ONTOGENIC MORPHOLOGY AND ENZYME ACTIVITIES OF THE INTESTINAL TRACT OF THE NILE TILAPIA, *Oreochromis niloticus*

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By

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Abstract

The gross intestinal configuration of the Nile tilapia intestine changed dramatically from a short, straight intestinal tube at hatch (day 0) to a very complex, coiling pattern first attained at 9 weeks post-hatch. During the developmental period, gut length increased from 90% to 410% of body length. The rate of increase in both intestinal and body lengths took place at an accelerating rate as the fish aged. The great intestinal length provides an advantage to the fish in digestion and absorption of nutrients present in the less energy-efficient herbivorous diet. Formulation of commercial diets to match the development of the fish's intestine may offer commercial advantage.

Appearance, localization and distribution of intestinal enzymes were observed in the fish at hatch and at mature stages using enzyme histochemistry. At hatch (day 0), most gut enzymes were already present in the intestinal brush border. As the fish matured, activities of the enzymes were widely distributed along the intestinal tract. The early appearance and broad distribution of activities of all studied intestinal enzymes may be one factor contributing to the rapid growth rate characteristic of tilapia, which differs markedly from other fish species.

To investigate the possibility of using alfalfa as a potential protein food replacement in tilapia, the effects of different levels of alfalfa in feeds on growth and intestinal enzyme

activities were observed in the fish aged 3-15 weeks. Results demonstrated that replacing 20% and 40% of a commercial diet with alfalfa had an overall negative effect on body and intestinal growth, as well as the intestinal enzyme activities from age 3-9 weeks. Thus, using alfalfa as a food replacement is not optimal for fish of these young ages, but may yet be suitable for older fish.

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DEDICATION

This dissertation is dedicated to my parents, Artith and Apiradee Tengjaroenkul, my sisters and brother, Pairach, Yauwaluk and Bhoriboon Tengjaroenkul for their loves, and strong support throughout my education. I wish that you all would enjoy every moment of my success.

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CHAPTER 1
INTRODUCTION

1.1: Tilapian fish

Tilapias are tropical cichlid teleosts native to Africa and the Middle East. They occupy a wide geographic area extending from 8°S to 32° N and from 0 to 6,000 feet (approximately 2,000 m) above sea level (Trewavas, 1983). Most tilapian fish are classified into two genera, *Tilapia* and *Oreochromis*. Fish in the genus *Tilapia* are macrophagous feeders, consuming mainly algae, macrophytes and other aquatic plants. These fish are substrate-spawners that can lay up to 7,000 eggs at a time. Fish in the genus *Oreochromis* are mainly omnivorous and microphagous, feeding largely on phytoplankton, zooplankton, and detritus organisms (Lim, 1989). These tilapia are mouth-brooders, producing about 500 eggs per spawning (Baylosis and Herrera, 1993). Only two species of the genus *Tilapia* (*T. zilli* and *T. rendalli*) and three species of *Oreochromis* (*O. niloticus*, *O. aureus* and *O. mossambicus*) are widely used in aquaculture.

Tilapia currently are becoming one of the most important cultured fish species in tropical and subtropical regions of the world as well as in the United States (Anonymous, 1999; Popma and Masser, 1999). Numerous characteristics of tilapia contribute to their suitability for rearing in aquaculture systems. These features include tolerance of relatively high water temperature (Allanson and Noble, 1964), low dissolved oxygen (Denzer, 1968), high ammonia levels (Stickney, 1986), and a wide range of salinity (Chervinski, 1966; Chervinski and Zorn, 1974; Lee and Newman, 1992); utilization of a wide variety of foods including commercial fish diets (Brown and Gratzek, 1980; Lim, 1989); attainment of market size in

less than a year (Baylosis and Herrera, 1993); resistance to diseases and stress of handling, and high palatability to the fish consumer (Lim, 1989; Baylosis and Herrera, 1993; Lovell, 1995).

Domestic tilapia production has increased dramatically in the last five years, with 1995 domestic production approximating 15 million pounds (Anonymous, 1996). Gross sales of tilapia in that year ranked first among other cultured freshwater fish in Virginia at a value of 2.88 million dollars (Kirkley and Oesterling, 1997; G.S. Libey, Virginia Polytechnic Institute and State University, personal communication). In 1998, total domestic production of tilapia further increased to nearly 18 million pounds (Anonymous, 1999), and the world production was around 1,800 million pounds (Popma and Masser, 1999). At present, tilapia's production is ranked second and fourth among cultured fish in the world and in the United States, respectively (Anonymous, 1999; Popma and Masser, 1999). Current projections anticipate continued increases in commercial production in domestic and worldwide tilapia production.

1.2: Gross anatomy of the piscine gastrointestinal tract

The morphology of a fish's gastrointestinal system commonly relates to its dietary habits. Generally, carnivorous fish have a large and distensible stomach, and a short intestine. Omnivores that tend toward animal diets also have a large stomach, but a longer intestine. Omnivores tending towards a plant diet typically have a smaller stomach and a long intestine. Truly herbivorous fish characteristically have either a small stomach or no stomach at all, and the longest and most complex intestine (Smith, 1991). Omnivorous fish are less efficient in utilizing plant foods than herbivorous fish, but more efficient than carnivores (Lim, 1989).

Adult tilapia are omnivores that generally consume more plant materials than animal foods. Thus, their relatively small stomach and long, complexly-arranged intestine fit the above generalization well. As a practical corollary to this gut configuration, continuous feeding behavior render them best suited for multiple daily feedings (Kabaryk, 1980; Coche, 1982; Jauncey and Ross, 1982).

Remarkably, the stomach is absent in almost 15% of fish species, including many fish in the family Cyprinidae, Blenniidae, and Cobitidae (Kapoor et al., 1975; Smith, 1989; Amin, Mortensen and Poppe, 1992). These fish families are obligate herbivores that characteristically consume tiny food items that pass directly to the intestine for further digestion. Despite the above exceptions, most fish do possess a stomach. As a muscular organ involved in storage and initial digestion of food, the stomach varies in size and structure depending on feeding behavior of individual fish species. Carnivorous salmonids possess a stomach that is relatively large and possesses pyloric ceca (Smith, 1989). The pyloric ceca are blind-ended tubes originating from the pylorus of the stomach and the cranial part of the intestine (Amin, Mortensen and Poppe, 1992). In contrast, the stomach of omnivorous/herbivorous fish, such as tilapia and catfish, are sac-like organs lacking pyloric ceca (Moriarty, 1973; Kapoor et al., 1975; Lovell, 1989; Smith, 1989). The stomach of the microphagous milkfish (*Chanos chanos*) is a tubular sac with a muscular gizzard (Smith, 1989).

The intestine plays the main role in the digestive and absorptive functions of the alimentary tract. Depending on diet, the intestine can vary morphologically from short and straight to long coiled and complexly arranged (Stroband, 1977; Zihler, 1982; Yamaoka, 1985; Govoni,

1986; Reinthal, 1989). Other anatomical specializations may be variably present among fish species, including the spiral valve in shark, paddlefish and other primitive bony fish, and pyloric ceca in salmonids and bluegills (Lagler et al., 1977; Smith, 1982; Hussain and Dutta, 1996; Moyle and Cech, 1996). As with other vertebrates, the gross morphology of the intestinal tract of fish generally correlates with the diet and feeding habits of individual fish species (Bucke, 1971; Douglas and Drewry, 1978; Geevarghese, 1983; Timmermans, 1987). In general, herbivorous fish have the greatest intestinal length, as well as a maximum number of intestinal loops and complexity of their disposition. Omnivorous fish possess intestines of intermediate length and looping, while carnivorous fish have a shorter intestine with minimal or even no looping. The ratio of intestinal length to body length in fish is less than one in carnivores, may increase to around two to three in omnivores, and is usually about four to six or higher in herbivores (Smith, 1982).

In tilapia, the intestine approximates 4 to 6 times the total body length (Hofer and Schiemer, 1981; Smith et al., 2000). The lengthened intestine provides a large surface area for nutrient digestion and absorption. In the Nile tilapia (*O. niloticus*), the elongation of gut is arranged into two major coils (proximal and distal coils, each comprised of centripetal and centrifugal portions) and two loops (hepatic and gastric loops) (Smith et al., 2000). Briefly, the Nile tilapia's intestine leaves the pyloric region of the stomach and forms an elongate loop that follows the borders of the liver. The intestine then passes into a spiral region having the form of a truncated cone. The spiral intestine consists of two major coils, each of which is composed of a centripetal and a centrifugal loop. A relatively short gastric loop is interposed between the two major coils. After the spiral region, the intestine straightens in its course and continues to the anus.

1.3: Microscopic structure of the fish gastrointestinal tract

The wall of the stomach and intestine of fish is generally similar to that of most other vertebrates, and is comprised of four distinct layers; the tunica mucosa, tunica submucosa, tunica muscularis and tunica serosa (Fange and Grove, 1979; Smith, 1989). Surface area may be increased by numerous mechanisms, such as mucosal folds in the stomach, pyloric ceca, diverticula located in the cranial intestine, folds situated along the gut length, and numerous microvilli projecting from each mucosal epithelial cell (Lagler et al., 1977; Amin, Mortensen and Poppe, 1992; Moyle and Cech, 1996; Gargiulo et al., 1998).

By means of distinctive characteristics of the epithelial lining of the tunica mucosa, the stomach can be microscopically differentiated into three regions: cardiac, proper gastric and pyloric (El-Shammaa et al., 1995; Caceci et al., 1997). The mucosa of the stomach is lined by a layer of simple columnar epithelium, with various stomach glands present beneath the epithelium. Numerous cardiac glands are embedded in the lamina propria of a relatively small region between the caudal part of the esophagus and the proper gastric gland region of the stomach. These glands are lined by cuboidal epithelium with flat nuclei positioned at the basal portion of the glandular cells. The proper gastric glands occupy the largest area of the stomach, and are distinguished by their columnar epithelium with prominent mucous cells. The cytoplasm of the glandular cells shows various cellular organelles, including secretory granules, rough endoplasmic reticulum, electron dense granules, and an abundance of mitochondria (Scocco et al., 1996). The mucous cells of the gastric gland typically present mucous granules with flat nuclei located toward the basal part of the cells. In this region, the

gastric glands themselves are situated at the base of the descending portions of gastric pits of the epithelium within the lamina propria of the mucosa. Unlike higher vertebrates, these glands in fish contain only one principle cell type, called a parieto-chief cell, which produces both pepsinogen (the digestive enzyme) and HCl (Smith, 1991). The pyloric gland region is covered by columnar epithelial cells possessing a few mucous glandular cells. The gastric pits may be absent in the cardiac as well as pyloric gland regions of the stomach in some fish species, including tilapia (Caceci et al., 1997). In addition to these digestive-related structures, numerous endocrine cells secreting substances such as gastrin and somatostatin, also have been reported in gastric epithelium of fish (Noaillac-Depeyre and Hollande, 1981; Noaillac-Depeyre and Gas, 1982).

The tunica submucosa of the stomach consists mainly of loose connective tissue, blood vessels, nerve plexuses and a thick layer of collagen fibers. A large number of eosinophilic granular cells also may be present in this layer (Amin, Mortensen and Poppe, 1992). As in other vertebrates, the tunica muscularis consists of inner circular and outer longitudinal layers of smooth muscle which are innervated by myenteric plexuses. The striated muscle layer may extend into this muscular layer of the caudal part of the stomach in some fish species (Al-Hussaini and Kholy, 1953; Mohsin, 1962; Caceci et al., 1997). The tunica serosa, the outermost layer, consists of mesothelium overlying a layer of loose connective tissue.

The tunica mucosa of the small intestine potentially includes three regions; the lamina epithelialis, lamina propria, and lamina muscularis mucosae. Numerous cell types characterize each layer. The lamina epithelialis (the innermost layer of the mucosa) contains various cell types, with the columnar absorptive cells being by far the most numerous. At the

electron-microscopic level, these cells are characterized by their prominent striated border (microvilli) along the apical cell surfaces. The regularly-arranged microvilli contain bundles of fine filaments which extend down into the terminal web of the cytoplasm. Like most cells in mammals, the surfaces of the enterocytes at the apical areas all attach to neighboring cells by intercellular junctions, such as tight junction, adherens junction and desmosomes. The cytoplasm of the enterocytes contains typical membrane-bound organelles, including rough endoplasmic reticulum, Golgi apparatus, mitochondria, free ribosomes and lysosomes. Mitochondria are distributed broadly throughout the cytoplasm including near the nuclei, whereas the lysosome-like structures are restrictedly to the supra-nuclear cytoplasmic region. The relatively large oval nuclei of the enterocytes are situated centrally or toward the base of the cells (Lagler et al., 1977; Gargiulo et al., 1998). The intestinal epithelium also contains numerous goblet cells producing mucus for protective and lubricant functions, secretory cells, and endocrine cells (Lagler et al., 1977; Noaillac-Depeyre and Hollande, 1981; Gargiulo et al., 1998). The mature goblet cells typically contain cisternae of rough endoplasmic reticulum and a Golgi apparatus in the cytoplasm, and nuclei localized at the basal part of the cells. The numerous endocrine cells of the intestine are characterized as flask/or spindle-shaped structures with abundant cytoplasmic granules broadly distributed throughout the gut epithelium (Noaillac-Depeyre and Hollande, 1981; Gargiulo et al., 1998). These endocrine cells are particularly numerous in the cranial gut region (Gargiulo et al., 1998). The lamina propria of the intestinal tunica mucosa is a thin connective tissue layer situated between the epithelium and muscularis mucosae. The lamina propria is separated from the base of the enterocytes by an amorphous basal lamina. Many blood vessels course through the submucosa, and form a dense capillary network close to the basal lamina of the epithelial layer. The muscularis mucosae of the intestinal tunica mucosa is a variably-developed layer

of smooth muscle that allows independent movement of the intestinal mucosa. This layer varies from quite robust in some fish to absent in others. Generally, in contrast to higher vertebrates, villi, simple tubular intestinal glands (crypts of Liberkuun) and acidophilic granular cells (of Paneth) are absent in this layer of the fish intestine (Kapoor et al., 1975; Lagler et al., 1977).

The tunica submucosa is composed of loose connective tissue containing blood vessels, nerve plexuses (Jobling, 1995), and adipose cells, as well as collagen and elastic fibers. Unlike mammals, most fish (except Gadidae and Macrouridae) lack glands in this layer (Lagler et al., 1977).

The tunica muscularis contains two distinct muscular layers, whose fibers are arranged perpendicularly to each other. The inner smooth muscle fibers are oriented in a circular direction, while the outer fibers run longitudinally parallel to the axis of the intestinal tract. In the Nile tilapia, the thickness of each muscular layer varies with region of the intestine (Smith et al., in preparation). Intestinal movement is stimulated by a nerve supply from the autonomic nervous system and stretch receptors located within the muscle of the intestine.

The outermost tunica serosa is the simplest of the layers, and consists of a thin band of loose connective tissue. In some places, this adventitial layer is covered by mesothelium, which appears as a single layer of cuboidal epithelial cells.

1.4: Feed and feeding

The natural geographic range of tilapian fish includes tremendous habitat diversity. This feature attests to the ability of tilapia to adapt to a broad range of environmental conditions, and also effectively utilize a wide variety of foods, ranging from insect larvae to algae, weeds and macrophytes (Allanson and Noble, 1964; Denzer, 1968; Stickney, et al., 1977). Young tilapia fry are largely carnivorous, hunting for zooplankton as well as aquatic larvae and terrestrial insects that have fallen into the water (Moriarty, 1973; Lowe-McConnell, 1975; Brown and Gratzek, 1980; Trewavas, 1983). As they grow, tilapia gradually begin taking more plant foods, including phytoplankton and macrophytes (Moriarty, 1973; Lowe-McConnell, 1975; Bowen, 1982; Trewavas, 1983; Lim, 1989). As adults, their diet is omnivorous to herbivorous. In culture, adult tilapia are similar to channel catfish in the ability to utilize protein and energy from fish meal, meat and bone meal. However, they have a greater capacity to digest and derive nutrients from highly fibrous feeds, such as alfalfa and coffee pulp, than channel catfish (Lovell, 1995).

1.5: Digestion

As in other animals, digestion in fish involves both mechanical and biochemical processes (Smith, 1991). Tilapia possess pharyngeal teeth capable of grinding (triturating) a wide variety of plant foods, especially algae, macrophytes and highly fibrous vegetables. This makes them able to utilize artificial feeds such as alfalfa and coffee pulp (Moriarty, 1973; Lagler et al., 1977; Trewavas, 1983; Lim, 1989). The 67% digestibility of protein found in alfalfa available to the Nile tilapia is similar to that of wheat bran (Popma, 1982). Tilapia also

have gill rakers protruding from each gill arch to prevent food from escaping and damaging the gill (Lagler et al., 1977). The stomach of tilapia secretes relatively strong acid that can break down the cell walls of certain vegetables (Moriarty, 1973). The long intestine of the tilapia provides a large surface area in this fish for nutrient digestion and absorption (Bowen, 1982; Smith et al., 2000).

1.5.1: Protein

Protein digestion in tilapia begins in the stomach, where endopeptidase activity from pepsin in gastric fluids (Fish, 1960; Nagase, 1964; Fange and Grove, 1979) assists in hydrolyzing large proteins into smaller peptide chains. The pepsins are secreted from the gastric glands in an inactive form (pepsinogen) which is subsequently activated by acids of the stomach (Fange and Grove, 1979). More distally along the digestive tract, the resulting polypeptides are broken down to low molecular weight peptides and amino acids suitable for the action of trypsin, chymotrypsin, and carboxypeptidases from the pancreas (Fish, 1960; Nagase, 1964; Cockson and Bourne, 1972; Moriarty, 1973; Fang and Chiou, 1989). Aminopeptidases from the intestinal secretion are also active in this regard (Keddis, 1957; Fange and Grove, 1979; Jobling, 1995; Tengjaroenkul et al., 2000). Though most protein digestion occurs in the intestinal lumen or along the enterocytes' brush border, small peptides are also hydrolyzed into free amino acids by the cytoplasmic enzymes of intestinal epithelial cells before being absorbed into the circulation. (Seligman et al., 1970; Overnell, 1973; Adibi and Kim, 1981; Hirji and Courtney, 1982).

1.5.2: Fat

Fish hydrolyze lipid substances (especially triglycerides) to fatty acids and monoglycerides by pancreatic lipase in the intestine (Vonk and Wertern, 1984). Pancreatic lipase becomes active in the presence of bile salts and colipase, a pancreatic enzyme which is activated by trypsin. Lipase can act mutually with pancreatic esterases or non-specific lipases in digestion of wax esters (Stevens and Hume, 1995) containing relatively short-chained, saturated fatty acids and alcohol. Phospholipids are broken down by the action of phospholipase in the presence of bile salts and calcium ions into fatty acids, alcohols and phosphoric acid. Like colipase, phospholipases are activated after a pro-enzyme is acted upon by trypsin in the gut lumen (Stevens and Hume, 1995).

Lipase and non-specific esterases are enzymes involved in digestion of glycerol esters of fatty acids. Activities of these enzymes in adult tilapia have been differently reported. One study reported an absence of lipase and esterase activity in the intestinal fluid of the Nile tilapia (Keddis, 1957), though other investigations (Al-Hussaini and Kholy, 1953) described lipase as distributed throughout the digestive tract of adult *T. nilotica* and *T. mossambica* (Nagase, 1964). Another study detected strong esterase activity, but no lipase activity, in intestinal fluid of mature *T. nilotica* (Moriarty, 1973).

1.5.3: Carbohydrates

Glycogen, starch, chitin, pectin and cellulose are the most common and important sources of carbohydrates in animals and plants. Chitin is a major structural carbohydrate in animals, whereas cellulose predominates in plants. Glycogen is the main storage form of carbohydrate in animals, whereas that of most plants is starch. Enzymes from the pancreas and the

intestinal epithelium hydrolyze poly- and oligosaccharides into monosaccharides. Amylase is a major pancreatic enzyme attacking α-1,4 linkages of starch and glycogen to convert these into oligosaccharides, maltose and isomaltose (Nagase,1964; Jobling, 1995; Horn, 1998). However, amylase cannot digest β-1,4 linkages of cellulose or α-1,4 linkages of pectin (Stevens and Hume, 1995). Oligosaccharides and disaccharides are hydrolyzed to monosaccharides, such as glucose and fructose, by maltase, sucrase and lactase of the intestinal cells (Lojda et al., 1979; Stevens and Hume, 1995; Tengjaroenkul et al., 2000). Cellulase has been reported found in a few fish species (Stickney and Shumway, 1974; Niederholzer and Hofer, 1979). Cellulase degrades plant cell walls, and may be produced by intestinal bacteria (Lindsay and Harris, 1980). Chitinase in the digestive tract of many fish is capable of breaking down chitin, the organic carbohydrate in the exoskeleton of crustaceans and insects (Fange et al., 1976).

1.6: Intestinal absorption

Absorption is the process by which digested food particles are transferred from the intestinal lumen to the blood vessels in the gut wall. The products of digestion can be absorbed into the enterocytes by various ways, such as diffusion or carrier-mediated transport (Kirschner, 1991; Jobling, 1995; Stevens and Hume, 1995). Though sugars, amino acids and water-soluble vitamins are transported to a limited degree by passive diffusion, most substances appear to be absorbed by carrier-mediated transport.

Many enzymes, such as ATPase and transferases, including alkaline phosphatase, also play an important role in nutrient absorption through the intestinal epithelium of fish. Intestinal

alkaline phosphatase is a hydrolytic enzyme considered to be involved in the absorption of lipid, glucose, calcium and inorganic phosphate (Malagelada et al., 1977; Roubaty and Portmann, 1988; Harris, 1989; Dupuis et al., 1991; Mahmood et al., 1994). The enzyme, localized in the brush border, is active in this regard. Detection of the enzyme in the supranuclear cytoplasm (Alpers et al., 1995; Tengjaroenkul et al., 2000) indicates the source of its production in the Golgi apparatus (Alpers et al., 1995).

1.6.1: Protein

Amino acids and peptides are absorbed against a concentration gradient using an active sodium-linked transport mechanism (Fange and Grove, 1979; NRC, 1993; Jobling, 1995; Stevens and Hume, 1995). The hydrolysis of ATP by the action of ATPase provides energy for transport. Previous studies have demonstrated that di- and tripeptides are transported more efficiently than free amino acids (Ash, 1980; Boge et al., 1981; Stevens and Hume, 1995). Protein and peptides may also be absorbed by pinocytosis or related processes without previous degradation (Hofer and Schiemer, 1981). The structure of the side chain of the amino acids is the major factor determining which transport system or systems are utilized (Prosser and De Villez, 1991; Stevens and Hume, 1995). Once inside the cell, transport to the blood over the cell's basal membrane is thought to occur by diffusion (Jobling, 1995). In addition, it is speculted that the function of dipeptidyl aminopeptidase IV involved in the absorption of small peptides across the intestinal lumen (Kim and Erickson, 1985).

1.6.2: Fat

Fatty acids and glycerides are absorbed differently than sugars and amino acids. Micelles are formed from small groups of bile salts, monoglycerols and long-chained fatty acids. When the

micelles contact the brush border, passive absorption of the monoglycerols and free fatty acids into the epithelial cells occurs (Stevens and Hume, 1995).

High levels of non-specific esterases have previously been reported in the intestine of tilapian fish (Li and Fan, 1997). Studies have demonstrated non-specific esterase reactions along the brush border and in the cytoplasm of the enterocytes of the intestine of the Nile tilapia, raising the possibility that lipid absorption and metabolism may involve both intra- and extracellular events (Deimling and Bocking, 1976; Van Lith et al., 1992).

1.6.3: Carbohydrates

Some glucose is absorbed by diffusion (Stevens and Hume, 1995). However, like amino acids, the absorption of glucose into enterocytes takes place against concentration gradients by means of secondary co-transport. Among fish, the absorption of sugar takes place by mechanisms similar to those of mammals. Teleost fish generally absorb carbohydrates in the form of monosaccharides, with herbivorous fish typically relying more on this pathway than carnivorous fish (Buddington et al., 1987; Buddington et al., 1997). However, in tilapian fish, disaccharides such as maltose and sucrose may be absorbed better than glucose (Shiau and Chuang, 1995). Nevertheless, absorption of glucose forms an important part of tilapian nutrition (Tung and Shiau, 1993).

1.7: Growth

Growth is a phenomenon resulting from a genetic-environment interaction that result in appropriate physiological and behavioral processes (Brette, 1979; Ricker, 1979) by which digestible energy from ingested food is transformed to body mass. The growth of fish can be

described by an asymmetric sigmoid curve (Soderberg, 1997). This descriptive model can be divided into three phases as exponential, linear and plateau phase. The exponential phase represents a surge increase of fish growth in the juvenile stage, which is followed by the linear phase during the maturation period before approaching the plateau phase where the fish grow close to its maximal size (Soderberg, 1997). In aquaculture, the exponential phase may be extended throughout the majority of the fish culture period, if the energy gained from food is converted largely to build fish body tissues, but not gonadal organs. This has been reported in the culture of rainbow trout; trout are not raised to a certain size, their body growth still follows an exponential growth curve (Soderberg, 1997). In contrast, tilapia generally reach maturation when they are relatively young (3-4 months of age) (Baylosis and Herrera, 1993). Therefore, tilapia potentially do not attain as large a market size as other cultured fish species such as trout, channel catfish and striped bass (Stickney, 1994). In order to solve this problem, numerous methods have been successfully used to increase the tilapia's ultimate size, including sex reversal using testosterone, monosex hybridization and cage culture (Stickney, 1994).

