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The differential role of Leydig cells in the skin and gills of *Lissotriton italicus* larvae

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Abstract

Larval urodeles are provided with external gills involved, along with the skin, in gas exchange and osmoregulation. Gills and skin epithelia are different, each showing a peculiar set of specialized cells but both provided with Leydig cells (LCs). Information on LCs in the gills is lacking as the literature has focused primarily on the epidermis. Contradictory and fragmentary results highlight that LCs origin, fate, and functions remain not fully understood. Here, we investigated the morpho-functional differences of LCs in the skin and gills of *Lissotriton italicus* larvae for the first time. LCs showed the same morphological and ultrastructural features in both tissues, even if LCs were significantly larger in the epidermis. Despite the uniform morphology within the LCs population, the proliferative ability was different. The putative diversity in the mucus composition was evaluated using a panel of 4 lectins as markers of specific carbohydrate moieties, revealing that sites of specific glycoconjugates were comparable in two tissues. To disclose the involvement of LCs in water storage and transport, immunofluorescence assay for aquaporin-3 has also been performed, demonstrating the expression of this protein only in gills epithelium. By demonstrating that LCs can multiply by cell division in gills, our results will also contribute to the discussion about their proliferative ability. Finally, we found that the LCs cytoplasm is rich in glycoconjugates, which are involved in many diverse and essential functions in vertebrates.

Research Highlights

- In gills LCs can multiply by cell division and express aquaporin-3 demonstrating a tissue-specific role of LCs.
- LCs cytoplasm is rich in glycoconjugates.
- LCs population show a uniform morphology in both gills and skin.

KEYWORDS

aquaporin, Leydig cells, Newts, respiratory epithelia

1 | INTRODUCTION

During larval life, urodeles typically present external gills, which disappear by the onset of metamorphosis, and are regarded as the sites of gas exchange and osmoregulation along with the skin (Duellman & Trueb, 1994; Lewinson et al., 1982, 1984). The stratified larval epithelia of gills and skin differ in the thickness and the presence of a peculiar set of specialized cells besides ordinary cells (Fox, 1986; Perrotta et al., 2012). However, in both cases, it is possible to recognize large Leydig cells (LCs) representing the most prominent feature in larval urodeles' epithelium (Greven, 1980; Leydig, 1876; Rosenberg et al., 1982). LCs differentiate during the early embryonic and larval stages, increasing in size during larval growth until they reach conspicuous dimensions and occupy most of the epithelial volume (Rosenberg et al., 1982). Mature LCs possess unique morphological ad ultrastructural features that include wide dimensions, a clear cytoplasm, and a diagnostic tonofilamentous network, also called Langerhans net, at the cytoplasm periphery (Jarial, 1989; Kato & Kurihara, 1987; 1988; Kelly, 1966; Rosenberg et al., 1982). It was previously believed that LCs were limited to the larval urodeles, but these cells have also been found in the epidermis of paedomorphic urodeles, such as axolotls and larval Caecilians (Fox, 1987, 1988; Jarial & Wilkins, 2003).

Over the years, LCs have attracted researchers' attention multiple times, and some specific characters of these cells have been investigated, leading, however, to contradictory and fragmentary results. First of all, the ontogenesis of LCs has been discussed several times. Although it is commonly assumed that LCs arise from undifferentiated basal cells (BCs; Fox, 1986; Leydig, 1876; Rosenberg et al., 1982), several authors suggest that LCs number increases during larval growth due to mitosis of fully differentiated LCs (Gerling et al., 2012 and references therein; Kelly, 1966). In addition, for what concerns the functional role of these cells, there is no general agreement. Based on available data from the oldest and most recent literature (Langerhans, 1873; Leydig, 1876; Lindinger, 1984; Quagliata et al., 2006; Seeger, 1933), LCs have often been presumed to be gland-like mucus-secreting cells. Whether mucus substances are released onto the epidermal surface has been questioned based on the absence of an open to the outer surface. Direct contact of LCs with the exterior through pores has been reported only in the skin of *Ambystoma mexicanum* Shaw, 1789 (Jarial, 1989) and in gills of *Salamandrina terdigitata* Bonnaterre, 1789, and *Triturus carnifex* Dubois & Breuil, 1983 (Brunelli et al., 2009). Otherwise, the mucous has been suggested to be released within the intercellular spaces providing an internal fluid reserve (Kato & Kurihara, 1988) or protection from viruses and bacteria (Jarial, 1989). One of the key features of mucus-producing cells in vertebrates is the presence of granules typically rich in glycoconjugates. Though the nature of the granules-content of LCs has been extensively discussed and the results regarding the glycoconjugates occurrence in LCs, evaluated through histochemical methods, are very conflicting (Greven, 1980; Jarial & Wilkins, 2003; Lewinson et al., 1987; Rosenberg et al., 1982; Warburg, Lewinson & Rosenberg, et al., 1994). Another proposed function for these cells is a water-retaining role supported by histochemical and ultrastructural evidence showing the electron-lucent content, presumably aqueous, of LCs vacuoles (Kelly, 1966; Warburg, et al., 1994). More recently, it has been suggested that a secretory role may be limited to the early stages of larval life while the ultrastructural features of mature cells (i.e., Langherans' network) indicate a mechanical and supportive role of these cells (Brunelli et al., 2009).

While the substantial past research effort, these cells are currently overlooked, except for a study on epidermal LCs in larval and paedomorphic *A. mexicanum* (Gerling et al., 2012) and the origin, fate, and function of these cells remain not fully understood. It should be emphasized that major contributions to literature concerning the properties, structure, and functional features of LCs come from studies conducted on the epidermis (Fox, 1988; Gerling et al., 2012; Jarial, 1989; Kato & Kurihara, 1987; Kelly, 1966). However, the complexity and diversity of body compartments complicate a generalized analysis, and the tissue-specific role of LCs has not received enough attention. According to Brown and Cai (2007), in amphibians, the same cell type can have a different fate depending upon its location. Moreover, differences in cellular composition between skin and gills suggest that, although similarities exist, these two epithelial tissues are perhaps more distinct than often acknowledged (Yoshizato, 1990). To the best of our knowledge, only a few investigations have been conducted on the LCs specific role in gills. Here, we investigated for the first time the morpho-functional differences of LCs in the skin and gills of *Lissotriton italicus* (formerly *Triturus italicus*, Peracca, 1898) larvae. We first assessed the histological and ultrastructural analysis of LCs in both tissues to disclose a putative structural variety of LCs population. In a second step, we applied histochemical methods to identify and visualize glycoconjugates as major components of mucous-substances. For this purpose, we selected a panel of 4 plant-based lectins that recognize specific carbohydrate moieties within glycoconjugates and are commonly used as tools for functional, histological, and biochemical studies (Chandrasekaran et al., 2016; Manalo et al., 2016).

