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A histological atlas of the tissues and organs of neotenic and metamorphosed axolotl

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ABSTRACT

Axolotl (*Ambystoma Mexicanum*) has been emerging as a promising model in stem cell and regeneration researches due to its exceptional regenerative capacity. Although it represents lifelong lasting neoteny, induction to metamorphosis with thyroid hormones (THs) treatment advances the utilization of Axolotl in various studies. It has been reported that amphibians undergo anatomical and histological remodeling during metamorphosis and this transformation is crucial for adaptation to terrestrial conditions. However, there is no comprehensive histological investigation regarding the morphological alterations of Axolotl organs and tissues throughout the metamorphosis. Here, we reveal the histological differences or resemblances between the neotenic and metamorphic axolotl tissues. In order to examine structural features and cellular organization of Axolotl organs, we performed *Hematoxylin & Eosin*, *Luxol-Fast blue*, *Masson's trichrome*, *Alcian blue*, *Orcein* and *Weigart's* staining. Stained samples from brain, gallbladder, heart, intestine, liver, lung, muscle, skin, spleen, stomach, tail, tongue and vessel were analyzed under the light microscope. Our findings contribute to the validation of the link between newly acquired functions and structural changes of tissues and organs as observed in tail, skin, gallbladder and spleen. We believe that this descriptive work provides new insights for a better histological understanding of both neotenic and metamorphic Axolotl tissues.

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1. Introduction

Metamorphosis term is used to define the innate process of amphibian transition from larval stage to adult form (Shi, 2000). This transformation provides an excellent model system to understand vertebrate organogenesis and remodeling of the organs. During and following this transformation, commonly observed phenotypical changes are anatomical and histological reconstitution of the organs as well as appendages to function properly in terrestrial life conditions. Regression, disappearing and/or remodeling of the existing organs as well as formation of new organs are the observed adjustments of metamorphosis (reviewed in (Brown and Cai, 2007)). For the description of changes at organ and system level, *Xenopus leavis* is the widely used organism among the amphibians (Burggren and Warburton, 2007; Colombo et al., 2015). Previous studies have demonstrated that from tadpole to adult frog transformation, most of the organs undergo remodeling such as skin (Yoshizato, 1996), lung (Dodd and Dodd, 1976) and liver (Atkinson et al., 1998). The external gills of the tadpoles, which are the primary site for respiration in aquatic environment, disappear at the end of the metamorphosis (Ishizuya-Oka et al., 2010). Bone marrow, functional limbs and glands in skin and stomach are the examples of newly formed cells, tissues and structures with metamorphosis. Timing and rate of this complex process is regulated by hormonal activity and several external factors such as temperature (Hayes et al., 1993), density of population (Semlitsch and Caldwell, 1982), threat of predator presence and food levels (Kupferberg et al., 1994). In terms of hormonal regulation, this remodeling cascade starts with production and secretion of thyroid hormones (THs). It has been found that, THs levels in amphibians are low at early larval stage and peak at metamorphic climax (Mondou and Kaltenbach, 1979).

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THs are formed by coupling of iodine with tyrosine residues and consequently condensation of two aromatic rings of the tyrosine (Hulbert, 2000; Nussey and Whitehead, 2013)). There are two active forms of THs; the thyroxine or T4 which has four iodine at aromatic rings (3, 5, 3′ and 5′ respectively) and T3, which has three iodine at aromatic rings (3, 5 and 3′). T4 hormone is the main hormone produced by thyroid gland in most of the species, and it is converted to T3 in peripheral organs (Nussey and Whitehead, 2013). Since T3 has almost 10 times higher affinity for its receptors than T4, it is accepted as biologically active TH (Hulbert, 2000). Secretion of THs to blood is pursued by uptaking into the cells. Once TH locates within a cell, it binds to its receptors called as thyroid hormone receptors (TRs) which are a sub class of nuclear receptor family proteins (Huang et al., 2010). In most of the vertebrates there are two paralogous of this gene; TRa and TRb (Escriva et al., 2002; Paris and Laudet, 2008). In the absence of TH, these receptors are suppressed by corepressor proteins and therefore target genes can not be transcribed. Whereas binding of TH to TRs brings about the conformational change of the TRs, and releasing of corepressor enhances binding of TRs to hormone response elements (HREs) on DNA by interacting with retinoid X receptor (RXR) (Kliewer et al., 1992). Binding to DNA triggers the recruitment of coactivator proteins, and hence, expression of the target genes in the presence of TH is achieved (Buchholz et al., 2006). Expression of the genes with TH is essential for remodeling of the organs during metamorphosis of amphibians, and according to microarray studies a large number of genes are differentially expressed with the increased TH activity (Das et al., 2006; Yen et al., 2003). Although TRs and RXR proteins are highly conserved among the vertebrates, TH induced gene expression profile remarkably differs between the animals (Bertrand et al., 2004). In spite of presence of conserved coactivator, corepressor and nuclear receptors between the amphibians and mammals (Furlow and Neff, 2006) limited overlap in physiological response to TH between these animals indicates specialized function of TH in amphibians.

Unlike the frogs, Axolotl represents larval characters beyond the larval stage, throughout its life. Inadequate conversion of T4 to T3, presence of inactive form of T3 (3,3′,5′- triiodothyronine) and expression of limited number of TRs contribute for lifelong lasting neoteny of Axolotl (Galton, 1992). Administration of T4 or T3 either by injection or immersion is sufficient to trigger the metamorphosis (Jacobs et al., 1988; Page and Voss, 2009). Weight loss, diminishment and disappearance of tail fin and gills and molting are the morphological signs for metamorphosis (Rosenkilde and Ussing, 1996). Availability of induction to metamorphosis offers the opportunity to utilize the metamorphosed Axolotl as a complementary system to Neotenic ones, since it is an accomplished model organism to study regeneration (Coots and Seifert, 2015; McCusker and Gardiner, 2011; Vincent et al., 2015), scarless wound healing (Denis et al., 2013; Seifert et al., 2012), cancer (Menger et al., 2010; Smith et al., 2000) and stem cells (Rodrigo Albors et al., 2015; Zielins et al., 2016). Particularly, remarkable regeneration capacity of this model holds a great promise to understand the molecular basis of regeneration and restoration considering its success in functional regeneration of the internal organs (Cosden-Decker et al., 2012), central nervous system (Amamoto et al., 2016; Maden et al., 2013; Zammit et al., 1993) and extremities (Kragl et al., 2009; Satoh et al., 2015) following the damage or amputation. Considering the evolutionary proximity between the amphibians and mammals, employment of Axolotl as a model system allows translation of acquired messages to Mammalians effectively. Although number of researches on Axolotl has been expanded, to our best knowledge, there is no extensive study to generate a histological map of its organs. Here, in this study we provide a histological atlas of Axolotl tissues and organs for both pre and post-metamorphic stages. All isolated tissues and organs were histologically analyzed for both neotenic and metamorphosed Axolotls. General and organ specific histological staining were performed to describe the similarities and variations between the pre and post-metamorphic animals. Our results demonstrate that remodeling of several organs is the primary source for adaptation to terrestrial life conditions. Disappearance of pre-existing and formation of new organs also maintains the sustainable survival in the new environmental conditions. In addition to revealing the histological differences and resemblances between pre and post-metamorphic Axolotls, this study serves as a general reference for histological information to use in further studies. It is well known that histological documentation of tissues and organs is tremendously useful to follow up the effects of any treatments at tissue and organ level. Therefore, we certainly believe that this reference map will be very beneficial and be widely used in Axolotl researches.

2. Materials methods

2.1. Ethical statement

Animal care and experimental procedures were approved by the Animal Research Ethics Committee of the Istanbul Medipol University (authorization number 38828770-E.2302) and the research was performed in accordance with the European Community guidelines for ethical animal care and use of laboratory animals.

2.2. Animal handling and induction of metamorphosis

Axolotls (*Ambystoma mexicanum*) were obtained from the Ambystoma Genetic Stock Center (AGSC) and bred in animal care facility of Istanbul Medipol University. Adult animals, 14–16 cm in length, were used in all experiments. Animals were maintained in individual aquarias at ~20 °C in Holtfreter's solution before sampling. Metamorphosis was induced by using L-thyroxine (Sigma-Aldrich, T2376) as described below: (Page and Voss, 2009). T4 solution with a final concentration of 50 nM was prepared by mixing L-thyroxine stock solution with modified Holtfreter's solution. Axolotls were transferred into containers (one Axolotl/container) having 50 nM T4 solution. T4 containing medium was changed every third day and animals were observed for morphological changes. After ~2–3 weeks of T4 administration, weight loss, disappearance of the fin and decrease in the gills size were apparent. Administration of the hormone was continued for another 3 weeks until fully metamorphosed Axolotls were obtained. Both neotenic and metamorphic Axolotls were sacrificed in 0.02% benzocaine (Sigma-Aldrich, E1501) and organs were isolated immediately after the sacrifice.