Under constant environmental conditions, fish length relates roughly linearly to fish age, while the relationship of the fish weight to the age is graphically exponential (Soderberg, 1997; Tengjaroenkul et al., in review). However, previous reports on practical pond culture of tilapia have shown that the tilapia’s body weight increased linearly with the age of fish (Anderson and Smitherman, 1978; Green and Teichert-Coddington, 1994). Moreover, Legendre and Albaret (1991) reported that growth rate is positively correlated with fish length and this relationship can be applied to fish in aquaculture.

Generally, the growth of fish is affected by two major factors: endogenous and exogenous. The endogenous factors relate directly to the genetics of fish (stocks, strain and species). In particular, genetics plays an important role in controlling maximal potential for growth in all living creatures, including fish grown for food under constant environmental conditions. The exogenous factors include quality and quantity of ration, feeding regime, social hierarchy, temperature, dissolved oxygen, level of nitrogenous waste, etc.

Regardless of individuals and species, the genetic factors have different impacts at different stages (phases) of fish growth. During the exponential phase, fish grow significantly faster than other phases because most of the ingested energy is transformed into body mass. While in the linear phase, a significant portion of the individual energy budget is diverted into development of reproductive organs, and thus somatic production occurs at a slower rate. Finally during the plateau phase, fish have very little growth as most of their energy is used mainly for maintenance, not for somatic growth (Rankin and Jensen, 1993).

Among fish exogenous factors, quality and quantity of ingested foods, greatly influences the growth of the fish by affecting the amount of energy and nutrients intake and their expenditure (Brette, 1979; Brett and Grove, 1979). Higher feed intake at optimal temperature and dissolved oxygen results in greater energy and nutrients intake, therefore fish grow at a faster rate. If energy expenditure approximates energy intake, fish have little energy left for growth, resulting in no growth or stunting.

Growth can be measured in any of three ways: anatomical, physiological, and biochemical. The anatomical mode is the simplest way to observe growth, by comparing size, length and weight of organs or body structures of the fish over a period of time (Brette, 1979; Ricker, 1979). Physiological measurements consider mainly fish bioenergetics, the outcome of net energy gained from metabolism (Brett and Grove, 1979; De Silva and Anderson, 1995). Biochemical manners determine the changes of the levels of RNA and oxidative enzymes directly responsible for protein synthesis (Jobling, 1993).

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CHAPTER 2

MORPHOLOGICAL DEVELOPMENT OF THE INTESTINAL TRACT OF THE TILAPIAN FISH, *Oreochromis niloticus*

2.1: ABSTRACT

Changes in length and configuration of the intestine during development of *Oreochromis niloticus* were described from hatch to week 14 post-hatch (definitive form). The morphological arrangement of the intestine over this time period was divided into seven stages based upon the presence and pattern of coiling. At hatch, the gut was simply a straight tube. Over time, the gut progressed into a complexly-coiled definitive form. The completed pattern, composed of two major coils and two intestinal loops, was first attained at 63 days post-hatch. During ontogeny, strong linear correlations were observed between fish size and gut length, as well as between the age of the fish and the ratio of gut length to standard length. During the developmental period, gut length increased dramatically from 90% to 410% of standard body length. Further, the rate of increase in both intestinal length and body length took place at an accelerating rate as the fish aged. The attainment of this great intestinal length, permitted only by the coiling, provides an advantage to the fish in digestion and absorption of food nutrients, and allows them to utilize a less energy-efficient herbivorous diet. Formulation of commercial diets to match the developmental stages of the intestine may be desirable in order to promote of increased feed efficiency and production performance of the fish.

2.2: INTRODUCTION

Change in gross morphology of the intestine during development is a common feature of numerous fish species (Stroband, 1977; Zihler, 1982; Yamaoka, 1985; Govoni, 1986; Reinthal, 1989). This change is usually demonstrated as alterations in length and configuration of the gut as a consequence of changes in functional demand in relation to the diet (Bucke, 1971; Douglas and Drewry, 1978; Geevarghese, 1983; Timmermans, 1987). Tilapias are a group of fish included in this general pattern. During growth from fry to adult, tilapia progress through a marked change in their dietary composition. As fry and young fingerlings, tilapia are carnivorous, depending largely on plankton and aquatic larvae. As the fish mature, they consume progressively more algae, weeds and macrophytes. As adult fish, plant material composes the larger part of their omnivorous diet (Moriaty, 1973; Lowe-McConnell, 1975; Bowen, 1982; Trewavas, 1983; Lovell, 1989). This alteration in feeding habits is accompanied by a considerable morphological change of the fish gut from a simple straight tube in the larval stage to a complex coiled pattern in the adult stage (Zihler, 1982; Baylosis and Herrera, 1993; Smith et al., 2000).

Though the intestinal morphology of adult tilapian fish has been studied to some degree (Zihler, 1982; Smith et al., 2000; Gargiulo et al., 1998), only one study of a single tilapian species, *Sarotherodon mossambicus*, has described changes in intestinal topography during the sub-adult and adult stages (Zihler, 1982). To date, no study has fully described changes of length and configuration of the gut during

development in the Nile tilapia, *Oreochromis niloticus*. Given the world-wide and increasing prominence of tilapia in aquaculture, this lack clearly extends beyond academic interest. In any fish so widely cultured, an understanding of morphological development is a requisite basis for distinction of conditions found at necropsy as normal or pathological. Further, such information also forms a foundation for improving management and production techniques. Toward this end, this study investigates the alteration of the relationship of the gut length to body size and age, as well as the ontogenetic progression of gut configuration of the Nile tilapia.

2.3: MATERIALS AND METHODS

2.3.1: Data collection

Approximately 150 Nile tilapia, *O. niloticus* Linnaeus, from the same brood were obtained and raised from hatch (week 0) to week 14 post-hatch in the Aquatic Medicine Laboratory of the Virginia-Maryland Regional College of Veterinary Medicine. The fish were reared in two 60-liter aquaria with a box filter system of dechlorinated municipal water. Water temperature and dissolved oxygen were maintained at 27 ±1°C and a range of 6 to 7 mg/L, respectively. Tilapia were fed ad libitum three times a day with a commercial fish diet (Salmon Starter, Zeigler Bros., Inc., Gardner, PA). Throughout the experiment, six fish were arbitrarily selected once a week for specimen preparation. Immediately upon collection, fish were anesthetized using tricaine methanesulfonate (Sigma Chemical Co., St. Louis, MO), the body cavity of each fish was opened, and the

entire fish fixed in 10% neutral-buffered formalin at 4°C for at least one week. At the time of examination, the gut mass was immersed in 5% methylene blue for 10 seconds to increase contrast among the coils. Drawings were made and photographs were taken from left, right and ventral aspects. In individuals in which the gut was coiled, the intestinal tract was carefully uncoiled to determine its course.

2.3.2: Data measurements

Gut length (GL) and body length of each fish were measured using calipers (Qing-gong, Republic of China) under a stereo-microscope. Total body length (TL) was measured from cranial end of the lower lip to the caudal end of the caudal fin; standard body length (SL) was measured from the cranial end of the upper lip to the base of the caudal fin of the fish (Trewavas, 1983).

2.3.3: Data analysis

Relationships between gut length and fish body size as well as the ratio (R) of gut length to standard length were expressed using linear regression models. Correlations between the body length as well as intestinal length and age in weeks (Wk) of the fish were plotted using exponential growth curves. Data were statistically calculated and analyzed using SAS (Statistical Analysis Software, SAS Institute Inc., NC). Results were also interpreted qualitatively, describing the increase of the gut length compared to the size and age of the fish during development.

2.4: RESULTS

2.4.1: Gross development of the intestinal tract

Not all fish within each weekly sample of six fish developed at the same rate. The number of fish in each sample of six that had developed to each particular stage is presented in Table 2-1.

The intestinal tract of hatchling *O. niloticus* was a simple, straight and undifferentiated tube. As the yolk sac reduced and body size increased associated with the stage in which active food searching behavior began, the growing intestine lengthen and progressively developed a more complex pattern. A minimum of 12 weeks (Table 2-1) was required for the gut to progress from the simple tube to the complexly-coiled definitive form as found in adult fish. The ontogenetic transition of the intestine in this study was divided into seven stages from A to G as follows:

A. The yolk sac was very large, and the fish were not free-swimming. The intestine as a part of alimentary tract formed a simple, straight and uncoiled or unlooped tube. Differentiation into regions was not possible (Figs. 2-1 and 2-5).

B. The yolk sac was dramatically reduced, and the fish began active swimming and searching for food. The intestine departed from the left side of the stomach and continued as the hepatic loop by passing along the edges of the liver before turning to the right side of the body cavity. Also, the gastric loop first developed in close physical proximity to blind end of the stomach (Figs. 2-1 and 2-5).

C. The two central flexures of each major coil were formed, the proximal one preceding and the distal one following the gastric loop. The hepatic and gastric loops increased in length but retained the same general form as in the previous stage (Figs. 2-2 and 2-5).

D. The elongating liver carried the hepatic loop caudally giving rise to distinct portions curving dorsally, caudally and ventrally along the borders of the liver. At this stage, both central flexures of the major coils began turning around each other in opposite directions. Parts of the intestine entering and leaving the gastric loop later became more recognizable as the proximal and distal major coils, respectively (Figs. 2-2 and 2-5).

E. The proximal and distal major coils increased in length and developed more coils, with the gastric loop interposed between the two major coils. The turning of both major coils was reciprocally related, with the central flexure of the proximal major coil coiling in a clockwise manner and the flexure of the distal major coil progressing in a counter-clockwise direction. Each of the major coils had progressed far enough in its coiling to clearly present its own centripetal (inward-turning), and centrifugal (outward-turning) loops. The proximal major coil was composed of about one and a half loops, while the distal major coil contained about two loops (Figs. 2-3 and 2-5).

F. The spiral intestine continued to rotate and develop additional loops. At this point, the proximal major coil was composed of about three and a half loops, with two centripetal loops and one and a half centrifugal loops. The distal major coil contained

about three loops, with one and a half loops in each of the centripetal and centrifugal coils (Figs. 2-3 and 2-5).

G. The cone-shaped mass of the major intestinal coils had essentially reached definitive form, composed of the hepatic loop, two major coils, and gastric loop. At this stage, the hepatic and gastric loops retained the same pattern as in the previous stages. The centripetal loops of both major coils spiraled inwardly in a clockwise direction, each composed of about two loops. The centrifugal loops lay anti-parallel to the centripetal loops, turning in a counter- clockwise direction, and each contained about two and a half loops (Figs. 2-4 and 2-5).

2.4.2: Length of the intestinal tract

During development, positive linear relationships were observed between gut length (GL) and fish length (TL and SL) including ratio of GL to SL on age of the fish (Table 2-3, Fig. 2-7). From week 1 to week 14, mean GL increased dramatically from 90% to 410% of SL. The relative GL ratio also increased directly with the fish age, changing from 0.9 in week 1 to around 4.1 in week 14 (Table 2-2, Fig. 2-7). The relationship of fish size and GL to age also increased in increasing rate, observed as exponential growth curves (Fig. 2-6). Standard deviation of the body length in the same fish group also became larger when the fish age increased (Table 2-2). Models, equations and parameters of the relationships between GL and body sizes as well as ratio of GL to SL on age (Wk) of the fish were summarized in Table 2-3.

2.5: DISCUSSION

2.5.1: Gross development of the intestinal tract

Changes in intestinal morphology of fish occur relatively rapidly compared to other organ systems (O'Connell, 1981). These alterations from a simple, straight tube progress to a more complex structure, and probably relate to increasing need for effective digestion and utilization food nutrients. Definitive patterns of intestinal coiling have been described in a number of cichlid fish (Zihler, 1982; Yamaoka, 1985; Reinthal, 1989). The arrangement of the tilapian intestine observed in this study resembles one of the developmental patterns proposed by Zihler (1982). For example, the intestine of the Nile tilapia first formed the gastric loop (a "flap-back" loop) as in *Haplochromis elegans* and *H. nigripinis*, and later, developed the two major coils by mutual folding of the central flexures. These major coils continued increasing their number of loops, and finally attained a definitive complex-coiling pattern similar to that found in *S. mossambicus.* From the anatomical features characterized above, the intestinal arrangement of the Nile tilapia observed in the present study appears most similar to that of the herbivorous tilapia, *S. mossambicus.* Trewavas (1983) had classified *O. niloticus* and *S. mossambicus* into the same genus using various criteria other than the gut coiling pattern. Further description of patterns of intestinal configuration as observed in our study might be taken into consideration as an additional character in taxonomic study of the cichlids.

Compared to other gut regions, the gastric loop of the Nile tilapia demonstrated little lengthening and no coiling, which is similar to numerous cichlid fish (Zihler, 1982; Yamaoka, 1985). Zihler (1982) speculated that this gut portion may develop differently

from its adjacent areas. In a work on the adult intestinal morphology of the Nile tilapia (Smith et al., 2000), the gastric loop also stood out from the other intestinal regions as being the most variable in terms of total percentage of intestinal length. Further, preliminary studies in our laboratory have shown this intestinal segment to express differences in appearance and intensity of digestive enzymes as compared to other regions.

The ontogenetic changes of the gut configuration seem to be influenced by the three major factors of body cavity, genetics and feeding habits. During growth from the fry stage, the degree and rate of intestinal growth far exceed that of the body cavity, and the limitation of space in body coelom in later stages causes the formation of intestinal loops and coils.

Genetics also has an influence on anatomical development, dictating definitive form in developmental processes. Despite feeding a highly-digestible commercial diet throughout the experiment, the gross morphology of the tilapia intestine still attains a definitive form intimately resembling the pattern in wild-caught Nile tilapia described by Al-Hussaini and Kholy (1953). This suggests that fish genetics probably has more influence on definitive gross intestinal morphology than the food resources.

Comparing fish of the same sizes and ages, considerable variations in intestinal coiling patterns were observed (Table 2-1). For example, at day 63 post-hatch, one fish demonstrated the more advanced development defined as in stage G rather than the less-developed pattern as in stage F. This likely reflects individual variation, and may also

imply differences in how aggressive these individuals were in feeding: more aggressive individuals securing more food may grow at a faster rate.

In natural populations, alterations in feeding habits of carnivorous tilapia fry to more herbivorous feeding habits when growing may relate to the development of the intestinal loops and coils (Moriaty, 1973; Lowe-McConnell, 1975; Zihler, 1982; Lovell, 1989; Smith et al., 2000). Progressing from a more digestible carnivorous diet to a relatively less-digestible herbivorous-omnivorous one, the progression to the complexly-coiled definitive form provides the advantage of packing more intestine into a relatively small body cavity and thus increasing surface area for the digestion and absorption of food particles.

2.5.2: Length of the intestinal tract

Zihler (1982) suggested that using intestinal weight length ratio for allometric calculations was useful in evaluation of different fish body shapes. However, relative gut length ratio (R) was more suitable for use in the present work than intestinal weight length because only one fish species was studied and different body shapes were not a factor.

At hatch, the fish intestine was undifferentiated from the alimentary tract and SL was difficult to measure. Therefore, SL, GL and R values of the fish in this stage cannot be determined (Table 2-2). As the fish grew, however, positive and strong relationships were observed between GL and fish size (r^2 =0.9608 for TL and r^2 =0.9606 for SL)

including relative GL ratio on age (r^2 =0.848) (Fig. 2-7, Table 2-3). Slopes of the equations of these correlations (Table 2-3) confirm that as the fish grew, the GL also increased. The GL of this fish lengthened about 3.63 and 4.67 times more rapidly than the TL and SL, respectively. Furthermore, mean GL from week 1 to week 14 increased dramatically from 90% to 410% relative to SL. This suggeats that increasing GL during development likely improves feed digestion and absorption of the fish, contributing to increased rate of fish growth.

Generally, growth is not uniform within fish populations. In this study, differences in mean body lengths developed immediately in the first week after hatch (Table 2-2). Further, standard deviations of the body length in a given group of fish were larger as the fish age increased. Similar variations have also been reported in other studies; such findings suggest that variables like fish size, stocking density, behavioral dominance and genetic influence can have significant impacts on growth rate of fish (Tave and Smitherman, 1980; Koebele, 1985; Jobling and Reinsnes, 1987; Thorpe, 1987; Francis, 1988; Zonneveld and Fadholi, 1991; Huang and Chiu, 1997). In the present study, larger fish most likely out-competed the smaller ones and thereby gained more access to food. That competitive feeding behavior develops as early as the second week after hatch is noteworthy, and suggests that in order to promote maximum growth in cultured fish, sufficient food must be provided to satiate the most aggressive fish and still leave food for the less competitive individuals.

The ratio of GL to SL increased linearly with the fish age (Fig. 2-7), this relationship being described as: $R = 0.8116 + 0.2581Wk$ (r^2 =0.848, n=84, $P<0.001$)(Table 2-3). The

reciprocal of the slope is the ratio of gut growth rate to the standard length growth rate; this value showed that the gut grew 3.87 times as fast as SL. The R value can be used as a criterion to attempted correlation with feeding habits of the fish. Based on this criterion, Bowen (1982) reported that the relative gut length ratios of the cichlids were shortest in carnivores, intermediate in omnivores and longest in herbivores. Buddington et al. (1997) stated that larvae of most fish are carnivorous, and tilapia fit this generalization. Trewavas (1983) found that the Nile tilapia fry, less than 6 cm TL, are still omnivorous and consume significant amounts of animal food. Based on gut morphology, the tilapia larvae in early stages of development in the present study (especially in the first two weeks) should be categorized as carnivorous, because the gut configuration was still arranged in a simple form with little or no looping (Figs. 2-1 and 2-5). The mean R value was less than 1.2 when the fish length was below 1.2 cm (Table 2-2).

The relationship of fish body size and the gut length on age was exponential in form (Fig. 2-6), suggesting that the growth of both fish size and gut length increased at a rate that itself increased with time. The rapid lengthening of the intestine during development contributed to this climbing rate of growth by increasing gut surface area, enhancing digestion and absorption, thereby raising energy available for growth, thereby increasing the body size of the fish.

2.6: CONCLUSION

This study investigated and described the ontogenic changes in coiling pattern and length of the intestinal tract of the Nile tilapia. The dramatic change from the straight intestinal

tube of the hatchling to the complex coiling pattern of the adult likely provides advantage to the fish by increasing both intestinal length and surface area. This results in increased food retention time and opportunity for continued enzymatic action on the food, allowing the fish to successfully derive sufficient energy from a more herbivorous diet as they mature. This predictable passage through the developmental stages suggests that, to maximize feed efficiency, diets should be formulated to appropriately match the morphological and physiological progression from carnivorous (first two weeks) to omnivorous (succeeding 10 weeks) to omnivorous-herbivorous (from 12 weeks onward).

2.7: ACKNOWLEDGEMENT

I would like to thank Sandy A. Brown for fish culture and Daniel Ward for statistical analysis.

2.8: BIBLIOGRAPHY

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TABLES

Table 2-1. Numbers of fish at each stage of intestinal development by a function of fish age in weeks

	Age of Fish (week)														
Stage	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
A	6														
B		6	1												
C			5	3	1										
D				3	4	1									
E					1	5	5	4	3	2					
F							1	2	3	3	1	1			
G										1	5	5	6	6	6

Numbers of fish in each column represent the number among the six fish in each weekly sample that had developed to each defined intestinal form.

Table 2-2. Mean total lengths (TL) (± SD), mean standard lengths (SL) (± SD), mean gut lengths (GL) (± SD), and R (ratio between gut length and standard length) (± SD) as a function of age of fish

Age (week)	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
TL (mm) ± SD	5.0 ±0.9	9.0 ±0.1	11.5 ±0.8	15.1 ±2.5	19.3 ±2.5	21.7 ±1.3	25.1 ±1.5	27.4 ±1.7	31.7 ±1.6	35.3 ±2.6	44.6 ±5.3	49.9 ±5.1	58.8 ±8.4	64.6 ±6.5	75.6 ± 5.4
SL (mm) ± SD	-	7.2 ±0.1	9.0 ±0.7	11.9 ±2.0	15.0 ±2.0	17.3 ±1.0	19.8 ±1.0	20.8 ±1.0	24.3 ±1.2	27.2 ±2.1	34.1 ±4.1	38.4 ±4.0	45.5 ±6.5	50.1 ±5.2	59.5 ±4.4
GL (mm) ± SD	-	6.8 ±0.5	10.2 ±1.9	17.8 ±5.4	23.4 ±2.8	38.0 ±4.5	51.2 ±8.8	62.4 ±17.4	76.1 ±13.8	88.8 ±7.9	119.6 ±24.8	144.6 ±34.6	177.9 ±34.4	198.8 ±44.0	242.9 ±27.4
R (mm) ± SD	-	0.9 ±0.1	1.1 ±0.1	1.5 ±0.2	1.6 ±0.2	2.2 ±0.2	2.6 ±0.6	3.0 ±0.6	3.1 ±0.5	3.3 ±0.3	3.4 ±0.5	3.7 ±0.6	3.9 ±0.3	3.9 ±0.7	4.1 ±0.5

Table 2-3. Summary of models, equations and parameters of the relationships between gut lengths and body sizes, as well as ratio of gut to standard length, body lengths and gut length on age of the fish.

Relationship	Model	Equation	r^2	N	P-value
GL to TL	Linear	GL = -36.9549 + 3.6334TL	0.9608	84	< 0.001
GL to SL	Linear	GL = -36.513 + 4.6694SL	0.9606	84	< 0.001
GL/SL to Age	Linear	R = 0.8116 + 0.2581Wk	0.8480	84	< 0.001
TL to Age	Quadratic	$TL = 8.88919e^{0.1569Wk}$	0.9693	84	< 0.001
SL to Age	Quadratic	$SL = 7.0512e^{0.1542Wk}$	0.9699	84	< 0.001
GL to Age	Quadratic	$GL = 7.5685e^{0.2653Wk}$	0.9444	84	< 0.001

GL= gut length; SL= standard length; TL= total length; Wk= week; R= ratio of gut length to standard length; r^2= coefficient of determination; n= number of fish sample

FIGURES

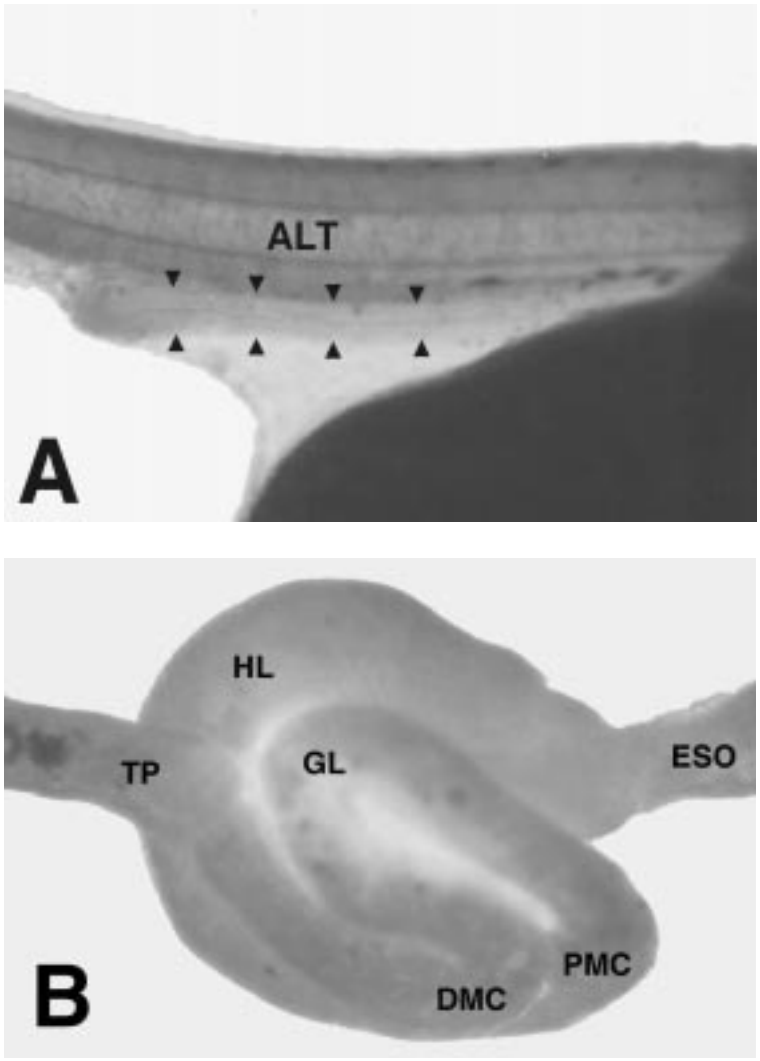

Fig. 2-1. *O. niloticus.* Photomicrographs of the stages A (at hatch) and B (at week 1 post-hatch) of the developmental morphology of the intestine. Right View. ALT= alimentary tract (arrows) ; DMC= distal major coil; ESO= esophagus; GL= gastric loop; HL= hepatic loop; L= liver; PMC= proximal major coil; S= stomach; TP= terminal portion of the intestine.

