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# Comparative histomorphology and histochemistry of the skin in different morphs of the Greek smooth newt *Lissotriton graecus* (Urodela: Salamandridae)

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**Abstract:** In the biphasic life cycle of the smooth newts, the aquatic larvae transform into terrestrial adults, the metamorphs. The Greek smooth newt *Lissotriton graecus* is characterized by facultative paedomorphosis in which 2 adult morphs can coexist. Paedomorphs are reproducing adults that live in an aquatic habitat and retain some larval characteristics. In this study, histological and histochemical characteristics of the skin of the *L. graecus* in all morphs were investigated: larvae, paedomorphs, and metamorphs. The most remarkable finding is that skin development in paedomorphic *L. graecus*, whose skin is a mosaic of larval and metamorphic features, is partially uncoupled from sexual maturity. Leydig cells in the epidermis and numerous clear/vacuolated cells in the skin glands are common features with larval, while the relative thickness and keratinized surface of the epidermis are common features with adult skin. Our results provide new insights into this neglected field, given the gap in the existing literature on skin development in paedomorphic urodeles. The results of this study emphasized the importance of histological investigation of the possible uncoupling of sexual and somatic development in different organ systems to achieve a deeper understanding of the complex process of paedomorphosis.

**Keywords:** skin; dermal glands; light microscopy; electron microscopy; paedomorphosis; Greek smooth newt *Lissotriton graecus*

## INTRODUCTION

Amphibians in general have a biphasic life cycle in which aquatic larvae undergo metamorphosis, a remarkable change in morphology, physiology and biochemistry to transform into sexually mature terrestrial adults [1]. In Urodela, individuals may reach sexual maturity before metamorphosis, and in some species, metamorphosis is absent. The resulting sexually mature paedomorphs retain their larval structures and live in an aquatic habitat [2]. In newts and salamanders, paedomorphosis is common, evidenced by external gills and other larval characteristics in reproducing adults [3]. The uncoupling of sexual maturity from metamorphosis demonstrates the phenotypic plasticity of species and is thought to improve fitness in variable habitats. For example, in some Urodela species such as *Ambystoma*, *Triturus* and *Lissotriton*, 2 adult morphs, paedomorphs and metamorphs, may be present in the same population, which is referred to as facultative paedomorphosis [4]. The difference between facultative and obligate paedomorphosis is that in facultative paedomorphosis, the organism can undergo normal metamorphosis, while in obligate paedomorphosis it remains permanently in the juvenile form but is capable of reproduction [2]. Although the uncoupling of sexual from somatic development is evident in Urodela, more in-depth studies are needed on the development of the different organ systems, especially during paedomorphosis. Moreover, this uncoupling has rarely been studied from a histological and/or histochemical point of view. To our knowledge, this has been done several times in reproductive tissues, e.g. in the oviducts of *Ambystoma talpoideum* [5], and only twice in somatic tissues: once in individuals from natural populations, in the skin of *Triturus alpestris apuanus* [6], and once when comparing naturally paedomorphic individuals with individuals with experimentally induced metamorphosis, in the skin of *Ambystoma mexicanum* [7].

The amphibian skin is a complex organ with a variety of functions in different morphs. Larvae live in water, and the skin plays an important role in respiration [8], while metamorphs live in terrestrial environments where the skin must be more robust and keratinized to prevent desiccation [9,10]. In general, the skin of urodeles consists of 2 layers: epidermis and dermis. The epidermis of the larvae consists of several cell layers containing predominantly Leydig cells as well as epithelial basal and apical cells [8,11,12]. Depending on the species, the dermis may be absent in larvae (*Hynobius retardatus*: [11]; *Salamandra salamandra*: [12]; *Triturus ivanbureschi*: [13]), or present (*Ambystoma mexicanum*: [14]; *Euproctus platicephalus*, *Salamandrina terdigitata*, *Triturus alpestris*, *Triturus cristatus*: [15]). The adult skin always consists of epidermis and dermis. The epidermis consists of keratinized stratified squamous epithelium, without Leydig cells, as these disappear during metamorphosis [16]. The dermis consists of the *stratum spongiosum* and the *stratum compactum* underlying the epidermis. The loose connective tissue of the *stratum spongiosum* contains blood vessels, skin glands and chromatophores, while the *stratum compactum* consists of dense connective tissue [16,17]. Various types of multicellular glands are a characteristic feature of the adult dermis [11,12,18]. Based on the chemical nature of the synthetic products, serous (granular), mucous and mixed glands can be recognized in abovementioned dermis-containing larvae and all adult metamorphs. Serous glands produce proteinaceous products, mucous glands synthesize various mucins, while mixed glands contain cells of both secretory lines [15,19]. However, the secretions of the skin glands may contain other chemical compounds, such as biogenic amines, alkaloids, steroids and lipids [20,21]. The skin of paedomorphic animals is a mosaic of larval and metamorphic characteristics. For example, Leydig cells are present in the epidermis, which is characteristic of the larval morph, while the dermis resembles that of metamorphic animals [6,7].

As the skin is one of the organs that changes the most during amphibian development [22,23], we investigated the histomorphology and histochemistry of the skin in all morphs of the Greek smooth newt *L. graecus*. The reason why we decided to study this species, apart from the fact that it is characterized by facultative paedomorphosis, is the fact that it is the most common species in most parts of its range [24] and has not yet been studied among the genera belonging to the group of modern Eurasian newts [25]. The primary objectives of this research were to provide, for the 1st time, a description of the histological and histochemical skin characteristics across all 3 *L. graecus* morphs, to determine whether skin development dynamics vary between paedomorphs and metamorphs, and to examine the potential uncoupling of sexual and somatic development.