Moreover, to evaluate the involvement of LCs in water storage and transport, immunofluorescence assay for aquaporin-3 (AQP3) detection has also been performed. The AQP3 belongs to the subfamily of aquaglyceroporins (AQPs) and is expressed in most types of epithelial cells where it is involved in the transport of water, glycerol, and hydrogen peroxide. Accumulating literature data indicates that AQP3 is involved in many cellular functions (Bollag et al., 2020). It has also been reported that AQP3 may play a pro-proliferative role, also increasing the expression of several differentiation markers (Guo et al., 2013; Hara-Chikuma & Verkman, 2008a, 2008b). To the best of our knowledge, the study presented here is the first report focusing on the morphofunctional differences of the LCs in urodeles epithelia, and it may contribute to fill a gap of knowledge on this topic.

2 | MATERIAL AND METHODS

2.1 | Study species

The newt *L. italicus* is an endemic species distributed in central and southern Italy, which colonizes a wide range of habitats, including

man-made water bodies, breeding preferential in lentic, or slow-flowing water (Sindaco et al., 2006). This species is listed in Appendix II of the Bern Convention and included in Annex IV of the EU Habitat Directive, as Least Concern (Rondinini et al., 2013), given its relatively wide distribution.

2.2 | Animals collection

All the larvae for the present study used were collected from a field in a natural pond near Cosenza (Calabria, Southern Italy) during the breeding season. Larvae were transferred to the laboratory using 2 L plastic containers filled with water taken from the original pond and then carefully transferred in 50 L glass aquaria filled with aerated and dechlorinated tap water at a median pH of 7.3, following the acclimation procedures. Larvae were kept at room temperature under natural light/dark cycle and fed on brine shrimp nauplii (*Artemia salina* Linnaeus, 1758) on alternate days.

Water quality parameters were regularly measured with a hand-held multi-parameter PCE-PHD 1 (PCE Instruments UK Ltd.) and keep constant.

The development stage was determined under a stereomicroscope (Leica MZ APO equipped with Canon camera) based on distinctive morphological characters according to larval development chronological tables (Bernabò & Brunelli, 2019). In *L. italicus*, gills appear as slightly protruding buds lengthen as the larval development proceeds. The branching of the first gill filament begins at stage 35 and gills reach the maximum development at stage 52a. Therefore, for the comparative analysis, we selected two stages of development during which the gills were well developed and not yet in regression: 51b (the larva is well developed and the hind limb have digits), and 52a (maximum development of gills).

For this study, six larvae at the stage 51b (mean body length: 23 mm ± 0.1) and six at stage 52a (mean body length: 28 mm ± 1) were used. Selected larvae were provided with a unique identification number and kept separate to estimate the putative influence of developmental stage and/or individual differences on the experiments' results. Larvae that have not been used for the present study were released at the capture site.

2.3 | Tissues preparation

All procedures for animal handling and tissues removal have been carried out with the most care to avoid any suffering or harm to the animals according to the recommendations of the Ethical Committee of the University of Calabria and with the approval of the "Ministero dell'Ambiente e della Tutela del Territorio (Direzione per la Protezione della Natura)", permit number 2004/30911.

Each larva, was euthanized by immersion in a solution of 0.1% tricaine methane sulfonate (MS-222, Sigma-Aldrich Chemicals Co., St. Louis, MO, USA), before the dissection phases. Gills and small pieces of skin, together with the underlying dermal and muscular layers, were quickly excised and placed into the appropriate fixative solution for subsequent steps of the processing procedures.

2.4 | Light microscopy and transmission electron microscopy

For conventional light microscopy (LM) and transmission electron microscopy (TEM), tissues were subjected to primary aldehyde fixation in 3% glutaraldehyde (in 0.1M phosphate buffer, pH 7.2; (Electron Microscopy Sciences, Hatfield, PA, USA) for 3 h at 4°C followed by postfixation in in buffered 1% osmium tetroxide. The samples were then dehydrated through a graded series of ethanol, soaked in propylene oxide and embedded in Epon-Araldite (Araldite 502/Embed 812, Electron Microscopy Sciences).

Blocks were cut using a Leica UltraCut UCT (Leica Microsystems, Wetzlar, Germany). Semithin sections (1–2 μm) for LM were stained with toluidine blue and were examined with a LM Leitz Dialux 20 EB (Leica Microsystems). Ultrathin sections (800 Å) were stained with uranyl acetate replacement, and lead citrate (Electron Microscopy Sciences) and observed under a Zeiss EM 10 electron microscope (Zeiss, Oberkochen, Germany).

2.5 | Confocal microscopy, immunohistochemistry, and histology

For immunohistochemical analysis, samples were fixed in Bouin's solution (freshly made) for 24 h at 4°C, dehydrated in ethanol, cleared in xylene, embedded in paraffin wax with a mean fusion point of 56°C and cut using a Leica RM2125 RTS rotary microtome (Leica Microsystems, Wetzlar, Germany). For aquaporin 3 detection, deparaffinized 8 μm sections were mounted on slides and then subjected to the indirect immunofluorescence technique. The sections were rinsed with distilled water and phosphate-buffered saline (PBS, pH 7.4), and incubated for 10 min in a moist chamber with 20% normal sheep serum to block nonspecific sites. Unwashed sections were incubated overnight at 4°C with a rabbit polyclonal antiwater channel AQP3 (Sigma-Aldrich Chemical Co., St. Louis, MO, USA; working dilutions of 1:100). To check the specificity of the immunostaining, the primary antiserum was substituted with normal sheep serum at a dilution of 1:100 in PBS in the control sections.

After several washes in PBS, the slides were incubated for 30 min at room temperature in the dark with fluorescein isothiocyanate (FITC)-conjugated antirabbit IgG (Sigma-Aldrich Chemical Co.) at working dilutions of 1:50. For nuclear counterstaining, propidium iodide (Sigma-Aldrich Chemical Co.; 1:200 in PBS), was applied for 30 s. Slides were then washed again in PBS and mounted.

For lectin-binding study, deparaffinized 8 μm sections were incubated for 30 min with FITC-conjugated lectins at working dilutions 50 μg/mL in PBS (Table 1; Vector Laboratories, Burlin-game, CA, USA), washed three times in PBS, and mounted. Lectin-binding

TABLE 1 Carbohydrate binding specificity of tested lectins

Source of lectin	Abbreviation nominal	Carbohydrate binding specificity
Maclura pomifera	MPA	α\\-acetylgalactosamine, > α\ \-galactosamine
Triticum vulgaris	WGA	*N*-acetyl-D-glucosamine, *N*-acetyl neuraminic acid
Triticum vulgaris	WGAs *Succinylated	*N*-acetyl-D-glucosamine
Ricinus communis	RCA-I	α\-D-galactose

specificity was tested by preincubation of the lectin conjugates with 0.2M solutions of the nominal specific sugars (Sigma). All staining procedures were conducted at room temperature in the dark. Observations were performed by using a Leica TCS SP2 Confocal Laser Scanning Microscope (Leica Microsystems).

For histological analysis, sections of 6 μm were deparaffinized and stained with hematoxylin and eosin (H&E; Bio-Optica, Italy) and observed using an LM Leitz Dialux 20 EB (Leica Microsystems).