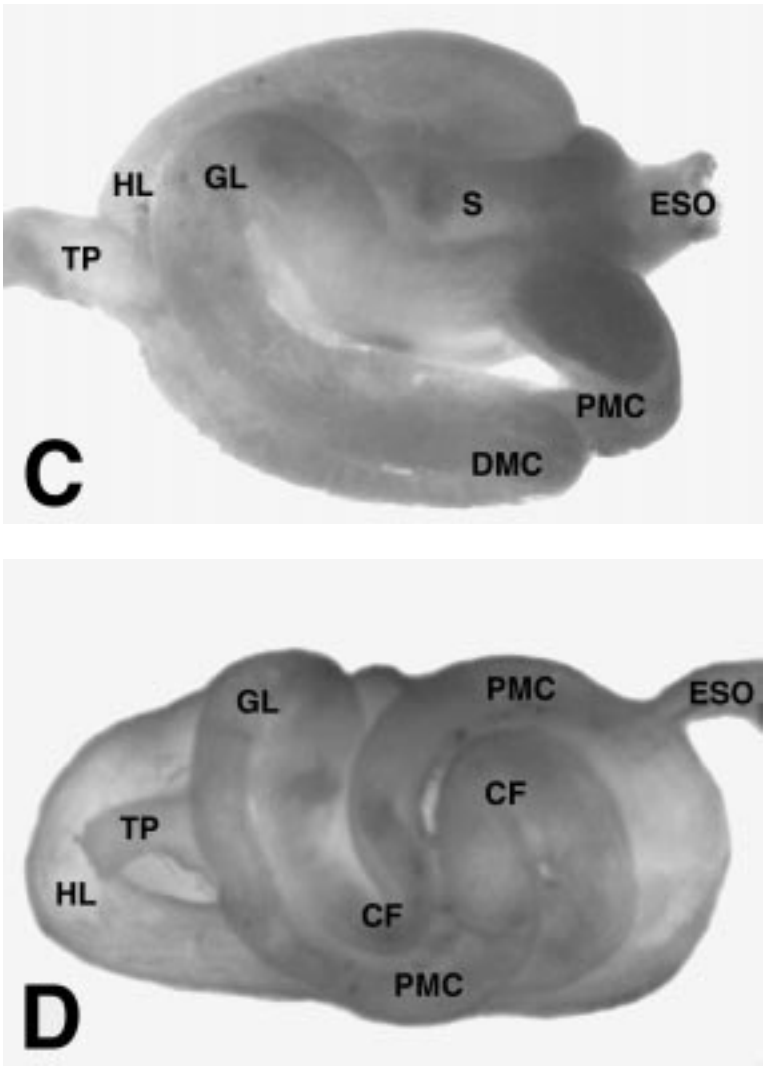

Fig. 2-2. *O. niloticus.* Photomicrographs of the stages C (at week 3 post-hatch) and D (at week 5 post-hatch) of the developmental morphology of the intestine. Right View. ALT= alimentary tract; CF= central flexure; DMC= distal major coil; ESO= esophagus; GL= gastric loop; HL= hepatic loop; L= liver; PMC= proximal major coil; S= stomach; TP= terminal portion of the intestine.

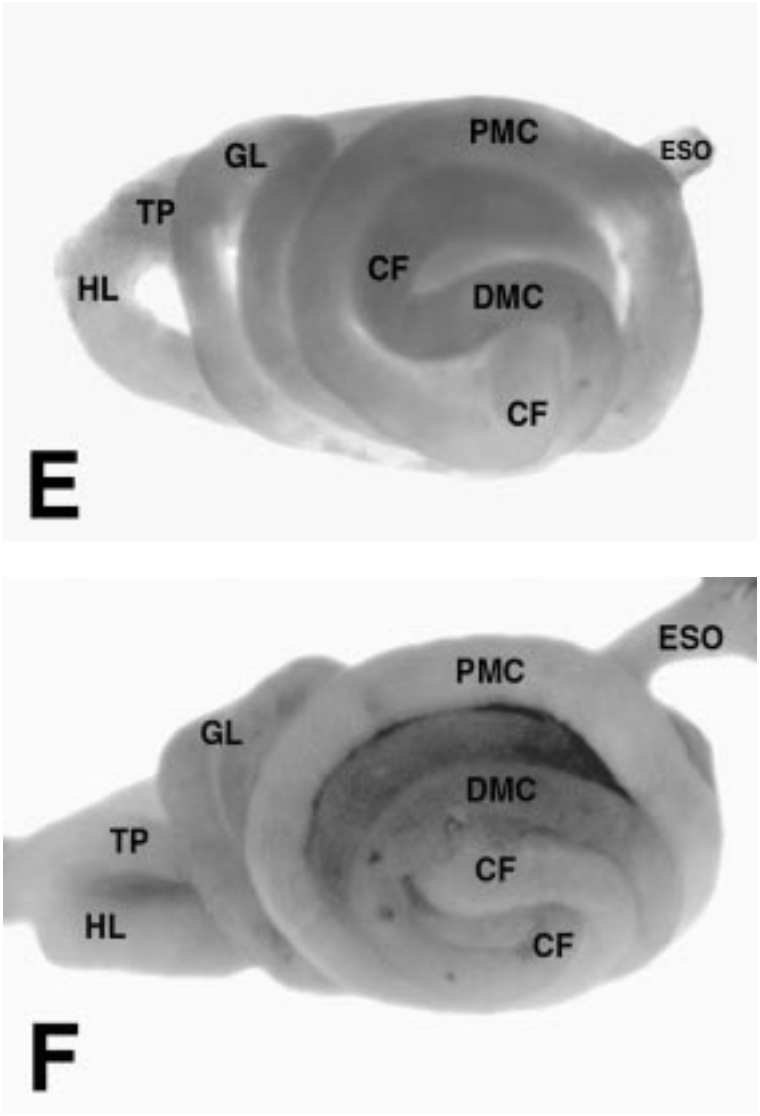

Fig. 2-3. *O. niloticus.* Photomicrographs of the stages E (at week 6 post-hatch) and F (at week 6 post-hatch) of the developmental morphology of the intestine. Right View. ALT= alimentary tract; CF= central flexure; DMC= distal major coil; ESO= esophagus; GL= gastric loop; HL= hepatic loop; L= liver; PMC= proximal major coil; S= stomach; TP= terminal portion of the intestine.

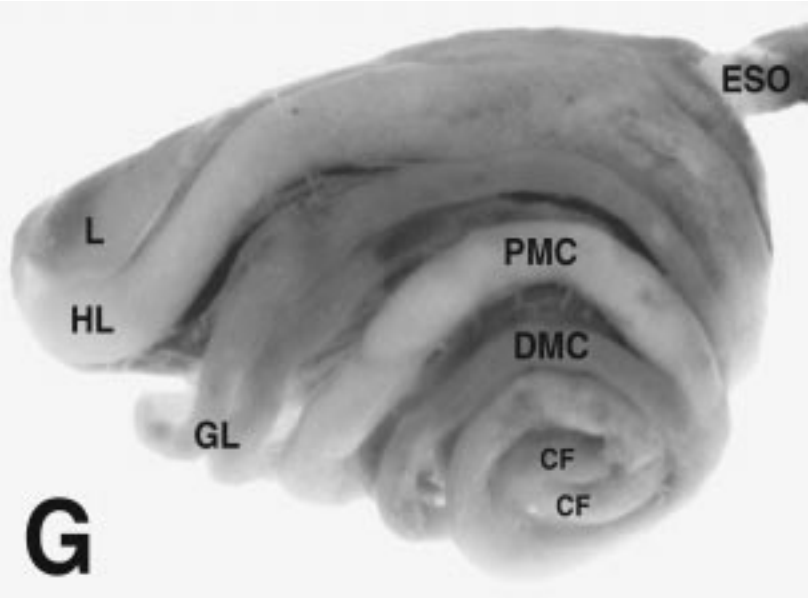

Fig. 2-4. *O. niloticus.* Photomicrographs of the stages G (at week 9 post-hatch) of the developmental morphology of the intestine. Right View. ALT= alimentary tract; CF= central flexure; DMC= distal major coil; ESO= esophagus; GL= gastric loop; HL= hepatic loop; L= liver; PMC= proximal major coil; S= stomach; TP= terminal portion of the intestine. (at week 5 post-hatch)

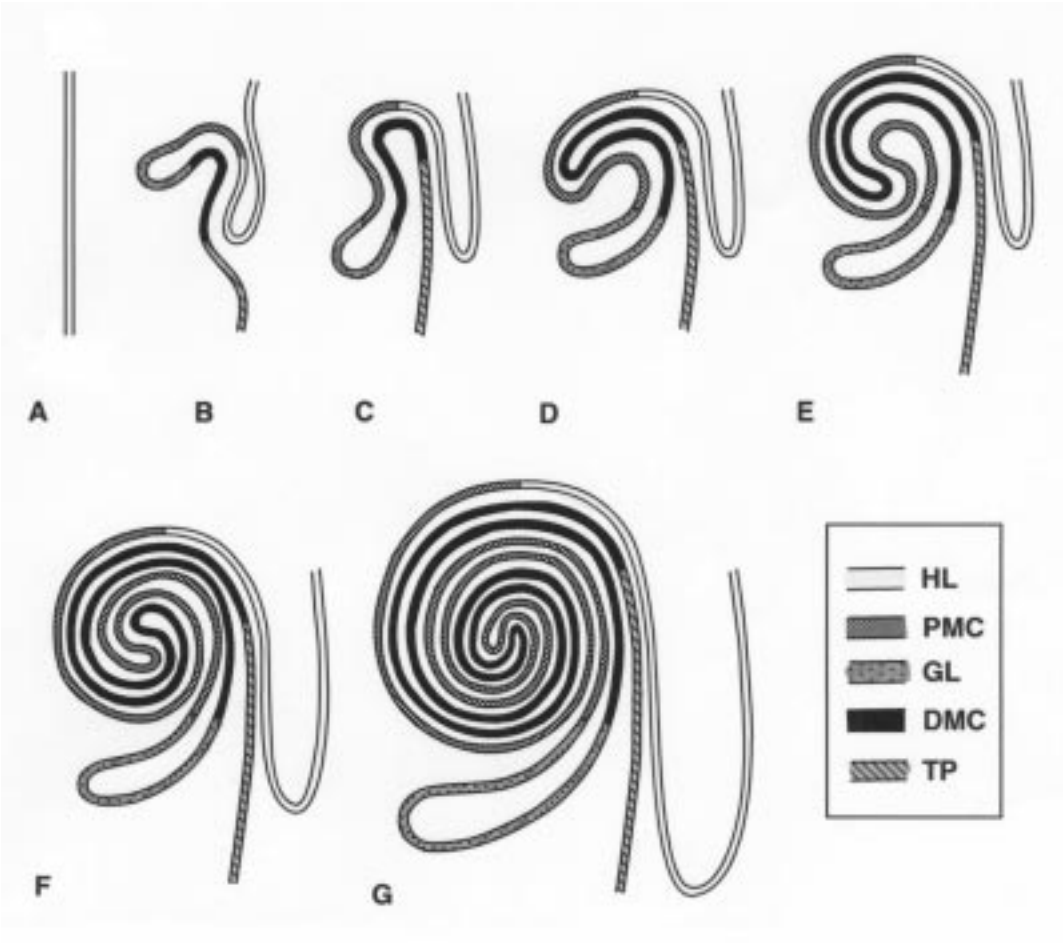

Fig. 2-5. *O. niloticus.* Schematic drawings of the seven stages (A to G) of the developmental morphology of the intestine. Ventral View. DMC= distal major coil; GL= gastric loop; HL= hepatic loop; PMC= proximal major coil; TP= terminal portion of the intestine.

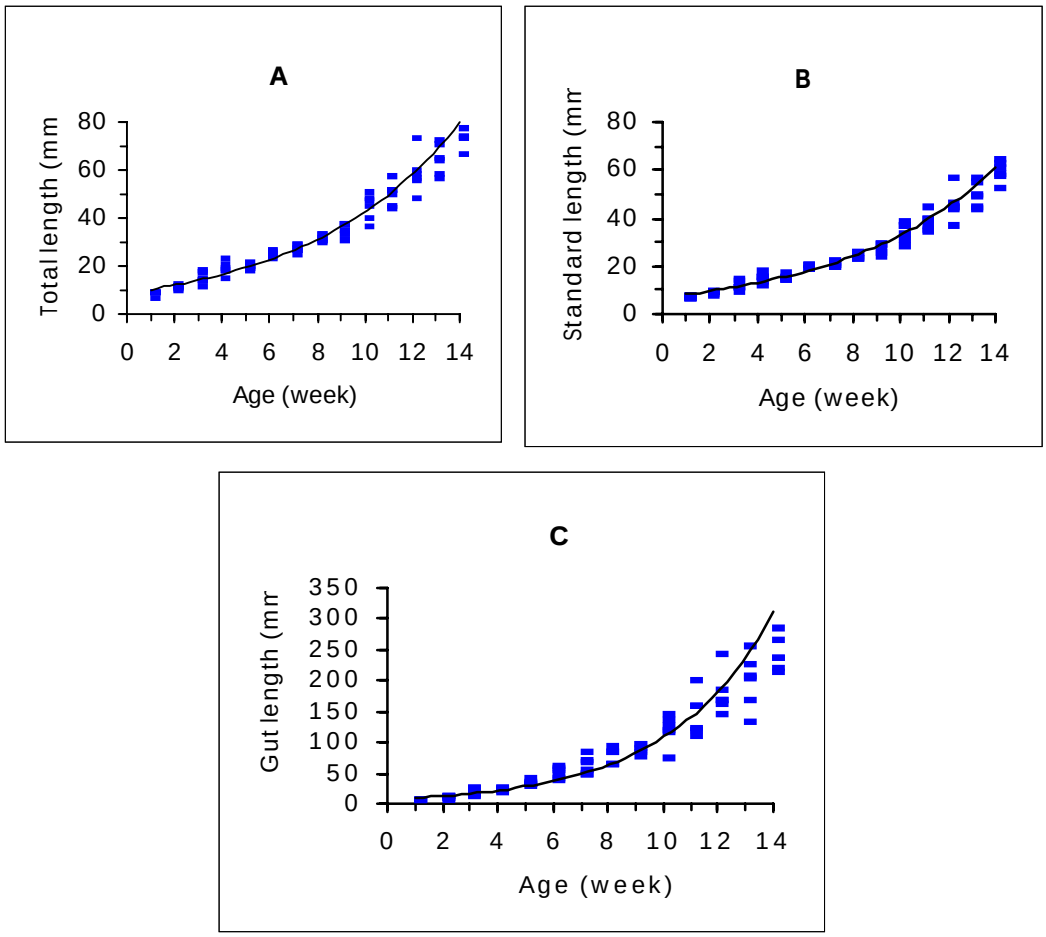

Fig. 2-6. *O. niloticus.* Scaling of fish length and gut length to age. Lines show exponential growth curves fitted to all data points. **A:** relationship between total length and age. **B:** scaling in standard length and age. **C:** relationship between gut length and age of the fish.

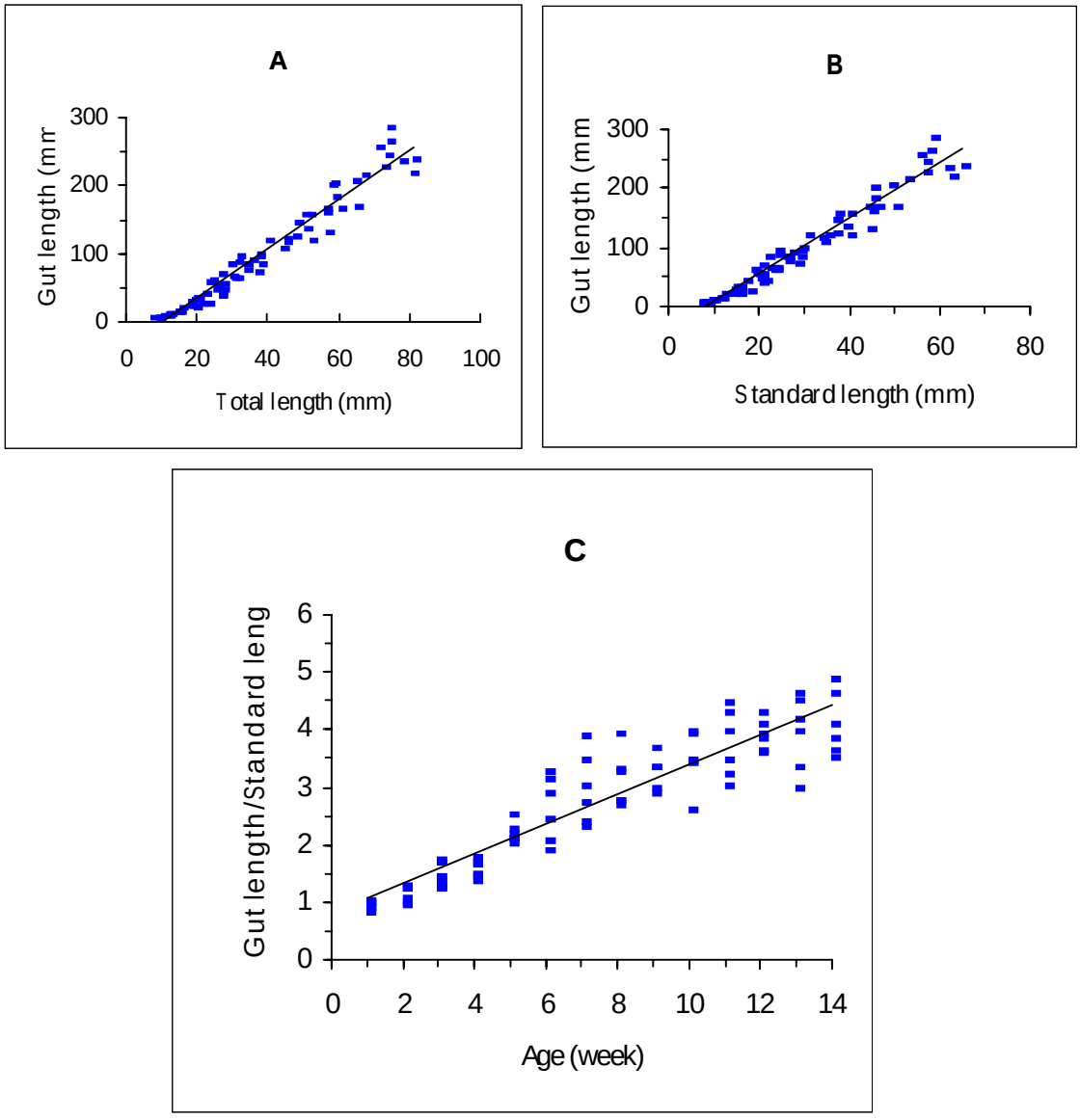

Fig. 2-7. *O. niloticus.* Scaling of gut length to fish length and relative gut length ratio to age. Lines are averages of regressions fit to all individuals. **A:** relationship between gut length and total length. **B:** scaling in gut length and standard length. **C:** relationship between ratio of gut length to standard length and age of the fish.

CHAPTER 3

DISTRIBUTION OF INTESTINAL ENZYME ACTIVITIES ALONG THE INTESTINAL TRACT IN *O. niloticus*[1]

3.1: ABSTRACT

Regional distribution of intestinal enzymes in Nile tilapia was investigated using enzyme histochemistry. Samples from adult fish were obtained from the five major intestinal segments. Activities of maltase, leucine aminopeptidase, dipeptidyl aminopeptidase IV, lipase, non-specific esterases, and alkaline phosphatase were examined in each segment. All enzymes were present at specific sites along the first four intestinal segments. Strong reaction for maltase was present in the third intestinal segment, while aminopeptidases and alkaline phosphatase were detected in the first three parts. The most intense activity for lipase was present in the first two parts, while non-specific esterases were observed in the first four portions. Activities of all these enzymes were demonstrated in the brush border. Non-specific esterases were also present in the cytoplasm of the enterocytes. In addition to its brush border localization in the cranial segments, dipeptidyl aminopeptidase IV was also observed in the basal lamina of all segments, including the terminal segment. These results suggest that the first four regions play the most important role in both digestion and absorption of nutrients. In addition, the great length of the portions of the intestinal tract particularly in digestion provides abundant surface area for nutrient absorption, and is likely in one factor involved in rapid growth rate characteristic of tilapian fish.

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3.2: INTRODUCTION

As in other vertebrates, the ability of fish to utilize ingested nutrients depends on the presence of appropriate enzymes in appropriate locations in the wall and along the lumen of the intestinal tract. Generally, distribution and intensity of intestinal enzyme activity along the gut varies with feeding habits and intestinal morphology (Cockson and Bourne, 1972; Hofer and Schiemer, 1981; Kuzmina, 1984; Kuzmina and Smirnova, 1992; Sabapathy and Teo, 1993). Though tilapian fish have been categorized as herbivorous, many are well-known for their ability to utilize a wide variety of foods including aquatic larvae and insects as well as algae, weeds and macrophytes (Lowe-McConnell, 1975; Bowen, 1982; Trewavas, 1983). Various intestinal enzymes involved in digestive and absorptive processes have been reported in tilapian fish, such as amylase, pepsin, trypsin, esterases and alkaline phosphatase (Nagase, 1964; Cockson and Bourne, 1972; Moriarty, 1973; Klaren et al., 1993; Li and Fan, 1997). As with other herbivorous fish, tilapia demonstrate greater activity of carbohydrase than protease, and a lesser lipase activity compared to carnivorous and omnivorous fish (Fish, 1960; Agrawal et al., 1975; Das and Tripathi, 1991; Opuszynski and Shireman, 1995).

Tilapias enjoy worldwide prominence in foodfish aquaculture, and are also growing in their importance as a laboratory animal. Recently, their complex gross intestinal morphology has been described as being composed of five major segments, a notable difference from other cultured fish (Smith et al., 2000). Neither the distribution along the intestinal tract nor the cellular location of their intestinal enzymes have been completely characterized. This study began this characterization by using enzyme histochemistry to investigate the occurrence, distribution and cellular localization of selected intestinal enzymes in the Nile tilapia.

3.3: MATERIALS AND METHODS

3.3.1: Preparation of tissue sections

Six mature Nile tilapia, *Oreochromis niloticus*, age 10-12 months, were obtained from the same brood at the Aquatic Medicine Laboratory of the Virginia-Maryland Regional College of Veterinary Medicine at Virginia Polytechnic Institute and State University. The fish were 15.7-18.1 cm (mean 16.95± S.D. 0.81 cm) in total length and weighed 72-97 g (mean 83.33± S.D. 9.05 g). Tilapia were fed two times a day with commercial fish diet (33% protein, 2.5% fat, 6% fiber, 10% ash) (Koi pond nuggets, PMI Feeds, Inc., St. Louis, MO). At time of tissue collection, fish were anesthetized with tricaine methanesulfonate (MS-222, Sigma Chemical Co., St. Louis, MO) and then killed by cervical separation. Intestinal samples were obtained from the five specific segments: the hepatic loop, proximal major coil, gastric loop, distal major coil, and the mid-portion of terminal part well proximal to the rectum (Plate I) (Smith et al., 2000). All tissue specimens were embedded in Optimal Critical Temperature Compound (VWR Scientific Products, Bridgeport, NJ), frozen in liquid nitrogen, and then sectioned on a cryotome at -15°C. Sections of 10 μm thickness were retrieved from the knife on positive-charged slides (Superfrost/plus, Fisher Scientific, Pittsburgh, PA).

3.3.2: Chemicals

Substrates, diazonium salts and conditions of incubation media for the enzyme studies are summarized in Table 3-1. All chemicals in this study were obtained from Sigma Chemical Co., St. Louis, MO. The buffers for each enzyme were specifically prepared as follows: 0.1M citric acid-phosphate buffer for maltase; 0.1M phosphate buffer for leucine aminopeptidase, dipeptidyl aminopeptidase IV, lipase, and non-specific esterases; and 0.1M Tris buffer for intestinal alkaline phosphatase.

3.3.3: Fixation, staining and enzyme examination

Before incubation in media, fresh cryostat sections intended for study of maltase (Malt), lipase (Lip), non-specific esterases (NSE) and intestinal alkaline phosphatase (IAP) were fixed for 5 min in 10% neutral buffered formalin. For detection of leucine aminopeptidase (LAP) and dipeptidyl aminopeptidase IV (DAP IV), tissue sections were fixed for 1 min in equal parts of cold absolute acetone and chloroform. Preparation of incubation media and procedures for the enzyme examinations followed established techniques using substituted naphthol methods (Lojda et al., 1979; Bancroft and Hand, 1987; Knospe and Plendl, 1997). After incubation, the tissue sections were coverslipped with mounting medium and examined using a light microscope. Enzyme activities were evaluated as strong (++), weak (+) or absent (-) depending on the staining intensity of the azo dye in the tissue (Hirji and Courtney, 1982; Gawlicka et al., 1995) using Q3 image analysis. Intestinal sections incubated without substrate were used as controls.

