Differential expression of aquaporin 3 in *Triturus italicus* from larval to adult epidermal conversion

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By using immunohistochemical techniques applied to confocal microscopy, the presence of aquaporin 3 water channel in the epidermis of *Triturus italicus* (Amphibia, Urodela) has been shown. We analysed the expression of aquaporin 3 (AQP3) during the larval, pre-metamorphic and adult phases; we also showed the localization of the water-channel protein AQP3 in free-swimming conditions and during aestivation in parallel with histological analysis of the skin, focusing on the possible relationship between protein expression and terrestrial habitats.

Our results indicate that aquaporin is produced as the epidermis modifies during the functional maturation phase starting at the climax. Moreover, our data suggest an increase in enzyme expression in aestivating newts emphasizing the putative functional importance of differential expression related to a distinct phase of the biological cycle.

Key words: water channel protein, epithelial cells, metamorphosis, aestivation, Amphibia, Urodela, confocal microscopy.

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The multilayered cornified epidermis which in Vertebrates functions as a mechanical and water permeable barrier, in amphibians also acts, to some extent, as a respiratory and an osmoregulatory surface (Spotila *et al.,* 1992). To accomplish its multiple functions, the amphibian skin needs to undergo dramatic changes, ranging from a cornified epidermis which reduces dessiccation to suitable rearrangement for gas exchange.

Amphibians are known to spend part of their life on land and return to water to reproduce; however, some urodeles spend their entire life in water, while others succeed in completely avoiding water even during reproduction. The wide environments that amphibians have colonized are reflected in skin permeability, considerable variation occurring in different species and in the same animal in different times and ecological and developmental conditions (Fox, 1986a). Since water loss from the skin severely challenges homeostasis, is of interest that an integument with low rates of evaporative water loss has evolved independently in taxonomically different species (reviewed by Toledo and Jared, 1993). Accordingly, an array of osmoregulatory mechanisms have evolved in amphibians depending on the species life history and its ecophysiological characteristics.

Recently, a family of water conducting transmembrane proteins, the aquaporins (AQPs) has been identified and characterized (Agre *et al.,* 1993, 2002; Engel *et al.,* 1994; Connolly *et al.,* 1998). Two subgroups of aquaporins: *water channel* and *water/glycerol channels* (Borgnia *et al.,* 1999; Hatakeyama *et al.,* 2001; Verkman and Mitra, 2000; Zheng and Bollinger Bollag, 2003) have been described. AQP3 belongs to aquaglyceroporins and is expressed in most types of epithelial cells of the urinary, digestive, respiratory tracts as well as in the epidermis.

Since there is a lack of knowledge in amphibians regarding skin remodelling during development in

relation with the aquaporin water channel, we have analyzed by immunohistochemical techniques applied to confocal microscopy, the presence, localization, and intensity of expression of AQP3 at different phases of the biological cycle of the urodele amphibian *Triturus italicus*.

The structure of amphibian epidermis has been the subject of several studies in different species of anuran, urodele and cecilian but only a few studies focus on epidermal modification throughout the entire metamorphic cycle (Pederzoli *et al.,* 2002; Warburg and Lewinson, 1977). In this work, preliminary histological analyses were carried out to better identify the difference in cellular composition of the skin between the different developmental stages of *Triturus italicus*.

Materials and Methods

Animals and tissue preparation

Both larvae, at various developmental stages, and adults of *Triturus italicus* were collected from a field near the city of Cosenza, Calabria, in southern Italy, in June. They were reared at room temperature in 50 L aquaria filled with water taken from the original pond. Animals were kept under a natural light/dark cycle and fed with *Artemia salina* on alternate days. Larvae were staged according to Gallien and Bidaud (1959). To obtain animals in the terrestrial phase of the cycle, which it is not easy to find in natural conditions, we manufactured and installed an aquaterrarium in the Botanical Garden of the University of Calabria; newts introduced in the aquaterrarium became well acclimatized and were supervised for an entire year.