To localize glycosaminoglycans and glycoproteins, tissue sections were stained by Periodic Acid - Schiff Method (PAS kit 04-130802, Bio-Optica, Milan, Italy) and observed using an LM Leica ICC50W (Leica Microsystems). The dimensions of LCs were measured on H&E stained sections using ImageJ software version 1.53a (National Institutes of Health, Bethesda, MD, USA, available at http://imagej.nih.gov/ij). For each animal both gills and skin were examined; four images and about five measures (width and length) per picture were made for both organs. Histological sections for measures were taken with a 50 μm distance between consecutive sections to avoid double-counting of LCs. Statistical analysis to compare LCs dimension in skin and gills sections was performed using GraphPad Prism 2007 version 8.0.2 for Windows (GraphPad Software, Inc., San Diego, CA, USA). Values indicating the dimension of LCs have been compared using the Student *t*-test. Kolmogorov–Smirnov test was used to evaluate the distribution of the data. *P*-values less than .01 were considered statistically significant.

3 | RESULTS

The organization of gills and skin was unvaried in larvae at the two selected developmental stages and no substantial differences in the histological and ultrastructural organization were detectable.

The morphology and ultrastructure of *L. italicus* gills and skin have previously been described in detail (Brunelli, 2018a, 2018b; Brunelli et al., 2007; Brunelli & Tripepi, 2005; Perrotta et al., 2012), revealing a general organization similar to other larval urodeles. The stratified epithelia covering the epidermis (Figure 1a,b) and the gills' main filament show different thickness, and each is provided with a particular set of specialized cells (Figure 1c,d). However, in both cases, it is possible to recognize a continuous layer of germinative BCs in direct contact with the basement membrane, an external layer consisting mainly of pavement cells (PVCs), while the middle layers are almost entirely occupied by large LCs (Figure 1a–d). The LCs, rounded or oval in shape, are arranged in 1–2 rows in the gills (Figure 1c,d), while in the skin (Figure 1a,b), they can originate more than three layers. LCs dimensions are significantly different (*p < .001), reaching dimensions of 29.78 ± 3.03 μm in width and 28.25 ± 3.51 μm in length in gills and a size of 35.17 ± 3.83 μm in width and 31.23 ± 4.32 μm in length in the skin (Table S1).

Several mitotic LCs have been observed only in gills epithelium (Figure 1d) while mitoses seem to be rare in LCs from epidermal tissue. LCs show a centrally located nucleus and a typical clear cytoplasm densely packed with large irregular vesicles (Figure 1a–d).

LM observation of semithin sections (Figure 1e), better than paraffin sections, allows to clearly recognize the content of LCs cytoplasm that appears completely filled by clear granules; it is also possible to recognize the accessory cells, with a high nucleus-cytoplasm ratio, that surround LCs. The LCs ultrastructure does not differ in samples from gills and skin (Figure 1f–h). LCs are characterized by a lobated or indented nucleus surrounded by a clear cytoplasm entirely occupied by numerous large vesicles of different dimensions and electron density. At the cytoplasm periphery, it is possible to recognize an electron-dense tonofilamentous network: the Langerhans net (Figure 1g). Coiled filament bundles forming the net are arranged in hexagonal units and could be better appreciated with a further enlargement (Figure 1h).

Using conventional glycoconjugate staining techniques, it could be confirmed that both the skin and gills contain a considerable amount of glycoconjugates. When the sections are subjected to the PAS reaction, it was found that the LCs, as well as the basal membrane, were distinctly stained, whereas no reaction on the PVCs or BC was detected (Figure 2a–d). With further magnification, it was possible to see that the PAS stain is distributed to form an extensive network intensely colored in magenta that branches out across the entire cytoplasm of the cell. The granules contents did not stain positively. We used four kinds of plant-based lectins in order to evaluate glycoconjugate expression patterns in the epithelial cells from gills and skin. The distribution pattern was similar in gills and skin and all the four lectins examined in the present study are expressed in epithelial cells. All tested lectins marked Leydig's cells (Figure 3), and only one also labeled the external layer of epithelium. More in detail, the lectin from wheat germ, specific to *N*-acetyl-D-glucosamine (GlcNAc) and *N*-acetyl-neuraminic acid (sialic acid), reacts strongly with both skin (Figure 4a) and gills (Figure 4b) epithelia. In the epithelial layers, the labeling was intense and restricted to LCs, whereas a less intense signal could also be seen in the basal lamina and connective tissue (Figure 4a,b). The signal was not affected by neuraminidase pretreatment in the epithelium of gills and skin, and accordingly, succinylated WGA shows a similar staining pattern (Figure 4c,d), and LCs were intensely stained, confirming the strong reaction with GlcNAc. On the contrary, a marked decrease in the signal was detected in the basal lamina and connective tissue when using the succinylated WGA, thus suggesting a reaction with sialic acid. In both