3.4: RESULTS

Intestinal enzymes demonstrated a marked difference in regional distribution and localization along the intestinal length (Table 3-2). Activities of various enzymes were detected at characteristic sites along the first four intestinal segments. Most of these activities localized along the brush border, but non-specific esterases were also present in the cytoplasm of the enterocytes, and DAP IV was also detected in the basal lamina of all segments.

Maltase activity was found in the brush border of the columnar epithelial cells (Fig. 3-1) in the first four intestinal segments, with the most intense staining observed in the third segment. Leucine aminopeptidase and DAP IV were detected in the microvilli of the enterocytes (Fig. 3-2) in the first four intestinal segments. Both peptidases demonstrated stronger activities in the first three intestinal segments than in the fourth. Weak DAP IV staining was also observed in the basal lamina (Fig. 3-2) of all intestinal segments. Lipase activity was detected in the brush border (Fig. 3-3) of the first three parts of the intestine, with the most intense enzyme reaction observed in the first two regions. Uniform non-specific esterase activity was found in the first four intestinal regions. The esterase reactions were present in the microvilli, and also in cytoplasm of the enterocytes (Fig. 3-3). Intestinal alkaline phosphatase activity was detected in the first four intestinal regions. The strongest reaction was observed in the first three intestinal segments, with a rapid decrease in intensity taking place at the transition from the third to the fourth segment. The enzyme reaction was localized in the brush border of the columnar

epithelial cells (Fig. 3-4). Higher magnification also demonstrated this enzyme activity in the supranuclear cytoplasm of the enterocytes (Fig. 3-4).

3.5: DISCUSSION

Digestion and absorption of food particles and molecules generally takes place along the brush border of the columnar epithelial cells, where numerous digestive and absorptive enzymes are localized. Examples of such enzymes include maltase, dipeptidases, lipase and alkaline phosphatase (Hirji and Courtney, 1982; Kuzmina and Gelman, 1997). In the Nile tilapia as in other teleost fish, these enzymes are variously distributed along the length of the intestine.

3.5.1: Maltase

Teleost fish generally absorb carbohydrates in the form of monosaccharides, with herbivorous fish typically relying more on this pathway than carnivorous fish (Buddington et al., 1987). Because maltase hydrolyses the disaccharide maltose to produce the monosaccharide glucose, the observation of the greatest maltase activity in the gastric loop suggests that the middle intestinal region is the most active region in formation of glucose. The resulting glucose may be absorbed there and/or more distally along the intestinal tract. Interestingly, the distribution of maltase activity observed in the present work corresponds well with the earlier work on amylase, the enzyme that hydrolyzes maltose to glucose (Stevens and Hume, 1995; Horn, 1998).

Nagase (1964) reported the greatest amylase activity from the middle portion of the intestine of *Tilapia mossambica*, similar to that observed here in the Nile tilapia for maltase. A similar topographic distribution pattern of these two enzymes has apparent functional significance: amylase would produce the substrate for maltase activity.

Variations in distribution of maltase activity along the intestinal length have been reported in numerous fish species. Bream (*Abramis brama*) demonstrated uniform enzyme reaction along the length of the intestinal tract (Kuzmina, 1985), whereas rainbow trout (*Oncorhynchus mykiss*) showed greater activity in the cranial than in the caudal region (Costanzo et al., 1983). Peak maltase activity in pike (*Esox lucius*) was present in the middle gut segment (Kuzmina, 1985), while in the ayu or sweet-fish (*Plecoglossus altivelis*) maximal activity was observed in the caudal intestinal region (Kawai and Ikeda, 1971). Optimal pH for maltase activity has also been reported to vary among fish. For the Nile tilapia, the optimum pH for this enzyme reaction has been reported as 6.9 (Keddis, 1957); for bream and roach, 7.0 to 8.0, and for pike, 8.0 (Kuzmina and Nevalenny, 1983). Intestinal pH in the immediate post-gastric segment of Nile tilapia has been reported as relatively low, about 5.5-6.0 (Moriarty, 1973), differing widely from the reported optimal pH of maltase activity at 6.9. Thus, weak maltase activity in the most cranial segment (the hepatic loop) of the tilapian intestine may relate at least in part to the pH of this segment.

3.5.2: Peptidases

Protein digestion in tilapia begins with the hydrolysis of proteins and polypeptides by the action of pepsin, trypsin and chymotrypsin (Fish, 1960; Nagase, 1964; Cockson and Bourne, 1972; Moriarty, 1973; Fang and Chiou, 1989). Long chain peptides are then further digested by peptidases such as LAP and DAP IV into smaller peptides and amino acids (Jobling, 1995). Our results show strong activities for both peptidases in the first three gut segments, which compose about 65% of total intestinal length (Smithet al., 2000). Thus, more than half of the intestinal length plays an important role in peptide degradation. These regions of the tilapian intestine contain peptidases capable of hydrolyzing peptides, particularly peptides in which the N-terminal position contains the amino acid proline (for dipeptidyl aminopeptidase IV) and all common amino acids except proline (for leucine aminopeptidase) (Lojda et al., 1979; Sakai and Kawatsu, 1983; Pearse, 1985). In addition, the function of DAP IV is speculated to be involved in the absorption of small peptides across the intestinal lumen (Kim and Erickson, 1985). Previous studies have also demonstrated that di- and tripeptides are transported more efficiently than free amino acids (Ash, 1980; Boge et al., 1981; Stevens and Hume, 1995). Our results suggest that the hydrolysis of polypeptides into peptides and amino acids, as well as the absorption of short peptides in Nile tilapia, takes place mainly in the first three gut regions. These findings are in agreement with Bowen (1981), who stated that proteins were completely digested and absorbed in the cranial half of the intestinal tract of *T. mossambica.*

The weak peptidase reactions in the fourth intestinal segment, and their absence from the brush border in the terminal segment, implies a relatively smaller role of these regions in peptide hydrolysis. The lower enzyme activities here likely relate to the effect of pH changes along the intestinal tract (Moriarty, 1973), as well as the resorption of the enzymes into the gut mucosa (Hofer and Schiemer, 1981).

The distribution and intensity of peptidases observed in this study correlate well with the presence and activity of protease enzymes reported in some other tilapia. Fish (1960) reported greater protease activity in the cranial part than in the caudal part of the intestine in *T. mossambica*. Similarly, *T. mossambica* demonstrated a higher trypsin activity in the cranial than caudal intestinal regions (Nagase, 1964), while *T. shirana* showed pepsin and trypsin activities strictly in the cranial intestinal segment (Cockson and Bourne, 1972). Thus, peptidases are present in the same location as protease, where they can immediately act upon the short-chain peptides produced by the proteases.

Interspecific differences have been noted in localization of peptidase activities in the enterocytes. Similar to the results seen here in the Nile tilapia, aminopeptidases in other teleost fish generally are present in the brush border (Hirji and Courtney, 1982; Vonk and Western, 1984; Gawlicka et al., 1995). However, leucine aminopeptidase in other vertebrates including fish has also been found in cytoplasmic organelles and vesicles of the columnar epithelial cells (Seligman et al., 1970; Overnell, 1973; Adibi and Kim, 1981; Hirji and Courtney, 1982). Complete digestion of peptide in those species apparently involves the enterocytic cytoplasm as well as the brush border.

3.5.3: Lipase

Early works on lipase activity in the intestinal tract of tilapia have reported varied results. Keddis (1957) and Moriarty (1973) found no lipase activity in the gut of *T. nilotica*. However, Al-Hussaini and Kholy (1953) and Nagase (1964) had previously demonstrated this enzyme activity in tilapia, with the activity most prominent in the proximal and middle parts of the intestine. These previous studies used tributyrin, an ester of butyric acid, as the enzyme substrate. However, tributyrin is not specific for lipase, and can also be hydrolyzed by esterases. Thus, those reports of lipase activity (Al-Hussaini and Kholy, 1953; Nagase, 1964) are not conclusive. In our work, α-naphthyl palmitate was used as the substrate. This compound gives a more specific reaction and results in a better enzyme localization, as reported by Knospe and Plendl (1997). Using this substance, our studies demonstrated positive lipase reactions in the first three gut segments. The staining was most intense in the first two parts, comprising 54% of the intestinal length (Smithet al., 2000). This suggests that lipolytic activity in this fish is indeed present, and occurs mainly in the cranial half of the intestinal tract. The observation of relatively restricted distribution of lipase enzyme in the Nile tilapia concurs with previous reports that lipase activity is lowest in herbivorous fish (Opuszynski and Shireman, 1995), related to the low fat content in plant materials naturally consumed by tilapia (Vonk and Western, 1984; Opuszynski and Shireman, 1995). These earlier reports, together with the observations in this study of the restricted distribution of lipase along the gut length, suggest that a low fat level may be more appropriate for adult tilapia diets.

3.5.4: Non-specific esterases

High levels of non-specific esterases have previously been reported in the intestine of tilapia (Li and Fan, 1997). The results of the present study concur with earlier work and localized esterase activity along the brush border. However, our studies also demonstrated non-specific esterase reactions in the cytoplasm of the enterocytes through the first four intestinal segments. This raises the possibility that lipid metabolism may involve events both intra- and extracellularly in the enterocytes of these gut regions (Deimling and Bocking, 1976; Wassmer et al., 1988; Van Lith et al., 1992).

3.5.5: Intestinal alkaline phosphatase

Intestinal alkaline phosphatase is considered to be involved in absorption of nutrients such as lipid, glucose, calcium and inorganic phosphate (Malagelada et al., 1977; Roubaty and Portmann, 1988; Harris, 1989; Dupuis et al., 1991; Mahmood et al., 1994). Enzymes localized in the brush border are active in this regard. Detection of the enzyme in the supranuclear cytoplasm indicates the source of its production, which has been shown to be in the Golgi apparatus located in that cellular region (Alpers et al., 1995). The widespread distribution of alkaline phosphatase throughout the first four segments, comprising 94% of the intestinal length (Smith et al., 2000), demonstrates that absorption of numerous forms of nutrients can take place along a tremendous surface area. Furthermore, the general distribution of the enzyme observed in this study correlates well with the localization and intensity of maltase, lipase and peptidases, including

amylase and proteases, reported in other tilapias (Fish, 1960; Nagase, 1964; Cockson and Bourne, 1972; Moriarty, 1973). Thus, this absorptive enzyme is present at the same location as the digestive enzymes, permitting absorption of the smaller particles as soon as they are produced.

Though the greater length of the gut is active in this regard, the stronger IAP activity in the first three parts (about 65% of the intestinal length) (Smith et al., 2000) probably indicates that the digestion and transport of nutrients in the intestine occurs mainly in the cranial and middle gut regions. The decrease in and subsequent loss of alkaline phosphatase activity in the fourth and terminal portions, respectively, suggests that nutrient absorption is most active in the more cranial intestinal regions, though absorption continues albeit at a reduced rate, through the greater proportion of intestinal length.

3.5.6: The terminal segment

The conspicuous lack of extensive enzyme staining in the terminal segment is noteworthy. Only DAP IV was detected here, where it was limited to the basal lamina (a site where it was also detected in all other segments). Previous work has suggested that DAP IV localized in the basal lamina is involved with metabolism of endogenous cellular structural proteins such as collagen, rather than with digestive processes of foodstuffs (Sakai and Kawatsu, 1983). The lack of digestive enzymes in the terminal segment suggests that this region is inactive in breakdown of nutrients influenced by these enzymes. Also, the absence of alkaline phosphatase activity in the terminal segment

further suggests that nutrient absorption is not an important function of this region. Other activities, such as resorption of electrolytes and/or water, likely predominate here.

3.6 CONCLUSION

These results suggest that the first four intestinal segments (from the hepatic loop through the distal major coil) represent the major regions of digestive and absorptive processes catalyzed by these enzymes. The first three segments appear to be the regions with the greatest participation in these functions. The decrease and loss of both digestive and absorptive enzyme activities in the distal major coil and terminal portion, respectively, suggest that other functions such as resorption of electrolytes and water likely predominate here. The combination of intestinal length (Hofer and Schiemer, 1981; Sugita et al., 1985; Smith et al., 2000) together with wide distribution of intestinal enzymes along that length enhance the fish's ability to utilize various food components and may be one factor contributing to the rapid growth rate of this fish.

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TABLES

Table 3-1. Summary of incubation techniques for enzyme histochemistry

Enzyme	Substrate	Diazonium salt	pH	Time (min.)
Malt	β-naphthyl-α-glucoside	Pararosaniline	6.9	60
LAP	L-Leucyl-4-M-2-N*	Fast Blue B	7.4	30
DAP IV	Glycyl-prolyl-4-M-2-N*	Fast Blue BB	7.4	30
Lip	α-naphthyl palmitate	Pararosaniline	7.4	60
NSE	Naphthol AS-D acetate	Pararosaniline	7.4	10
IAP	Naphthol AS-MX phosphate	Fast Red TR	8.5	10

M-2-N*: methoxy-2-naphthylamide; Malt= Maltase; LAP= Leucine aminopeptidase; DAP IV= Dipeptidyl aminopeptidase IV; Lip= Lipase; NSE= Non-specific esterases; IAP= Intestinal alkaline phosphatase

Table 3-2. Distribution and localization of the enzymes along the intestinal tract of the Nile tilapia

	Intestinal segment				
	Hepatic loop	First major coil	Gastric loop	Distal major coil	Terminal part
Brush border enzymes:					
Malt	+	+	++	+	–
LAP	++	++	++	+	–
DAP IV	++	++	++	+	–
Lip	++	++	+	–	–
NSE	++	++	++	++	–
IAP	++	++	++	+	–
Cytoplasmic enzyme:					
NSE	++	++	++	++	–
Basal enzyme*:					
DAP IV	+	+	+	+	+

Malt= Maltase; LAP= Leucine aminopeptidase; DAP IV= Dipeptidyl aminopeptidase IV; Lip= Lipase; NSE= Non-specific esterases; IAP= Intestinal alkaline phosphatase

Level of the staining intensity: ++ (strong), + (weak), – (absent)

*: in the basal lamina

FIGURES

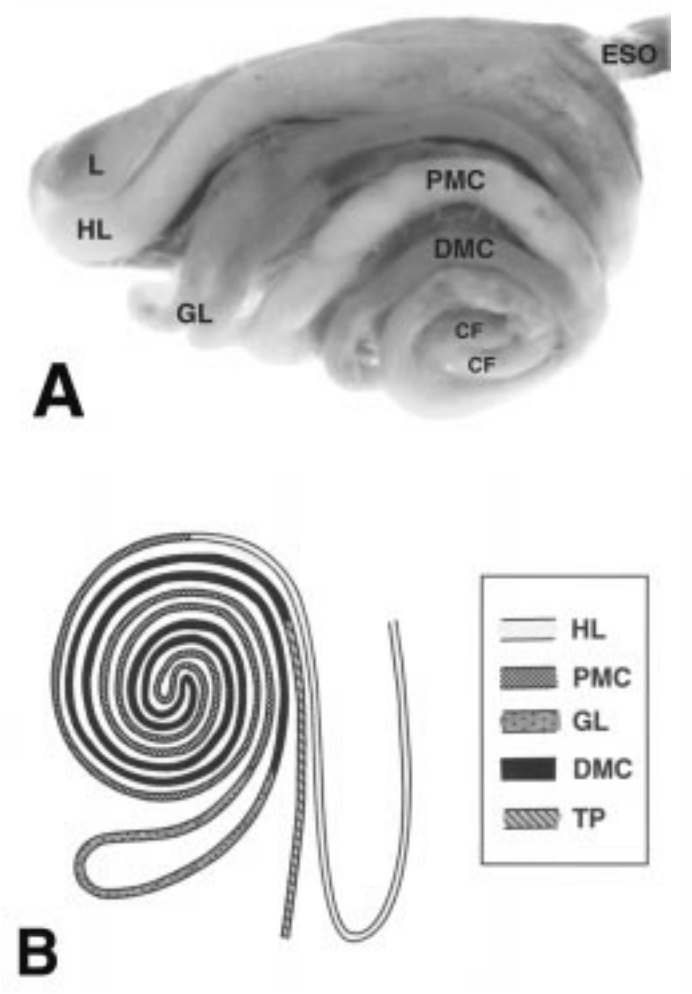

Figure 3-1. Photomicrograph (Right View) (A) and schematic drawing (Ventral View) (B) of the five intestinal segments. CF, central flexure; DMC, distal major coil; ESO, esophagus; GL, gastric loop; HL, hepatic loop; L, liver; PMC, proximal major coil; TP, terminal portion of the intestine.

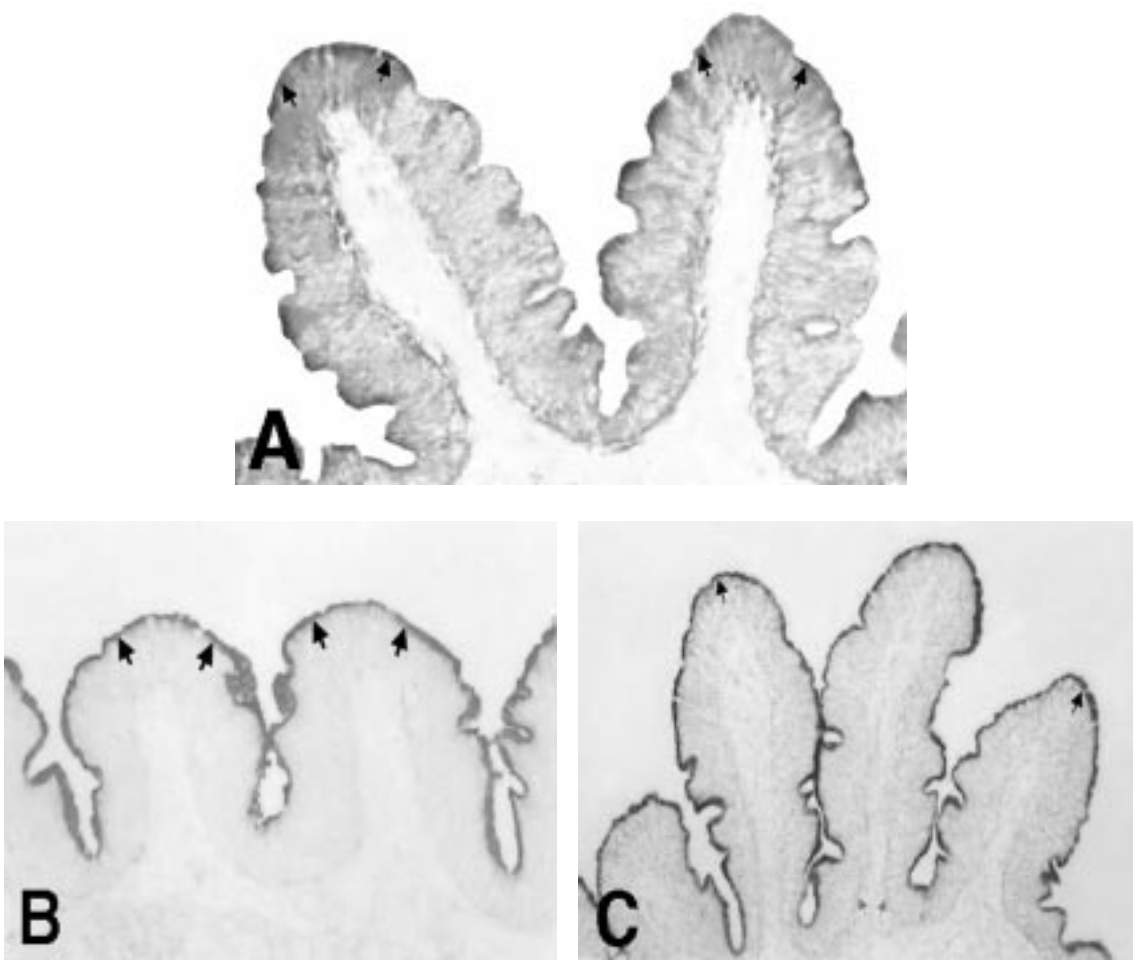

Figure 3-2. Intestinal sections showing enzyme staining (arrows). (A) Maltase in the microvilli of the first major coil (66x). (B) Leucine aminopeptidase in the brush border of the second major coil (50x). (C) Dipeptidyl aminopeptidase IV with strong reaction (in black) in the microvilli in comparison to the weaker intensity (in gray) in the basal lamina. Counterstained with hematoxylin (40x).

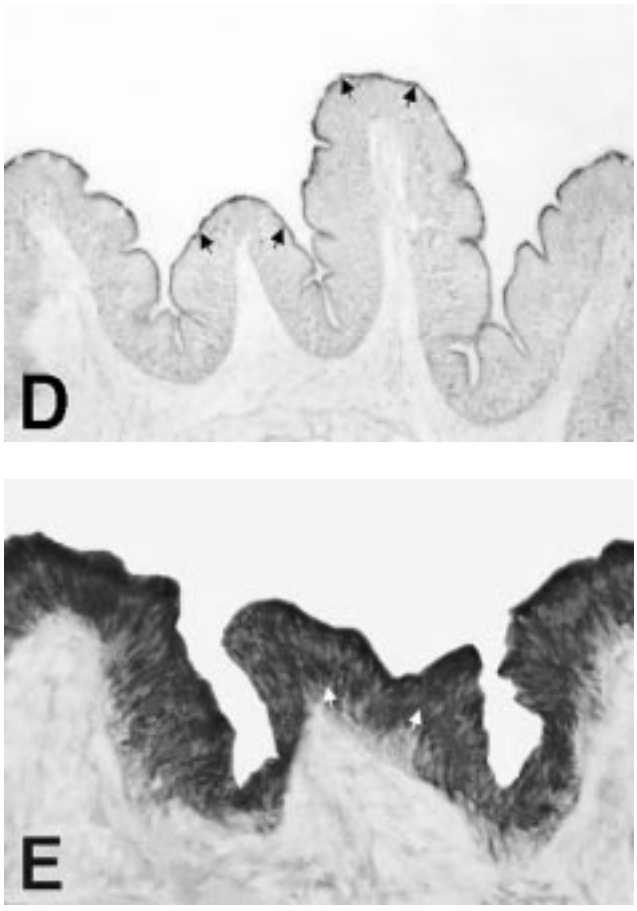

Figure 3-3. Intestinal sections showing enzyme staining (arrows). (A) Lipase in the brush border of the first major coil (40x). (B) Non-specific esterases in the brush border and cytoplasm of the columnar epithelial cells of the gastric loop (100x).

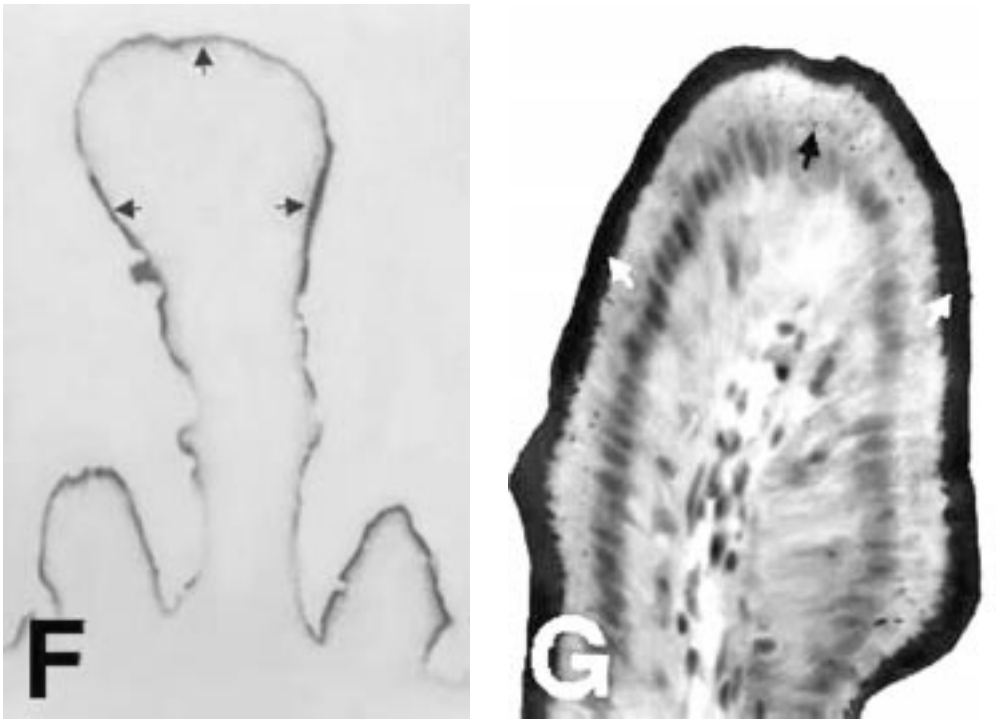

Figure 3-4. Intestinal sections showing enzyme staining (arrows). (A) Alkaline phosphatase in the microvilli of the gastric loop (40x). (B) Alkaline phosphatase in the brush border (in white) and supranuclear cytoplasm (in black) of the enterocytes of the gastric loop. Counterstained with hematoxylin (330x).

CHAPTER 4
ONTOGENIC DEVELOPMENT OF THE INTESTINAL ENZYMES IN *O. niloticus* L.