Three developmental stages were selected: larvae from 50 to 54 Gallien and Bidaud stages, premetamorphic Gallien and Bidaud stage 55c, postmetamorphic or adult. Furthermore, free swimming and aestivating adult/phases were distinctly studied.

The study was conducted on 5 larvae, 5 pre-metamorphic individuals, 5 adults in aquatic phase and 5 adults in aestivating phase. Small pieces of skin, including the underlying dermal and muscular layers, were quickly removed from the ventral and dorsal regions of the body. The animals were anesthetized with tricaine methane sulfonate, MS 222 (Sandoz, Sigma, St. Louis, MO) and decapitated. Animals were handled according to the recommendations of the Ethical Committee and under the supervision of authorized investigators. The removed skins were prepared for light microscopy and confocal microscopy.

Light microscopy

After removal, the specimens for light microscopy were fixed with 3% glutaraldehyde in 0.05 M phosphate buffer, pH 7.5, at 4°C for 2 h and postfixed for 1 h at 4°C in 1% osmium tetroxide, in the same buffer. After washing with the same buffer, the specimens were dehydrated in graded ethanol and embedded in Epon-Araldite resin. One micrometer-thick sections were stained with the technique described by Humphrey and Pittman (1974), observed and photographed by a LEITZ Dialux EB 20 light microscope.

Morphological measurements were taken with an image analysis program (NIH Image); all measurements were performed by using LM images. We analyzed 6 specimens for each developmental stage; 5 images were taken for each sample and about 10 measures for each image.

Immunohistochemistry

Bouin-fixed samples were dehydrated in ethanol, cleared in xylene and embedded in paraffin wax.

Sagittal 7 μm sections were cut and mounted on gelatine coated slides. Immunofluorescence staining was carried out as described by Coons *et al.* (1955). Briefly, sections were dewaxed and endogenous peroxidase was blocked with 2% H_2O_2 in 0.1 phosphate-buffered saline (PBS). After washing with PBS, the sections were then incubated for 10 min with normal goat serum in a moist chamber at 20°C. Unwashed sections were incubated overnight at 4°C with anti-water channel aquaporin-3 (AQP3) developed in rabbit (1:100, Sigma-Aldrich); after several washes in PBS, they were incubated with fluorescein-isothiocyanate-conjugated γ-globulins goat anti-rabbit (1:200, Sigma-Aldrich) for 30 min at room temperature. For nuclear counterstaining, propidium iodide was used at working dilutions of 1:200. Finally the sections were washed with PBS and then mounted in Vectashield (Vector Lab. Burlingame, CA). To check the specificity of the immunostaining (negative control), the primary antiserum was substituted with non immune goat serum at a dilution of 1:200 in PBS in control sections. Specimens were examined with a Leica TCS SP2 Confocal Laser Scanning Microscope.

Western blot analysis

The frozen tissues (four adult and four larvae) were homogenized in cell lysis buffer [100 mL Tris-HCl (pH 7.5), 5% sodium dodecyl sulfate (SDS), 1 mL phenylmethylsulfonyl fluoride, 1 mL aprotinin] and centrifuged in a microcentrifuge for 10 min to remove insoluble materials.

Protein concentration was determined by Bradford reagent according to the manufacturer's recommendations (Sigma, Milan Italy). The supernatant protein (10 µg) was denatured at 70 C for 10 min in denaturation buffer comprising 2% sodium dodecyl sulfate, 25 mM Tris-HCl (pH 7.5), 25% glycerol, and 0.005% bromophenol blue, subjected to electrophoresis on a 12% polyacrylamide gel, transferred to a nitrocellulose membrane (GE Healthcare, Milan, Italy), probed overnight at 4°C with the antibody against AQP3 (Sigma, Milan, Italy), and then revealed using the ECL system (GE Healthcare).

Results

General histological organization

The dorsal epidermis of *Triturus italicus* larva at stages 50-54 of Gallien and Bidaud (1959) is composed of 2-3 layers of cells with a thickness of 86.58±0.79 µm (Figure 1A); the epithelial surface appears to be formed by squamous pavement cells (PVCs). PVC are flattened with a rounded or elongated nucleus.

