measure in the duodenum from two to sixteen days

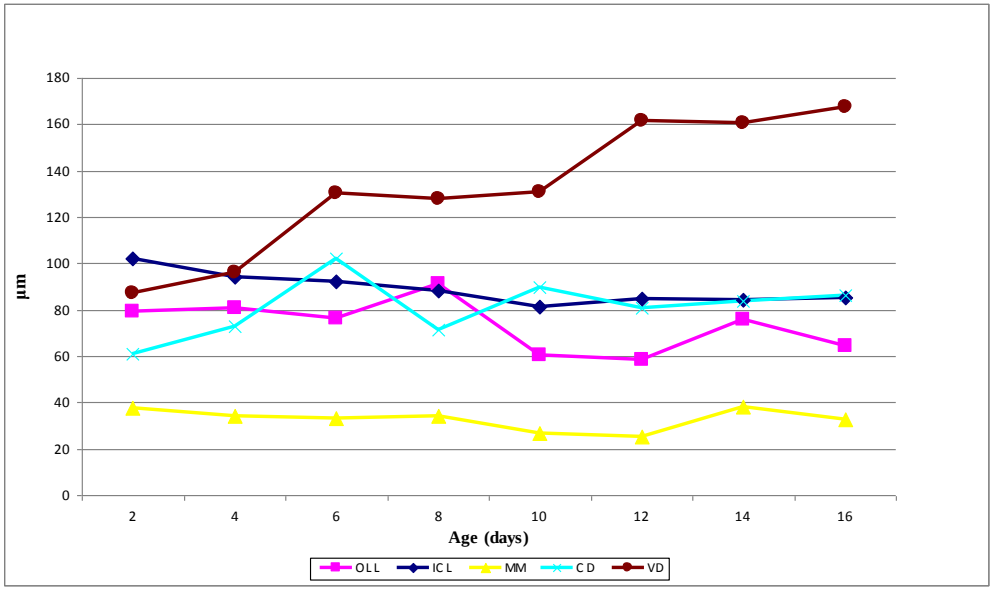


Figure 20 Trends of the various layers measure in the duodenum from two to sixteen days

Jejunum

The dimensional changes that occurred in the various tissue layers and in the villi of the jejunum measured over the 16 day period are reflected in Figures 21 and 22. Standard errors are not indicated on Figure 22 as their inclusion makes the graph difficult to interpret. The length of the jejunal villi increased significantly ($P<0.05$) from day two to 16, with the biggest increase in length occurring on days six and 10 (Figure 21). The villi also appeared to increase significantly ($P<0.05$) in diameter (VD) over the 16 day trail period (Figure 22). Crypt depth (CD) showed a slight, but significant ($P<0.05$), increase from day two to 16. Similar to changes observed in the duodenum, crypt depth in the jejunum also showed a significant ($P<0.05$) peak on day six, but dropped significantly ($P<0.05$) on day eight, only to remain constant for the remainder of the trial period (Figure 22).

The width of the *muscularis mucosae* (MM) remained relatively constant between days two and 16, with two significant ($P<0.05$) peaks manifesting on days four and 14 (Figure 22). The inner circular layer (ICL) of the *tunica muscularis* showed a significant ($P<0.05$) increase in thickness on day four, but declined significantly ($P<0.05$) on day eight, and then remained relatively constant until day 16 (Figure 22). The outer longitudinal layer (OLL) showed a significant ($P<0.05$) decline in thickness on day 10, but gradually recovered significantly ($P<0.05$) to reach its original width by days 14 to 16 (Figure 22).

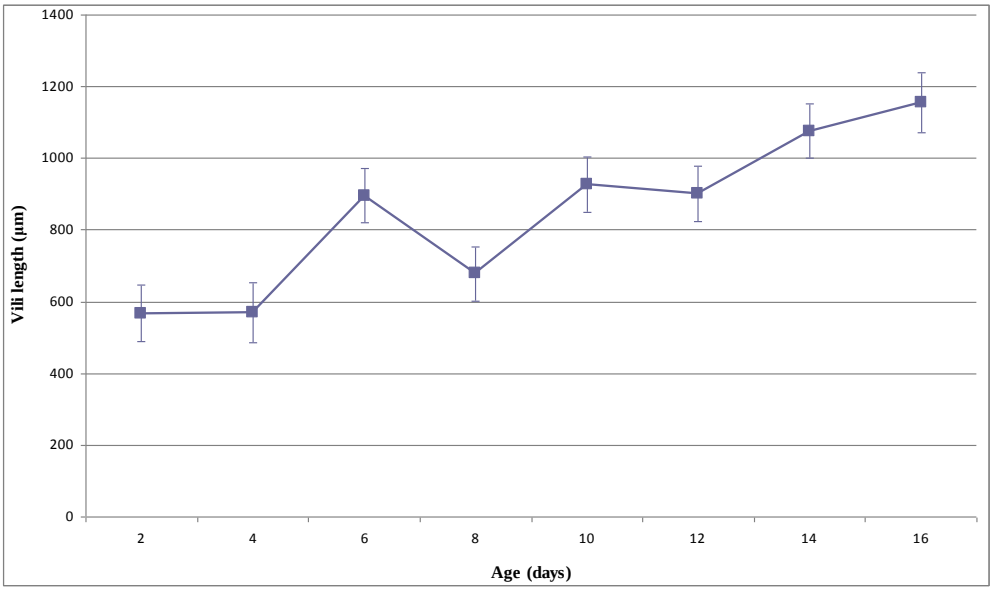


Figure 21 Trends of the villi length (± SE) measure in the jejunum from two to sixteen days

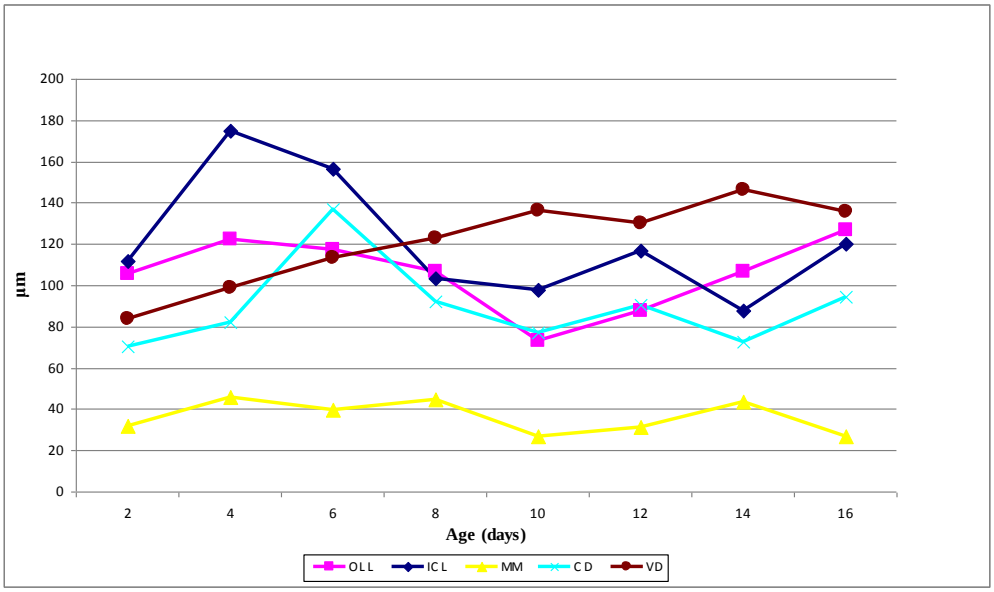


Figure 22 Trends of the various layers measure in the jejunum from two to sixteen days

Ileum

The dimensional changes that occurred in the various tissue layers and in the villi of the ileum measured over the 16 day period are reflected in Figures 23 and 24. Standard errors are not indicated on Figure 24 as their inclusion makes the graph difficult to interpret. Villus length (VL) in the ileum showed a gradual yet significant ($P<0.05$) increase over the trial period (Figure 23). A similar significant increase ($P<0.05$) was reflected in villus diameter (VD) over the 16 day period (Figure 24). Crypt depth (CD) remained constant from day two to day 16, except for a significant ($P<0.05$) peak on day six (Figure 24).

The width of the *muscularis mucosae* (MM) did not differ significantly ($P<0.05$) between days two and 16, but showed a significant ($P<0.05$) decrease in width between days eight and 14 (Figure 24). The

dimensions of the inner circular layer (ICL) of the *tunica muscularis* fluctuated markedly over the trial period, with significant (P<0.05) differences in thickness being noted between days six and eight and between days 12 and 14. There were, however, no differences (P<0.05) between the ICL thickness on days two and 16 (Figure 24). The outer longitudinal layer (OLL) showed a similar pattern of fluctuation to that of the ICL, with significant increases (P<0.05) in thickness being observed on days four and 14 (Figure 24).

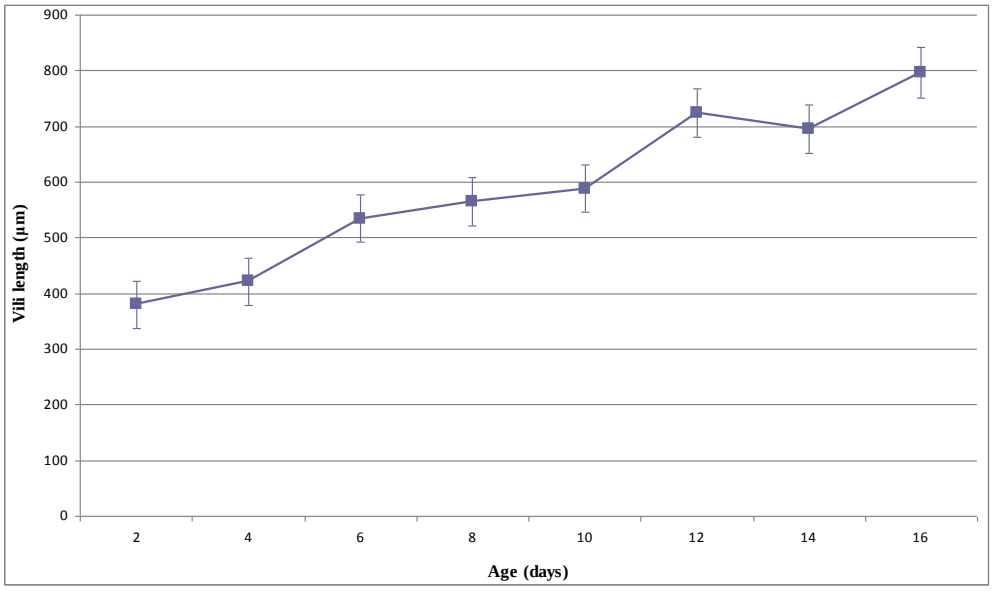


Figure 23 Trends of the villi length (± SE) measure in the ileum from two to 16 days

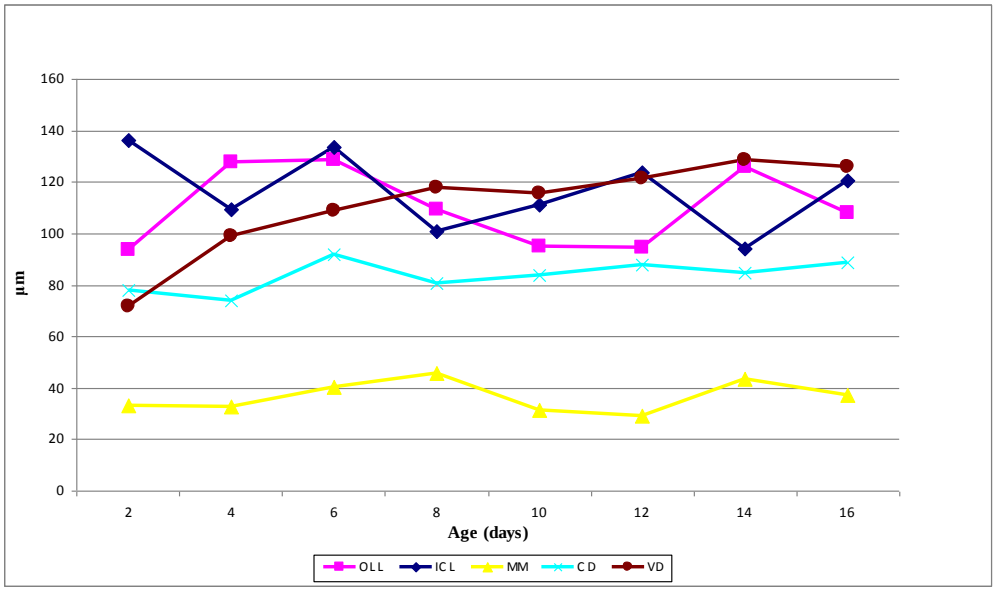


Figure 24 Trends of the various layers measure in the ileum from two to sixteen days

The V:C ratio of the duodenum, jejunum and ileum are reflected in Figure 8. Standard errors are not indicated on Figure 25 as their inclusion makes the graph difficult to interpret. There was a significant decline (P<0.05) in the V:C ratio of the duodenum from day two to day four post-hatch. This initial decline

was followed by an increase in the V:C ratio which differed significantly (P<0.05) between days six and eight, and again between days ten and 12 post-hatch. The V:C ration of the jejunum also displayed a significant (P<0.05) decline from day two to day four post-hatch, followed by a fluctuating yet significant (P<0.05) pattern of increases and decreases in the ratio between days ten and 16 post-hatch. The basic trend was that of an increasing ratio. The V:C ratio of the ileum showed a steady increase (P<0.05) from day two to day 16 post-hatch.

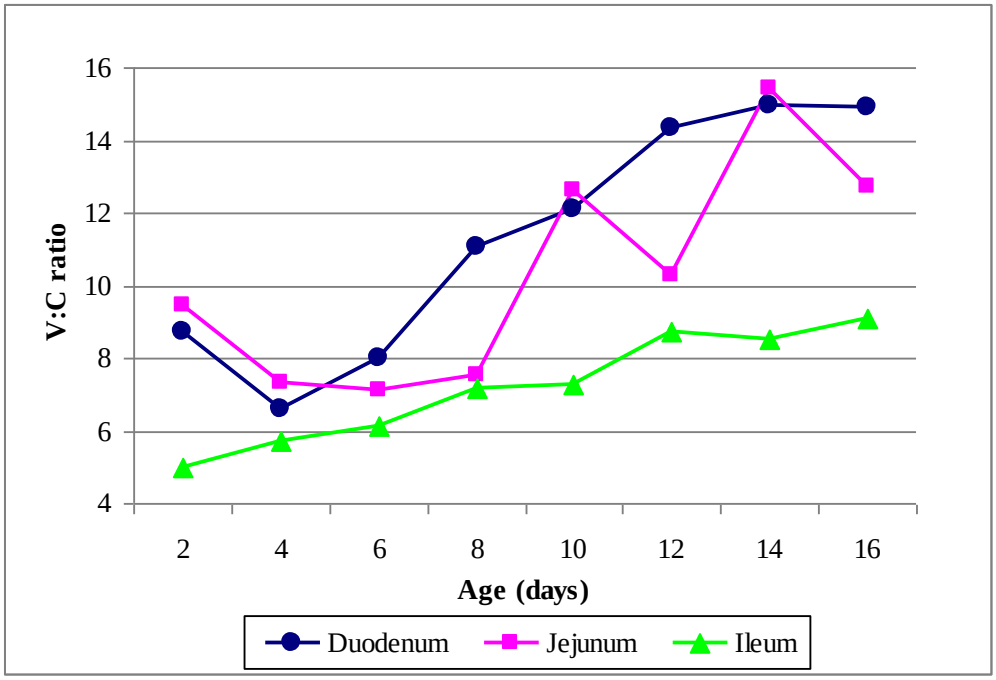


Figure 25 V:C ratio for the duodenum, jejunum and ileum

Discussion

Previous studies on the morphological development of the avian small intestine have shown that rapid changes occur during the first ten days to two weeks after hatch (Uni *et al.*, 1995, Uni *et al.*, 1998) and that these changes are already evident from day 14 of incubation in the chicken (Uni *et al.*, 1996). These changes involve the growth in height, volume and number of the intestinal villi (together with a concomitant increase in the number of enterocytes), as well as an increase in crypt formation (Sklan, 2001; Uni 2006) and goblet cell numbers (Uni *et al.*, 2000, 2003). Villus growth, both in size and number, provides a larger absorptive surface per unit of intestine and during the critical early growth phase of the chick ensures optimal uptake of essential nutrients (Uni, 2006; Wang & Peng, 2008). This increase in villus length and diameter during development of the small intestines has been reported for chickens (Iji *et al.*, 2001; Noy *et al.*, 2001) and turkey poults (Uni *et al.*, 1999). A similar sequence of rapid villus development in the small intestine has also been described in the ostrich (Wang & Peng, 2008), although this study did not investigate possible changes occurring during the first few weeks post-hatch.

The present study confirmed that villus length and diameter increases markedly in all segments of the small intestine of ostrich chicks during the first 16 days of development post-hatch, with the greatest degree

occurring in the duodenum and jejunum and the least in the ileum. It was noteworthy that a difference in villus length was observed in all parts of the small intestine between one day old (Wang & Peng, 2008) and two day old ostrich chicks (this study). In both the duodenum and jejunum the villus length was greater in two day old chicks (486.05 µm vs. 371 µm and 567.83 µm vs. 283 µm, respectively). In contrast, the length of the villi in the ileum was shorter in the two day old chicks (380 µm vs. 506 µm), but reached a length of 534.65 µm by day six. Villus diameter in the duodenum was similar in both studies, while that of the jejunum and ileum was greater in the two day old chicks than the dimensions reported by Wang & Peng (2008) (84.08 µm vs. 65.03 µm and 71.92 µm vs. 54.75 µm, respectively). While these differences may reflect actual villus growth between days one and two post-hatch, it should also be considered that genetic and other factors could be responsible for the variation in villus dimensions. For example, De Verdal *et al.* (2010) conducted a study to compare histological and morphological adaptations in two broiler lines (D- and D+) which were selected according to their ability to digest a wheat-based diet. The authors reported that the D- line birds displayed a greater villus height and area compared to the D+ birds, resulting in an increased absorption surface area. Yet the D- line birds had a slower growth rate than the D+ birds. This was ascribed to the possibility that nutrient availability may have been lower in the D-line, which would compromise nutrient absorption.

The present study also confirmed the existence of two types of villi during the early stages of small intestine development in birds. During the 16 day trial period long finger-like villi alternated with short dagger shaped villi in all segments of the small intestine of the ostrich. Those in the ileum, however, were not as conspicuous or ordered as those in the other two segments. This phenomenon of alternating villi is already apparent at 17 days embryonic age in broiler breeds, although the villi are described as pear-shaped (the longer type) and rocket-shaped (the shorter type) (Uni, 2006). Two types of villi have also been described in one day old broiler chicks, namely broad finger-like and narrow plate-like villi (Bayer *et al.*, 1975). In the ostrich the alternating long and short villi are generally no longer obvious in older birds (see Chapter 7).

The histological structure of the villi in ostrich chicks reflected the typical characteristics previously described for the avian small intestine (Calhoun, 1954; Hodges, 1974; McLelland, 1979) including that of ratites (Feder, 1972; Herd, 1985; Bezuidenhout, 1990; Illanes *et al.*, 2006). The rounded form of intestinal enterocytes described in poultry at hatch (Sklan, 2001) were not observed in the two day old ostrich chicks which displayed a simple high columnar epithelium with a distinct brush border. Although a degree of branching was present in the duodenum at day 16, the profuse branching described by Bezuidenhout & Van Aswegen (1990) in the small intestine of adult ostriches was not observed. As previously described in birds (Calhoun, 1954; Hodges, 1974; McLelland, 1979), the lamina propria formed the connective tissue core of the villi and supported a rich capillary network. There was no indication in the present study of lymphatic vessels within the villus core. A similar observation was made in adult ostriches (Bezuidenhout & Van Aswegen, 1990). Central lymphatic lacteals are reported to be absent in birds (Graney 1967, Hodges 1974), although lymph vessels (Hodges, 1974) and lacteals (Calhoun, 1954) are described within the connective

tissue core of the fowl. The existence of prominent lymphatic vessels in the subserosa, submucosa and between the layers of the *tunica muscularis* in all segments of the ostrich chick small intestine would suggest, however, that an effective lymphatic drainage system is indeed present in the mucosa. Lymphoid aggregations as described for adult ostriches (Bezuidenhout & Van Aswegen, 1990) and in two month old birds (see Chapter 7), were not observed in the ostrich chicks studied and only occasional lymphocytes were identified within the epithelium and lamina propria.

The development of the crypts of Lieberkühn (the glandular layer of the small intestine) is known to parallel the development of the villi in poultry, and become well-defined by days 2 to 3 post-hatch (Iji *et al*., 2001; Sklan, 2001). A similar trend was observed in ostrich chicks which displayed rudimentary crypt formation at day two, but which by day four displayed a well-established glandular layer. However, the glandular layer (crypt depth) showed a relatively small increase in depth from day 4 to day 16. Crypt depth has been shown to increase incrementally in the duodenum and jejunum in the ostrich over a longer period (1 to 334 days), although the ileum showed a fluctuating pattern (Wang & Peng, 2008). As was the case with villus length, crypt depth was far greater in two day old chicks examined in this study than that reported by Wang & Peng (2008) for day old ostrich chicks: (duodenum 61.22 µm vs. 13.05 µm, jejunum 70.18 µm vs. 12.67 µm and ileum 77.99 µm vs. 26.83 µm, respectively) and similar factors may be involved in explaining this discrepancy. The crypts represent the region in the small intestine where stem cells divide to both replace and supplement the lining cells of the villi (McLelland, 1979; Wang & Peng, 2008). This was clearly illustrated in the present study by the large numbers of mitotic figures observed in the crypts in all segments of the small intestine and during all stages of the trial period. The presence of a well-developed glandular layer is therefore an indication of a high demand for villus tissue synthesis and fast tissue turnover (Xia *et al.*, 2004). The width of the glandular layer may also be an important factor in the ability of the crypts to maintain villus structure, as well as sustain growth in villus length and diameter (Poole *et al.*, 2003). According to De Verdal *et al.* (2010) this explains why larger villi have larger crypts, an observation supported by the present study where peaks in villi length throughout the small intestine coincided with maximum development of the glandular layer. Of interest in the present study is the indication that multiple crypts open between the villi during the early post-hatch period. In poultry hatchlings a single crypt is present per villus in hatchlings and by the end of the second week post-hatch three to four crypts are observed per villus (Uni, 2006), a situation which is also present in ostrich chicks.

The important morphological events leading to enlargement of the absorptive surface of the small intestine during the critical post-hatch period in the chick hinge around the establishment of the crypt-villus axis (Uni, 2006). This morphological phenomenon can be quantified by dividing villus length by crypt depth (the width of the glandular layer) to determine the Villus (V) : Crypt (C) ratio. According to Wu *et al.* (2004) an increase in this ratio is associated with improved nutrient absorption, a decrease in secretion throughout the gastro-intestinal tract, improved disease resistance and faster growth. In the present study an increase in the V:C ratio was indicated for each of the segments of the small intestine during days 1 to 16, although the jejunum displayed a fluctuating pattern. In contrast, Wang & Peng (2008) observed a decrease

in the V:C ratio from day one to day 45 in the duodenum, jejunum and ileum, although an increase was again evident from day 45 to 90 in the duodenum and ileum. Based on this evidence, Wang & Peng (2008) suggest that "the intestine gradually develops from day 1 to 90 and is in a primitive state before day 45". The present study would suggest that dynamic growth, particularly in villus length, takes place in the ostrich small intestine during the first 16 days of development post-hatch. This is partly supported by the study of Wang *et al.* (2008) in broiler chicks who noted that the V:C ratio is higher in the duodenum at the age of 22 days than at 42 days, although age did not affect the ratio in the jejunum or ileum. The discrepancy between the results of this study and that of Wang & Peng (2008) may therefore simply reflect the fact that the crucial developmental changes taking place during the first few weeks post-hatch were not measured in the latter study. A further important observation from the present study was that the V:C ratio of the duodenum and jejunum was very similar and that both regions had a higher V:C ratio than the ileum. This would suggest that both the duodenum and jejunum play an important role in the early uptake of nutrients in ostrich chicks. The day one results of Wang & Peng (2008) would appear to indicate a similar trend.

An increase in V:C ratio is associated with higher epithelial cell turnover and better nutrient absorption, resulting in faster growth (Fan *et al.*, 1997; Wu *et al.*, 2004; Wang *et al.*, 2008), therefore the conclusion can be drawn that an increase in V:C ratio would benefit the growth of the ostrich chick, especially in the crucial post-hatch period. In the study by De Verdal *et al.* (2010) referred to above there was no significant ($P<0.05$) differences between the V:C ratio of D- and D+ lines, as both villus height and crypt depth were equally greater in the D- line than in the D+ line. The authors suggested that the D- birds have to maintain a greater absorptive surface compared to the D+ birds, which may lead to a higher nutrient requirement for maintenance and therefore a lower digestive efficiency in these birds (De Verdal *et al.*, 2010).

The muscle layer controls the motility of the intestines, as well as the progression of food down the intestines. A thick muscular layer would support sufficient contact of the mucosa and the intestinal content, resulting in higher absorption rate (De Verdal *et al.*, 2010). As typically described for birds (Calhoun, 1954; Hodges, 1974; McLelland, 1979), the muscle layer in the day 2 to day 16 ostrich chicks consisted of a relatively thin *muscularis mucosae* and a thicker *tunica muscularis*, the two being separated by a rudimentary submucosa. The muscle fibres in the *muscularis mucosae* were oriented longitudinally with no obvious sign of the circular and longitudinal fibres described by Bezuidenhout & Van Aswegen (1990) in older ostriches. The *tunica muscularis* was divided into inner circular and outer longitudinal which were similar in width. In birds the outer longitudinal layer is reported to be generally thinner than the inner layer (Hodges, 1974; McLelland, 1979), a situation also apparent in adult ostriches (Bezuidenhout & Van Aswegen, 1990). The observation that the muscular tunic in the ostrich is best developed in the ileum (Bezuidenhout & Van Aswegen, 1990) was confirmed in the present study. In the ostrich, the muscle layer was reported to increase in thickness from day one to day 90 for each segment of the small intestine (Wang & Peng, 2008). In the present study the thickness of this layer remained relatively constant over the 16 day period. The muscular layer was also much thicker in two day old chicks in this study than that reported by

Wang & Peng (2008) for day old ostrich chicks: duodenum (181.47 µm vs. 0.28 µm), jejunum (217.24 µm vs. 0.30 µm) and ileum (230.17 µm vs. 0.18 µm). This wide difference in dimensions remains unexplained.

The gradual segmentation of both the inner and outer layers of the *tunica muscularis* from the duodenum to the ileum observed in the present study represents a significant finding, the importance of which is not clear.

Conclusion

The results in this study indicate that the histological and morphological growth of the small intestine of the ostrich chick favours increased absorption with an increase in age. Although there are similarities between the histology and morphology of the small intestine of ostrich chicks and poultry, the same amount of research regarding the co-evolution of growth performance and intestinal morphology still needs to be done on ostrich chicks as is done on chickens and poults.