2.3. Histological analysis

Isolated organs (brain, gallbladder, heart, intestine, liver, lung, spleen, stomach and tongue), skin and tail were fixed in 10% neutral buffered formalin (NBF) for 48 h. Following the removal of fixative by washing the samples with tap water for 1 h, the organs were incubated in ascending alcohol series (70%, 90% and 100% ethanol) for 1 h at 60 °C. Incubation in 100% ethanol was repeated two more times. Then, samples were incubated in toluene for 30 min at room temperature twice. As a next step, samples were embedded to paraffin. Microtome was used to section the paraffin embedded organs in 4 μm thick tissue sections. Then, the sections were deparafinized by incubation in toluene (30 min at 60°), descending alcohol series (100%, 96% and 70%; 1 min at RT) and distilled water (1 min at RT). Paraffin sections of all organs were stained with Hematoxylin and Eosin (Bio-Optica Mayer's Hematoxylin and Eosin Y Plus) according to manufacturer's protocol to identify general

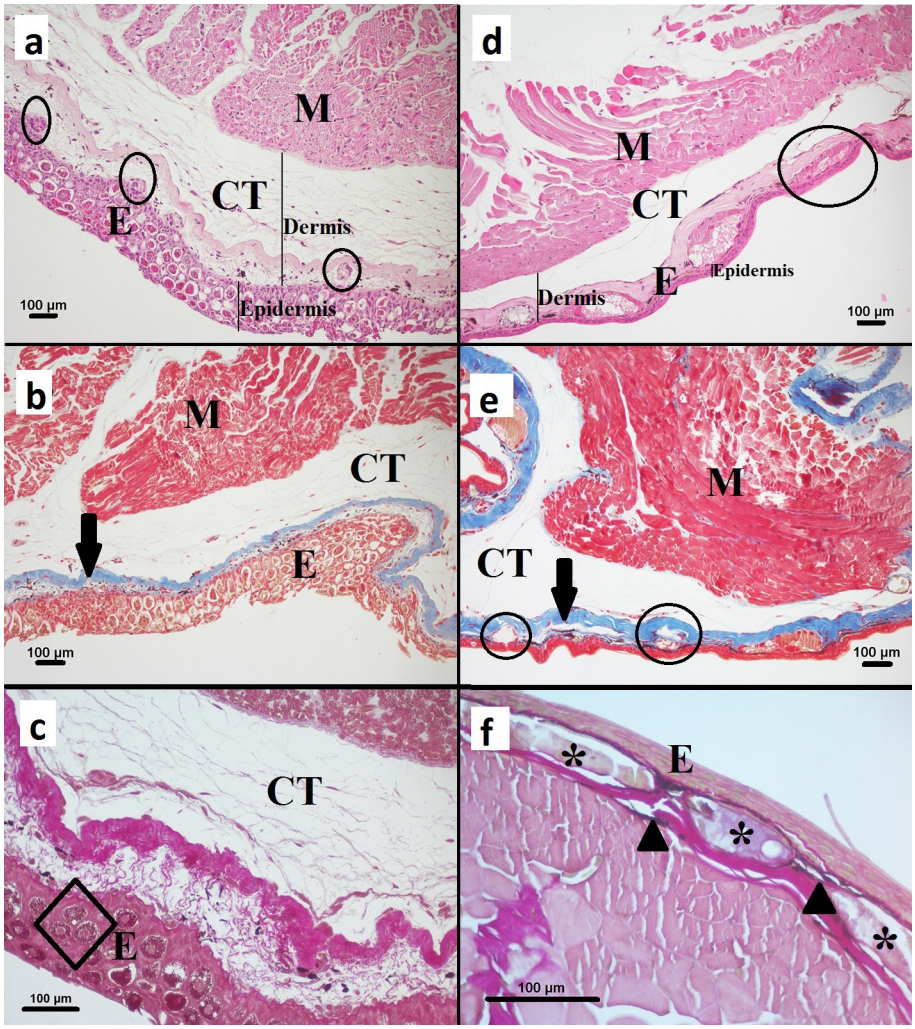

Fig. 1. Axolotl skin histology.
Microscopic examination of neotenic Axolotl's skin.
A (10X) and d (10X) (hematoxylin and eosin staining, bar = 100 µm) E:Epithelium, M:Musculus, CT:Connective Tissue
b and e (10x) (masson trichrome staining, bar = 100 µm) black arrow: collagen fibers.
c (20x) and f (40x) (weigert staining, bar = 100 µm) black triangle: elastic fibers, circles and *: mucous gland, quadrangle frame: Leydig cells.

histological structures. Specific tissues in organs were characterized by Luxol Fast Blue (KIT, Luxol Fast Blue Kluver ve Barrera, Bio Optica, 04-200812), Masson's Trichrome (KIT, Masson Trichrome with aniline blue, Bio Optica, 04-010802), Alcian Blue (KIT, Alcian Blue Acid Mucopolusaccharides staining, Bio-Optica, 04-160802), Weigert (KIT, WEIGERT-VAN GIESON for elastic fibers and connectivum, Bio Optica, 04-053812) and Orcein (KIT, Orcein for Elastic Fibers, Bio Optica, 04-055802) by following the manufacturer's suggested protocol. Bio-mount solution was used to cover the all stained samples with cover-slide. The imaging was performed by using the NIKON DS-Fi2-U3 Digital Camera and Image Analysis Software System.

3. Results

The process of amphibian metamorphosis is characterized by plenteous morphological and biochemical transitions which are mainly responsible for adapting the new terrestrial environment. In this distinctive phase of their life most organs and extremities such as spleen, liver, skin and tail are remodeled so that the aquatic organism accustoms to be a terrestrial. Tissues and organs are classified into two groups based on the major or minor remodeling events.

3.1. Major remodeling events

3.1.1. Skin

Skin samples were obtained from the back skin of the animals. Histological examination of the skin presents two main layers; epidermis and dermis. Thickness of these layers of the skin is dependent to and an indicator of regional variation. Neotenic axolotl epidermis is called pseudo-stratified epithelium and contains epithelial and Leydig cells (Fig. 1a–c). Secreting glands formed by invagination of epidermal layer through dermis (Fig. S1a). The dermis is constructed by irregular loose connective tissue. Furthermore, collagen fibers and a trace of fibroblasts are observed in dermis (Fig. 1b, c, S1b).

Noticeable differences in skin organization of metamorphosed Axolotl are observed (Fig. 1d). As a sign of adaptation to terrestrial life conditions, the epidermis is formed of keratinized stratified squamous epithelium (Fig. 1d–f) like the other terrestrial organisms. In comparison to neotenic counterparts (Fig. 1a) thinner