## MATERIALS AND METHODS

### Ethics statement

The animals used in this study were collected before the formal establishment of the Ethics Committee of the University of Belgrade, Faculty of Biology. Sampling was conducted according to historical collection standards, with care taken to minimize the number of animals removed from the natural population.

### Animals

Individuals of *L. graecus* collected in Velika Osječenica, Montenegro (42° 58' N, 14° 40' E; altitude 990 m) in 1989 were used for this study. At that time, the tissue samples were prepared for microscopy, and the specimens embedded in resin have been stored since then in the tissue bank managed by the Department of Cell and Tissue Biology, University of Belgrade, Faculty of Biology. Trunk skin samples from 3 different morphs (larvae, paedomorphs, metamorphs; 1 individual of each morph, 3 tissue blocks per each animal) were used for histological analysis. The shape of the cloaca and the presence of external gills were used to precisely distinguish between the 3 morphs [26].

### Specimen preparation for microscopy

Tissue samples of approximately 1 $mm^3$ in size were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and post-fixed in 1% osmium tetroxide. The samples were dehydrated through a series of ethanol solutions with increasing concentrations (50-100%). The ethanol was gradually replaced by propylene oxide, which allowed the resin (Araldite) to penetrate the tissue. Semi-fine (1-2 μm thick) and ultra-thin (<0.5 μm thick) sections were prepared using the

Leica EM UC6 ultramicrotome (Leicamicrosystem, Wetzlar, Germany) equipped with either a glass or a diamond knife (Diatome Ltd., Switzerland). Semi-fine sections were mounted on slides and stained as described below. Grids with ultra-thin sections, obtained after selection from semi-fine sections stained with toluidine blue, were contrasted with uranyl acetate and lead citrate and observed on the Philips CM12 transmission electron microscope (Eindhoven, The Netherlands). The electron micrographs were taken with the SIS MegaView III digital camera (Olympus Soft Imaging Solutions, Germany).

### Staining methods

The 2 histochemical staining methods, AB (alcian blue)-PAS (periodic acid-Schiff reagent) and mercuric bromophenol blue (MBPB), were used both to study the chemical composition of the secretory product of the skin glands and to visualize the general histological organization of the skin. After resin removal, the sections were hydrated in ethanol solutions of decreasing concentrations and distilled water ($dH_2O$).

In the AB-PAS staining method, tissue sections were stained in AB (pH 2.5), rinsed in $dH_2O$ and incubated with 1% periodic acid. After washing in $dH_2O$, the sections were treated with Schiff reagent and sodium metabisulfite solution, rinsed in $dH_2O$, stained with hematoxylin and washed in tap water. In the MBPB staining method, the sections were stained with BPB in a mercuric chloride-ethanol solution, rinsed in acetic acid and tap water. All stained samples were air-dried and mounted in DPX (distyrene, plasticizer, and xylene) mounting medium.

AB stains acidic polysaccharides (certain types of mucopolysaccharides) turquoise blue, while PAS stains neutral polysaccharides (mucosubstances such as some glycoproteins, glycolipids and mucins) magenta [27]. MBPB detects the presence of proteins by staining them dark blue [28].

### Morphometry

All histological sections were analysed and photographed using a Leica DMLB light microscope with Leica DFC295 camera and LAS Core software. Morphometric measurements were performed using ImageJ 1.45 software [29]. The morphometric measurements included the thickness of the epidermis and dermis, the number of Leydig cells in the epidermis and the number and diameter of the glands in the dermis. The thickness of the epidermis and dermis was measured orthogonally from the basement membrane upwards and downwards, respectively. For both epidermis and dermis thickness, 5 measurements were systematically performed on each micrograph and reported as the mean value for each image. The proportion of epidermis and dermis in the total thickness of the skin in all 3 morphs was calculated. The mean gland diameter was expressed as the arithmetic mean of the longest and shortest diameters measured perpendicular to each other. The thickness of the epidermis, dermis and diameter of the glands were measured on photomicrographs at 40x magnification (15-20 measurements for each parameter per morph) taken from AB-PAS-stained sections. The number of Leydig cells and glands, both per unit area (1 $mm^2$) of the skin section, was determined from 7-10 photomicrographs per morph at 20x magnification.

### Statistical analysis

All collected data were analyzed statistically. The mean and standard error of the mean were calculated using Excel 2016. Although multiple sections and measurements were taken, these are pseudoreplicates from the same individual, not biological replicates. Therefore, biological variability cannot be assessed, and no statistical comparisons were made.

