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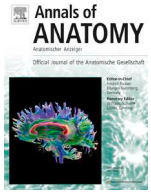

Research Article

Histological changes of the skin during postembryonic development of the crested newt *Triturus ivanbureschi* (Urodela, Salamandridae)

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ABSTRACT

Background: Amphibian skin has been studied for many decades, especially the metamorphic changes in the skin of frogs. Less attention has been paid to salamander skin. Here, we describe changes in the skin structure during postembryonic development in a salamandrid species, the Balkan crested newt *Triturus ivanbureschi*.
Method: Using traditional histological techniques we examined the skin in the trunk region of three pre-metamorphic larval stages (hatchling, mid larval and late larval) and two postmetamorphic stages (juvenile, just after metamorphosis, and adult).
Results: In larval stages, skin consists only of the epidermis, which gradually develops from the single epithelial cell layer in hatchlings, to a stratified epidermis with gland nests and characteristic Leydig cells at the late larval stage. During metamorphosis, Leydig cells disappear, and the dermal layer develops. In postmetamorphic stages, skin is differentiated on stratified epidermis and the dermis with well-developed glands. Three types of glands were observed in the skin of the postmetamorphic stages: mucous, granular and mixed. Gland composition appears to be stage- and sex-specific, with juveniles and adult female being more similar to each other. In juveniles and adult female, there are a similar proportion of glands in both dorsal and ventral skin, whereas in adult male granular glands dominated the dorsal skin, while mixed glands dominated the ventral skin.
Conclusion: Our results provide a baseline for future comparative research of skin anatomy in salamanders.

1. Introduction

The skin of amphibians is a morphologically and physiologically complex organ with numerous vital functions, including respiration, osmoregulation, ion and water transfer, chemical defence, and thermoregulation (Duellman and Trueb, 1994; Erspamer, 1994; Daly, 1995; Larsen, 2021). Amphibian skin is unique among tetrapods as it is relatively fragile, thin, and semipermeable with function in water and gas exchange (Frolich, 1997; Lillywhite, 2006). Amphibian skin has been studied for over a century, but most works focused on frogs (Dawson, 1920; Voute, 1963; Parakkal and Matoltsy, 1964; Billett and Gould, 1971; Fox, 1981; Regueira et al., 2016; Ponssa et al., 2017) while data on salamanders'skin is limited.

Skin structure and thickness may vary across different regions of the body (Centeno et al., 2015), are sexually dimorphic (Wenying et al., 2011), and are subject to seasonal variation (Kobelt and Linsenmair, 1986). As in all other vertebrate groups, the amphibian skin consists of epidermis and dermis. The epidermis is formed by a stratified epithelium, designated as "germinative" (Duellman and Trueb, 1994), "common epithelium" or "keratinized stratified squamous epithelium" (Page et. al, 2009), with the outermost layer consisting of flattened, cornified cells – *stratum corneum*. Generally, the amphibian epidermis is divided into four cell layers: the innermost *stratum germinativum*, the *stratum spinosum*, the *stratum granulosum*, and the outermost layer, the *stratum corneum*. According to Fox (1986), several cell types have been described in the epidermis, including Leydig cells, which are typical of the larval stages and disappear during metamorphosis. The dermis consists of two layers: the upper layer, *stratum spongiosum*, which contains loose connective tissue, chromatophores, blood vessels, and various types of glands, and the *stratum compactum* below, which contains

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alternating layers of collagen fibers (Fox, 1986; Duellman and Trueb, 1994).

Characteristic features of amphibian skin are multicellular dermal glands that secrete their products to the surface through epithelial ducts. These glands are characterized by different secretory products, by which they are classified into two main types: mucous and granular glands, also known as serous or venomous glands (Toledo and Jared, 1995). Mucous glands secrete mucus that enables gas exchange, maintains water balance and is a barrier against pathogens. These glands are smaller than the granular ones and widely distributed over the skin. Granular glands synthesize various chemical compounds such as proteins, lipids, catecholamines, and alkaloids with defence functions (Toledo and Jared, 1995; Antoniazzi et al., 2013). Mixed glands with combined mucus and granular secretion have also been described in amphibian skin, and these glands are common in salamanders (Nicoglu, 1893; Furlotti, 1909; Dawson, 1920; Delfino et al., 1986) and rare in frogs, toads and caecilians.

One of the most dramatic phases during the amphibian life cycle is metamorphosis. During metamorphosis, remarkable changes occur at the morphological, physiological, and biochemical level, including changes in the skin. Therefore, most of the anatomical studies of the amphibian skin are related to the changes during metamorphosis (Kemp, 1963; Fox, 1985; Amano et al., 1995; Tamakoshi et al., 1998), including changes between larval and adult skin in salamanders (Warburg and Lewinson, 1977; Fox, 1988; Wakahara and Yamaguchi, 1996). Studies of skin development and ontogenetic changes are sparse (Brown et al., 1981; Warburg et al., 1994; Chammas et al., 2015), especially for salamanders (Warburg and Lewinson, 1977; Rosenberg and Warburg, 1997; Pederzoli et al., 2002).

