



A histo-morphometric study on the developing gastrointestinal tract of the axolotl, *Ambystoma mexicanum*

Gamal M. Badawy

E. mail: Gamalbadawy940@yahoo.com

Faculty of Science Menoufiya University, Shebeen El-Koom, Egypt

ABSTRACT

The histological structure of the developing gastrointestinal tract (GIT) of the axolotl, *Ambystoma mexicanum*, was investigated during embryonic, larval and juvenile stages. The earliest histological events witnessed the formation of the archenteron as a more or less circular shaped cavity at stage 13. Concurrent with the first significant elongation of the embryo, the gut was evidently subdivided into foregut, midgut and hindgut at stage 16 after which there was a progressive but slow differentiation up to stage 37. Very active cellular movements occurred and resulted in the anatomical differentiation of the stomach at stage 40. During the endogenous feeding phase, two prominent features characterized the histological structure of the developing GIT. Firstly, the endodermal yolk mass resorption which occurred in a proximo-distal (PD) direction and lasted up to six days after hatching. Secondly, the progressive PD cellular differentiation during which both the endodermal and mesodermal components showed a co-ordinated and concomitant histological differentiation towards the establishment of the gastrointestinal wall. Subsequent stages displayed active cellular proliferation accompanied with progressive histogenesis, which eventually led to a well-developed GIT in the 2.5 cm long larvae. The histological structure of the mature GIT is described and illustrated. Furthermore, certain morphometric parameters were measured during development.

Keywords: Anatomy; Histology; Embryonic development; Gastrointestinal tract; *Ambystoma mexicanum*.



Council for Innovative Research

Peer Review Research Publishing System

Journal: Journal of Advances in Biology

Vol. 6, No. 1

editorsjab@gmail.com , editor@cirjab.com



Abbreviations

Ap, Animal pole; **Ar**, Archenteron; **Bl**, Blastocoel; **BMSU**, Biomedical services unit; **Bp**, Blastopore; **Bv**, Brain vesicle; **C**, Circular muscle layer; **Cg**, Cement gland; **Ct**, Connective tissue; **Df**, Dorsal fin; **E**, Ectoderm; **Ec**, Endodermal cell; **Eym**, Endodermal yolk mass; **Fg**, Foregut; **G**, Gill; **Gl**, Gastric lumen; **GIT**, Gastrointestinal tract; **H**, Heart; **Ha**, Heart anlage; **H&E**, Haematoxylin and eosin; **Hg**, Hindgut; **Hm**, Head mesoderm; **IR**, Immunoreactive; **K**, Kidney; **L**, Longitudinal muscle layer; **Ld**, Liver diverticulum; **Lil**, Large intestinal lumen; **Lpm**, Lateral plate mesoderm; **Lps**, Lamina propria submucosal; **Lv**, Liver; **M**, Mesodermal cells; **Mc**, Mucosa; **Mf**, Mucosal folds; **Mg**, Midgut; **Mm**, Muscularis mucosa; **MTS**, Mallory triple stain; **Nc**, Neural crest cells; **Ng**, Neural groove; **Nt**, Notochord; **Ntc**, Notochordal cells; **Oe**, Oral invagination; **Pa**, Pancreas; **PD**, Proximo-distal; **Pli**, Presumptive large intestine; **Plv**, Presumptive liver; **Psi**, Presumptive small intestine; **Pst**, Presumptive stomach; **S**, Serosa; **Sil**, Small intestinal lumen; **Sm**, Submucosa; **Smc**, Somatogenic mesoderm; **So**, Somite; **Sp**, Spinal cord; **Tb**, Tail bud; **Tg**, Thyroid gland; **Vm**, Ventral mesoderm; **Vp**, Vegetable pole; **Yp**, Yolk plug.

INTRODUCTION

Amphibians occupy a central position in taxonomy between aquatic and terrestrial vertebrates and are widely used as model systems. They consequently play an important role in the field of vertebrate developmental biology. The greatest advantage of using amphibian embryos over their tetrapod counterparts is that they offer the opportunity to study ontogenesis in free living organisms. Different sorts of ontogenetic studies are therefore preferably performed on amphibians on the basis that their tissue and organ morphogenetic patterns are homologous to those of higher vertebrates. Anatomically, the vertebrate GIT is well known for its remarkable ability to adapt in response to the nature of feeding habits. In anurans, this adaptation is clearly reflected in the general pattern of the developing GIT where switching from a herbivorous to a carnivorous diet is followed by extensive intestinal remodeling (Kupferberg 1997; Badawy et al., 2012; Sakr et al., 2014).

During their early histological development, all vertebrate species pass through three definite phases of programmed cellular events. The first is the cleavage and blastulation phase, which provides the basic cellular material required for performing the prospective morphogenetic movements. The second is the gastrulation phase which comprises a set of morphogenetic movements by which the three distinct germ layers, namely; ectoderm, mesoderm and endoderm are laid out and lead to the establishment of the basic body plan (Warga and Kimmel 1990; Arendt and Nubler-Jung 1999). The third is the organogenesis phase which witnesses the differentiation of different body organs. Within amphibians, the gastrulation is modified to a variable extent by the presence of a yolk-laden cells in the vegetal hemisphere of the developing embryo. This modification leads, at least partly, to some differences although the basic plan of gastrula among amphibians is generally similar.

It is widely accepted that the general pattern of the early histological structure of the developing GIT is homologous among different vertebrates although species variations do exist as a result of differences in intrinsic and/or extrinsic factors (Stevens and Hume, 1995; Gilbert and Raunio 1997; Zorn and Wells 2009). The general rule is that the gut originates from splanchnic mesoderm and visceral endoderm with the mesoderm initially encircling the underlying endoderm to form a simple tubular structure. By this way, the endoderm gives rise to the inner epithelial sheet, which lines the gastric and intestinal lumen, while the mesenchyme provides the outer connective and muscular tissues (Warga and Nusslein-Volhard 1999; Wells and Melton 1999; Grapin-Botton and Melton 2000; Zorn and Wells 2009). The mutual interaction between the mesoderm and endoderm plays a crucial role in the patterning of the whole gut and it is well documented that without this interaction, the gut cannot develop normally (Yasugi and Mizuno 1990; Yasugi 1993; Montgomery et al., 1999). Later in development, the gut components exhibit characteristic features specific for each vertebrate group. The morphological organization of the developing gastrointestinal mucosa of vertebrates is one example for this phylogenetic variation. Thus, while adult fish, amphibians and reptiles are characterized with longitudinal mucosal folds, birds and mammals are characterized with villi.

Among lower vertebrate classes, fishes have uniquely attracted a large number of biologists to investigate the histological structure of their developing GIT. As a consequence, the literature regarding that is notably vast (Sarasquete et al., 1995; Gisbert et al., 1998; Veggetti et al., 1999; Morrison et al., 2001; Senarat; et al., 2013). Three main reasons contribute to the high interest with respect to investigating the development of the GIT of fishes. Firstly, their commercial importance for either fishery exploitation or intensive aquaculture. Secondly, an understanding of the ontogeny of the GIT is essential for information on the abilities of the developing fish to cope with specific ingredients of commercial food and for a detailed comparison between species. Thirdly, it is believed that the limited success of intensive rearing of certain fish species is, at least partly, due to a poor knowledge of morphological and functional development of the GIT.