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Part 2

Influence of various pre-starter diets on growth and the development of the small intestine of ostrich chicks

Chapter 5

A growth and digestibility study of ostrich (*Struthio camelus* L.) chicks on eight different pre-starter diets

Introduction

Information about the nutritional requirements of ostriches, especially compared to that available for other domestic animals, is scarce (Ullrey & Allen, 1996). Research regarding ostrich nutrition has taken giant leaps since 1995 (Cilliers *et al.*, 1998; Brand *et al.*, 2002; Cooper, 2004), when ostrich diets were formulated based on both the energy value of the ingredients and the nutritional requirements of other poultry, such as chickens and turkeys and ingredients based on nutritive values obtained with poultry were used (Angel, 1996; Brand *et al.*, 2002). This led to ostrich nutrition being based on assumptions from other species (Ullrey & Allen, 1996). These diets usually overestimated ostrich nutritional requirements and underestimated the ostrich's ability to effectively digest certain feedstuffs (Gandini *et al.*, 1986; Cilliers *et al.*, 1998; Brand *et al.*, 2000a). This has been costly for the ostrich industry as 70-80% of total intensive production costs can be attributed to feeding costs (Brand *et al.*, 2002).

Cilliers *et al.* (1994) and Cilliers (1995) were the first authors to report values for true metabolisable energy in ostriches, and five years later Brand *et al.* (2000b) compared the efficiency of digestion of ostrich diets with that of pigs and poultry. These authors concluded that ostriches demonstrated a better ability to utilise low quality raw material than the other two monogastric species.

The ostrich digestive tract undergoes dramatic changes in the first two to three months post-hatching (Iji *et al.*, 2003; Van der Walt *et al.*, 2003; Wang & Peng, 2008), but as the digestive tract of the ostrich is not yet fully developed at three months of age (Angel, 1993), Gandini *et al.* (1986) suggested that high-quality diets should be fed to young ostriches up to three months of age. No differences in production on diets varying between 16% and 20% protein were found, but birds on these diets performed better than birds on a diet containing 14% protein (Gandini *et al.*, 1986).

Unfortunately, between 30% and 50% of all mortalities also occur during the first three months of age (Smith *et al.*, 1995a; Verwoerd *et al.*, 1998). Cloete *et al.* (2001) reported that the high mortality rate of chicks up to 90 days post-hatching could be due to an inability to adapt to the extensive rearing environments, hence resulting in stress. Low weights at 28 days post-hatching were also a common factor in chick mortalities before 90 days of age (Cloete *et al.*, 2001).

Even though various studies have been conducted to determine nutritional requirements for growing ostriches (Smith *et al.*, 1995b; Cilliers *et al.*, 1998; Brand *et al.*, 2000a, 2000b, Brand *et al.*, 2003), little scientific literature or specifications are available for energy and protein levels in pre-starter diets for ostrich chicks. The predicted energy level suggested is 14.65 MJ/kg ME (Du Preez, 1991, Smith *et al.*, 1995), while a crude protein level of 22.89% is indicated by Smith *et al.* (1995). It therefore remains a constant challenge

for nutritionists to fully comprehend the nutritional physiology and nutritional requirements of ostriches, especially 90 days post-hatching.

In this study eight pre-starter diets were fed to ostrich chicks. The performance, growth and physiological development of the chicks on high and low variations of different nutritional components were examined. The project had ethical approval from the Onderstepoort Animal Use and Care Committee (Protocol 36-5-623).

Material and Methods

Animals

A trial was set up with 105 South African Black Ostrich (*Struthio camelus* L.*)* chicks (58 males and 47 females). The ostrich chicks were obtained from the Oudtshoorn Experimental Farm of the Department of Agriculture Western Cape, South Africa. After being weighed, they were transported as day-old chicks to the Kromme Rhee Experimental Farm of the Department of Agriculture Western Cape, near Stellenbosch, South Africa. The transportation over the 500 km distance was carried out in plastic containers with sufficient airflow in a closed vehicle. The average weight of the day-old chicks was 0.837 kg. Chicks were randomly divided into eight trial groups: 13 chicks per group, except for the high protein group, which had 14 chicks. Of the 105 chicks that started in the trial, 64 chicks survived till the end of the trial. Post-mortems were not performed on any of the dead chicks, but conclusions were drawn on apparent causes of mortalities based on observations. Ten chicks were found dead the day after an extraordinary hot day; eight chicks were found dead in the water troughs; six chicks were in poor condition from the onset of hatching and died three days into the trial; four chicks were found dead in the feeding buckets; four chicks lost weight and died; six chicks were found dead in the morning with no indication to any possible causes; one chick broke its leg and was put down; and two chicks, fed the high protein diet, died in the two days between the end of the growth trial and the commencement of slaughtering, also with no indication to the possible cause of death.

Treatments

The eight groups of ostrich chicks were fed diets with high and low levels of protein, fat, sugar and starch. The pre-starter diets were formulated using the Mixit 2+ feed formulation program (Agricultural Software Consultants, P.O. Box 32, Kingsville, Texas 78363, USA). The raw material used and the calculated nutrient composition of the eight trial diets are presented in Tables 1 and 2.

Table 1 Raw material composition of eight pre-starter trial diets fed to ostrich chicks

Ingredients	High protein %	Low protein %	High fat %	Low fat %	High starch %	Low starch %	High sugar %	Low sugar %
Maize meal	25.55	63.86	50.17	58.04	29.68	23.78	24.72	53.05
Soybean oilcake meal	43.26	8.25	20.00	27.20	22.48	11.66	21.26	20.00
Maize starch	—	—	—	—	20.00	—	—	—
Full-fat soya	16.78	12.00	12.10	—	12.00	12.00	12.00	12.00
Lucerne hay	10.00	10.00	10.00	10.00	10.00	37.74	17.26	10.00
Sugar	—	—	—	—	—	—	20.00	—
Wheat bran	—	—	—	—	—	10.00	—	—
Limestone	2.27	2.96	2.99	3.03	2.89	1.18	1.78	2.25
Plant oil	1.00	1.00	3.00	—	1.00	2.00	1.00	1.00
Mono calcium phosphate	0.49	1.28	1.07	1.06	1.26	0.94	1.28	1.04
Fine salt	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Mineral and vitamin mixture	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Synthetic methionine	—	—	0.02	0.02	0.04	0.05	0.05	0.01

Table 2 Calculated nutrient composition of eight pre-starter trial diets fed to ostrich chicks

Nutritional composition	High protein %	Low protein %	High fat %	Low fat %	High starch %	Low starch %	High sugar %	Low sugar %
Crude protein	29.83	15.21	19.51	19.02	18.89	19.27	19.05	19.71
Dry matter	89.46	88.20	88.86	88.30	71.41	89.36	91.31	88.51
ME (MJ/kg feed)	14.5	14.5	14.8	13.5	14.5	13.0	14.5	14.5
Crude fibre	6.35	5.18	5.47	5.34	5.14	12.96	6.93	5.53
Crude fat	6.01	6.06	7.71	2.91	4.97	6.81	4.99	5.83
Starch	19.50	41.43	33.48	38.23	38.22	19.00	17.78	35.28
Sugar	6.54	2.99	4.03	3.58	4.61	5.30	24.43	4.04
Lysine	1.78	0.75	1.06	1.00	1.08	1.00	1.08	1.06
Methionine	0.43	0.25	0.32	0.32	0.32	0.34	0.33	0.32
Cysteine	0.46	0.29	0.34	0.34	0.30	0.29	0.30	0.34
Methionine + Cystine	0.89	0.54	0.66	0.66	0.62	0.63	0.62	0.66
Threonine	1.18	0.59	0.76	0.74	0.74	0.76	0.75	0.77
Tryptophan	0.39	0.17	0.24	0.23	0.24	0.26	0.25	0.24
Arginine	2.12	0.94	1.29	1.24	1.29	1.18	1.28	1.30
Isoleucine	1.43	0.66	0.89	0.85	0.88	0.85	0.89	0.89
Leucine	2.42	1.47	1.73	1.73	1.59	1.53	1.57	1.76
Histidine	0.80	0.42	0.53	0.52	0.50	0.49	0.50	0.54
Phenylalanine	1.43	0.72	0.93	0.90	0.90	0.93	0.92	0.94
Tyrosine	1.20	0.57	0.76	0.76	0.74	0.73	0.75	0.76
Phenylalanine + Tyrosine	2.62	1.29	1.68	1.66	1.64	1.65	1.66	1.70
Valine	1.53	0.78	1.00	0.99	0.97	0.98	0.98	1.01
Glycine + Serine	2.79	1.39	1.80	1.75	1.75	1.77	1.77	1.82
Calcium	1.20	1.49	1.49	1.49	1.49	1.19	1.18	1.22
Phosphorus	0.60	0.65	0.63	0.63	0.64	0.66	0.64	0.63

Treatment groups were kept in separate 4 m^2 pens. For the first eight days of the trial the floor was covered with green shade cloth and the surface area within the pens was divided into three smaller areas, i.e. reduced to 1.77 m^2 each, with round hardboard dividers. This was done in order to prevent crushing in corners and to ensure equal opportunity for access to the feed and water. After the initial eight days the hardboard dividers were removed and the shade cloth replaced with rubber mats for the rest of the trial. Water and feed were supplied *ad libitum* for the duration of the trial. Chromium oxide (0.5%) was added to the feed as a marker during the last week of the trial, in order to calculate the digestibility of the feed (Schürch *et al.*, 1950; McGuire *et al.*, 1966; Angel, 1993).

Measurements and Calculations

The growth and feed intake of the chicks was measured over 58 days. Chicks were weighed every second day until slaughter. Pens were checked for mortalities twice daily and mortalities were weighed back for consideration in calculation of feed conversion ratios. Possible cause of death was noted. Feed intake was calculated by weighing all the feed given to the chicks, as well as weighing the remaining uneaten feed every second day. Wet or contaminated feed was replaced after weighing. During the part of the trial when the digestibility was estimated, samples of feed containing chromium oxide marker were taken from every batch of feed mixed. At slaughter, faeces was collected from the distal part of the colon and frozen immediately at -20ºC. The following week the faecal samples were defrosted overnight in a refrigerator at 4ºC and dried at 50ºC in a Labotec oven for 72 hours, where after it was sent to the laboratory for proximate analyses.

Slaughtering commenced on day 60 of the trial and was done over a five-day period. Two chicks from each treatment group were slaughtered each day, starting with a different treatment group on the different days and also selecting the chicks randomly. The hatching- and slaughter weights of the chicks in the different treatment groups are presented in Table 3.

Table 3 Hatching- and slaughter weights for ostrich chicks fed eight different pre-starter diets

Treatment groups	Number of birds at slaughter	Average hatching weight (g)	Average slaughter weight (g)
High protein	2	873.3^{a}	5612.5^{bc}
Low protein	8	825.6^{a}	5395.6^{c}
High fat	9	794.3^{a}	8773.0^{a}
Low fat	10	853.1^{a}	7993.7^{ab}
High starch	7	814.7^{a}	9266.7^{a}
Low starch	10	806.8^{a}	8504.3^{a}
High sugar	8	862.3^{a}	7602.3^{abc}
Low sugar	8	821.6^{a}	9137.9^{a}

$^{a, b, c}$ Column means with different superscripts differ significantly at $P<0.05$

Chromium oxide concentrations within faecal and feed samples were determined by using perchloric and nitric acid for chromic oxide digestion (AOAC, 2000). The absorbance of the chromium oxide was obtained according to the directions of the standard methods for the Varian AA Spectrophotometer (Varian Australia Pty Ltd, Mulgrave, Victoria, Australia). The apparent digestibility (AD) values were then calculated according to Equation 1 reported by Fastinger & Mahan (2003), using the chromium oxide concentrations in the faecal and feed samples.

Equation 1 Calculation of apparent digestibility

$AD = [(N_D/N_F) \times (Cr_F/Cr_D) \times 100]$.

N_D = nutrient concentration in the faecal sample

N_F = nutrient concentration in the feed sample

Cr_F = chromium oxide concentration in the feed sample

Cr_D = chromium oxide concentration sample in the faecal sample

Feed conversion efficiency (FCE) was calculated by dividing the total feed intake by the total weight gain of the chicks within each treatment.

Average daily gain (ADG) within each treatment was calculated by subtracting the average starting weight from the average end weight and dividing it by the total amount of days of the trial period.

Faecal and feed samples were analysed for ash, dry matter (DM), crude protein (CP) and fat content, measured as ether extract (EE), by standard methods (AOAC, 1995). Average proximate analysis values are presented in Table 4.

Table 4 Proximate analysis values for feed and faecal samples of ostrich chicks fed eight different pre-starter diets

Nutritional composition	High protein %	Low protein %	High fat %	Low fat %	High starch %	Low starch %	High sugar %	Low sugar %
Feed								
Moisture	7.32	8.34	7.83	8.16	7.75	6.78	13.26	7.64
Crude protein	27.41	13.06	20.02	19.39	22.22	18.79	23.99	17.39
Crude fibre	6.51	4.68	5.32	5.16	5.33	14.13	8.53	5.52
Crude fat	5.15	4.59	5.58	2.62	4.18	5.10	5.33	5.28
Ash	9.80	7.88	10.58	9.98	9.51	11.13	13.14	9.07
Gross energy (MJ/kg)	18.27	17.68	17.84	17.09	17.76	17.70	17.61	17.76
Faeces								
Moisture	99.97	99.97	99.98	99.97	99.98	99.98	99.97	99.97
Crude protein	14.33	11.01	13.12	13.23	15.11	10.96	14.47	11.72
Crude fibre	-	19.53	17.81	19.70	22.79	36.22	28.29	17.76
Crude fat	1.86	1.48	1.72	1.75	1.86	1.35	1.88	1.44
Ash	43.47	38.43	40.06	35.85	39.73	23.07	35.14	45.14
Gross energy (MJ/kg)	13.07	12.57	12.36	13.22	14.20	15.55	14.11	11.74

All values are expressed as a percentage of total DM

Statistics

The experimental design was completely random with eight diets fed to groups of 13 randomly selected ostrich chicks. Chicks were weighed every second day from hatching up to 60 days, after which they were slaughtered.

Survival was compared for chicks on the low and high levels of each nutritional component. Owing to very low frequencies, Fisher's Exact Test was used for this purpose (Siegel, 1956).

Weight change displayed an exponential increase (Height=A*e^{B*Week}) over time. In exponential growth, the rate of change increases over time, in other words, the rate of the growth becomes faster as time passes. These trends are depicted in graphs, together with the appropriate non-linear functions. To facilitate interpretation, all weights were transformed to natural logarithms (Ln(Weight)=A+B*Week)) which would allow growth patterns to be analysed as linear functions. For each chick (experimental unit) a linear regression function was fitted on weight change over the periods 0-58 days. Results from the linear regression analyses were subjected to analysis of variance to compare regression parameters (intercepts and slopes) for the eight diets.

Analysis of variance, as well as non-linear regression was performed using SAS version 9.2 (SAS, 2000). Levene's test was performed to test for treatment homogeneity of variance (Levene, 1960). The Shapiro-Wilk test was performed to test for non-normality (Shapiro & Wilk, 1965). Student's t-Least Significant Difference was calculated at the 5% confidence level to compare treatment means (Ott, 1998).

Results and Discussion

The growth and feed intake, as well as parameters measured and calculated for the surviving chicks, are presented in Table 5. From the data in Table 5 it was clear that no differences in chick survival existed between the high and low fat diets, the high and low starch diets, as well as the high sugar and low sugar diets. However, there was a tendency for the survival of chicks fed the high protein diet to be poorer than those fed the low protein diet (4/14 = 0.286 *vs.* 8/13 = 0.615; Fisher's Exact Probability = 0.091). Significant differences ($P<0.05$) might have been obtained with a bigger data set. This tendency could possibly be because of the high metabolic strain put on the ostrich chicks due to the increased need for de-amination of the high protein diet. Swatson *et al.* (2000) also suggested that any surplus protein in the broiler chick's diet has an adverse effect on biological performance.

A chi-squared (χ_1^2) test was conducted to test whether mortality percentage is independent of level of protein, fat, starch and sugar respectively in pre-starter diets. None of the P-values for the chi-squared test (P=0.0850, 0.6584, 0.2162, 1.000 respectively) indicated significant (95% probability) evidence against independence.

Table 5 Growth and feed intake parameters for ostrich chicks fed eight different pre-starter diets

	High protein	Low protein	High fat	Low fat	High starch	Low starch	High sugar	Low sugar
Number of chicks at beginning of trial	14	13	13	13	13	13	13	13
Number of chicks which survived throughout trial	4	8	9	10	7	10	8	8
Mortalities (%)	71.4%	38.5%	30.8%	23.1%	46.2%	23.1%	38.5%	38.5%
Average hatching weight of survived chicks (g)	873.3^{a}	825.6^{a}	794.3^{a}	853.1^{a}	814.7^{a}	806.8^{a}	862.3^{a}	821.6^{a}
Average slaughter weight of survived chicks (g)	5613^{bc}	5396^{c}	8773^{a}	7994^{ab}	9267^{a}	8504^{a}	7602^{abc}	9138^{a}
ADG (survived chicks) (g/day)	34.66^{e}	35.57^{de}	45.78^{abc}	41.32^{bc}	46.49^{ab}	44.79^{abc}	40.49^{cd}	47.05^{a}
Cumulative weight gain (g)	13312	38936	67499	69868	57995	69809	51833	63365
Cumulative feed intake (g)*	31209	96705	134477	133514	98686	150141	103398	129346
FCR (whole group) (g)*	2.34	2.48	2.04	1.91	1.74	2.15	2.02	2.04

$^{a, b, c}$ Row means with different superscripts differ significantly at $P<0.05$

* Individual feed intake were not recorded and statistical analysis were therefore not possible for these parameters

ADG = Average daily gain

FCR = Feed conversion ratio

In the case of the amount of fat and sugar in the diet, there appeared to be divergence in growth. This could be subtracted when the regression coefficient (Figure 1) of the data, in the linear form, were compared. The slopes of the natural log transformed data differed ($P<0.05$) for fat and sugar (Table 6). Chicks fed a high fat diet showed a higher ($P<0.05$) growth rate (slope = 0.046) than chicks fed a low fat diet (slope = 0.041), while chicks fed a low sugar diet had a higher ($P<0.05$) growth rate (slope = 0.047) than chicks fed a high sugar diet (slope = 0.040). When a regression of mean weights on age was performed using the exponential growth function, the observed R^2 values were high (R^2 >0.98), as was expected for a normal growth curve.

There are few reports on dietary fat digestibility or suggested fat content in starter diets for ostriches (Cooper, 2004). Angel (1993) reported a fat digestibility of 44.1% for ostrich chicks 3 weeks of age. This

increased to 74.3% at 6 weeks of age. In this study chicks on the high fat (5.14% fat) diet showed a faster growth ($P<0.05$) than those on the low fat (2.41%) diet (slope = 0.046 *vs.* 0.041).

Angel (1993) reported that 3-week old ostrich chicks have a fibre digestibility of 6.5%, which increases to 27.9% in chicks that are 6 weeks old. High mortalities were reported by Sato *et al*. (1994) in 6-week old chicks fed lucerne hay, due to impaction of lucerne hay and maize in the proventriculus and gizzard. In this study, only three mortalities occurred in the low starch group, where the diet consisted of 37.74% lucerne hay and had a crude fibre content of 13.17%. There was also no difference ($P>0.05$) in growth rate between the chicks on the high starch (slope = 0.046) and on the low starch (slope = 0.045) diets. Chicks on the lower sugar diet had a faster growth ($P<0.05$, slope = 0.047) than on the higher sugar (slope = 0.047) diet. The high sugar diet also had a higher lucerne hay inclusion (17.26%), and hence higher crude fibre content (8.53%), than the low sugar (10% lucerne inclusion) diet (5.52% crude fibre content).

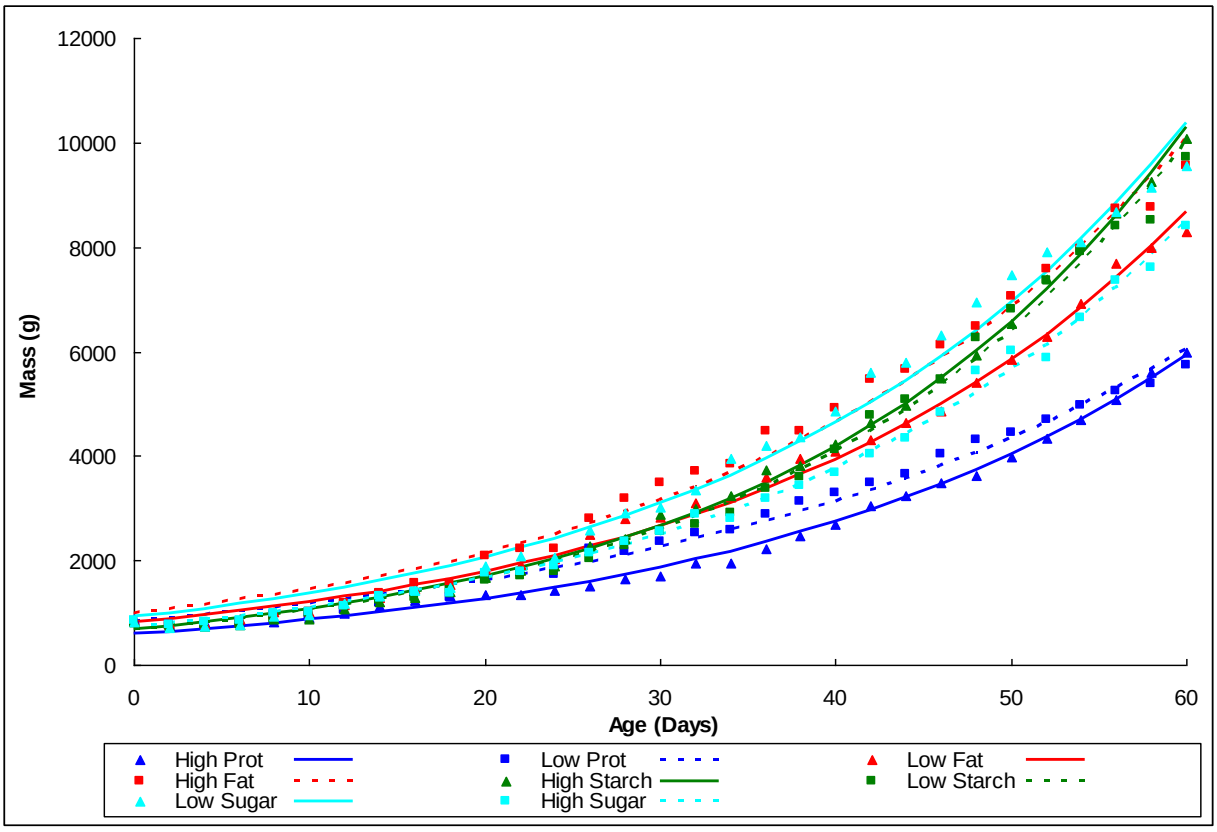

Figure 1 Exponential growth curves for ostrich chicks fed eight different pre-starter diets

Table 6 Linear regression values for ostrich chicks fed eight different pre-starter diets

Treatment	Intercept (SE)	Slope (SE)
Protein		
High	6.511 ± 0.165[a]	0.035 ± 0.005[a]
Low	6.595 ± 0.143[a]	0.036 ± 0.005[a]
Fat		
High	6.598 ± 0.061[a]	0.046 ± 0.002[b]
Low	6.618 ± 0.114[a]	0.041± 0.004[a]
Starch		
High	6.451 ± 0.170[a]	0.046 ± 0.005[a]
Low	6.462 ± 0.080[a]	0.045 ± 0.007[a]
Sugar		
High	6.579 ± 0.100[a]	0.040 ± 0.005[a]
Low	6.523 ± 0.119[a]	0.047 ± 0.004[b]

[a, b] Column means with different superscripts differ significantly at P<0.05

(Ln(y) = a + bx, where y = the natural logarithm of live weight at a specific age, a = the intercept, b = the linear regression coefficient (or the slope) and x = age at live weight recording (days).)

Even though multiple feed samples for each diet and faecal samples from all slaughtered birds were taken, a specific faecal sample could not be linked to a specific feed sample, as all the birds in a treatment were fed as a group and not as individual birds. All values, therefore, were pooled and averages calculated for each treatment. Data were not statistically analysed and are presented as is (Table 7), while tendencies are discussed.

Table 7 Apparent digestibility of proximate analysis parameters for ostrich chicks fed eight different pre-starter diets

	High protein (%)	Low protein (%)	High fat (%)	Low fat (%)	High starch (%)	Low starch (%)	High sugar (%)	Low sugar (%)
Digestibility								
Crude Protein	85.7	82.4	87.4	85.7	91.2	71.3	71.5	79.3
Crude fat	90.2	93.3	94.1	86.0	94.2	86.9	83.3	91.6
Dry matter	72.7	79.1	80.7	79.0	87.0	50.9	52.8	69.2

Differences in digestibility of dry matter, crude protein and crude fat between chicks receiving high and low diets for the different nutrient components were observed in the high and low starch diets, as well as the high and low sugar diets. Chicks fed the high starch diet had higher digestibility values for crude protein (91.2% *vs*. 71.3%), fat (94.2% *vs*. 86.9%) and dry matter (87% *vs*. 50.9%) than chicks on the low starch diet. This tendency was reversed for birds receiving the high and low sugar diets. Birds fed the high sugar diet had a tendency for lower digestibility for protein (79.3% *vs*. 71.5%), fat (91.6% *vs*. 83.3%) and dry matter intake (69.2% *vs*. 52.8%) than birds fed the low sugar diet. This could be due to the higher crude fibre content in the low starch diet (14.13% *vs*. 5.33%) (Table 4) and high sugar diet (8.53% *vs*. 5.52%) (Table 4).

Conclusion

It was clear from this trial that ostrich chicks performed differently on diets formulated with different nutritional components. The results appear to indicate that a high fat and low sugar content is of advantage in ostrich pre-starter diets. There is no clear indication as to specific reasons why chicks died in the different trial groups, as the causes of mortalities were not investigated. This is a factor that should be explored in future studies.

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Chapter 6

Enzyme activity in the small intestine of ostrich (S*truthio camelus* L.) chicks on eight different pre-starter diets

Introduction

In contrast to the wealth of data available on mammalian digestive enzymes, only limited information is available on digestive enzymes in the intestines of ratites (Hartley *et al.*, 1987; Angel, 1996). Some work on the characterisation of pancreatic enzymes has been done in ostriches (Hartley *et al*., 1987; Van der Westhuizen, 1988; Sutherland, 1990; Oosthuizen *et al*., 1992; Smith, 1993; Bodley *et al*., 1995), as well as on the characterisation of certain enzymes in the duodenal mucosa (Naudé *et al.*, 1993; Roos *et al.*, 1993) and the proventriculus (Streicher, 1984; Pletschke *et al*., 1995) of this species. There is, however, no information available on the changes in digestive enzyme activity resulting from changes in diet or due to age (Angel, 1996) in ratites.