The Eurasian genus *Triturus* has been established as a model system for evolutionary and developmental studies (e.g. Ivanović et al., 2007; Vučić et al., 2019; Ajduković et al., 2021). However, data on *Triturus* morphology and anatomy are still incomplete, including the information on skin morphology. The histology of the skin of the Southern crested newt *Triturus karelinii* (Bingol-Ozakpinar and Murathanoglu, 2011; Gürcü et al., 2004), has been documented, but information on changes during ontogeny is still missing. The aim of this study is to describe changes in skin structure and gland composition during postembryonic development, from the hatching to the adult stage, in the Balkan crested newt (*Triturus ivanbureschi*).

2. Material and methods

2.2. Model species

The genus *Triturus* forms a well-supported monophyletic clade of newts within the family Salamandridae and consists of two major groups, the marbled and the crested newts, which have separated from each other by 24 mya (Steinfartz et al., 2007; Wielstra et al., 2019; Rancilhac et al., 2020). According to the current taxonomy, the genus consists of nine species: two marbled (*T. marmoratus* and *T. pygmaeus*) and seven crested newts (*T. anatolicus*, *T. carnifex*, *T. cristatus*, *T. dobrogicus*, *T. ivanbureschi*, *T. karelinii* and *T. macedonicus*) (Wielstra and Arntzen, 2016). These newts have a complex life cycle with an aquatic larval stage and terrestrial postmetamorphs (juveniles and adults), which return to the aquatic habitat during the breeding period. The aquatic period of *T. ivanbureschi* adults is estimated to be three months (Arntzen, 2003; Wielstra et al., 2019). *Triturus ivanbureschi* has a relatively robust body and body size (snout to vent length) ranges from 67 to 82.3 mm for males (Lukanov and Tzankov, 2016), and 60.5 to 91.0 mm for females (Lukanov and Tzankov, 2016; Vučić et al., 2020). In adults, the dorsal and lateral sides of the skin are granulated, while the ventral side is smooth and bright orange with black dots (Wielstra et al., 2013).

Three larval stages were selected for this study: hatchling (stage 42), mid larval stage (stage 47) and late larval stage (stage 62). Larval stages were based on limb development according to the staging table for *Triturus* newts by Glücksohn (1931). At the hatchling stage, the first two digits begin to form on the forelimbs, and there is no evidence of hind limbs. At the mid larval stage, the third digit of the forelimbs is cone-shaped, while the first and especially the second digits are elongated. The hind limbs are not yet developed. The gills are long and branched. The late larval stage is characterized by fully developed front and hind limbs and the gills reach their maximum. From stage 62, the larvae increase in size until metamorphosis.

Metamorphic changes include numerous changes in internal and external morphology. Complete metamorphosis and transition from larval to juvenile stage is marked by the resorption of the gills and closure of the gill slits, when juveniles were collected.

Adult female and male were taken from natural populations in 2014 at the end of the breeding season, while they were in the aquatic phase, and immediately sacrificed to obtain skin samples.

2.3. Experimental settings

Adults were brought from the population of Zli Dol in Serbia (42.423° N, 22.434° E) to the laboratory of the Institute for Biological Research "Siniša Stanković" in 2014 and used for breeding experiments over the years. To obtain material for this study, females and males were crossed in common containers (500 l, filled with dechlorinated tap water) in spring 2021, after overwintering in the refrigerator at a constant temperature. Females laid eggs on plastic strips and eggs were collected daily. Then eggs were transferred to Petri dishes filled with dechlorinated tap water to further develop at controlled laboratory conditions. After hatching, larvae were transferred to 1-litre plastic boxes filled with dechlorinated tap water and five larvae were kept per plastic box. The water was changed every other day. Larvae were fed ad libitum with *Artemia* sp. at early stages, and *Tubifex* sp. at later stages.

Larvae, juveniles and adults were euthanized with ethyl 3-aminobenzoate methanesulfonate, MS 222 (CAS number: 886–86–2; Sigma, St. Louis, MO, USA). After euthanasia, the adult animals were decapitated.

2.4. Tissue preparation and staining methods

Two animals per developmental stage (hatchling, mid larval, late larval and juvenile) and one adult female and male were used. A minimal number of adults were used for skin analysis because adults were derived from a natural population. Hatchlings and larvae were fixed as intact individuals, while dorsal and ventral skin samples were dissected from the trunk region of juveniles and adults and then fixed (Fig. 1A, B). All specimens were fixed in 4% neutral phosphate buffered formalin solution at pH 7.2–7.4.

After washing under running tap water, tissue samples were dehydrated through a graded ethanol series (30–100%), cleared in xylene, and embedded in paraffin wax. Tissue sections (5 µm thick) were stained by the Alcian blue periodic acid-Schiff method (AB-PAS) using a commercially available kit (04–163802 Bio-Optica, Milano S.p.A.) according to the manufacturer's instructions and with bromophenol blue (BPB) according to Mazia et al. (1953). For specificity of staining methods and corresponding results, see Table 1.