The basal cells (BCs), arranged in a continuous layer, were connected to the basal lamina. They appeared cubic in shape with a large nucleus surrounded by a little cytoplasm.

The Leydig cells (LCs) were organized in up to 3 layers; they never reached the epithelium surface and were in contact with the underlying basal cells, on one side, and with the surface pavement cells, on the other. In the clear cytoplasm, different kinds of secretory granules and vacuoles were present; the lobated nucleus was centrally located. Mitochondria-rich cells were sometimes observed in the surface epithelium.

Larval ventral skin of *T. italicus* showed a thickness of 73.09±0.90 µm with a cellular composition almost identical to that observed in the dorsal one; no substantial differences could be recognized in the structural features or in the number and distribution of cellular types.

At the climatic stage (stage 55c Gallien and Bidaud, 1959) the typical larval cells (Leydig cell, mitochondria-rich cell) disappeared and the larval skin becomes similar to the adult one (Figure 1B). The number of layers increased and the epidermis being composed of 5-7 layers with a thickness of 33.16±0.88 µm in the dorsal skin and 35.78±0.86 µm in the ventral one.

Glandula primordia appeared during premetamorphic stages when keratinization and sloughing mechanism begin.

In both premetamorphic and adult skin (Figure 1B-E) it was possible to recognize the typical pattern of keratinization. In adult skin 4 layers can be indentified: the stratum germinativum, the spinosum, the granulosum, and the corneum. The cell shape, columnar in the proliferative basal layer, becomes flattened in the outermost one. The stratum germinativum lies above the basement lamella.

The thickness of dorsal and ventral epithelium during aquatic phase is 31.71±0.92 µm and 26.52±0.76 µm respectively. The epidermal surface in dorsal (Figure 1C) and ventral skin (Figure 1D) during the aquatic phase showed a smooth profile while during the aestivating period it was characterized by a great number of tubercles. The thickness of dorsal (Figure 1E) and ventral skin (Figure 1F) become equal to 18.61±1.00 µm and 20.85±0.88 µm respectively.

### Aquaporin 3 immunohistochemistry

Immunofluorescence staining performed on larval skin revealed absence of immunopositive reactions for AQP3 (Figure 2A).

In contrast, in premetamorphic stages the localization of AQP3 was evident in all the epidermal layers (Figure 2B); at the basolateral membrane of cells. No differences in intensity of labelling were observed between dorsal and ventral skin at these stages.

In adult dorsal skin during the aquatic phase (Figure 2C), germinative basal cells immunopositive for AQP3 were detected with the labelling always found in the basolateral membrane of the cells (Figure 2c). The labelling, which was less intense, was also found in the spinosum and granulosum strata and disappear in the external corneum layer.

In the ventral skin (Figure 2D) the aquaporin pattern is similar to that observed in the dorsal one but the staining intensity is higher.

