Number and distribution of Leydig cells (LC) in the epidermis of the growing axolotl, *Ambystoma mexicanum* (Amphibia: Urodela)

Sonja Gerling, Jochen D'Haese & Hartmut Greven

Institut für Zoomorphologie und Zellbiologie der Heinrich-Heine-Universität Düsseldorf,
Universitätsstr. 1, D-40225 Düsseldorf, Germany.
Grevenh(at)uni-duesseldorf.de
(corresponding author)

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> Abstract

The epidermal Leydig cells (LC) of larval and paedomorphic Urodela (= Caudata) are highly specialized cells, which are characterized by a complex peripheral cytoskeleton (Langerhans' net) and numerous inclusions usually named secretory granules. We studied number, distribution and development of these cells in larvae up to 100 days after hatching and in some adults of the paedomorphic axolotl (*Ambystoma mexicanum*). With the exception of a short period after hatching, relation between age and total length of larvae was linear. The tail grew positively, the width of the head negatively allometric. Keeping larvae in groups resulted in a somewhat slower growth, in deviations from a strict linearity of some morphological parameters, and in a delayed increase of the number of LC, which is interpreted as crowding effect. LC could be identified already before hatching and developed first in the head, then in the trunk, and finally in the tail. Number of LC increased highly disproportionally during larval growth. Within 6 months, LC differentiated from relatively small cells (20 × 10 µm) with a vacuolated appearance to large round cells (diameter ca. 65 µm) with distinct and stainable granules and a prominent Langerhans' net forming several layers within the epidermis. LC neither rested directly upon the basal lamella nor reached the epidermal surface. They showed a moderate mitotic activity in all age groups examined. Number of mitoses appeared too small to explain the high number of LC in the epidermis and to guarantee continuous replacement. Mature, most superficially located LC appear to be shed.

> Kurzfassung

Die epidermalen Leydigzellen (LC) larvaler und paedomorpher Urodelen (= Caudata) sind hochspezialisierte Zellen mit einem komplexen peripheren Zytoskelett (Langerhans-Netz) und zahlreichen Einschlüssen, die meist als Sekretgrana bezeichnet werden. Wir haben Anzahl, Verteilung und Entwicklung dieser Zellen bei Larven bis zum 100. Tag nach dem Schlüpfen und bei einigen Adulten des paedomorphen Axolotls (*Ambystoma mexicanum*) untersucht. Mit Ausnahme einer kurzen Periode nach dem Schlupf ist die Beziehung zwischen Alter und Länge linear. Das Wachstum des Schwanzes ist positiv, das der Kopfbreite negativ allometrisch. Die gemeinsame Haltung mehrerer Larven hat ein langsameres Wachstum, Abweichungen von der Linearität mancher morphologischer Parameter und eine verzögerte Entwicklung der LZ zur Folge. LZ lassen sich bereits kurze Zeit vor dem Schlupf identifizieren und erscheinen zuerst am Kopf, dann im Rumpf und zuletzt im Schwanz. Die Anzahl der LZ nimmt mit fortschreitendem Alter und zunehmender Länge der Tiere überproportional zu. Innerhalb von 6 Monaten differenzieren sich die LZ aus relativ kleinen (20 × 10 µm) vakuolisierten Zellen zu großen Zellen (Durchmesser ca. 65 µm) mit zahlreichen distinkten und stark anfärbbaren Grana und einem ausgeprägten Langerhans-Netz, die in mehreren Lagen übereinander liegen. LC stoßen niemals unmittelbar an die Basallamina und erreichen normalerweise nicht die Epidermisoberfläche. In allen untersuchten Altersgruppen zeigen die LZ eine moderate Mitoseaktivität. Diese scheint jedoch zu gering zu sein, um daraus die hohe Anzahl von LC und deren kontinuierlichen Ersatz zu garantieren. Reife, distal gelegene LZ werden offensichtlich abgestoßen.

> Key words

Ambystoma mexicanum. Epidermis, Leydig cells, uneven distribution, amplification, crowding.

Introduction

Leydig cells (LC) in the epidermis of larval and paedomorphic Urodela (= Caudata) are known since the century before last. They were discovered by the German anatomist Franz von Leydig (LEYDIG, 1853), who characterized these cells in his textbook of histology as "Schleimzellen …, die bei gewissen constant im Wasser lebenden Wirbelthieren … gefunden werden …, unter den Batrachiern wurden sie beobachtet beim Proteus und den Larven des Landsalamanders" (LEYDIG, 1857, p. 96). Some years later, PAULICKI (1885) studying the skin of the axolotl (*Ambystoma mexicanum*) suggested the term Leydig cells for these noticeable cells.

LC do not appear to be limited to the epidermis of Urodela, but have also been described in the epidermis of larval Gymnophiona. Homology of the specialized "Riesenzellen", "Kugelzellen", "goblet cells", "clear cells" or "skein cells" in the larval anuran epidermis to LC has been questioned (e.g., POSKA-THEISS, 1930; FOX, 1988; DELFINO *et al*., 2007).

Since their discovery numerous histological and ultrastructural studies have been published to analyse development, structure and possible function of the urodele LC. The classical articles have been repeatedly summarized (e.g. DAWSON 1920; POSKA-THEISS, 1930; THEIS, 1932; SEEGER, 1933; GERSCH, 1942). Some more recent data have been reviewed by FOX (1986, 1988). A more updated synthesis, however, is missing.

Mature LC are considerably larger than the surrounding 'normal' keratinocytes. They are characterized by a highly elaborated peripheral cytoskeleton (Langerhans' net) and are crowded with numerous granules leaving only a small space around the centrally located nucleus. These granules are usually named secretory granules, although discharge of their content is a matter of debate (e.g. DAWSON, 1920; THEIS, 1930; FÄHRMANN, 1971a; KELLY, 1966; JARIAL, 1989; GERLING *et a*l., in preparation). The small, central space of LC (named "hofplasma" or "hofbereich"; e.g., GERSCH, 1942; KELLY, 1966; FÄHRMANN, 1971 a; GREVEN, 1980; KANTOREK & CLEMEN, 1991) is occupied by the cell organelles and further components of the cytoskeleton.

It is generally assumed that LC first arise from undifferentiated epidermal cells. In *A. mexicanum*, GERSCH (1942) postulated three centres of development with distinct location ("Bildungsherde"), from which LC are suggested to spread over the body. Then, LC increase in size and number, accumulate granules in their cytoplasm and subsequently undergo a kind of maturation (e.g. SEEGER, 1933; THEIS, 1932; GERSCH, 1942; KANTOREK & CLEMEN, 1991). Replacement and further increase of their number during larval growth may take place by the mitotic activity of fully differentiated LC (GERSCH, 1942; KELLY, 1966) and/or by differentiation of epidermal precursor cells. Development of LC may depend on neuronal signals as they disappear in limbs after treatment with cholinolytic drugs or after limb denervation (HUI & SMITH, 1976).