Microscopic preparations were analyzed with a Leica DMRB light microscope and photographed with a Leica DFC295 camera (Leica Microsystems, Wetzlar, Germany). The histological organization of the skin of larval stages was analyzed on two nonconsecutive AB-PAS stained slides for each animal. For juveniles and adults, non-adjacent AB-PAS stained sections taken at least 250 µm apart (2–4 slides) were used for both histological and morphometric analysis. Morphometric measurements included the thickness of the epidermis and dermis and the total number and size of glands. The epidermis thickness was measured orthogonally from the basement

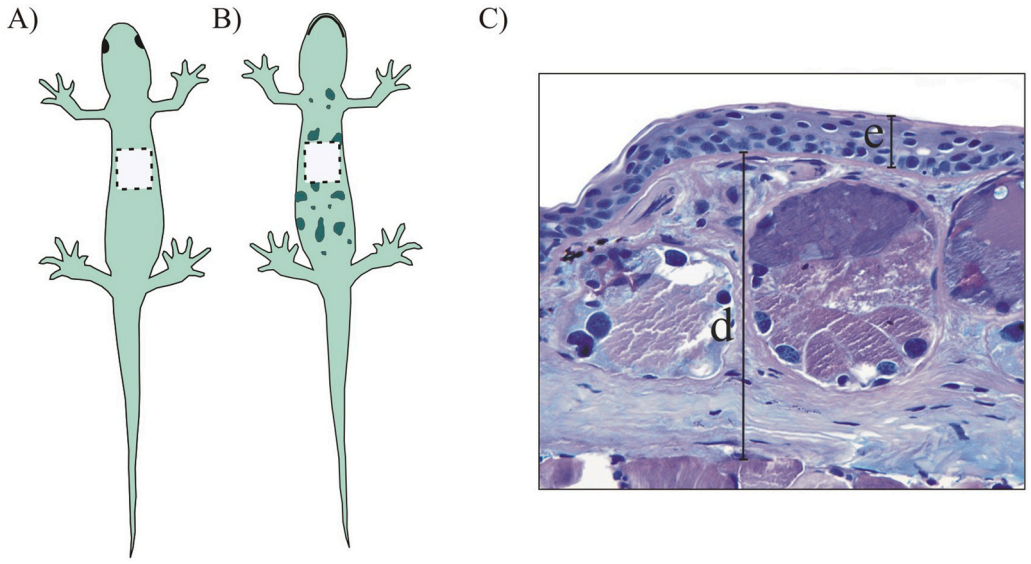

Fig. 1. Detailed description where the skin samples were taken from dorsal (A) and ventral (B) side of the juveniles and adults. Micrograph (C) is showing how morphometric measurements of the epidermis (e) and dermis (d) were taken.

membrane, while a line orthogonal to the orientation of the collagen fibers in the dermis was used to measure dermis thickness (Fig. 1C). The distance of 250 µm, which corresponds to the maximum gland diameter, was used for histological evaluation and morphometric analysis to avoid redundant recording of the glands. Comparable skin areas were analyzed in juveniles, adult female and male (Table 2). On each photomicrograph five measurements have been systematically performed both for epidermis and dermis thickness, expressed as mean value for every image, and presented as the repeated measures for each stage. For this purpose, between 35 and 52 photomicrographs (original magnification 20×) for adults and 70–88 for juveniles per animal and skin region were used for morphometric measurements using the ImageJ 1.45 software (Rasband, 2018).

2.5. Statistical analyses

The proportion of epidermis and dermis to total skin thickness was calculated for postmetamorphic stages (juveniles and adults). To analyse the frequency of gland types in the dorsal and ventral skin, the proportion of each type of gland relative to the total number of glands was also calculated. Statistical significance of differences in skin thickness and the gland's proportion among stages and sexes was tested using the proportion test for pairwise comparisons with Bonferroni correction for multiple comparisons. All statistical analyses were performed in R version 4.2.1 (R Core Team, 2022).

3. Results

3.1. Histology results

For this study, glands are classified according to the predominant synthetic product that prevails in them. Mucous glands are filled with acidic or neutral mucins (stained with AB or PAS). Granular glands contain proteinaceous synthetic products, while at the same time they do not contain acid mucins (negative for AB) but may occasionally contain neutral mucins and/or carbohydrates (positive for PAS). Mixed glands are composed of cells separately dedicated to the production of mucins and proteins. Although some synthetic products appear to be mucous, based on their PAS positivity, sections of the same glands stained with BPB show that cells containing neutral mucins/carbohydrates are simultaneously positive for proteins. Thus, overlapping staining for PAS and BPB on closely adjacent sections demonstrates the presence of glycosylated proteins (Fig. 2).

Table 2
The examined skin area and the total number of glands observed in dorsal and ventral skin of *Triturus ivanbureschi* postmetamorphic stages. N – number of mucous glands (mg), granular glands (gg) and mixed glands (mixg).

	Juveniles	Female	Male
Dorsal skin			
examined skin area (µm^2)	7.90E+06	5.55E+06	7.33E+06
total gland area (µm^2)	2.59E+06	1.68E+06	2.70E+06
total gland area/examined skin area	0.33	0.30	0.37
N mg	115	49	19
N gg	67	33	68
N mixg	54	55	63
Ventral skin			
examined skin area (µm^2)	1.05E+07	4.54E+06	6.88E+06
total gland area (µm^2)	2.51E+06	1.26E+06	2.19E+06
total gland area/examined skin area	0.24	0.28	0.32
N mg	180	56	48
N gg	31	15	41
N mixg	99	51	72

3.1.1. Hatchling (stage 42)

The skin of the hatchlings consists entirely of the epidermis. It contains one row of flattened epithelial cells covered with a thin continuous layer of acid mucins. Beneath the epidermis, melanophores form a discontinuous layer (Fig. 3A).