4.1: ABSTRACT

Ontogenic development of intestinal enzymes in *Oreochromis niloticus* was investigated using enzyme histochemistry. Intestinal samples were obtained from hatch to day 14 post-hatch. Tissue was cryomicrotome-sectioned, fixed using procedures appropriate for each specific enzyme, and examined using a light microscope. Most enzymes demonstrated activity along the brush border. In addition, non-specific esterases were found in the cytoplasm of the epithelial cells. Positive reactions for maltase, leucine aminopeptidase, dipeptidyl aminopeptidase IV, non-specific esterases, and alkaline phosphatase were already present in the brush border of the intestine at hatch (day 0). Only lipase demonstrated a delayed appearance, being first detected in the brush border of the enterocytes at day 3 post-hatch. Activities of all enzymes were widely distributed along the intestinal tract with no detectable difference between cranial and caudal segments. The early appearance and broad distribution of activities of these intestinal enzyme activities likely indicates the functional importance of these enzymes (digestion and absorption of food nutrients) at the first feeding, and differs markedly from what has been reported in other species.

4.2: INTRODUCTION

The ontogenic development of digestive and absorptive enzymes during the post-hatch period has been described in many fish species (Vu, 1983; Buddington, 1987; Pedersen et al., 1987; Zambonino Infante and Cahu, 1994; Gawlicka et al., 1995; Baglole et al., 1998; Gisbert et al., 1998), and has yielded information applicable to understanding their pattern of food utilization. For species cultivated in aquaculture, knowledge of the temporal appearance of the key enzymes in the gut can assist in more accurate formulation of feeds. Alhough tilapia are a species of current and increasing importance in aquaculture, information of the ontogenic appearance of enzymes in this group has not been available. Adjusting feed formulation to best suit the character of the tilapian digestive tract at given stages of growth could result in increased income to the producer, by decreasing loss due to feed wastage, as well as by improving the fishes' health and growth. Thus, the objective of this portion of the study was to document the ontogenic appearance of selected digestive and absorptive enzymes in the intestinal tract of the Nile tilapia using enzyme histochemical techniques.

4.3: MATERIAL AND METHODS

4.3.1: Culture Methods

Oreochromis niloticus Linnaeus, the Nile tilapia, were obtained from the same brood and raised in the Aquatic Medicine Laboratory of the Virginia-Maryland Regional College of Veterinary Medicine. The young fry were reared in 60-liter aquarium with a box filter

system using dechlorinated municipal water. Water temperature and dissolved oxygen were maintained at 27±1°C and a range of 6 to 7 mg/L, respectively. Automated surface lighting (12-12 hour day/night cycle) was provided throughout this project. Tilapia were fed daily with a commercial fish meal (Zeigler Bros., Inc., Gardner, PA). At the time of collection, eight fish were sampled daily from hatch (day 0) to day 14 post-hatch.

4.3.2: Preparation of Tissue Sections

For tissue collection, fish were anesthetized with MS-222 (tricane methanesulfonate, Sigma Chemical Company, St. Louis, MO), and then killed by cervical separation. The body cavity of each fish was immediately opened. Whole fish specimens were immersed in Optimal Critical Temperature Compound (VWR Scientific Products, Bridgeport, NJ). Immediately after the embedding compound completely surrounded the intestinal tissue, the samples were frozen in liquid nitrogen for about 15 seconds, and then serial 10 μm sections were prepared from the cranial to caudal end of the abdomen using a cryomicrotome at -15°C. Sections were retrieved from the knife on positive-charged slides (Superfrost/plus, Fisher Scientific, Pittsburgh, PA).

4.3.3: Fixation and Enzyme Staining

All chemicals for preparing media for each process were obtained from Sigma Chemical Company, St. Louis, MO. Before incubation in medium, fresh cryostat sections intended for the study of maltase (Malt), lipase (Lip), non-specific esterases (NSE) and intestinal alkaline phosphatase (IAP) were fixed for five minutes in 10% neutral buffered formalin

(Lojda et al., 1979). For detection of leucine aminopeptidase (LAP) and dipeptidyl aminopeptidase IV (DAP IV), tissue sections were fixed for one minute in equal parts of cold absolute acetone and chloroform (Lojda et al., 1979; Bancroft and Hand, 1987). Procedures for the enzyme examinations followed established techniques using substituted naphthol methods as follows (also see Appendix A: General Methods for Enzyme Histochemistry):

1. Method for maltase: Lojda et al., 1979
2. Method for leucine aminopeptidase: Lojda et al., 1979
3. Method for dipeptidyl peptidase IV: Lojda et al., 1979; Bancroft and Hand, 1987
4. Method for lipase: Knospe and Plendl, 1997
5. Method for nonspecific esterases: Lojda et al., 1979; Bancroft and Hand, 1987
6. Method for alkaline phosphatase: Lojda et al., 1979; Bancroft and Hand, 1987

4.3.4: Enzyme Examination

After incubation, tissue sections were coverslipped with mounting medium and examined using a light microscope. Enzyme activities were blindly evaluated semi-quantitatively as strong, weak or absent depending on the staining intensity in the tissue (Hirji and Courtney, 1982; Gawlicka et al., 1995). Intestinal sections incubated without substrate were used as controls.

4.4: RESULTS

Results showed that all six enzymes were present in the intestine of tilapia larvae before the onset of exogenous feeding (about 6 days post-hatch) (Table 4-2). However, differences in appearance and localization of these enzyme reactions were observed (Table 4-2 and Figs. 4-2 to 4-5). Activities of maltase, leucine aminopeptidase, dipeptidyl aminopeptidase IV, non-specific esterases, and alkaline phosphatase were first detected at hatch (day 0). Lipase was the sole enzyme surveyed that was not detected at hatch: this enzyme was first observed at day 3 post-hatch (Fig. 4-4). Activities of most of the enzymes were detected along the brush border. In addition, non-specific esterases alone were found in the cytoplasm of the epithelial cells (Fig. 4-5). Further, distributions of all enzyme activities were demonstrated with no detectable difference between cranial and caudal segments of the intestine.

4.5: DISCUSSION

The detection of activities of maltase, leucine aminopeptidase, dipeptidyl aminopeptidase IV, lipase, non-specific esterase and intestinal alkaline phosphatase in the intestine of the tilapia larvae before the first feeding (day 6 post-hatch) was particularly striking. Even more so was the observation that all but one of these enzymes were actually present at hatch. This is in contrast to many other species of fish (Vu, 1983; Buddington, 1985; Pedersen et al., 1987; Gawlicka et al., 1995). In the turbot, *Scophthalmus maximus* L., aminopeptidase, non-specific esterases and alkaline phosphatase were detected in the intestine within the first two days after hatch, while lipase was not found until day 15

post-hatch (Cousin et al., 1987). The yolk sac of the gilthead sea bream, *Sparus aurata* L., showed trypsin and alkaline phosphatase activities at hatch, but enzyme activities in the digestive tract did not develop until 3 days post-hatch (Gawlicka et al., 1995). In the white sturgeon, *Acipenser transmontanus*, dipeptidyl peptidase IV, nonspecific esterases and alkaline phosphatase were detected in the intestine at day 11 post-hatch (1 day before exogenous feeding) with their activities in the gut increasing after the second day of exogenous feeding (Sarasquete et al., 1993). Moreover, the intestinal brush border of the Siberian sturgeon (*Acipenser baeri*) demonstrated nonspecific esterases and alkaline phosphatase at day 4 post-hatch, while aminopeptidase M and lipase were not detected until day 9 after hatch (Gisbert et al., 1999). That the Nile tilapian intestine differs so markedly from so many other species likely suggests that at least this species of tilapia is rather uniquely prepared to utilize feedstuffs at the onset of exogenous feeding.

The positive maltase reaction observed here possibly indicates an extremely early development of the pancreas. Maltase hydrolyses the disaccharide maltose, which is in turn produced by the digestion of pancreatic amylase on long chain carbohydrates (Prosser and Devillez, 1991; Stevens and Hume, 1995). Thus, pancreatic function is apparent at this time. Maltase activity as observed in this study suggests that tilapia are also able to utilize dietary carbohydrate at a very early stage of life.

Digestion of protein and polypeptides in tilapia and other species (Overnell, 1973; Hirji and Courtney, 1982) begins by the action of pepsin in the stomach, and is furthered by the effects of trypsin and chymotrypsin secreted by the pancreas into the intestine (Fish, 1960; Nagase, 1964; Cockson and Bourne, 1972; Moriarty, 1973; Fang and Chiou, 1989). Other

reports demonstrated the functional development of stomach and intestinal enterocytes in the first few days post-hatch (Baylosis and Herrera, 1995). Long chain peptides are then further digested by intestinal peptidases such as LAP and DAP IV. Leucine and dipeptidyl aminopeptidase are exopeptidases hydrolyzing the amino terminal peptide bonds present in long chain peptides into smaller peptides and amino acids (Jobling, 1995). The detection of both peptidase activities in this study likely suggests that the stomach, pancreas and intestine are well-prepared for digestion of protein food at the first exogenous feeding.

Lipid and non-specific esterases are involved in digestion of glycerol esters of fatty acids in most vertebrates including fish (Stevens and Hume, 1995). Detection in the present work of these enzymes within 3 days post-hatch, still prior to the first exogenous feeding, is further evidence that the pancreas, the organ responsible for lipase production, becomes functional either before or at least at this time. Further, this suggests that lipid digestion and metabolism (Deimling and Bocking, 1976; Wassmer et al., 1988; Van Lith et al., 1992) may be important very early in the life of fish larvae.

Intestinal alkaline phosphatase is considered to be involved in absorption of nutrients such as lipid, glucose, calcium and inorganic phosphate (Malagelada et al., 1977; Roubaty and Portmann, 1988; Harris, 1989; Dupuis et al., 1991; Mahmood et al., 1994). The Golgi apparatus has been shown to be the source of enzyme production in the enterocytes (Alpers et al., 1995), from where the enzyme then moves to the intestinal brush border which is its active site. Moreover, the appearance and localization of this absorptive enzyme as observed in this study correlates well with those of other key enzymes including maltase, lipase and

peptidases. The adjacent distribution of alkaline phosphatase (an absorptive enzyme) and the digestive enzymes then likely permits efficient absorption of the smaller particles at the immediate same location.

The detection of enzymes in the brush border is widely accepted as an indication of intestinal function (Zambonino Infante and Cahu, 1994; Gawlicka et al., 1995). Timing of functional differentiation is thought to contribute to the growth rate, feed efficiency and survival of fish larvae during transformation to the later stages of life (Jauncey and Ross, 1982; Santiago et al., 1982). Therefore, the appearance of these enzymes in tilapia larvae in the first week post-hatch indicates that the intestine is functional in both digestion and absorption of nutrients at this very early age. Moreover, this feature likely contributes to the exceptionally fast growth characteristic of tilapia in aquaculture. Numerous studies have demonstrated that tilapia grow faster in culture than channel catfish and striped bass. This phenomenon occurs probably due to the outstanding digestive mechanisms of tilapias contributing to their abilities to utilize nutrients from a wide variety of natural foods. These mechanisms include pharyngeal teeth, gill rakers, strong stomach acid and a broad distribution of hydrolytic enzymes along the fish intestinal tract (Lovell, 1995; Smith et al., 2000; Tengjaroenkul et al., 2000). Tilapia can reach 1-2 pounds in less than a year, whereas catfish and striped bass require 1-2 years and 1.5 years, respectively (Shepherd and Bromage, 1988).

Since intestinal enzyme development of the Nile tilapia as observed in the present study was not affected by dietary intake, the onset of enzyme production and activities may be inferred to take place solely because of genetic factors (Zambonino Infante and Cahu,

1994). Age-specific adjustment of dietary formulation in an attempt to better suit the composition of the diet to the types of enzymes present at this young age would therefore appear unnecessary.

4.6: CONCLUSION

The presence of all six enzyme activities and their wide distribution throughout the intestinal tract before the onset of exogenous feeding differ significantly from the pattern reported in other species. Tilapia are thus uniquely adapted to maximize digestion and absorption of food nutrients at the first feeding. Since this phenomenon is not stimulated by diet, the development of these enzymes seems influenced solely by genetic factors.

4.7: ACKNOWLEDGEMENT

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TABLES

Table 4-1. Summary of incubation techniques for enzyme histochemistry

Enzyme	Substrate	Diazonium salt	pH	Time (min.)
Malt	β-naphthyl-α-glucoside	Pararosaniline	6.9	60
LAP	L-Leucyl-4-M-2-N*	Fast Blue B	7.4	30
DAP IV	Glycyl-prolyl-4-M-2-N*	Fast Blue BB	7.4	30
Lip	α-naphthyl palmitate	Pararosaniline	7.4	60
NSE	Naphthol AS-D acetate	Pararosaniline	7.4	10
IAP	Naphthol AS-MX phosphate	Fast Red TR	8.5	10

M-2-N*= methoxy-2-naphthylamide; Malt= Maltase; LAP= Leucine aminopeptidase; DAP IV= Dipeptidyl aminopeptidase IV; Lip= Lipase; NSE= Non-specific esterases; IAP= Intestinal alkaline phosphatase

Table 4-2. Ontogeny of the enzymes along the intestinal tract of the Nile tilapia

Enzyme	First appearance (Days post-hatch)
Brush border enzymes:	
Malt	0*
LAP	3
DAP IV	0
Lip	0
NSE	0
IAP	0
Cytoplasmic enzyme:	
NSE	0

Malt= Maltase; LAP= Leucine aminopeptidase; DAP IV= Dipeptidyl aminopeptidase IV; Lip= Lipase; NSE= Non-specific esterases; IAP= Intestinal alkaline phosphatase

*0: immediately upon hatch

FIGURES

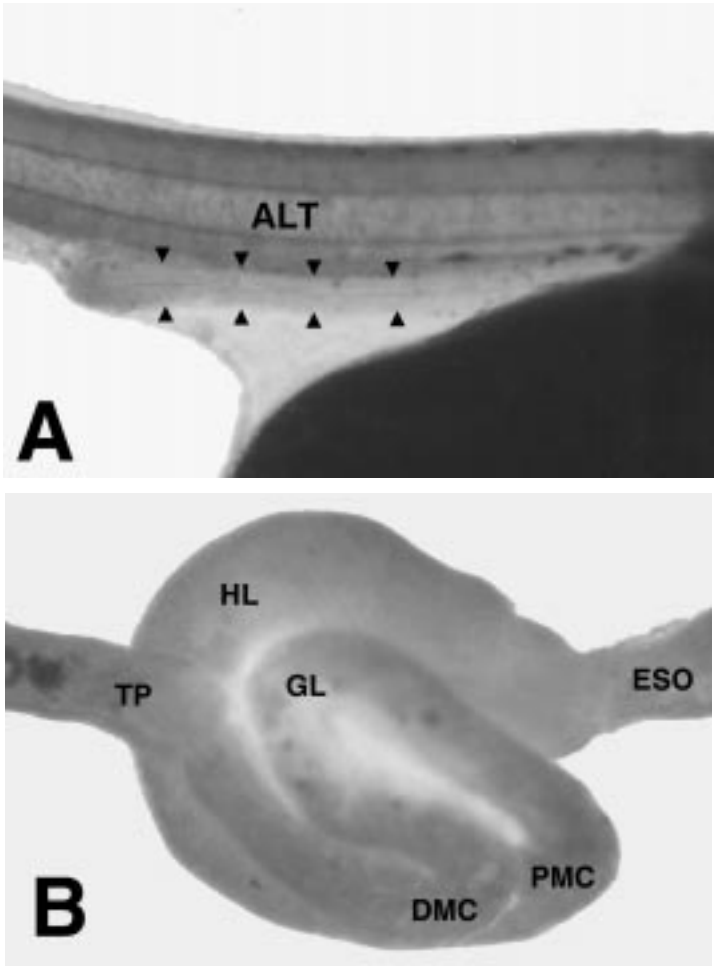

Figure 4-1. Photomicrograph (Right Lateral View) of the intestine at hatch (A) and within the first week post-hatch (B). ALT, alimentary tract (arrows); HL, hepatic loop; PMC, proximal major coil; GL, gastric loop; DMC, distal major coil; TP, terminal portion of the intestine.

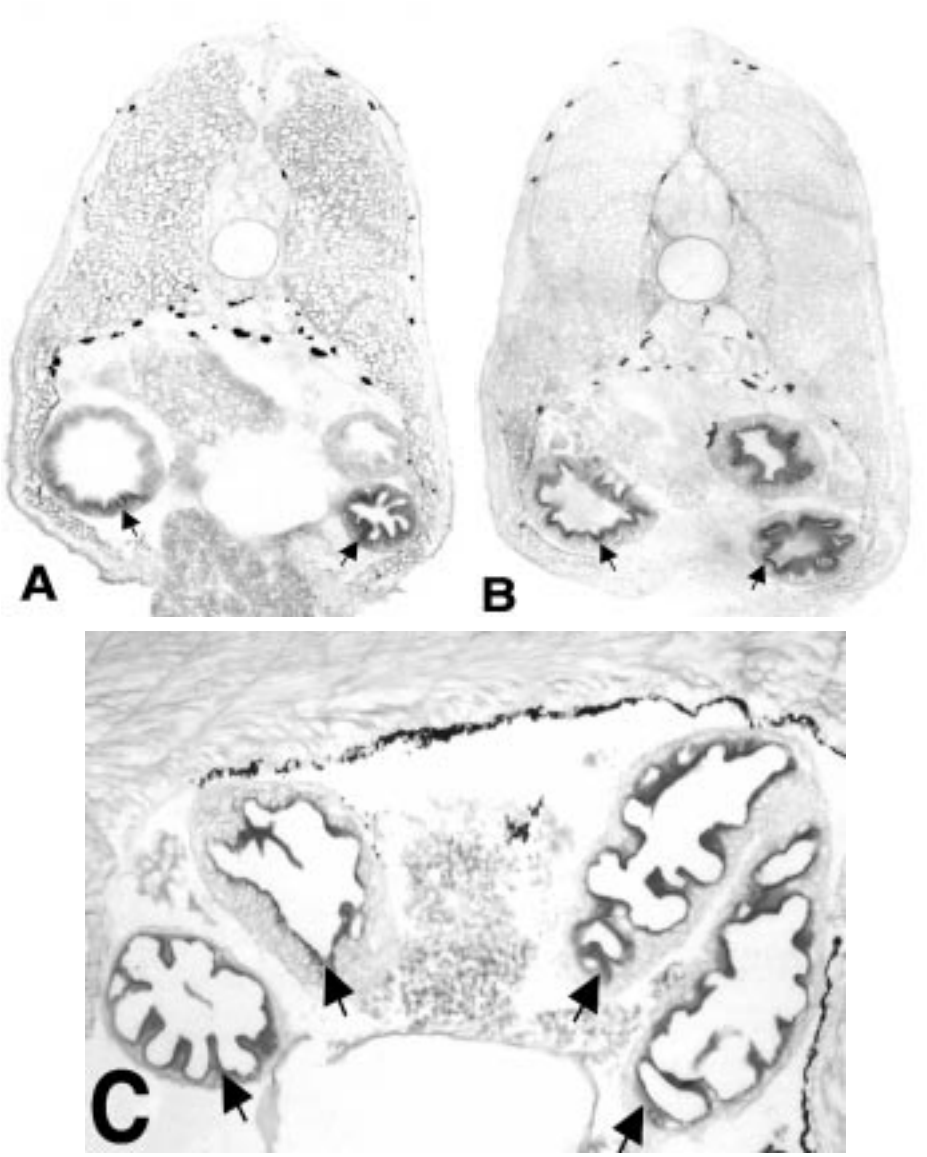

Figure 4-2. Intestinal sections showing enzyme staining (arrows). (A) At day 1 post-hatch, maltase in the microvilli of the intestine (cross section, 66x). (B) At day 1 post-hatch, leucine aminopeptidase in the brush border (cross section, 50x). (C) At day 2 post-hatch, leucine aminopeptidase in the brush border (longitudinal section, 100x).

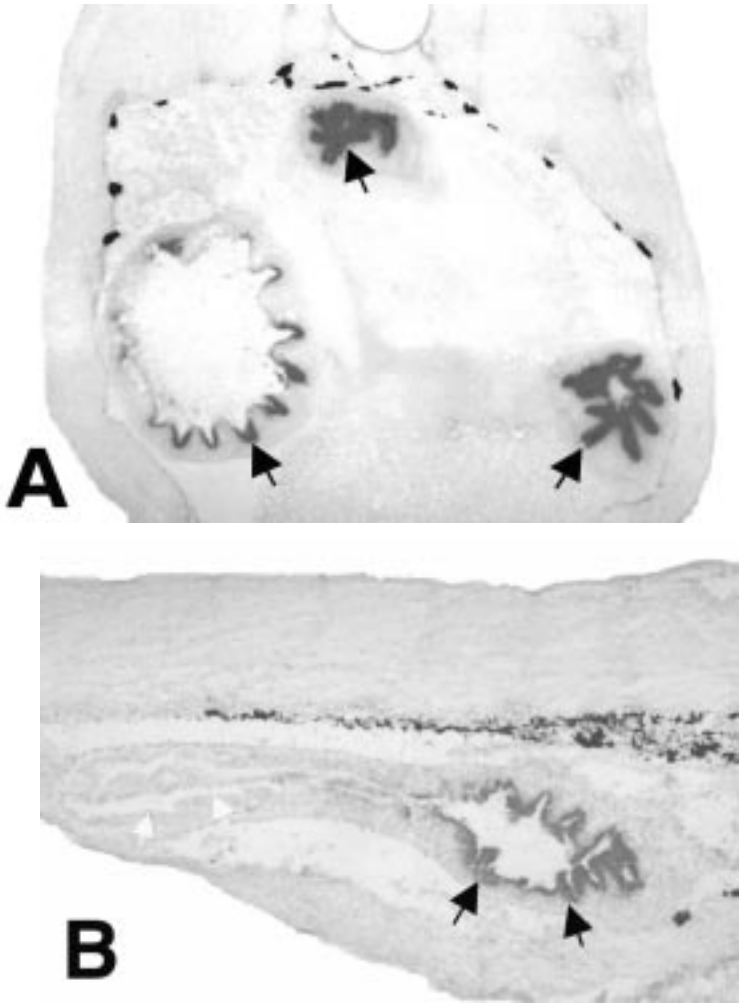

Figure 4-3. Intestinal sections showing enzyme staining (arrows). (A) At day 1 post-hatch, dipeptidyl aminopeptidase IV reaction in the microvilli (cross section, 60x). (B) At day 2 post-hatch, dipeptidyl aminopeptidase IV reaction in the brush border of the caudal region (arrows in black) in comparison to the disappearance of the enzyme activity in the anal region (arrows in white) (longitudinal section, 80x).

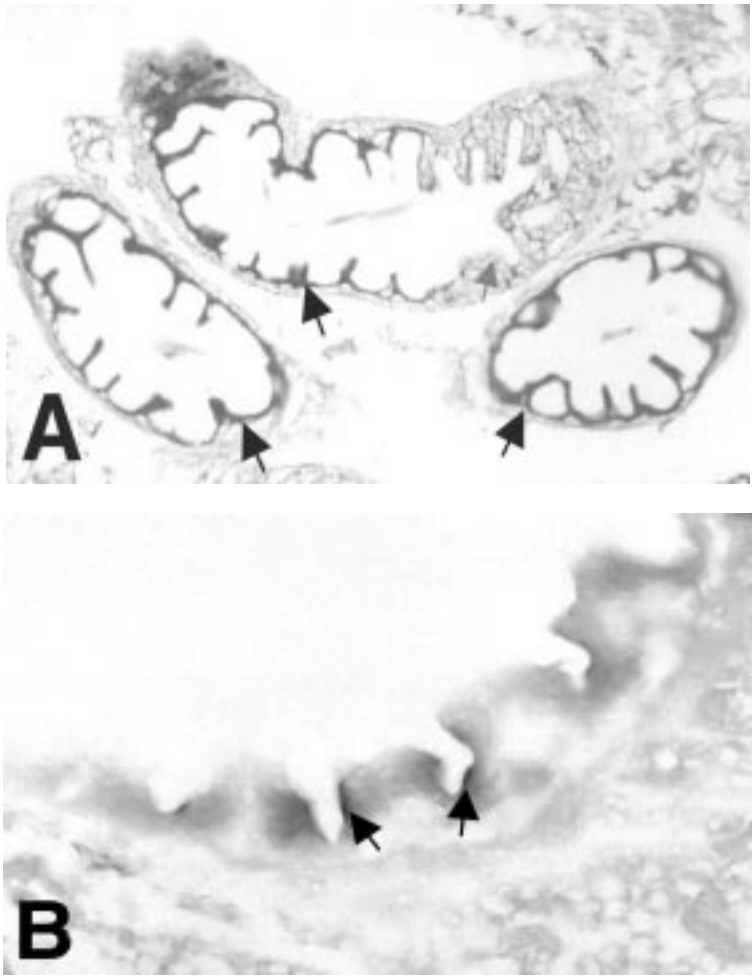

Figure 4-4. Intestinal sections showing enzyme staining (arrows). (A) At day 3 post-hatch, lipase activity in the brush border of the caudal region (arrows in black) in comparison to the disappearance of the enzyme activity in the anal region (arrows in gray) (longitudinal section, 60x). (B) At day 3 after hatch, lipase reaction in the microvilli (arrows in black) (cross section, 200x).