During naturally or artificially induced metamorphosis, LC undergo apoptosis (OHMURA & WAKAHARA, 1998) and remnants appear to be removed by macrophages (ROSENBERG *et al*., 1982). In paedomorphic species, however, they are present throughout life. LC have been considered an adaptation to aquatic life like the "Kugelzellen" in Anura, which are believed to may act as a kind of stiffness modules, in which the peripheral cytoskeleton (Langerhans´ net) withstands the turgor pressure exerted by the hydrated cytoplasm (e.g. DELFINO *et al*., 2007).

The above described "life cycle" of the urodele LC has been reconstructed from studies of only few species, namely the metamorphosing *Triton* (=*Lissotriton*) *vulgaris* (SEEGER, 1933), *Salamandra salamandra* (Salamandridae) (e.g. THEIS, 1932; ROSENBERG *et al*., 1982) and the paedomorphic axolotl *Ambystoma mexicanum* (Ambystomatidae) (e.g. GERSCH, 1942; FÄHRMANN, 1971 a–c; KANTOREK & CLEMEN, 1991).

Despite the considerable wealth of literature, knowledge of LC in Urodela remained sketchy at best, and results are in part contradictory. In addition, literature does not consider possible species-specific differences with regard to LC or differences between paedomorphic adults and larval species.

Therefore, we have again taken up work on LC of the axolotl to gain a basis for further studies on these cells. In the present article, we broaden previous findings combining morphometric data and histology. In a forthcoming article we will examine characteristic constituents of LC by ultrastructure and cytochemistry (GERLING *et al*., in preparation).

Material and methods

Larvae and adults of the wild type axolotl, white axolotl and Humphrey axolotl were obtained from various private breeders.

To study the growth, 10 larvae (series I) were isolated immediately after hatching, kept singly in small containers (0.5 l) and measured in defined intervals (see below).

To locate distribution and to count numbers of LC during growth, larvae were held up to 100 days after hatching either in small groups of initially five individuals in 12 l-plastic containers (altogether 33; series II) or as a single large group in a 12 l-container (31 individuals; series III). While analysing the data,

some differences between series II and III became obvious. Therefore, some findings will be described separately.

The first 30 days after hatching larvae were fed once a day with *Artemia*-nauplii. Later they were fed with chopped frozen larvae of *Chironomus* sp. For comparison, some juveniles and adults were used (see below), which were fed with shrimp pellets (Vitakraft, Germany) and frozen *Chironomus*-larvae. Water temperature in all containers was approximately 25 °C. Parts of the water were changed several times per week.

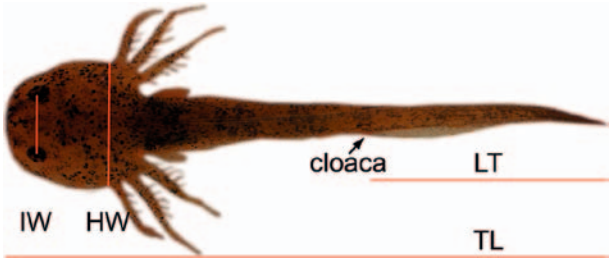

Fig. 1. Measurements taken from the growing larvae: total length (TL), length of the tail (LT), interocular width (IW), and head width (HW) at gills.

Histology

For histology embryos and larvae of different age were used. From series II (n=33) we examined three embryos (16 days after fertilization) and larvae (three per age) at day 0 (immediately after hatching); 5; 10; 15; 20; 25; 35; 50; 65; 80; and from series III (n=31) three larvae at day 0; 5; 3; 7; 10; 15; 20; 30; 50; 70; and two larvae at day 85 and 100.

Further, pieces of the dorsal (between or immediately behind the eyes) and ventral skin (a short distance behind the heart region) and of the lateral regions of the tail of juvenile and adult axolotls (6 and 12 months) were examined.

Animals were anaesthetized with MS 222. Embryos and whole larvae were fixed 6–12 h in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 at 4 °C, postfixed in 2 % osmiumtetroxide in the same buffer, dehydrated and embedded in resin (Spurr, 1969). Sections of approx. 1 µm thickness were taken with a Histo-Diamond knife (Ultramicrotom Om U3; Fa. Reichert) and stained with 1 % toluidine blue in 1 % borax. The heads of the two 100 days-old individuals were fixed in Bouin, embedded in Paraplast, sectioned at approximately 10 µm and stained with trichrome after Goldner (Mulisch & Welsch, 2010).

Mitoses of LC were counted in all 5059 semithin sections of series III (31 individuals). In addition their occurrence was checked in some semithin section of the six month and one year old specimen and in paraplast sections of the 100 day old larva (see above).

Morphometrics

Growth and body proportions: From the 10 larvae (series I) various parameters were measured at day 0; 5; 10; 15; 20; 30; 40; 50; 60; 70; 80; 90; and 100 (Fig. 1). For measurements specimens were transferred in a small petri dish filled with water, and photographed (Olympus ZH Binocular and Olympus C 3030 Zoom digital camera) together with graph paper at the bottom of the dish. Measurements (total length, head width at gills, interocular width, and length of the tail from the cloaca until the tip of the tail) were taken with the programme Olympus DP-Soft. Proportions of the body regions were determined by relating the total length to the length of the tail and the width of the head. For convenience, only the total length was measured from the animals used for histology. Allometry was calculated according to $y = b\ x^a$ and $\log y = \log b + a \log x$ (see Huxley, 1950; Gould, 1971; Alsleben, 2002).

Number and distribution of LC: For counting the LC, 50 to 60 semithin sections (1 µm) per larva from series II (33 specimens) and III (31 specimens) were taken from the head (immediately behind the eyes), the trunk (shortly behind the heart, s.o.) and the tail at the beginning of the second third (Fig. 2). Five sections with a distance of 100 µm from each other were used per body region and per specimen, to avoid double counting of LC. Particular attention was paid to mount sections always in the same position to unambiguously distinguish the left and right side of the specimen. We distinguished the three regions in the head (dorsal, lateral and ventral), three region in the trunk (fine edge, lateral and ventral), and two (lateral and tail edge) in the tail (Fig. 2). The mean number of LC per axolotl and body region from both series was calculated for each specimen (mean number of LC per section and body region) and plotted against the total length and the age of the animals.

Further, mean values of LC in the different body regions of the animals of series II and III were summed up (mean number of LC defined as LC in the total animal) and also plotted against the total length and the age of the animal. The significance of differences in the number of LC was calculated using a univariate significance test and a Tukey HSD test (Programme Statistika, Version 8, Fa. StatSoft). ($p > 0.05$ not significant, $p \leq 0.05$ significant, $p \leq 0.001$ highly significant).