Compared with fishes, amphibians display some differences in their early development which may result from structural changes in yolk organization, accompanied with an increase in the size of the developing egg (Elinson 1987). These differences are probably due to the fact that minor phylogenetic differences in the original construction of the developing egg may have great influence on the later embryonic development (Gilbert and Raunio 1997). Amphibians are generally characterized by spontaneous metamorphosis which occurs between the latest larval stage and adulthood. In this regard, the axolotl is an exception because of its inability to metamorphose naturally into a terrestrial salamander; and



thus it remains neotenuous, i.e. keeping larval morphological features during its entire lifetime (Kuhn and Jacobs, 1989). Because of this neotenic character, the development of the axolotl GIT is anticipated to deviate from that of *Xenopus*.

Extensive investigations on the morphogenesis of the GIT in amphibians is largely based on studies of *Xenopus* as the principal amphibian model (Chalmers and Slack 1998; Wacker et al., 2000; Slack 2001), however, information for other amphibians, especially urodeles is limited and sporadic. Study of alternative species may provide valuable information on how much variation and similarity actually exist in the early development between different species within a given group. The present descriptive study on the histological development of the GIT of the axolotl, *Ambystoma mexicanum*, has thus been conducted on this basis. The previous staging studies on the axolotl did not include either a description of the GIT development or any related illustrations (Schreckenber and Jacobson 1975; Bordzilovskaya et al., 1989). Furthermore, they ended at hatching (stage 44) and thus did not extend to the later post-hatching stages during which the major part of GIT development occurs. The lack of histological investigation of the axolotl GIT is probably due to the technical difficulties associated with sectioning early stages (Kurth et al., 2012). The main target of the present study was therefore to describe the histological development of the axolotl GIT.

MATERIALS AND METHODS

Animals and staging

Embryonic, larval and juvenile stages of the axolotl, *Ambystoma mexicanum*, were obtained from natural spawning at the Biomedical Services Unit (BMSU), School of Biosciences, Birmingham, UK. Principles of animal care and use as regulated by the BMSU were followed. Animals were reared in tanks aerated by running dechlorinated tap water at a temperature of $19\pm 1^\circ\text{C}$ under alternate 12 h periods of light and darkness. Considering the objective of the study, the sampling started from mid blastula according to the criteria of Bordzilovskaya et al., (1989) onwards. The de-jellying of the early embryos was performed in urodele saline (0.8% NaCl) following the procedure of Asashima et al. (1989). While de-jellying of the early stages up to the late gastrula (stage 12 ½) was feasible, it was difficult to free the post-gastrula stages up to stage 33 from the jelly coats. The latter stages were therefore de-jelled during gastrulation and kept in dechlorinated tap water in Petri dishes until reaching the desired stage for fixation. However, stages from 34 up to 43 were de-jelled without difficulty immediately before fixation.

Because the normal table of the axolotl development end at hatching (Schreckenber and Jacobson 1975; Bordzilovskaya et al., 1989), both time and total body length were considered for determining the post-hatching stages. These included 1, 3 & 6 (external feeding stage) days after hatching; 1.5 cm long (aged 12 days after hatching), 2.5 cm long (aged 24 days after hatching) larvae and 12 cm long (aged 158 days after hatching) juveniles. Six days after hatching, the larvae were fed on *Daphnia* and then switched to fresh chopped and intact *Tubifex*. Prior to fixation, animals were anaesthetized with 0.01% tricaine methanesulphonate (MS222). Individuals which matched the desired developmental stage for fixation were killed with long immersion in the anaesthesia. For both 1.5 and 2.5 cm long larvae only the trunk region was processed in order to ensure better fixation. Whenever necessary, lengths of the early investigated specimens were measured, after fixation and transferring to 70% ethanol, using eye piece micrometer fitted in a dissecting binocular microscope and were given as mean value \pm standard error of the mean.

Fixation, embedding and sectioning

Early intact specimens and the trunk region of 1.5 and 2.5 cm long larvae were fixed *in toto* in three different fixatives, namely, neutral buffer formalin, Altman fixative and Bouin's fluid. The time of fixation varied according to the fixative used, being 24 h for the first and 2-4 h for the other two at room temperature depending on the size of the specimen. In case of the juvenile stage, the GITs were excised and tissue samples were taken from the stomach, small and large intestines and fixed in either Bouin's fluid or neutral buffer formalin for a period of 24 h at room temperature. After fixation and washing in 70% ethanol, the specimens were then transferred to 80% ethanol, dehydrated and embedded according to Badawy et al. 2012.

Further processing, investigation and photographing

Sections were dried overnight, de-waxed, re-hydrated, and stained conventionally with either haematoxylin and eosin (H&E) or Mallory triple stain (MTS) and eventually mounted in DPX using standard histological techniques (Bancroft and Gamble, 2005). Some histological sections were photographed using Olympus microscope.

Morphometry

Different morphometric parameters including diameter, wall thickness and height of mucosal folds of the sectioned GIT were measured using image analysis system. The slides were placed under a Leitz Ortholux II microscope on which was mounted a standard 625 line monochrome TV camera connected to a Magiscan (Joyce-Loebl) image analysis system.



Statistical analysis

All data sets were expressed as mean \pm standard error of the mean (SEM). The data were analyzed statistically for normal distribution (student's t test) and homogeneity of variances (Levene test) using statistical program of social sciences (SPSS) software for windows, version 11. Differences were considered significant at $P < 0.05$. (P had two values i.e. < 0.005 for all except in case of the wall thickness where $P < 0.05$ at the developmental stages aged 6 and 12 after hatching.

RESULTS

A- Histological investigation

The present descriptive investigation for the GIT of the axolotl started at mid-blastula stage and ended with a well developed juvenile stage. Figures 1-4 illustrate the histogenesis of the axolotl GIT at different stages of development. Early morphogenetic movements resulted in rapid changes in the organization of the blastoderm. Thus, unlike its thick shape in mid blastula stage (Fig. 1A); the blastoderm was evidently reduced in thickness at late blastula stage (Fig. 1B). In both stages, the blastoderm was a multi-layered sheet three to four irregular cell layers in depth but the tightness of the cells differed, being more closely applied in the late blastula stage. After the thinning of the blastoderm, its cells began to spread in order to perform the morphogenetic movements of epiboly. As a result, the vegetal hemisphere started to be covered with animal cells and the inner yolk vegetal cells were shifted upwards and began to take up a new, deeper position thus initiating the cell movements leading to invagination. The latter consisted of a series of cellular movements which occurred simultaneously in a regulated manner as can be seen at the early gastrula stage where the blastopore lip is located closer to the vegetal pole (Fig. 1C). Therefore, epiboly seemed to start first, then the two morphogenetic movements showed a co-ordinated succession towards the mid gastrula stage (Fig. 1D). At late gastrula stage, while invagination was occurring at the blastopore lips, the ectodermal cells were expanding over the entire embryo. These co-ordinated cellular movements ended with a construction where the ectoderm (E) constituted the outermost layer, the archenteron (Ar) was bounded by the endodermal yolk mass from the bottom and the two lateral sides while the archenteron roof was free of any endodermal cells (Fig. 1E). The animal pole cells seemed to exhibit more active movement than the vegetal cells. The endodermal and mesodermal cells were undistinguishable morphologically; however, there was a slight border between the ectoderm (E) and the underlying mesoderm.

At the mid neurula stage (Fig. 1F); one can distinguish the outermost epidermal layer, the population of spreading cells which is the precursor of the somatogenic mesoderm (Smc), lateral plate mesoderm (Lpm) and the ventral mesoderm (vm). The notochordal cells (Ntc) were flattened and form the archenteron roof. The latter was evidently free of endodermal cells and only the notochordal cells were located between the archenteron and neural plate. Laterally to the neural folds at the junction between the neuroectoderm and the ectoderm, a band composed of specific neuroectodermal cells was seen; these are the neural crest cells (Nc) which can be distinguished from the neighboring cells on the basis of their different morphology.