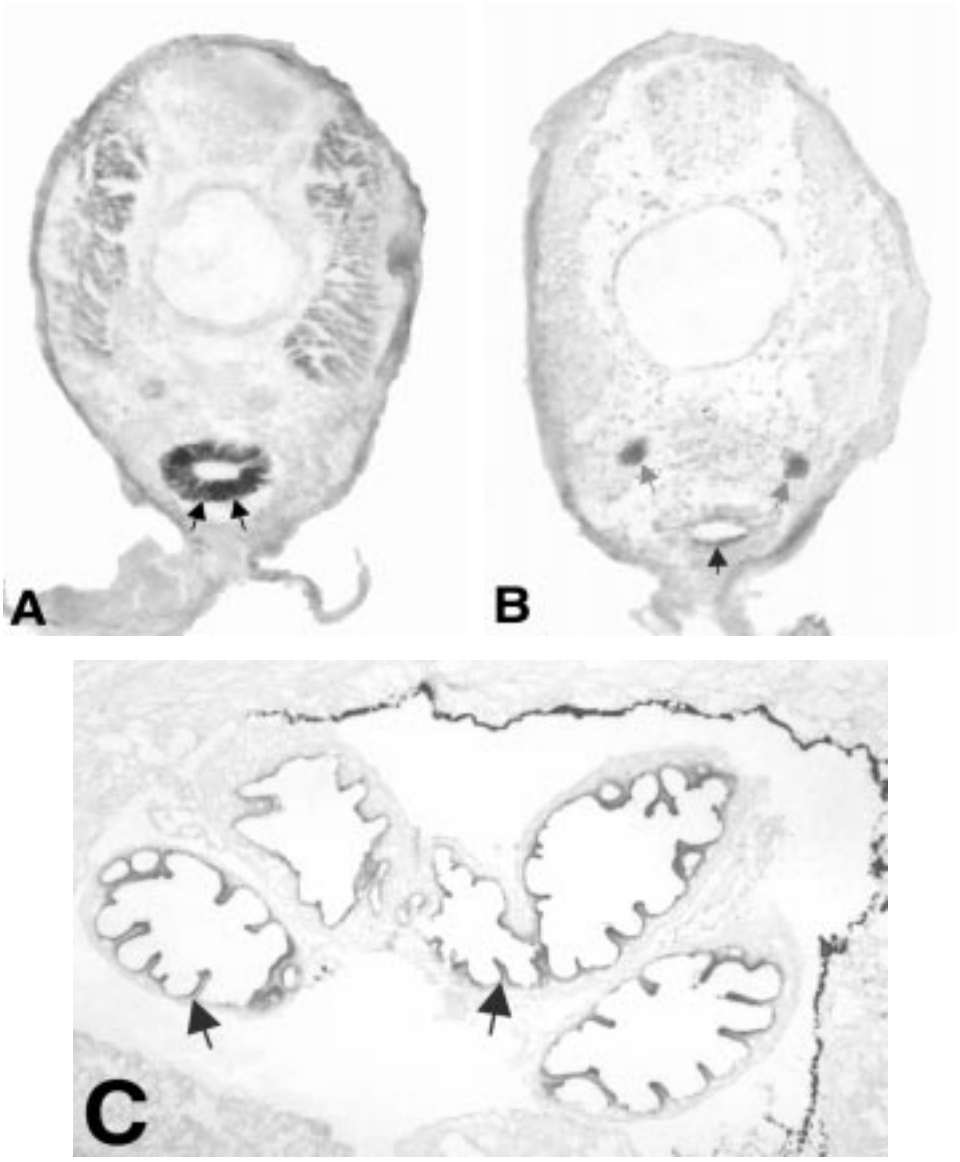

Figure 4-5. Intestinal sections showing enzyme staining (arrows). (A) At hatch stage, non-specific activity in the brush border and cytoplasm of enterocytes (cross section, 50x). (B) At hatch stage, alkaline phosphatase reaction in the microvilli (arrows in black) and kidney tissue (arrows in gray) (cross section, 50x). (C) At day 2 post-hatch, alkaline phosphatase activity in the brush border (longitudinal section, 100x).

CHAPTER 5

EFFECTS OF ALFALFA AS A FOOD REPLACEMENT ON GROWTH AND INTESTINAL ENZYME ACTIVITIES IN *O. niloticus*

5.1: ABSTRACT

Three feeding formulae containing different levels of alfalfa meal were prepared and used in five different feeding trials to determine the effects of varying concentrations of alfalfa on growth and intestinal enzyme activities in the Nile tilapia, *Oreochromis niloticus*. From the ages of week 3 to week 15 post-hatch, using different percentages of alfalfa (0%, 20% and 40%) in the diet at different ages of fish significantly affected body size, weight, intestinal weight and length, as well as the activities of maltase, leucine aminopeptidase and alkaline phosphatase.

The greatest body weight and fish length occurred in control fish, which were fed only a commercial fish diet (CD) containing 44% crude protein (CP). In the alfalfa feeding trials, a negative correlation was observed between percentage of alfalfa in the diet and both fish growth and enzyme activities. The fish fed a mixture of 20% alfalfa in CD (38% CP), increased average weight, body length, as well as intensities of the measured intestinal enzymes at a rate intermediate between the other two diets. Even on the same alfalfa concentrations, fish receiving the alfalfa diets at the younger age (3 weeks) grew more slowly and demonstrated decreased enzyme activities than those first fed alfalfa at the older age (9 weeks). Despite exhibiting a reduced rate of growth and enzyme activity,

the fish consuming a higher percentage of alfalfa in the ration demonstrated an increase the intestinal weight and length, most likely as an adaptive response to enhance their ability to utilize this less-than-optimal diet.

Activities of the intestinal enzymes increased significantly with fish age. Tilapia fed only the commercial fish diet showed highest activities of aminopeptidase, whereas the fish fed a diet with 40% alfalfa demonstrated greater intensities of maltase and alkaline phosphatase. These diet-specific alterations in enzyme activities suggest that dietary alfalfa affects enzyme production and physiological adaptation, possibly by changing the number as well as rate of protein synthesis of the enzyme secreting cells. In conclusion, this study demonstrated that replacing either 20% and 40% of a commercial diet with alfalfa had an overall negative effect on growth and certain intestinal enzyme activities from age 3-9 weeks. Thus, using alfalfa as a food replacement is not optimal for fish of these young ages.

5.2: INTRODUCTION

Tilapia are omnivorous fish having an ability to utilize a wide variety of foods. At body lengths less than 6 cm, tilapia fry are largely carnivorous, hunting for zooplankton and aquatic larvae (Lowe-McConnell, 1975; Trewavas, 1983). As they grow, tilapia gradually begin taking more plant foods including phytoplankton and macrophytes (Moriarty, 1973; Lowe-McConnell, 1975; Bowen, 1982; Lim, 1989), and using pharyngeal teeth and strong stomach acid to assist their digestion of various plant foods (Moriarty, 1973; Bowen, 1982; Lovell, 1989; Popma and Masser, 1999). Tilapia also possess a long

intestine with a broad distribution of digestive and absorptive enzymes distributed along the greatest part of its length. (Bowen, 1982; Smith et al., in press; Tengjaroenkul et al., 2000). These features likely contribute to the tilapias' known capacity to digest and derive nutrients from fibrous plant foods including alfalfa (Lovell, 1989).

Animal food sources generally provide excellent quality protein for commercial fish diets, but are quite expensive and also prone to spoilage. Thus, for economic and practical reasons, several attempts have been made to examine the possibility of replacing animal protein sources with less expensive plant sources (Jackson et al., 1982; Lovell, 1989; Falaye, et al., 1999). Alfalfa has been suggested as one such possible source, having been shown to contain 15-25% crude protein (Hardy, 1989). Popma (1982) mentioned that percentage digestibility for protein in alfalfa by tilapia was close to that for wheat bran, approximately 67%. Despite these promising characteristics, the possibility of using alfalfa as a potential protein food replacement in tilapian fish has not yet been fully investigated. Therefore, the objective of this study was to use various levels of alfalfa in the feed to study the effects on growth and intestinal enzyme activities in the Nile tilapia.

5.3: MATERIAL AND METHODS

5.3.1: Culture Methods

Oreochromis niloticus Linnaeus, the Nile tilapia, were obtained and raised from the same brood in the Aquatic Medicine Laboratory of the Virginia-Maryland Regional College of Veterinary Medicine. The young fry were reared in 60-liter aquaria with a box filter

system using dechlorinated municipal water. Water temperature and dissolved oxygen were maintained at 27±1°C and a range of 6 to 7 mg/L, respectively. Automated surface lighting (12-12 hour day/night cycle) was provided throughout this project. When the fry reached 3 weeks of age, feeding trials were begun. The trials lasted 12 weeks, during which time the fish attained the age of 15 weeks.

5.3.2: Experimental Procedures

5.3.2.1: Feed Formulation

Control feed: Small and large crumbles (sizes 0.3-0.5 mm and 0.5-1.0 mm, respectively) of a commercial fish diet (44% protein, 5.3% fat, 4.3% fiber, 8.5% ash, 9% moisture) (Rangen Extra 400, Rangen Inc., South Buhl, Idaho) were provided to fish in Trial I throughout the experiment.

Test feeds: Due to relatively small volume of food samples used in this work, pulverizing and re-combining food pellets by a commercial feed mill was not possible. Thus, small and large crumbles of alfalfa (15% protein, 1% fat, 37% fiber, 7% moisture) were selected using mesh screens, and then mixed with the same sizes of crumbles of the commercial fish diet at different concentrations (Trials II-IV) (Tables 5-1 and 5-2). Menhaden fish oil (Omega Protein, Reedville, VA) was also added into all diets at 5% of the food weight including the control diet according to established procedure (Popma, 1989) in order to increase palatability by covering alfalfa taste and smell.

5.3.2.2: Dietary Manipulation

Fish fry were fed with the ground commercial fish diet (powder) from the first exogenous feeding to 21 days (3 weeks) of age. During the first six weeks of the experiment (age 3-9 weeks), fish were fed the small food crumbles (size 0.3-0.5 mm) at a feeding rate of 12% of fish body weight. During the second six weeks of the study (age 9-15 weeks), fish received the larger food crumbles (size 0.5-1.0 mm), but the feeding rate was decreased to 6% of body weight (Jauncey and Ross, 1982; NRC, 1993; Tacon, 1990). All prepared diets in all feeding trials were delivered six times a day, every two hours from 8:00 AM to 6:00 PM using automatic feeders (Sweeney Enterprises, Inc., Boerne, TX). Individual groups of fish in this study were reared on each of the specific diets from week 3 to week 15 post-hatch.

Control Group: Fish in the control group (Trial I) were fed throughout the experiment with the commercial diet (Rangen Extra 400, Rangen Inc., South Buhl, Idaho) (Tables 5-1 and 5-2).

Test Groups: Fish in four test groups (Trials II-V) were arbitrarily selected to receive the different diets from the higher- to the lower-protein levels (44 to 32%) by adding a specific amount of alfalfa to the commercial fish food (Tables 5-1 and 5-2). Feed composition as well as percentage protein were later adjusted at week 9 post-hatch, since this is the time when morphology of the fish intestine first attains the definitive form (herbivorous/omnivorous type) (Tengjaroenkul et al., in review). During the first 6 weeks of the experiment (from 3 to 9 weeks of age), tilapia fingerlings were placed on small

crumbles of commercial fish diet (Trial II), containing alfalfa meal at concentrations of either 20% (Trials III, IV) or 40% (Trial V) (Tables 5-1 and 5-2). In the second 6 weeks of the study (from 9 to15 weeks of age), fish in all four feeding groups (Trials II-IV) were fed larger crumbles. Fish in only two feeding trials (Trials II and IV) received the lower-protein diets by increasing the percentage of alfalfa to 20% (for Trials II) and 40% (for Trial IV) (Tables 5-1 and 5-2).

5.3.2.3: Sampling Procedure

Samples consisting of eight arbitrarily-selected fish were taken from each group at 6-week intervals for the study of growth and intestinal enzyme activities. A sample of eight arbitrarily-selected fish was also taken from the control group at the same point in time. The following procedures were performed on each group of eight fish.

5.3.2.3.1: Study of Growth

Immediately upon collection, fish were anesthetized using tricane methanesulfonate (Sigma Chemical Co., St. Louis, MO) and then killed by cervical separation. Individual whole fish were weighed using an electronic balance (Ohaus AP 1105, Analytical Plus, Ohaus Corporation, Florham Park, NJ). The intestine was immediately remove *en masse* from the fish's body cavity and weighed using the same balance. The body length and gut length of each fish were then measured using calipers (Qing-gong, China).

5.3.2.3.2: Enzyme Assays

Enzyme assays were performed on fish at ages 3, 9 and 15 weeks post-hatch. Fish were anesthetized with MS-222 (Sigma), before being killed by cervical separation. After body lengths and weights were recorded, the intestine of each fish was immediately removed from the body cavity, weighed, and homogenized with distilled water using a sonicator (Model W-225 R, Heat Systems–Ultrasonics, Inc., Plainville, N.Y.) to give a 1:5 homogenate. For the enzyme assays, specific substrate, buffer and incubation conditions were provided that were appropriate for each enzyme (Table 5-3) (Hycarinen and Nikkila, 1962; Dahlqvist, 1969; Hanninen and Hartiala, 1965; Bergmeyer and GraBl, 1983). All chemicals for the enzyme assays were obtained from Sigma Chemical Co., St. Louis, MO. The buffers for each enzyme were specifically prepared as follows: 100 mM sodium maleate buffer for maltase (Malt); 50 mM sodium phosphate buffer for leucine aminopeptidase (LAP); and 50 mM Tris buffer for intestinal alkaline phosphatase (IAP). The reagents without standard enzymes or mucosal dilution were used as control.

5.3.2.4: Data Measurements

5.3.2.4.1: Study of Growth

Body weight (BW) and gut weight (GW) were measured from each fish using an electronic balance (OHAUS AP 1105, Analytical Plus, Ohaus Corporation). The gut length (GL) and total body length (TL) of each fish was measured using calipers. Total body length was measured from the cranial end of the lower lip to the caudal end of the caudal fin (Trewavas, 1983).

5.3.2.4.2: Enzyme Assays

5.3.2.4.2.1: **Maltase** (from Hycarinen and Nikkila, 1962; Dahlqvist, 1969)

Maltase activity was determined in 100 mM sodium maleate buffer (0.1 ml) which was mixed with 56 mM maltose at pH 6.0. The reaction was started by adding 0.1 ml mucosal dilution (1:50). After 40 min of incubation, the reaction was stopped with 1.0 ml of 1% trichloroacetic acid and the proteins were sedimented by centrifugation (2500 g for 10 min). O-Toluidine color reagent (4.5 ml) was added into 0.5 ml of the supernatant in order to detect free glucose. The sample tubes were subsequently placed in a boiling water bath for 8 min. Thereafter they were cooled and the color was measured using a Beckman DU 640B spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) at 630 nm wavelength.

5.3.2.4.2.2: **Leucine Aminopeptidase** (from Bergmeyer and GraBl, 1983)

An activated mixture for leucine aminopeptidase was prepared from 50 mM phosphate buffer (2.0 ml), 25 mM $MnCl_2$ (0.2 ml) and mucosal solution (0.1 ml). Leucine aminopeptidase activity was measured by adding 0.1 ml of the activated mixture into the substrate solution containing 60 mM L-leucine-ρ-nitroanilide (2 ml), 62.5 mM $MgCl_2$ (0.2 ml) and 50 mM phosphate buffer (0.7 ml). The enzyme reaction was started by using 1:50 tissue solution, and incubated at pH 7.4 for 2 hours. Control tubes were incubated

without leucine aminopeptidase. The color was measured spectrophotometrically at 238 nm wavelength.

5.3.2.4.2.3: Alkaline Phosphatase (from Hanninen and Hartiala, 1965; Bergmeyer and GraBl, 1983)

Alkaline phosphatase activity was measured in a mixture consisting of 50 mM Tris buffer (0.4 ml), 10 mM magnesium chloride (0.05 ml) and 20 mM (ρ-nitrophenyl-phosphate in Tris buffer (0.1 ml). The reaction was started using 0.1 ml of mucosal solution (1: 50) incubated at pH 8.5 for 10 min at 25°C. The reaction was stopped by 20 mM NaOH (5.0 ml), and absorbency then measured spectrophotometrically at 405 nm.

5.3.2.5: Data Analysis

5.3.2.5.1: Study of Growth

Relationships between TL, GL and the ratio (R) of gut length to body length and fish age in weeks (Wk) were expressed using linear regression models. Correlations between the BW, GW and age of the fish were plotted using exponential growth curves. Data from the control and each of the treatment groups were analyzed using analysis of variance and Duncan's multiple range test (Duncan, 1955). Specific growth rate (SGR), a growth parameter, was calculated on the basis of the formula: SGR = $100(\log_e \text{weight gain})/\text{day}$ (Tacon, 1990). The growth rate index (GRI) or condition factor reflecting the nutritional state of each feeding trial was calculated using the formula: GRI = $\text{weight}/(\text{length})^3$ (Busacker et al., 1990). Results were also interpreted qualitatively, describing the

changes of the BW, BL, GW and GL on age of the fish in each feeding group.

5.3.2.5.2: Enzyme Assays

Data were analyzed using analysis of variance and Duncan's multiple range test (Duncan, 1955). Results were also interpreted quantitatively, describing the effect of alfalfa on the intestinal enzyme activities of each fish group.

5.4: Results

5.4.1: Feeding Behavior

In contrast to fish in the control group (Trial I), some tilapia fed the test diets (Trials II-V) often expelled and ignored alfalfa mixed in the diets consisting mainly of stem material, leaving it at the bottom of the aquarium. As characteristic for tilapias, at the time of feeding, the larger fish in all feeding trials aggressively approached the food and often chased the smaller fish away.

5.4.2: Growth of fish

All fish fed the various diets grew and gained weight during the 12 weeks of the study. However, fish fed diets containing alfalfa consistently performed less well than fish fed the commercial diet alone (Tables 5-4 to 5-9). For instance, fish fed the greatest amount of alfalfa over the largest period of time (Trial V) gained 0.92% of their body weight per

day, while fish fed the least amount of alfalfa gained over 2.00% of body weight per day over the 12 weeks of the experiment (Table 5-7).

At the end of the first six weeks of the study, significant differences were observed in body weights and lengths of fish (Table 5-4). Significant differences ($P<0.05$) in body weight gained during these six weeks were observed only between fish fed mainly the commercial fish diet (Trials I, II) and those the 40% alfalfa mixed diet (Trial V). Body length showed significant differences among all three feeding formulae: commercial diet (Trials I, II), 20% alfalfa (Trials III, IV), and 40% alfalfa (Trial V) (Table 5-4).

After 12 weeks of the experiment, weight, length and growth rate (specific growth rate and growth rate index) were significantly different among all five fish groups ($P<0.05$) (Tables 5-4 and 5-7, Fig. 5-6). These decreases from control were greatest in the fish fed the highest percentage of alfalfa meal. Mean body weight and length at the beginning of the study were 0.036 g (± S.D. 0.0068) and 1.346 cm, respectively (Table 5-4). Mean juvenile weight and length at the termination of the feeding trials varied from 2.85 g (Trial V, 40% alfalfa) to 6.26 g (Trial I, 0% alfalfa), and 4.63 cm (Trial V, 40% alfalfa) to 7.49 cm (Trial I) (Table 5-4). Fish fed the commercial diet alone (Trial I) demonstrated the highest average weight gain (2.04% per day or 0.518 g per week) and growth rate (2.682 SGR and 1.487 GRI) compared to all other feed formulations in the experiment; the smallest increases in these parameters were demonstrated by fish fed the most (40%) alfalfa in the diet (Table 5-4). Among fish receiving alfalfa in the diet, Trial II (20% alfalfa) showed the best growth (weight gain 1.73% per day or 0.44 g per week, 2.595 SGR and 1.757 GRI) (Table 5-7).

Analysis of variance of date for weight and length at the end of the trials showed a highly significant heterogeneity in weight and length among diets ($F=6.8808$, $P<0.001$, and $F=11.4451$, $P<0.001$, respectively). A comparison of means among treatments demonstrated a significantly higher growth rate for fish fed the commercial diet when compared to any of the alfalfa mixed diets, particularly those incorporating alfalfa at the onset of feeding (Trials III to V) (Table 5-7). Among Trials III to IV, a trend toward decreasing body weight with increasing percentage of dietary alfalfa was observed (Table 5-4). However, this trend was not significant.

In all feeding trials, variation in body weight and length increased greatly during the 12 weeks of the study. At the onset of the study (week 3 post-hatch), the standard deviation of the fish weight and length ranged between 0.029 to 0.044 g and 1.295 to 1.397 cm, respectively. At the end of the trials, the variation in body weight and length were higher when the fish were fed only the commercial diet (Trial I) (2.297 g and 1.163 cm) compared to fish fed the alfalfa meal (Trial V) (0.858 g and 0.737 cm).

5.4.3: Relationship between intestine and growth

After the first six weeks of the experiment, significant differences ($P<0.05$) in gut weight were found between fish groups fed the diets containing either the commercial fish food alone (Trials I, II) and the 20% alfalfa supplemented diet alfalfa diet (Trials III, IV). In addition, the gut length showed significant variation among fish groups fed all three feeding formulae (Table 5-5). However, there was no significant difference in the ratio

(R) between gut length and total body length among the 5 feeding trials (Table 5-6).

After 12 weeks of the study, some significant differences in intestinal weight and length were found. The groups that were fed higher concentrations of alfalfa in the diet (except Trial V) showed greater increase in the gut weight and length as compared to those fed the lower alfalfa levels ($P<0.05$) (Table 5-5).

Positive linear relationships were observed between fish length (TL) and GL as well as the ratio of GL to TL on age (R) (Tables 5-6, 5-8 and 5-9, Fig. 5-1). During the 12 weeks of the study (i.e., between the ages of 3 and 15 weeks), the relationship of fish weight and GW increased dramatically, observed as exponential growth curves (Tables 5-8 and 5-9, Fig. 5-2). The standard deviation of the body length in the same group of fish became larger as the fish age increased. Models, equations and parameters of the relationships between TL, GL, BW, GW as well as ratio of GL to TL on age (Wk) of the fish are summarized in Tables 5-8 and 5-9.

5.4.4: Enzyme assays

The intestinal enzyme activities showed marked trends among fish groups fed different percentages of alfalfa; however, statistical significance was demonstrated only among ages of fish within the same trials ($P<0.05$) (Table 5-10). Across the full 12 weeks of the study, activities of maltase, leucine aminopeptidase and alkaline phosphatase increased with the age of fish in all trials. The activity of IAP showed the greatest increase across time, while maltase showed the least.

After the first six weeks of the experiment, the activities of leucine aminopeptidase and alkaline phosphatase were highest in the control group, were significantly lower in the feeding trial containing 20% alfalfa (Trials III, IV) and showed the lowest level in the 40% alfalfa meal (Trial V) (Table 5-10). In contrast, maltase activity was highest in fish fed the 20% alfalfa diet and lowest in the group consuming 40% alfalfa.

In the last six weeks of the experiment, the levels of maltase and alkaline phosphatase dramatically increased and reach a maximum in the tilapia fingerlings fed 40% alfalfa (Trial IV), increased only moderately for 20% alfalfa (Trials II, III), were relatively low in Trial V, and were lowest in the control diet (Trial I) (Table 5-10). The activity of leucine aminopeptidase was highest in the control group and lowest in the fish consuming 40% alfalfa meal.

Fish fed the highest concentration of alfalfa for the longest period of time showed the greatest increase in maltase and alkaline phosphatase activities, but a lesser increase in aminopeptidase compared to those fed lower alfalfa levels and for a shorter period of time (Table 5-10).

5.5: DISCUSSION

5.5.1: Feeding Behavior

The selective feeding behaviors demonstrated in some fish by expulsion and abandonment of alfalfa, mainly the stem materials on the bottom of the aquarium were

likely caused by the relatively coarse texture and lower palatability of alfalfa meal. Adding Menhaden fish oil into tilapia diets has been reported (Popma, 1989; Tacon, 1990), and described to increase digestible energy and palatability of the diets. Thus, this procedure was also adopted in the present study in an effort to increase palatability of the alfalfa meal. The behavior of the fish suggests that these efforts were only partially successful.

Moreover, behavioral dominance among the fish has been shown to make a significant impact on feeding behavior as well as food consumption of each individual (Francis, 1988; Jobling and Reinsnes, 1987; Koebele, 1985). In this work, the more aggressive fish out-competed the more submissive ones, and thus demonstrated a faster growth rate. This feature, combined with the selective feeding that left the smaller fish with less commercial feed and more alfalfa, are likely major components in the increased standard deviation in length/weight among fish later in the study. A pellet diet that incorporated the alfalfa into the pellet might have increased consumption. Such a possibility invites further investigation.

5.5.2: Growth of Fish

Growth is a phenomenon resulting from a genetic-environment interaction that result in appropriate physiological and behavioral processes (Brette, 1979; Ricker, 1979) that produce by which digestible energy from ingested food is transformed to body mass. The growth of fish can be described by an asymmetric sigmoid curve (Soderberg, 1997). This descriptive model can be divided into three phases as exponential, linear and plateau phase.