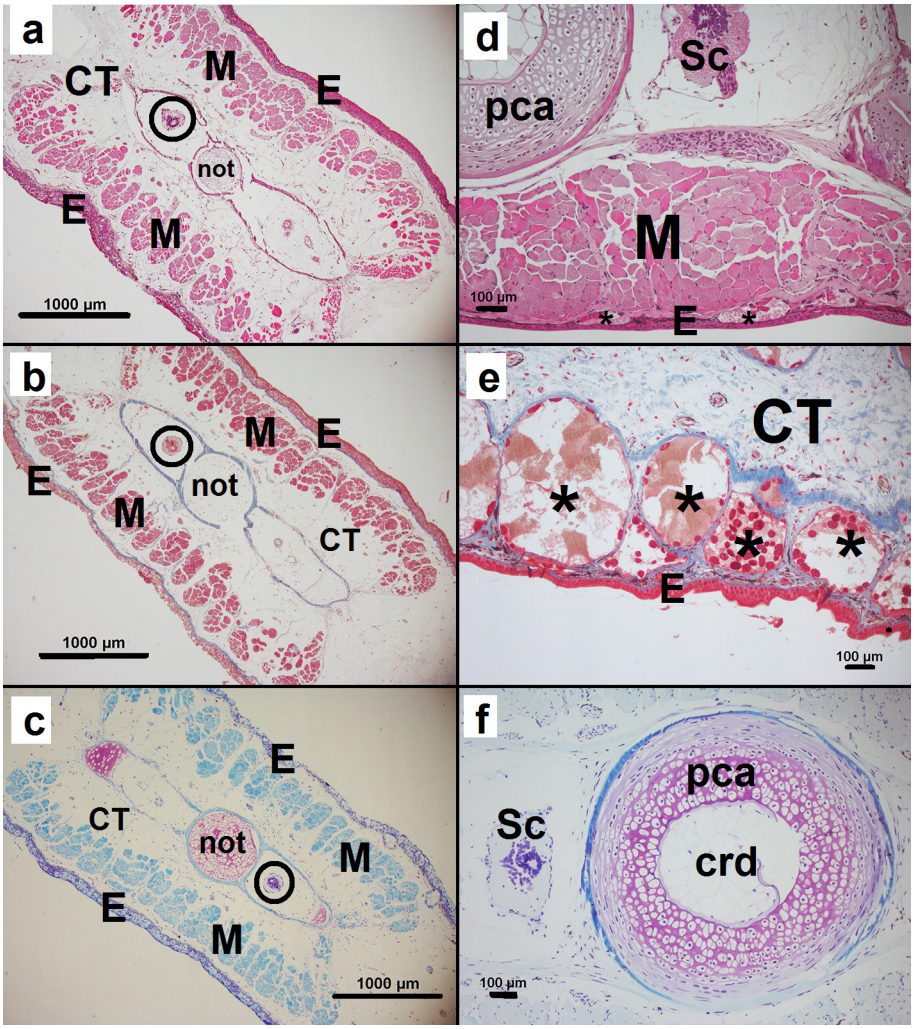

Fig. 2. Axolotl tail histology.
Remodelling of tail during metamorphosis. Morphological alterations between the neotenic Axolotl's tail (a–c) and metamorphic Axolotl' tail (d–f) are shown on taken sections.
a (4x, bar = 1000 µm) and d (10X, bar = 100 µm) (hematoxylin and eosin staining) E:Epithelium, M:Musculus, CT:Connective Tissue, black circle and Sc:spinal cord, not:notochord,
*: mucous and granular glands, pca: perichordal cartilage
b (4x, bar = 1000 µm) and e (10x, bar = 100 µm) (masson trichrome staining) E:Epithelium, M:Musculus, CT:Connective Tissue, black circle:spinal cord, not:notochord, *: mucous and granular glands,
c (4x, bar = 1000 µm) and f (10x, bar = 100 µm) (Luxol fast blue staining) E:Epithelium, M:Musculus, CT:Connective Tissue, black circle and Sc:spinal cord, not:notochord, pca: perichordal cartilage, crd: chordoid cells/tissue.

epidermis without skin appendages (hair, sebaceous glands, sweat glands) is observed. It is noted that Leydig cells disappeared and the epidermis contained a well-defined stratum spinosum, granulosum and corneum (Fig. S1c, d). Dermal papilla is not observed. Mucous glands are found at high numbers (Fig. 1d–f). Alignment of collagen fibers in the dermis is observed more closely and intensely (Fig. S1d) than the neotenic skin sample. Especially the allocation of elastic fibers is recognized around the mucus-producing glands (Fig. 1f).

3.1.2. Tail

Neotenic Axolotls' tail has pseudo-stratified epithelium which includes epithelial and Leydig cells similar to skin epithelium (Fig. 2a). Beneath the epithelium, in connective tissue, there are skeletal muscles shaped as significant fascicules (Fig. 2a, b) encompassed by a kind of connective tissue called perimysium (Fig. 2a, b). In the middle of the tail section, notochord is noticed (Fig. 2a–c) and the chordoid cells/tissue in the center of the notochord [structures are described in (Jonasson et al., 2012; Schnapp et al., 2005)]. Chondrocytes can be detected in cartilage tissue (Fig. 2c). Moreover, the spinal cord is also marked in tail section as expected (Fig. 2c).

As a result of macroscopic alteration, fin is disappeared after metamorphosis. Based on light microcopy results, the main adaptation in tail epithelium is conversion to keratinized stratified squamous epithelium (Fig. 2d, e). Presence of mucous glands is recognized underneath the epithelium (Fig. 2e). Spinal cord, notochord, and at the center of notochord the chordoid cells/tissue are observed in metamorphic Axolotls tail section which resembles the neotenic tail sample section (Fig. 2f).

3.1.3. Spleen

In neotenic Axolotls spleen we observed intensive cell regions as a white pulp – probably includes lymphocytes – which are placed around the central vein (Fig. 3b) as described elsewhere (Lopez et al., 2014). Red pulps are also noticed which consists of red blood cells (Fig. 3a, b). In metamorphosed Axolotl's spleen sections, we

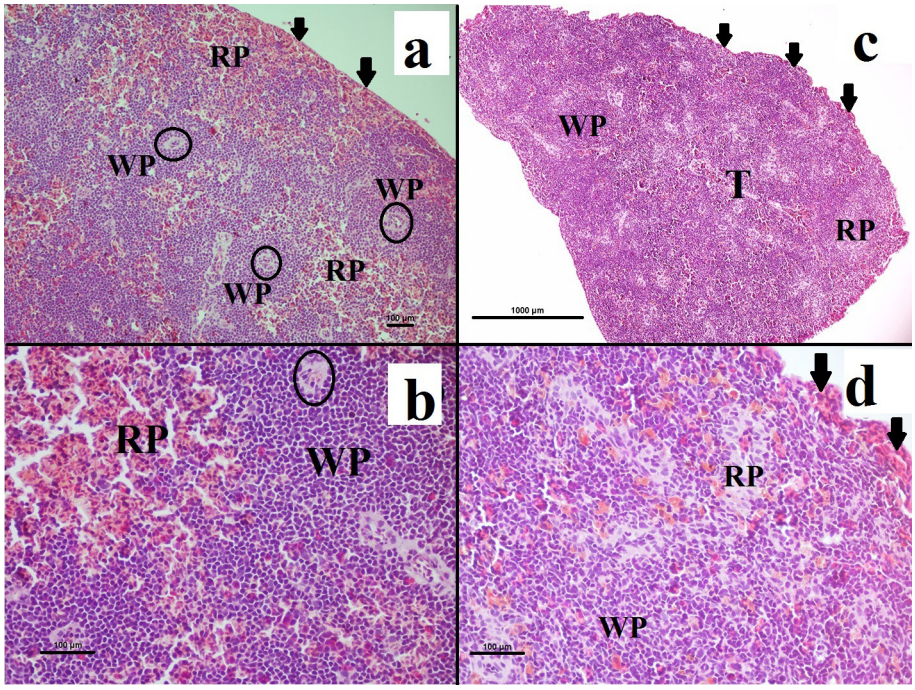

Fig. 3. Axolotl spleen histology.
Microscopy of neotenic (a and b), and metamorphic Axolotl's spleen (c and d).
Histological slices were stained with hematoxylin and eosin and presence of blood cells (a) red and white pulps in neotenic spleen was noticed (b).
a (10x, bar = 100 μm) and c (4X, bar = 1000 μm) (hematoxylin and eosin staining) C:Cortex, black arrow: blood cells, T: Trabecula.
b (20x) and d (20x) (hematoxylin and eosin staining, bar = 100 μm) black arrow: blood cells, RP: Red pulp, WP: White pulp. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

could detect blood cells and trabecula structure (Fig. 3c, d). However, we could not significantly recognize white and red pulps (Fig. 3d).

3.1.4. Gallbladder

Gallbladder has several layers; the mucosa, muscularis, perimuscular and serosa. Our result suggests that there are certain structural differences between neotenic and metamorphic Axolotls gallbladder.

First of all, limited mucosal folds are observed in the lumen of the neotenic Axolotls gallbladder (Fig. 4a) whereas number of mucosal folds increases after metamorphosis (Fig. 4b). Although epithelium and loose connective tissue exist, there is no muscle layer beneath the epithelium for neotenic gallbladder (Fig. 4a). On the other hand in metamorphosed gallbladder circular smooth muscle layer dispersed in vessel rich connective tissue is observed (Fig. 4b, c). Moreover, Neotenic Axolotl's epithelium is characterized by single-layered cubic/single-layered prismatic cells and after metamorphosis gall bladder epithelium of Axolotl change to multilayered from single-layered (Fig. 4b). Furthermore, unlike neotenic Axolotl, Rokitansky-Aschoff sinuses formed by mucosal folds can be seen in metamorphic organism (Fig. 4b).