## RESULTS

### Histology and histochemistry

### Larva

The skin of the larvae was smooth and consisted of epidermis and dermis of approximately the same thickness (Fig. 1A, F). The epidermis consisted of 3-5 cell layers. The epidermal surface was covered with a thin, continuous AB-stained layer of acid mucins (Fig. 1A). Superficial, roughly cuboidal cells were not stained with MBPB, indicating that they were not keratinized (Fig. 1F). They contained acid mucins mainly in the supranuclear cytoplasm (Fig. 1A). In

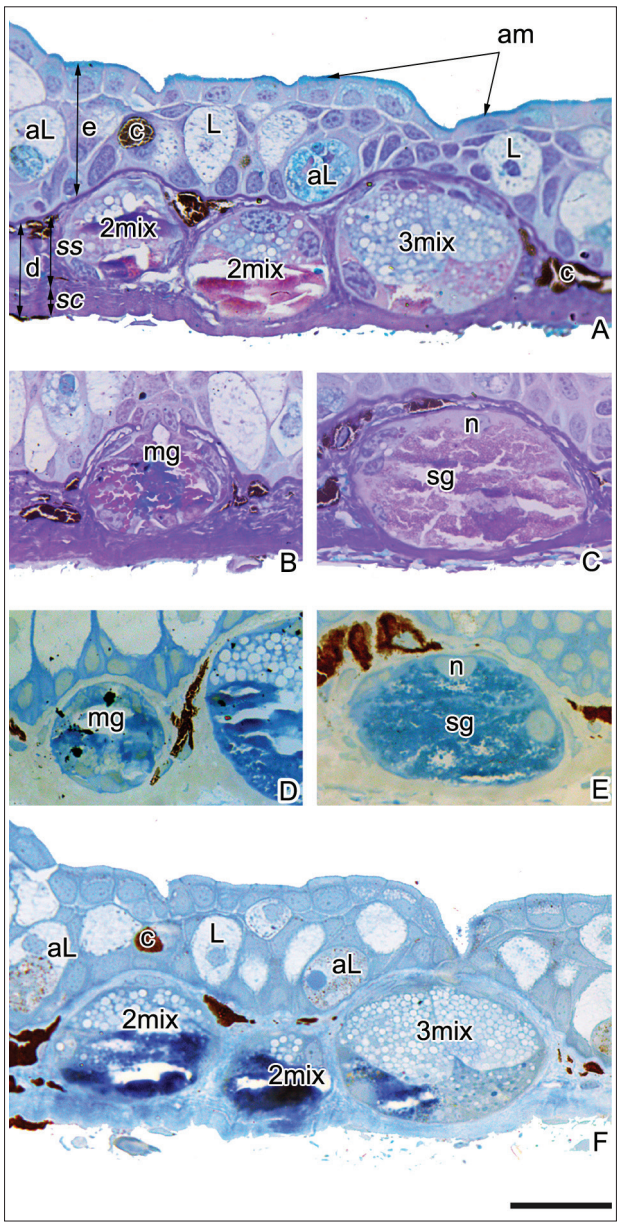


**Fig. 1.** The histological organization of the skin of *L. graecus* larva is shown in closely adjacent sections stained with AB-PAS (**A**, **B**, **C**) and MBPB (**D**, **E**, **F**). The skin consists of epidermis (e) and dermis (d) (**A**). *Stratum spongiosum* (*ss*) and *stratum compactum* (*sc*) are clearly demarcated areas of the dermis. The stratified non-keratinized epidermis is covered with acid mucins (AB-positive, am) and harbors numerous Leydig cells (L). Some Leydig cells are altered, i.e. they are partially or completely filled with acid mucins (aL). Various glands are located in the *stratum spongiosum*. The serous glands (sg) are filled with protein-containing (MBPB-positive) products (**E**), which are simultaneously negative for acid and neutral mucins (**C**). Mucous glands (mg) contain centrally arranged acid mucins and laterally positioned neutral mucins in the form of carbohydrate-decorated proteins, indicated by overlapping PAS and MBPB staining on closely adjacent sections (**B**, **D**). Mixed glands can consist of 2 (2mix) or 3 (3mix) different cell types: cells containing proteinaceous granules and clear/vacuolated granules (with or without acid mucous AB-positive lace-like material within) are the common to all mixed glands, while cells filled with glycoprotein-containing granules (PAS- and MBPB-positive) together with clear/vacuolated granules are additionally found in 3-component glands (**A**, **F**). c – chromatophores, n – nucleus. Color coding: light blue = AB-positive = acid mucins; magenta = PAS-positive = neutral mucins; dark blue = MBPB-positive = proteins. Original magnification 40×, scale bar 50 μm.

the intermediate region, there were numerous large spheroidal or elongated, almost translucent Leydig cells in 1 or more rows (Fig. 1A, F). The Leydig cells were characterized by a centrally located nucleus and abundant granules. Altered Leydig cells were also observed, showing diffuse or localized acid mucins (Fig. 1A). Discrete chromatophores were rarely present in the epidermis. The basal layer rested on the basement membrane and contained 1 row of cuboidal or columnar cells (Fig. 1A, F). The dermis consisted of the *stratum spongiosum* and the *stratum compactum*, which differed in the relative abundance and arrangement of cells and fibers, with the *stratum spongiosum* being looser and harboring numerous multicellular glands and chromatophores (Fig. 1A). The 3 different types of glands were present in the larval skin. A detailed description of the skin glands of all morphs can be found in a separate section below.

## Paedomorph

In the paedomorphs the skin surface was undulated, with discrete ridges and grooves. The dermis was obviously thicker than the epidermis (Fig. 2A, F). The epidermis consisted of 3-5 cell layers. Superficial MBPB-positive staining showed a continuous layer of keratinized squamous epithelial cells (Fig. 2A, F). Leydig cells, which were arranged in 1 row in the intermediate region of the epidermis, were less abundant than in the larval stage. They had a centrally positioned nucleus and a supporting Langerhans' net (cytoskeleton) in their peripheral cytoplasm (Fig. 2A, F). The dermis showed a typical histological organization and contained various skin glands (Fig. 2). Serous glands were absent in this morph, and mucous and mixed glands were present in approximately equal amounts.