Table 1
Specificity of staining methods and corresponding results.

Histological stain	Staining results	Chemical composition of stained tissue
AB	Turquoise blue, purple, or dark blue	Acid mucins
PAS	Magenta red	Neutral mucins and carbohydrates
BPB	Dark blue	Proteins

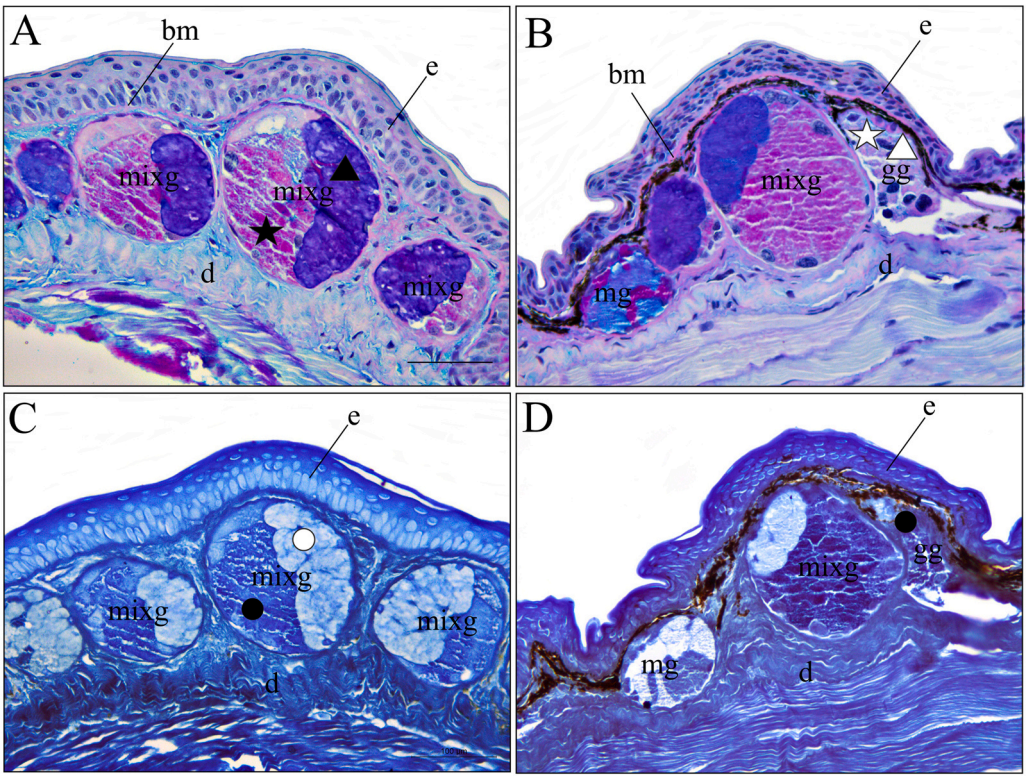

Fig. 2. Closely adjacent skin sections of postmetamorphic *Triturus ivanbureschi* stained with AB-PAS (A, B) and BPB (C, D). The staining outcomes are marked by symbols: AB- (△), AB+ (▲), PAS- (☆), PAS+ (★), BPB- (○), BPB+ (●). Comparison reveals that (i) AB-positive products (▲) are at the same time negative for BPB (○), hence they are acid mucins; (ii) PAS-positive products (★) are at the same time positive for BPB (●), therefore they are glycosylated proteins; (iii) both AB- and PAS-negative products (△, ☆) are positive for BPB (●), i.e. they are proteins. Epidermis (e), dermis (d), basement membrane (bm), mucous gland (mg), granular gland (gg), mixed gland (mixg). Original magnification 20×, bar= 100 µm.

3.1.2. Mid larval stage (stage 47)

At the mid larval stage, the skin consists of two layers of flattened epithelial cells with an acid mucous external coating. Within the epithelium, aggregations of cells with ellipsoidal nuclei can be seen, oriented perpendicular to the free surface of the skin. These aggregations of undifferentiated cells are referred to as nests of future skin glands. Individual melanophores can be seen beneath the stratified epithelium (Fig. 3B).

3.1.3. Late larval stage (stage 62)

Compared to earlier stages, the late larval skin is characterized by a thicker epidermis with epithelial cells arranged in four layers: the lower three rows of cells have large and voluminous nuclei, and the uppermost row consists of flattened cells with correspondingly shaped nuclei. Large translucent Leydig cells are located in 1–2 layers positioned in the middle of the epidermis, i.e. they are surrounded by epithelial cells. They have an oval to ellipsoidal shape with centrally located nuclei. Numerous gland nests can be seen at the lowest level of the epidermis. Similar to the previously described stages, there are superficial acid mucins above the epidermis and individual melanophores below the epidermis (Fig. 3C).