3.2. Minor remodeling events

3.2.1. Cerebrum

We could not detect dramatic differences between the neotenic and metamorphic Axolotls brain sections. The axolotls brain is characterized by the presence of a narrow, one-to three cell layered VZ (matrix zone) contiguous to the ventricle (Fig. 5b). The VZ is encompassed by wide region of uniformly spherical neurons ((Maden et al., 2013); Fig. 5b). As shown in Fig. 5, Granule cell layer (GcL), Mitral cell Layer (McL) and Glomerular Layer exist in both neotenic and metamorphic Axolotls brain sections (a–d). The olfactory bulb (Fig. 5a) and Anterior Olfactory Nucleus (Fig. 5b) are noticed on neotenic Axolotls brain section.

3.2.2. Tongue

As shown in the figures no significant structural diversity is observed between the neotenic and metamorphic Axolotl tongue sections (Fig. 6a–d). In both of the samples, a non-stratified squamous epithelium covers the loose connective tissue (Fig. 6a–d). Likewise to skin epithelium, Leydig cells exist within the epithelium of tongue (Fig. 6b,c). Both tongue sections have a vessel rich connective tissue (Fig. 6b,c) which is also site for the skeletal muscle fibers (Fig. 6c,d). Furthermore, the hyalin cartilage areas are notably observed in both neotenic and metamorphic Axolotls tongue sections (Fig. 6a,d).

3.2.3. Heart

As shown in Fig. 7 there is no dramatic difference between neotenic and post metamorphic Axolotls heart sections in terms of cardiomyocyte dispersion and organization of the tissue. Cardiomyocytes can be observed in random pattern in both animals (Fig. 7a,c). These cells have a central oval nucleus and they form the striated heart muscle structure (Fig. 7b,d). As distinct from post metamorphic animal's heart section, the connective tissue (endomysium), surrounded by muscle fibers, is more noteworthy in neotenic one (Fig. 7b,d).

3.2.4. Lung

According to our result, central air space of Axolotl lung is divided into smaller air pockets by alveolar folds (Fig. 8a–c). Blood vessels, alveolar folds and smooth muscle tissues form a network to facilitate the gas exchange. Thin epithelium of the alveolar folds lines the lumen of the lung (Fig. 8b). This epithelium consists of

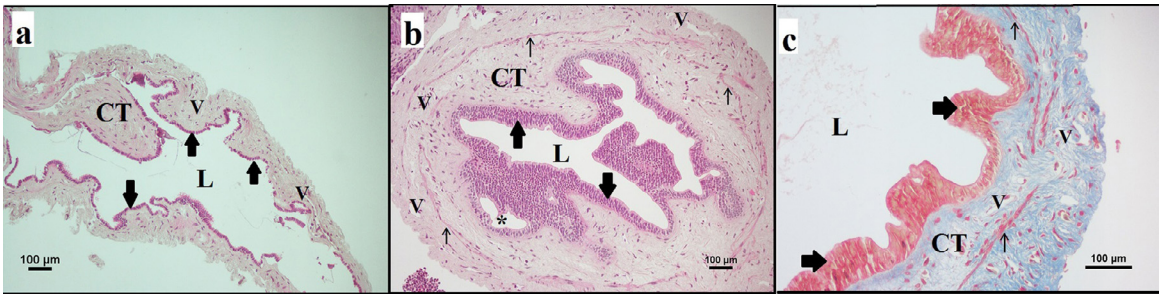

Fig. 4. Axolotl gallbladder histology.
Neotenic (a) and metamorphic Axolotl's gall bladder (b and c). Epithelial and muscle tissues are formed/reformed during the metamorphosis process (b and c).
a (10X), and b (20X) (hematoxylin and eosin staining, bar = 100 μm).
thick black arrow:epithelium, CT:connective tissue, thin black arrow:smooth muscle,
*: Rokitansky–Aschoff sinuses.
c (20X) (masson trichrome staining, bar = 100 μm), v:vessel.

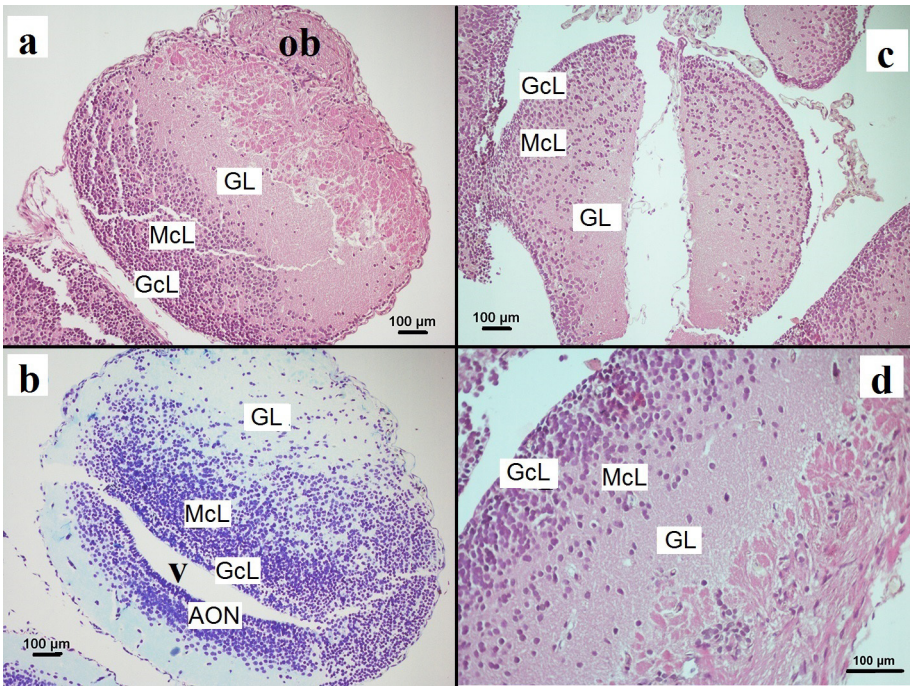

Fig. 5. Axolotl cerebrum histology.
Microscopic examination of neotenic (a and b) and metamorphic Axolotl's cerebrum (c and d).
a (10X), c (10X) and d (20X) (hematoxylin and eosin staining, bar = 100 μm) ob: olfactory bulb, GcL: Granule cell layer, McL:Mitral cell layer, GL:Glomerular layer, AON:Anterior olfactory nucleus.
b (10X) (luxol blue staining, bar = 100 μm) v:ventricle.

three different cell types which are pneumocytes, ciliated cells and goblet cells (Dierichs and Dosche, 1982). While Pneumocytes are located on one side of capillary (Fig. 8b), the two other groups of the cells, ciliated and goblet cells, cover the smooth muscle cells and do not take part in the respiration process directly (Fig. 8a,c). Blood cells are detected in the large blood vessels and voluminous connective tissue is recognized (Fig. 8b, c).

We observed some similarities and variations in neotenic and metamorphic Axolotl's lung samples. The major noticed difference is an obvious decrease in the amount of connective and smooth muscle tissues (Fig. 8d,f). Furthermore, although the pneumocytes and ciliated cells are recognized around the airpockets (Fig. 8e), we could not detect any goblet cells (Fig. 8f) on the section. There are also many alveoli which has a function in pulmonary respiration in metamorphic Axolotl as in mammalian lungs (Fig. 8f).

3.2.5. Liver

The microscopic images of histological slides of neotenic and metamorphic Axolotls' liver are depicted in Fig. 9. As shown in the figure, there are no voluminous differences between the liver of neotenic and metamorphic organism in terms of tissue composition and cell types. The observed differences can be listed as following: First of all, neotenic hepatocytes (shown with 'h' letter on figures) are polyhedral and have a rounded nucleus (Fig. 9a,b), while the hepatocytes of metamorphic organisms are seen as either polyhedral or rounded (Fig. 9c,d). Furthermore, neotenic hepatocytes' nuclei are located in the center of cytoplasm or approach to one side of cytoplasm. On the other hand, after metamorphosis hepatocytes become more regular and nuclei of the cells are commonly located in the middle of cytoplasm. The most significant histological variation between neotenic and metamorphosed liver is recognized