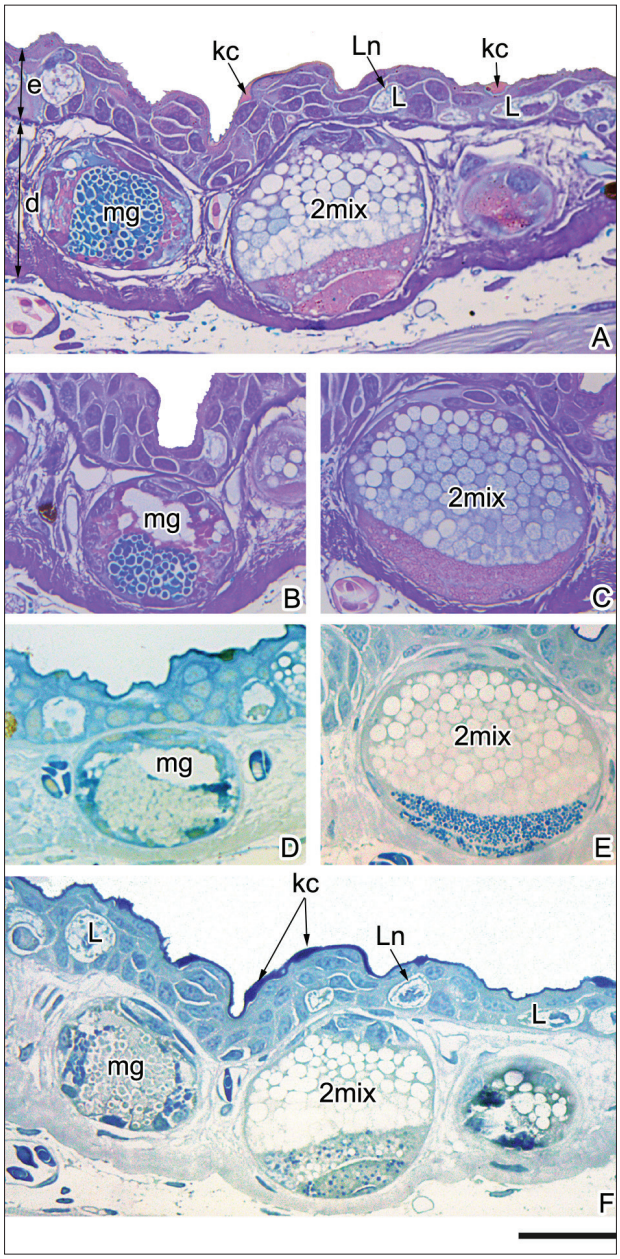


**Fig. 2.** The histological organization of the skin of paedomorphic *L. graecus* is shown in closely adjacent sections stained with AB-PAS (**A**, **B**, **C**) and MBPB (**D**, **E**, **F**). The skin consists of epidermis (e) and dermis (d) (**A**). The stratified epidermis is characterized by a superficial layer of keratinized squamous epithelial cells (MBPB-positive, kc) and rare Leydig cells (L) with Langerhans' net (Ln) (**A**, **F**). Mucous glands (mg) with visible lumen (**B**, **D**) or without lumen (**A**, **F**) as well as mixed glands (2mix) (**A**, **F** and **C**, **E**) are present in the dermis. Colour coding: light blue = AB-positive = acid mucins; magenta = PAS-positive = neutral mucins; dark blue = MBPB-positive = proteins. Original magnification 40×, scale bar 50 μm.

## Metamorph

The skin surface of the metamorphs was undulated, with a thicker dermis compared to the epidermis, similar to the skin of paedomorphs (Fig. 3A, D). The epidermis consisted exclusively of keratinocytes arranged in 3-5 layers (Fig. 3A, D). The superficial cells were MBPB-positive, i.e. keratinized, and flattened, except in the area previously occupied by the nucleus (Fig. 3D). As in the paedomorphs, the underlying dermis harbored all gland types except the serous glands, with the proteinaceous synthetic product dominating in the mixed glands (Fig. 3D).

## Dermal glands

The glands were spherical or slightly ellipsoidal and consisted of several cells with large euchromatic nuclei in the basal region. Histochemical staining revealed 3 types of glands in the dermis of *L. graecus*, based on the staining properties of their granules: acidic and neutral mucins were stained turquoise blue with AB and magenta with PAS, respectively, while proteins were stained dark blue with MBPB. Table 1 summarizes the gland type and function in each morph.

The 1st type of gland contained only cells with protein-containing granules and were therefore referred to as serous glands (Fig. 1C, E). The margins of the individual cells were indistinguishable, i.e. these glands were rather syncytially organised and had no obvious lumen. Serous glands were only present in larvae.

In the 2nd type of gland with a more or less distinct lumen, acid mucins were present in centrally located cells, whereas glycosylated proteins (detected by overlapping PAS and MBPB staining on closely adjacent sections) were found in laterally located cells (Fig. 1B, D, 2B, D, 3B, C). Hence, this 2nd type was predominantly mucous glands, and are herein referred to as mucous glands. They were found in all morphs.