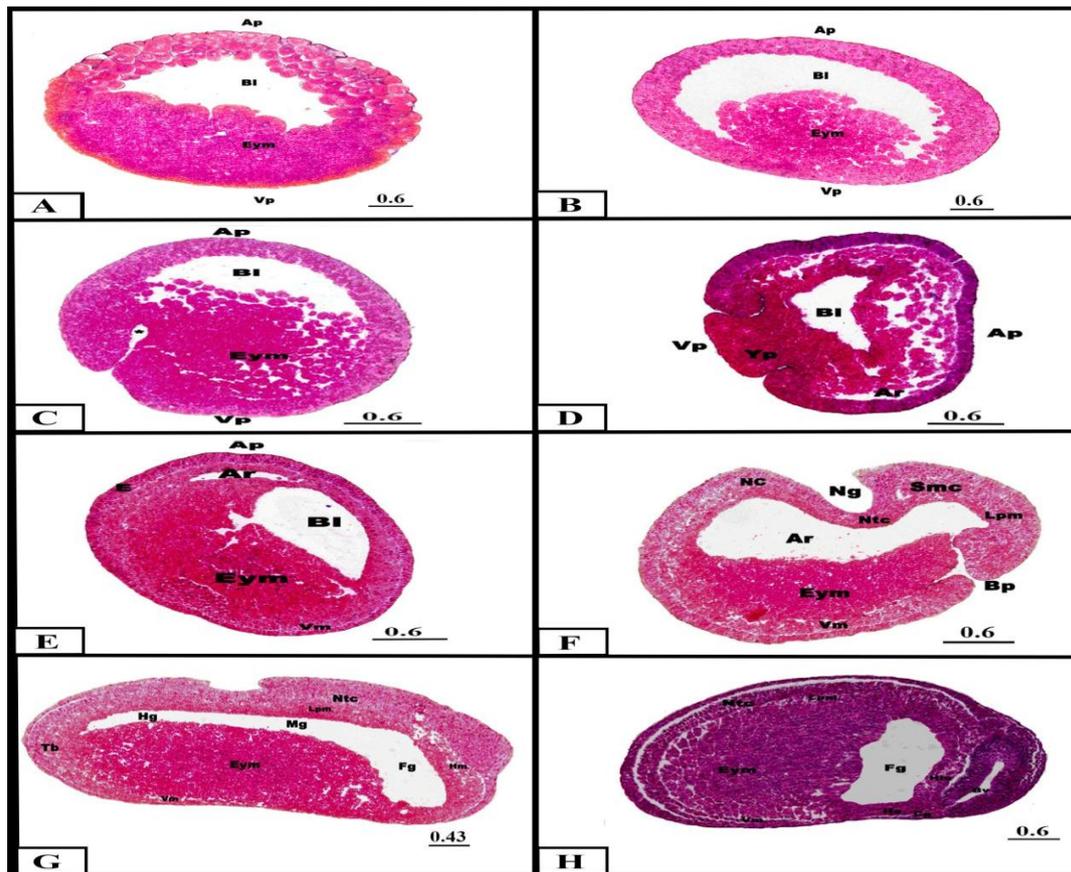


Fig. 1: **A:** A photomicrograph of a transverse section through the mid blastula (2-3 h after stage 8). **B:** A photomicrograph of a transverse section through the late blastula (stage 9). **C:** A photomicrograph of a transverse section through the early gastrula (stage 10½). The blastopore leads to the prospective archenteron (indicated by asterisk). **D:** A photomicrograph of a transverse section through the mid gastrula (stage 11). **E:** A photomicrograph of a transverse section through the late gastrula (stage 12½). The yolk plug, not shown in the section, reached its minimum size. **F:** A photomicrograph of a transverse section through the mid neurula (stage 13). **G:** A photomicrograph of a sagittal section through stage 16. **H:** A photomicrograph of a sagittal section through stage 20. All stained with MTS except H with H&E. Scale bars = mm.

At stage 16, the embryo exhibited the first significant elongation which was accompanied with the first indication of the subdivision of the archenteron into its three parts. These are the anterior expanded foregut (Fg) with wider diameter than the rest of the presumptive GIT, the narrow midgut (Mg) situated dorsal to the yolk mass and the posterior hindgut (Hg) (Fig. 1G). The successive tail bud stages up to the developmental stage 29 displayed the same primitive condition seen at stage 16 (Figs. 1H & 2A). However, at the developmental stage 30 (Fig. 2B), more advanced features emerged by the subdivision of foregut into two main pocket like structures. These were the anterior oral invagination (Oe) which made its first appearance at stage 20 and the posterior liver diverticulum (Ld) as a precursor for both the liver and pancreas. Developmental stages from 31 to 37 showed very little changes in terms of the histological structure of the developing GIT. Cement gland (Cg) which enables substrate-spawning species to attach themselves to the substrate appeared at stage 20 and degenerated by stage 38, presumably because it had no function.

Stage 38 witnessed very active developmental changes in terms of the GIT histogenesis (Fig. 2C). The migration of the mesodermal cells occurred gradually and progressively in a PD direction. An extensive elongation of the entire body especially in the anterior region was evident. Thus, the average body length at stage 37 was 51.1 ± 1.76 mm, while at stage 38 it reached 63.3 ± 2.42 mm. This significant elongation resulted in a general reduction in the overall diameter of the GIT cavity. The most anterior region developed into the pharyngeal cavity as the ventral epithelium expanded laterally into pouches, which constituted the pharyngeal external gills (G). In the foregut region, the presumptive stomach (Pst) started to be distinguished concurrently with the appearance of the presumptive liver (Plv). The active migration of the mesodermal cells (M) in the trunk region of the embryo made the gastric region distinguishable from the intestinal one. These morphogenetic changes were accompanied with the migration of the splanchnic mesoderm (M) both dorsally and ventrally of the embryo. From stage 38 onwards, the mutual interaction between the mesoderm and the endoderm was evident and expressed by their concomitant cellular behavior.

At stage 40 (with total body length 71.05 ± 1.92 mm) (Fig. 2D), in a preliminary step towards the formation of the mouth, the archenteron expanded anteriorly into the head region in order to make contact with the ectoderm. Meanwhile, a very active cellular movement in the gastric region was underway. The mesodermal cells (M) from both dorsal and ventral sides started to migrate towards the longitudinal axis of the GIT until they met at the middle. This in turn led to the anatomical differentiation of the stomach from the intestine. In the presumptive intestinal region, the migration of the mesodermal cells (M) continued to occur in a PD direction. At stage 43 (with total body length 81.9 ± 2.47 mm) (Fig. 2E), a mesodermal layer of cells can be seen around the presumptive large intestine which was, unlike the other GIT parts, characterized by the presence of dense endodermal yolk.