Naudé *et al*. (1993) conducted a study on the characterisation of enterokinase from the ostrich duodenal mucosa. Enterokinase plays a key role in activating pancreatic trypsinogen to trypsin. Trypsin, in turn, activates pancreatic chymotrypsinogen to chymotrypsin (Anderson *et al*., 1977). Naudé *et al*. (1993) reported that ostrich enterokinase has a similar amino acid composition to its mammalian counterpart and that it showed the highest activation of bovine trypsinogen at pH 5.2 – 5.7.

Alkaline phosphatase is expressed in active and mature mucosal enterocytes and has therefore been used in enzyme studies as an enterocyte maturation marker (Wieser, 1973; Traber *et al*., 1991). In a study by Toyoda *et al.* (1985) the authors found that proteases from the pancreas caused the release of enterokinase and alkaline phosphatase from rat enterocytes and that chymotrypsin, specifically, causes a selective release of enterokinase over that of alkaline phosphatase.

Hartley (1986) suggested that ostrich trypsin performs the same function in ostrich intestinal digestion as that of mammalian trypsin. Harley *et al*. (1987) reported an optimum activity of ostrich trypsin in the pH range 8.1 to 8.4. Smith (1993) reported that when using ostrich trypsin to activate ostrich chymotrypsinogens, it resulted in a slower rate compared to that of bovine trypsin. Van der Westhuizen (1988) also suggested that the properties of ostrich chymotrypsinogen in intestinal digestion are similar to those of mammalian chymotrypsinogen. Ostrich α-amylase appears to be closely related to α-amylase in other animals, with a special close resemblance to chicken α-amylase, with an optimum activity for starch hydrolysis at pH 7.5 (Oosthuizen *et al*., 1992).

Knowledge of the activity of digestive enzymes in the intestines could be beneficial to the ostrich industry, especially with regards to nutritional composition of diets and the inclusion of certain feedstuffs

that may have an influence on digestibility. For this study eight pre-starter diets were fed to ostrich chicks from hatch until they were slaughtered at 60 days of age. The possible effects of the different diets on enzyme activity were determined. The project had ethical approval from the Onderstepoort Animal Use and Care Committee (Protocol 36-5-623).

Materials and Methods

Animals

105 South African Black Ostrich (*Struthio camelus* L.*)* chicks (58 males and 47 females) were obtained from the Oudtshoorn Experimental Farm of the Department of Agriculture Western Cape, South Africa, where they were hatched and weighed. They were transported as day old chicks to the Kromme Rhee Experimental Farm of the Department of Agriculture Western Cape, near Stellenbosch, South Africa. Their transportation over the 500 km distance was carried out in plastic containers with sufficient airflow in a closed vehicle. The average weight of the day-old chicks was 0.837 kg. Chicks were randomly divided into eight trial groups: 13 chicks per group, except for the high protein group, where 14 chicks were used. During the 58 day trial period, 39 of the 105 chicks that started the trial died and another two mortalities occurred before commence of slaughter. The number of chicks slaughtered for each trial group is given in Table 2. Apparent causes of death are discussed in Chapter 5, pg. 82. All the remaining birds were utilised for the trial.

Treatments

Ostrich chicks were fed diets with varying high and low levels of protein, fat, sugar and starch. The raw material and calculated nutrient composition of the eight trial diets are presented in Table 1. Regarding the proximate values of the diets obtained by the different analyses, see Chapter 5, Table 4, pg. 86.

Table 1 Raw material and calculated nutrient composition of trial diets

Ingredients	High protein %	Low protein %	High fat %	Low fat %	High starch %	Low starch %	High sugar %	Low sugar %
Maize meal	25.55	63.86	50.17	58.04	29.68	23.78	24.72	53.05
Soybean oilcake meal	43.26	8.25	20.00	27.20	22.48	11.66	21.26	20.00
Maize starch	—	—	—	—	20.00	—	—	—
Full-fat soya	16.78	12.00	12.10	—	12.00	12.00	12.00	12.00
Lucerne hay	10.00	10.00	10.00	10.00	10.00	37.74	17.26	10.00
Sugar	—	—	—	—	—	—	20.00	—
Wheat bran	—	—	—	—	—	10.00	—	—
Limestone	2.27	2.96	2.99	3.03	2.89	1.18	1.78	2.25
Plant oil	1.00	1.00	3.00	—	1.00	2.00	1.00	1.00
Mono calcium phosphate	0.49	1.28	1.07	1.06	1.26	0.94	1.28	1.04
Fine salt	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Mineral and vitamin mixture	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Synthetic methionine	—	—	0.02	0.02	0.04	0.05	0.05	0.01
Nutrient composition								
Crude protein (%)	29.83	15.21	19.51	19.02	18.89	19.27	19.05	19.71
Dry matter (%)	89.46	88.20	88.86	88.30	71.41	89.36	91.31	88.51
ME (MJ/kg feed)	14.5	14.5	14.8	13.5	14.5	13.0	14.5	14.5
Crude fibre (%)	6.35	5.18	5.47	5.34	5.14	12.96	6.93	5.53
Crude fat (%)	6.01	6.06	7.71	2.91	4.97	6.81	4.99	5.83
Starch (%)	19.50	41.43	33.48	38.23	38.22	19.00	17.78	35.28
Sugar (%)	6.54	2.99	4.03	3.58	4.61	5.30	24.43	4.04

Collection of Material

Slaughter commenced on day two after completion of the growth trial and was carried out over a five-day period. Two chicks from each treatment group were slaughtered each day, starting with a different treatment group every day. Chicks were randomly selected from each treatment group for slaughtering. The hatching- and slaughter weights of the chicks in the different treatment groups are presented in Table 2.

Table 2 Hatching- and slaughter weights for ostrich chicks fed eight different pre-starter diets

Treatment groups	Number of birds at slaughter	Average hatching weight (g)	Average slaughter weight (g)
High protein	2	873.3[a]	5612.5[bc]
Low protein	8	825.6[a]	5395.6[c]
High fat	9	794.3[a]	8773.0[a]
Low fat	10	853.1[a]	7993.7[ab]
High starch	7	814.7[a]	9266.7[a]
Low starch	10	806.8[a]	8504.3[a]
High sugar	8	862.3[a]	7602.3[abc]
Low sugar	8	821.6[a]	9137.9[a]

[a, b, c] Column means with different superscripts differ significantly at $P<0.05$

Chicks were euthanized with CO_2 in a closed container. The digestive tracts were immediately removed from the carcasses after death. Each section of the digestive tract was identified. Samples of the wall of the small intestine were taken from the duodenum (ascending or distal limb of the duodenal loop, just before the secondary distal loop); jejenum (before the vitelline diverticulum, where the yolk sac stalk enters the jejunum) and ileum (approximately 10 cm before the ileo-ceacal junction). The samples were approximately 2.5 cm in length and weighed between 0.42 and 4.07 g. These samples were used for enzyme analyses.

Tissue samples were rinsed with ice-cold saline (0.9% NaCl), using a syringe to flush out any intestinal content. The samples were then cut open lengthwise and placed on ice with the luminal surface facing away from the surface of the ice. An 18 gauge (18G) needle was attached to a syringe and the tissue surface was thoroughly rinsed, taking special care not to damage the mucosal layer of the wall. The tissue samples were wrapped in pre-marked pieces of aluminium foil and stored in liquid nitrogen, in a thermos flask. When all the tissue samples had been collected, they were transferred to pre-marked plastic bags and stored in a freezer at approximately minus 85°C.

Measurements and Calculations

Brush-border membrane vesicles were isolated from the intestinal tissues according to the method described by Shirazi-Beechey *et al.* (1991) with the following additions: A crushed ice-bed of about 5 cm thick was prepared in a flat container (24 cm by 30 cm). All instruments and buffers were kept cold on crushed ice. All the steps of the procedure were completed on the ice bed. Samples (eight per day) were taken from the freezer and placed on the ice-bed to prevent thawing. Each sample was quickly weighed, cut into small pieces, with a pair of sharp scissors while still frozen, and immediately transferred to pre-marked (for identification) conical plastic tubes, which was again placed in the ice-bed in an upright position. Depending on the weight of the tissue sample, the appropriate amount of pre-cooled Buffer A (Table 3) was

added to the tissue sample (< 1-1 g tissue weight use 10 ml of Buffer A; 1-2 g use 20 ml; 2-3g use 30 ml; 3-4 g use 40 ml). Each tube was sealed with parafilm to prevent spillage. Each sample was vibromixed with a Vortex tube mixer (Heidolph REAX top) at maximum speed for 60 seconds and filtered through a 70 mm diameter Buchner funnel, where after each sample was homogenized individually for 30 seconds with an ULTRA-Turrax T25 homogenizer at 13 500 r.p.m. To prevent contamination, the homogenizer dispersing head was spun in clean distilled water and wiped dry after each sample. Immediately after mixing, the homogenate was divided (± 600 µl each), into three 1.5 ml Eppendorf tubes, recapped firmly and kept on the ice-bed. After preparation of all the samples, they were placed in a freezer at -85°C until they could be analysed for alkaline phosphatase and protein content.

The remaining homogenate was weighed (to the nearest 0.5 g) into centrifuge tubes. The weights of the samples to be placed opposite each other in the centrifuge were balanced by adding cold Buffer A (Table 3). This dilution was carefully noted. Where 10 ml of Buffer A was added at the start of the procedure (corresponding with a ± 1 g cut sample), 50 µl of $MgCl_2.7H_2O$, from a 2.5M Stock Solution (50.825g/100ml) was added to each sample. Accordingly, 100 µl, 150 µl and 200 µl of $MgCl_2.7H_2O$ was added if the sample volume was above 20 ml, 30 ml and 40 ml, respectively. The exact amount added was taken into account during enzyme activity calculations. After the $MgCl_2.7H_2O$ was added, each sample was vibromixed briskly and allowed to stand for 20 minutes on the ice-bed to aid subsequent sedimentation during centrifugation. Suspensions were centrifuged at high speed, using a Du Pont Refrigerated Sorvall Ultra-Centrifuge RC 6, Rotor SS-34, at 3 000 g (5 000 r.p.m.) at 6°C for 15 minutes. The supernatant was then transferred to clean pre-marked tubes, discarding the pellets this time, then centrifuged at 30 000 g (14 500 r.p.m.) at 6°C for 30 min. The supernatant was removed and each pellet re-suspended in cold Buffer B (Table 3). Homogenization was performed this time using a 18G needle. Samples were again centrifuged at 30 000 g (14 500 r.p.m.) at 6°C for 45 minutes. The final pellets were re-suspended in 500 µl of cold Buffer C (Table 3) and passed through a 25G needle to obtain a homogenous suspension. The suspension was divided and transferred to four 1.5 ml Eppendorf tubes kept on ice. These homogenized tissue samples were stored at minus 85°C for later analyses for chymotrypsin, trypsin, amylase and lipase activity.

Table 3 Chemical composition of Buffers A, B and C (Shirazi-Beechey *et al.*, 1991)

Buffers	A	B	C
Mannitol	100mM (18.22g/l)	100mM (18.22g/l)	300mM (13.665g/250ml)
	2mM	2mM	20mM
Hepes &	(0.477g Hepes/l)	(0.477g Hepes/l)	(1.192g Hepes/250ml)
Trizma/HCl	(0.315g Trizma/HCl/l)	(0.315g Trizma/HCl/l)	(0.788g Trizma/HCl/250ml)
$MgSO_4$	-	0.1mM (0.012 g/l)	0.1mM (0.003g/250ml)
NaN_3	-	-	0.02% (0.05g/250ml)
pH	7.1	7.4	7.4

Alkaline phosphatase was analysed according to the method described by Forstner *et al*. (1968) and Holdsworth (1970). Protein content was determined according to the method described by Bradford (1976). Chymotrypsin amidase and trypsin amidase were determined according to the method described by Servière-Zaragoza *et al.* (1997).

Amylase content was determined using the ACE™ Amylase Reagent (Reagent number AE2-5) intended for the quantitative determination of alpha (α) amylase activity in serum using the ACE™ clinical chemistry system. This method makes use of a modified p-nitrophenyl-maltoheptaoside as substrate. A multifunctional glucosidase cleaves the amylase reaction products and releases the p-nitrophenol. The terminal glucose of the substrate is chemically blocked preventing cleavage by the indicator enzyme. The rate of release of pNP is monitored at 408 nm and is proportional to the α-amylase activity in the sample.

Lipase content was determined using the ACE™ Lipase Reagent (Reagents 1 and 2, Catalog number 11821792), intended for the quantitative determination of lipase activity in serum using the ACE™ clinical chemistry system. The method which was used is based on a 1,2-O-dilauryl-rac-glycero-3-glutaric acid-(6-methylresorufin) ester as substrate. The chromogenic lipase substrate 1,2-O-dilauryl-rac-glycero-3-glutaric acid-(6-methylresorufin) ester is cleaved by the catalytic action of alkaline lipase solution to form 1,2-O-dilauryl-rac-glycerol and an unstable intermediate glutaric acid-(6-methylresorufin) ester. This decomposes spontaneously in alkaline solution to form glutaric acid and methylresorufin. The colour intensity of the red dye formed is directly proportional to the lipase activity and was determined photometrically.

Enzyme activity can either be expressed as mole substrate hydrolysed/mg protein/min (Shirazi-Beechey *et al.* 1991; Iji *et al.*, 2001) or as Units/mg protein (Pletschke *et al.*, 1995; Servière-Zaragoza *et al.* 1997). After analysis the enzyme activity was expressed as mmole product/min/mg protein.

Statistics

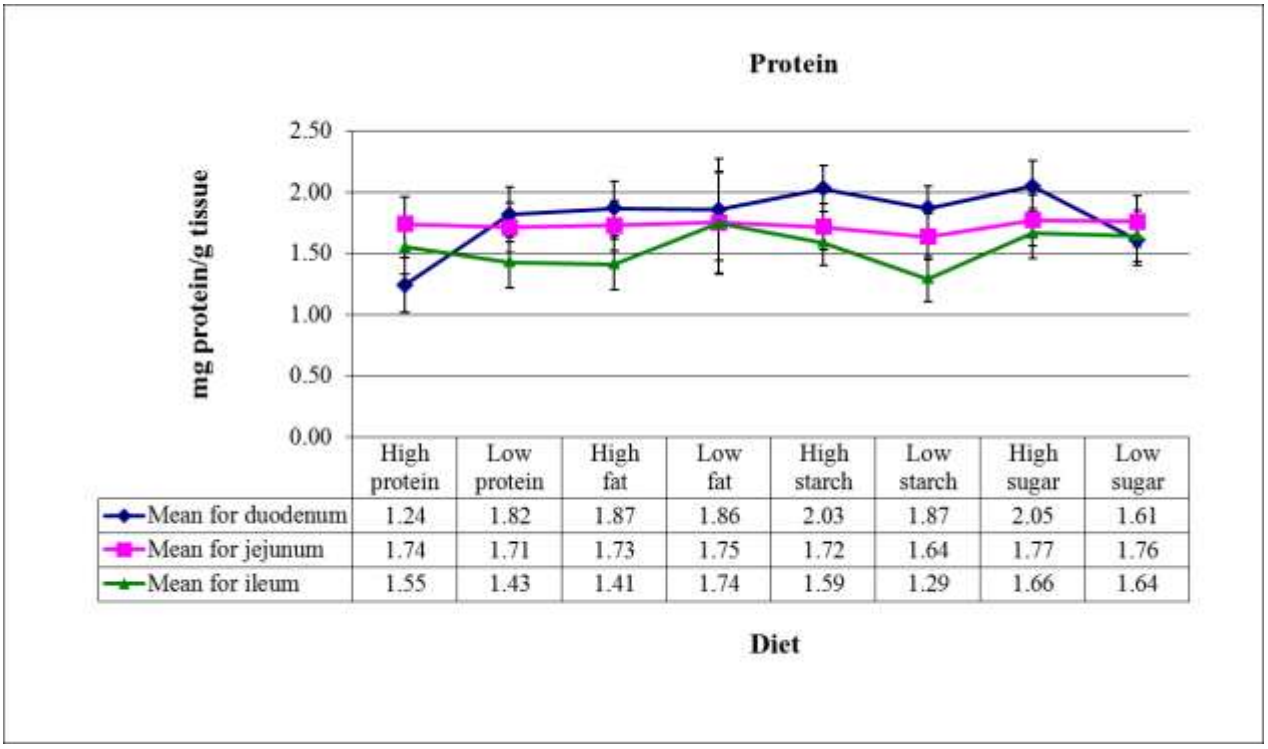
The data was analysed according to a standard eight (diets) x three (region of small intestine) factorial analysis (Snedecor & Cochran, 1980) for protein of the brush border membrane and the enzymes alkaline phosphatase, trypsin and chymotrypsin. For the enzymes amylase and lipase a standard eight (diets) x two (region of small intestine) factorial analysis was done, as activity of these two enzymes were only tested in the duodenum and ileum. Repeated records from the same experimental units assessed for different parts of the small intestines were accounted for by adding the random effect of animal in the mixed model analysis (Harvey, 1990). Although the interaction between diets and region of the small intestine was not significant ($P>0.05$) in all analyses, these interactions are provided to depict a clear picture of the results.

Results

Protein

In the following section the value for protein content is expressed as milligram per gram tissue (mg/g tissue). The protein content of the brush-border membrane (BBM) showed a tendency to decrease along the

length of the small intestine, but only significantly (P<0.05) for birds fed the high fat and low starch diets. Although protein content of the BBM was lower in the duodenum for birds fed the high protein diet than in the other two regions and differed compared to that of birds fed the other diets, the difference was not significant (P<0.05) due to the large standard error (SE ± 0.41) (Figure 1). In the duodenum the protein content of the BBM was similar for most of the trial groups, except for birds fed the high protein diet (1.24 ± 0.41), which was lower (P<0.05) than that of birds fed the high starch (2.03 ± 0.22), high sugar (2.05 ± 0.22), low starch (1.87 ± 0.19) and low fat (1.85 ± 0.19) diets. There were no differences in protein content of the BBM of the jejunum of birds fed the different diets. The only difference (P<0.05) in protein content of the BBM of the ileum was between birds fed the low starch diet (1.29 ± 0.19) which was lower than the protein content in the BBM of birds fed the low fat diet (1.74 ± 0.19).



	High protein	Low protein	High fat	Low fat	High starch	Low starch	High sugar	Low sugar
Mean for duodenum	1.24	1.82	1.87	1.86	2.03	1.87	2.05	1.61
Mean for jejunum	1.74	1.71	1.73	1.75	1.72	1.64	1.77	1.76
Mean for ileum	1.55	1.43	1.41	1.74	1.59	1.29	1.66	1.64

Figure 1 Protein content (± SE) of the BBM in the small intestines of ostriches fed eight different diets

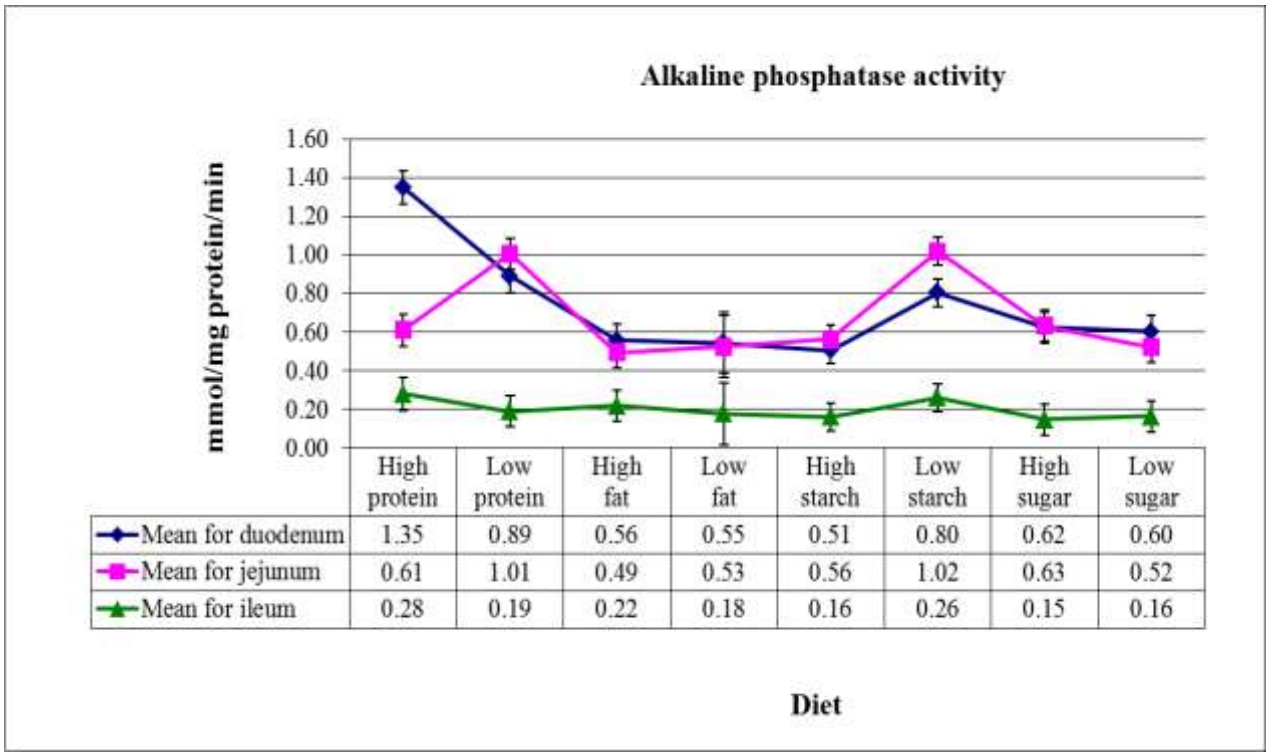
Alkaline Phosphatase

For the rest of this section the values for enzyme activity are expressed as millimole product per minute per milligram protein (mmole product/min/mg protein).

Alkaline Phosphatase (AP) activity decreased (P<0.05) from the first two regions of the small intestine to the ileum over all the trial diets. The activity of AP remained constant in the duodenum and jejunum for birds on most of the diets, except for birds that received the low starch diet, which showed an increase (P<0.05) of AP activity from the duodenum (0.81 ± 0.07) to the jejunum (1.02 ± 0.07). The birds that received the high protein diet, demonstrated a decrease (P<0.05) in the AP activity from the duodenum (1.35 ± 0.16) to the jejunum (0.61 ± 0.16) (Figure 2).

The activity of AP remained constant in the duodenum in five of the eight diets, but was higher ($P<0.05$) for birds fed the high protein (1.35 ± 0.16), low starch (0.81 ± 0.07) and low protein (0.89 ± 0.08) diets.

Alkaline Phosphatase activity also remained constant in the jejunum for most of the diets in the trial, except for birds fed the low starch (1.02 ± 0.07) and low protein (1.01 ± 0.08) diets, which had a higher ($P<0.05$) activity. There were no differences in the AP activity in the ileum for all the trial diets.

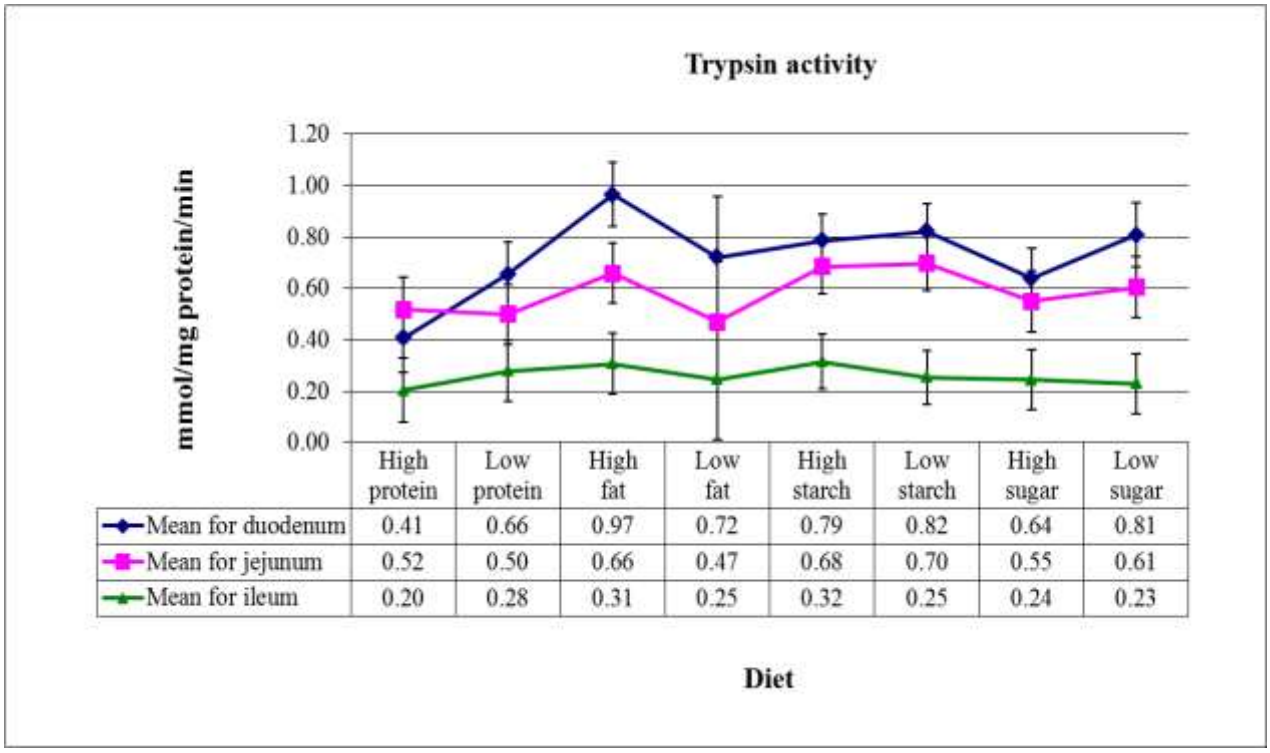


	High protein	Low protein	High fat	Low fat	High starch	Low starch	High sugar	Low sugar
Mean for duodenum	1.35	0.89	0.56	0.55	0.51	0.80	0.62	0.60
Mean for jejunum	0.61	1.01	0.49	0.53	0.56	1.02	0.63	0.52
Mean for ileum	0.28	0.19	0.22	0.18	0.16	0.26	0.15	0.16

Figure 2 Alkaline Phosphatase activity (± SE) in the small intestines of ostriches fed eight different diets

Trypsin

The activity of trypsin decreased ($P<0.05$) along the length of the small intestine for all the treatments, except for birds fed the high protein diet (Figure 3). The activity of trypsin in the duodenum was lower ($P<0.05$) for birds fed the high protein diet (0.41 ± 0.24), than for birds fed the high (0.79 ± 0.14) and low (0.82 ± 0.11) starch, high fat (0.97 ± 0.13) and low sugar (0.81 ± 0.12) diets. There were no differences in trypsin activity in the ileum between the various diets, while trypsin activity in the jejunum only showed a difference ($P<0.05$) for birds fed the low starch (0.70 ± 0.11) diet and birds fed the low fat (0.47 ± 0.11) diet.