The 3rd type of gland observed in all morphs contained either 2 or 3 different cell types, which is why these glands were referred to as 2-component or 3-component mixed glands. In the 2-component glands, there were cells with proteinaceous product at the base of the gland, sometimes not arranged in individually recognisable granules (Fig. 1F, 2E, F, 3D). The other cell type, with clear/vacuolated granules was

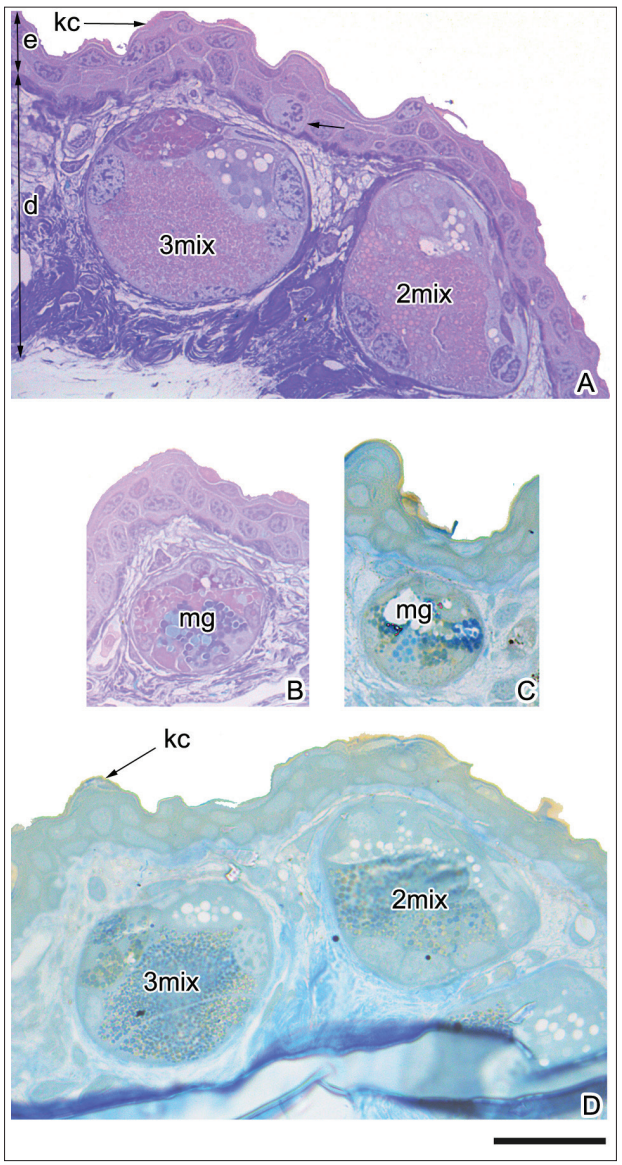


**Fig. 3.** The histological organization of the skin of metamorphic *L. graecus* is shown in closely adjacent sections stained with AB-PAS (**A**, **B**) and MBPB (**C**, **D**). The skin consists of epidermis (e) and dermis (d) (**A**). As in paedomorph, the stratified epidermis is characterized by a superficial layer of keratinized squamous epithelial cells (MBPB-positive, kc), but Leydig cells are not present (**A**, **D**). Mucous glands (mg) (**B**, **C**) and mixed glands (2mix and 3mix) (**A**, **D**) are found in the dermis. In the mixed glands, proteins are the predominant synthetic product, in contrast to the mixed glands of the other morphs. Colour coding: light blue = AB-positive = acid mucins; magenta = PAS-positive = neutral mucins; dark blue = MBPB-positive = proteins. Original magnification 40×, scale bar 50 μm.

located above. Clear/vacuolated granules were either unstained or with acid mucous material in them (Fig. 1A, 2A, C, 3A). These 2 cell types were about equally represented in the mixed 2-component glands of the larvae, whereas cells with clear/vacuolated granules dominated in paedomorphs, while proteinaceous secretory products predominated in the metamorphs. In addition to the cell types just described, the 3-component glands contained another cell type that is morphologically intermediate. These cells were filled with glycoprotein-containing granules, with (larvae) or without (metamorphs) clear/vacuolated granules (Fig. 1A, F, 3A, D), giving the appearance of a gradual change of secretory products towards the apex of the gland. In the 3-component glands, cells filled with clear/vacuolated granules predominated in the larvae (Fig. 1A, F), whereas proteinaceous secretory cells were in the majority in the metamorphs (Fig. 3A, D).

Fig. 4 is an electron microscopic image showing parts of different cell types in various glands. Proteinaceous granules were the most electron-dense (Fig. 4A). Glycoprotein-containing granules were also uniformly electron-dense, while acid mucous granules were larger and characterized by dense core(s) in the electron-translucent background (Fig. 4B, Supplementary Fig. S1). In some clear/vacuolated granules, lacy, electron-dense material was visible (Fig. 4C).

## Morphometric analysis

The results of a morphometric analysis are presented in Table 2. These data show that the skin was thickest in the larvae and thinnest in the paedomorphs, due to a thicker epidermis in the larvae and a thinner epidermis in the paedomorphs, while dermis thickness was similar in all morphs. Nevertheless, Fig. 5 shows that the proportion of epidermis and dermis in total skin thickness was similar in paedomorphs and metamorphs, with the ratio of epidermis to dermis being approximately 1:2. In contrast, the larval skin had an approximately equal proportion of epidermis and dermis. The mean diameter and the number of glands per unit area of skin were the same in all morphs. Finally, the number of Leydig cells per unit area of skin was higher in the larvae than in paedomorphs, whereas these cells were practically absent in the metamorphs.