In the present study, fish consuming lower protein levels due to increased alfalfa concentration in the diets demonstrated a lower growth rate than those having higher protein content with lower alfalfa concentration (Tables 5-4 to 5-6, Figs. 5-1 and 5-2). More sub-commercial standard in growth performance was observed in tilapia fed the high (40%) percentage of alfalfa. This phenomenon likely results from a combination of various factors, including lower food intake, low quality and availability of protein, and anti-nutritional factors (anti-protease, anti-vitamin E and phytate) in the alfalfa meal (Tacon, 1992).

The amount of food intake is an important element in producing maximal growth by providing sufficient energy and nutrients to the fish. This is particularly important in regard to protein (Bowen, 1982). Higher protein level with a greater protein quality has been proven to result in greater weight gain and growth rate in fish (Mazid et al., 1999). Replacement of commercial fish diet with alfalfa meal likely dilutes the concentration and availability of food nutrients, particularly when replacing animal protein in the food (Hardy, 1989; Lovell, 1998; NRC, 1993). This combination of factors likely causes a decrease in nutrients and energy available for growth and thus results in a lesser growth rate and reduction of body weight and length of the fish (Lovell, 1998; NRC, 1993). Though tilapia are capable of utilizing food nutrients in plant foodstuffs including alfalfa (Popma, 1989), the coarse texture and lesser palatability of the alfalfa meal likely contribute to the decrease in food intake of the fish in this study. In addition, high protein levels in the diet have been shown to relate to the secretion of cholecystokinin, a neuroendocrine hormone of the intestinal wall, which itself directly regulates feed intake and secretion of growth hormone in fish (Peng and Peter, 1997; Holloway and

Leatherland, 1998). Thus, the higher protein intake in fish (i.e., lesser alfalfa concentration) likely provides greater protein availability, and also stimulates the higher secretion of the growth hormone, and therefore influences the faster growth in the fish.

Although alfalfa contains numerous food nutrients including protein, lipid and vitamins (Bickoff et al., 1972; Hardy, 1989), this plant food has also been reported to contain several anti-nutritional factors, such as protease inhibitor, anti-vitamin E and phytate (Tacon, 1992). Protease inhibitor has been found to directly affect protein availability in the diet by forming a complex with trypsin and chymotrypsin causing low protein digestion as well as reduction of growth and feed efficiency (NRC, 1983; Hendricks and Bailey, 1989; Lovel, 1989; Tacon, 1992). Anti-vitamin E factor, especially at high levels may initiate deficiency signs such as anemia, growth reduction, poor feed efficiency, and mortality (Roem, Stickney and Kohler, 1990). Moreover, phytate in plant foods can complex with proteins and minerals in the diet and reduce their availability to fish, thereby causing reduction in growth and feed efficiency (O'Dell and Savage, 1960; Smith, 1977; Spinelli et al., 1983). In addition, because alfalfa contains a high amount of fiber which is usually indigestible by fish, its high level in the diet may increase fecal waste, create poor water condition and thereby affect health as well as growth of the fish (Hardy, 1989; Lovell, 1998).

Gender and metabolism also play an important role on growth rate of fish. Generally, male tilapia tend to out-compete females in food consumption and thus grow at a faster rate (Meyer, 1992; Lovell, 1993). Males have been shown to perform better in their protein, carbohydrate and fat metabolism by a greater deposition of protein in the body, a higher metabolic rate and more fat utilization than females (Guyton and Hall, 1996).

Typically, growth is not uniform within fish populations. This generality was observed in the present work, where standard deviations of body length in a given group of fish became larger as fish age increased. Similar variations have also been reported in other studies; such findings suggest that variables like fish size, stocking density, behavioral dominance and genetic influence can have significant impacts on growth rate of fish (Francis, 1988; Huang and Chiu, 1997; Jobling and Reinsnes, 1987; Koebele, 1985; Tave and Smitherman, 1980; Zonneveld and Fadholi, 1991).

5.5.3: Relationship between Intestine and Growth of Fish

Positive and strong relationships were observed between TL, GL ratio of GL to TL (R) and age of fish in all feeding trials (Tables 5-4, 5-6, 5-8 and 5-9). Slopes of the equations describing these correlations confirm that the TL, GL and R also increased linearly with the fish age (Tables 5-8 and 5-9). The differences in the increase of body size and GL were found to differ significantly among ages of fish ($P<0.05$), but not among feeding treatments (Table 5-7). The R-value can be used as a criterion to attempt to correlate a factor with feeding habits of the fish. Based on this criterion, Bowen (1982) reported that the relative gut length ratios of the cichlids were shortest in carnivores, intermediate in omnivores and longest in herbivores. The tilapia larvae at week 3 post-hatch in the present study should be categorized as carnivorous, because the gut configuration was still arranged in a simple form with little looping (Tengjaroenkul et al., in review), and the mean R value was around 1.0 with the fish length below 1.35 cm (Table 5-6). At the end of the study, this value increased dramatically, particularly in the fish groups fed the

higher level of alfalfa meal (Table 5-6, Fig. 5-5). This implies that increased GL of fish during development, particularly when on the highly fibrous foods (lower food nutrients) likely improved feed efficiency, contributing to increased growth rate of fish. Moreover, the slope of the equations of R-value (Table 5-9) relating gut growth rate to the body length growth rate showed the highest value in Trials II and IV. This likely confirms that this characteristic is directly influenced by the level of alfalfa meal in the diet. This suggests considerable phenotypic plasticity for this trait.

The relationship of fish body weight and the gut weight on age was exponential graphically (Figs. 5-1 and 5-2), suggesting that the growth of both fish size and gut length increased at a rate that itself increased with time. The rapid increase of the intestinal weight during development contributed to this climbing rate of growth probably by increasing the number of enterocytes, enhancing digestion and absorption, thereby raising energy available for growth, and thus increasing the body weight of the fish.

5.5.4: Enzyme Activity

Intestinal enzyme activities of tilapia were found to vary significantly among the different alfalfa levels at different fish ages ($P<0.05$); however, no significant difference was present among all alfalfa treatments ($P<0.05$) (Table 5-10). This suggests that regulation of the intestinal enzymes of tilapia appear to be age-dependent at least to a certain degree.

After the first six weeks, fish fed the highest percentage of alfalfa meal (with lower total protein levels) demonstrated lower activities of Malt, LAP and IAP. However, in the last

six weeks, the Malt and IAP increased to a higher in the fish fed on the high percentage of alfalfa, whereas LAP reached a maximum in the control group consuming the commercial fish diet alone. These alterations of the enzyme activities likely indicate that the different concentrations of total protein and alfalfa in the diets played a major role in enzyme production and physiological adaptation, probably by changes in number as well as rate of protein synthesis of enterocytes. This also suggests that even in the same treatments, the activities of Malt and IAP have different regulatory mechanisms as compared to LAP. Moreover, Malt and IAP seem to develop at a slower rate than LAP during growth from fry to fingerlings (weeks 3 to 9), but faster from fingerlings to juveniles (weeks 9 to 15).

Notable variations in gut enzyme levels were observed among fish on similar diets. This suggests that while enzyme activities are to a large degree induced by dietary factors, are nonetheless also to a certain degree under genetic control as well as non-genetic factors such as size. In addition, exogenous factors such as nutrient components, and many endogenous factors (particularly microorganisms) may also affect mucosal hydrolytic activities. Microorganisms in the intestine of some fish species are important in fermentation of highly fibrous plant materials (Niederholzer and Hofer, 1979; Rimmer and Wiebe, 1987). However, the microflora of the tilapia intestine is only minimally functional in fermentation (Bowen, 1976; Stickney and Shumway, 1974). Tilapian intestinal microbes thus likely have a minimal effect on the levels and variations of the enzyme activities.

5.6: CONCLUSION

These results have demonstrated the effect of alfalfa meal on growth and intestinal enzyme activities of the Nile tilapia. In regard to its palatability to and consumption by the fish, protein digestibility as well as several other undesirable effects, using alfalfa as protein food replacement in young tilapia that are still in the exponential phase of growth produces sub-commercial standard growth rate, and thus is unsuitable. Providing alfalfa at levels less than 20% of the ration in the fish aged more than 9 weeks once having curved into linear or even plateau stage of growth, may or may not be more acceptable, and awaits further investigation.

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TABLES

Table 5-1. Ratio of commercial fish crumbles (44% protein) and alfalfa crumbles (15% protein) used in five feeding trials of *O. niloticus* during the twelve week study

Feeding trial	First six weeks (aged 3-9 weeks)	Second six weeks (aged 9-15 weeks)
I	100% C	100% C
II	100% C	80% C + 20% A
III	80% C + 20% A	80% C + 20% A
IV	80% C + 20% A	60% C + 40% A
V	60% C + 40% A	60% C + 40% A

C: Commercial fish crumbles (44% protein), A: Alfalfa crumbles (15% protein)

Table 5-2. Total percentage of protein used in five feeding trials of *O. niloticus* during the twelve weeks of the study

Feeding trial	Total percentage of protein	
	First six weeks (aged 3-9 weeks)	Second six weeks (aged 9-15 weeks)
I	44	44
II	44	38
III	38	38
IV	38	32
V	32	32

Table 5-3. Substrates, buffers and conditions of enzyme assays for homogenized intestine of *O. niloticus*

Enzyme	Substrate	Substrate concentration (mM/ ml)	Buffer	Incubation	
				Time (min)	pH
Malt	Maltose	56	sodium maleate	40	6.9
LAP	L-leucine-ρ-nitroanilide	60	sodium phosphate	120	7.4
IAP	ρ-nitrophenylphosphate	20	Tris	10	8.5

Malt = maltase; LAP = leucine aminopeptidase; IAP = intestinal alkaline phosphatase

Table 5-4. Average body weight (g) and length (cm) of *O. niloticus* in different feeding trials (I to V) during the twelve week study

Feeding		Mean Body Weight (g)			Mean Body Length (cm)		
Trial	Age of fish	Wk 3	Wk 9	Wk 15	Wk 3	Wk 9	Wk 15
I		0.036^{a}	0.802^{a}	6.256^{a}	1.346^{a}	4.640^{a}	7.493^{a}
II		0.036^{a}	0.802^{a}	5.312^{b}	1.346^{a}	4.640^{a}	$6.712^{a,b}$
III		0.036^{a}	$0.665^{a,b}$	$3.663^{b,c}$	1.346^{a}	3.831^{b}	$5.772^{b,c}$
IV		0.036^{a}	$0.665^{a,b}$	3.342^{c}	1.346^{a}	3.831^{b}	$5.277^{c,d}$
V		0.036^{a}	0.555^{b}	2.848^{c}	1.346^{a}	3.374^{c}	4.632^{d}

$*^{abcd}$: Mean values with the same superscripts in the same column are not significantly different at $p<0.05$.

Table 5-5. Average gut weight (g) and length (cm) of *O. niloticus* in different feeding Trials (I to V) during the twelve weeks of the study

Feeding		Mean Gut Weight (g)			Mean Gut Length (cm)		
Trial	Age of fish	Wk 3	Wk 9	Wk 15	Wk 3	Wk 9	Wk 15
I		0.0025^{a}	0.0441^{a}	0.252^{ac}	1.397^{a}	8.903^{a}	20.075^{a}
II		0.0025^{a}	0.0441^{a}	0.3073^{a}	1.397^{a}	8.903^{a}	22.18^{ab}
III		0.0025^{a}	0.035^{bc}	0.206^{bc}	1.397^{a}	6.941^{b}	13.668^{c}
IV		0.0025^{a}	0.035^{bc}	$0.241^{a,c}$	1.397^{a}	6.941^{b}	15.281^{c}
		0.0025^{a}	0.032^{c}	0.1734^{c}	1.397^{a}	5.394^{c}	11.824^{c}

*^{abc}: Mean values with the same superscripts in the same column are not significantly different at $p<0.05$.

Table 5-6. Ratio between gut length and body length of the tilapia during the trial period

Ratio between gut length and body length									
Age of Fish	Week 3			Week 9			Week 15		
Feeding Trial	GL	TL	R	GL	TL	R	GL	TL	R
I	1.397	1.346	1.038[a]	8.903	4.640	1.919[a]	20.07	7.493	2.679[b]
II	1.397	1.346	1.038[a]	8.903	4.640	1.919[a]	22.19	6.712	3.306[a]
III	1.397	1.346	1.038[a]	6.941	3.831	1.812[a]	13.67	5.772	2.367[b]
IV	1.397	1.346	1.038[a]	6.941	3.831	1.812[a]	15.28	5.277	2.89[ab]
V	1.397	1.346	1.038[a]	5.394	3.374	1.599[a]	11.82	4.632	2.553[b]

GL= gut length; TL= total body length; R= ratio between gut length and body length

*[ab]: Mean values with the same superscripts in the same column are not significantly different at $P<0.05$.

Table 5-7. Body and intestinal growth of *O. niloticus* in different feeding trials (I to V) containing different levels of alfalfa during experimental period

	Feeding Trial Number				
	I	II	III	IV	V
Weight Gain (g/wk)	0.518^{a}	0.440^{ab}	0.302^{c}	0.276^{c}	0.234^{c}
% Weight Gain per day	2.04^{a}	1.73^{ab}	1.19^{c}	1.09^{c}	0.92^{c}
SGR (% per day)[1]	2.6816^{a}	2.5947^{ab}	2.4061^{c}	2.3581^{c}	2.276^{c}
Body Length Gain (cm/wk)	0.512^{a}	0.447^{ab}	$0.369b^{c}$	0.328^{cd}	0.274^{d}
Growth Rate Index[2]	1.487^{a}	1.757^{ab}	$1.905b^{c}$	2.275^{d}	2.865^{e}

[1]: Specific Growth Rate (SGR) was calculated on the basis of the formula:
SGR = 100 ($\log_e$ of weight gain)/day.

[2]: Growth Rate Index/or condition factor (GRI) was calculated according to the formula:
GRI = (weight x 100)/(length)^3.

$*^{abcde}$: Mean values with the same superscripts in the same row are not significantly different at $P<0.05$.

Table 5-8. Summary of models, equations and parameters of the relationships between body weight and its length on age of the fish

Relationship	Feeding Trial	Model	Equation	r^2
BW and Age	I	Quadratic	$BW = 0.0118e^{0.4298\ Wk}$	0.9864
	II	Quadratic	$BW = 0.0126e^{0.4162\ Wk}$	0.9807
	III	Quadratic	$BW = 0.0139e^{0.3852\ Wk}$	0.9777
	IV	Quadratic	$BW = 0.0144e^{0.3776\ Wk}$	0.9732
	V	Quadratic	$BW = 0.0145e^{0.3642\ Wk}$	0.9793
TL and Age	I	Linear	TL = 0. 5123 Wk - 0.1173	0.9983
	II	Linear	TL = 0.4472 Wk + 0.2082	0.9830
	III	Linear	TL = 0.3688 Wk + 0.3302	0.9950
	IV	Linear	TL = 0.3276 Wk + 0.5364	0.9772
	V	Linear	TL = 0.2738 Wk + 0.6528	0.9820

BW= body weight; TL= body length; Wk= week; r^2= coefficient of determination

Table 5-9. Summary of models, equations and parameters characterizing relationships between gut weight, gut length and ratio of gut length to fish length and age of the fish

Relationship	Feeding Trial	Model	Equation	r^2
GW and Age	I	Quadratic	$GW = 0.001e^{0.3845\ Wk}$	0.9805
	II	Quadratic	$GW = 0.0009e^{0.401\ Wk}$	0.9877
	III	Quadratic	$GW = 0.001e^{0.3676\ Wk}$	0.9873
	IV	Quadratic	$GW = 0.0009e^{0.3806\ Wk}$	0.9920
	V	Quadratic	$GW = 0.001e^{0.3533\ Wk}$	0.9865
GL and Age	I	Linear	GL = 1.5565 Wk - 3.8835	0.9875
	II	Linear	GL = 1.7325 Wk - 4.7635	0.9749
	III	Linear	GL = 1.0226 Wk - 1.8679	0.9969
	IV	Linear	GL = 1.157 Wk - 2.54	0.9867
	V	Linear	GL = 0.8689 Wk - 1.6153	0.9822
R and Age	I	Linear	R = 0.1368 Wk + 0.6476	0.9982
	II	Linear	R = 0.189 Wk + 0.3866	0.9837
	III	Linear	R = 0.1108 Wk + 0.7417	0.9911
	IV	Linear	R = 0.1548 Wk + 0.5217	0.9908
	V	Linear	R = 0.1262 Wk + 0.5937	0.9780

GW= gut weight; GL= gut length; R= ratio of gut length and fish length; Wk= week; r^2= coefficient of determination

Table 5-10. Levels of intestinal enzyme activities for each feeding trial in the experiment

Feeding		Malt (U/ 1 ml chyme)*			LAP (U/ 1 ml chyme)*			IAP (U/ 1 ml chyme)*		
Trial	Fish Age	Wk 3	Wk 9	Wk 15	Wk 3	Wk 9	Wk 15	Wk 3	Wk 9	Wk 15
I		0.039^{a}	0.117^{b}	0.210^{c}	1.21^{a}	4.19^{b}	8.27^{c}	1.164^{a}	2.353^{b}	10.368^{c}
II		0.039^{a}	0.117^{b}	0.282^{c}	1.21^{a}	4.19^{b}	6.88^{c}	1.164^{a}	2.353^{b}	13.678^{c}
III		0.039^{a}	0.126^{b}	0.348^{c}	1.21^{a}	4.05^{b}	6.23^{c}	1.164^{a}	2.098^{b}	13.496^{c}
IV		0.039^{a}	0.126^{b}	0.361^{c}	1.21^{a}	4.05^{b}	5.90^{c}	1.164^{a}	2.098^{b}	15.848^{c}
V		0.039^{a}	0.105^{b}	0.264^{c}	1.21^{a}	3.88^{b}	4.75^{c}	1.164^{a}	1.790^{b}	14.143^{c}

U: unit of enzyme that can hydrolyze 1.0 μMole of substrate per minute at pH 6.8 and 37°C (for Maltase), pH 8.5 and 25°C (for leucine aminopeptidase), and pH 9.8 and 38°C (for alkaline phosphatase).

*: average enzyme activity (unit) measured from 1 ml of chyme of each fish and at ratio 1: 50 of intestinal weight and distilled water.

^{*abc}: mean values not having a common superscript differ significantly at $P<0.05$.

FIGURES

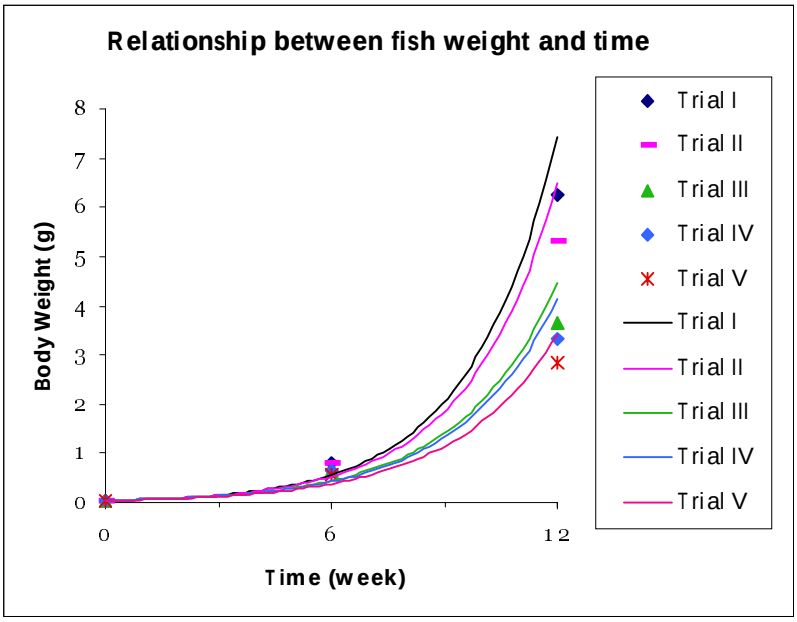

Fig. 5-1. *O. niloticus.* Relationship of fish weight to time. Lines of exponential growth curves fit to all feeding trials.

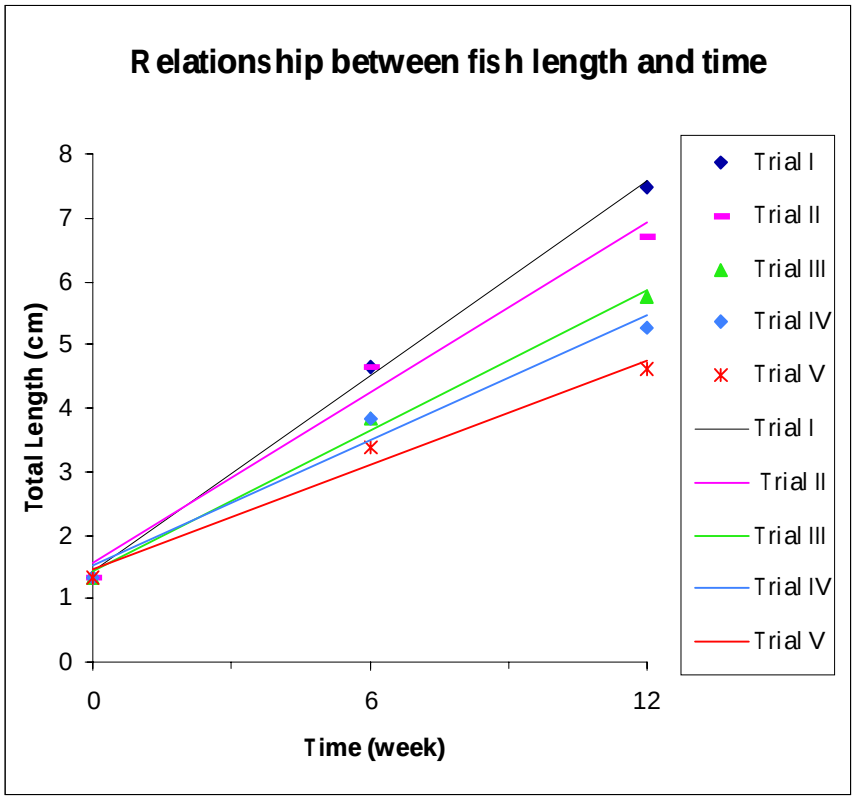

Fig. 5-2. *O. niloticus.* Relationship of fish length to time. Lines are regressions fit to data from all feeding trials.

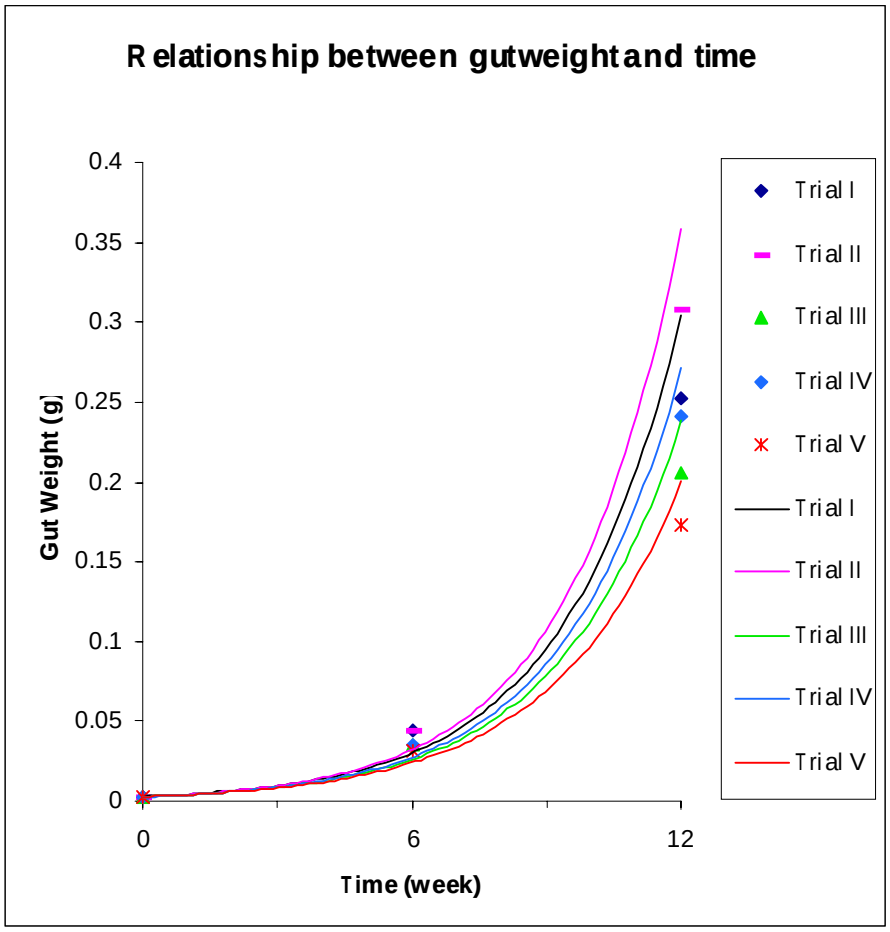

Fig. 5-3. *O. niloticus*. Relationship of gut weight and time. Exponential growth curves fitted to all data from all feeding trials.