	High protein	Low protein	High fat	Low fat	High starch	Low starch	High sugar	Low sugar
Mean for duodenum	0.41	0.66	0.97	0.72	0.79	0.82	0.64	0.81
Mean for jejunum	0.52	0.50	0.66	0.47	0.68	0.70	0.55	0.61
Mean for ileum	0.20	0.28	0.31	0.25	0.32	0.25	0.24	0.23

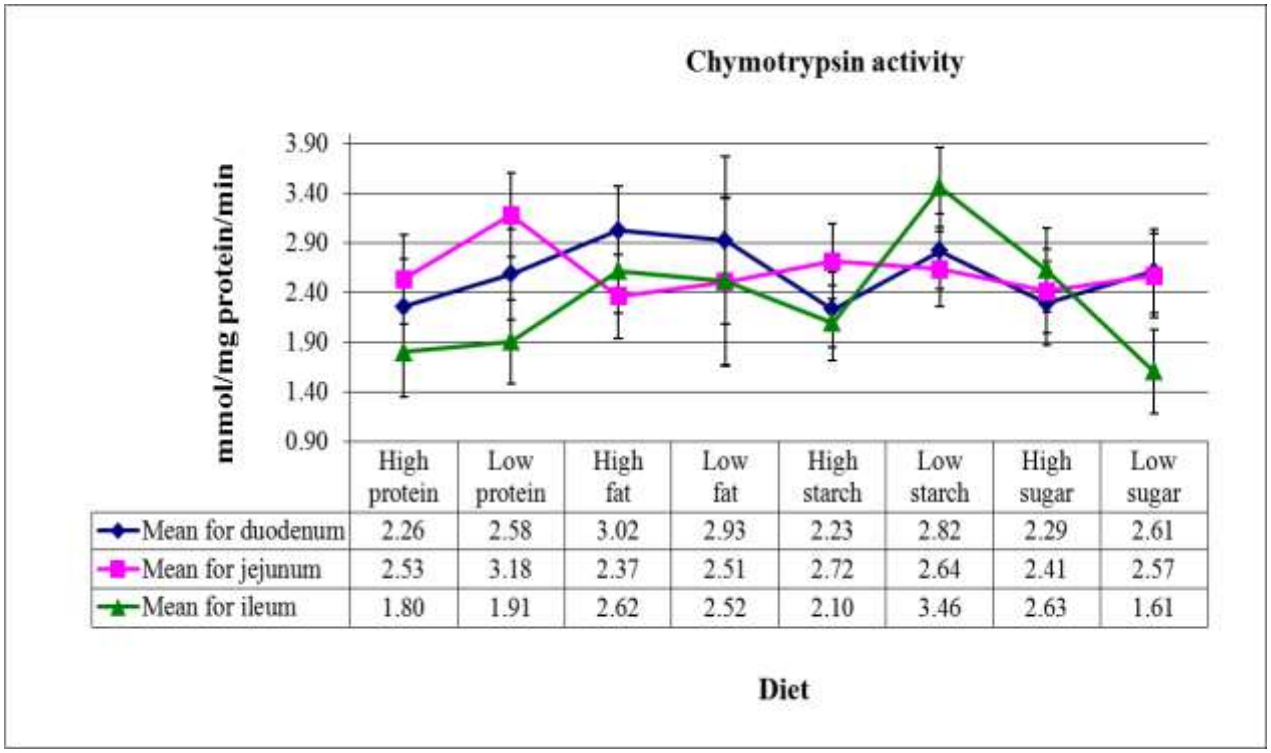
Figure 3 Trypsin activity (± SE) in the small intestines of ostriches fed eight different diets

Chymotrypsin

The activity of chymotrypsin did not differ between the different regions of the small intestine for birds on five of the trial diets. However, birds fed the low starch diet showed an increase ($P<0.05$) in the activity of chymotrypsin from the jejunum (2.64 ± 0.38) to the ileum (3.46 ± 0.38), while birds fed the low sugar (LSu) and low protein (LP) diets had a lower ($P<0.05$) chymotrypsin value in the ileum (LSu = 1.61 ± 0.42; LP = 1.91 ± 0.42) than in the jejunum (LSu = 2.57 ± 0.42; LP = 3.18 ± 0.42) (Figure 4).

There were no differences in the activity of chymotrypsin in the duodenum and jejunum of birds across all the trial diets.

The activity of chymotrypsin in the ileum was lower ($P<0.05$) for birds fed the low sugar diet (1.61 ± 0.42), than for birds fed the high fat (2.62 ± 0.42), high sugar (2.63 ± 0.42), low starch (3.46 ± 0.38) and low fat (2.52 ± 0.40) diets. Chymotrypsin activity in the ileum was also lower ($p<0.05$) for birds fed the high protein (1.80 ± 0.85) diet, than birds fed the low starch (3.46 ± 0.38) diet.



	High protein	Low protein	High fat	Low fat	High starch	Low starch	High sugar	Low sugar
Mean for duodenum	2.26	2.58	3.02	2.93	2.23	2.82	2.29	2.61
Mean for jejunum	2.53	3.18	2.37	2.51	2.72	2.64	2.41	2.57
Mean for ileum	1.80	1.91	2.62	2.52	2.10	3.46	2.63	1.61

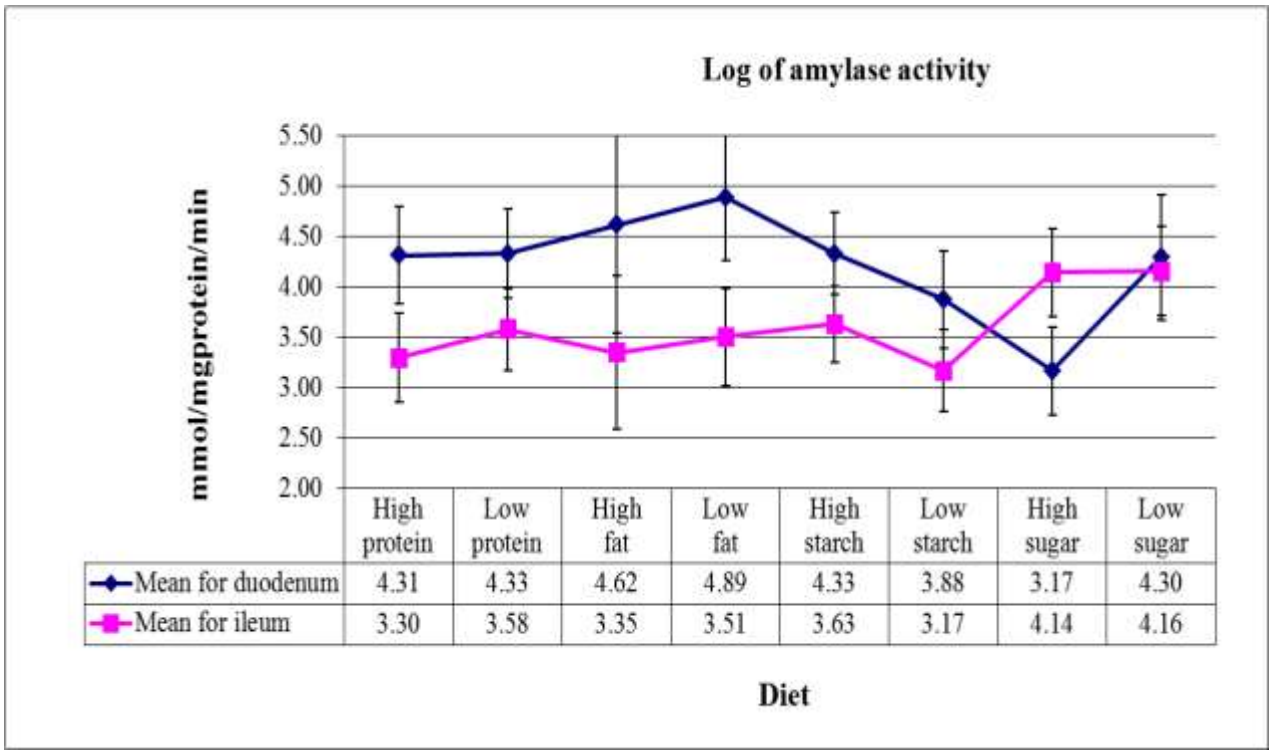
Figure 4 Chymotrypsin activity (± SE) in the small intestines of ostriches fed eight different diets

Amylase

The graph for amylase (Figure 5) is presented on the natural log scale, as data was transformed to ensure a normal distribution and equal variances during the treatment period. Values in the text are back transformed to geometric means for ease of interpretation. Amylase activity showed a tendency to decline from the duodenum to the ileum in almost all the trial diets, but the only significant decline ($P<0.05$) was observed between the two regions for birds fed the high fat (101.1 ± 45.87; 28.40 ± 11.88) and low fat (132.8 ± 66.45; 33.35 ± 13.95) diets (Figure 5).

The only difference in amylase activity noted in the duodenum was between birds fed the low fat (132.8 ± 66.45) diet, which reflected higher ($P<0.05$) amylase activity than birds fed the high sugar (23.70 ± 30.75) and low starch (48.27 ± 20.19) diets.

Amylase activity remained constant in the ileum for almost all the trial diets, except for birds fed the low sugar (63.80 ± 28.96) diet, which displayed a higher ($P<0.05$) amylase activity than birds fed the low starch (23.80 ± 9.28) diet.



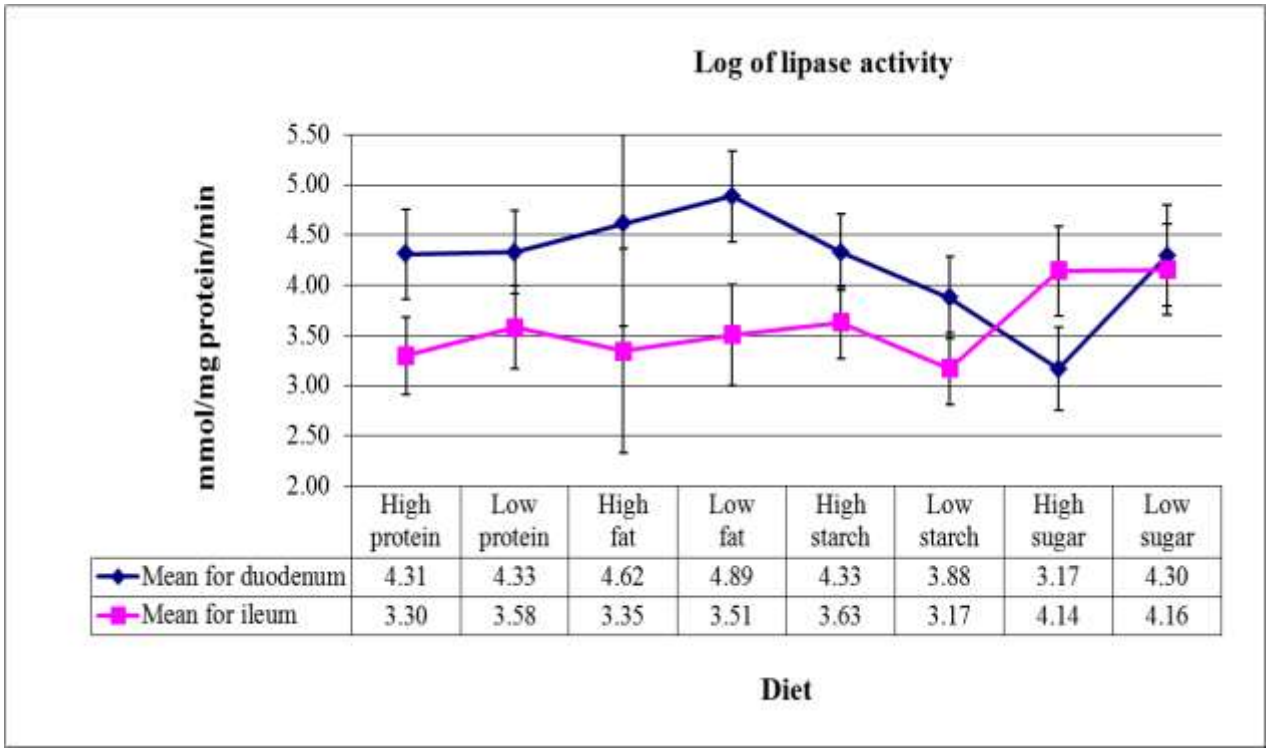
	High protein	Low protein	High fat	Low fat	High starch	Low starch	High sugar	Low sugar
Mean for duodenum	4.31	4.33	4.62	4.89	4.33	3.88	3.17	4.30
Mean for ileum	3.30	3.58	3.35	3.51	3.63	3.17	4.14	4.16

Figure 5 Amylase activity (± SE) in the small intestines of ostriches fed eight different diets

Lipase

The graph for lipase (Figure 6) is presented on the natural log scale, as data was transformed to ensure a normal distribution and equal variances during the treatment period. Values in the text are back transformed to geometric means for ease of interpretation. Lipase activity also showed a tendency to decline from the duodenum to the ileum in almost all the trial diets, but the only significant decline ($P<0.05$) was observed between the two regions for birds fed the high starch (91.51 ± 42.42; 38.22 ± 15.10) and low fat (92.76 ± 38.90; 30.56 ± 11.14) diets (Figure 6). The only exception was the lipase activity of the high sugar diet that showed an increase in lipase activity from the duodenum (42.67 ± 51.46) to the ileum (94.50 ± 113.96). This increase was, however, not significant ($P<0.05$), due to the large standard errors for lipase activity measured in both regions.

There were no differences in the lipase activity in the duodenum between all the various trial diets and in the ileum only birds fed the low starch (25.77 ± 9.39) diet had a lower lipase activity than birds receiving the low sugar (61.34 ± 28.40) diet. Lipase activity between the other diets remained constant.



	High protein	Low protein	High fat	Low fat	High starch	Low starch	High sugar	Low sugar
Mean for duodenum	4.31	4.33	4.62	4.89	4.33	3.88	3.17	4.30
Mean for ileum	3.30	3.58	3.35	3.51	3.63	3.17	4.14	4.16

Figure 6 Lipase activity (± SE) in the small intestines of ostriches fed eight different diets

Discussion

Protein of the BBM is measured to determine the active digestive and absorption capability within a fixed amount of tissue in a certain region of the digestive tract. The protein content, which remained relatively similar between the different trial diets, may suggest that the amount of tissue protein is fixed and does not increase or decrease due to different diets. The only way, therefore, to increase absorption and digestion is to increase the amount of tissue within the intestine. This can be done by either increasing the length of the intestine or by increasing the surface area of the villi. Protein of the BBM showed a tendency to decline along the length of the small intestine. This could be an indication of a general trend in the size of the villi which decrease in length from the duodenum to the ileum (Chapter 7, Table 4, pg.130). These results differ from those of Iji *et al*. (2003) who reported a higher protein content of the BBM of the jejunum and ileum than those of the duodenum. The authors contributed their findings to the general trend in the growth of microvilli.

The only noticeable difference in protein content of the BBM was that of the duodenum of birds fed the high protein diet. This low protein content could be ascribed to a number of reasons. Firstly, there were only two birds left in the group receiving the high protein diet at the time of slaughter, compared to at least seven birds in the other groups (Table 2). This may have unfortunately skewed the data. Secondly, the slaughter weight of the birds in this group was lower than in the other groups, which could result in a lighter or less physiological mature digestive tract (Table 2). Thirdly, in Chapter 5 (pg. 87), it was reported that there was a tendency for survival of chicks fed the high protein diet to be poorer than birds fed the other

diets, and this could be attributed to the high metabolic strain put on the ostrich chicks as a result of the increased need for de-amination of the high protein diet. This could therefore also result in a poorly developed digestive tract in these birds.

Alkaline Phosphatase (AP) is used as a maturation marker of enterocytes (Wieser, 1973; Traber *et al.*, 1991) and therefore the excretion of AP and enterokinase from enterocytes in mammalian enterocytes can be linked (Toyoda *et al.*, 1985). As several authors reported that ostrich enzymes are similar to their mammalian counterparts (Van der Westhuizen, 1988; Naudé *et al.*, 1993; Smith, 1993), it was assumed that AP activity can be used as an indication of the enterokinase activity within the small intestines of the ostrich.

The decrease ($P<0.05$) of AP activity from the first two regions of the small intestine to the ileum could be an indication that AP is not released in the last part of the small intestine and is only measured as it moves down the small intestines with the digesta. Similar observations were made by Majumdar *et al.* (1988) for White Rock cockerels, by Dupuis *et al.* (1990) for the rat and by Iji *et al.* (2001) in broiler chicks. The high AP activity in the duodenum of birds fed the high protein diet (1.35 ± 0.16) could be an indication that a large amount of enterokinase had also been excreted by the enterocytes in the duodenum. This could be due to the high protein content (29.83%, Table 1) of the high protein diet, as an increase in substrate will result in an increase in enzyme secretion. The high protein content would therefore trigger the release of protease enzymes from the pancreas and as enterokinase functions to activate inactive trypsinogen to its active form, trypsin. An increase in trypsinogen might again lead to an increase in enterokinase content.

Soya beans contain a trypsin inhibitor, which is an anti-nutritional factor that attaches to the trypsin enzyme and inhibits its activity (Birk *et al.*, 1963). The trypsin inhibitor is denatured when soya beans are heat treated and its activity is reported to decrease over time with a constant temperature of heat treatment (Onyeike *et al.*, 1991). Soybean oilcake meal is a by-product of the soybean oil industry and is subjected to very high temperatures during the extraction process. It could therefore be assumed that most of the trypsin inhibitor has been destroyed in this feedstuff. With full-fat soya the whole bean is toasted (Cooper & Benson, 2000) and not exposed to the same amount of heat as soybean oilcake meal, which may result in the trypsin inhibitor not being completely inactivated. Perilla *et al.* (1997) reported that full-fat soya beans that have been wet extruded at 118°C and 120°C resulted in significant lower body weights for broiler chickens than full-fat soya beans extruded at 122°C and 126 °C. Rachis *et al.* (1985) report toasted soya bean flour to have a trypsin inhibitor activity of 15.9 mg/g protein. In the present study all of the diets were formulated using two soya products, namely, oilcake meal and full-fat soya, except for the low fat diet which contained only soybean oilcake meal. As most of the diets contained only 12% full-fat soya, the trypsin inhibitor would be present in small enough amounts not to have an impact on trypsin activity. The high protein diet, however, contained 16.78% full-fat soya, which might allow the trypsin inhibitor to have an influence on trypsin activity. This could explain the low trypsin activity (0.41 ± 0.24) noted for birds fed the high protein diet. It is possible that a large amount of trypsin might be present in the duodenum as a result of the high amount of protein substrate, but its activity cannot be measured due to its binding with the trypsin inhibitor.

The decrease in trypsin activity along the length of the small intestine was to be expected as trypsin is excreted as trypsinogen by the pancreas in the duodenum, changed by enterokinase to trypsin and believed to start functioning as soon as it is activated. It will then move along the length of the intestine with the digesta and the concentration would decrease as it spreads down from the excretion point along the duodenum. Additionally, as trypsin is also a protein it would be digested by other proteases along the digestive tract.

It did not seem as if the type of diet had any influence on the chymotrypsin activity as it remained relatively constant in both the duodenum and the jejunum across all the trial diets. The only differences noted in chymotrypsin activity were in the last region of the small intestine, but the differences were inconsistent and could not be linked to any factor which differed between the diets. Chymotrypsin is not usually digested by other proteases of the digestive tract and is often used as a marker for pancreatic activity. The chymotrypsin activity for all the diets was much higher than reported by Iji *et al.* (2003) for ostrich chicks aged 55 and 72 days of age. The chymotrypsin in the latter study, however, was measured in the pancreas and not in the brush border membrane as was the case for this study.

Amylase and lipase activity both showed a tendency to decline down the length of the small intestine, but the decline was not significant ($P<0.05$) for most diets due to large standard errors. Furthermore, budget constraints unfortunately limited the number of samples ($n = 41$) which could be analysed for the activity of these enzymes. Values for amylase and lipase activity were much higher than reported by Iji *et al.* (2003). This can be contributed to the site where enzyme activity was measured. These authors reported that amylase activity tended to be lower in ostrich chicks than in poultry and attributed the lower activity of amylase, compared to that of poultry, to the low concentration of soluble sugars in the diet of the ostrich. Lipase activity in broiler chicks increased with age, but is less marked and occurs at a later age than does the changes in trypsin and amylase activity (Noy & Sklan, 1995; Noy & Sklan, 1998).

Conclusion

Differences in composition of the eight pre-starter diets did not seem to have an obvious influence on the protein content or enzyme activity of the small intestines of ostrich chicks. An anti-nutritional factor within certain feedstuffs may, however, have had an influence on enzyme activity. Choosing the right combination of feedstuffs to compile a ration is therefore just as important as formulating the ration to meet the nutritional requirements of the ostrich chick. Various authors (Iji *et al.*, 2003; Van der Walt *et al.*, 2003; Wang & Peng, 2008) indicated that the change in the ostrich digestive tract from a typical monogastric animal to a hindgut fermenter occurs within the first 70-80 days post-hatching, with changes already accelerating at 55 days post-hatching. Anti-nutritional factors which have an influence on monogastric animals, such as the trypsin inhibitor in soya beans, should however still be taken into account when formulating an ostrich pre-starter ration, as the microbes that can inactivate this anti-nutritional factor are not present in the small intestine. Even though there did not seem to be many differences in the enzyme activity between different diets in this trial, a great deal of research into enzyme activity in growing ostriches is still required. As the ostrich changes from a monogastric animal to a hindgut fermenter, the question arises as to

how effective digestion in the small intestine remains if much of the chemical digestion is replaced by microbial digestion in the caeca. Should ostrich digestion continue to be compared to that of other avians, or should the focus shift to comparing it with other monogastric hindgut fermenters, like the horse and rabbit?

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Chapter 7

A histological and morphometric study of the intestinal tract of ostrich (*Struthio camelus* L.) chicks on eight different pre-starter diets

Introduction

The growth rate of an animal is dependent on the efficiency of the gastrointestinal tract (GIT) to effectively digest feedstuffs and provide a continuous supply of balanced nutrients to its tissue (Bedford, 1996). The influence of different diets on the anatomy of the GIT of the ostrich has been previously reported. In a study by Baltmanis *et al.* (1997), differences were observed in the gross anatomy of the intestines of ostriches fed a high forage diet and a complete diet provided as pellets. The colon of the ostriches fed higher forage diets were heavier, while the caeca and ileum were heavier in the ostriches fed on a complete diet provided as pellets (Baltmanis *et al.*, 1997). Viljoen *et al.* (2004) reported a reduction in the length of the large intestines, small intestines and caeca with an increase in dietary energy concentration. This was attributed to the higher fibre concentrations of low energy diets (Viljoen *et al*., 2004), suggesting that the higher fibre concentration of low energy diets results in the development of longer intestinal tracts (Viljoen *et al*., 2004).

In order to fully appreciate the capacity of the small intestines of the ostrich to digest feedstuffs and thereby absorbing nutrients, it is important to have a thorough understanding of the morphological changes that occur within the small intestines (Wang & Peng, 2008). The changes in the morphology of the GIT associated with different feedstuffs have been investigated in wild gallinaceous birds as well as domestic fowls by various authors (Leopold, 1953; Savory, 1992; Bedford, 1996; Langhout *et al.*, 1999; Cowieson *et al.*, 2003). In contrast, only a single study has investigated the morphological changes occurring in the small intestine of the ostrich. Wang & Peng (2008) examined the morphological development of the small intestines of ostriches on post-hatch days 1, 45, 90 and 334. These authors suggested that the small intestine of the ostrich develops gradually from day 1 to day 90 post-hatch and that day 1 to day 45 post-hatch is marked by significant developmental changes in the parameters associated with digestive capacity, such as intestinal weight, length and surface area of the intestines, as well as in the number of goblet cells. The authors further suggested that feed management should be enhanced between days 1 and 45 post-hatching (Wang & Peng, 2008). However, this study did not compare the effect of various diets on the morphological development of the intestinal tract.

The large intestine also plays an important role in digestion. The mature ostrich has two well-developed caeca and an unusually long colon that is divided into a thicker proximal section and an extensive but thinner distal section (Bezuidenhout, 1986). The colon contributes 57% and the caeca 6% of the total digestive tract of the mature ostrich (Cho *et al.*, 1984). Ostriches are hatched as monogastric digesters and

change to hindgut fermenters after 70 to 90 days, with changes noticeable from day 55 post-hatch (Iji *et al.*, 2003; Van der Walt *et al.*, 2003; Wang & Peng, 2008). Microbial fermentation of cellulose and hemicellulose in the caeca produce large amounts of volatile fatty acids, which are absorbed in the caeca and large colon (Swart *et al.*, 1993). These authors reported that the energy contribution from microbial fermentation in the hindgut can contribute up to 76% of the ME requirement of the growing ostrich. Bezuidenhout (1993) described the spiral fold of the caecum and suggested that the fold plays an important role in the absorption of volatile fatty acids and other metabolites produced by microbial fermentation of cellulose and hemicellulose. This absorptive ability of the hindgut of the ostrich is further supported by observations from Bezuidenhout & Van Aswegen (1990) that the caecal mucosa has an absorptive epithelium and large veins which are present in the lamina propria.

Bezuidenhout & Van Aswegen (1990) described the villi in the distal part of the digestive tract to diminish in size and numbers, until only a few small villi were observed in the caeca and colon. They further described the crypts between the villi to be short and that they reached the peak of their development in the proximal part of the small intestine and gradually decrease in size and number towards the large intestine.

Some work has been done on the anatomy of the large intestine on different diets (Baltmanis *et al.*, 1997; Viljoen *et al.*, 2004), but these studies concentrated on the effect of various levels of fibre. No research has been done on the effect of different diets on the histological development of the large intestines of the ostrich during this crucial time of development.

The general histological structure of the various parts of the avian intestinal tract has been thoroughly described, particularly in poultry (Calhoun, 1954; Hodges, 1974; McLelland, 1979). The lumen is lined by a mucosa which is thrown into numerous villi that vary in shape, number and size and which consist of a simple columnar epithelium supported by an extension of the underlying lamina propria that forms the core of the villus. Between the *muscularis mucosae* and the base of the villi lie the crypts of Lieberkühn that open into the lumen between the villi. The final component of the mucosa is the *muscularis mucosae*, a thin but obvious layer of smooth muscle that extends fibres into the core of each villus. The submucosa is poorly developed in the small intestines of the chicken and its presence is only obvious where cell bodies of the sub-mucosal nerve plexus or an occasional large blood vessel increases the thickness of the layer. The *tunica muscularis* consists of a poorly developed outer longitudinal layer and a much thicker inner circular layer of smooth muscle. The muscular tunic is covered externally by a serosa supported by a loose connective tissue sub-serosa (Calhoun, 1954; Hodges, 1974; McLelland, 1979). These basic structural features have also been confirmed in ratites (Feder, 1972; Herd, 1985), including the ostrich (Bezuidenhout, 1990; Illanes *et al.*, 2006).

Although previous studies have provided important information on the morphological development (Wang & Peng, 2006) and structural features (Bezuidenhout, 1990; Illanes *et al.*, 2006) of the ostrich intestinal tract, the possible effects of variations in diet on the small intestine of ostrich chicks during the critical first two months have not been addressed. In this chapter the possible histological and morphometric

changes in the intestinal tract of ostrich chicks fed eight different pre-starter diets over a two month period were studied.