3.1.4. Juveniles

In juveniles, the epidermis consists of the stratified squamous non-keratinized epithelium, in which 4–5 layers of epithelial cells can be seen. The basal layer of epidermal cells is cuboidal to low

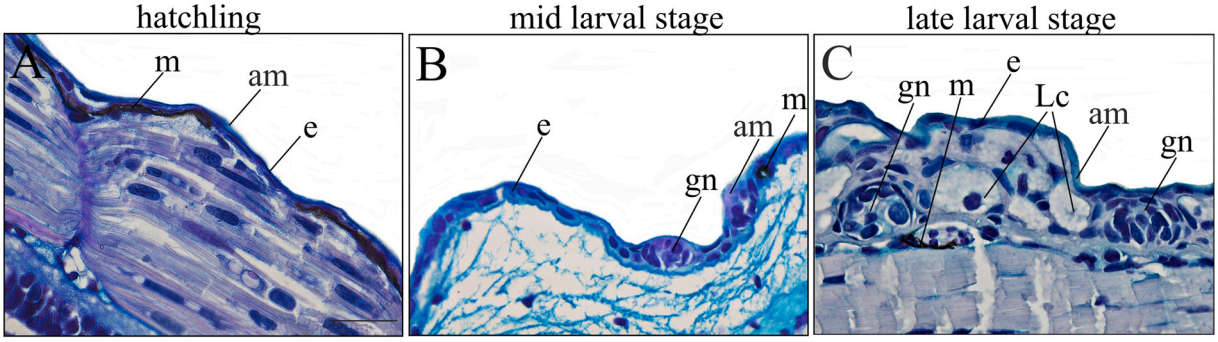

Fig. 3. Histological organization of the skin in premetamorphic *Triturus ivanbureschi* stained with AB-PAS. In hatchlings, stage 42 (A), mid larval stage, stage 47 (B), and late larval stage, stage 62 (C) the skin is composed solely of epidermis (e), which is covered with acid mucus (am). Melanophores (m) are positioned within discontinuous layer underlining the epidermis in all investigated stages. Skin consists of single layer of flattened cells in hatchlings, and two-cell high epidermis with rare gland nests (gn) at mid larval stage. At the late larval stage numerous voluminous Leydig cells (Lc) are evident between epithelial cells, which are occasionally organized into gland nests. Original magnification 40×, bar = 50 µm.