In both dorsal (Figure 2E) and ventral skin
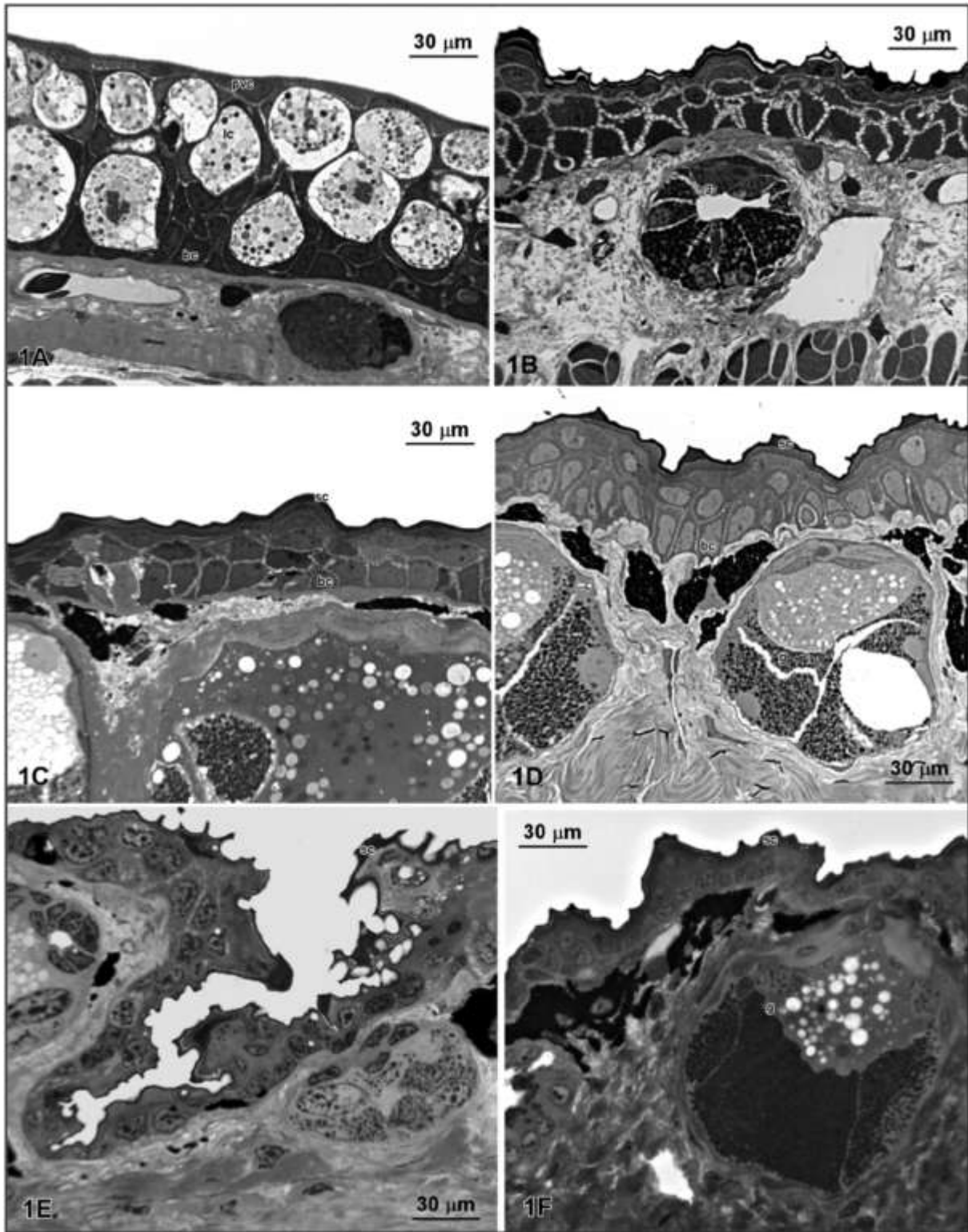
Figure 1. (A) LM micrographs of *Triturus italicus* larval skin. bc = basal cells; pvc = pavement cells; lc = Leydig cells. (B) *Triturus italicus* pre-metamorphic skin filament, observed with LM. Note the appearance of dermal glands. g = glands. (C) Semithin section through dorsal skin of the adult aquatic phase. Note the presence of an outermost stratum corneum. bc = basal cells; sc = stratum corneum. (D) Semithin section through ventral skin of the adult aquatic phase. The epidermis consists of about four to six epithelial layers with an outer covering of flattened cornified cells. bc = basal cells; g = gland; sc = stratum corneum. (E) Semithin section through dorsal skin of the adult terrestrial phase. The epidermal surface is characterized by its velvety nature. A large number of tubercles can be distinguished. sc = stratum corneum. (F) Semithin section through ventral skin of the adult terrestrial phase. Stratum corneum (sc) consists of a rather thick outer keratinized layer. Note the well organized and compact epidermis and the dermal glands (g).
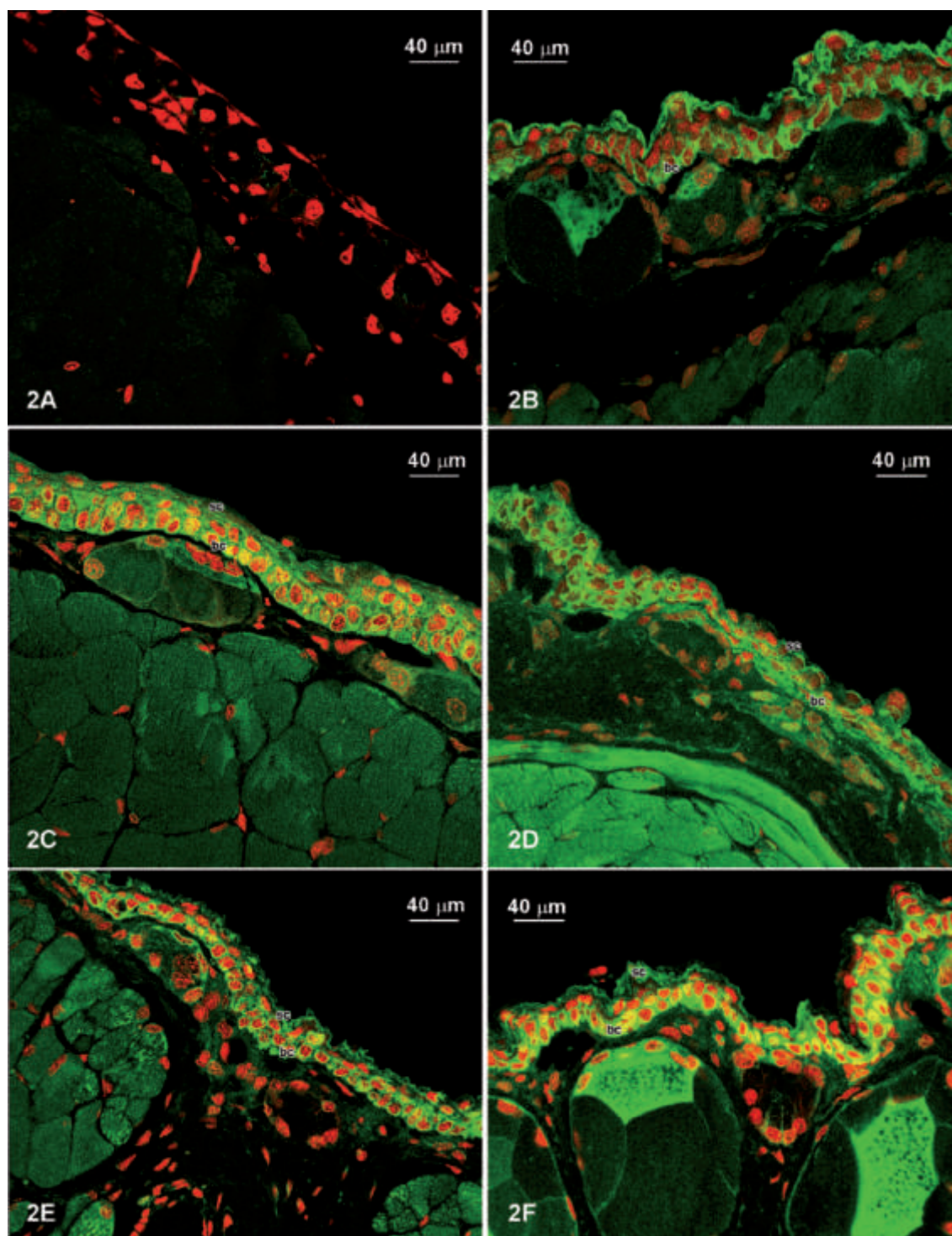