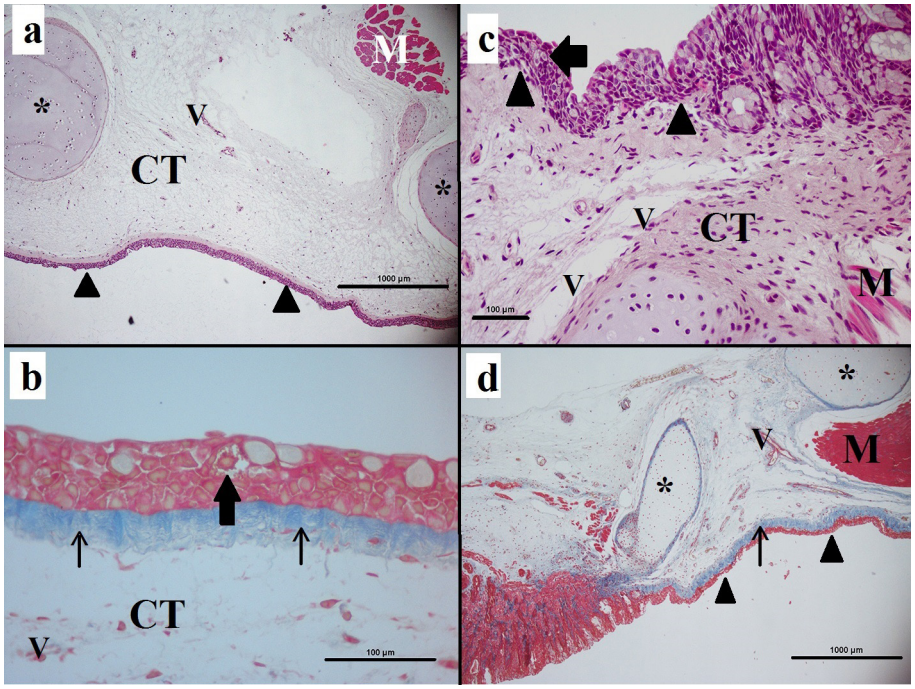

Fig. 6. Axolotl tongue histology.
Microscopy of neotenic (a and b) and metamorphic Axolotls tongue (c and d).
a (4x, bar = 1000 μm) and c (20X, bar = 100 μm) (hematoxylin and eosin staining) black triangle Epithelium, *:Cartilage, CT:Connective Tissue, M: Musculus, black arrow: Leydig cell.
b (40x) and d (10x) (masson trichrome staining,bar = 100 μm) black triangle: Epithelium, *:Cartilage, CT:Connective Tissue, M: Musculus, black arrow: Leydig cell, V:Vessel, thin arrow:dense regular connective tissue.

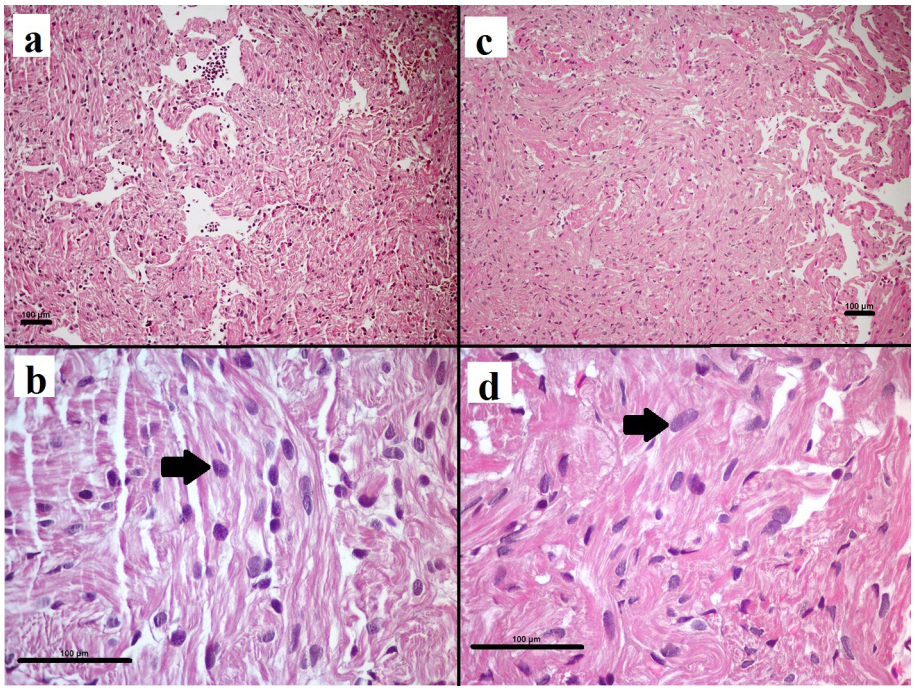

Fig. 7. Axolotl cardiac tissue histology.
Morphological analyses of neotenic (a and b) and metamorphic Axolotl's cardiac tissue (c and d).
a (10x) and b (10X) (hematoxylin and eosin staining, bar = 100 μm).
c (40x) and d (40x) (hematoxylin and eosin staining,bar = 100 μm) black arrow indicates the central nucleus of cardiac muscle cells.

in cytoplasmic staining. In neotenic liver tissue absence of regular stained areas is presumably due to cytoplasmic glycogen and lipid storage. Additionally, a large number of melanin granules assemblies can be noticed in the metamorphic Axolotl's liver parenchyma, as opposed to neotenic (Fig. 9c). Central vein of neotenic and metamorphosed liver samples resemble each other and elastic fibers are noticed around the central vein in both tissues (Fig. 9b,d). Our findings regarding Neotenic Axolotl's liver are in agreement with

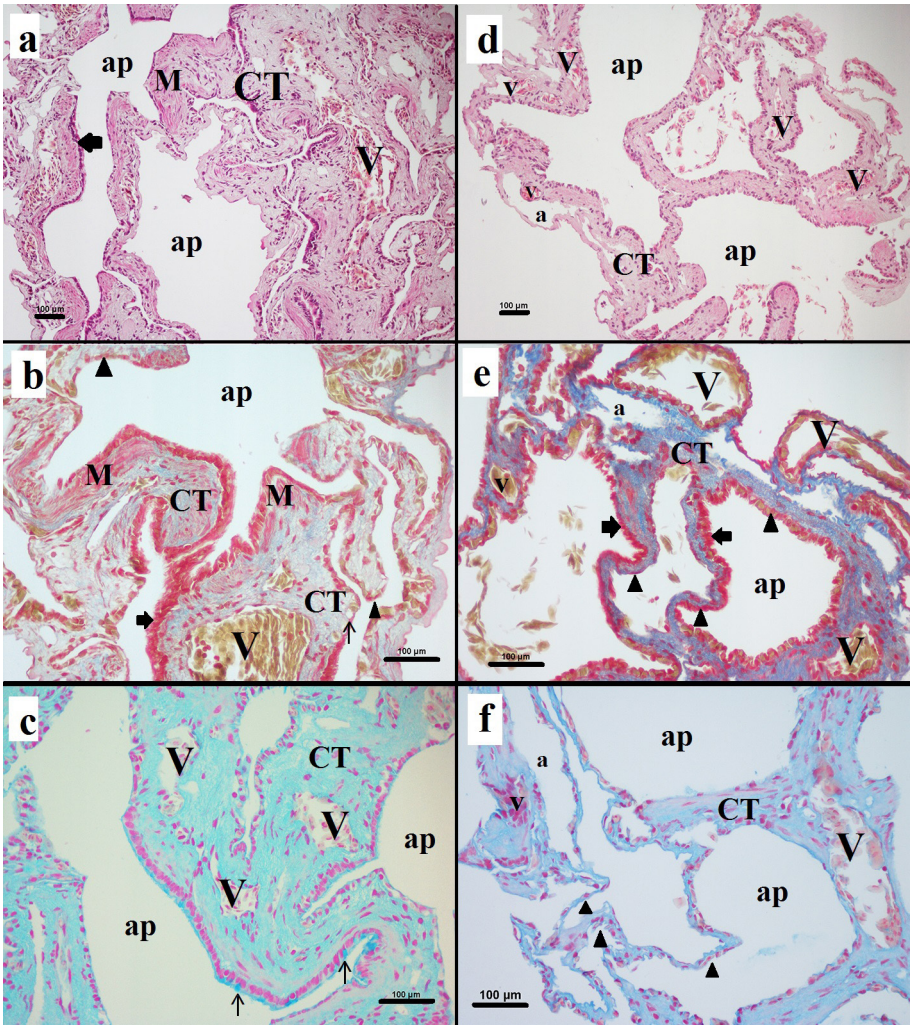

Fig. 8. Axolotl lung histology.
Neotenic (a,b and c) and metamorphic Axolotl's lung sections (d–f) indicates structural similarities and variations before and after the metamorphosis.
a (10X) and d (10X) (hematoxylin and eosin staining, bar = 100 μm) V: Vessel, ap: air pocket, M: smooth mussle, a: alveol, black arrow: ciliated and cuboidal cells, CT:connective tissue.
b (20X) and e (20X) (masson trichrome staining, bar = 100 μm) V: Vessel, ap: air pocket, a: alveol, black arrow: ciliated and cuboidal cells, black triangle: pneumocytes M: musculus.
c (20x) and f (20X) (alcian blue staining, bar = 100 μm) V: Vessel, thin black arrow: goblet cell, ap: air pocket, a: alveol. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the previously observed and reported images and/or description (Lopez et al., 2014).