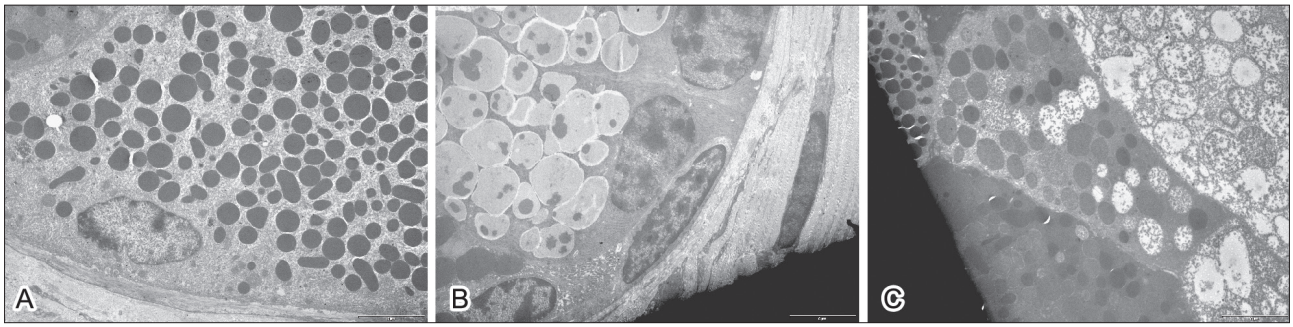


**Fig. 4.** Electron micrographs show parts of different cell types in various skin glands of *L. graecus.* The proteinaceous granules are the most electron-dense and relatively small (**A**). Mucous granules are larger; see mucous glands in Figs. 1-3 and Supplementary Fig. S1: glycoprotein-containing granules are uniformly electron-dense, while acid mucous granules have dense core(s) within the electron-translucent background (**B**). Clear/vacuolated granules have more or less obvious lacy, electron-dense material (**C**). Please note the gradual change in granule composition within neighbouring cells from left to right in C. Original magnification 3000×, scale bar 5 µm.

**Table 1.** Overview of dermal gland types and their functions in different morphs of *Lissotriton graecus*

| **Dermal gland** | | **Larva** | **Paedomorph** | **Metamorph** |
|---|---|---|---|---|
| **Type** | **Function of secretory products** | | | |
| Serous | Protection against predators and infections; communication through scent marking | Present | Not present | Not present |
| Mucous | Maintaining a moist, slippery surface protects against physical damage and serves as a selective barrier against pathogens | Present | Present | Present |
| Mixed | All of the above | Present | Present | Present |

**Table 2.** Results of morphometric analysis of the skin in different morphs of *Lissotriton graecus*

| | | **Larva** | **Paedomorph** | **Metamorph** |
|---|---|---|---|---|
| Thickness (µm) | epidermis | 65.9±4.2 | 30.4±1.4 | 36.5±2.4 |
| | dermis | 61.6±3.0 | 63.8±3.4 | 69.2±5.3 |
| | skin (e+d) | 127.4±5.2 | 94.3±3.4 | 105.8±4.0 |
| Gland diameter (µm) | | 83.6±5.4 | 68.8±5.4 | 80.6±4.0 |
| Gland N/mm$^2$ of skin section | | 43.0±6.0 | 60.2±6.0 | 54.8±3.9 |
| Leydig cells N/mm$^2$ of skin section | | 296.5±29.9 | 137.2±22.7 | 4.4±4.4 |

Data are presented as the mean±SEM

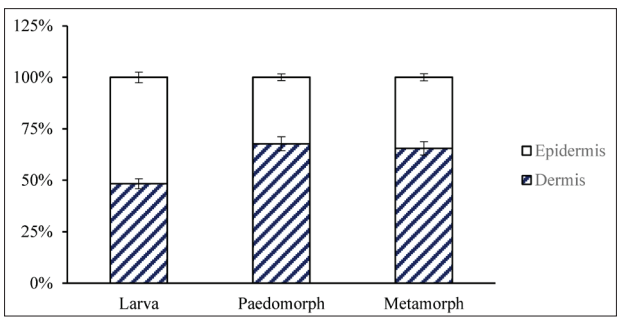


**Fig. 5.** Proportion of epidermis and dermis in the total skin thickness of larva, paedomorph and metamorph of *L. graecus*. In larva, the proportions of epidermis and dermis are approximately equal, whereas in paedomorph and metamorph the dermis predominates.

## DISCUSSION

Studies dealing with histological and histochemical changes in amphibian skin during its development, especially in urodeles, are very rare [11,13,23]. In addition, the histological organization of the skin in paedomorphic amphibians has not yet been sufficiently investigated [6]. Therefore, the present study focused on the histological and histochemical characteristics of the skin of the Greek smooth newt, *L. graecus*. In addition to the canonical biphasic life cycle, which includes an aquatic larval stage that metamorphoses into a terrestrial adult stage [30], smooth newts are characterized by facultative paedomorphosis, where 2 adult morphs may coexist in the same population. Like the metamorphs, the paedomorphs also develop from aquatic larvae, but become sexually mature without metamorphosis (they retain larval characteristics such as gills) and remain in the aquatic habitat [2]. However, this study should be considered exploratory histological documentation, specifically a descriptive histomorphological and histochemical case study, because biological

variability cannot be assessed due to the critically small sample size (N = 1 individual per morph).