Materials and Methods

Animals

105 newly hatched South African Black Ostrich (*Struthio camelus* L.) chicks (58 males and 47 females) were obtained from the Oudtshoorn Experimental Farm of the Department of Agriculture, Western Cape Province, South Africa. The chicks were weighed and transported as day old chicks by road to the Kromme Rhee Experimental Farm of the Western Cape Department of Agriculture, located near Stellenbosch, Western Cape Province, South Africa. The chicks were transported in plastic containers with sufficient airflow in a closed vehicle for the 500km journey. The average weight of the day old chicks was 0.837 kg. Chicks were randomly divided into eight trial groups, 13 chicks per group, except for the high protein group, which had 14 chicks. Of the 105 chicks originally utilised in the trial, 62 chicks survived until the end of the trial period (see Table 2).

Treatments

The 8 groups of ostrich chicks were fed diets reflecting high and low levels of protein, fat, sugar and starch, respectively. The raw material used and the calculated nutrient composition of the eight trial diets are presented in Table 1.

Table 1 Raw material and calculated nutrient composition of trial diets

Ingredients	High protein %	Low protein %	High fat %	Low fat %	High starch %	Low starch %	High sugar %	Low sugar %
Maize meal	25.55	63.86	50.17	58.04	29.68	23.78	24.72	53.05
Soybean oilcake meal	43.26	8.25	20.00	27.20	22.48	11.66	21.26	20.00
Maize starch	—	—	—	—	20.00	—	—	—
Full-fat soya	16.78	12.00	12.10	—	12.00	12.00	12.00	12.00
Lucerne hay	10.00	10.00	10.00	10.00	10.00	37.74	17.26	10.00
Sugar	—	—	—	—	—	—	20.00	—
Wheat bran	—	—	—	—	—	10.00	—	—
Limestone	2.27	2.96	2.99	3.03	2.89	1.18	1.78	2.25
Plant oil	1.00	1.00	3.00	—	1.00	2.00	1.00	1.00
Mono calcium phosphate	0.49	1.28	1.07	1.06	1.26	0.94	1.28	1.04
Fine salt	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Mineral and vitamin mixture	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Synthetic methionine	—	—	0.02	0.02	0.04	0.05	0.05	0.01
Nutritional composition								
Crude protein (%)	29.83	15.21	19.51	19.02	18.89	19.27	19.05	19.71
Dry matter (%)	89.46	88.20	88.86	88.30	71.41	89.36	91.31	88.51
ME (MJ/kg feed)	14.5	14.5	14.8	13.5	14.5	13.0	14.5	14.5
Crude fibre (%)	6.35	5.18	5.47	5.34	5.14	12.96	6.93	5.53
Crude fat (%)	6.01	6.06	7.71	2.91	4.97	6.81	4.99	5.83
Starch (%)	19.50	41.43	33.48	38.23	38.22	19.00	17.78	35.28
Sugar (%)	6.54	2.99	4.03	3.58	4.61	5.30	24.43	4.04

Collection and preparation of samples

Slaughter started on day 60 of the trial and continued on a daily basis for five days. Two chicks from each treatment group were slaughtered every day, starting with a different treatment group each day. Chicks were randomly selected from each treatment group for slaughtering. The hatching and slaughter weights of the chicks in the different treatment groups are presented in Table 2.

Table 2 Hatching- and slaughter weights for ostrich chicks fed eight different pre-starter diets

Treatment groups	Number of birds at slaughter	Average hatching weight (g)	Average slaughter weight (g)
High protein	2	873.3[a]	5612.5[bc]
Low protein	8	825.6[a]	5395.6[c]
High fat	9	794.3[a]	8773.0[a]
Low fat	10	853.1[a]	7993.7[ab]
High starch	7	814.7[a]	9266.7[a]
Low starch	10	806.8[a]	8504.3[a]
High sugar	8	862.3[a]	7602.3[abc]
Low sugar	8	821.6[a]	9137.9[a]

[a, b, c] Column means with different superscripts differ significantly at $P<0.05$

Chicks were euthanized with CO_2 in a closed chamber and the gastrointestinal tract immediately removed from the carcasses. The gastrointestinal tract was spread open for identification of the different regions of the small intestine as described for the ostrich by Bezuidenhout (1986, 1999). Tissue samples representing transverse sections of the duodenum, jejunum and ileum were taken from the specific parts of the intestinal tract outlined in Table 3.

Table 3 Regions of the intestinal tract sampled for histological and morphometric analyses

Region	Specific point of sample collection
Duodenum	Samples taken from the ascending limb of the duodenal loop, immediately proximal to the secondary loop of the ascending limb.
Jejunum	Samples taken approximately 5cm proximal to the point where the vitelline duct (Meckel's diverticulum) enters the small intestine.
Ileum	Samples taken approximately 5 cm proximal to the ileo-caecal junction.
Caecum	Samples taken approximately at the 5th spiral fold from the ileo-caecal junction.
Proximal part of colon	Samples taken approximately 5 cm distal to the ileo-caecal junction.
Distal part of colon	Samples taken approximately 5 cm proximal to the cloaca.

Tissue samples were thoroughly rinsed with saline (0.9%NaCl) to remove adhering intestinal contents and mucus and immersion-fixed in 10% neutral buffered-formalin for a minimum period of 48 hours. The samples were dehydrated through 70, 80, 96, and 2X 100% ethanol and further processed through 50:50 ethanol : xylol, 2X 100% xylol and 2X paraffin wax (60–120 min per step) using a Shandon Excelsior Automatic Tissue Processor (Shandon, Pittsburgh, PA, USA). Tissue samples were then imbedded manually into paraffin wax in plastic moulds. Sections were cut at 4-6 µm, stained with haematoxylin and

eosin (H&E), viewed and micrographed, using a light microscope (Olympus CX-31) equipped with a digital camera.

Morphometric analysis

The following parameters were determined for each region of the small intestine in each of the eight groups of chicks by light microscopy of the H&E-stained tissue sections: Villus length (VL) and width (diameter) (VD), crypt depth (CD) (thickness of the glandular layer essentially representing that part of the lamina propria, adjacent to the *muscularis mucosae* housing the crypts of Lieberkühn), thickness of the *muscularis mucosae* (MM) and thickness of the inner circular (ICL) and outer longitudinal (OLL) layers of the *tunica muscularis*. The thickness of the mucosa and *tunica muscularis* was determined for each region (caecum, thick proximal section and thin distal section of the colon) of the large intestine. The measurements were made using a light microscope (Olympus CX-31) equipped with the AnalySIS™ (Soft Imaging System, 1999) software package. The specific tissue layers/structures measured are indicated in Figure 1A and Figure 1B. Ten measurements were obtained for each layer in each of the chicks in each group. Similarly, the length and width of ten villi were determined for each chick. Villus width was determined by averaging three measurements taken near the base, at the middle and towards the tip of the villus. The data was analysed according to a standard eight (diets) x three (region of intestine) factorial analysis (Snedecor & Cochran, 1980). Repeated records from the same experimental units assessed for different parts of the intestine were accounted for by adding the random effect of animal in the mixed model analysis (Harvey, 1990). Although the interaction between diet and region of the intestine was not significant ($P>0.05$) in all analyses, these interactions are provided to depict a clearer picture of the results.

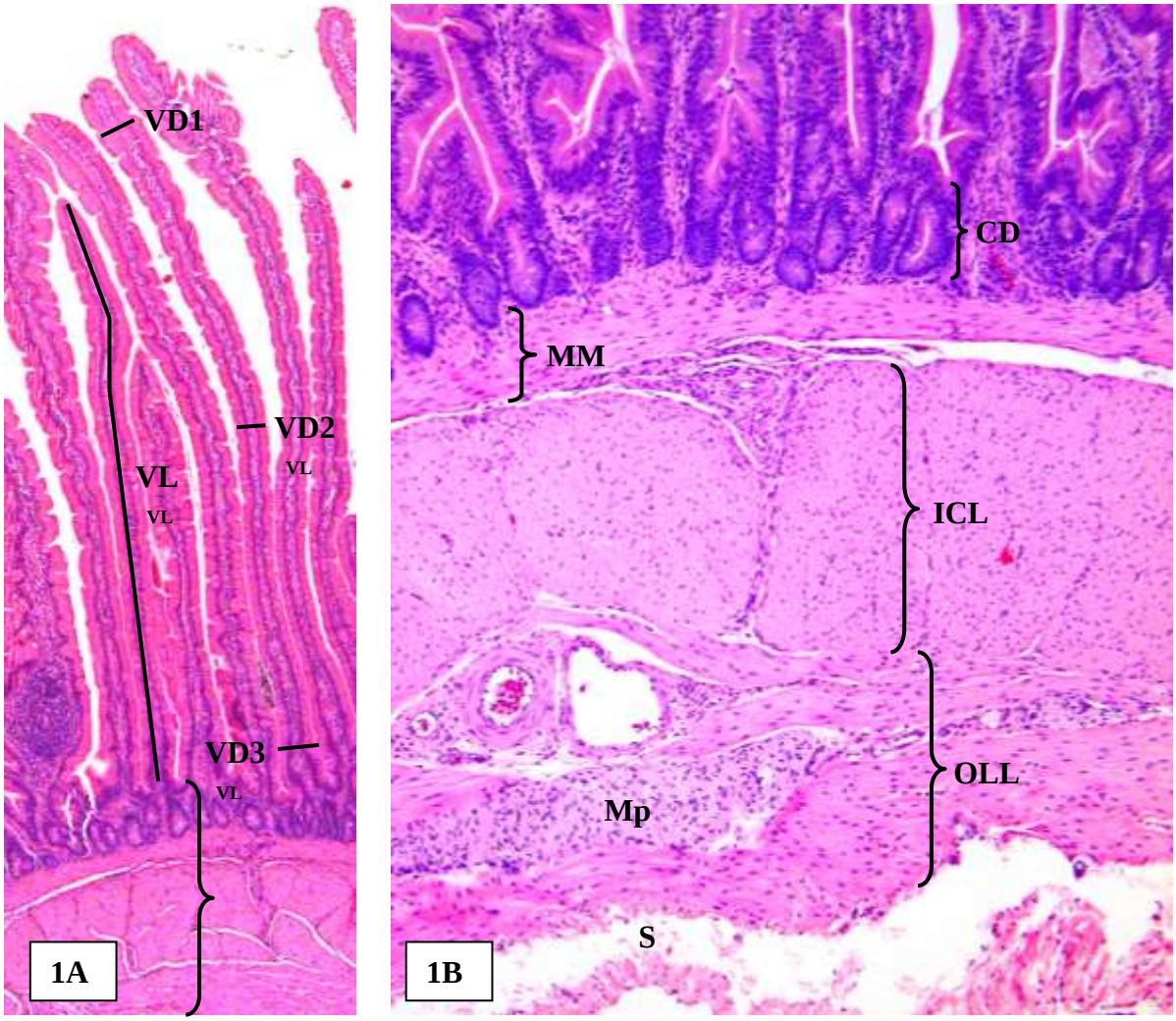


Figure 1A A section through the duodenum of a two month old ostrich (low sugar diet) illustrating the linear measurements taken of villus length (VL) and diameter (VD1 – 3). Layers bracketed at the base of the villi are similar to those shown in Figure 1B. **B** An enlargement of the base of the villi and the muscle layers of the duodenum in a two month old ostrich (high starch diet) indicating the measurements taken of crypt depth (CD) and the thickness of the *muscularis mucosae* (MM) the inner circular layer (ICL) and outer longitudinal layer (OLL) of the *tunica muscularis* and the subserosa (S). Myenteric plexus (Mp)

Results

Histological structure of the small intestine

The basic structure of the three segments of the small intestine was essentially similar to that described for 16 day old chicks (see Chapter 4) and no specific diet-related differences were observed in the histological features. Where structural features were similar, particularly between the duodenum and jejunum, references to figures are not restricted exclusively to the specific segment of the small intestine being described.

Duodenum

The duodenum displayed long, finger-like villi that extended into the lumen. All the villi were of similar length although a few shorter forms were sometimes apparent (Figure 2). They were generally single structures although a degree of branching was observed (Figure 6). Small indentations were apparent at regular intervals along the length of the villi, giving them a scalloped appearance (Figures 2, 5, 6, 7). They were tightly packed with narrow intervening inter-villous spaces (Figure 2), although localized, more loosely arranged areas were occasionally observed. The villi were lined by a simple columnar epithelium. The enterocytes consisted of chief cells and goblet cells (Figure 7). Chief cells displayed a round, vesicular nucleus situated in the basal third of the cell and a wide zone of apical cytoplasm. Higher magnification revealed a conspicuous brush border on the free surface of the chief cells. The brush border was continued onto the surface of the indentations on the villi (Figure 7). Typical pale-staining goblet cells were scattered at regular intervals between the chief cells and exhibited a narrow basal portion containing the nucleus and a swollen apical part containing secretory material (Figure 7). As in younger birds (see Chapter 4), the central core of the villus was formed by an extension of the lamina propria (Figure 7). No obvious lymphatic vessels were present but a rich capillary network extended throughout the length of the villus. A well-developed glandular layer was present in the lamina propria at the base of the villi (Figures 2 and 3). The numerous crypts of Lieberkühn were deep and closely packed, and displayed numerous mitotic figures. A number of crypts were seen to open into each inter-villous space although this was often masked by the close compaction of the individual villi. The *muscularis mucosae* were well-formed and in most sections consisted exclusively of longitudinally oriented smooth muscle fibres. However, in some sections small numbers of circularly arranged fibres were encountered adjoining the interface with the lamina propria and formed an indistinct inner circular layer of the *muscularis mucosae* (Figure 3). Tracts of smooth muscle were seen to extend from the *muscularis mucosae* into the base of the villi. The presence of lymphoid tissue was a prominent feature throughout the small intestine of the two month old birds. In the duodenum it occurred in the form of large accumulations of diffuse lymphoid tissue located within the lamina propria. Nodular lymphoid tissue was not observed in the material studied. The diffuse lymphoid tissue was most frequently encountered near the base of the villi which were distorted to form bulbous structures (Figure 2). The distended epithelial lining covering the lymphoid aggregations was smooth and lacked the convolutions present elsewhere on the villi (Figures 2 and 11). The lymphocytes were contained within the lamina propria. However, a greater number of individual lymphocytes were observed in the epithelial layer in the immediate vicinity of the lymphoid tissue than throughout the rest of the epithelium. The number of migrating lymphocytes was greater than that observed in the younger ostrich chicks (see Chapter 4). Lymphoid aggregations also extended between the crypts completely isolating them. Fewer crypts were observed in these regions.

As in the young ostrich chicks (see Chapter 4), a sub-mucosa was virtually non-existent and could only be identified due to the presence of occasional blood vessels (Figures 3, 4, 8, 12). However, large nerve and vascular plexuses were observed (Figures 3, 4, 12). Again, there was a tendency in some sections for the

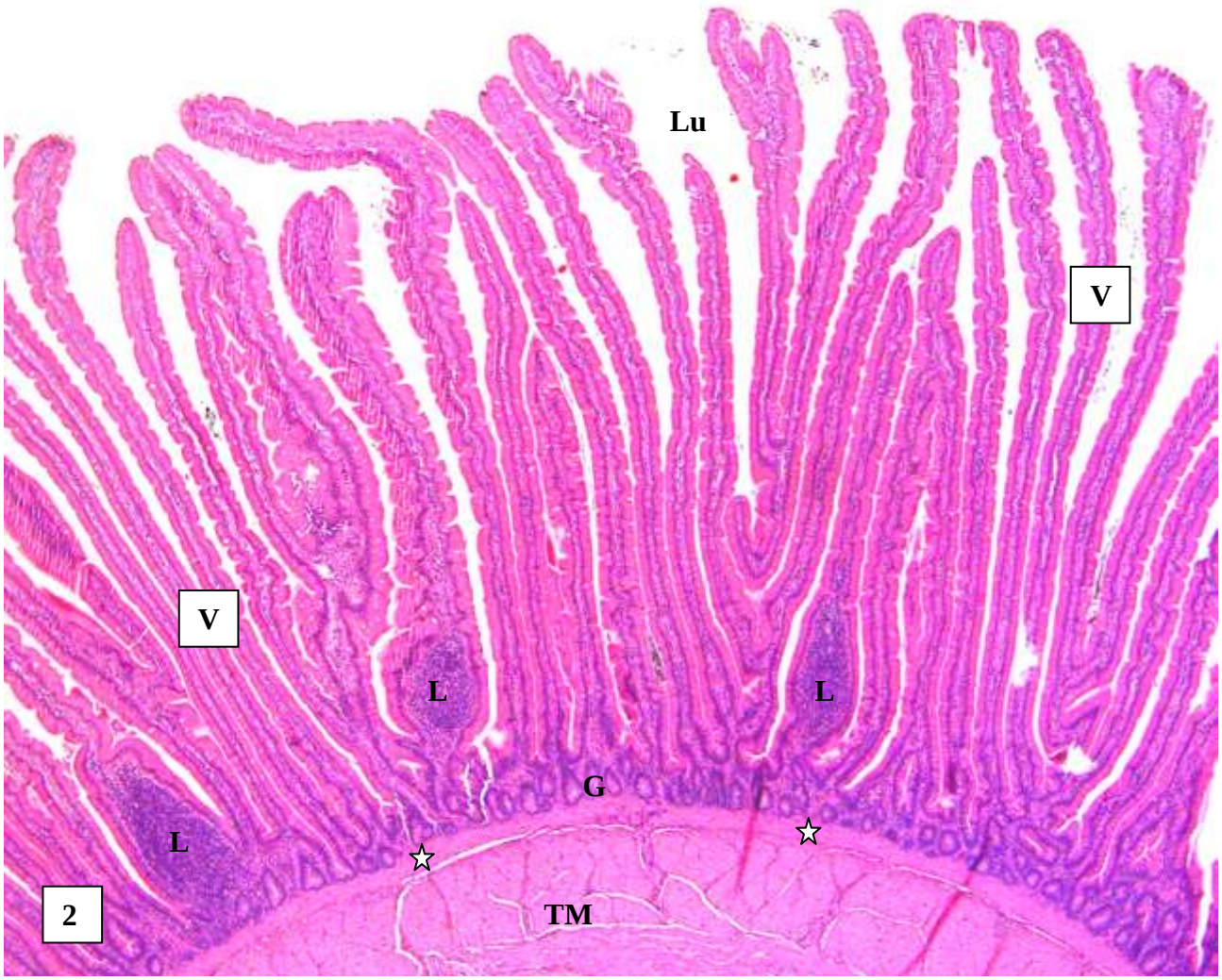
tissue layers to separate at the sub-mucosa. The thick inner circular layer (ICL) of the *tunica muscularis* displayed longitudinally oriented smooth muscle fibres. In transverse sections of the duodenum this layer appeared continuous, but in longitudinal sections (indicated by the smooth muscle fibres appearing in cross-section) it was seen to consist of large tissue blocks demarcated by thin strands of connective tissue containing blood vessels and nerves (Figure 4). The outer longitudinal layer (OLL) was visibly thinner than the circular layer (see morphometric data below) and was composed of numerous muscle bundles. Large nerve (non-medullated) and vascular plexuses were positioned between the ICL and OLL (Figures 1B and 4). In some instances large blood vessels and elements of the myenteric plexus separated the muscle bundles of the OLL and occupied part of the subserosa. Blood vessels from this region extended through the ICL of the *tunica muscularis* to form a vascular plexus in the submucosa (Figure 4). From here vessels entered the lamina propria, where they formed a plexus of smaller vessels at the base of the villi before branching into the core of the villi. The subserosa varied in thickness and was composed of a layer of loose, almost mucus-like connective tissue covered externally by a serosa (Figure 13). The subserosa displayed conspicuous vascular collections consisting of a prominent artery and vein and attendant lymphatic vessels and nerves (Figure 13).

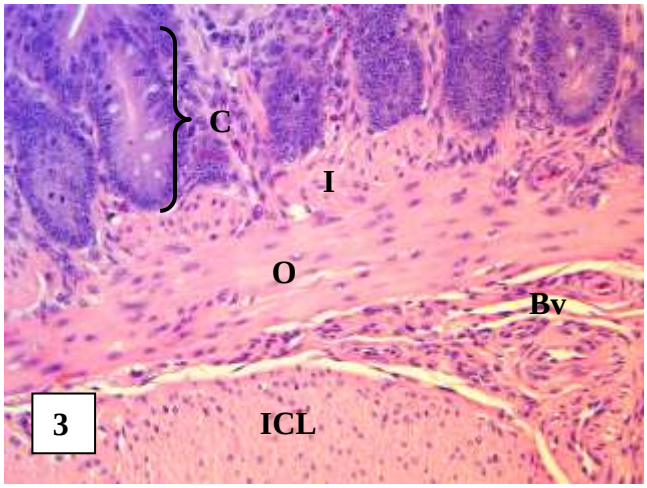
Jejunum

The histological features of the jejunum were similar in most respects to those of the duodenum (Figures 5, 6, 7, 8, 9). In some specimens a high degree of branching of the villi was observed (see Figure 6), although this was not connected to a particular diet. There also appeared to be a higher concentration of goblet cells in the epithelial lining but this was highly variable (see Figures 6 and 8). The glandular layer appeared as well-developed as that of the duodenum. Both layers of the *tunica muscularis* displayed a high degree of partitioning and were composed of numerous individual muscle bundles separated by strands of connective tissue in similar fashion to that observed in the ileum (see below).

Ileum

As was already obvious in the 16 day old chicks (see Chapter 4), the ileum displayed a number of important structural differences when compared to the duodenum and jejunum. The villi were visibly shorter (see morphometric data below) than those of the other components of the small intestine and appeared in a variety of forms, from stubby, finger-like projections to club-shaped and highly folded extensions (Figure 10). The glandular layer (crypt depth) was prominent but displayed a low density of crypts (Figure 10). Lymphoid tissue was particularly conspicuous in the ileum (Figures 10 and 11). When present, the inner circular layer of the *muscularis mucosae* appeared to be best developed in the ileum. Both layers of the *tunica muscularis* were discontinuous and consisted of numerous muscular bundles separated by strands of connective tissue (Figures 12 and 13). This was particularly obvious in the outer longitudinal layer where the connective tissue was continuous with the subserosa (Figure 13).





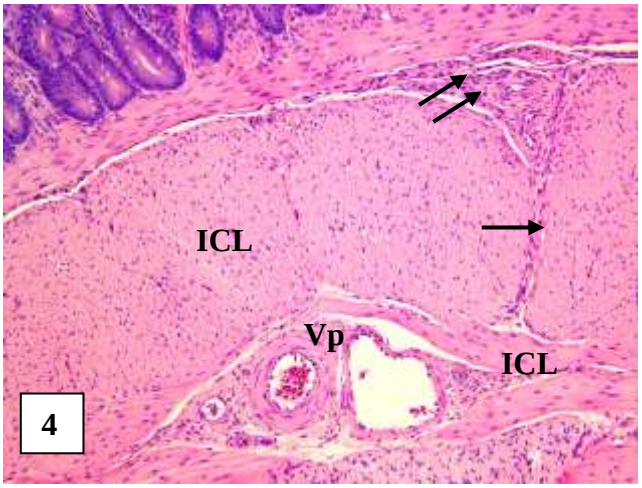
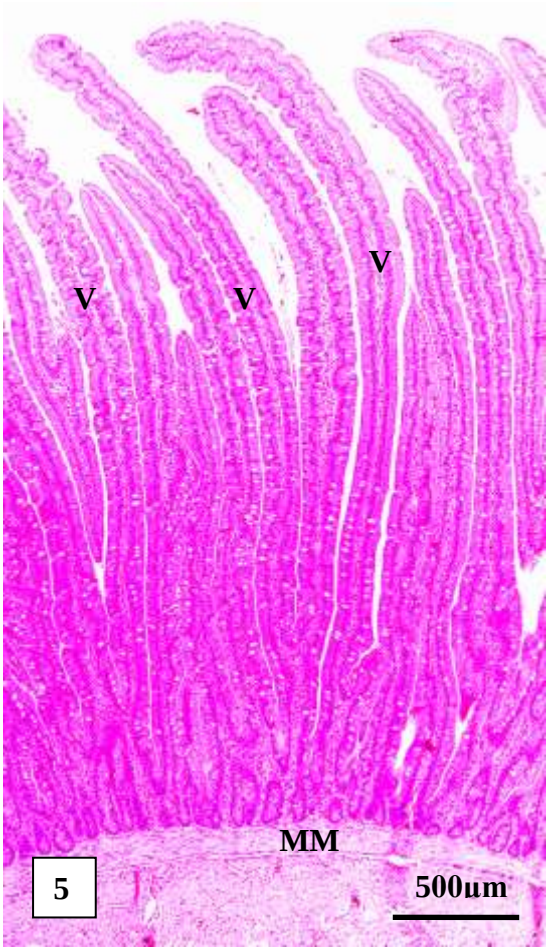
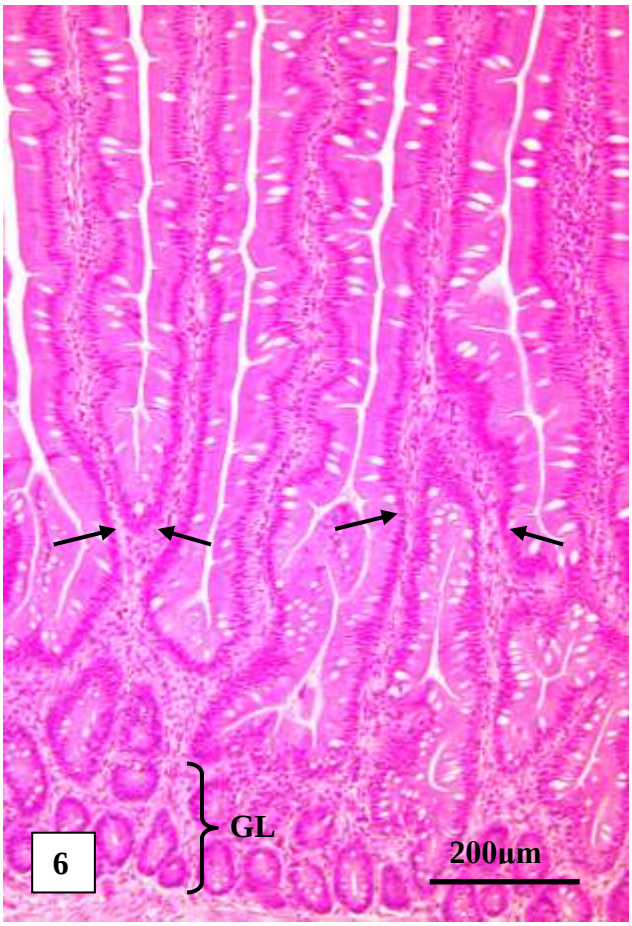