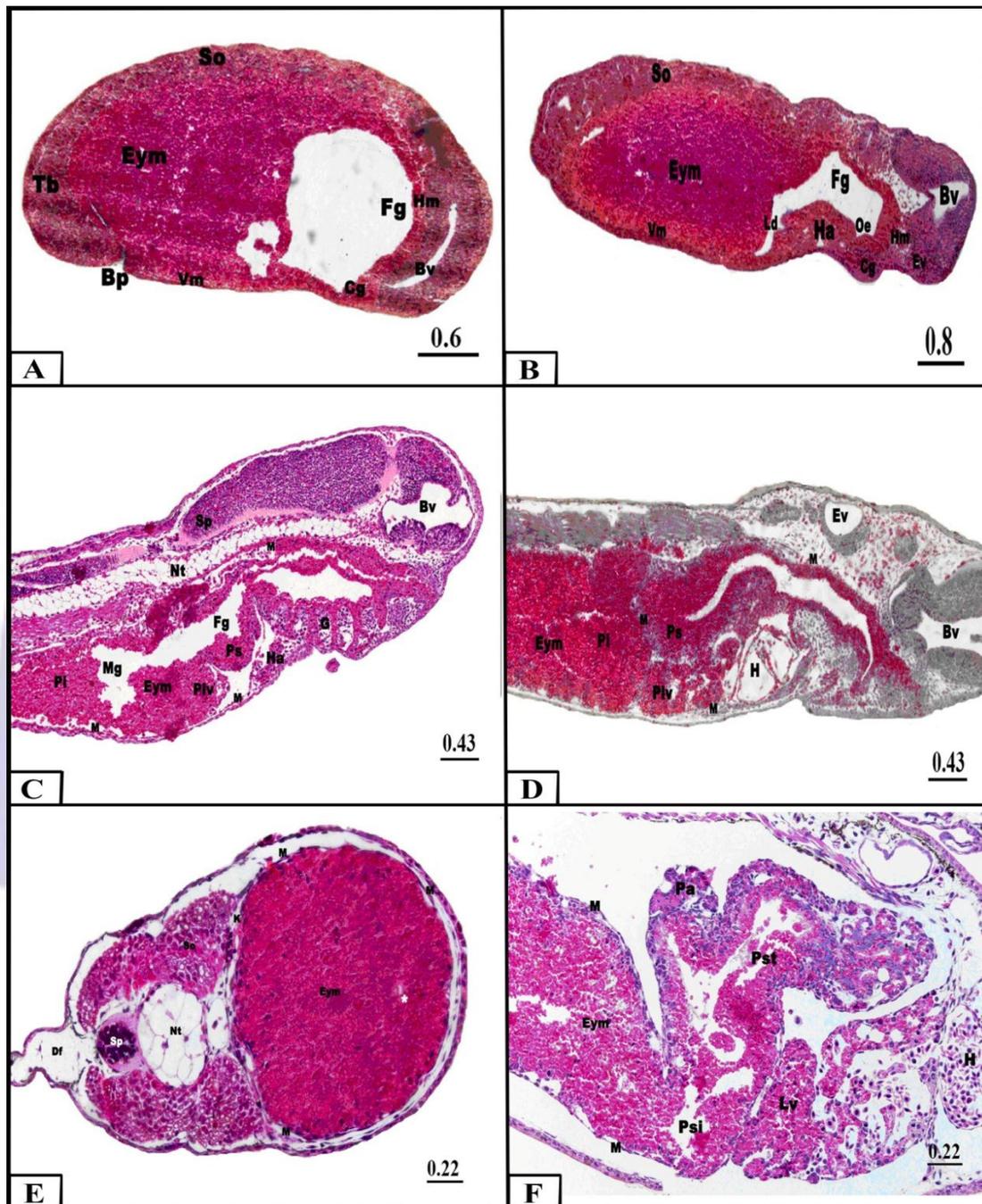


Fig. 2: **A:** A photomicrograph of a sagittal section through stage 23. **B:** A photomicrograph of a sagittal section through stage 30. **C:** A photomicrograph of a sagittal section through the proximal region of stage 38. **D:** A photomicrograph of a sagittal section through the proximal region of stage 40. **E:** A photomicrograph of a transverse section through the distal region of the large intestine of stage 43. The intestinal lumen is indicated by an asterisk. The active migration of the mesodermal cells (M) was accompanied with the progressive differentiation of the epithelial cells. **F:** A photomicrograph of a sagittal section through the trunk region of the late hatching stage (44). A, B & D stained with MTS while C&F stained with H&E. Scale bars = mm.



The hatching stage (44) (with total body length 87.65 ± 6.16 mm) was characterized by having the mouth open and the very early decline in the endodermal yolk mass (Eym) progressively in a PD direction. As can be seen in Figs. (2F, 3A), although the GIT was poorly differentiated at this stage, it exhibited marked histological progress. Liver (Lv) and pancreas (Pa) were not yet differentiated into distinct organs. However, the GIT itself showed two main developmental changes. First, the yolk mass (Eym) showed clear depletion in the gastric region and as a result, the presumptive stomach (Pst) with its new lumen was considerably expanded. Second, there were a gradual arrangement of the endodermal epithelial cells (EC) and concurrent migration of the mesoderm (M) in a PD direction in order to establish the wall of the developing GIT. Less histogenesis can be seen posteriorly compared to the proximal region (Fig. 3A).

One day after hatching (with total body length 12 mm) (Fig. 3B), the subsequent cellular differentiation of the developing GIT was accompanied with the progressive intercellular consumption of the yolk in a PD direction. Meanwhile, there was a continuous histogenesis along the developing GIT in the same direction. While the intestinal wall was flat at this stage, some mucosal folds started to appear in the gastric wall as a result of the rapid epithelial cell proliferation. The latter went concurrently with the migration of the mesodermal cells which differentiated to loose connective tissue (Ct) under the gastric epithelial cells. This connective tissue made up the fold axis and the thin layer that surrounded the epithelium. The latter showed more differentiated state in the gastric than in the intestinal portion of the GIT and therefore displayed a PD transition into a simple columnar epithelium.

Three days after hatching (with total body length 12 mm) (Fig. 3C), the two processes of cell proliferation and the underlying connective tissue accumulation went concurrently with each other in a PD direction. While the epithelial cells were proliferated rapidly to form the mucosal folds (Mf), there was a concurrent change in the mesenchymal cells underneath. The connective tissue (Ct) started to enter the thickened points of the epithelium as a sign of intestinal mucosal folds formation. Meanwhile, the intestinal epithelial cells started to show the columnar shape. Nests of intestinal proliferative cells were seen in this stage. The mucosal folding can also be seen in the intestinal region of this stage owing to the active epithelial proliferation.

Six days after hatching (with total body length 12 mm) (Fig. 3D), active myogenesis have been seen at higher magnification especially at the gastric region. Furthermore, the mesenchymal cells showed more PD differentiation and segregation into two major distinct areas. These were cells found at the periphery of the mesenchyme became oriented circularly giving rise to the muscle layers through myogenesis and a subset of mesenchymal cells became close to the epithelium and placed parallel to it giving rise to the connective tissue. The yolk mass showed a complete absorption anteriorly at the gastric region as the larvae started feeding just after this stage.

At 1.5 cm long larval stage (aged 12 days after hatching), due to continuous epithelial cell proliferation, the gastric as well as the anterior intestinal mucosal folds increased in number and modified into zigzag pattern (Fig. 3E). The invagination of the mesodermal cells into the mucosal folds was clear at this stage and resulted in the construction of the lamina propria submucosa (Lps). However, in the large intestinal region the number of folds was very few compared to that of the small intestine. The underlying connective tissue layer, more developed than in the previous stage, was surrounded by a circular layer of striated muscle cells and a longitudinal muscle layer in the gastric and small intestinal regions. The circular muscle layer exhibited earlier differentiation than the longitudinal one and both can be clearly observed in the gastric region. The larvae of this stage had started feeding and therefore yolk was evidently absent.

At 2.5 cm long feeding stage (aged 24 days after hatching); the GIT was well-developed with the advanced differentiation of the whole intestine which, to a large degree, reached its definitive morphology and structure. As can be seen in Fig. (3F), the gastric and intestinal folds increased in number due to epithelial proliferation. The spaces between the mucosal folds underwent reshaping to form crypts by the penetration of the epithelial cells into the underlying connective tissue. The intestine showed many mucosal folds which gradually decreased in height in a PD direction. The posterior end of the large intestine was consisted of somewhat straight part with a thickened epithelial wall. The connective and muscular tissues were well established at this stage.