The epidermis of aquatic morphs, larvae and paedomorphs of *L. graecus* was characterized by Leydig cells. However, they showed different predominant structures in the cytoplasm: granules in the larvae and Langerhans' net (peripheral cytoskeleton) in paedomorphs. This indicates different functions of these cells in the different morphs. Literature data suggest that Leydig cells have both secretory and mechanical roles [19]. Based on the presence of secretory granules in the typical Leydig cells and of acid mucins in altered Leydig cells, we assume that secretion is the main role of these cells in the larvae of *L. graecus*. Since the granules were not observed to discharge onto the skin surface, their contents may be released within the epidermis and provide antimicrobial protection [31]. On the other hand, synthetic products from altered Leydig cells may contribute to the continuous layer of acid mucins on the skin surface. In addition, the source of the acid mucous layer was superficial epidermal cells with obvious acid mucins in their apical cytoplasm. This is consistent with previous findings that the superficial cells of larvae continuously produce a mucous coat [32]. In the paedomorphs of *L. graecus*, the Langerhans' net was a prominent feature of the Leydig cells. In this morph, the Leydig cells provide the structural stability to the epidermis, based on both the turgor pressure of the hydrated cytoplasm and the restriction by the Langerhans' net [19]. The reduced number of Leydig cells and the change in their function in the paedomorphs of *L. graecus* compared to the larvae go hand in hand with the observed processes of keratinization and the emergence of more numerous keratinocytes with tonofilaments in the paedomorphs. Although one might expect that, due to common environmental niche, the epidermis of paedomorphs resembles that of larvae, our histological results showed a keratinized skin surface in paedomorphs. While the epidermis of adult paedomorphic axolotls is not keratinized [7], it was also reported that paedomorphs of *Triturus alpestris apuanus* showed larval aspects in some cases, while in others they resembled metamorphic newts [6]. It is possible that keratinization in paedomorphic *L. graecus* was merely the embodiment of delayed metamorphosis, as previously suggested [6]. The ongoing transformation of the epidermis should allow adequate adaptation to the terrestrial environment, with the main function being protection against desiccation [9,10]. The epidermis of the terrestrial morph in the present study consisted of keratinized stratified squamous epithelium and was virtually devoid of Leydig cells. The loss of Leydig cells after metamorphosis is well known in urodeles [12,33-35] and is associated with thyroid activity [11].

In the present study, the dermis was well developed in all 3 morphs and showed various skin glands. Dermal glands can develop during or shortly after metamorphosis in Urodela [12,13,21], but they can also develop at the larval/premetamorphic stage [11,14,15], as was the case in the present study in *L. graecus*. Our results showed the presence of 3 different types of glands: serous, mucous and mixed. Serous glands were observed only in larvae, while mucous and mixed glands were found in all morphs. The absence of serous glands in the sexually mature morphs could be attributed to a developmental shift of the protein-secreting activity into the mixed glands, which is particularly evident in the terrestrial morph. According to some authors, the mixed glands could represent an intermediate stage in the transformation of serous glands into mucous glands [18]. Serous gland secretions can be chemically very diverse and serve as protection against predators and infections or are used for scent marking [36,37].

The results of our study show that there are different types of mucous-secreting cells inside the same mucous gland. Since 1 cell type was responsible for the production of acid mucins and the other for the synthesis of neutral mucins complexed with proteins, these glands were considered to be predominantly mucous. Literature data also support the presence of different types of mucins within the same mucous gland in urodeles [13,18]. The secretory products of the mucous glands are important for maintaining a moist, slippery surface that prevents mechanical damage and as a defence barrier against pathogens [36], similar to the mucus that originates from the epidermis.

According to the literature, mixed glands imply the existence of mucous insert of different sizes and location in the granular gland [15,37,38]. However, in our study, the mixed glands exhibited a more complex organization. Based on the number of different cell types within the same gland, we classified them as 2- or 3- component mixed glands. Common to all mixed glands in our study were cell types containing proteins, and clear/vacuolated granules with or without acid

mucous material within. These 2 cell types were the only components of the 2-component mixed glands of *L. graecus*. Histomorphologically, they resembled the serous glands previously reported, where proteins were proposed to be stored either in very dense secretory granules (defence strategy against predators) or in translucent secretory vesicles (regulatory role) [15,21,39]. However, using specific staining for proteins and 2 different mucins, histochemistry demonstrated the presence of acid mucins only, in some clear/vacuolated granules. Taken together, these results indicate that the products stored in the clear/vacuolated granules are at least partially acid mucous in nature and not proteinaceous. The chemical composition of the product(s) in the unstained clear/vacuolated granules remains elusive, but it is certain that they contain non-proteinaceous and non-mucous substances, as indicated by the negative histochemical staining results in our study. Therefore, the traditional definition of the mixed gland is not perfect, implying that the list of secretory cells is not exhausted by the presence of protein- and mucin-containing cells alone. We hypothesise that unstained clear/vacuolated granules may contain biogenic amines, alkaloids, steroids, or lipids, as dermal glands have been reported to contain these compounds [20,21], but further *in situ* studies are required. The mixed 3-component glands had the same cell types as the 2-component glands, plus the additional type with glycoprotein-containing granules, sometimes together with clear/vacuolated granules. In the aquatic morphs examined in this study, clear/vacuolated granules (some of which contained acid mucins) were the predominant component of the mixed glands, whereas in terrestrial morph protein synthesis prevailed. We speculate that this is likely related to the increased need for distinct secretions due to the different physical properties of the habitats.