Figure 2 Longitudinal section of the duodenum of a two month old chick showing the tightly packed villi (V) of similar length extending into the lumen (Lu). Lymphoid aggregations (L) expand the base of some villi. Glandular layer (G), *Muscularis mucosae* (stars), *Tunica muscularis* (TM)
Figure 3 Enlargement of the intestinal crypts (C) and adjacent muscle layers illustrated in Figure 2. The *muscularis mucosae* (MM) demonstrates inner circular (I) and outer longitudinal (O) layers. The submucosa is widened by a plexus of blood vessels (Bv). Inner circular layer of the *tunica muscularis* (ICL)
Figure 4 The inner circular layer (ICL) of the *tunica muscularis* forms large tissue blocks. The vascular plexus (Vp) between this layer and the outer longitudinal layer (OLL) extends between the muscle blocks (arrow) to lie in the submucosa (double arrow). *Muscularis mucosae* (MM)





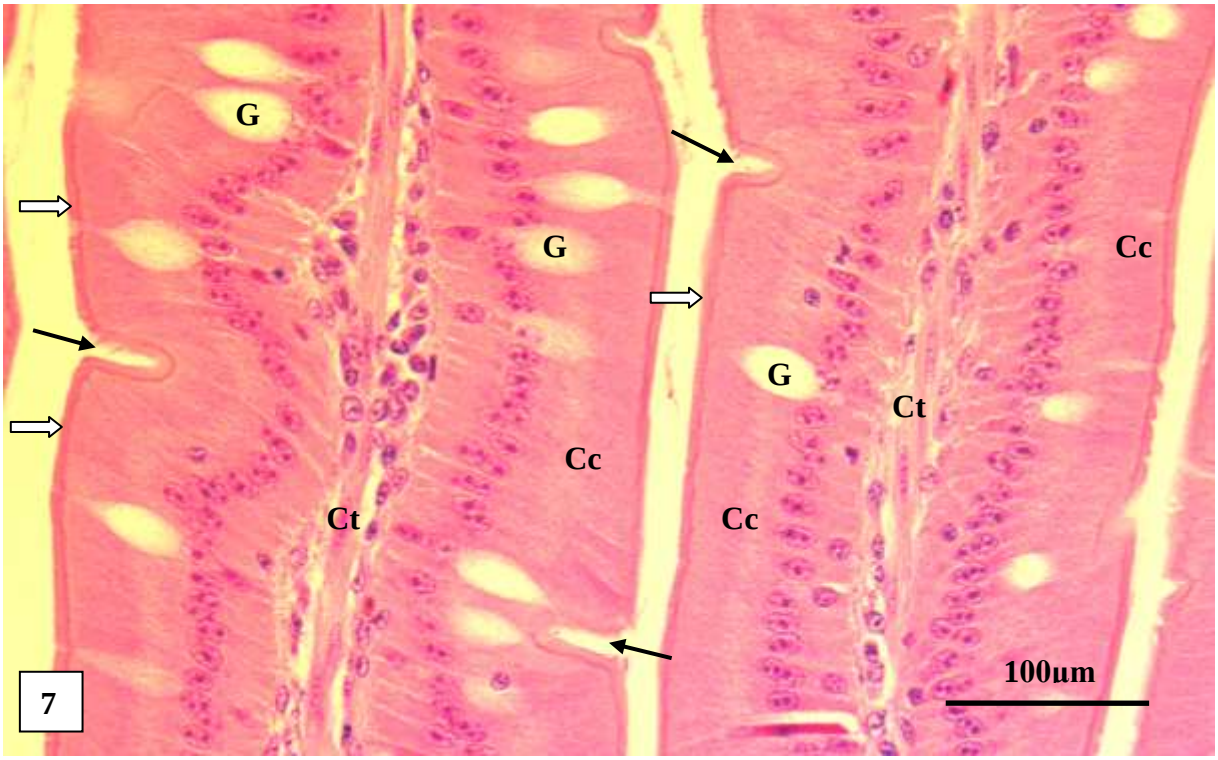
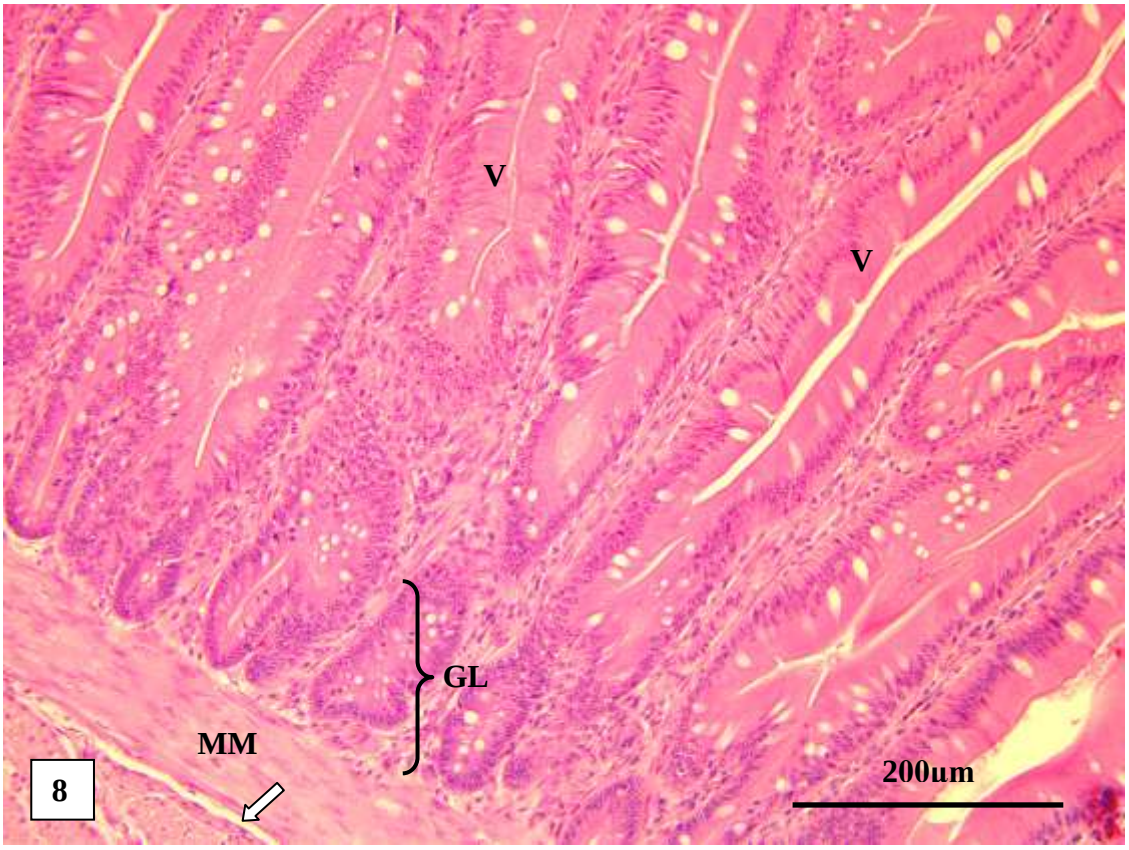


Figure 5 Typical long, finger-like villi (V) found in the jejunum. Note the similarity to the duodenal villi shown in Figure 2. No lymphoid tissue is apparent. *Muscularis mucosae* (MM)
Figure 6 Higher magnification of the base of the duodenal villi. Note the branching of the villi (arrows) and the depth of the glandular layer (GL) housing the crypts
Figure 7 Two adjacent duodenal villi lined by tall simple columnar chief cells (Cc) and numerous goblet cells (G). The connective tissue core (Ct) and brush border (white arrows) are obvious. Epithelial clefts (black arrows)



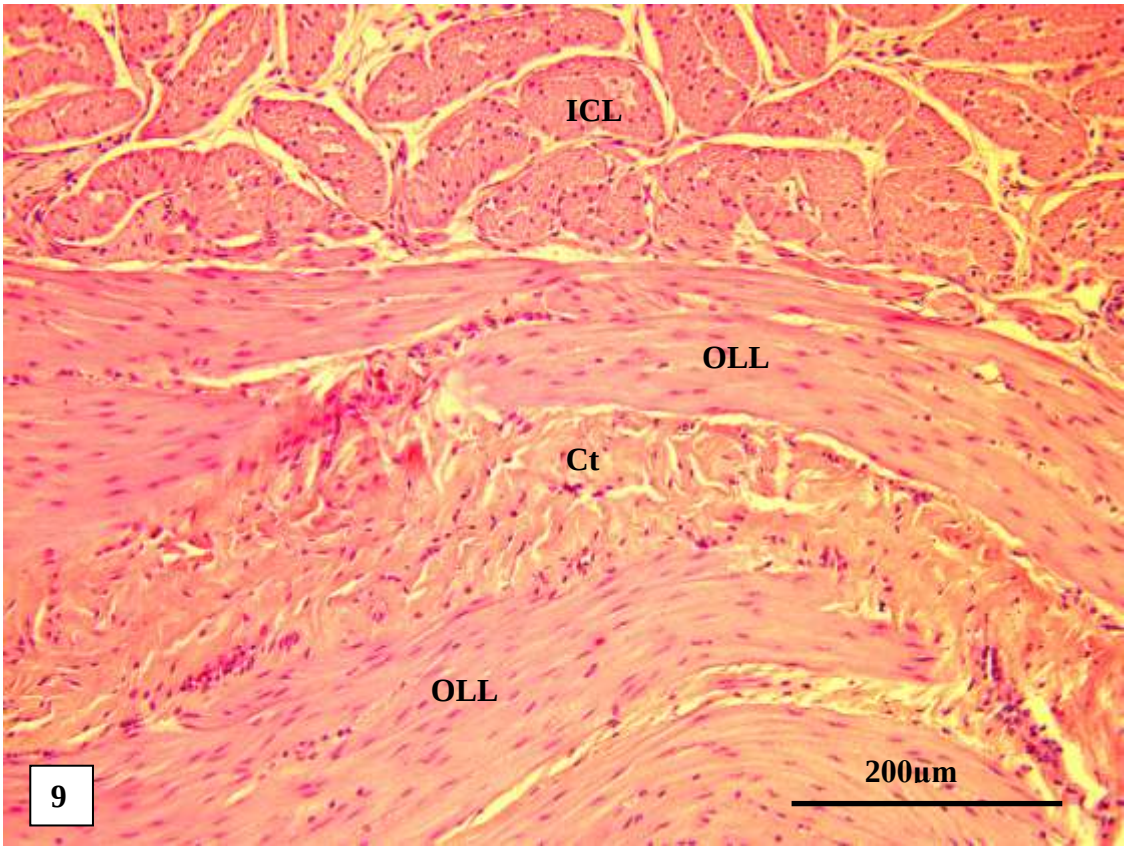
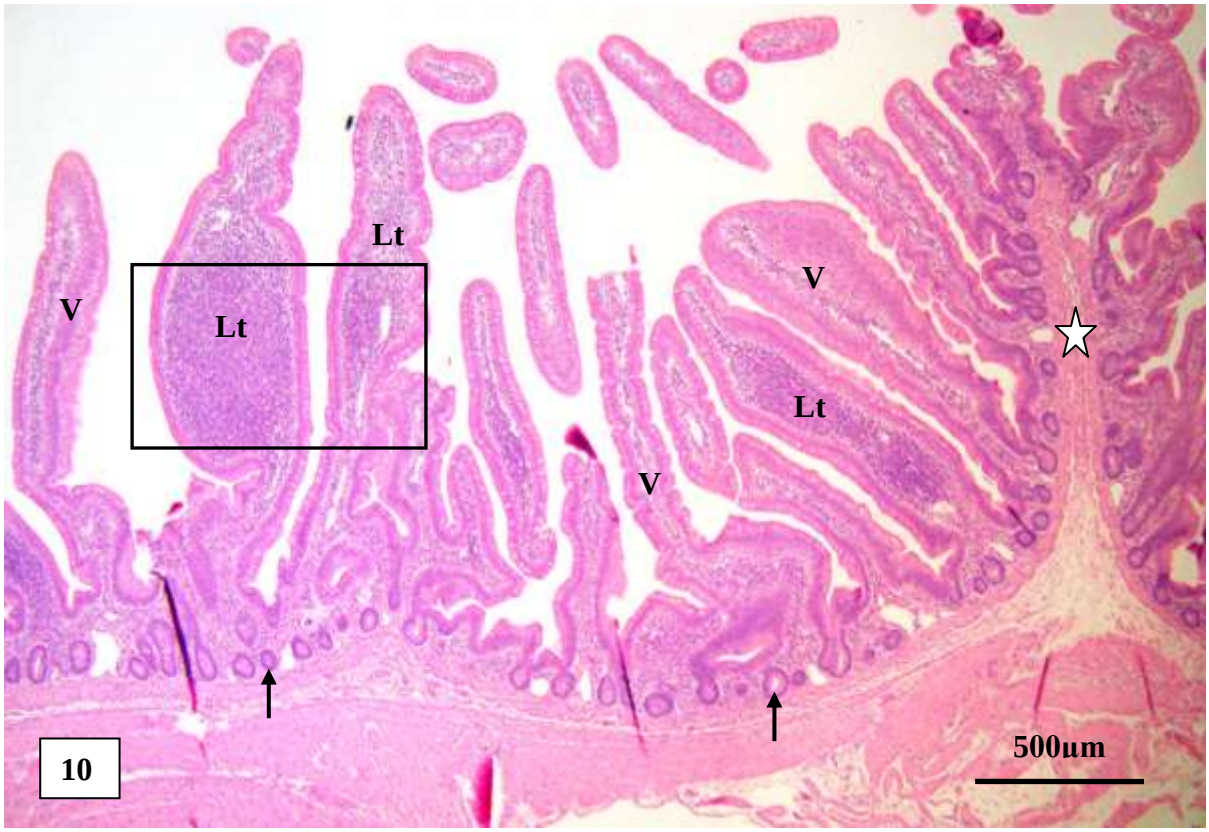


Figure 8 Base of the jejunal villi (V) showing the glandular layer (GL) resting on the *muscularis mucosae* (MM) which in turn is separated from the ICL of the *tunica muscularis* by a thin submucosa (white arrow)
Figure 9 Longitudinal section of the *tunica muscularis* illustrating the broken nature of both the inner circular (ICL) and outer longitudinal (OLL) layers. Intervening tracts of connective tissue (Ct) are particularly obvious in the OLL



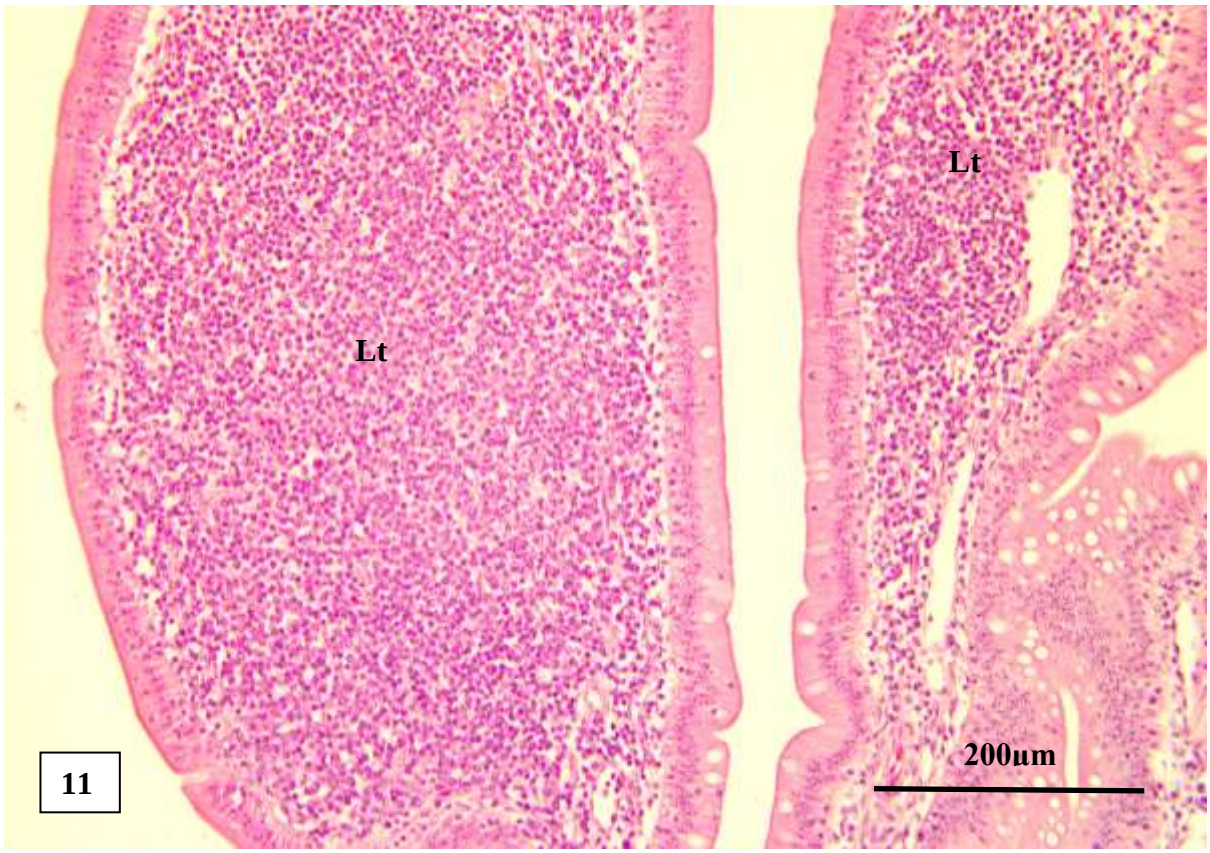
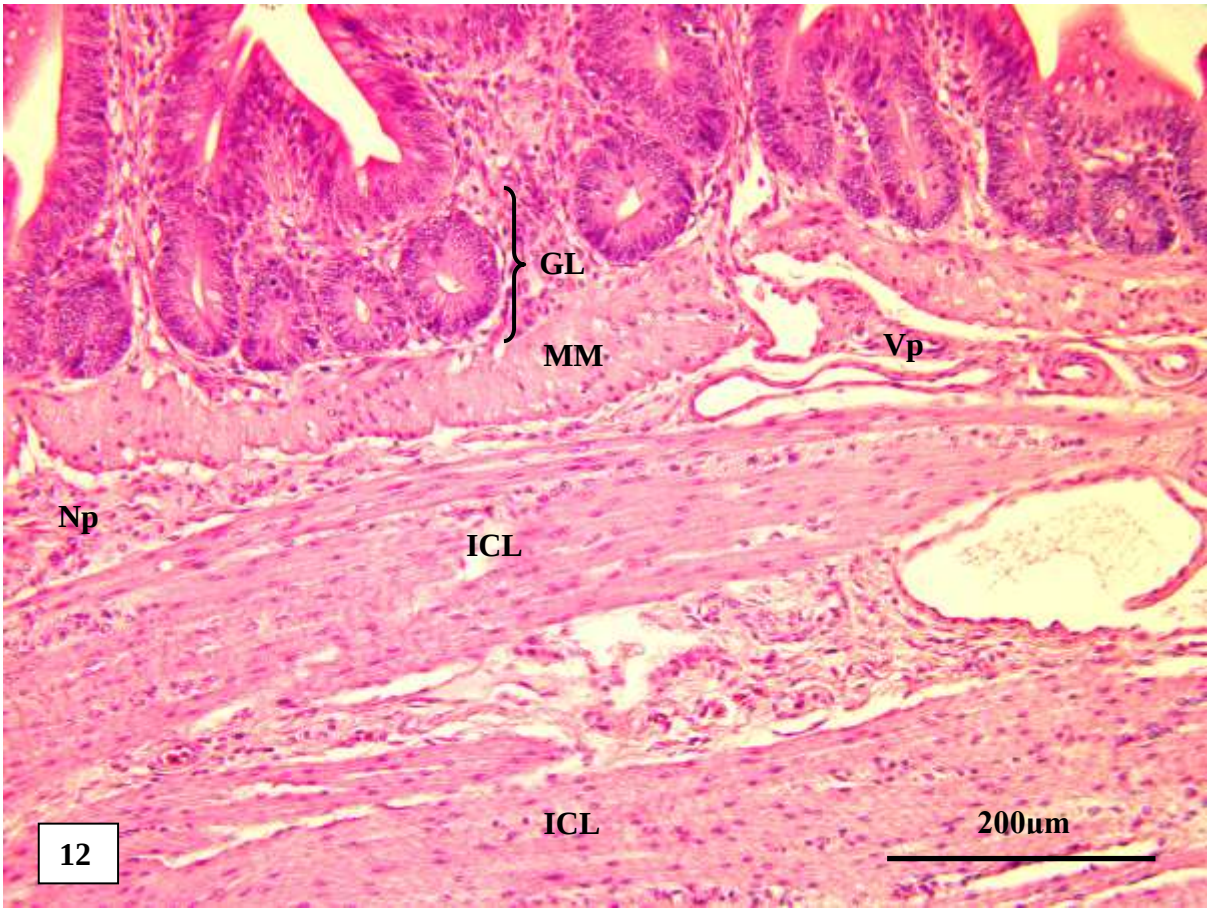


Figure 10 Low magnification of the wall of the ileum. Note the short, variably shaped villi (V), the large mucosal fold (star), lymphoid tissue (Lt) and the sparsely arranged crypts (arrows) in the glandular layer

Figure 11 An enlargement of the rectangle in Figure 10 demonstrating the accumulation of diffuse lymphoid tissue (Lt) in two adjacent villi



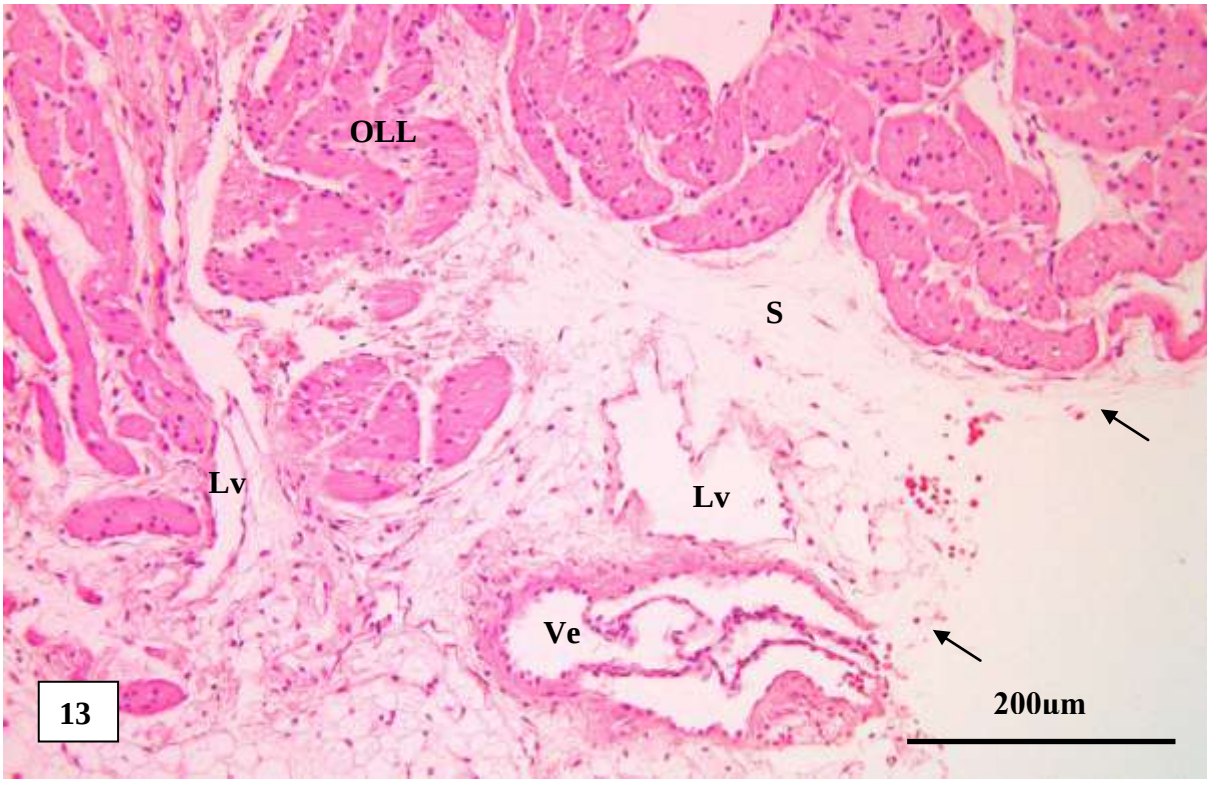


Figure 12 Transverse section of the base of the mucosa in the ileum showing the glandular layer (GL), the *muscularis mucosae* (MM), the submucosa with a nerve (Np) and vascular (Vp) plexus, and the inner circular layer (ICL) of the *tunica muscularis*
Figure 13 The outer longitudinal layer (OLL) of the *tunica muscularis* and the subserosa (S) which displays lymphatic vessels (Lv) and a vein with a valve (Ve). Note the broken appearance and longitudinal orientation of the smooth muscle cells of the OLL. Serosa (arrows)

Histological structure of the large intestine

No obvious histological differences in the structure of the large intestine were observed between the various diets and the following description is based on the general observations of all the material.

Caeca and proximal colon

The caeca (Figure 14) and the thick part of the colon (Figure 15) were similar in appearance. Short, broad villi protruded from the mucosal surface and in places seemed to flow into each other. The villi were lined by a high simple columnar epithelium which displayed only occasional goblet cells (Figure 16). Occasional lymphocytes were scattered throughout the epithelium. However, large aggregations of lymphoid tissue were rarely observed in the material studied. Extending between the villi were short crypts of Lieberkuhn which formed a relatively thin glandular layer within the lamina propria. The crypts extended to the base of the lamina propria where they lay against the *muscularis mucosae* (Figure 14). Twin crypts were sometimes observed to open between the villi (Figure 14). In sharp contrast to the surface epithelium, the lining of the crypts was dominated by goblet cells (Figure 14). The supporting lamina propria was cell-rich and extended into the intestinal villi carrying small blood vessels which were often seen at the base of the villi. The underlying *muscularis mucosae* was generally relatively thin and formed a single layer of longitudinally oriented smooth muscle fibres. However, in some areas there was evidence of an inner circular layer of smooth muscle fibres in addition to the longitudinal layer (Figure 14 & 15). In comparison to the small intestine, the submucosa formed a substantial layer between the *muscularis mucosae* and the *tunica muscularis*. The loose connective tissue of this layer contained a well-developed vascular plexus and nerves that supplied the overlying mucosa. The *tunica muscularis* consisted of a thick inner circular and a thinner outer longitudinal layer of smooth muscle. Both layers displayed a degree of compartmentalization with blocks of tissue being demarcated by connective tissue strands. A well-developed myenteric and vascular plexus was positioned between the two muscle layers. Nerves and blood vessels from these plexuses coursed through the inner circular muscle layer via the connective tissue strands to form the submucosal plexuses described above. The serosa was formed by a simple squamous epithelium (mesothelium) and was supported by a substantial subserosa which was more fibrous in nature than that observed in the small intestine. Both these segments of the large intestine were characterised by the presence of large folds (plicae), particularly in the caeca. The folds displayed the basic morphological features outlined above except for the absence of the outer longitudinal layer of the *tunica muscularis*, the serosa and subserosa.