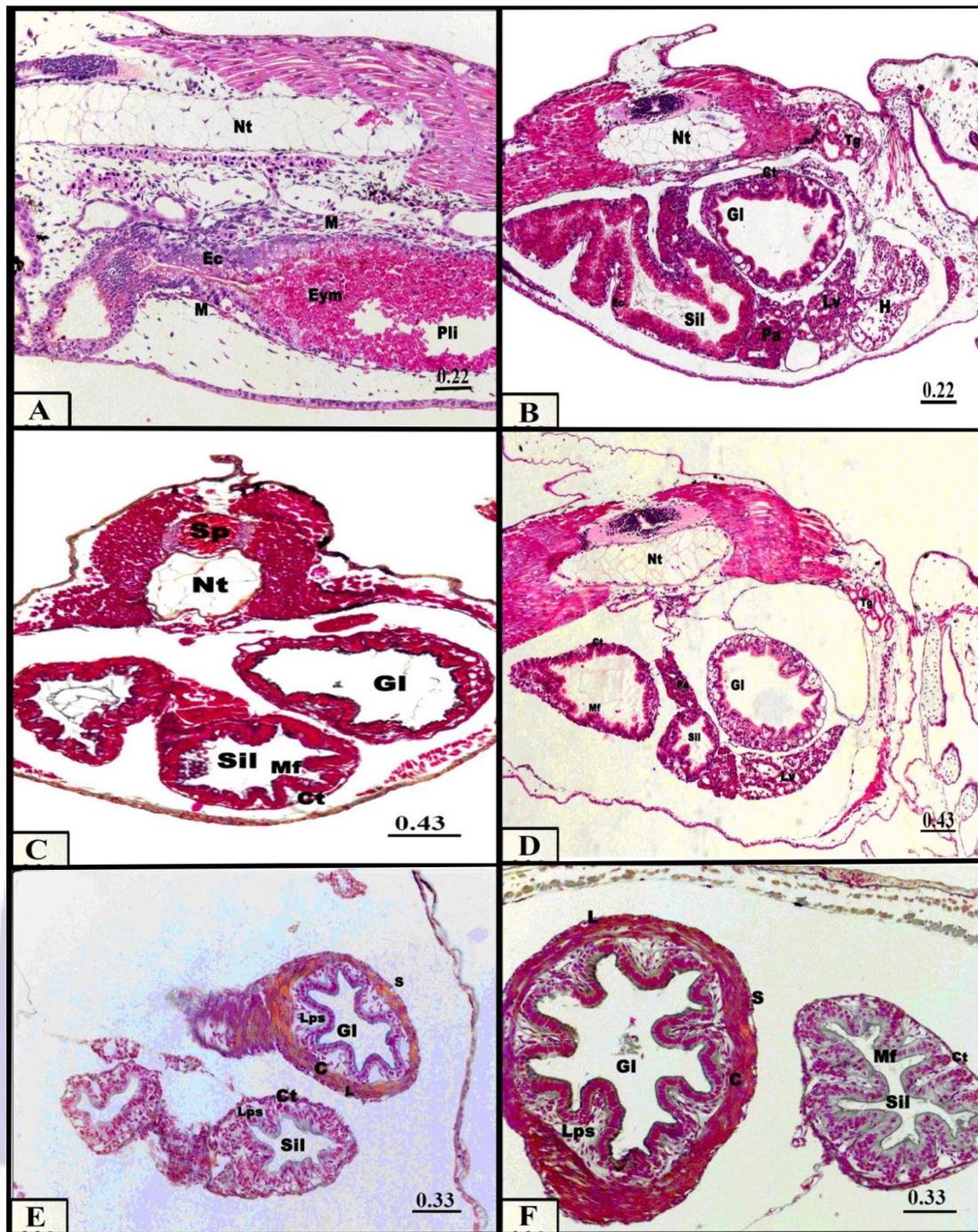


Fig. 3: **A:** A photomicrograph of a sagittal section through the distal region of the late hatching stage (44). The empty site within the yolk mass is an artefact resulted from the sectioning process. **B:** A photomicrograph of a sagittal section through the trunk region one-day after hatching. **C:** A photomicrograph of a transverse section through the trunk region three days after hatching. **D:** A photomicrograph of a transverse section through the trunk region six days after hatching. **E:** A photomicrograph of a transverse section through the trunk region of 1.5 cm long larva. **F:** A photomicrograph of a transverse section through the trunk region of 2.5 cm long larva. All stained with MTS except A with H&E. Scale bars = mm.

As illustrated in Figs. (4A, B & C), the fully developed histological structure of the juvenile axolotl GIT follows the same general pattern as that of other vertebrates. It, therefore, exhibits a 4-layers structure:

- (1) the innermost layer is the mucosa (Mc), composed of the lining epithelial, and the adjacent overlying mesoderm, a lamina propria of loose connective tissue rich in blood and lymph vessels. Under the mucosa, the muscularis mucosa (Mm) consists of a thin inner circular and an outer longitudinal layer of smooth muscle cells separating the mucosa from the submucosa. The muscularis mucosa promotes the movement of the mucosa independent of the other movements of the GIT, thus increasing its contact with the food. It is located in both the stomach and the small

intestine but more evident in the stomach (Fig. 4A). The gastric mucosal folds are inter-connected and branched in a complex way (Fig. 4A). Unlike the large intestinal folds, the small intestinal ones are longer and seem to occupy most of the lumen area (Fig. 4B). The large intestinal mucosa has a large number of goblet cells (Fig. 4C) which are interspersed between the absorptive intestinal cells;

- (2) the next layer is the submucosa (Sm) characterizes by the occurrence of both loose connective and vascular tissues in the lamina propria submucosa (Lps). The submucosa accommodated blood vessels and lymphatic vessels in addition to the other nervous components which cannot be seen with the utilized stain;
- (3) the next layer is the muscularis layer composed of smooth muscle cells which are spirally oriented and divided into two sublayers according to the main direction of the muscle cells. In the internal sub layer, which is close to the lumen, the orientation is generally circular giving rise to the circular muscle layer (C), while it is mostly longitudinal in the external sub layer constituting the longitudinal muscle layer (L). While the blood and lymph vessels can also be seen in the connective tissue between the muscle sub layer at a higher magnification, the other nervous elements are invisible;
- (4) the outermost layer is the serosa which appears as a very thin single layer of epithelial squamous cells continuous with the mesenteries supporting the GIT.