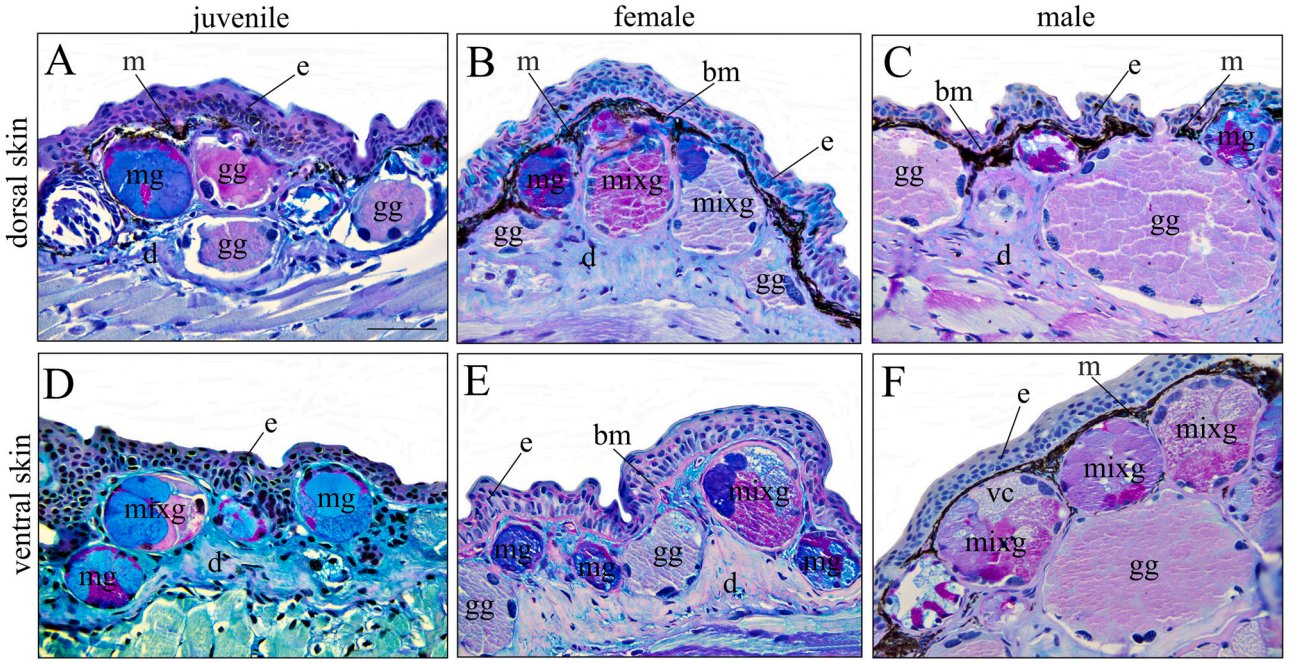

Fig. 4. Histological organization of the dorsal and ventral skin in postmetamorphic *Triturus ivanbureschi* stained with AB-PAS. In juveniles (A, D), and both adult female (B, E), and male (C, F) skin is composed of epidermis (e) and dermis (d) which is composed of connective tissue that houses the discontinuous subepidermal layer of melanophores (m). The epidermis is composed of stratified squamous non-keratinized epithelium and is not covered with acid mucus. Basement membrane (bm) is visible between epidermis and dermis in adult individuals only. Three types of skin glands are present within the dermis in postmetamorphic animals: mucous gland (mg), granular gland (gg) and mixed gland (mixg). Distinct vacuolated cells (vc) are more often observed within glands positioned in ventral skin. Original magnification 20 ×, bar= 100 µm.

columnar, while the uppermost cells are flattened with correspondingly shaped nuclei. The superficial acid mucous layer is not detectable, as opposed to the larval stages. The dermis is composed of connective tissue that houses the discontinuous subepidermal layer of melanophores (Fig. 4A, B, C, F). The most prominent histological feature of the dermis, in both dorsal and ventral skin, is the presence of numerous multicellular skin glands. All three types of glands are present and usually arranged in a single row, but glands in two rows may also be found. In juveniles, the majority of cells in the mucous glands appear to be filled with AB-positive acid mucins (Fig. 4A, D).

3.1.5. Adults

In adults, the stratified squamous non-keratinized epithelium consists of 5–6 layers of epithelial cells that gradually change shape from the base to the top of the epidermis. A thin, well-defined PAS-positive basement membrane is clearly visible beneath the epidermis (Fig. 4B, C, E). Similar to juveniles, the melanophores form a more or less continuous layer under the epidermis. The connective tissue of the dermis appears to be better developed than in juveniles, especially under the glands. The dermal glands may be arranged in two rows, but this arrangement is observed more frequently in male. As in juveniles, all three types of glands are present (Fig. 4B, C, E, F). The nucleus of the AB- and PAS-positive cells is usually flattened against the base of the cell by accumulated secretory product. On the contrary, the nucleus of the BPB-positive cells is typically round or oval (Fig. 2C, D). The contours of individual secretory cells are commonly discernible, although syncytial organization is also present in the whole gland or just in part of it. The syncytial organization is mostly seen in glands packed with BPB-positive synthetic products. Also, particular gland cells with cytoplasm filled with numerous translucent granules characterise adult glands. These cells are here referred to as vacuolated cells and are found primarily in the ventral skin of male (Fig. 4F). In the mucous glands, neutral mucous cells (PAS-positive) appear to predominate, which is especially evident in male, while acid mucous cells (AB-positive) are not as common, unlike in juveniles. Summarised results of the skin histological organization during postembryonic development, from hatchling stage to adult, are presented in Table 3.

3.2. Morphometric results

Statistically significant differences in the relative thickness of the epidermis and dermis for dorsal and ventral skin are not found between juveniles and adults (Table 4; Fig. 5, $P > 0.05$, in all pairwise comparisons).

Table 3
Overview of changes in the skin histological organization of *Triturus ivanbureschi* during postembryonic development starting from hatchlings (stage 42), over premetamorphic, mid larval (stage 47) and late larval (stage 62) to postmetamorphic stages (juveniles and adults).

	Premetamorphic stages			Postmetamorphic stages	
	Hatchling	Mid larval	Late larval	Juveniles	Adults
Epidermis / free surface	Acid mucins	Acid mucins	Acid mucins	Flattened cells	Flattened cells
Non keratinized epithelium	Simple squamous	Stratified squamous	Stratified squamous	Stratified squamous	Stratified squamous
Number of cell layers	1	1	4	4-5	5-6
Leydig cells	Not present	Not present	Present	Not present	Not present
Gland nests	Not present	Present	Present	Present	Not present
Basement membrane	Not visible	Not visible	Not visible	Not visible	Visible
Dermis / Glands	Not present	Not present	Not present	Present	Present

Table 4
Mean thickness of the epidermis and dermis in micrometers (μm) for dorsal and ventral skin of postmetamorphic stages (juveniles, female and male). N – the number of repeated measures for each stage.

	Dorsal skin		Ventral skin	
	N	Mean ± StdDev	N	Mean ± StdDev
Epidermis				
Juveniles	70	40.4 ± 11.8	88	34.4 ± 4.0
Female	43	42.1 ± 6.7	35	48.7 ± 7.3
Male	50	32.0 ± 6.0	52	35.1 ± 7.3
Dermis				
Juveniles	54	160.6 ± 65.1	55	117.3 ± 35.5
Female	29	191.4 ± 50.1	29	179.5 ± 49.6
Male	42	254.8 ± 50.3	45	251.0 ± 49.6

The proportion of mucous, granular, and mixed glands relative to the total number of glands in the dorsal and ventral skin of juveniles, adult female and male is shown in Fig. 6. At all postmetamorphic stages, mucous glands are more numerous in the ventral than in the dorsal skin. Opposite is the state of granular glands, which are more abundant in the dorsal skin than in the ventral. The proportion of mixed glands is similar in the dorsal and ventral skin of adult animals, but in juveniles, mixed glands are more abundant in the ventral skin (Fig. 6). Comparisons of gland proportion between postmetamorphic stages (Fig. 6) revealed that juveniles and female do not differ ($P > 0.05$, in all pairwise comparisons), while there is a significant difference between juveniles and male in gland proportion in both, dorsal and ventral skin ($P < 0.001$, in all pairwise comparisons). Adult female and male differ in proportions of mucous and granular glands in dorsal skin ($P < 0.001$, in both comparisons), with no difference in other gland's proportions ($P > 0.05$, in all pairwise comparisons; Fig. 6).