Distal colon

This region displayed the same general arrangement of tissue layers to that seen in the other segments of the large intestine (Figure 17). However, a number of important differences were noted. The villi in this region were slightly narrower and appeared more separated, creating the impression that fewer villi were present (Figure 18). The crypts were also longer although in most instances they did not extend to the

muscularis mucosae. The most striking difference was observed in the composition of the surface epithelium which was characterized by large numbers of goblet cells (Figure 18). The lamina propria appeared less cellular than that of the other two regions and the *muscularis mucosae* formed a conspicuous layer composed of inner circular and outer longitudinal layers of smooth muscle. The inner and outer layers of the *tunica muscularis* were approximately of similar width. This part of the large intestine contained no folds (Figure 18).

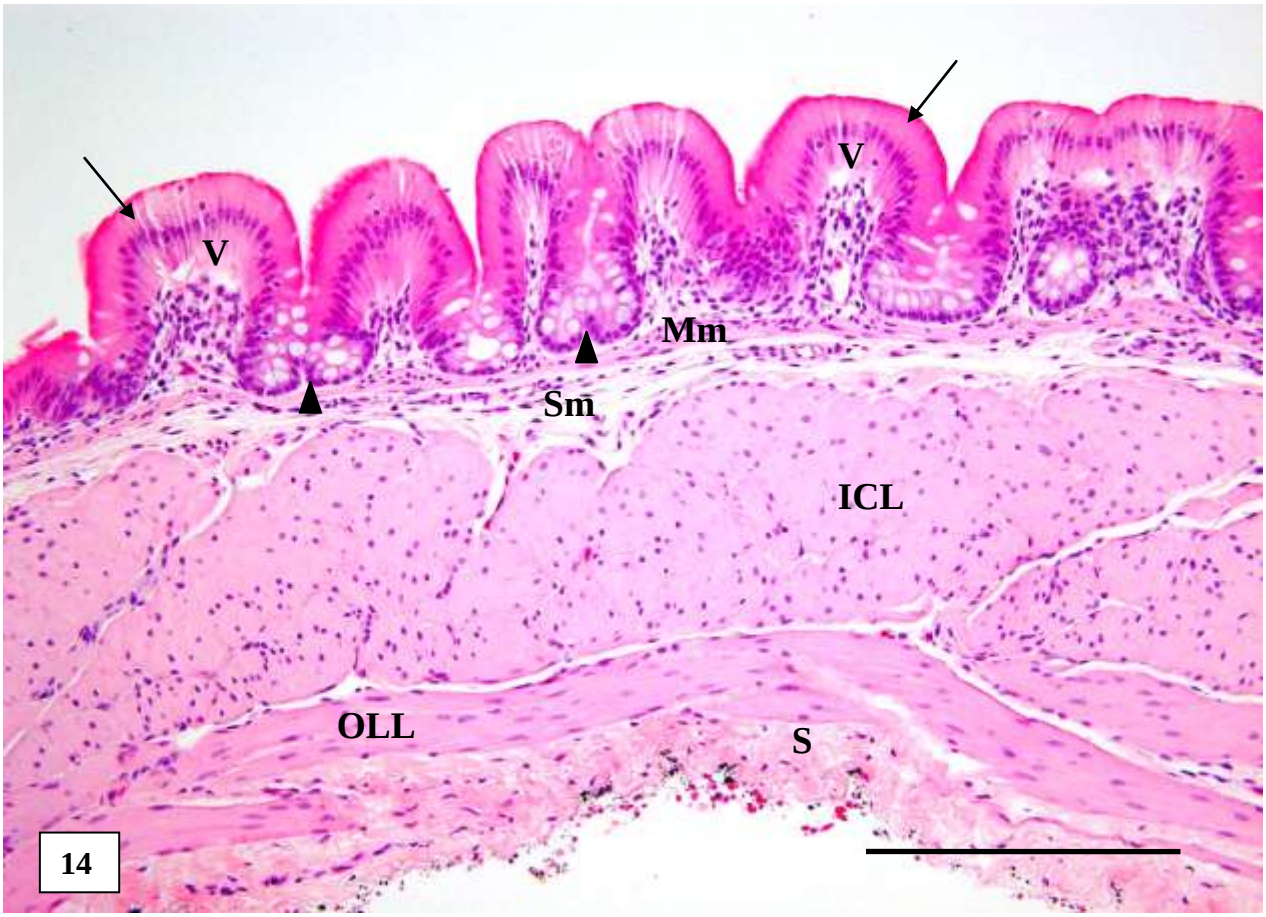


Figure 14 Longitudinal section through the caecum of an ostrich fed a high starch diet. Note the short, broad villi (V), the simple columnar epithelium free of goblet cells (arrows), the crypts of Lieberkühn lined by many goblet cells (arrowheads) and the thin outer longitudinal layer of the *tunica muscularis* (OLL). Lamina propria (Lp), *muscularis mucosae* (Mm), submucosa (Sm), thick inner circular layer of the *tunica muscularis* (ICL), subserosa (S). Bar = 200 µm

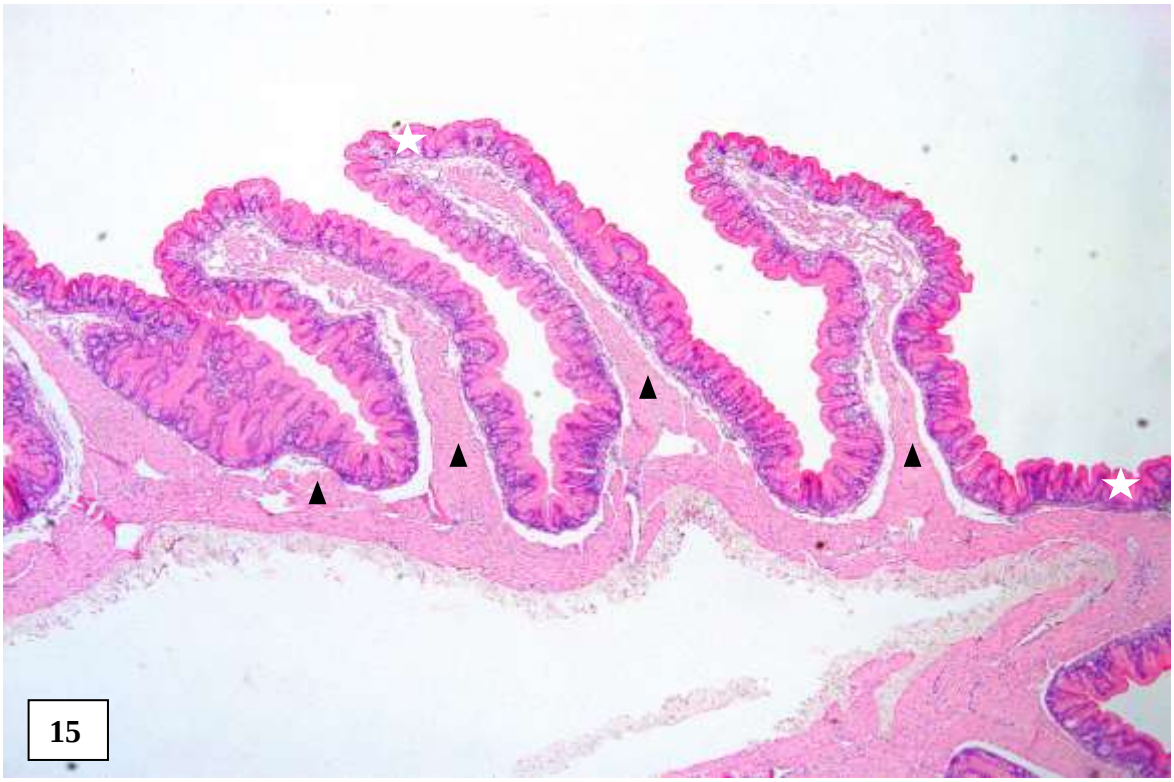


Figure 15 Low power micrograph showing the folds (arrows) in the proximal colon of a two-month old ostrich chick fed a high starch diet. Note how the inner circular muscle layer of the *tunica muscularis* extends into the folds. The villi covering the folds (stars) are similar to those on the surface of the colon. Bar = 1000 µm

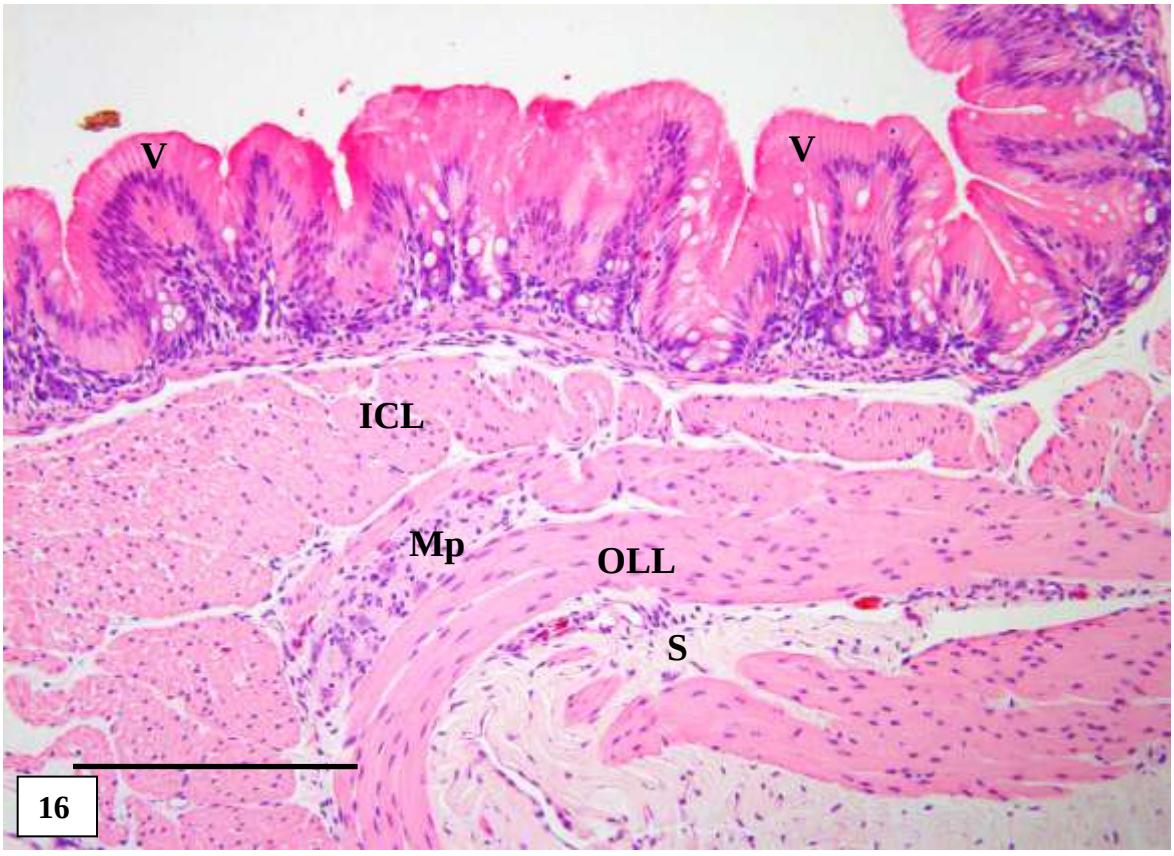


Figure 16 Higher magnification of the proximal colon of a two month old ostrich chick fed a high starch diet. Note the short, broad villi (V), the inner circular layer (ICL) and outer longitudinal layer (OLL) of the *tunica muscularis*. Subserosa (S), myenteric plexus (Mp). Bar = 200 µm

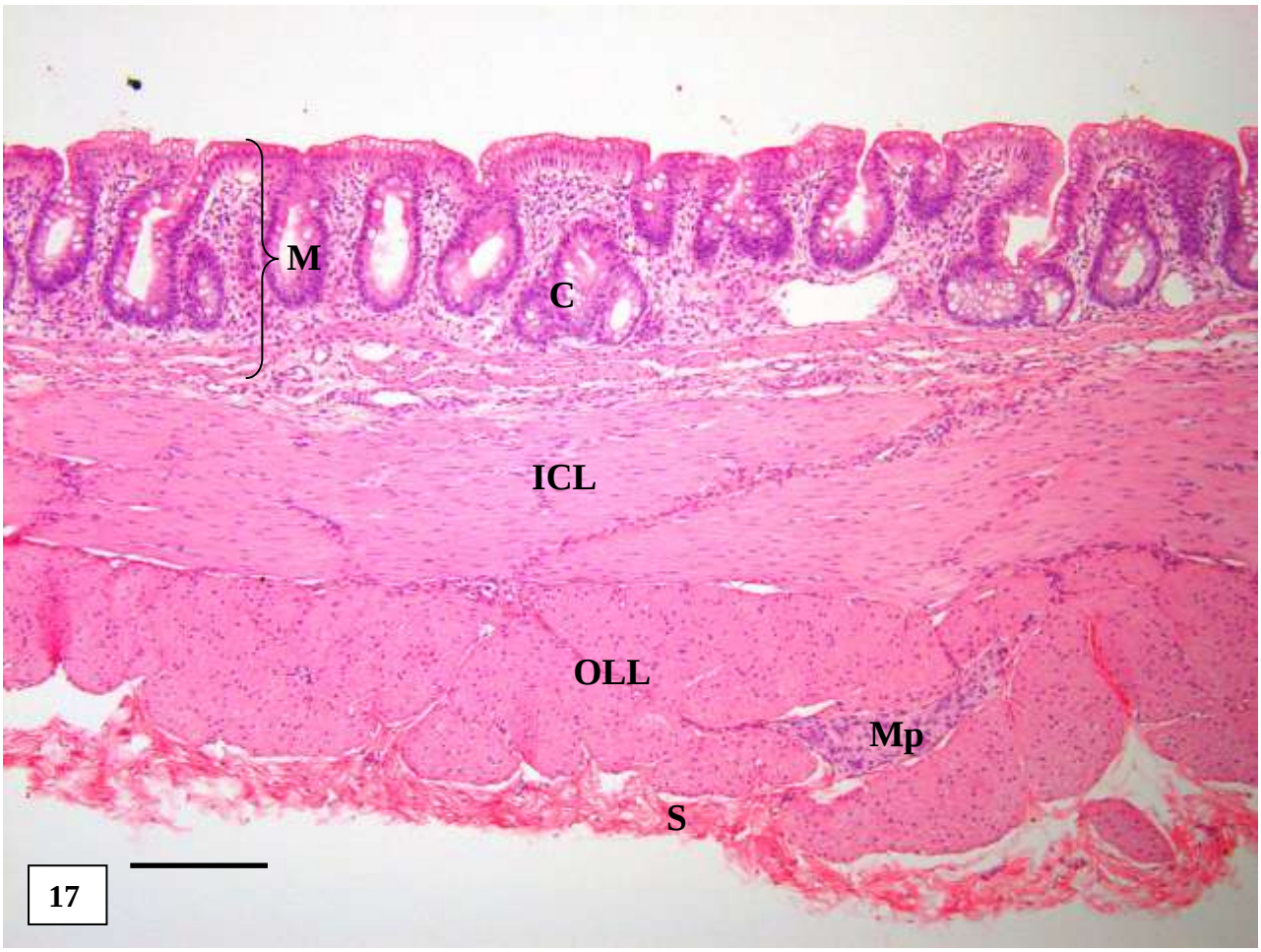


Figure 17 Longitudinal section through the distal colon of a two month old ostrich chick fed a low sugar diet. Mucosa (M), crypts of Lieberkühn (C). The outer longitudinal layer (OLL) and inner circular layer (ICL) of the *tunica muscularis* are of similar thickness. Subserosa (S), myenteric plexus (Mp). Bar = 200 µm

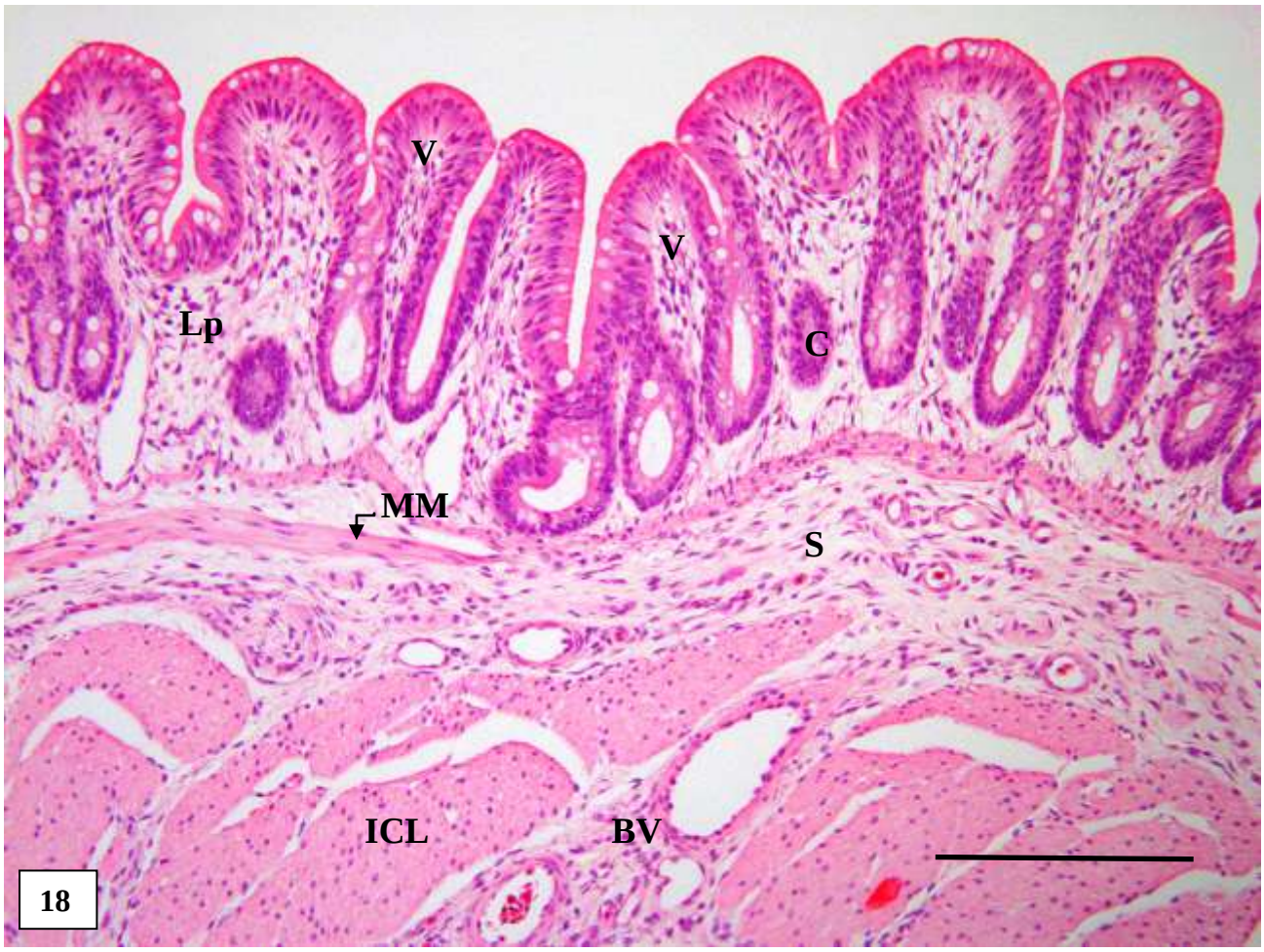


Figure 18 Longitudinal section through the distal colon of a two month old ostrich chick fed a low fat diet. The villi (V) are narrower and appear separated, and the crypts of Lieberkühn (C) do not extend to the *muscularis mucosae* (MM). The *muscularus mucosae* is composed of inner circular and outer longitudinal layers of smooth muscle. The thick inner circular layer of the *tunica muscularis* (ICL) is visible with blood vessels (Bv) coursing through a connective tissue strand. Note the thick submucosa (S). Bar = 200 µm

Morphometric analysis

The dimensions measured in the various parts of the small intestines for the eight diets are indicated in Table 4.

Table 4 Means (± SE) of the various layers measured of the small intestine

Diet	VL	VD	CD	MM	ICL	OLL	V:C ratio
Duodenum							
High protein	1793.2 ± 283.1^{ab}	104.7 ± 15.9^{a}	108.3 ± 23.4^{ab}	57.1 ± 10.0^{abc}	145.4 ± 80.1^{a}	83.4 ± 70.7^{a}	14.4 ± 3.25^{a}
Low protein	1878.0 ± 120.6^{b}	155.6 ± 6.70^{b}	108.1 ± 8.48^{b}	60.2 ± 3.63^{abc}	295.8 ± 40.4^{bcd}	170.6 ± 36.6^{ab}	19.9 ± 1.22^{b}
High fat	2166.1 ± 125.6^{ac}	155.9 ± 7.00^{b}	130.8 ± 8.79^{ac}	57.0 ± 3.65^{ab}	279.4 ± 44.5^{bc}	170.8 ± 39.9^{ab}	17.4 ± 1.28^{a}
Low fat	2142.9 ± 108.8^{ac}	137.3 ± 6.05^{c}	140.1 ± 7.39^{c}	67.2 ± 3.17^{ac}	348.3 ± 33.8^{bd}	188.2 ± 32.5^{b}	16.2 ± 1.10^{a}
High starch	2106.8 ± 126.8^{abc}	160.2 ± 7.04^{b}	151.0 ± 9.10^{c}	56.7 ± 3.79^{ab}	270.9 ± 40.4^{c}	205.8 ± 36.6^{bc}	18.0 ± 1.35^{ab}
Low starch	2299.9 ± 111.0^{c}	166.0 ± 6.19^{b}	135.5 ± 7.97^{ac}	67.0 ± 3.17^{ac}	311.3 ± 36.0^{bcd}	228.3 ± 32.4^{bc}	18.1 ± 1.20^{ab}
High sugar	2283.9 ± 117.2^{c}	151.1 ± 6.37^{b}	154.2 ± 8.26^{c}	63.0 ± 3.55^{abc}	357.6 ± 37.8^{bd}	268.2 ± 34.2^{c}	16.3 ± 1.18^{a}
Low sugar	1954.9 ± 118.9^{abc}	133.1 ± 6.59^{c}	145.5 ± 8.52^{c}	61.3 ± 3.65^{abc}	384.3 ± 53.5^{d}	195.4 ± 48.4^{abc}	15.2 ± 1.20^{a}
Jejunum							
High protein	1433.4 ± 229.4^{a}	180.8 ± 12.7^{a}	125.2 ± 23.4^{abcd}	96.1 ± 10.0^{a}	170.3 ± 75.6^{a}	149.5 ± 68.4^{ad}	8.71 ± 3.25^{a}
Low protein	1654.2 ± 115.2^{a}	129.5 ± 6.37^{b}	114.8 ± 8.26^{abd}	61.2 ± 3.55^{b}	379.3 ± 43.3^{bc}	255.2 ± 38.0^{ab}	16.8 ± 1.16^{b}
High fat	1739.4 ± 121.1^{ab}	153.4 ± 6.73^{cd}	110.2 ± 8.52^{ab}	67.4 ± 3.65^{bd}	373.5 ± 51.2^{bc}	225.1 ± 43.6^{ab}	16.5 ± 1.22^{bc}
Low fat	1637.3 ± 103.1^{a}	142.7 ± 5.70^{c}	121.2 ± 7.39^{abd}	75.8 ± 3.23^{c}	353.1 ± 40.7^{bc}	110.3 ± 35.6^{d}	14.6 ± 1.04^{c}
High starch	2047.0 ± 129.3^{c}	146.2 ± 7.18^{cd}	129.3 ± 8.83^{ad}	69.2 ± 3.79^{cd}	303.1 ± 45.5^{b}	260.8 ± 42.2^{b}	16.9 ± 1.31^{bc}
Low starch	1995.3 ± 106.2^{c}	150.9 ± 5.89^{cd}	153.8 ± 7.79^{ac}	64.5 ± 3.17^{bd}	398.0 ± 38.7^{c}	427.7 ± 33.8^{c}	15.6 ± 1.09^{bc}
High sugar	1910.6 ± 118.2^{cb}	151.1 ± 6.55^{cd}	124.8 ± 8.48^{abd}	66.3 ± 3.64^{bd}	354.9 ± 38.1^{bc}	215.2 ± 34.3^{ab}	15.6 ± 1.19^{bc}
Low sugar	1919.8 ± 129.5^{cb}	162.7 ± 7.24^{ad}	133.6 ± 8.79^{ad}	78.2 ± 3.76^{c}	561.8 ± 62.5^{d}	194.3 ± 53.1^{abd}	15.8 ± 1.32^{bc}
Ileum							
High protein	1270.1 ± 289.5^{a}	186.6 ± 16.3^{a}	131.5 ± 23.4^{a}	61.3 ± 10.0^{a}	133.4 ± 80.1^{a}	107.0 ± 70.7^{a}	9.38 ± 3.32^{a}
Low protein	661.9 ± 118.3^{b}	159.4 ± 6.56^{b}	95.7 ± 8.26^{b}	52.1 ± 3.55^{abd}	322.4 ± 42.2^{b}	288.5 ± 37.5^{b}	6.68 ± 1.19^{ab}
High fat	858.1 ± 114.7^{bc}	135.4 ± 6.34^{cd}	94.4 ± 8.26^{b}	55.9 ± 3.55^{acd}	631.5 ± 55.9^{d}	448.9 ± 46.1^{c}	9.57 ± 1.15^{ac}
Low fat	805.5 ± 102.6^{bc}	142.9 ± 5.67^{cd}	95.6 ± 7.39^{b}	59.7 ± 3.17^{ac}	470.3 ± 40.7^{c}	514.5 ± 37.5^{c}	9.02 ± 1.03^{ac}
High starch	890.4 ± 122.6^{bc}	143.2 ± 6.78^{cd}	99.2 ± 8.83^{b}	54.7 ± 3.79^{abcd}	525.7 ± 43.3^{ce}	453.4 ± 38.2^{c}	9.50 ± 1.23^{ac}
Low starch	912.3 ± 102.7^{c}	166.4 ± 5.68^{ab}	111.5 ± 7.79^{ab}	57.1 ± 3.23^{acd}	251.6 ± 106.9^{ab}	279.6 ± 96.7^{b}	8.87 ± 1.09^{abc}
High sugar	960.5 ± 114.7^{ac}	146.1 ± 6.34^{bcd}	97.8 ± 8.26^{b}	47.5 ± 3.55^{ac}	566.6 ± 40.8^{de}	330.6 ± 35.7^{b}	10.03 ± 1.15^{ac}
Low sugar	863.8 ± 114.7^{bc}	151.5 ± 6.34^{bc}	111.2 ± 8.26^{ab}	62.3 ± 3.55^{b}	589.7 ± 62.5^{de}	283.6 ± 53.1^{b}	8.16 ± 1.15^{abc}

$^{a, b, c, d, e}$ Column means with different superscripts differ significantly at $P < 0.05$

Discussion

Histologically the small intestine resembled that of the 16 day old chicks (see Chapter 4) and further confirmed that the morphological features of the birds used in this trial were similar to those recorded for birds in general (Calhoun, 1954; Hodges, 1974; McLelland, 1979) and specifically for the ostrich (Bezuidenhout & Van Aswegen, 1990; Bezuidenhout, 1993). No obvious diet-related differences were observed.