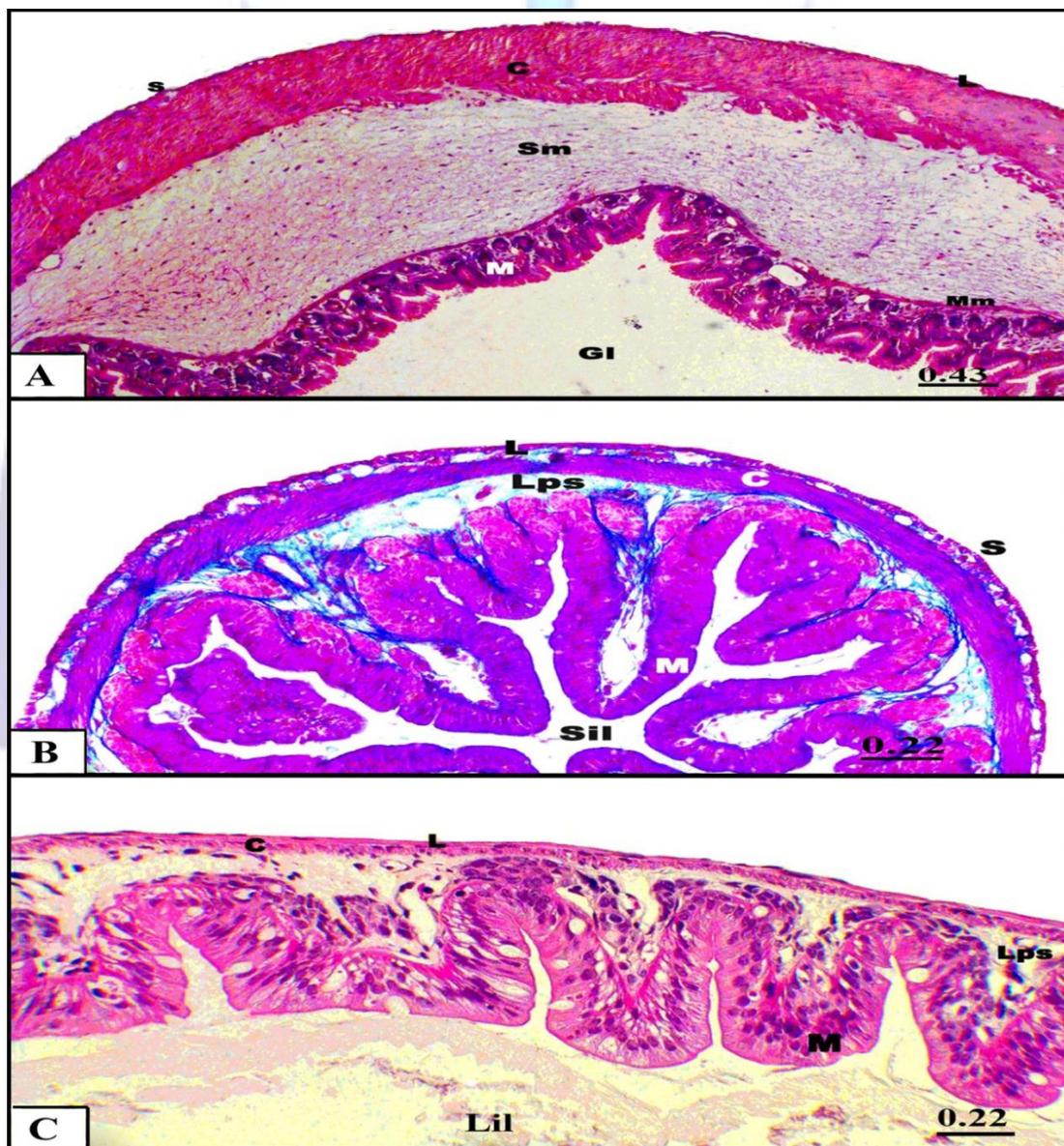


Fig. 4: **A:** A photomicrograph of a part of transverse section through the pyloric stomach of the juvenile stage. **B:** A photomicrograph of a major part of transverse section through the small intestine of the juvenile stage. **C:** A magnified part of transverse section through the large intestine of the juvenile stage. MTS. Scale bars = mm.

B- Morphometric parameters

B.1. The diameter of the GIT

Both gastric and intestinal diameters exhibited a highly significant but gradual increase with the advancement of the developmental stage (Fig. 5A).

B.2. The wall thickness of the GIT

The GIT of the investigated developmental stages displayed a progressive increase in its wall thickness (Fig. 5B). This increase was gradual until 12 days after hatching, and then was moderate at 24 days after hatching after which the increase was dramatically evident at 158 days after hatching i.e. juvenile stage. P values were highly significant (<0.005) except after 6 and 12 days (<0.05) where P values were moderately significant.

B.3. The height of Mucosal folds

In a similar trend as the increase in the wall thickness, the height of mucosal folds displayed a progressive but slowly gradual increase until 24 days after hatching, after which the height of mucosal folds displayed a dramatic increase at the juvenile stage.

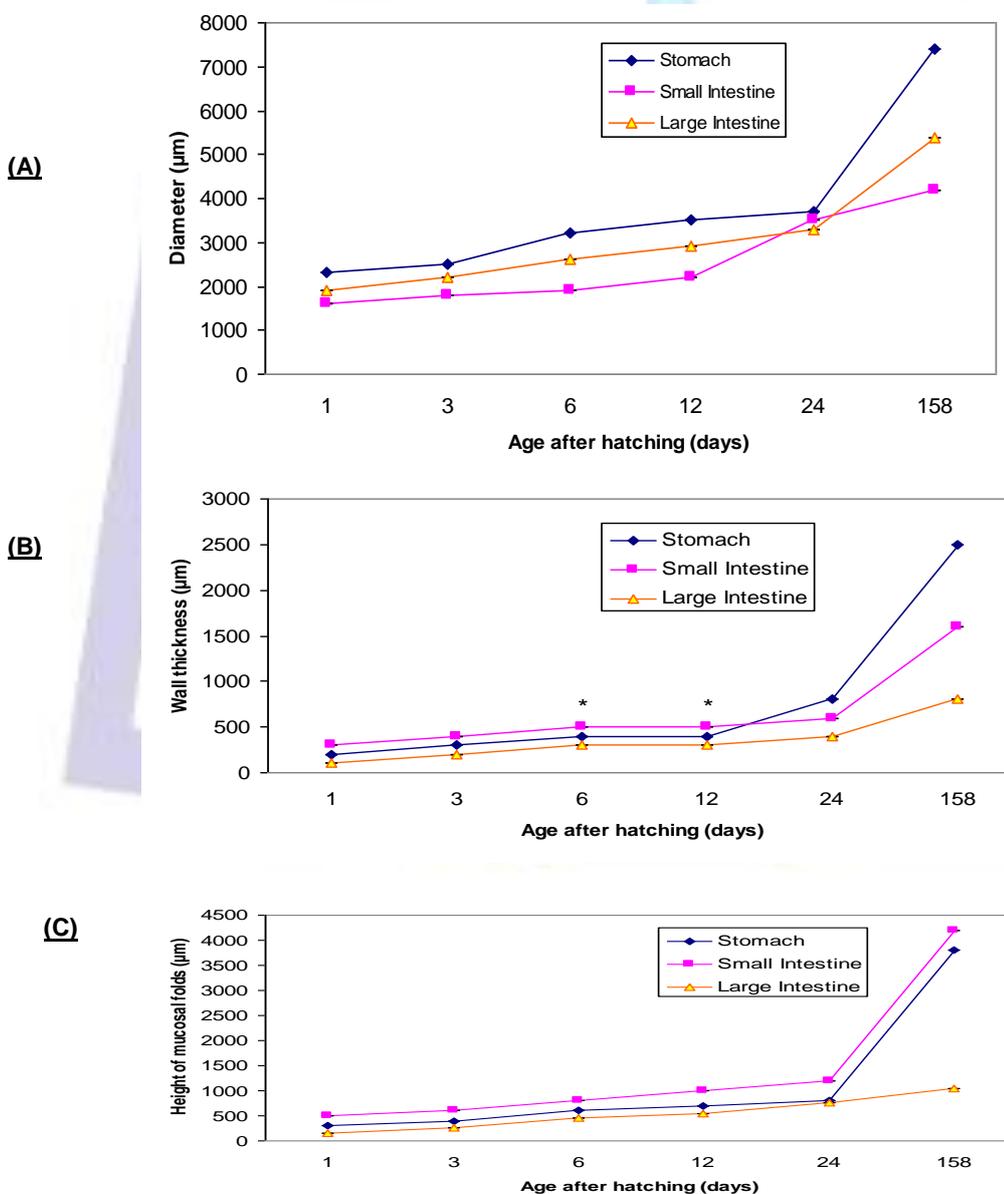


Fig. 5: Graphs showing developmental changes in different morphometric parameters in six of the investigated stages. (P had two values, P for all <0.005 except * where P <0.05).