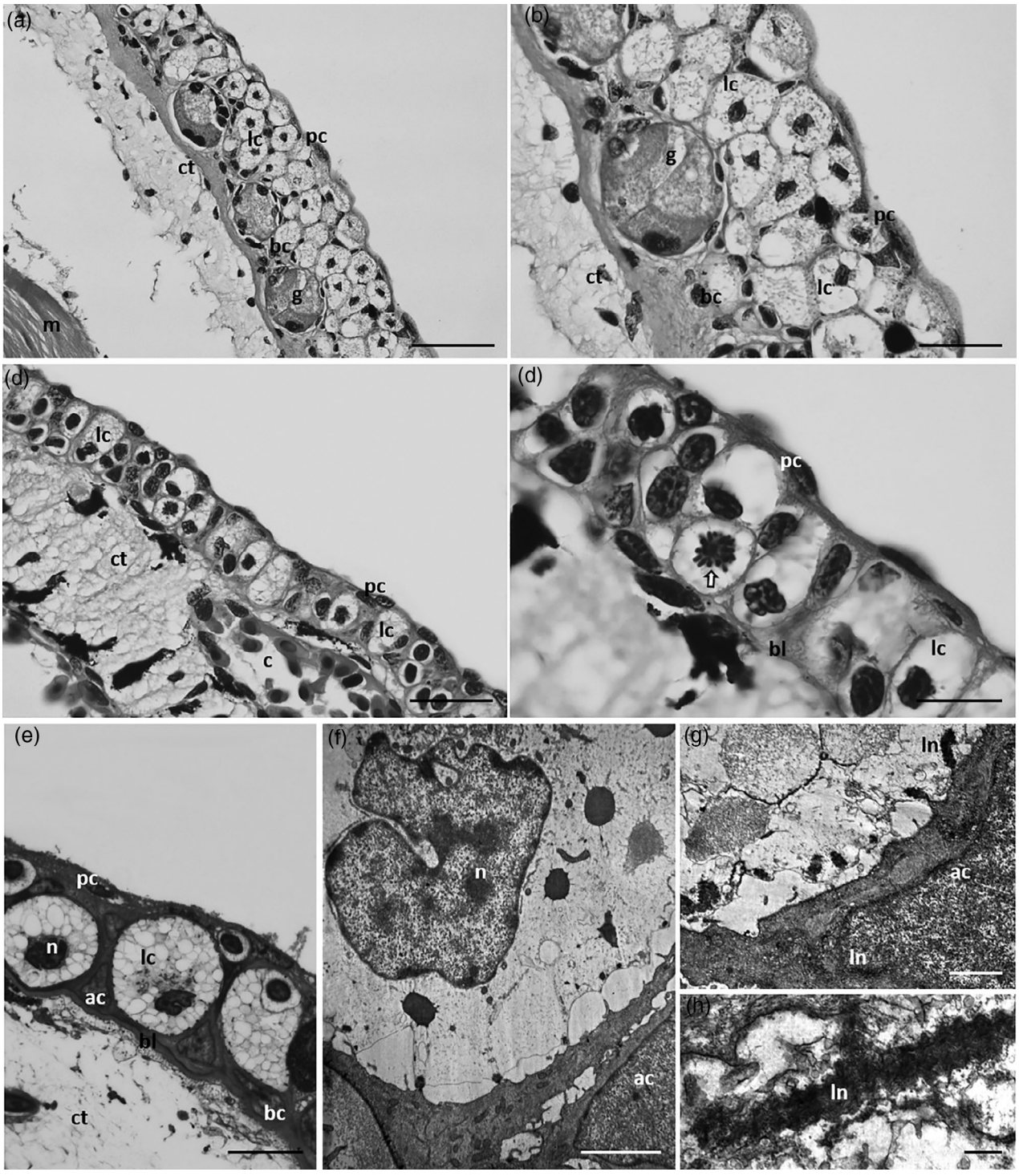

FIGURE 1 Light and electron micrographs of *Lissotriton italicus* skin and gill. (a) Light micrograph (H&E stained section) showing the stratified epidermis. Note in the outermost layer the pavement cells (pc), in the intermediate layer the large Leydig cells (lc), and in the innermost layer the basal cells (bc). ct, connective tissue; g, dermal gland; m, muscle; scale bar = 100 μm. (b) With further enlargement, it is possible to note the Leydig cells (lc) arranged in layers. pc, pavement cell; bc, basal cell; g, dermal gland; ct, connective tissue; scale bar = 50 μm. (c) Light micrograph (H&E stained section) showing the organization of the gill epithelium: pc, pavement cell; lc, Leydig cells; bc, basal cell; ct, connective tissue; c, capillary; scale bar = 50 μm. (d) Further enlargement of gill epithelium. Note a mitotic figure in Leydig cell (arrow). The basal cells lie on the underlying basal lamina (bl). pc, pavement cell; scale bar = 20 μm. (e) Light micrograph (semithin section stained with toluidine blue) of gills' epithelium. Observe the clear granules filling the cytoplasm of Leydig cell (lc) and the associated accessory cells (ac). pc, pavement cell; n, nucleus; bc, basal cell; bl, basal lamina; ct, connective tissue; scale bar = 50 μm. (f) TEM micrograph showing a Leydig cells and the adjacent accessory cell (ac); a lobated nucleus (n) and numerous vesicles could be seen in the cytoplasm of the Leydig cell.; scale bar = 4 μm. (g) High magnification micrograph showing the Langerhans net (ln) in the peripheral cytoplasm of the Leydig cell. ac, accessory cell; scale bar = 4 μm. (h) Detail of the Langerhans net. Note the typical bundles of filaments arranged in hexagonal units; scale bar = 250 nm

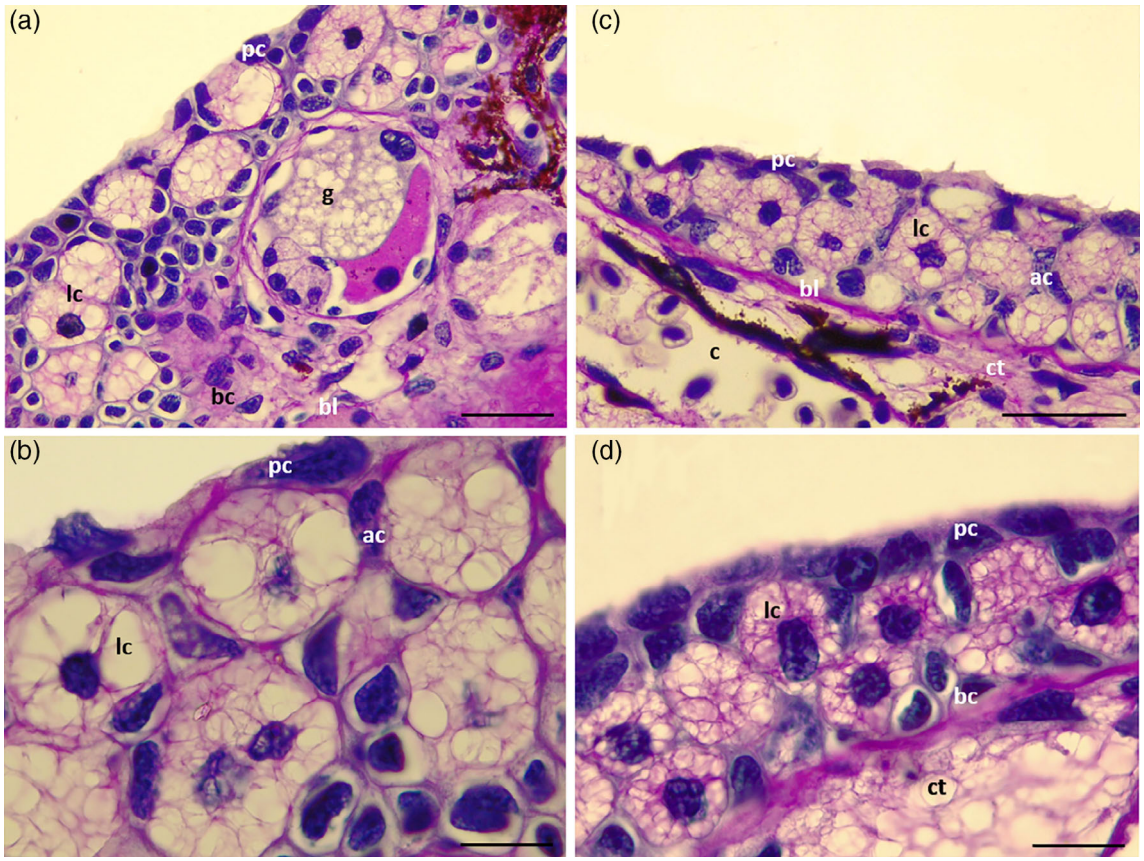

FIGURE 2 Periodic acid Schiff (PAS) staining in skin and gill. In both skin (a, b) and gills (c, d) the PAS reaction shows positive staining (magenta-colored) in Leydig cells (lc) and basal lamina of larval skin and gill. Note the absence of PAS reaction in pavement cells (pc) and basal cells (bc); g, dermal gland; ac, accessory cell; ct, connective tissue; c, capillary; scale bars = 50 μm. High magnification micrographs in the skin (b) and gill (d) epithelium. pc, pavement cell; lc, Leydig cell; ac, accessory cell; bc, basal cell; ct, connective tissue; scale bars = 20 μm

skin and gills, the D-galactose binding lectin RCA-I was highly expressed, showing diffuse staining in the cytoplasm of LCs (Figure 4e,f); no labeling was distinguishable in the other epithelial cells. The only lectin which strongly labeled the basal lamina of both skin and gills epithelia was MPA (α-GalNAc specific lectin; Figure 4g, h). This lectin labeled LCs and the cytoplasm of PVCs in the external layer of epithelium and the periphery of dermal glands.