Duodenum

Villus length (VL) in the duodenum was similar for most of the diets, except for high sugar and low starch diets, where villi were significantly ($P<0.05$) longer than villi from the low and high protein diets. Longer villi reportedly result in an increased absorptive surface area (De Verdal, 2010) which conceivably would promote growth (Yamauchi *et al.*, 2006). Villus diameter (VD) was also similar for most of the diets although the villi from the high protein and low sugar diets were significantly ($P<0.05$) narrower than those from the other diets. Crypt depth (CD), as for VL, showed significantly lower values ($P<0.05$) for the high and low protein diets when compared to the other diets. A similar tendency was reported by De Verdal (2010) in a study conducted on broiler chickens. The width of the *muscularis mucosae* (MM) remained relatively similar, although the values for the low fat and low starch diets were significantly ($P<0.05$) higher than those for the high fat and high starch diets. The histological features of the duodenal mucosa appeared similar for all the diets although the epithelial convolutions were a noteworthy feature when compared to the 16 day old chicks (see Chapter 4). Similar convolutions have, however, been illustrated in the small intestine of the chicken (Hodges, 1974) and would therefore appear to represent a normal feature in older birds. The smooth nature of the duodenal villi in young birds illustrated by Calhoun (1954) would appear to confirm this observation although sample preparation technique possibly may also play a role.

The muscular tunic of the duodenum displayed similar histological features irrespective of diet. The thickness of the inner circular layer (ICL) of the *tunica muscularis* displayed little difference between the various diets, although the low fat, low sugar and high sugar diets demonstrated a significantly ($P<0.05$) thicker ICL than that of the high protein and high starch diets. Similarly, the outer longitudinal layer (OLL) differed little in thickness between the different diets, only showing significant ($P<0.05$) differences between the low protein, low fat, high fat diets and the high sugar diet. The mean values for the thickness of the ICL and OLL for all the diets confirmed the observation that in birds the OLL is relatively thin compared to the ICL (McLelland, 1979; Hodges, 1974). The only difference ($P<0.05$) in the villus length:crypt depth (V:C) ratio was between the high and low protein diets, where the ratio was lower in the high protein diet. A high V:C ratio reportedly corresponds with increased nutrition digestion, a lower secretion in the gastro intestinal tract, higher disease resistance and an overall greater growth (Wu *et al.*, 2004). It was clear from this study that a high protein diet has a detrimental effect on the development of the small intestine as demonstrated by

the low V:C ratio, as also demonstrated by the high mortality rate in birds fed a high protein diet (see Chapter 5).

Jejunum

The mean length of the villi in the jejunum (1792.1 µm) was shorter (P<0.05) than that for the duodenum (2078.2 µm), although the diameter of the villi remained similar (152.2 µm and 145.5 µm for the jejunum and duodenum respectively). This observation is in agreement with the general trend that villus height decreases from the duodenum to the ileum (Iji *et al.*, 2001; Wang & Peng, 2008; Wang *et al.*, 2008; Khambualai *et al*., 2009; De Verdal *et al*., 2010). However, birds on the high and low starch diets and the high and low sugar diets had significantly (P<0.05) longer villi than those on the high and low protein and high and low fat diets. Based on the occurrence of relatively long villi in the jejunum and on the similarity in the V:C ratio of the jejunum and duodenum, it would appear that both these segments of the small intestine might be equally important for digestion in the ostrich. This phenomenon was also apparent in younger ostrich chicks (see Chapter 4) and in broiler chicks where it was noted by Iji *et al.* (2001) that the jejunum might be an important site of digestion and absorption. The diameter of the villi (VD) of birds on the high protein diet was significantly greater than that reflected in the other diets, except for the low sugar diet, while the VD of the low protein diet was significantly (P<0.05) less than that of the other diets.

The low starch diet demonstrated a significantly (P<0.05) thicker CD than the other diets, except for the high protein diet (probably due to the large standard error of the high protein diet values). The high fat diet showed a significantly (P<0.05) thinner CD than those of the high starch and low sugar diets. A larger glandular component (demonstrated by an increase in CD) reportedly indicates greater cell proliferation and is usually associated with longer villi (De Verdal, 2010). However, this was not apparent in the jejunum in the present study where the amount of glandular tissue remained similar to that seen in the duodenum but was accompanied by an average decrease in villus length. The exception was that of the low starch diet where the jejunum displayed a wide glandular layer associated with long villi. This may be attributed to the higher fibre content (12.96%) of this diet, which could result in a faster turnover rate due to increased cell loss from the villi (Jin *et al.*, 1994). Sections of the glandular layer revealed that numerous mitotic figures were present in the crypts in both the jejunum and duodenum irrespective of the diet thus indicating on-going maintenance of the villus epithelium. The width of the MM in birds on the high protein diet was significantly (P<0.05) higher than those on the other diets, whereas the low fat and low sugar diets demonstrated a significantly (P<0.05) higher MM width than the low protein, high fat, low starch and high sugar diets. When compared to the width of the MM in the duodenum it was clear that no particular pattern was obvious in the thickness of the MM between the various diets.

A similar trend (no particular pattern) was noted in the thickness of the ICL, with only the low sugar diet reflecting a significantly (P<0.05) thicker layer than that of the other diets. The outer longitudinal layer of the *tunica muscularis* also displayed variable thicknesses with the low fat diet showing a significantly (P<0.05) thinner OLL and the low starch diet a significantly (P<0.05) wider OLL than the other diets. In

both the jejunum and duodenum the total thickness of the *tunica muscularis* was markedly thinner for the high protein diet. To what extent this phenomenon would have influenced intestinal function (bearing in mind the high mortality of the high protein diet birds) remains to be determined. The significantly ($P<0.05$) low C:V ratio of the high protein diet birds, when compared to that of the other diets further reflects the detrimental influence of the high protein diet. As a higher V:C ratio is associated with an optimum gastro-intestinal tract for growth (Wu *et al*., 2004), it might be an indication that the jejunum of the ostriches on the high protein diet was under higher digestive strain compared to the other diets.

Ileum

VL in the ileum was similar for most of the diets, except for the high protein diet which demonstrated significantly ($P<0.05$) longer villi than for the other diets. VD of the high protein, low protein and low starch diets, were significantly ($P<0.05$) higher than the high fat, low fat, high sugar, low sugar and high starch diets. The longer and thicker villi in the ileum for the high protein diet would clearly indicate a larger villus area for this diet. However, villus length in the ileum showed a further incremental decrease when compared to the jejunum and duodenum, thus further confirming that the digestive function of the small intestine decreases progressively from the duodenum and jejunum to the ileum (Iji *et al.*, 2001; Wang & Peng, 2008; Wang *et al.*, 2008; Khambualai *et al*., 2009; De Verdal *et al*., 2010). Although the histological observations indicated that the villi in the ileum appeared to be shorter and stubbier, the morphometric data revealed that although shorter, the villi displayed a similar average diameter (153.9 µm) to those in the jejunum (152.2 µm) and duodenum (145.5 µm). A larger villus surface area was also reported by Khambualai *et al*. (2009) in a study on the effect of dietary chitosan on intestinal histology in broiler chickens. Chitosan, however, is regarded as a dietary fibre of animal origin (Maezaki *et al.*, 1993) and the authors suggested that intestinal villi might have been activated by the added chitosan, which resulted in increased cell mitosis (Khambualai *et al.*, 2009). This did not affect villus height, as cell loss was higher than in the control group, but it increased the villus area. In addition, birds on the high protein diet also displayed a significantly ($P<0.05$) thicker glandular layer than birds on most of the other diets, except the low starch and low sugar diets, due to the large standard error of the high protein diet CD values.

Both layers of the *tunica muscularis* of the ileum were significantly thicker (ICL = 436.4 µm and OLL = 338.3 µm) than the corresponding layers in the other parts of the small intestine (duodenum: ICL = 299.1 µm and OLL = 188.8 µm; jejunum: ICL = 361.8 µm and OLL = 229.8 µm), confirming the light microscopic observations. Much variation was observed in the thickness of the ICL, with significant ($P<0.05$) differences being observed between most of the diets. However, the ICL was significantly ($P<0.05$) thinner for the high protein, low protein and low starch diets compared to the other diets, with that of the high protein diet being significantly ($P<0.05$) thinner than those of the other two diets as well. The low fat, high fat and high starch diets demonstrated significantly ($P<0.05$) higher values for the thickness of the OLL than that of the other diets. Even though villus area was larger for the low protein diet, the CD was also greater, which resulted in

a significantly (P<0.05) lower V:C ratio for this diet compared to the high fat, low fat, high starch and high sugar diets.

The dimensions measured in the various parts of the large intestine for the eight diets are indicated in Table 5.

Table 5 Means (± SE) of the various layers measured of the large intestine

Diet	Mucosa	*Tunica muscularis*	Mucosa:TM
Caecum			
High protein	217.5 ± 19.7^{ac}	209.9 ± 74.4^{a}	1.10 ± 0.14^{a}
Low protein	188.5 ± 9.9^{ab}	346.3 ± 37.2^{bc}	0.66 ± 0.07^{b}
High fat	220.8 ± 10.5^{c}	289.8 ± 39.8^{ab}	0.87 ± 0.07^{c}
Low fat	200.6 ± 9.0^{ab}	371.8 ± 33.7^{bc}	0.57 ± 0.06^{b}
High starch	184.5 ± 10.9^{b}	335.6 ± 40.6^{bc}	0.61 ± 0.07^{b}
Low starch	194.4 ± 9.0^{ab}	365.0 ± 34.5^{bc}	0.62 ± 0.06^{b}
High sugar	206.7 ± 10.1^{abc}	288.7 ± 37.9^{ab}	0.76 ± 0.07^{bc}
Low sugar	188.0 ± 10.1^{ab}	468.6 ± 37.9^{d}	0.53 ± 0.07^{b}
Proximal part of Colon			
High protein	254.9 ± 19.7^{a}	341.2 ± 74.4^{abc}	0.97 ± 0.14^{a}
Low protein	201.0 ± 10.2^{bc}	242.7 ± 37.9^{bc}	0.90 ± 0.07^{a}
High fat	194.9 ± 11.4^{cd}	387.9 ± 41.7^{a}	0.59 ± 0.08^{b}
Low fat	207.6 ± 9.0^{bc}	411.3 ± 33.8^{a}	0.57 ± 0.06^{b}
High starch	219.1 ± 10.5^{b}	395.8 ± 39.8^{a}	0.74 ± 0.07^{ce}
Low starch	207.5 ± 9.0^{bc}	404.5 ± 33.9^{a}	0.64 ± 0.06^{bc}
High sugar	176.4 ± 10.1^{d}	354.6 ± 37.9^{a}	0.60 ± 0.07^{bc}
Low sugar	196.9 ± 9.9^{bc}	262.3 ± 37.2^{bc}	0.82 ± 0.07^{ae}
Distal part of Colon			
High protein	369.9 ± 23.4^{ae}	445.5 ± 83.3^{ab}	1.04 ± 0.15^{a}
Low protein	271.0 ± 9.9^{b}	437.9 ± 37.2^{b}	0.72 ± 0.07^{b}
High fat	348.2 ± 10.5^{ce}	516.5 ± 39.8^{a}	0.72 ± 0.07^{b}
Low fat	390.0 ± 8.8^{a}	538.0 ± 33.3^{a}	0.81 ± 0.06^{b}
High starch	283.6 ± 10.5^{bf}	445.5 ± 39.8^{ab}	0.69 ± 0.07^{b}
Low starch	337.3 ± 9.0^{c}	664.2 ± 33.7^{c}	0.52 ± 0.06^{c}
High sugar	317.4 ± 9.9^{d}	470.6 ± 37.2^{ab}	0.74 ± 0.07^{b}
Low sugar	298.8 ± 9.9^{df}	452.4 ± 37.2^{ab}	0.69 ± 0.07^{b}

$^{a, b, c, d, e}$ Column means with different superscripts differ significantly at P < 0.05

Caeca

The thickness of the mucosa layer demonstrated a significant (P<0.05) thicker layer for the high fat diet than the low protein, low fat, high and low starch and low sugar diets. The high starch diet had a significant (P<0.05) thinner mucosa layer than the high protein and high fat diets.

The high protein diet had a significant (P<0.05) thinner *tunica muscularis* (TM) layer than the other diets, except for the high fat and high sugar diets. The low sugar diet had a significant (P<0.05) thicker TM layer than the other diets.

In the mucosa : *tunica muscularis* (M:TM) ration, the high protein diet was the only diet with a ration higher than one. This diet also had a significant ($P<0.05$) higher ration than the other diets. Similarly, the high fat diet had a higher ($P<0.05$) M:TM ratio than the low protein, low fat, high and low starch and low sugar diets.

Proximal part of the colon

The mucosa layer in the proximal part of the colon was significantly ($P<0.05$) thicker for the high protein diet than all the other diets, while the high sugar diet had a thinner ($P<0.05$) mucosa layer than the other diets, except for the high fat diet.

The width of the TM remained relatively similar, although the values for the low protein and low sugar diets were significantly ($P<0.05$) lower than the other diets, except for the high protein diet.

All the values for the M:TM ratio in the proximal part of the colon was below one. The M:TM ratio for the high and low protein diets was significant ($P<0.05$) lower than the other diets, except for the low sugar diet. The ratio for the low sugar diet was also higher than the high and low fat, low starch and high sugar diets.

Distal part of the colon

The mucosa layer of the distal part of the colon showed much variation between the different diets. The high protein and low fat diets had a significant ($P<0.05$) thicker mucosa than most of the other diets, while the low protein and high starch diets had a significant ($P<0.05$) thinner mucosa than most of the other diets.

The TM layer was similar for most of the diets, except for the low starch diet which demonstrated a significant ($P<0.05$) thicker layer than the other diets.

Similarly to the results for the M:TM ratio in the caeca, the high protein diet had a ration above one, which was significantly ($P<0.05$) higher than the ratio for the other diets. Low starch had a significant lower M:TM ration compared to the other diets.

Large intestine

Throughout the large intestine, and in all birds irrespective of their diet, the structural features of this part of the digestive tract appeared normal and reflected the basic morphology previously described for this species (Bezuidenhout & Van Aswegen, 1990; Bezuidenhout, 1993). No overt lesions indicative of enteritis were observed in any of the material examined. This study confirmed that the lining of the caeca was of the intestinal type as defined by Ziswiller & Farner (1972), indicating an absorptive function for this part of the large intestine. This important role of the caeca has also been emphasised by Swart *et al.* (1987) and confirmed by Bezuidenhout (1990). This study has thus revealed that the proximal (thick) part of the colon was remarkably similar to that of the caeca and therefore likely to perform a similar function. The paucity of

goblet cells in these regions further emphasised the absorptive function of the lining epithelium. The study confirmed that the spiral folds of the caeca and the folds in the proximal part of the colon as previously described for the ostrich (Bezuidenhout & Van Aswegen, 1990; Bezuidenhout, 1993), would significantly increase the absorptive area of this part of the digestive tract. The distal part of the colon, although composed of a simple columnar epithelium, displayed large numbers of goblet cells, seemingly indicating that this region was less involved in absorption and more with the passage of excreta.

According to Bezuidenhout & Van Aswegen (1990) the *muscularis mucosae* throughout the intestinal tract is composed of longitudinally and circularly arranged smooth muscle fibres. However, in this study the *muscularis mucosae* of the small intestine was composed almost exclusively of longitudinally oriented smooth muscle fibres. In contrast, the various regions of the large intestine demonstrated both arrangements of the smooth muscle, particularly in the distal part of the colon. This part of the large intestine also differed from other parts of the intestinal tract in the observation made that the the two layers (inner circular and outer longitudinal) of the *tunica muscularis* were often of equal thickness.

General

As noted above, all eight diets demonstrated a decline in VL along the length of the small intestine. High and low fat, low starch and high sugar diets produced a significant ($P<0.05$) difference in VL between all the regions, while low protein, high starch and low sugar diets only showed a significant ($P<0.05$) difference between the jejunum and ileum. The high protein diet likewise, showed a progressive decrease in VL from the duodenum to the ileum, although the decrease was not significant ($P<0.05$).

Low fat and high sugar diets showed no difference in VD along the small intestine. Low protein and low starch diets reflected a significant ($P<0.05$) increase in VD from the duodenum to the jejunum and a significant ($P<0.05$) decrease from the jejunum to the ileum. Both high protein and low sugar diets showed a significant ($P<0.05$) increase in VD from the duodenum to the jejunum, but no difference between the VD of the jejunum and ileum. High fat and high starch diets showed a decline in VD with significant ($P<0.05$) differences being noted between the jejunum and ileum for the high fat diet and significant ($P<0.05$) differences between the duodenum and ileum for the high starch diet. Although the low starch (12.96%) and high sugar (6.93%) diets contained the highest crude fibre content (Table 1), this did not appear to have an effect on either VL or VD. In contrast to these findings, Jin *et al.* (1994) reported short villi in pigs receiving high dietary fibre, and concluded therefore that short villi might be induced by a quick turnover rate due to increased cell loss from the villi. In the study by Khambualai *et al.* (2009) on broiler chickens fed a diet with added chitosan, only the ileal villus area was increased with higher dietary fibre. The apparent increase in villus area, particularly in the ileum, of the high protein group, could be attributed to the high protein content in the intestinal lumen requiring an increased absorptive surface. This corresponds with the findings of Yamauchi (2002) and Khambualai *et al*. (2009) that the ileum is the most reactive site for changes in villus area for coping with nutritional changes or increased nutrient content.

A significant (P<0.05) decrease in the thickness of the glandular layer as measured by crypt depth (CD) was observed between all three regions of the small intestine for low fat, high starch and high sugar diets. Low sugar and high fat diets also showed a decrease in CD, but this was only significant (P<0.05) between the duodenum and ileum. In contrast, the low starch diet resulted in a significant (P<0.05) increase in the thickness of the glandular layer from the duodenum to the jejunum, although a significant (P<0.05) decrease in thickness was noted between the jejunum and ileum. This decrease was so marked, that CD in the ileum was significantly (P<0.05) lower than CD in the duodenum. This increase in the thickness of the CD from the duodenum to the jejunum could be due to an increased demand for villus tissue synthesis, as the higher fibre content in the low starch diet could led to faster tissue turnover (Xia *et al.*, 2004). This again emphasizes the apparent importance of the jejunum for digestion in ostrich chicks as noted above. CD remained constant throughout the small intestines for both protein diets.

High and low fat, high protein, high starch and low sugar diets showed a significant (P<0.05) increase in the width of the MM from the duodenum to the jejunum, with a significant (P<0.05) decrease being observed from the jejunum to the ileum. The low protein, low starch and high sugar diets also resulted in a decline in MM width along the small intestines, although this was only significant (P<0.05) between the jejunum and ileum.

Low and high fat, high starch as well as low and high sugar diets showed an increase in the thickness of the ICL of the *tunica muscularis* along the small intestine. This increase was significant (P<0.05) between the first two regions and the ileum for the high and low fat, high starch and high sugar diets and between the duodenum and last two regions for the low sugar diet. The high and low protein diets did not reveal any significant (P<0.05) differences between the three regions. The low starch diet showed a significant (P<0.05) increase in ICL thickness between the duodenum and jejunum and a significant (P<0.05) decrease between the jejunum and the ileum. However, no significant (P<0.05) difference was observed in the width of the ICL between the duodenum and ileum. This was due to the large standard error for the ICL diameter value of the ileum.

Low protein, low and high fat and high starch diets showed an increase in the width of the OLL along the small intestine, with significant (P<0.05) differences being apparent between all three regions for the low fat diet, between the duodenum and the distal two regions for the low protein diet and between the first two regions and the ileum for the high fat, high sugar and high starch diets. High and low sugar diets demonstrated an increase in OLL width from the duodenum to the ileum, although this was not significant (P<0.05). The low starch and high protein diets showed a significant (P<0.05) increase in thickness of the OLL of the duodenum and jejunum, with a decrease in thickness being observed from the jejunum to the ileum, although this was only significant (P<0.05) for the low starch diet. There was no difference in the thickness of the OLL of the duodenum and ileum for these two diets.

There was no difference in the V:C ratio for most of the diets between the duodenum and the jejunum (again indicating the importance of this segment for digestion), but most diets displayed a significant

($P<0.05$) decrease from the jejunum to the ileum. Wang & Peng (2008) also reported a decline in the V:C ratio from the duodenum to the ileum for ostrich chicks. The only diet which differed in this regard was the high protein diet, which demonstrated a significant ($P<0.05$) decline in the V:C ratio between the duodenum and the jejunum, but no difference between the jejunum and the ileum.

Conclusion

This study demonstrates that the eight pre-starter diets had no obvious morphological effects on the microscopic structure of the small intestine in two month old ostrich chicks. It seem clear, however, that the birds fed a high protein diet were poorly equipped, based on the V:C ratio and the unusually thin muscular tunic, for effective digestion. These birds seemed to be under higher digestive stress than birds on the other diets, although, due to the high mortality rate of the birds fed the high protein diet, too little data was available to draw definitive conclusion. Although no discernable morphological pattern could be seen indicating the advantage of a particular diet, it is suggested that the application of additional parameters such as determining the density of goblet and chief cells, and establishing the number of villi and crypts per fixed area, may provide more accurate data. Transmission electron microscopy (TEM) may also reveal subtle ultra-structural changes associated with the various diets. Future research should furthermore take into account the role of organs closely associated with the digestive tract, such as the liver, to determine whether other organs may also be affected by variations in diet.

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Chapter 8

General conclusion and future perspective

Individual chapters included in this thesis already carry conclusions. This chapter therefore summarizes the objectives and outcomes generated in this study. It will also provide suggestions regarding future research on ostrich chick nutrition.

In Chapter 1, the following hypotheses were posed regarding the early period of the ostrich's life:

8. The nutrients supplied from the yolk content of ostrich chicks need to be supplemented with external feed post-hatching.
9. In spite of similarities between the anatomy of the gastrointestinal tract of ostrich chicks and poultry during the first weeks post-hatching, the external nutrient requirements differ between these species from hatching.
10. Enzyme activity in the digestive tract of ostrich chicks changes during the first two weeks post-hatching to adapt from the digestion of yolk content to that of exogenous feed.
11. The histology of the small intestines develops over the first two weeks post-hatching to accommodate absorption of exogenous feed.
12. The composition of pre-starter diets has an influence on the growth of ostrich chicks.
13. The composition of diets has an influence on the development of enzyme activity. Enzyme activity will adjust according to the composition of the diet.
14. The composition of diets has an influence on the development of the digestive tract. Higher growth rate will correspond with an increased surface area of the gastrointestinal tract.

The following conclusions were reached:

Part 1: Yolk utilisation and the development of the small intestines

The measurements of yolk content from starved and fed ostrich chicks indicated that yolk absorption was more rapid in starved ostrich chicks than in fed ostrich chicks. Protein was assimilated from the yolk sac in both cases, while it appeared that fat was absorbed at a much slower rate. Fat content, however, decreased faster over the first eight days of the fed ostrich chicks, than the starved ostrich chicks, which could be an indication that external feed had a positive influence on the absorption of fat from the yolk. Changes in the fatty acid composition of the yolk fat content were observed, which may indicate that ostrich chicks have the ability to withdraw certain fatty acid components, especially unsaturated fatty acids. Further studies should be done with pre-starter diets, where amino acids, especially histidine and valine are included, as well as

adding mono-unsaturated fatty acids during the first seven days post-hatch. This may result in an increased utilisation of yolk lipids as energy source until the ostrich chick can effectively utilise dietary fat.

The digestive tract of ostrich chicks is generally compared with that of broiler chicks, as they are similar in appearance immediately post-hatching, only changing in character to that of a hindgut fermenter around 55 days of age (Iji *et al.*, 2003; Van der Walt *et al.*, 2003; Wang & Peng, 2008). Most of the enzyme activity measured in this trial had the same tendencies than reported for broilers, except for amylase activity, which remained constant in the duodenum and declined in the ileum over the first two week post-hatching. This differed from broilers where amylase activity is reported to increase with an increase in body weight, especially during the first few days post-hatching. The differences observed in amylase activity between the birds in this trial and poultry could be due to genetical differences between species. Poultry used in trials are also genetically much more homogenous than ostrich chicks, which have not been subjected to the same extent of advanced breeding progress than poultry up to date. Another reason for this difference could be due to different methods of determining enzyme activity, as well as the time period of the trials. Lipase activity measured in ostrich chicks showed the same tendencies than lipase activity reported in poultry, although the time period at which activity changes occur was much shorter for poultry than measured in ostrich chicks.

As ostrich production is advancing in the same direction than poultry production, more studies should follow to fully comprehend the development and functioning of the digestive tract of this ratite species. This will enable the nutritionist to formulate ostrich diets that will not only benefit initial growth of the ostrich chick, but also result in a stronger chick which will have higher resistance to external stresses that may cause the high mortalities which is still a problem in ostrich production.

Part 2: Influence on various pre-starter diets on growth and the development of the small intestines

It was clear in this trial that ostrich chicks performed differently on the diets formulated with different nutritional components. It seems that a high fat and low sugar content of the diet is of advantage in ostrich pre-starter diets. There is no clear indication as to why chicks died in the different trial groups, as the causes of mortalities were not investigated. This is definitely a factor that should be explored in future studies.

Differences in composition of eight pre-starter diets did not seem to have an influence on the protein content or enzyme activity of the small intestines of ostrich chicks. An anti-nutritional factor within certain feedstuffs may, however, have had an influence on enzyme activity. Choosing the right combination of feedstuffs to compile a ration is therefore just as important as formulating the ration to meet the nutritional requirements of the ostrich chick. Changes in the digestive tract of the ostrich from a monogastric animal to a hindgut fermenter occur within the first 70-80 days. Anti-nutritional factors which have an influence on monogastric animals, such as the trypsin inhibitor in soy beans, should however still be taken into account when formulating an ostrich pre-starter ration, as the microbes that can inactivate this anti-nutritional factor

is not present in the digestive tract till after the small intestines. Even though there did not seem to be many differences in the enzyme activity between different diets in this trial, there is still a lot of work to be done on enzyme activity in growing ostriches. Similar results were obtained for the histological and morphological parameters measured for the different diets. There was no singular diet that promoted intestine development, although it seemed as if the small intestine of the chicks fed the high protein diet were under higher digestive strain than the other diets. As the ostrich changes from a monogastric animal to a hindgut fermenter, the question arises as to how effective the small intestine digestion remains if a big part of the chemical digestion is replaced by microbial digestion in the caeca? Should one continue to compare it to the digestion of other avians, or should the focus shift to comparing it with other monogastric hindgut fermenters, like the horse and the rabbit?

Recommendations

Ostrich chick nutrition remains an interesting and challenging subject. There is still a vast field of research necessary to fully comprehend the digestion and absorption of these primitive birds, compared to what is already known for poultry. The author recommends that changes in the small intestines should be measured continually, as the chicks change from a monogastric animal to a hindgut fermenter, aided with changes occurring in the caeca, colon and digestive organs. Further studies should also be undertaken to test possible changes in growth with nutritional components, like varying levels of fatty acids and amino acids. As the breeding of ostriches lead to a more homogenous group of animals which can be used for nutrition trials, the author suspect that data obtained in future studies will lead to more precise conclusions.

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