- A- Developmental relationship between age and GIT diameter.
- B- Developmental relationship between age and GIT wall thickness.
- C- Developmental relationship between age and GIT height of mucosal folds.

DISCUSSION

Histological studies at different stages of development provide useful descriptive information on how precisely regulated cell division results in the construction of different body organs. Taking the axolotl GIT as a paradigm, the present study describes the histological structure of the developing GIT. Before the start of exogenous feeding, the developing GIT of most fish and amphibians is not fully differentiated (Sarasquete et al., 1995). However, they must bring their GIT to a differentiated and functional state by the time they start the external feeding even if its anatomical structure is not fully developed (Ribeiro et al., 1999). During the endogenous feeding phase of fishes, gut differentiation proceeds in a distal-proximal direction owing to the important role of the intestinal portion in the digestion of yolk which in turn contributes to the early differentiation of the intestinal epithelium compared to the gastric one (Gisbert et al., 1998). This is in contrast to the present observation which agrees with previous observation in anurans in which the differentiation of the GIT follows the PD direction (Hourdry et al., 1996). It was also evident from the present investigation that the yolk mass was utilized in differentiation and organogenesis rather than in growth as during the endogenous feeding phase, larvae aged one and six days after hatching had the same body length.

The development of the GIT results from the association of intrinsic genetic factors, endogenous regulatory mechanisms and environmental influences. During the early histological development, the vertebrate GIT is established as an endodermal structure surrounded by mesenchyme. In *Xenopus* tadpole, it is composed of an outer smooth muscle layer derived from the mesoderm, an inner epithelial layer derived from the endoderm and a connective tissue in between (Chalmers and Slack 1998). Since the pioneer work of Yasugi and Mizuno (1990) it has been well established that the morphogenetic processes within the vertebrate GIT are dependent on interactions and mutual co-operation between the epithelium and mesenchyme. It has been reported that patterning of the gut involves reciprocal inductive signalling between the endoderm and mesoderm which plays a permissive role in the morphogenesis and cytodifferentiation of the gut endoderm (Yasugi 1993; Wells and Melton 1999, Zorn and Wells 2009). In anurans, the endoderm acquired a latent ability to undergo a limited degree of differentiation on its own in the absence of the mesoderm but for full morphogenesis and cytodifferentiation interaction with the mesenchyme is required (Henry et al., 1996). More recently, Horb and Slack (2001) examined the role of mesodermal endodermal interactions during gut formation in *Xenopus* using explants of mesoderm and endoderm from early developmental stages. Utilizing endodermal and mesodermal molecular markers, their investigation revealed that endoderm formation is autonomous but regional specification and subsequent differentiation occur only when there is concurrent formation of mesoderm. Equally similar, the differentiation of the mesoderm into smooth muscle is dependent on signals from the adjacent endoderm (Grapin-Botton and Melton 2000). The present morphogenetic movements displayed by both the mesoderm and endoderm during the histogenesis of the axolotl GIT suggested a probable reciprocal relationship between the two components. The stratified endoderm formed of undifferentiated epithelial cells underwent a gradual PD differentiation and at the same time the mesodermal cells exhibited a concurrent spontaneous arrangement underneath the epithelium in order to establish the GIT wall. The current descriptive study illustrated the early histogenesis of the axolotl GIT from as early as the mid blastula stage. It elucidated the dual origin of the GIT i.e. from both the endoderm and mesoderm apart from the neuroectodermal participation. The two components displayed associated and concurrent differentiation which went in a clear PD direction until a well-developed GIT was established. The manner of behaviour exhibited by the endodermal and mesodermal cells during the histogenesis of the GIT provided indirect evidence for their mutual interaction. The prominent feature of the axolotl GIT is its simple tubular shape and gradual development unlike the highly coiled nature of the developing *Xenopus* gut. Finally, the study showed that the fully developed GIT of the axolotl has no particular characteristic feature which could be seen as a deviation from the general histological pattern of other vertebrate species.

The histological structure of the developing GIT of the axolotl if compared with that of anurans may be better understood in the context of the life history (Badawy et al., 2012). In contrast to *Xenopus* which has accurate timetable of developmental stages until the completion of metamorphosis at stage 66 (Nieuwkoop and Faber 1994), studies which determine the axolotl developmental stages did not exceed the hatching stage (44) (Schreckenber and Jacobson 1975; Bordzilovskaya et al., 1989). This is mainly because there are no clear-cut morphological criteria upon which the staging can depend. As the result of the absence of metamorphic events and therefore the lack of switching the feeding habit in the axolotl, the development of the GIT exhibited a gradual nature with no recognizable separations and consequently cannot be phased in contrast to *Xenopus*. However, in agreement with the studies conducted on *Xenopus*, the inner circular layer was always thicker and developed earlier than the outer longitudinal one. Also the increase in thickness of the muscle layers along the GIT during this period of development was mainly due to that of the circular muscle layer.



The morphogenesis of the GIT in the spontaneously metamorphosed salamander, *Ambystoma maculatum* has been previously investigated (Harris 1967). Beside its morphogenetic parameters uniqueness, the present investigation showed two main points of similarities compared with that of Harris (1967). First, the embryo elongated and showed a dramatic cellular activity at the same developmental stage (i.e. 38). Second, the differentiation of the GIT occurred in a clear PD direction. However, beside the main difference in the nature of development in terms of metamorphosis between the two species, i.e. *Ambystoma maculatum* and *Ambystoma mexicanum*, the present study covered much wider range of developmental stages compared to the study of Harris (1967).

The fully developed GIT of the axolotl showed a general histological pattern similar to that of other vertebrate species: an arrangement of the smooth muscle cells in an outer comparatively thin layer of longitudinally oriented cells and a thicker inner layer of circularly oriented cells which seem to be common for all vertebrates. Smooth muscle cells also form a muscle layer in the submucosa with a thickness which varies according to the species. This muscularis mucosa, which was clearly detectable in the stomach of the axolotl, is mainly involved in the control of the surface area for absorption over the mucosa. In contrast to this finding, species variations do occur in the GIT of certain animals, i.e. the presence of an inner incomplete third muscle layer of oblique orientation in the mammalian stomach (Kelly 1981) and of two longitudinal layers separated by a ganglionated plexus in the GIT of the spiny dogfish, *Squalus acanthias*, and (Holmgren and Nilsson 1983).

Among the metamorphic transformations in anurans is the extensive remodeling of the intestine, which involves specific degeneration of larval tadpole tissues and concurrent development of adult ones (Badawy et al., 2012). The period of intestinal remodeling in *anurans* is accompanied the ontogenetic change from herbivorous to carnivorous feeding habits (Tolosa and Diamond 1990) and characterized by a complete stopping of external feeding but this is followed by a natural carnivorous feeding. In the axolotl, metamorphic induction renders the animal to stop feeding after an average period of ten days from the beginning of thyroid hormone treatment until exhibiting the signs of morphologically complete metamorphosis (own observations). Thereafter, if survives, the animal cannot feed naturally and therefore, must be fed by force. No study to date has described the possible histological change, if any, in the intestine of the axolotl during this critical period of transformation. In conclusion, the present study represents a histological guide to the normally developing GIT in the axolotl, *Ambystoma mexicanum*.