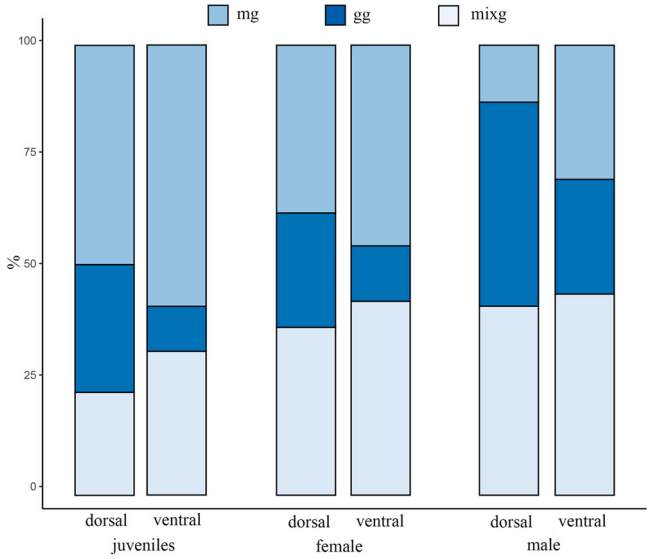

Fig. 6. Frequency distribution of the three gland types in the dorsal and ventral skin at different postmetamorphic stages (juveniles, female, male), expressed as mean percentage of a particular gland type on the total number of glands.

4. Discussion

We documented remarkable changes in skin structure during the postembryonic development of *Triturus ivanbureschi*. At early and mid-larval stages, skin only consists of one (hatchlings) or two layers of epithelial cells (mid larval) and is covered with continuous acid mucins. Glands gradually develop and gland nests, from which future glands will be formed, are clearly visible in the epidermis at mid larval stage. Leydig cells are visible only at the late larval stage. In some salamander species such as *Hynobius retardatus* (family Hynobidae), Leydig cells appear at the hatchling stage (Ohmura and Wakahara, 1998), much earlier than in *T. ivanbureschi* larvae. These cells occupy most of the epithelial volume of the larval skin and there is no general agreement on their role (Brunelli et al., 2022). According to some authors (Kelly, 1966; Warburg et al., 1994), the function of Leydig cells may be water retention related to aquatic life during the larval stage. Other authors claim that the main function of these cells is mucus secretion (Leydig, 1876; Seeger, 1933; Quagliata et al., 2006). Leydig cells increase in size during larval growth and during metamorphosis they are replaced by stratified squamous epithelium as an adaptation to terrestrial life (Kelly, 1966; Kato and Kurihara, 1987; Ohmura and Wakahara, 1998). The late larval stage is the one with the thickest epidermis due to increase of epidermal cell layers and the presence of Leydig cells.

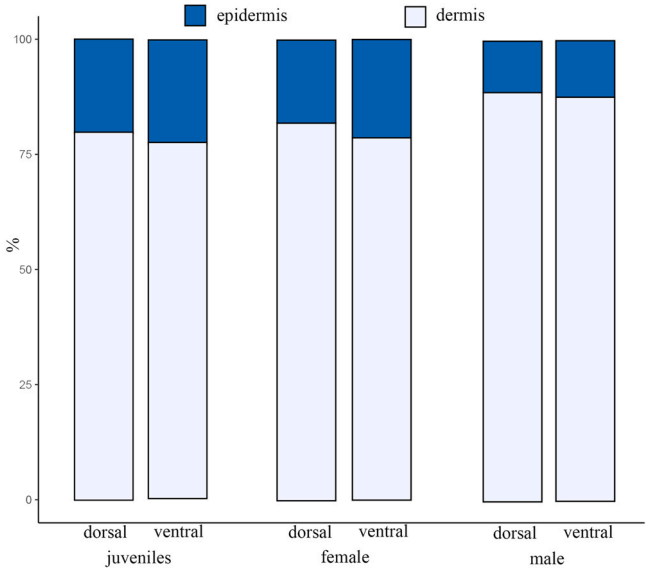

Fig. 5. Proportion of epidermis and dermis, expressed as mean percentages of total skin thickness, in postmetamorphic stages (juvenile, female and male). Total N are reported in Table 4.

Clear differentiation of skin into epidermis and dermis is evident in juveniles and adults. The epidermis is formed of stratified, squamous, non-keratinized epithelium, constructed of epithelial cells, but without clear differentiation into four distinct layers as described in the smooth newt, *Lissotriton vulgaris* (Perrotta et al., 2012). However, the absence of differentiation of the epidermis was also recorded in the phylogenetically closely related *Triturus karelinii* (Gürcü et al., 2004). It seems that the absence of differentiation of the epidermis and unclear demarcation between the two dermal layers, *stratum spongiosum* and *stratum compactum* characterise crested newts (Gürcü et al., 2004, this study).