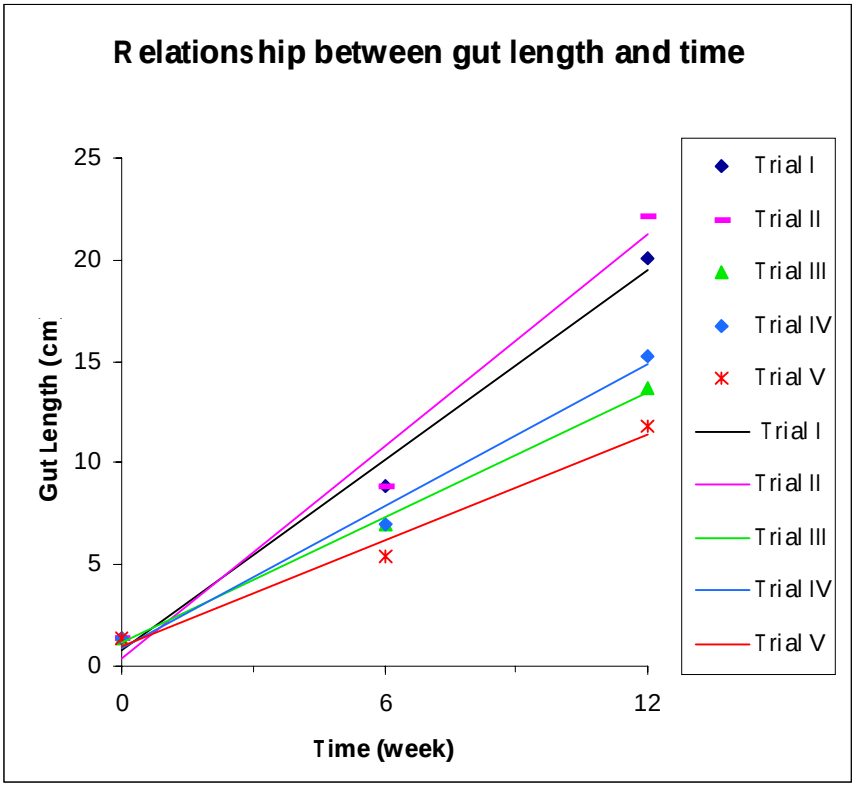

Fig. 5-4. *O. niloticus.* Relationship of gut length to time. Lines are regressions fitted to data from all feeding trials.

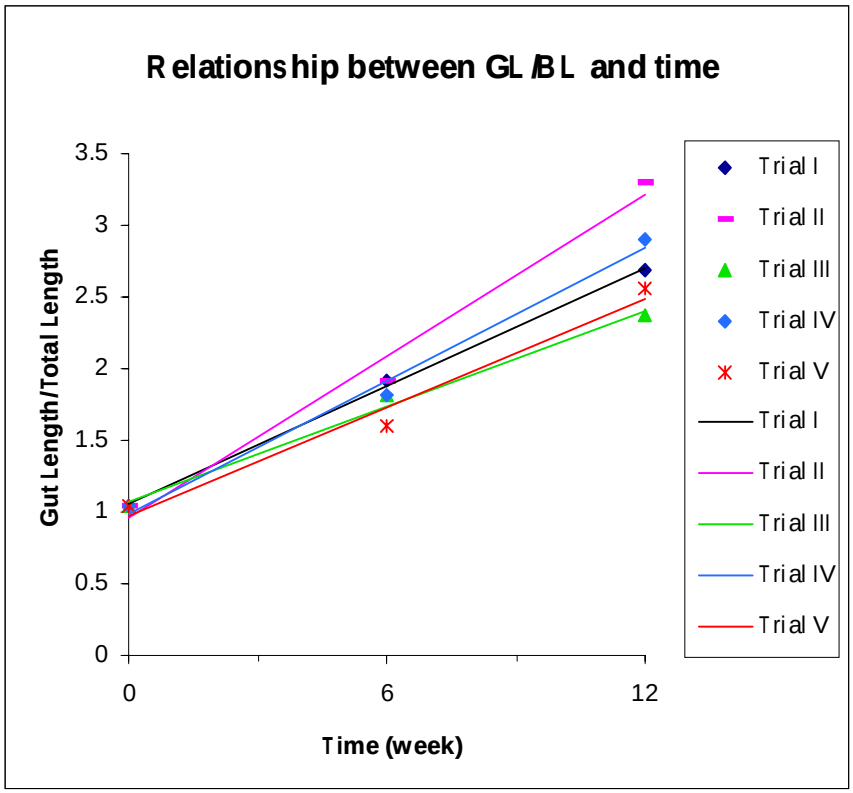

Fig. 5-5. *O. niloticus*. Relationship of ratio of GL to TL over time. Lines are regressions fit to data from all feeding trials.

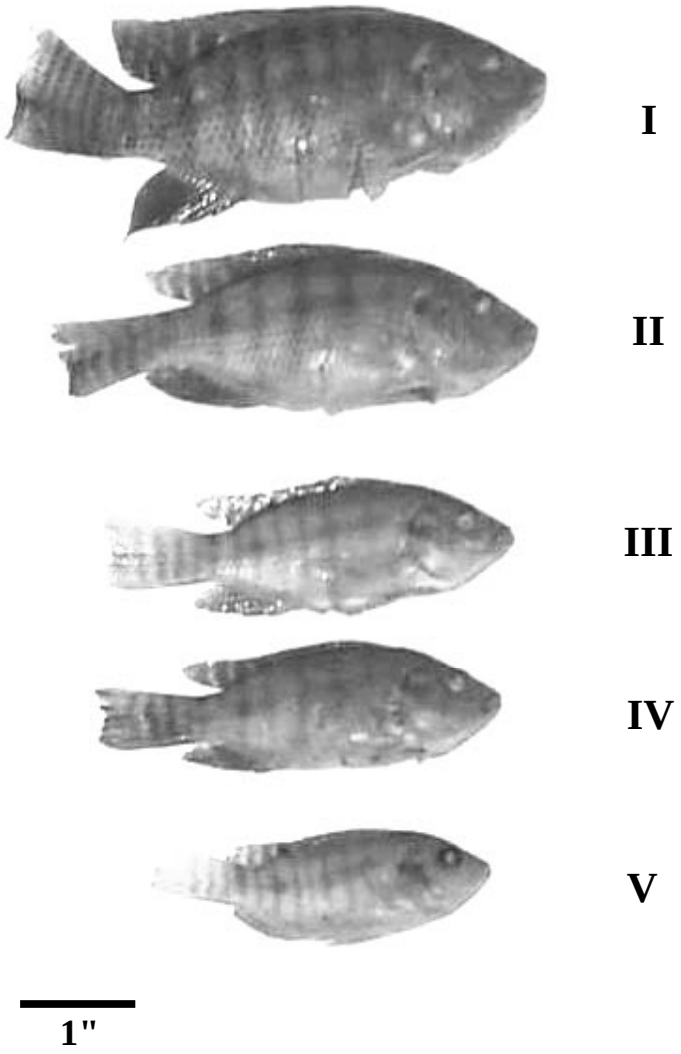

Fig. 5-6. Comparison of tilapia sizes of Nile tilapia among five feeding trials (Trials I-V) after twelve weeks of the study.

APPENDIX A: GENERAL METHODS FOR ENZYME HISTOCHEMISTRY

A. Preparation of Tissue Sections

For tissue collection, fish were anesthetized with MS-222 (tricane methanesulfonate, Sigma Chemical Company, St. Louis, MO), and then killed by cervical separation. The intestine of each fish was immediately removed from the body cavity. In fish of an age in which mature intestinal morphology had not yet developed, samples were taken from the cranial and caudal regions of the intestinal tube. In fish with mature intestinal morphology, samples were taken from the hepatic loop, first major coil, gastric loop, second major coil and the terminal portion. All tissue specimens were later be embedded in Optimal Critical Temperature Compound (VWR Scientific Products, Bridgeport, NJ), frozen in liquid nitrogen, and then sectioned on a cryomicrotome at -15°C. Sections of 10 μm thickness were retrieved from the knife on positive-charged slides (Superfrost/plus, Fisher Scientific, Pittsburgh, PA).Before incubation in medium (all chemicals for preparing media available from Sigma Chemical Company, St. Louis, MO), fresh cryostat sections intended for study of maltase (Malt), lipase (Lip), non-specific esterases (NSE) and intestinal alkaline phosphatase (IAP) were fixed for five minutes in 10% neutral buffered formalin. For detection of leucine aminopeptidase (LAP) and dipeptidyl aminopeptidase IV (DAP IV), tissue sections were fixed for one minute in equal parts of cold absolute acetone and chloroform formalin. Sections incubated without the substrate were served as controls.

B. Chemicals Preparation:

Substrates, diazonium salts and conditions of incubation media for the enzyme studies were summarized in Table 1. All chemicals in this study were obtained from Sigma Chemical Co., St. Louis, MO. The buffers for each enzyme were: 0.1M citric acid-phosphate buffer for maltase; 0.1M phosphate buffer for leucine aminopeptidase, dipeptidyl aminopeptidase IV, lipase, and non-specific esterases; and 0.1M Tris buffer for intestinal alkaline phosphatase.

C. Fixation and Enzyme Staining

Before incubation in medium, fresh cryostat sections were fixed as described in Section B, Preparation of Tissue Sections. Preparation of incubation media and procedures for the enzyme examinations were followed established techniques using substituted naphthol methods. After incubation, the tissue sections were coverslipped with mounting medium and blindly examined using a light microscope. Enzyme activities were evaluated semi-quantitatively as strong, weak or absent depending on the staining intensity in the tissue.

1. **Simultaneous azo-coupling method for maltase** (adapted from Lojda et al., 1979)

The fixed cryostat sections were incubated for 60 minutes at 37°C in incubating medium consisting of 0.1 M citric acid-phosphate buffer (12 ml, pH 6.5), β-naphthyl-α-D-glucopyranoside (10 mg) as the substrate, hezaxonium-ρ-rosaniline (0.5 ml) as the diazonium salt, and N,N-dimethylformamide (0.5 ml) as the solvent. Specimens were rinsed in distilled water and then placed in 4% formaldehyde at room temperature for 2 hours to reduce formation of gas bubbles. Tissue samples were rinsed again for 5 minutes in distilled water.

Finally, specimens were mounted in glycerin jelly and coverslipped. Light microscopy showed enzyme-active sites as orange, while the background will show yellow.

2. Simultaneous azo-coupling method for leucine aminopeptidase

(modified from Lojda et al., 1979)

The fixed cryostat sections were incubated at 37°C for 30 minutes in incubating medium consisting of 0.1 M phosphate buffer (10 ml, pH 7.4), L-Leucyl-4-methoxy-2-naphthylamide (5 mg) as the substrate, Fast Blue B (10 mg) as the diazonium salt, and N,N-dimethylformamide (0.5 ml) as the solvent. The medium was then be poured off and the specimens were rinsed in distilled water. Finally, tissue samples were mounted with glycerin jelly and coverslipped. Light microscopy showed enzyme-active sites to be stained red.

3. Simultaneous azo-coupling method for dipeptidyl peptidase IV (modified from Lojda et al., 1979; Bancroft and Hand, 1987)

The fixed cryostat sections were incubated at 37°C for 30 minutes in incubating medium consisting of 0.1 M phosphate buffer (4 ml, pH 7.2), glycyl-proline-4-methoxy naphthylamide (2 mg) as the substrate, Fast Blue BB (5 mg) as the diazonium salt in distilled water (1 ml), and N,N-dimethylformamide (0.3 ml) as the solvent. The medium was then be poured off and the specimens were rinsed in distilled water. Finally, tissue samples were mounted with glycerin jelly and coverslipped. Light microscopy showed enzyme-active sites to be stained red.

4. **Naphthol method for Lipase (E.C.3.1.1.3.)** (modified from Knospe and Plendl, 1997)

The fixed cryostat sections were incubated at 37°C for 60 minutes in incubating medium containing 64 mg of Triton X-100 in 0.1 M phosphate buffer (8 ml, pH 7.4), α-naphthyl palmitate (1.6 mg) as the substrate in 0.1 ml of N,N-dimethylacetamide, and Hezaxonium-ρ-rosaniline (0.24 ml) as the diazonium salt. Tissue sections was then be washed in distilled water for 5 minutes, mounted on slides with glycerin jelly, and finally coverslipped. Light microscopic observation indicated sites of lipase activity by the presence of red-brown dye.

5. **Simultaneous azo-coupling method for nonspecific esterases** (Lojda et al., 1979; Bancroft and Hand, 1987).

The fixed cryostat sections were incubated for 10 minutes at 25°C in incubating medium consisting of 2.8% disodium phosphate buffer (10 ml, pH 6.5), 1% naphthol AS-D acetate in acetone (0.1 ml) as the substrate, and hezaxonium-ρ-rosaniline (0.3 ml) as the diazonium salt. Specimens were rinsed in distilled water and then placed in 4% formaldehyde at room temperature for 2 hours to reduce formation of gas bubbles. Tissue samples were rinsed again for 5 minutes in distilled water. Finally, specimens were mounted in glycerin jelly and coverslipped. Light microscopy showed enzyme-active sites to appear brown, while the background demonstrated yellow.

6. **Simultaneous azo-coupling method for alkaline phosphatase** (Lojda et al., 1979; Bancroft and Hand, 1987).

The fixed cryostat sections were incubated for 10 minutes at room temperature in incubating medium consisting of stock naphthol AS-BI solution (10 ml) and Fast Red TR (10 mg) as the diazonium salt. The stock of naphthol solution was prepared from naphthol AS-BI phosphate as the solvent (1.25 mg), N,N-dimethylformamide (0.5 ml), 15.5 ml of distilled water, and 0.2 M tris-HCl buffer (10 ml, pH 8.3). Tissues were then washed in water for 2 minutes, and counterstained with 1% Mayer's hematoxylin for 1 minute. Finally, specimens were mounted with glycerin jelly, and coverslipped. Light microscopic evaluation presented enzyme-active sites to be stained red.

D. Enzyme Examination

After incubation, the tissue sections were coverslipped with mounting medium and examined using a light microscope. Enzyme activities were blindly evaluated semi-quantitatively as strong, weak or absent depending on the staining intensity in the tissue.

Summary of incubation techniques for enzyme histochemistry

Enzyme	Substrate	Diazonium salt	pH	Time (min.)
Malt	β-naphthyl-α-glucoside	Pararosaniline	6.9	60
LAP	L-Leucyl-4-M-2-N*	Fast Blue B	7.4	30
DAP IV	Glycyl-prolyl-4-M-2-N*	Fast Blue BB	7.4	30
Lip	α-naphthyl palmitate	Pararosaniline	7.4	60
NSE	Naphthol AS-D acetate	Pararosaniline	7.4	10
IAP	Naphthol AS-MX phosphate	Fast Red TR	8.5	10

M-2-N*= methoxy-2-naphthylamide; Malt= Maltase; LAP= Leucine aminopeptidase; DAP IV= Dipeptidyl aminopeptidase IV; Lip= Lipase; NSE= Non-specific esterases; IAP= Intestinal alkaline phosphatase

APPENDIX B: ENZYME ASSAYS

A. Maltase (modified from Hycarinen and Nikkila, 1962; Dahlqvist,1969)

Maltase activity was determined in 100 mM sodium maleate buffer (0.1 ml) which was mixed with 56 mM maltose. The pH was 6.0 and the reaction was started by 0.1 ml mucosal dilution (1:50). After 40 min incubation the reaction was stopped by 1% trichloroacetic acid (1.0 ml) and the proteins was sedimented by centrifugation (2000 rpm for 10 min). O-Toluidine color reagent (4.5 ml) was added into 0.5 ml of the supernatant in order to detect free glucose. Tubes was subsequently placed in boiling water bath for 8 min. Thereafter they were cooled and the color was measured by a Beckman DU 640B spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) at 630 nm wavelength.

B. Leucine Aminopeptidase (modified from Bergmeyer and GraBl, 1983)

Activated mixture for leucine aminopeptidase was prepared from 50 mM phosphate buffer (2.0 ml), 25 mM $MnCl_2$ (0.2 ml) and mucosal solution (0.1 ml). The leucine aminopeptidase activity was measured by adding 0.1 ml of the activated mixture into the substrate solution containing 60 mM L-leucine-ρ-nitroanilide (2 ml), 62.5 mM $MgCl_2$ (0.2 ml) and 50 mM phosphate buffer (0.7 ml). The enzyme reaction was started by using 1:200 tissue solution, and was incubated for 2 hours at pH 7.4. Control tubes were incubated without leucine aminopeptidase. The color was measured using a Beckman DU 640B spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) at 238 nm wavelength.

C. Alkaline Phosphatase (modified from Hanninen and Hartiala, 1965; Bergmeyer and GraBl, 1983)

Alkaline phosphatase activity was measured in a mixture consisting of 50 mM Tris buffer (0.4 ml), 10 mM magnesium chloride (0.05 ml) and 20 mM ρ-nitrophenyl-phosphate in Tris buffer (0.1 ml). After pre-incubation, the reaction was started using 0.1 ml of mucosal solution (1: 500) incubated for 10 min at pH 8.5 and 25°C. The reaction was stopped by adding 20 mM NaOH (5.0 ml) and absorbency immediately measured using a Beckman DU 640B spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) at 405 nm wavelength.

APPENDIX C: EQUIPMENT USED

Study 1: Morphological development of the intestinal tract of the tilapian fish, *O. niloticus*

- fish net
- dissection equipments (scissors, forceps, knife, blades)
- dissection trays
- a stereo microscope
- calipers (Qing-gong, China)
- droppers
- petri-dishes
- glass test tubes & glass bottles
- test tube racks
- 4°C refrigerator

Study 2: Distribution of intestinal enzyme activities along the intestinal tract in *O. niloticus*

- fish net
- dissection equipments (scissors, forceps, knife, blades)
- dissection trays
- a compound microscope
- a cryo-microtome
- embedding blocks

- droppers
- glass test tubes & glass bottles
- thermostat incubator (37°C)
- hot plate
- magnetic stirrer
- positive-charged slides (Superfrost-plus, Fishers Scientific,PA)
- cover glasses

Study 3: Ontogenic development of intestinal enzymes of *O. niloticus*

- fish net
- dissection equipments (scissors, forceps, knife, blades)
- dissection trays
- a compound microscope
- a cryo-microtome
- embedding blocks
- droppers
- glass test tubes & glass bottles
- thermostat incubator (37°C)
- hot plate
- magnetic stirrer
- positive-charged slides (Superfrost-plus, Fishers Scientific, PA)
- cover glasses

Study 4: Effects of alfalfa as a food replacement on growth and intestinal enzyme activities in *O. niloticus*

- fish net
- dissection equipments (scissors, forceps, knife, blades)
- dissection trays
- a steromicroscope
- calipers (Qing-gong, China)
- weighing balance
- plastic droppers
- glass test tubes & test tube racks
- glass bottles & beakers
- centrifuge
- sonicator
- hot plate
- magnetic stirrer
- incubator (37°C)
- stainless steel screens
- automatic pipettes (0.1, 1.0, 5.0 ml) & pipette tips
- spectrophotometer (Beckman Instruments, Inc., Fullerton, CA)

APPENDIX D: REAGENTS AND CHEMICALS USED

Study 1: Morphological development of the intestinal tract of the tilapian fish, *O. niloticus*

- MS-222 (Tricane methanesulfonate) (Sigma Chemical Co., St. Louis, MO)
- 10% neutral buffered formalin
- methylene blue

Study 2: Distribution of intestinal enzyme activities along the intestinal tract in *O. niloticus*

- MS-222 (Tricane methanesulfonate) (Sigma Chemical Co., St. Louis, MO)
- 10% neutral buffered formalin
- chloroform
- acetone
- Optimal Critical Temperature Compound (VWR Scientific Products, Bridgeport, NJ)
- liquid nitrogen
- citric acid-phosphate buffer, pH 6.9
- phosphate buffer, pH 7.4
- Tris buffer, pH 8.5
- β-naphthyl-α-glucoside (Sigma Chemical Co., St. Louis, MO)

- L-Leucyl-4-methoxy-2-naphthylamide (Sigma Chemical Co., St. Louis, MO)
- Glycyl-prolyl-4-methoxy-2-naphthylamide (Sigma Chemical Co., St. Louis, MO)
- α-naphthyl palmitate (Sigma Chemical Co., St. Louis, MO)
- Naphthol AS-D acetate (Sigma Chemical Co., St. Louis, MO)
- Naphthol AS-MX phosphate (Sigma Chemical Co., St. Louis, MO)
- Pararosaniline (Sigma Chemical Co., St. Louis, MO)
- Fast Blue B (Sigma Chemical Co., St. Louis, MO)
- Fast Blue BB (Sigma Chemical Co., St. Louis, MO)
- Fast Red TR (Sigma Chemical Co., St. Louis, MO)

Study 3: Ontogenic development of intestinal enzymes of *O. niloticus*

- MS-222 (Tricane methanesulfonate) (Sigma Chemical Co., St. Louis, MO)
- 10% neutral buffered formalin
- chloroform
- acetone
- Optimal Critical Temperature Compound (VWR Scientific Products, Bridgeport, NJ)
- liquid nitrogen
- citric acid-phosphate buffer, pH 6.9
- phosphate buffer, pH 7.4
- Tris buffer, pH 8.5

- β-naphthyl-α-glucoside (Sigma Chemical Co., St. Louis, MO)
- L-Leucyl-4-methoxy-2-naphthylamide (Sigma Chemical Co., St. Louis, MO)
- Glycyl-prolyl-4-methoxy-2-naphthylamide (Sigma Chemical Co., St. Louis, MO)
- α-naphthyl palmitate (Sigma Chemical Co., St. Louis, MO)
- Naphthol AS-D acetate (Sigma Chemical Co., St. Louis, MO)
- Naphthol AS-MX phosphate (Sigma Chemical Co., St. Louis, MO)
- Pararosaniline (Sigma Chemical Co., St. Louis, MO)
- Fast Blue B (Sigma Chemical Co., St. Louis, MO)
- Fast Blue BB (Sigma Chemical Co., St. Louis, MO)
- Fast Red TR (Sigma Chemical Co., St. Louis, MO)

Study 4: Effects of alfalfa as a food replacement on growth and intestinal enzyme activities in *O. niloticus*

- MS-222 (Tricane methanesulfonate) (Sigma Chemical Co., St. Louis, MO)
- 10% neutral buffered formalin
- sodium maleate buffer, pH
- sodium phosphate buffer, pH
- Tris buffer, pH
- maltose
- 1% trichloroacetic acid (Sigma Chemical Co., St. Louis, MO)
- O-Toluidine color reagent (Sigma Chemical Co., St. Louis, MO)

- $MnCl_2$
- $MgCl_2$
- NaOH
- L-leucine-ρ-nitroanilide (Sigma Chemical Co., St Louis, MO).
- ρ-nitrophenyl-phosphate (Sigma Chemical Co., St. Louis, MO)

APPENDIX E: INTESTINAL pH OF MATURE TILAPIA

Table showing pH measured from five different intestinal segments of mature tilapia at three hours after feeding

	pH of Intestinal Segment				
Fish Number	**I**	**II**	**III**	**IV**	**V**
1	6.19	6.87	8.16	8.52	9.06
2	6.48	7.19	8.2	8.04	7.52
3	6.71	7.41	8.05	8.62	8.87
4	6.73	7.18	7.89	8.25	8.24
5	6.75	6.85	7.52	8.19	8.34
6	6.76	7.28	7.35	8.25	7.91
7	6.18	7.01	8.25	8.47	8.14
8	6.81	7.65	7.86	8.5	7.93
9	6.75	7.12	7.67	8.03	7.98
10	6.03	6.71	7.88	7.94	7.96
11	6.68	6.84	7.64	7.63	7.54
12	6.85	7.15	8.19	7.83	8.29
13	6.93	7.28	8.11	8.02	7.54
14	6.76	7.02	7.68	7.98	8.21
15	6.72	7.02	7.43	8.41	8.21
16	7.12	7.76	7.64	8.85	8.85
17	7.14	7.25	7.74	9.01	8.16
18	7.38	8.36	8.08	8.22	8.96
19	7.84	7.72	8.15	7.49	7.85
20	7.65	7.72	8.41	8.98	9.98
Average	**6.82**	**7.27**	**7.90**	**8.26**	**8.28**
Standard Deviation	**0.451**	**0.40**	**0.30**	**0.41**	**0.51**

I= hepatic loop, II= proximal coil, III=gastric loop, IV=distal coil, V= terminal segment

VITA

Bundit Tengjaroenkul, son of Artith and Apiradee Tengjaroenkul, was born on December 8, 1965, in Khon Kaen, Thailand. He has younger sisters, Pirat and Yauwaluk Tengjaroenkul; and a younger brother, Boriboon Tengjaroenkul. He graduated from Satith High School, Khon Kaen, Thailand in 1984.

Dr. Tengjaroenkul earned his DVM degree from Chulalongkorn University, Bangkok, Thailand in 1990. He served for five years as an instructor in the Department of Veterinary Anatomy, Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen, Thailand. In August 1995, he received scholarship from the Thai government to pursue a doctoral degree in Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University. He worked with Dr. Bonnie J. Smith dealing with the ontogenic development of the morphology and intestinal enzyme activities in the tilapian fish.

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