To check, whether the number of LC increased absolutely or relatively (depending on growth), we meas-

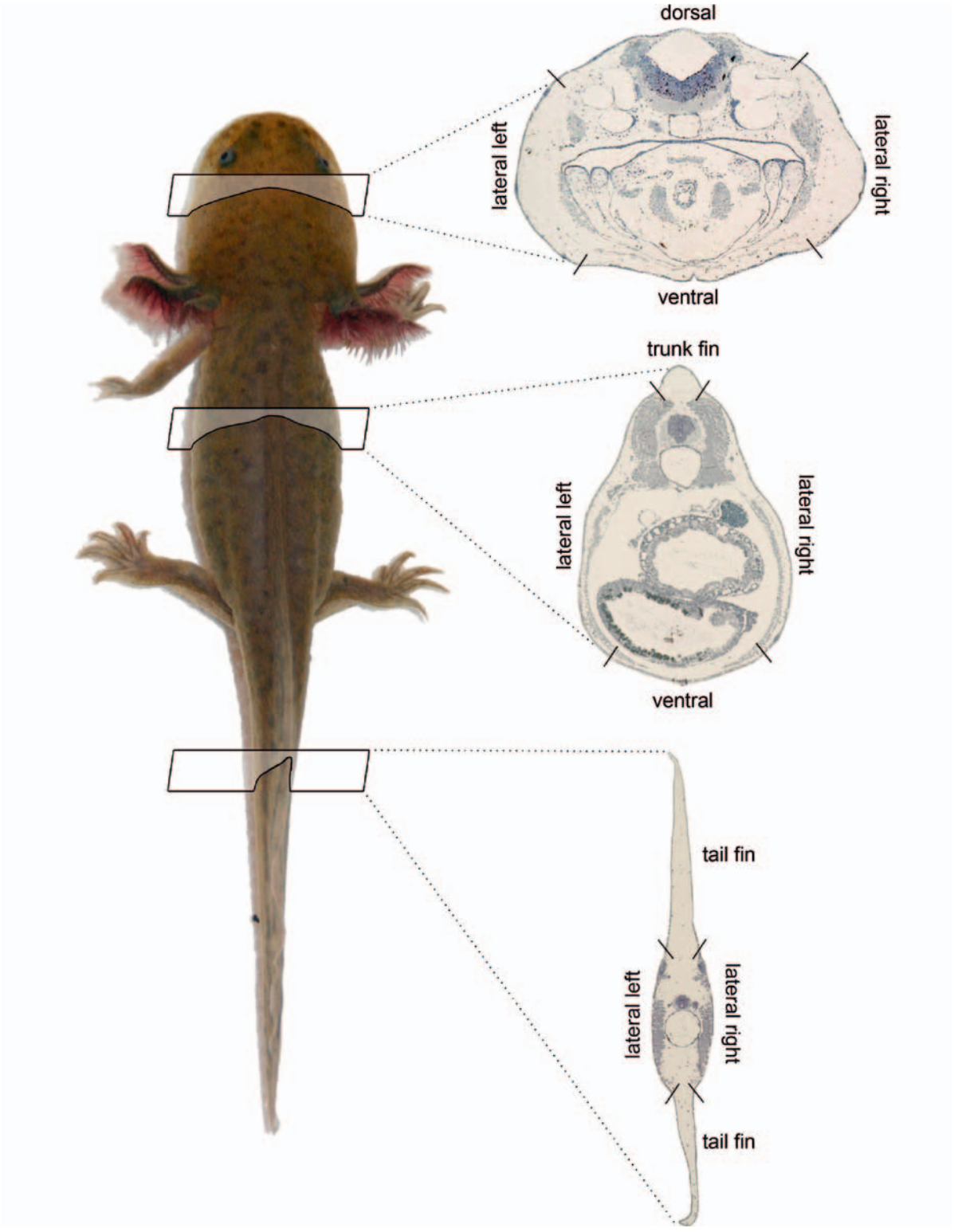

Fig. 2. Body regions, in which Leydig cells were counted.

ured the girth (in mm) of the head, the trunk and the tail of the specimens of series II (programme Olympus DP-Soft). Then we calculated the mean of the circumference for the respective age group (n=33) and its percent rise based on the calculations from embryos to day 80 and did the same for the number of LC. The percent rise of both, the circumference and the number of LC was plotted against the age and total length of the animals.

Finally, we calculated the mean number of LC per 1 mm² of the sectioned epidermis of the three body regions of the individuals of series II by counting the number of LC per 1 mm length of the epidermis and extrapolating it to 1 mm².

Results

Growth and proportional changes of larvae

Total length: Figure 3 shows that increase of total length in series I was low and rather constant in all larvae in the first days after hatching. Approximately at day 15, standard deviations (data not shown) increase indicating a more irregular growth among individuals. Generally, however, the increase of total length was largely linear from day 15 to day 100.

Changes of body proportions: Compared to the total length the tail grows positively allometric (value of allometry 1.21). This is most clearly seen in the curve during the first 30 days (Fig. 4 A). The width of the head grew negatively allometric (value of allometry 0.87) (Fig. 4 B). The interocular width increases faster compared to the width of the head. (allometry value 1.25) (Fig. 4 C).

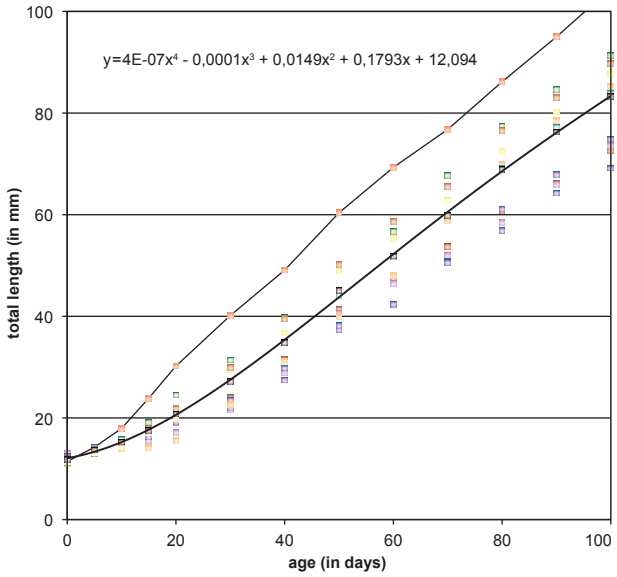

Fig. 3. Increase of total length of 10 axolotl larvae held singly (series I) from hatching to an age of 100 days (mean and individual values). Note the largely linear course from day 16–100.

Number and distribution of LC

Number of LC in the „total animal“: With increasing length the mean number of LC increased linearly in series II (Fig. 5) and series III (data not shown). After 100 days the largest specimen in series III measured 48 mm in length with a “total” of 1250 LC, whereas in series II already after 80 days the largest specimen measured 54 mm with a “total” of 1204 LC indicating a faster growth in this series.

Mean number of LC plotted against the age reveals, however, deviations from linearity in both series (data not shown).

Distribution of LC on the head, the trunk, and the tail: Increase of the mean number of LC per section plotted against total length is again linear in both series for the three regions studied, exemplarily shown for series II (Fig. 6).