Confocal microscopy observations revealed that no immunoreactivity for AQP3 was recognizable in sections of skin (Figure 4a,b) even if LCs periphery showed a weak fluorescence signal due to unspecific binding. On the contrary, the immunofluorescence staining performed on gills (Figure 4c–e) revealed an intense signal mainly in the cytoplasm of basal epithelial cells and the cytoplasm and peripheral membrane of the LCs.

4 | DISCUSSION

Our observations clearly showed that in *L. italicus* larvae, the LCs show the same morphological traits in both gills and skin, and it was possible to recognize the typical ultrastructural characteristics already known in the literature (Greven, 1980; Jarial, 1989; Kato & Kurihara, 1987, 1988; Leydig, 1876; Rosenberg et al., 1982). The only difference highlighted is a significantly larger size of the LCs in the epidermis compared with the gills.

Knowledge about the process of differentiation and growth of LCs throughout larval development was summarized by Gerling et al. (2012). It has been shown in several species that the number of LCs in the epidermis increase as larval growth proceeds and then decrease at the onset of metamorphosis undergoing apoptosis (Greven, 1980; Kato & Kurihara, 1987; Kelly, 1966; Wakahara & Yamaguchi, 1996; Warburg, et al., 1994). Established that a chronological increase in the number of LCs and/or their replacement occurs, the origin of the newly formed LCs appears to be more uncertain. Although there is broad agreement on the basal layer's proliferative role in the epidermis of adult amphibians, there is no demonstration that this is actually true for larvae or that this is true for all larval tissues. The ability of LCs to divide by mitosis has been questioned several times (Fox, 1986; Rosenberg et al., 1982; Warburg & Rosenberg, 1997; Warburg, et al., 1994), and all previous papers refer to very ancient literature when mentioning the origin and proliferative capacity of these cells (Dennert, 1924; Kelly, 1966; Pfitzner, 1879). Mitoses in LCs in the epidermis are considered absent (Rosenberg et al., 1982) or rare in both paedomorphic and metamorphosing

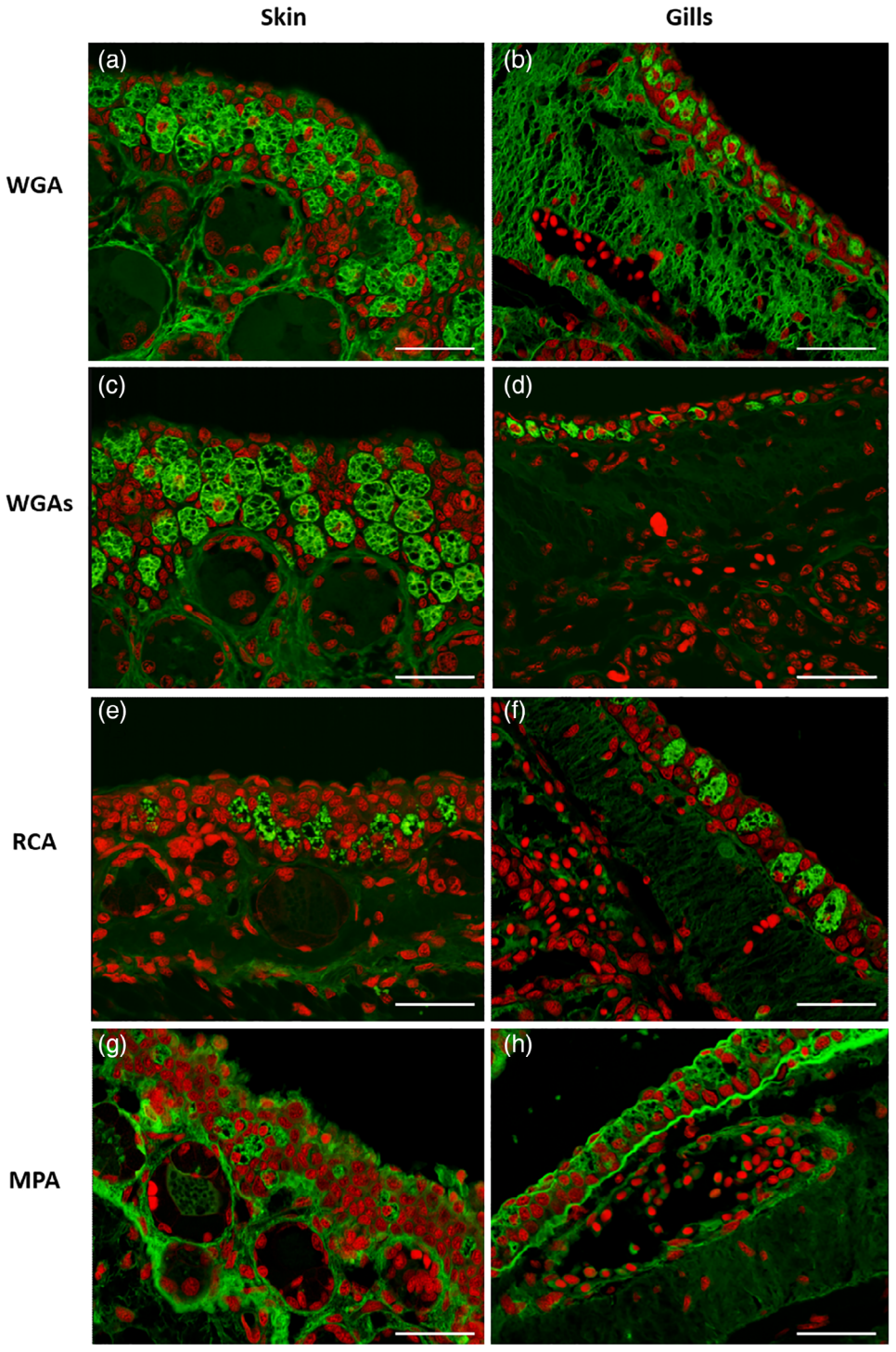

FIGURE 3 Lectins-binding pattern in skin and gills. Confocal micrographs of lectins-binding pattern (FITC-conjugated; green signal); nuclei are stained with propidium iodide (red signal). All bars = 75 μm. (a) Skin and (b) gills sections labeled with WGA. Note the intense immunoreactivity in the cytoplasm of Leydig cells; a less intense labeling is detected in the basal lamina and connective tissue. (c) Skin and (d) gills sections labeled with WGAs. An intense reactivity is detected in Leydig cells. (e) Skin and (f) gills sections labeled with RCA-I. An intense staining is evident exclusively in the Leydig cells cytoplasm. (g) Skin and (h) gills section labeled with MPA. The cytoplasm of Leydig cells and PVC are intensely stained. Note the staining in the basal lamina and dermal glands

species (Gerling et al., 2012; Greven, 1980), but in *Taricha torosa* Rathke, 1833, the number of mitotic figures observed appears to be conspicuous (Kelly, 1966). In the gill epithelium of *L. italicus*, we showed the presence of mitotic figures in the cytoplasm of LCs, thus supporting the hypothesis that during the larval stages, some epithelial cells can multiply by cell division. Although, we did not detect any histological or ultrastructural differences in the LCs population, the proliferative ability of these cells may also be different in different body districts due to tissue-specific roles. It is noteworthy that AQP3 is exclusively expressed in gill epithelium labeling basal layer and LCs. Indeed, it has been demonstrated that AQP3 exerts a pro-proliferative role, inducing the expression of several differentiation markers in human keratinocytes (Guo et al., 2013; Hara-Chikuma & Verkman, 2008a, 2008b). In *L. italicus* (formerly *T. italicus*) AQP3 becomes expressed in the epidermis at the metamorphic climax, but low pH exposure induces an increase in AQP3 expression and the precocious maturation of skin in adult-type. In this light, it is legitimate to speculate that the AQP3 expression in gills may play a role