Figure 2 (A) Confocal micrograph of larval skin section labeled with a rabbit monoclonal antibody against AQP3: no positive labeling is seen. Nuclei are stained with propidium iodide. (B) Confocal micrograph of pre-metamorphic skin section labeled with a rabbit monoclonal antibody against AQP3: AQP3 protein is detected in all epidermal layers. bc = basal cells. (C) Confocal micrograph of adult aquatic phase skin through dorsal epidermis labelled with a rabbit monoclonal antibody against AQP3: protein is localized to the plasma membrane of the keratinocytes in the basal and intermediate layers of epidermis. bc = basal cells; sc = stratum corneum. (D) Confocal micrograph of adult aquatic phase skin through ventral epidermis labeled with a rabbit monoclonal antibody against AQP3. Note the more intense labeling of the ventral skin. bc = basal cells; sc = stratum corneum. (E) Confocal micrograph of adult terrestrial phase skin through dorsal epidermis labeled with a rabbit monoclonal antibody against AQP3: the label for AQP3 gradually decreases as the cells differentiate and completely disappears in the external stratum corneum. bc = basal cells; sc = stratum corneum.
(F) Confocal micrograph of adult terrestrial phase skin through ventral epidermis labeled with a rabbit monoclonal antibody against AQP3: along ventral epidermis AQP3 protein was hardly detected. Labeling intensity decreases towards the surface from the basal cells. bc = basal cells; sc = stratum corneum.