From the very first day there was a specific distribution pattern of LC in several body regions of all specimens (Fig. 7). Despite of some variations in the increase of the number of LC in these regions, some trends could be recognized. Immediately before and after hatching the number of LC per mm² in the dorsal head region was the highest in the whole animal, followed by the ventral region of the trunk. The tail had the lowest number of LC. Later also the number of LC in the ventral region of the head increased, but was always lower than in the back. In the flanks number of LC was always smaller than in ventral and dorsal regions. In the tail increase of the number of LC was delayed until approx. the 10th day. In the edge of the

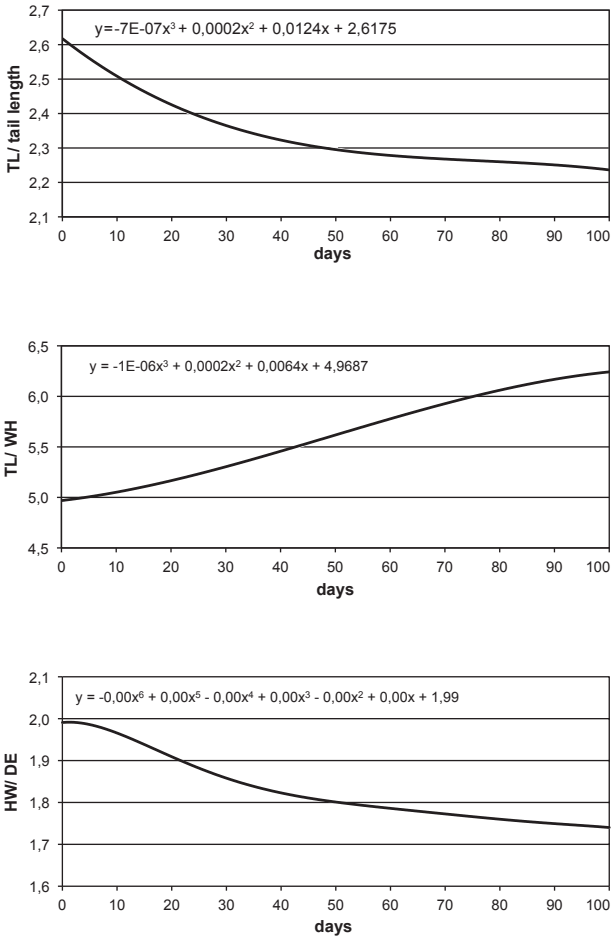

Fig. 4. Changes of body proportions during the growth of 10 axolotl larvae (series I) up to an age of 100 days. **A**: Age (in days) and ratio of total length (TL) to tail length (LT). **B**: Age and ratio of TL to width of the head (HW). **C**: Age and ratio of HW to interocular width (IW).

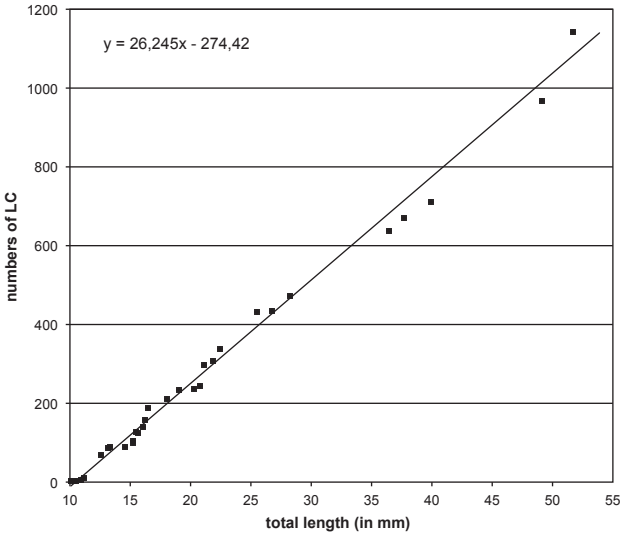

Fig. 5. Mean number of LC ("total animal") of axolotl larvae of series II (plotted against the total length).

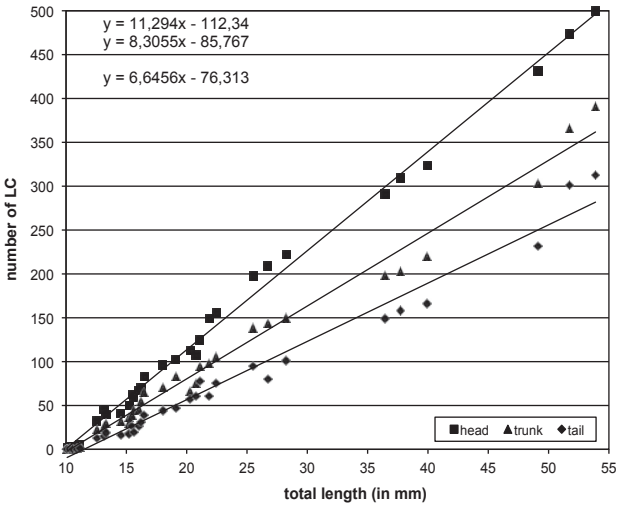

Fig. 6. Mean number of LC per section in three body regions of axolotl larvae of series II plotted against the total length.

tale number of LC grew most slowly and remained lower throughout life.

Beyond a given length, differences in the distribution of LC between the two series were always significant ($p < 0.05$) to highly significant ($p < 0.001$). In series II numbers of LC in a 13.3 mm-long larva was higher in the head than in the trunk, in a 14.1 mm larva number was higher in the trunk than in the tail. In series III the difference between head and trunk was significant at a length of 15.2 mm and between the trunk and the tail at 12.5 mm, i.e. already an increase of few millimetres resulted in a significant difference of the LC-numbers.

The mean number of LC per section plotted against the age of the animals shows again a deviation from linearity (Fig. 8) that was even more pronounced in series III (not shown).

Data of series II show exemplarily that in all three body regions the percent rise of the number of LC clearly exceeds the percent increase of the body girth.

During linear growth from 10 to 52 mm, increase of the girth of the head was appoximately 5 fold, of the trunk 3 fold and of the tail 2.7 fold. However, increase of the number of LC in the head region was approx. 190 fold, in the trunk 485 fold, and in the tail approx. 3800 fold. This enormous increase, seemingly disproportional, is due to the fact that in the smallest larvae, number of LC was extremely low in the trunk and also in the tail. The increase in percent is shown in Fig. 9.