3.3. Stomach

The typical histology of stomach is comprised of four layers. As demonstrated in Fig. 10, from the inside out, these layers are mucosa, submucosa, muscular layer and serosal layer; respectively. The mucosa is formed of mucous-secreting columnar epithelium and numerous gastric cells (Fig. 10a,d). Gastric chief cells are one of the main components of mucosa layer for both neotenic and metamorphic animal stomach (Fig. 10b,e) Between the mucosa and epithelium in lamina propria, serous and mucous glands are noticed (Fig. 10b, e). Mucin-secreting goblet cells are found in metamorphic Axolotl stomach sample (Fig. 10f). Connective tissue gets more organized after metamorphosis as shown in submucosa (Fig. 10e) In contrary, for the neotenic ones, connective tissue elements dispersed more between the gastric cells than the metamorphosed animals (Fig. 10b, e) Submucosa, characterized by vascular and undifferentiated connective tissues, is detected in both neotenic and metamorphic Axolotl (Fig. 10a–f). Intense vascularization is noticed in submucosa in both organisms (Fig. 10c, f). Finally, the last observation about both neotenic and metamorphic Axolotl stomach is, tunica muscularis (shown in the figure as ME) composed of circular sheet.

3.4. Intestine

As shown in Fig. 11, luminal side of the intestine is composed of multi layer of epithelial cells. The epithelium is surrounded by thin layers of connective tissue and outer muscles. There is numerous epithelial fold. The neotenic intestinal tract resembles a typical vertebrate intestine. The epithelium is into a thick temporary multi cellular lining (Fig. 11a–c). The epithelium has abundant goblet cells (Fig. 11b, c).

Intestine of metamorphosed animal shares similarities with neotenic ones. For instance, the epithelium of the intestine is developed into the multiply folded structure (Fig. 11d). Crypt and villi structures are observed as in neotenic samples (Fig. 11d) and plen-

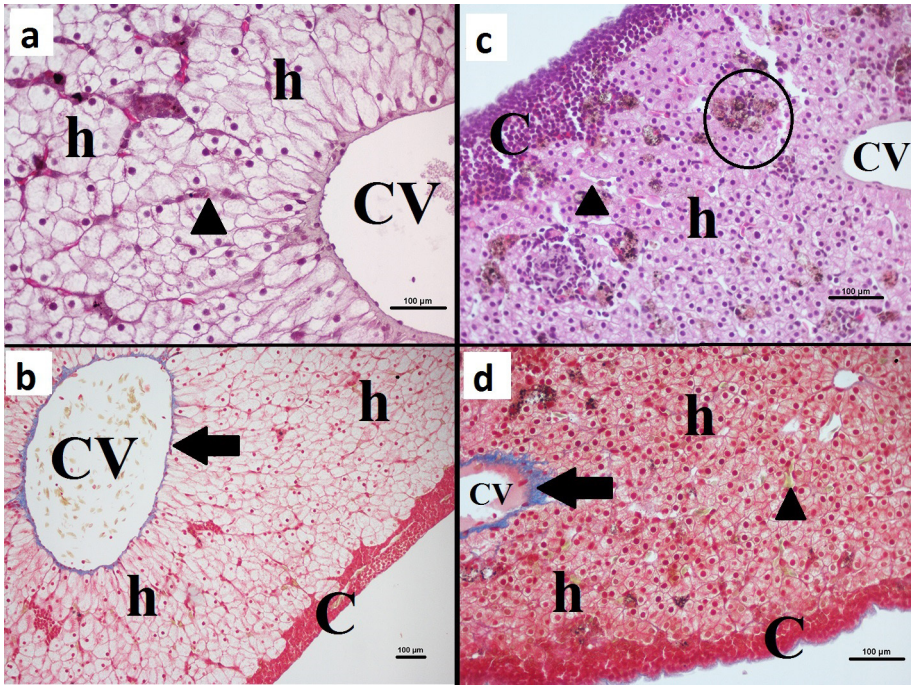

Fig. 9. Axolotl liver histology.
Microscopic examination of neotenic (a and b) and metamorphic Axolotl's liver (c and d) on taken sections.
a (20x) and c (20X) (hematoxylin and eosin staining, bar = 100 μm) h:hepatocytes, CV:central vein, black triangle:sinusoid, C:Cortex, black circle: Brown pigment granules.
b (10x) and d (20x) (masson trichrome staining,bar = 100 μm) black arrow: basement membrane, h:hepatocytes, CV:central vein, black triangle:sinusoid. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tiful goblet cells are detected in metamorphosed samples as well (Fig. 11e, f).

Isolated intestine size for the metamorphosed one is significantly shorter than the neotenic one (data not shown).

3.5. Skeletal muscle, vessel and bone tissues

Axolotls skeletal muscle sections are indistinguishable from the mammalian skeletal muscles in terms of the nucleus localization. As shown in the figure of transversal and longitudinal muscle sections, the nucleus is placed the periphery of cell (Fig. S2 a,c). Muscle fascicles are surrounded by connective tissue (Fig. S2b, d) and metamorphic muscle fascicles are more compact than the neotenic ones (Fig. S2b, d) as defined elsewhere (Monaghan et al., 2014). On metamorphic section, there are more intensive connective tissue than in neotenic one, and as a striking difference, loss of edema in the connective tissue is observed (Fig. S2 b,d).

Beside analyzed muscle tissue, no significant alterations are noticed for vessel (Fig. S3) and bone (Fig. S4) sections. For vessel samples, artery, vein, erythrocytes, endothelium and fibroblast cells are detected in both neotenic and metamorphic Axolotls (Fig. S3a–d). In the same way, we could not recognize crucial differences in bone samples. Vessel, osteocytes, collagen and cartilage tissue are found in both neotenic and metamorphic bone sections (Fig. S4a, b).

4. Discussion

4.1. Skin

Skin is the protective coverage for the body surface and it conducts many vital functions. For aquatic organisms, skin permits the transition of oxygen and water between the organism and environment to maintain the homeostasis. In neotenic Axolotl, oxygen transport through the skin contributes to respiration and penetration of water into the skin fulfills the need to water. The most striking changes with the induction of Axolotl to metamorphosis occur in the skin. In previous studies removal of pre-metamorph epidermis and construction of metamorph epidermis as a consequence of THs induction was examined (Page et al., 2009; Seifert et al., 2012) and their results are agrees well with our observations. Structure of metamorphosed Axolotl skin resembles other terrestrial vertebrates' keratinized stratified squamous epithelium. This adaptation is crucial to reduce the water loss and protect the organism from physical damage. Humidity level of the skin is maintained by continuous mucus secretion from the increased number of glands. Moreover, this increase may account for providing a protective layer. Additional roles that mucus secretion plays are protection from pathogens such as bacteria, cooling the body via evaporation and functioning as a part of excretion system.

4.2. Tail

Changings between neotenic and metamorphic Axolotls tail sections are similar to alterations in the skin. The epithelium turns into keratinized stratified squamous epithelium and mucous glands are formed to act in required mucous secretion for the terrestrial life conditions. Since aquatic animals do not need to humidify their skin, appearance of mucous glands after metamorphosis is essential to prevent water loss. Beside the humidity related remodeling and loss of fin, no significant modifications are noticed.

4.3. Spleen

The spleen is one of the lymphoid system organs and until fifth month of fetal life it acts as a part of hematopoietic system. On postpartum period in vertebrates, spleen has various tasks in terms of blood filtration and storage, phagocytosis, destruction of old erythrocytes, iron metabolism, blood cells and antibody production.