Figure 3. Characterization of anti-AQP3 serum by Western blot analysis. Immunoreactive band is seen at about 29.0 kDa in an extract of adult skin (lane 1). No bands are visible with the extract of larval skin (lane 2).

Table 1. Qualitative and semi-quantitative ratings of the degree of AQP3 labelling in *Triturus italicus* skin. Skin was rated according to the strength of labelling as either none (-), some (+), moderate (++), strong (+++).

Stage	AQP3
Larva: dorsal and ventral skin	-
Premetamorphic stages: dorsal and ventral skin	+
Aquatic adult dorsal skin	+
Aquatic adult ventral skin	++
Terrestrial adult dorsal skin	++
Terrestrial adult ventral skin	+++

(Figure 2F) of terrestrial individuals the same staining gradation was evident: the basal cells were heavily stained, while in the surface they were moderately or weakly stained. As observed in the aquatic phase: the ventral skin was more intensely stained than the dorsal counterpart.

Antibody specificity

To test the specificity of the anti-AQP3 antibody toward the *Triturus* skin Western blot analysis was performed. By immunoblotting with the anti-AQP3 antibody, an immunopositive band was detected in the homogenates of adult skin at about 29.0 kDa (Figure 3 lane 1). No band was detectable when the extracts of larval skin were examined (Figure3, lane 2).

Discussion

The skin of *Triturus italicus* during larval and adult stages exhibits a general organization and a cellular composition similar to that of other urodeles (*Triturus cristatus*, Pillai 1962; Lawker 1972; Necturus, Lindinger 1984; *Tritutrus vittatus*, Warburg and Rosenberg 1997; Rosenberg and Warburg 1997; *Salamandra salamandra*, Greven 1980; Lewinson *et al.* 1987; Warburg *et al.* 1994).

Metamorphosis represents a switchpoint during the life cycle of Amphibians during which several morphological and biochemical changes occur (Fox, 1986b).

During the larval phase, the epidermis is still *porous* and plays a major role in gas exchange (Fox, 1986a,b; Warburg *et al.,* 1994; Rosenberg and Warburg, 1997). Numerous changes take place in larval skin during the metamorphic cycle, when typical larval cells (Leydig cells, MRCs) disappear in coincidence with basement lamella reorganization. The number of epidermal cell layers increases

and at metamorphic climax the outermost layers flatten and keratinize. The major role of adult integument is to provide protection allowing, at the same time, the selective transport of gases, water and solutes through it (Wittouck, 1974; Warburg *et al.,* 1994).

We have shown that a marked difference in the expression and intensity of AQP3 (Table 1) is present among all developmental stages i.e., larval, premetamorphic and both aquatic and terrestrial adult. The expression of AQP3 was first detected at the metamorphic climax when epidermal transition from larval to adult type occurs.