Development and maturation of LC

LC can be recognized already before hatching by their position between the two epidermal cell layers characteristic for this stage; the slight vacuolization (Fig. 10 A) of LC and presence of widened intercellular spaces is variable. In freshly hatched larvae the bi-layered head epidermis is slightly thicker than in the trunk and noticeably thicker than in the tail. In the trunk the epidermis is ventrally stronger than laterally. The thinnest epidermis is found in the edge of the tail. These relationships remain the whole life. At this stage LC form a discontinuous intermediate layer and have a size of approx. 20 × 10 µm. LC in freshly hatched larvae LC can be clearly identified by their "vacuolized" appearance. All LC increase in size dependent on their location in the head, trunk, or tail (see Fig. 11 A–C). They become more roundish forming a discontinuous layer in the epidermis (Fig. 11 A, D). In the fin edge, LC are extremely rare. When LC grow larger, vacuoles, which are not stainable with toluidine blue, increase in size and number (Fig. 10 B–E). At an age of approx. 20 days the peripheral Langerhans´ net becomes visible (Fig. 10 E) and around day 35 some of the smaller vacuoles became stainable. At this time, nuclei are smaller than before (Fig. 10 F). In course of further differentiation of LC, the vacuolization is replaced by smaller granules. Stainabilty of these granules increases continuously (Fig. 10 G–L) and around the nucleus the "hofplasma", i.e. the central region containing the nucleus and most cell organelles, can be recognized (Fig. 10 I). In addition, size and thickness of the Langerhans' net considerably increases. Fully differentiated LC are crowded with toluidine blue-positive granules and exhibit a prominent net (Fig. 10 M–O). LC of all body regions are fully developed after approx. 6 months. During growth of the larvae LC considerably contribute to the thickness of the epidermis (Fig. 11 A–F). For example, in larvae at an age of six months the head epidermis is up to 140

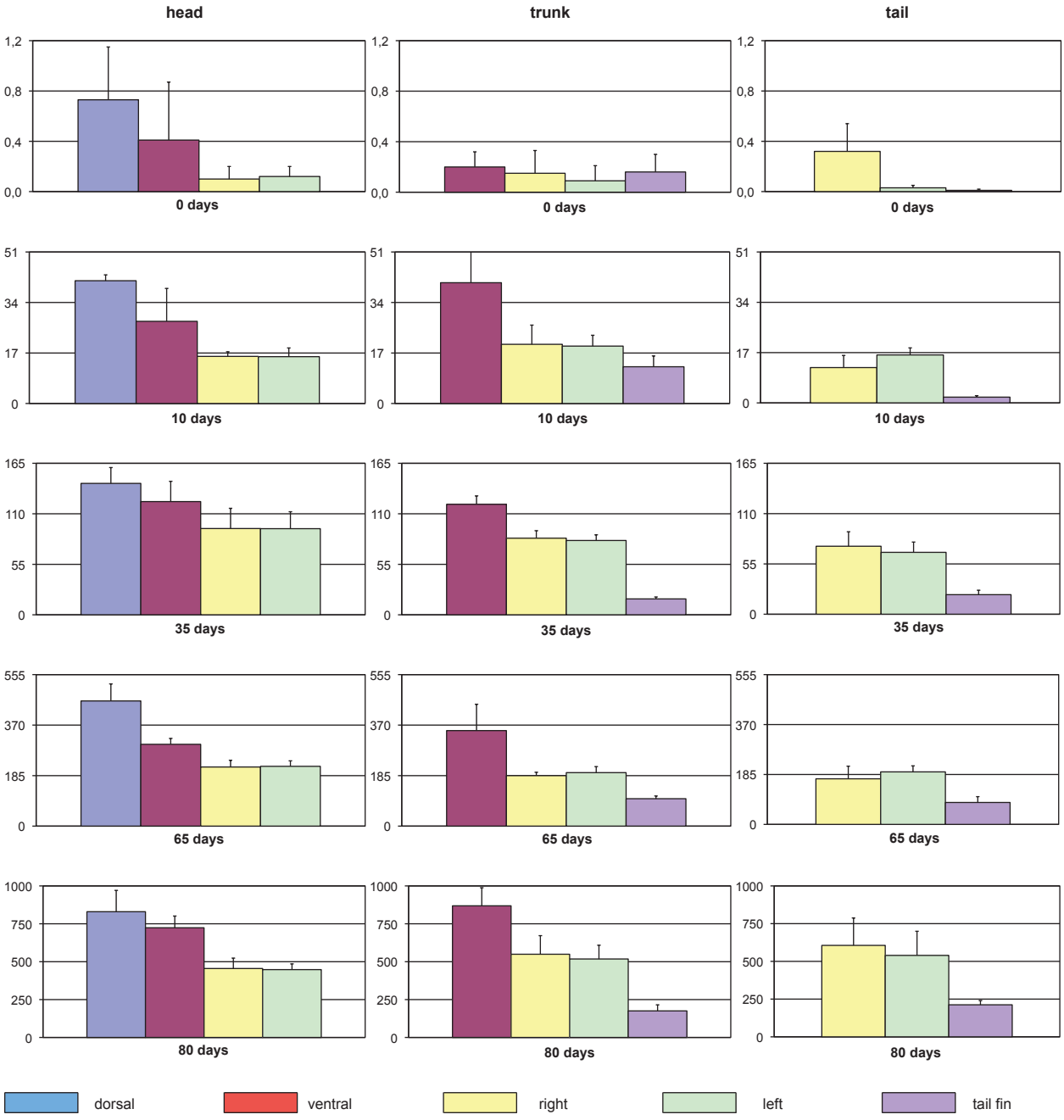

Fig. 7. Mean number plus SD of LC per 1 mm^2 epidermis of the body regions examined (series II).

µm thick containing 2 to 4 layers of LC in a section. Size may vary, the largest LC may reach. 60–65 µm in diameter. In specimens at an age of one year the head epidermis reaches a thickness of approx. 200 µm with four layers of LC, and in an eight year old specimen a thickness of 385 µm with 8 to 12 layers of LC (not shown).

LC were separated from one another and from the basal lamina by epidermal keratinocytes and never reached the surface of the epidermis. However, in mature specimen, but not in the larvae, we repeatedly observed shedding of the most distally located LC (Fig. 11 G, H).

Mitoses

We checked 5059 semi-thin and some paraplast sections obtained from 31 axolotls ranging from an age of 0 (hatchlings) to 100 days and several sections from individuals of an age of 6 months, 12 months and eight year for mitoses. In the semithin sections of the axolotls at an age of 0 to 100 days we detected altogether 54 LC in mitosis (Fig. 11 I–K), 21 mitoses in the head epidermis, 23 in the trunk epidermis and 10 in the tail epidermis. Mitoses in the „normal“ epidermis cells were more frequent (not counted) and were found in all layers, the stratum basale, the stra-

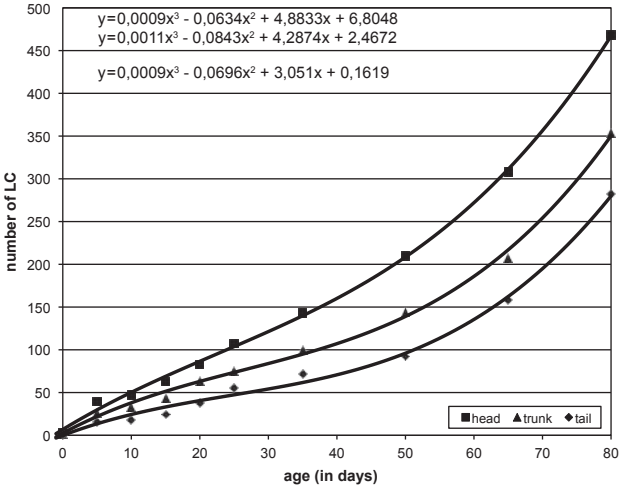

Fig. 8. Mean number of LC per section in three body regions of axolotl larvae of series II plotted against the age.

tum intermedium, and the superficial pavement cells. Mitoses of LC were also found in the older specimens mentioned.