To summarise, in *L. graecus* there is cellular heterogeneity (coexisting divergent cell types) within the mucous glands (producing both acid and neutral mucins, the latter in association with proteins) and the mixed glands (producing proteins, acid mucins and sometimes neutral mucins, the latter in association with proteins) in all morphs studied. We assume that the purpose of this heterogeneity may be that 1 type of gland can be replaced by or transformed into the other as an adaptation to sudden environmental changes, similar to what has been previously reported [15].

In this study, archival material was used. Despite this, no potential long-term storage artifacts were detected, as there were no issues in making ultra-thin sections, resulting in preserved quality and good electron micrographs. A serious limitation of this study, apart from the aforementioned small sample size, is the lack of detailed metadata (sex, developmental stage, body region) for the tissues used. Therefore, all morphometric results and comparisons are uncertain. With all this in mind, we nevertheless attempted to provide a rough survey of some differences in the measured parameters. In the larval skin, the epidermis and dermis each accounted for about 50% of the total skin thickness. The abundance of Leydig cells in the larval epidermis undoubtedly contributed to this high proportion of the epidermis. In contrast, the epidermis was thinner in both adult morphs, where a reduction or absence of Leydig cells occurred, resulting in the dermis comprising about two-thirds of the total skin thickness. This is consistent with the results for postmetamorphic *Triturus ivanbureschi* [13]. Similar gland frequency and size in the morphs studied is of qualitative rather than quantitative importance, since some urodeles lack dermis and dermal glands entirely in the larval stage [11-13]. Because dorsal and ventral skin can differ in the proportion of gland types and chromatophore distribution [13], we did not investigate these in our study. However, it is important to note that pronounced sexual or body-regional differences are not always present. For example, data indicate a similar proportion of all 3 gland types in both female and male *Triturus karelinii* [18], and there is no difference in the relative thickness of the epidermis and dermis between dorsal and ventral skin in *Triturus ivanbureschi* [13]. Based on the limited literature on developmental changes in facultatively paedomorphic Urodela, and considering that the pond in Velika Osječenica from which the animals were collected no longer exists, we believe that our study is both historically important and a good starting point for future histological research on paedomorphic species.

## CONCLUSIONS

This study provides a detailed histological and histochemical description of the skin of *L. graecus*. Skin development in *L. graecus* paedomorphs is partially uncoupled from sexual maturation. The histological

organization of the paedomorphic skin is a mosaic of larval and metamorphic skin. Leydig cells in the epidermis and many clear/vacuolated cells in the dermal glands are common features with larval skin, while the epidermis/dermis ratio and keratinized surface are common aspects with metamorphic skin. The present results should provide a framework for future studies on the possible uncoupling of sexual and somatic development in various organ systems to gain a deeper understanding of complex development in facultative paedomorphic species.

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**Data availability:** The data supporting this article are available in the online dataset https://www.serbiosoc.org.rs/NewUploads/Uploads/Ukropina%20et%20al_Dataset.pdf

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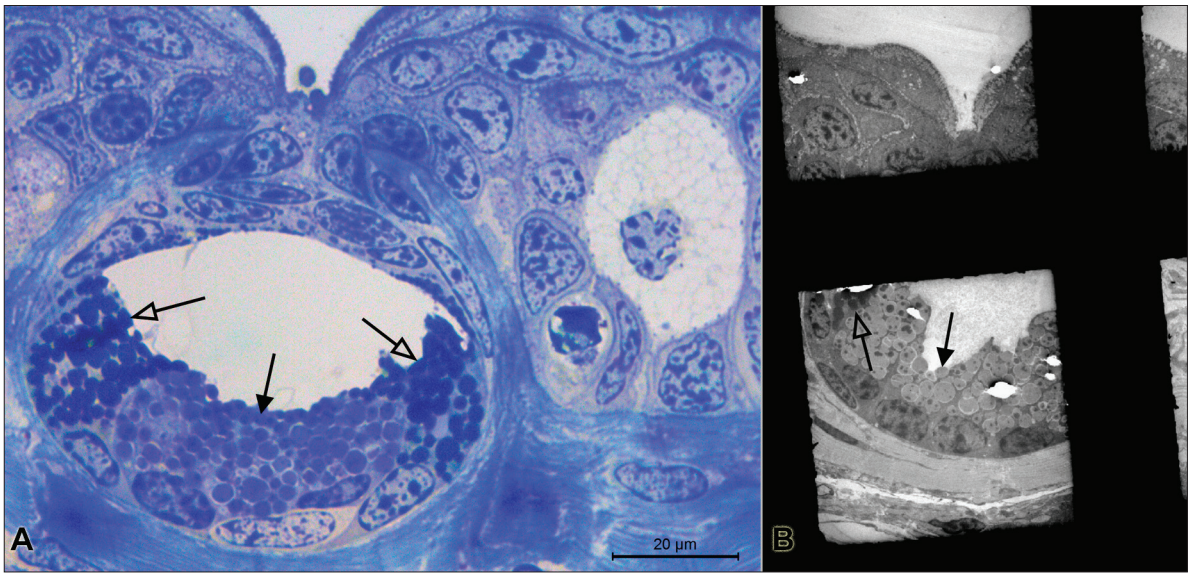
## SUPPLEMENTARY MATERIAL




**Supplementary Fig. S1.** Correlation of the appearance of the mucous glands of *L. graecus* in light (**A**) and electron (**B**) microscopy. Centrally located acid mucous granules (➞) differ from laterally located glycoprotein-containing (➞) granules (see mucous glands in Figs. 1-3). **A** – toluidine blue staining, original magnification 100×; **B** – original magnification 800×.