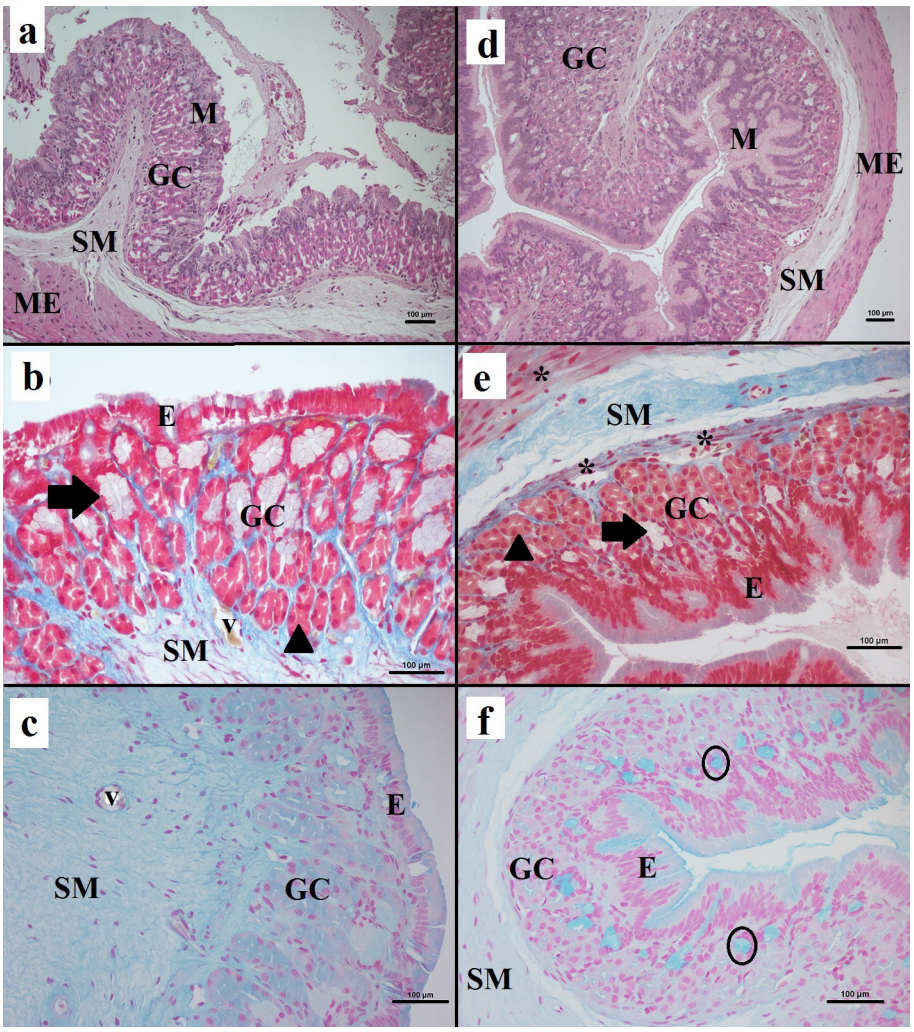

Fig. 10. Axolotl stomach histology.
Microscopy of neotenic (a,b and c) and metamorphic Axolotl's stomach (d–f) on taken sections.
a (10X) and d (10X) (hematoxylin and eosin staining, bar = 100 μm) M:Mucosa, GC:Gastric cells, SM:Submucosa, ME:Muscularis externa.
b (20X) and e (20X) (masson trichrome staining, bar = 100 μm) E:Epithelium, black arrows show parietal cells and black triangle indicates chief cells.
c (20X) and f (20X) (alcian blue staining, bar = 100 μm) circle: goblet cells, *vessel. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The spleen is surrounded by a capsule and from this capsule, thin connective tissue compartments (trabecula) split off and these compartments proceed to depth of the organ with blood cells. Capsule and trabeculae form the stroma of the spleen. The spleen parenchyma settled in this stroma is called the pulp. The pulp is shaped by cell rich connective tissue and divided into two groups; white and red pulp. White pulp consists of cords formed by lymphoid tissue. Red pulp is a special lymphoid tissue includes reticulum cells, macrophages, plasma cells, lymphocytes, and platelets (Eroschenko and Di Fiore, 2013)

In neotenic Axolotls spleen, white pulp and erythrocytes rich red pulp areas indicate that spleen takes an active role in hematopoiesis. After metamorphosis notwithstanding, we considered the spleen may already have completed this function.

4.4. Gallbladder

We observed major differences in different layers of the gallbladder before and after the metamorphosis. The mucosa layer of gallbladder consists of a simple columnar epithelium and underline the epithelium there is connective tissue. No submucosa is defined in gallbladder. Change of epithelium organization and increase in the mucosa folds might point out the fact that bile is concentrated in gallbladder after production in the liver for the metamorphosed animal but not for the neotenic one. The muscularis layer is composed of scattered bundles of smooth muscle (Rajguru et al., 2013). Adventita locates in the muscularis and dense connective tissue of Adventita binds the gallbladder to the liver. Gallbladder surface and abdominal cavity is interrupted with serosa layer which comprises blood vessels, nerves and a lymphatic network (Frierson, 1989). The existence of muscle layer after metamorphosis can be interpreted as a crucial alteration in order to concentrate bile secretion. Lack of muscle layer in gallbladder of neotenic Axolotl could be due to the unnecessity of active bile secretion in this period. Rokitansky-Aschoff sinuses are pseudo-diverticula in the wall of gallbladder and formation of them might be related to increased pressure (Rajguru et al., 2013).

4.5. Cerebrum

In mammals, THs are crucial for brain development. Regarding the pre and post metamorphic stages, amphibians represent an excellent model to investigate the THs roles on brain development to extend the current understanding on mammalian brain develop-

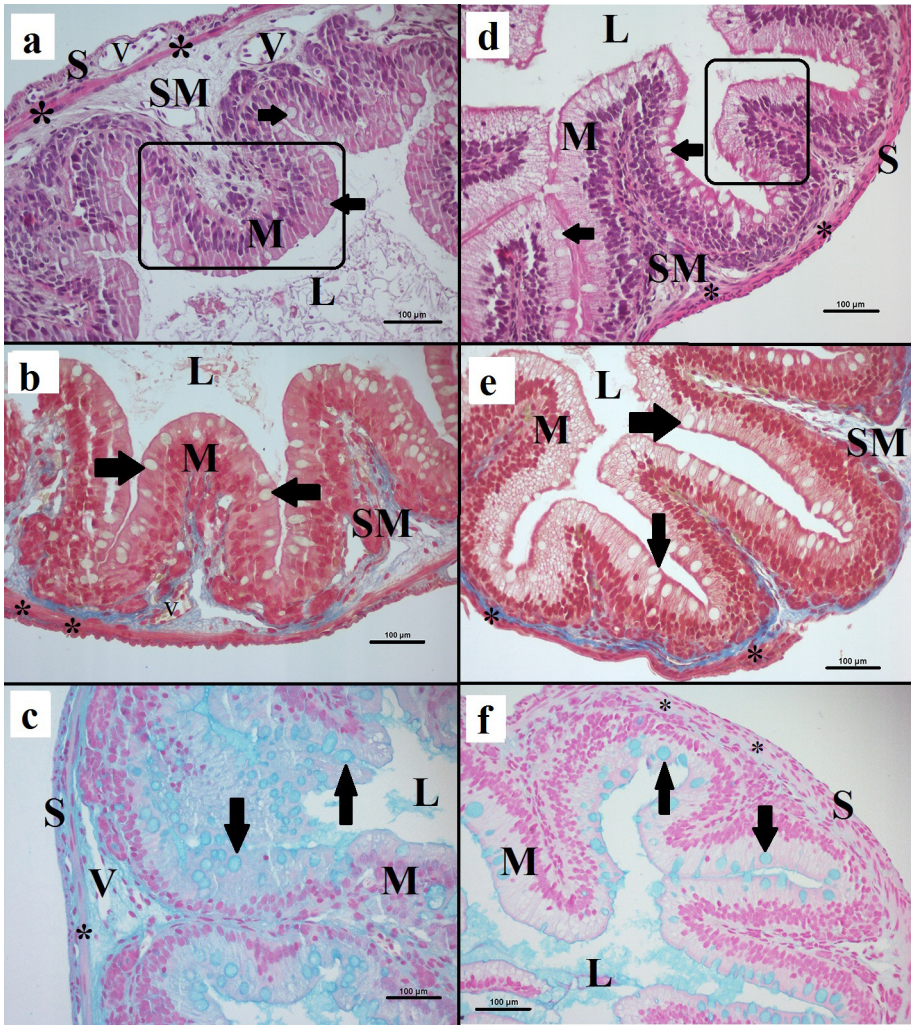

Fig. 11. Axolotl intestine histology.
Neotenic (a–c) and metamorphic Axolotl's intestine sections (d–f) specifies structural resemblances and differences before and after the metamorphosis.
a (20X) and d (20X) (hematoxylin and eosin staining, bar = 100 μm) S: Serosa, SM: Submucosa, M: Mucosa, V: Vessel, L: Lumen, black frame: villi.
b (20X) and e (20X) (masson trichrome staining, bar = 100 μm) SM: Submucosa, M: Mucosa, L: Lumen, black arrow: goblet cells, *: musculus.
c (20x) and f (20X) (alcian blue staining, bar = 100 μm) S: Serosa, M: Mucosa, V: Vessel, L: Lumen, black arrow: goblet cells, *: musculus.

ment. Additionally, amphibians Ventricular zone (VZ) and Neural progenitor cells (NPCs) are considered to proliferate throughout adulthood which is not common for higher vertebrates. The amphibian telencephalon includes a dorsal and thicker ventral matrix (ventricular) zone that reveals higher proliferative and regenerative capacity than the teleost and reptile telencephalon VZ (Maden et al., 2013). In comparison to anuran models, an important benefit in employing Axolotl is the ability to induce the onset of metamorphosis in juvenile or adults (Huggins et al., 2012). From the sections we examined, we did not observe any drastic changes between neotenic and metamorphic brain slices. This might be the result of early modeling of brain with limited THs present in neotenic Axolotl. In order to test this hypothesis, it is worth to analyze brain of younger juvenile Axolotls. Moreover, histological resemblances of metamorphosed brain are encouraging to inspect more detailed for regeneration capacity as described elsewhere (Amamoto et al., 2016).