Discussion

Growth and changes of proportions of larvae

In amphibians embryonic and larval development are affected by various parameters such as size of eggs, food abundance, etc. and – of particular significance – temperature (e.g., DEMPSTER, 1930; BIZER, 1978; PETRANKA, 1984; for summary see BRADFORD, 1990). Thus, the axolotl larvae, held by us at 25 °C hatched later (after 17 ± 1 days on average) than axolotl larvae held at 29 °C, which hatched 11 days after oviposition (BORDZILOVSKAYA & DETTLAFF, 1979; BORDZILOVSKAYA *et al*., 1989).

Growth curves of amphibians (growth defined as an increase in the mass of living substance) often use measurements of the snout-vent length plotted against the age to calculate mass (e.g., SEMLITSCH, 1983; HUTCHERSON *et al.,* 1989). Contrary to this practice, we measured the total length of the larvae as we focused on number and distribution of LC in the whole body. Nevertheless, also the curve obtained, showed a short postembryonic phase with a lag phase and the period of nearly exponential growth typical for amphibian growth curves (WILBUR & COLLINS, 1973; for review see HOTA, 1994; HARRIS, 1999) not finished at an age of 100 days.

With respect to changes of body proportions during postembryonic development we noticed (as expected) a positive allometric growth of the tail (compared to the total body length), a decrease of the relative width of the head and a slight expansion of the interocular width. Largest alterations regarding total length/tail length and width of the head/interocular distance take place within the first 20 days after hatching. To our knowledge such data are missing for other urodele species. However, tail growth, and decrease of the width of the head can be seen by the mere inspection of the external appearance of larvae in developmental staging tables (e.g., in *Triturus* (= *Ichthyosaura*) *alpestris*: EPPERLEIN & JUNGINGER, 1982; *Batrachuperus gorganensis* (Hynobiidae): EBRAHIMI *et al.*, 2004).

Laboratory and field studies have documented density-dependent effects on larval populations, especially in anuran tadpoles, but also in urodele larvae. Rising larvae under crowded conditions resulted in slower growth rates and higher mortality (summarized and discussed in WELLS, 2007; *Ambystoma* spp.: e.g. WILBUR, 1976; STENHOUSE *et al*., 1983; SCOTT, 1990, 1996), but also no effects have been observed (PETRANKA, 1984). Albeit we did not intend to study the crowding effect in the present study, the slower growth of the larvae from series III and even the deviations from a roughly linear relationship of several parameters plotted against the age (LC/age) seen in both series (mostly stronger in series III) may be interpreted in this way. Crowding results in a delayed growth, i.e. larvae remained smaller. Thus, a linear relationship between length and age is not guaranted in the both series.

The successive removal of specimens from the crowded containers in course of the study could not entirely neutralize this effect indicating a considerable sensitivity of axolotl larvae to this kind of stress.

Number and distribution of LC

LC in larval Urodela can definitely be identified immediately after hatching (e.g. in *Necturus maculosus* (DAWSON, 1920), *Ambystoma mexicanum* (GERSCH, 1942), and *Hynobius dunni*: KATO & KURIHARA, 1987), but even some days before hatching as shown herein. Increase of the number of LC during development and their gradual decrease around metamorphosis have been described qualitatively in a number of naturally metamorphosing urodeles (e.g. *Hynobius* spp.: KATO & KURIHARA, 1987; WAKAHARA & YAMAGUCHI, 1996; *A. mexicanum*: GERSCH, 1942; KANTOREK & CLEMEN 1991; *Salamandra salamandra*: DENNERT, 1923; THEIS, 1932; WARBURG & LEWINSON, 1977; GREVEN, 1980; *Taricha torosa*: KELLY, 1966; *Triton* (= *Ichthyosaura*) *alpestris*: HADORN & CHEN, 1953) and was noted in the paedomorphic axolotl after artificially induced metamorphosis (GERSCH, 1942; FÄHRMANN, 1971 c;

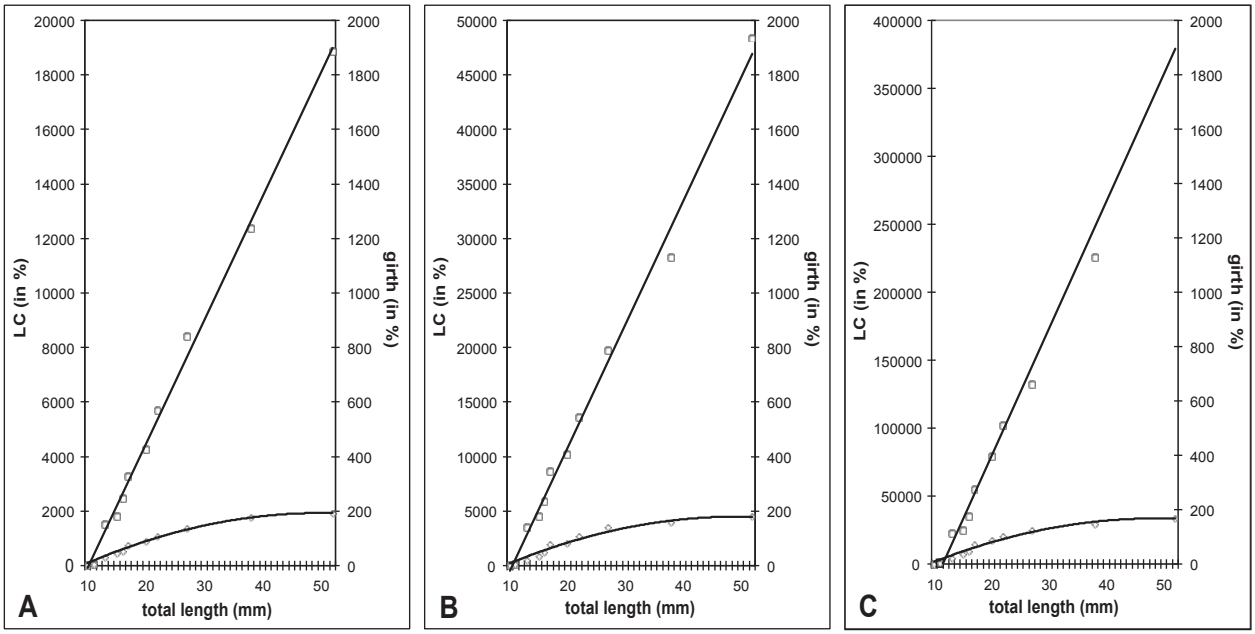

Fig. 9. Increase (in %) of the girth of the head (**A**), the trunk (**B**) and the tail (**C**) (right abscissa) of axolotl larvae of series II related to the length and the mean number of LCs (left abscissa) per section in these body regions.