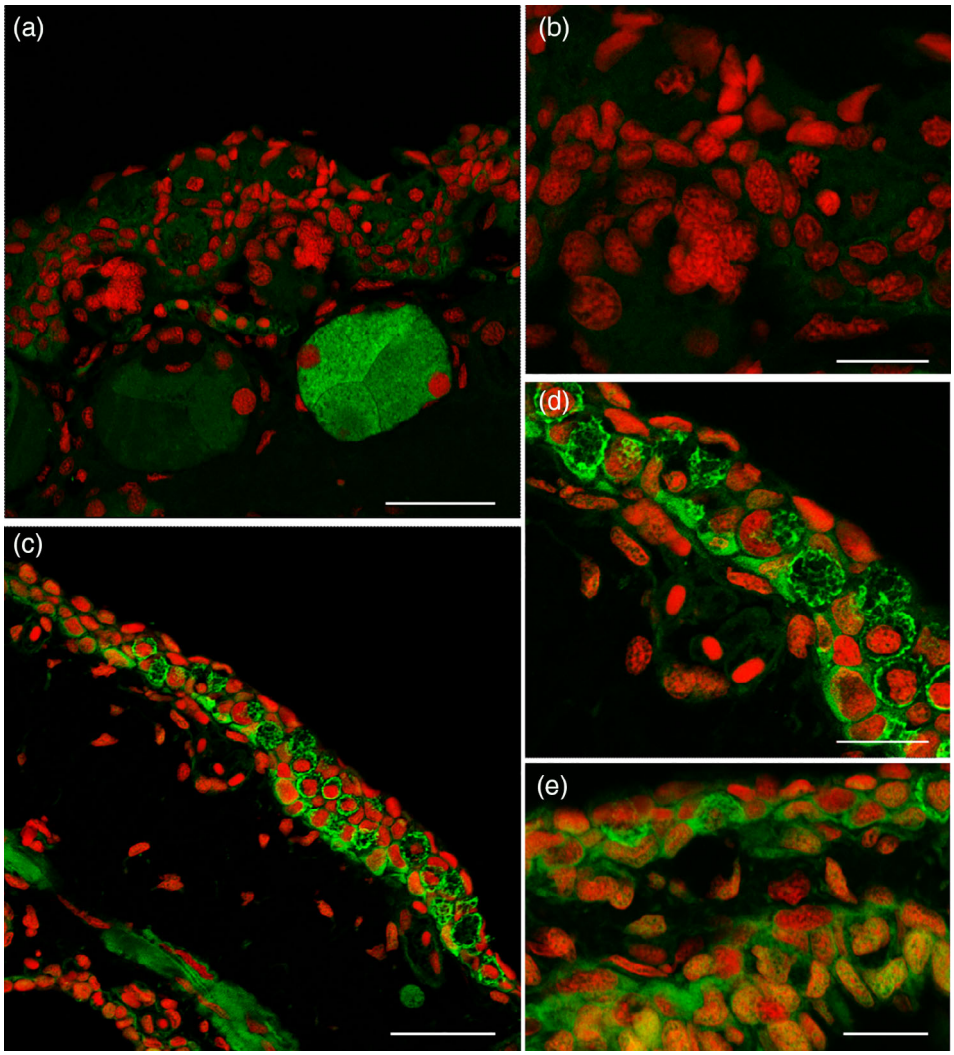

FIGURE 4 Aquaporin-3 localization in skin and gills. Confocal micrographs of skin and gill labeled with an antibody against water channel aquaporin-3 (FITC; green signal); nuclei are labeled with propidium iodide (red). (a, b) No immunostaining is observed in the epidermis; a weak unspecific binding is visible at Leydig cells' periphery. Scale bars = 75 and 30 μm, respectively. (c) In the gill, an intense immunoreaction is evident in cytoplasm of basal cells and Leydig cells. Scale bar = 75 μm. (d, e) The immunolabeling is better appreciable with further enlargement. Scale bars = 30 μm

in epithelial cell proliferation and, more specifically, in LC mitotic activity in gills. As recently outlined by Taylor (2018), physiologists have overlooked amphibians' gills, although their involvement in ion regulation is well recognized, mainly when skin transport systems are still poorly developed. Therefore, a more obvious explanation for the differential expression of AQP3 in respiratory epithelia is the need for a fine permeability regulation to maintain osmotic and ion homeostasis in gills epithelium.

4.1 | Lectin-binding pattern

Conventional PAS staining techniques confirmed that both the skin and gills contain a considerable amount of glycoconjugates. Glycans are abundant and ubiquitous to all cells in nature, mediating a large variety of functions essential for development, growth, or organism survival (Varki, 2017). To identify putative differences in the mucus composition of LCs here we evaluated the localization of 4 lectins in the two epithelial districts. Our overall binding results showed that sites of specific glycoconjugates were similar in two tissues. At epithelial level, all tested lectins marked Leydig's cells, three exclusively whereas one (MPA, α-GalNAc specific lectin) also labeled the cytoplasm of external cells corresponding to PVCs, suggesting the presence of mucin in both cell types.

Interestingly the high-resolution confocal microscopy observations showed that the labeling was not uniform throughout LCs cytoplasm. Therefore, it did not exclude the possibility that the granules' content is partly aqueous, as previously suggested by several authors (Kelly, 1966; Warburg et al., 1994). According to Kaltenbach et al. (2004), in amphibians, sugar residues of glycoconjugates are important for regulating water balance; however, their mechanism of action remains poorly understood. One interesting extension of our observations regards the role of *N*-acetylglucosamine moiety (O-GlcNAc), recognized by WGA/WGAs binding, in posttranslational modifications. Since its discovery, O-GlcNAc modification of proteins has been shown to contribute to numerous cellular functions (Bond & Hanover, 2015; Chatham et al., 2021). In *Xenopus laevis* Daudin, 1802 marked changes in the O-GlcNAc proteome have been reported as key regulators in crucial events such as cell migration and differentiation during embryogenesis (Dehennaut et al., 2009). Comparing these results with those reported in previous papers is difficult. This is partly due to the scarcity of specific data on the amphibian larval epithelium.

Although the adult's epidermis has received considerable attention, the distribution of glycoproteins among amphibian species is highly heterogeneous, and no common pattern could be established (Kaltenbach et al., 2004; Zaccone et al., 1999). Perhaps further characterization of the LCs glycoconjugate content may foster a better comprehension of their functional role.