The pattern of labelling distribution is the same in premetamorphic newts and adults (during both aquatic and terrestrial phases): the label for AQP3 is more intense in the basal and intermediate layer, where it was localized to the plasma membrane of the keratinocytes. The label for AQP3 gradually decreases as the cells differentiate and move upward to the surface; while it completely disappears in the stratum corneum.

The function of AQP3 was studied previously in mammalian epithelia where it has been postulated to participate in the supply of water from intermediate and basal cells to the cells located above; such a role for this protein has also been suggested for fish epithelium (Lignot *et al.,* 2002; Matsuzaki *et al.,* 1999, 2000). It is well known that Aquaporin 3 is a key water channel protein in mammalians (Agren et al, 2003; Ma *et al.,* 2002), amphibians (Sturla *et al.,* 2003; Tanii *et al.,* 2002) and fish (Cutler and Cramb, 2002; Lignot *et al.,* 2002).

Our observations suggest that in *Triturus italicus* skin aquaporin is produced as the epidermis modifies from larval to adult type. This ontogenic change of AQP3 expression is probably due to the fact that the larva is at low risk for osmotic loss while after

metamorphosis the newt is potentially exposed to the air and to the risk of dehydration through the body surface.

The observed AQP3 pattern could therefore be related to the morphological and functional modification of the epidermis and in particular with the keratinization process, that starts at the climax; in metamorphosing skin, the thickness of the epithelium decreases but cornification mechanisms begin and make the uptake of water from environment more difficult. The principal role of AQP3 at the plasma membrane of keratinocytes could be to provide a water entry route into epithelial cells from the sub-epithelial side to maintain intracellular osmolarity and cell volume if the cell undergoes water loss (Matsuzaki *et al.,* 1999).

Therefore, on the basis of our data, it is conceivable that AQP3 may exert a similar function in the amphibian epidermis.

Switching from aquatic to terrestrial habitats is another critical event in the amphibian's life which appears to affect the morphology of the epidermis (Warburg and Rosenberg, 1997). For example, the epithelium architecture in free swimming and aestivating *T. italicus* differs in the number of tubercles and in the epithelium thickness which significantly decreases in terrestrial aestivating newts. Clearly, this remodelling includes at the molecular level the activity of aquaporin 3, which appears more intense in aestivating newts than in aquatic ones.

The amphibian antidiuretic hormone, arginine vasotocin (AVT), is released as the amphibians leave the aquatic medium and migrate to a terrestrial environment (Bentley, 1971; Heller, 1974). AQP3 and AQP2 in amphibian skin were shown to be vasotocin-regulated AQPs and that they play a role in water balance of the body (Hasegawa *et al.,* 2003). The skin of the terrestrial phase was found to be over twice as much responsive to AVT as that of the aquatic phase (Warburg, 1971; Lodi *et al.,* 1995).

Conceivably, the increased enzyme expression observed in *T. italicus* may be related to passage from a free-swimming aquatic condition to a terrestrial aestivating one. Our observations suggest that the increase in AQP3 expression has neither relation to the number of layers nor to skin thickness, but is probably related to keratinization phenomena and to vasotocin regulation. Further study is necessary to clarify the induction mechanism of AQP expression.

Tanii and colleagues (2002) recently demonstrated that in frogs, AQP3 is specifically expressed in the plasma membrane of principal cells in ventral pelvic skin. They also demonstrated that the expression of this protein increases as the cell differentiates, reaching a maximum in the outermost layer of the stratum granulosum.

Despite the differences existing between anuran and urodele skin structure, the strong expression of AQP3 in ventral skin suggests a major role for this district in water and glycerol transport.

The difference existing in water permeability of amphibian skin has long been known to be species-specific (Bentley, 71; Hillyard, 1999), moreover, regional differences in skin permeability are well documented (McClanahan and Baldwin, 1969; Bentley and Main, 1972; Marrero and Hillyard, 1985). The differences observed in regional localisation of AQP3 in *Hyla* and *Triturus italicus* could be related to different water balance strategies among Amphibian orders.

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