Hackford *et al*., 1977; Page *et al*., 2009). Only in *Triton* (= *Lissotriton) vulgaris*, a species that undergoes metamorphosis increase of LC has been documented quantitatively. First the relative number of LC per mm^2 increased up to a length of larvae of 20 mm (approx. 20 per mm^2 and largely comparable in the different body regions) and then remained more or less constant until moulting in course of metamorphosis (Seeger, 1933). Our data including those of the number of LC per mm and per mm^2 of the epidermis confirm increase of LC.

Decrease of LC before metamorphosis was mentioned in *T. torosa*, when the epidermis thickens (Kelly, 1966). The statement of Rosenberg *et al*. (1982), that in larvae of the lecithotrophic viviparous *Salamandra salamandra* (verry probably *infraimmaculata*) number of LC begins to decrease already after birth needs to be confirmed, especially as their number seems to increase in the closely related *S. salamandra* after birth (Dennert, 1923) but a relative decrease was observed in older larvae (Dennert 1923). Interestingly, larvae of the pueriparous *Salamandra atra* possess only a very small number of LC (Dennert, 1923; Guex & Greven, 1994).

In the paedomorphic axolotl, we observed a short time after hatching a sudden increase of LC. Increase continued far beyond an age of 100 days. Generally, increase of LC is linear when plotted against the body length. When the number of LC was plotted against the age, deviations (more distinct in series III) from linearity were obtained, which may be caused by external influences, such as crowding (see above). The mean number of 400 LC per section occurred in series III in 45 mm-long specimens at an age of 100 days, in series II, however, in 53 mm-specimen at an age of 80 days, indicating that their increase depends largely on growth and less on age. Nevertheless, the possible effect of crowding, does not question the general results of the present study.

The order of LC appearance follows a distinct pattern. First of all LC are seen on the head, then in the trunk and finally in the tail. In the same order also a second layer of LC develops. Number of LC on the head is larger than in the trunk and numbers in the trunk are higher than in the tail. These differences appear to persist throughout life as indicated by the same disproportion in an 8-year old axolotl. Already Dawson (1920: 502) reported on a "tendency toward local grouping" in *Necturus maculosus*. Gersch (1942) postulated three "Bildungsherde" (centres of development) of LC in larval axolotls: between the eyes, in the heart region, and around the cloaca. We can confirm at least the first and in part the second center (our ventral region behind the heart). Around the cloaca we occasionally found only a higher number of LC.

The obvious faster increase of the number of LC on the head cannot be ascribed to a faster growth of the head related to the body, as the growth of the head slightly lags behind the growth of the body so that number of LC increases disproportionately. From day 5 to 7 after hatching density of LC on the venter was always larger than in the other trunk regions. The large difference in the number of LC between the ventral and lateral trunk between day 50 to 70 after hatching is caused by the fact that a second layer of LC is developed in the ventral trunk at that time. Compared

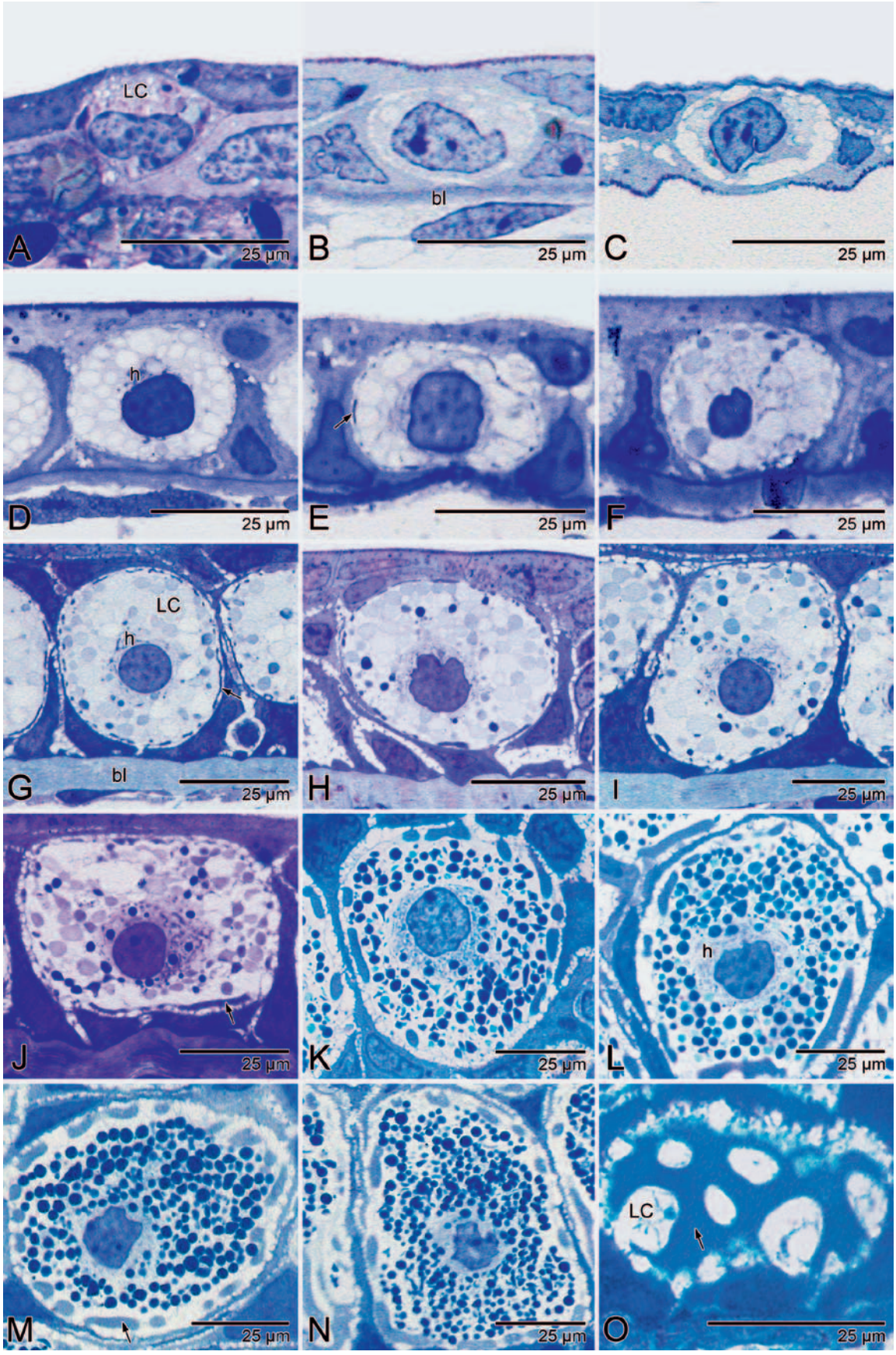

Fig. 10. Differentiation of epidermal LC in larvae and adults. 16 days after fertilization (embryo) (**A**). Immediately after hatching (**B**). 3 days (**C**). 10 days (**D**). 20 days (**E**). 35 days (**F**). 50 days (**G**). 65 days (**H**). 85 days (**I**). 100 days (**J**). 6 months (**K**). 12 months (**L**). 12 months (**M**). 8 years (**N, O**). Note changes in the appearance and size of granules; Langerhans´ net (arrow), basal lamina plus basement lamella (bl), "hofplasma" (h) Leydig cell (LC).