In the case of *T. ivanbureschi*, there is no difference in the relative thickness of the epidermis and dermis between dorsal and ventral skin in the trunk region of juveniles and adults. In some species, skin thickness, especially of the epidermis, can vary across different regions of the body, during the season, between the sexes and during different phases of the life cycle (Warburg and Lewinson, 1977; Brown et al., 1981; Warburg et al., 1994; Lili et al., 2013). Sexual dimorphism in skin thickness and variation in this trait indicates that the two sexes might differ in their microhabitat preferences, particularly with regard to temperature and moisture requirements (VanBuren, 2017). Also, according to VanBuren et al. (2019), differences in skin thickness could be explained by body size. However, the absence of sexual dimorphism in relative thickness obtained in this study should be interpreted with caution due to the low sample

size and to the fact that adults were still in aquatic phase. The absence of keratinized layer, *stratum corneum* in both, juveniles and adults can be also explained by aquatic phase.

Basement membrane separates the epidermis from the underlying connective tissue in the dermis. The functional role of the basement membrane is to provide physical and biochemical cues to the overlying cells (Khalilgharibi and Mao, 2021). This membrane characterizes both the larval and adult skin of salamanders (*Ambystoma punctatum* and *A. opacum*) and anurans (*Rana pipiens* and *Xenopus laevis*) (Weiss and Feriss, 1954; Kemp, 1961). However, during the postembryonic development of *T. ivanbureschi* skin, this membrane is clearly visible only in adults, but not in juveniles, despite largely similar skin organisation. As all epithelia rest on the basement membrane (Ross and Pawlina, 2006), it is very likely that in *T. ivanbureschi* basement membrane is present at larval and juvenile stages, but as thin and not very prominent. As such, it could be difficult to detect it by the light microscopy.

Analysis of the skin structure of *T. karelinii* revealed the presence of all three types of glands, with a similar proportion in females and males (Bingol-Ozakpinar and Murathanoglu, 2011). Our observation of *T. ivanbureschi* skin also reveals the presence of all three gland types, but with some differences in their proportions among postmetamorphic stages. The most prominent differences were between juveniles and adult male in the proportion of all three types of glands. Adult female was similar to juveniles and differed from adult male only in proportion of mucous and granular glands in the dorsal skin. It is interesting to note that in another newt species, *Tylototriton verrucosus*, skin consists of mucous and granular glands and there are no mixed glands at all (Wanninger et al., 2018).

The functional role of mixed glands in urodele skin is still unclear, but some authors propose that these glands might represent an intermediate stage from granular into mucous glands conversion (Dawson, 1920; Bingol-Ozakpinar and Murathanoglu, 2011). Dawson (1920) suggests that amphibians need much more mucous secretion to preserve skin moisture in unfavourable environment and this condition can be achieved by conversion of granular to mucous cells. According to Delfino et al. (1982), ultrastructural analysis of mixed glands reveals that they originate from specific Anlagen (gland nests) in early larval stages in several salamander species including *Triturus cristatus*. Comparative study of skin, particularly mixed glands, of crested newt species with different habitat preferences (for example, largely aquatic *T. dobrogicus*) and more distantly related marbled newts (even more terrestrial than *T. ivanbureschi*) during postembrionic development will shed light on the evolution of these glands and their adaptive significance.

5. Conclusion

Changes in skin histological organization during postembryonic development in *Triturus ivanbureschi* are evident, and the most prominent differences are between the mid- and late larval stage related to the development of Leydig cells, as well as changes related to metamorphosis. In postmetamorphic stages, all three types of skin glands (mucous, granular and mixed) are well differentiated, with some sex- and stage specific differences in gland proportions. There are no differences in relative skin thickness among postmetamorphic stages. We describe and summarize skin histological organization as a baseline for further comparative research. The data on skin development in various salamandrid species would provide a more detailed insight into ontogenetic and evolutionary changes of salamander skin.

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Ethical statement

The collection of *T. ivanbureschi* adults from a natural population was approved by the Ministry of Energy, Development and Environmental Protection of the Republic of Serbia (Permit No. 353–01–75/2014–08). The experimental procedure was approved by the Ethics Committee of the Institute for Biological Research "Siniša Stanković" and the Veterinary Administration of the University of Belgrade and Ministry of Agriculture, Forestry and Water management of the Republic of Serbia (Decision No. 323–07–01372/2021–05). The animals were handled in accordance with the European Directive (2010/63/EU) on the protection of animals used for experimental and other scientific purposes.

Author contribution

Maja Ajduković: Writing – original draft, Investigation, Visualization, Writing – review & editing. **Mirela Ukropina:** Investigation, Visualization, Writing – review & editing. **Milena Cvijanović:** Investigation, Writing – review & editing. **Tijana Vučić:** Investigation, Formal analysis, Visualization, Writing – review & editing. **Ana Ivanović:** Supervision, Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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