4.2 | Limitations of the study

It is important to note a critical limitation of our study. This study aimed to point out the differential role of LCs cells in different body districts. Still, the research design was not a histometric study: the reduced number of sacrificed animals and the resulting low number of sections for measurements were undersized for a comprehensive statistical analysis (i.e., evaluation of mitotic index). Although qualitative, the observed results reveal some dynamics of noteworthy relevance regarding the role of LCs.

5 | CONCLUSIONS

In conclusion, our results provide new information on such unique and peculiar cells: the larval amphibians LCs. For the first time in this study, we reported a differential expression of AQP3 in gills and skin epithelia, thus supporting a major role of gills in osmotic and ion homeostasis and specifically a tissue-specific role of LCs. Our results will also contribute to the discussion about the proliferative ability of LCs. Moreover, evidence of the richness of LCs in sugar residues suggests putative roles in many diverse and essential functions. These observations need to be complemented by future studies focused on determining the LCs glycoconjugate content accurately. As a whole, our results suggest that the hypothesis of a mechanical and supportive role of these cells may be an oversimplification.

AUTHOR CONTRIBUTIONS

EB planned and conducted all experiments, wrote and edited the article, and conducted the ultrastructural analysis and confocal microscopy examinations. RM performed the histological examinations and contributed to the experimental design and the writing of the article VC performed sample collections and contributed to the histological examinations. BD performed sample collections and contributed to the analysis and interpretation of the histological data. AD contributed to the design of the experiment and a review of the article. All authors have read and approved this article at submission.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

Bernabò, I., & Brunelli, E. (2019). Comparative morphological analysis during larval development of three syntopic newt species (Urodela: Salamandridae). *The European Zoological Journal*, *86*(1), 38–53. https://doi.org/10.1080/24750263.2019.1568599

Bollag, W. B., Aitkens, L., White, J., & Hyndman, K. A. (2020). Aquaporin-3 in the epidermis: More than skin deep. *American Journal of Physiology-Cell Physiology*, *318*(6), C1144–C1153. https://doi.org/10.1152/ajpcell.00075.2020

Bond, M. R., & Hanover, J. A. (2015). A little sugar goes a long way: The cell biology of O-GlcNAc. *Journal of Cell Biology*, *208*(7), 869–880. https://doi.org/10.1083/jcb.201501101

Brown, D. D., & Cai, L. (2007). Amphibian metamorphosis. *Developmental Biology*, *306*(1), 20–33. https://doi.org/10.1016/j.ydbio.2007.03.021

Brunelli, E. (2018a). Histological and ultrastructural alterations of the Italian newt (*Lissotriton italicus*) skin after exposure to ecologically relevant concentrations of nonylphenol ethoxylates. *Environmental Toxicology and Pharmacology*, *60*, 17–27. https://doi.org/10.1016/j.etap.2018.04.007

Brunelli, E. (2018b). Morphological and immunohistochemical reactions of the larval epidermis in the Italian newt (*Lissotriton italicus*) after exposure to low pH. *Zoology*, *126*, 20–28. https://doi.org/10.1016/j.zool.2018.01.003

Brunelli, E., Perrotta, I., Bonacci, A., & Tripepi, S. (2007). Differential expression of aquaporin 3 in *Triturus italicus* from larval to adult epidermal conversion. *European Journal of Histochemistry*, *51*(1), 25–32. https://doi.org/10.4081/1008

Brunelli, E., Sperone, E., Maisano, M., & Tripepi, S. (2009). Morphology and ultrastructure of the gills in two Urodela species: *Salamandrina terdigitata* and *Triturus carnifex*. *Italian Journal of Zoology*, *76*(2), 158–164. https://doi.org/10.1080/11250000802484869

Brunelli, E., & Tripepi, S. (2005). Effects of low pH acute exposure on survival and gill morphology in *Triturus italicus* larvae. *Journal of Experimental Zoology Part A: Comparative Experimental Biology*, *303*(11), 946–957. https://doi.org/10.1002/jez.a.221

Chandrasekaran, E. V., Xue, J., Xia, J., Khaja, S. D., Piskorz, C. F., Locke, R. D., Neelamegham, S., & Matta, K. L. (2016). Novel interactions of complex carbohydrates with peanut (PNA), *Ricinus communis* (RCA-I), *Sambucus nigra* (SNA-I) and wheat germ (WGA) agglutinins as revealed by the binding specificities of these lectins towards mucin core-2 O-linked and N-linked glycans and related structures. *Glycoconjugate Journal*, *33*(5), 819–836. https://doi.org/10.1007/s10719-016-9678-y

Chatham, J. C., Zhang, J., & Wende, A. R. (2021). Role of O-linked N-acetylglucosamine protein modification in cellular (patho) physiology. *Physiological Reviews*, *101*(2), 427–493. https://doi.org/10.1152/physrev.00043.2019

Dehennaut, V., Lefebvre, T., Leroy, Y., Vilain, J. P., Michalski, J. C., & Bodart, J. F. (2009). Survey of O-GlcNAc level variations in *Xenopus laevis* from oogenesis to early development. *Glycoconjugate Journal*, *26*(3), 301–311. https://doi.org/10.1007/s10719-008-9166-0

Dennert, W. (1924). Über den Bau und die Rückbildung des Flossensaums bei den Urodelen. *Zeitschrift für Anatomie und Entwicklungsgeschichte*, *72*(3), 407–462.

Duellman, W. E., & Trueb, L. (1994). *Biology of amphibians*. JHU Press, McGraw-Hill Book Company.

Fox, H. (1986). The skin of amphibian: Epidermis. In J. Bereiter-Hahn, A. G. Matoltsy, & K. S. Richards (Eds.), *Biology of the integument* (pp. 78–135). Springer, Heidelberg.

Fox, H. (1987). On the fine structure of the skin of larval, juvenile and adult *Ichthyophis* (Amphibia, Caecilia). *Zoomorphology*, *107*(2), 67–76.

Fox, H. (1988). Riesenzellen, goblet cells, Leydig cells and the large clear cells of *Xenopus*, in the amphibian larval epidermis: Fine structure and a consideration of their homology. *Journal of Submicroscopic Cytology and Pathology*, *20*(2), 437–451.

Gerling, S., D'Haese, J., & Greven, H. (2012). Number and distribution of Leydig cells (LC) in the epidermis of the growing axolotl, *Ambystoma mexicanum* (Amphibia: Urodela). *Vertebrate Zoology*, *62*(1), 97–111.

Greven, H. (1980). Ultrastructural investigations of the epidermis and the gill epithelium in the intrauterine larvae of *Salamandra salamandra* (L.) (Amphibia, Urodela). *Zeitschrift fur Mikroskopisch-Anatomische Forschung*, *94*(2), 196–208.

Guo, L., Chen, H., Li, Y., Zhou, Q., & Sui, Y. (2013). An aquaporin 3-notch1 axis in keratinocyte differentiation and inflammation. *PLoS One*, *8*(11), e80179. https://doi.org/10.1371/journal.pone.0080179

Hara-Chikuma, M., & Verkman, A. S. (2008a). Aquaporin-3 facilitates epidermal cell migration and proliferation during wound healing. *Journal of Molecular Medicine*, *86*(2), 221–231. https://doi.org/10.1007/s00109-007-0272-4

Hara-Chikuma, M., & Verkman, A. S. (2008b). Prevention of skin tumorigenesis and impairment of epidermal cell proliferation by targeted aquaporin-3 gene disruption. *Molecular and Cellular Biology*, *28*(1), 326–332. https://doi.org/10.1128/MCB.01482-07

Jarial, M. S. (1989). Fine structure of the epidermal Leydig cells in the axolotl *Ambystoma mexicanum* in relation to their function. *Journal of Anatomy*, *167*, 95–102.