ACKNOWLEDGMENTS

I thank Professors Manfred Reinecke (Division of Neuroendocrinology, Institute of Anatomy, University of Zürich, SWITZERLAND), Ted Taylor (School of Biosciences, University of Birmingham, UK), Tobias Wang (Department of Zoophysiology, University of Aarhus, DENMARK).

REFERENCES

- [1] Arendt D, Nubler-Jung K (1999) Rearranging gastrulation in the name of yolk: evolution of gastrulation in yolk-rich amniote eggs. *Mech Dev*, 81: 3-22.
- [2] Asashima M, Malacinski G, Smith S (1989) Surgical manipulation of embryos. In: Armstrong J, Malacinski GM (eds) *Developmental biology of the axolotl*. Oxford Univ. Press, New York/ Oxford, pp 255-263.
- [3] Badawy G, Sakr S, El-Borm H (2012): Morphological and histological remodeling of the gastrointestinal tract of the toad *Bufo regularis* during ontogeny. *Egypt. J. Exp. Biol. (Zool.)*, 8: 67–81.
- [4] Bancroft J, Gamble M. (2005) *Theory and Practice of Histological Techniques*. Philadelphia, Churchill Livingstone.
- [5] Bordzilovskaya N, Dettlaff T, Duhon S, Malacinski G (1989) Developmental-stage series of axolotl embryo. In: Armstrong J, Malacinski GM (eds) *Developmental biology of the axolotl*. Oxford Univ. Press, New York/ Oxford, pp 201-219.
- [6] Chalmers A, Slack J (1998) Development of the gut in *Xenopus laevis*. *Dev Dyn*, 212: 509-521.
- [7] Elinson R (1987) Change in developmental patterns: embryos of amphibians with large eggs. In: Raff R, Raff E (eds) *Development as an evolutionary process*. Alan R Liss, New York, pp 1-21.
- [8] Gilbert S, Raunio A (1997) *Embryology: Constructing the organism*. Sinauer Ass, Sunderland, MA.
- [9] Gisbert E, Rodriguez A, Castello-Orvay F, Williot P (1998) A histological study of the development of the digestive tract of Siberian sturgeon (*Acipenser baeri*) during early ontogeny. *Aquaculture*, 167: 195-209.
- [10] Grapin-Botton A, Melton D (2000) Endoderm development, from patterning to organogenesis. *Trends Genetics*, 16: 124-130.
- [11] Harris T (1967) The morphogenesis of the stomach and intestine in the salamander *Ambystoma maculatum*. *J Morph*, 122: 345-366.
- [12] Henry G, Rivanlou I, Kessler D, Hemmati-Brivanlou A, Melton D (1996) TGF- β signals and a prepattern in *Xenopus laevis* endodermal development. *Development*, 122: 1007-1015.



- [13] Holmgren S, Nilsson S (1983) Bombesin-, gastrin/CCK-, 5-hydroxytryptamine-, neurotensin-, somatostatin-, and VIP-like immunoreactivity and catecholamine fluorescence in the gut of the elasmobranch, *Squalus acanthias*. *Cell Tissue Res*, 234: 595-618.
- [14] Horb M, Slack J (2001) Endoderm specification and differentiation in *Xenopus* embryos. *Dev Biol*, 13: 1-14.
- [15] Hourdry J, Hermite A, Ferrand R (1996) Changes in the digestive tract and feeding behaviour of anuran amphibians during metamorphosis. *Physiol Zool*, 96: 219-251.
- [16] Kelly K (1981) Motility of the stomach and gastroduodenal junction. In: Johnson L et al. (eds) *Physiology of the gastrointestinal tract*. Raven Press, New York, pp 393-410.
- [17] Kuhn E, Jacobs G (1989) Metamorphosis. In: Armstrong J, Malacinski G (eds) *Developmental biology of the axolotl*. Oxford Univ. Press, New York/ Oxford, pp 187-197.
- [18] Kupferberg S (1997) The role of larval diet in anuran metamorphosis. *Amer Zool*, 37: 146-159.
- [19] Kurth T, Weiche S, Vorkel D, Kretschmar S, Menge A (2012) Histology of plastic embedded amphibian embryos and larvae. *Genesis*, 50:235–250.
- [20] Montgomery R, Mulberg A, Grand R (1999) Development of the human gastrointestinal tract: Twenty years of progress. *Gastroenterology*, 116: 702-731.
- [21] Morrison C, Miyake T, Wright J (2001) Histological study of the development of the embryo and early larva of *Oreochromis niloticus* (Pisces: *Cichlidae*) *J Morphol*, 247: 172-195.
- [22] Nieuwkoop P, Faber J (1994) Normal table of *Xenopus laevis* (Daudin). A systematical and chronological survey of the development from the fertilized egg till the end of metamorphosis. Garland Publishing. Inc New York, London.
- [23] Ribeiero L, Sarasquete C, Dinis M (1999) Histological and histochemical development of the digestive system of *Solea senegalensis* larvae. *Aquaculture*, 171: 293-308.
- [24] Sakr S, Badawy G, El-Borm H (2014) Ultrastructural and Molecular Changes in the Developing Small Intestine of the Toad *Bufo regularis*. *The Scientific World Journal*, 2014: 1-13.
- [25] Sarasquete M, Polo A, Yufera M (1995) Histology and histochemistry of the development of the digestive system of larval gilthead sea bream, *Sparus aurata*. *Aquaculture*, 130: 79-92.
- [26] Schreckenberg G, Jacobson A (1975) Normal stages of development of the axolotl *Ambystoma mexicanum*. *Dev Biol*, 42: 391-400.
- [27] Senarat S, Yenchum W, Poolprasert P (2013) Histological study of the intestine of Stoliczkae's barb *Puntius stoliczkanus* (Day, 1871) (Cypriniformes: Cyprinidae) *Kasetsart J. (Nat. Sci.)* 47 : 247-251.
- [28] Slack J (2001) *Essential developmental biology*. Blackwell Science Ltd, Oxford.
- [29] Stevens C, Hume I (1995) *Comparative physiology of the vertebrate digestive system*. Cambridge Univ Press, Cambridge.
- [30] Toloza E, Diamond J (1990) Ontogenetic development of nutrient transports in bullfrog intestine. *Am J Physiol*, 258: G760-G769.
- [31] Veggetti A, Rowlerson A, Radaelli G, Arrighi S, Domeneghini C (1999) Post-hatching development of the gut and lateral muscle in the sole. *J Fish Biol*, 55: 44-65.
- [32] Wacker S, Grimm K, Joos T, Winklbauer S (2000) Development and control of tissue separation at gastrulation in *Xenopus*. *Dev Biol*, 224: 428-439.
- [33] Warga R, Kimmel C (1990) Cell movements during epiboly and gastrulation in zebrafish. *Development*, 108: 569-580.
- [34] Warga R, Nusslein-Volhard C (1999) Origin and development of the zebrafish endoderm. *Development*, 126: 827-838.
- [35] Wells J, Melton D (1999) Vertebrate endoderm development. *Annu Rev Cell Dev Biol*, 15: 393-410.
- [36] Yasugi S (1993) Role of epithelial-mesenchymal interactions in differentiation of epithelium of vertebrate digestive organs. *Dev Growth Differ*, 35: 1-9.
- [37] Yasugi S, Mizuno T (1990) Mesenchymal-epithelial interactions in the organogenesis of digestive tract. *Zool Sci*, 7: 159-170.
- [38] Zorn A, Wells J (2009) Vertebrate endoderm development and organ formation. *Annu Rev Cell Dev Biol*, 25: 221-251.