4.6. Tongue

As seen in some other organs, there is no detectable difference between the neotenic and metamorphic Axolotl tongue samples based on histological staining and light microscopy results. Absence of lingual papillae in both samples is a noteworthy observation in comparison to mammalian tongue structure. The mammalian tongue is organized as core of muscle covered by-non stratified/stratified squamous epithelium. This epithelium layer is covered with lingual papillae characterized by various irregularities and elevations. (Billingham and Silvers, 1967). Lack of Leydig cells in larval Axolotl and appearance during the development may correlate with partial reorganization of tissues *via* secretion of THs (Wistuba et al., 1999). We detected Leydig cells in both neotenic and metamorphosed Axolotl's tongue sections. Remarkable finding about the metamorphosed Axolotl tongue is presence of the Leydig cells since the ones in the skin disappear during metamorphosis.

4.7. Heart

We did not observe considerable variations between the neotenic and metamorphic heart sections regarding cardiomyocytes. However, Malvin and Heisler pointed out occurrence of several morphological differences of heart after metamorphosis (Malvin and Heisler, 1988). The major alteration that they demonstrated was reshaping of vessels during metamorphosis due to

change in primary perfusion path to the lungs (Malvin and Heisler, 1988). Since we did not focus particularly on vessels in heart sections, this might be the explanation to not to obtain the similar outcomes.

4.8. Lung

In neotenic stage, the Axolotl is aquatic and uses its skin, gills and lungs for gas exchange. However, after metamorphosis the gills disappear and for respiration it can use only its skin and lungs. While, approximately %45 of total O_2 uptake actualizes with pulmonary respiration in neotenic stage, after metamorphosis this proportion increases significantly and becomes approximately %65 (Malvin and Heisler, 1988). Presumably, the observed similarity between the neotenic and metamorphic Axolotls lung sections is due to active usage of lungs during the neotenic stage. This is another layer of evidence to support the partial remodeling of the neotenic organs with the produced T3 hormone whose level is normally inadequate to induce metamorphosis.

However, the transition from aquatic to terrestrial life still obliges several crucial adaptations regarding respiration. For example, the loss of buoyancy requires more energy for movement and consequently an increased metabolism with increased gas exchange. Since terrestrial animals' skin is adapted to diminish the water loss, this alteration brings about the decrease in gas exchange through the skin. Loss of gill during metamorphosis is another reason to be restricted to use lungs more efficiently for the metamorphosed animals. To overcome the difficulties coming with terrestrial life conditions, number of alveolar increases and shows more folding to increase the surface area in order to carry out an effective respiration.

4.9. Liver

The largest internal organ is liver and it has crucial roles in protein synthesis, storage of metabolites, bile secretion and detoxification. Lobule structure of liver is formed from hepatocytes and capillary network called as sinusoids which are localized in between hepatic plates. According to light microscopy results, we did not detect remarkable differences between neotenic and metamorphosed Axolotl liver samples. The main distinction noticed is in cytoplasmic staining pattern, which can be due to glycogen and lipid storage in neotenic liver tissues and not in metamorphosed ones. Variations in feeding regime with metamorphosis and/or temperature difference in and out of aquatic environment may be the source of observed difference.

Clustering of melanin granules in metamorphic liver samples may be related to higher phagocytic activity since hepatocytes with melanin pigments are considered as the part of reticulohistiocytic system. Difficulties in removal of cytotoxic ions and free radicals from the body in terrestrial life conditions may account for higher phagocytic activity and therefore clustering of melanin pigments.

4.10. Stomach

The stomach is placed in the posterior foregut and its morphology identified with thickened muscle and unique glands. Thickened muscle is essential for peristaltic movements and elastic distention when the stomach is filled with large quantity of food (Smith et al., 2000).

According to previous researches, most anuran tadpoles generally indicates lacking a "true" stomach and proteolytic enzymes (Smith et al., 2000). However, gastric chief cells which produce proteolytic pepsinogen enzyme can be numerously seen in neotenic Axolotl stomach. It could be the result of being a carnivore born species, and therefore required enzymes to hydrolyze the proteins are produced in and secreted by the gastric chief cells. This is not frequently observed for the most of the amphibians' neotenic stage. According to this observation, we can conclude that the formation or maturation of gastric chief cells is not strictly controlled by THs induce alterations in stomach during the Axolotl metamorphosis.

Tadpoles do not possess a submucosa till the transformation to adult frog (Rovira et al., 1995), but for both neotenic and metamorphic Axolotl a well-defined submucosa is observed. Since the submucosa consists of connective tissue, it might have structural roles in addition to functional roles in digestion.

4.11. Intestine

Intestine is one of the highly plastic organs that functions mainly in food processing and nutrient absorption. It owns its reestablishment to its plasticity and this is highly affected from THs. Postembryonic development and remodeling of intestine is a commonly observed process among vertebrate. In mouse, formation of crypt-villi structure takes few weeks after the birth since T3 production and its release to blood is required for remodeling the intestine (Hasebe et al., 2013). T3 is also crucial for amphibians' intestine establishment post-embryonically and its climax structures the crypt-villi formation in intestinal epithelium of amphibians (Sun and Shi, 2012).

Based on our light microscopy results, there is no significant difference between the neotenic and metamorphic Axolotl intestine, unlike the frog. It has been documented that T3 is produced by the time of early juvenile stages in neotenic Axolotl but it is not enough to stimulate the metamorphosis (Badawy, 2011; Rosenkilde and Ussing, 1996). Since the animals we used were older than 6 months, the most plausible scenario is secreted T3 amount is too low to transform the animals; however it is high enough to remodel the intestine. In that respect, post-embryonic intestinal establishment of neotenic Axolotl should occur at very early stages after the birth of the animal.

4.12. Skeletal muscle, vessel and bone tissues

In both neotenic and metamorphic animals skeletal muscle and connective tissues exhibit similarities rather than differences in terms of found cells and structural organization. The main alteration in these tissues is loss of edema with THs climax. Resemblances of these tissues between pre and post metamorphic animals might be due to conserved structural roles of these tissues among vertebrates.

5. Concluding remarks

Tissue renewal and restoration capacity of Axolotls makes them promising model to explore the molecular mechanisms that have crucial roles in regeneration. Availability of metamorphosed Axolotls by administration of THs and alterations in experimental characteristics after metamorphosis offers a dual model system to perform stem cell, regeneration and cancer studies. Reliable evolutionary proximity between amphibians and mammals may facilitate the digestion of messages from salamander studies and it may enhance the translation of messages from amphibians to mammals. In this study we present a comparison of neotenic axolotl with metamorphic ones in terms of detailed histological analysis which may contribute to gain new perspectives to link the renewable capacity of tissues and their organization for the future studies. Our findings suggest that remodeling of organs and tissues with THs induction facilitate the adaptation to terrestrial life. Surprisingly, we have noticed extensive similarities between the neotenic and metamorphic Axolotl tissues. Production and secretion of THs

in early developmental stages may account for remodeling of several tissues and organs of Neotenic Axolotl due to being responsive to trace amounts of THs. To test this possibility, a more comprehensive research by inspection of organs from younger animals at different developmental stages can be carried out. Moreover, a more detailed analysis using electron microscopy to examine intra and intercellular components would be advantageous to distinguish resembling structures. Furthermore, cell and tissue specific immune-staining would provide a valuable data to observe the similarities and differences between the organs and tissues of neotenic and metamorphosed Axolotl.

Conflict of interest statement

The authors have no conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.acthis.2016.07.006.

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