Jarial, M. S., & Wilkins, J. H. (2003). Ultrastructure of the external gill epithelium of the axolotl, *Ambystoma mexicanum* with reference to ionic transport. *Journal of Submicroscopic Cytology and Pathology*, *35*(4), 445–455.

Kaltenbach, J. C., Faszewski, E. E., Nytch, K. E., Potter, C. H., Shanthakumar, N., & Fakin, A. (2004). Glycoconjugate localization in larval and adult skin of the bullfrog, *Rana catesbeiana*: A lectin histochemical study. *Journal of Morphology*, *261*(2), 184–195. https://doi.org/10.1002/jmor.10237

Kato, S., & Kurihara, K. (1987). The intracellular supporting network in the Leydig cells of larval salamander skin. *Zoological Science*, *4*(1), 187–191.

Kato, S., & Kurihara, K. (1988). Electron microscopic studies of the external gill epithelium of the salamander, *Hynobius dunni* Tago, followed throughout larval life. *Okajimas Folia Anatomica Japonica*, *65*(4), 177–201. https://doi.org/10.2535/ofaj1936.65.4_177

Kelly, D. E. (1966). The Leydig cell in larval amphibian epidermis. Fine structure and function. *The Anatomical Record*, *154*(3), 685–699. https://doi.org/10.1002/ar.1091540314

Langerhans, P. (1873). Ueber die Haut der Larve von *Salamandra maculosa*. *Archiv für Mikroskopische Anatomie*, *9*(1), 745–752.

Lewinson, D., Rosenberg, M., & Warburg, M. R. (1982). Mitochondria-rich cells in salamander larva epidermis: Ultrastructural description and carbonic anhydrase activity. *Biology of the Cell*, *46*, 75–84.

Lewinson, D., Rosenberg, M., & Warburg, M. R. (1984). 'Chloride-cell'-like mitochondria-rich cells of salamander larva gill epithelium. *Experientia*, *40*(9), 956–958.

Lewinson, D., Rosenberg, M., & Warburg, M. R. (1987). Ultrastructural and ultracytochemical studies of the gill epithelium in the larvae of *Salamandra salamandra* (Amphibia, Urodela). *Zoomorphology*, *107*(1), 17–25. https://doi.org/10.1007/BF00312125

Leydig, F. (1876). Über die allgemeinen Bedeckungen der Amphibien. *Archiv für Mikroskopische Anatomie*, *12*(1), 119–241.

Lindinger, M. I. (1984). Fine structure of the abdominal epidermis of the adult mudpuppy, *Necturus maculosus* (Rafinesque). *Cell and Tissue Research*, *238*(2), 395–405. https://doi.org/10.1007/BF00217313

Manalo, T., May, A., Quinn, J., Lafontant, D. S., Shifatu, O., He, W., Gonzalez-Rosa, J. M., Burns, G. C., Burns, C. E., Burns, A. R., & Lafontant, P. J. (2016). Differential lectin binding patterns identify distinct heart regions in giant danio (*Devario aequipinnatus*) and zebrafish (*Danio rerio*) hearts. *Journal of Histochemistry & Cytochemistry*, *64*(11), 687–714. https://doi.org/10.1369/0022155416667928

Perrotta, I., Sperone, E., Bernabò, I., Tripepi, S., & Brunelli, E. (2012). The shift from aquatic to terrestrial phenotype in *Lissotriton italicus*: Larval and adult remodelling of the skin. *Zoology*, *115*(3), 170–178. https://doi.org/10.1016/j.zool.2011.10.004

Pfitzner, W. (1879). Die Leydig'schen Schleimzellen in der Epidermis der Larve von *Salamandra maculosa* (Doctoral dissertation, Medicinische Facultät zu Kiel).

Quagliata, S., Malentacchi, C., Delfino, C., Brunasso, A. M., & Delfino, G. (2006). Adaptive evolution of secretory cell lines in vertebrate skin. *Caryologia*, *59*(2), 187–206. https://doi.org/10.1080/00087114.2006.10797915

Rondinini, C., Battistoni, A., Peronace, V., & Teofili, C. (2013). Lista rossa IUCN dei vertebrati italiani. *Comitato Italiano IUCN e Ministero dell'Ambiente e della Tutela del Territorio e del Mare, Roma*.

Rosenberg, M., Lewinson, D., & Warburg, M. R. (1982). Ultrastructural studies of the epidermal Leydig cell in larvae of *Salamandra salamandra* (Caudata, Salamandrida). *Journal of Morphology*, *174*(3), 275–281. https://doi.org/10.1002/jmor.1051740304

Seeger, P. G. (1933). Untersuchungen an den Leydigschen zellen der Urodelenhaut. *Zeitschrift für Zellforschung und Mikroskopische Anatomie*, *19*(3), 441–487. https://doi.org/10.1007/BF00393355

Sindaco, R., Doria, G., Razzetti, E., & Bernini, F. (2006). *Atlante degli anfibi e dei rettili d'Italia/Atlas of Italian Amphibians and Reptiles*. Societas Herpetologica Italica, Edizioni Polistampa.

Taylor, P. M. (2018). Water permeability of the gill epithelium: Salinity and temperature relations. In G. Benga (Ed.), *Water transport in biological membranes* (pp. 211–232). CRC Press.

Varki, A. (2017). Biological roles of glycans. *Glycobiology*, *27*(1), 3–49. https://doi.org/10.1093/glycob/cww086

Wakahara, M., & Yamaguchi, M. (1996). Heterochronic expression of several adult phenotypes in normally metamorphosing and metamorphosis-arrested larvae of a salamander *Hynobius retardatus*. *Zoological Science*, *13*(3), 483–488. https://doi.org/10.2108/zsj.13.483

Warburg, M. R. Lewinson, D., Rosenberg, M. (1994). *Ontogenesis of amphibian epidermis*. In: H. Heatwole, G. T. Barthalmus (Eds), Amphibian Biology, Vol. 1: The Integument, Sydney: Surrey Beatty & Sons.

Warburg, M. R., & Rosenberg, M. (1997). Structure of gill epithelium in *Triturus vittatus* larvae. *Annals of Anatomy-Anatomischer Anzeiger*, *179*(1), 57–64. https://doi.org/10.1016/S0940-9602(97)80137-9

Yoshizato, K. (1990). Biochemistry and cell biology of amphibian metamorphosis with a special emphasis on the mechanism of removal of larval organs. *International Review of Cytology*, *119*, 97–149. https://doi.org/10.1016/S0074-7696(08)60650-6

Zaccone, G., Fasulo, S., Gabbay, S., Mauceri, A., & Katz, U. (1999). Lectin binding patterns in amphibian skin epithelium. *Acta Histochemica*, *101*(3), 317–326. https://doi.org/10.1016/S0065-1281(99)80032-4

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