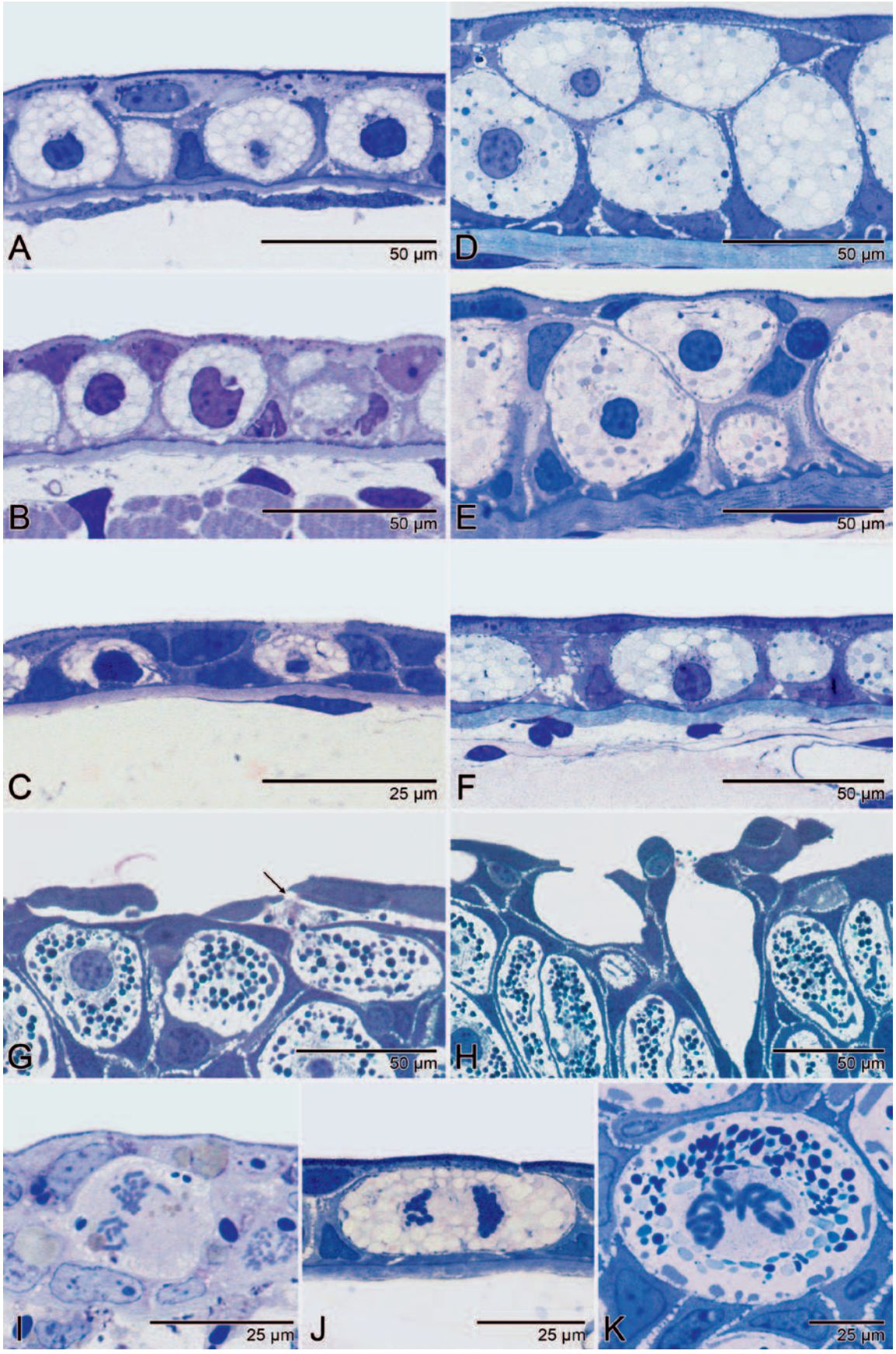

Fig. 11. Differences in size and number of LC in the epidermis of the head (**A**, **D**), the ventral trunk (**B**, **E**) and the fin edge of the tail (**C**, **F**) in a 15 day old larva (**A**, **B**, **C**), and a 85 day old larva (**D**, **E**, **F**). Shedding of LC in the trunk of a six mounth old specimen. Note detached superficial cells (arrow) (**G**) and holes previously occupied by LC (**H**). Mitoses of epidermal LC in axolotls of different age; embryo, head (**I**), 50 days after hatching, edge of the tail (**J**), 12 month, trunk (**K**).

to the head and the trunk the tail-epidermis showed the lowest number of LC. This is not due to a delayed growth of the tail, which grows positively allometric. Contrary to GERSCH (1942) we found LC also in the fin edge, where they appear last, but generally this region remains poor in these cells or LC are entirely absent (e.g. PAULICKI, 1885; DENNERT, 1923; SEEGER, 1933; GERSCH, 1942; KELLY, 1966). Information on developmental centres of LC in other larval Urodela is doubtful. In *Triton* (=*Lissotriton*) *vulgaris* SEEGER (1933) first recognized LC in the neck region of embryos of 3–6 mm and then two dorsal strands of LC spreading caudally and ventrally. In fully developed larvae LC appear to be distributed more or less uniformly. This may indicate species-specific differences not studied yet.

In brief: The number of LC in the epidermis of the paedomorphic axolotl increases after hatching very probably throughout life. Until the 100. day after hatching at least in the head (dorsally and ventrally), and in the trunk (ventrally) increase of number is disproportionate resulting in higher numbers than in other body regions. This disproportionate distribution appears to persist a long time (as shown in a 8-year old axolotl) or even throughout life.

Origin, development, maturation and replacement of LC

Undoubtedly, LC arise from undifferentiated stratum basale cells during ontogeny (e.g., THEIS, 1932; SEEGER, 1933; GERSCH, 1942 and further readings herein). The occurrence of "Bildungszentren" suggests a preferential formation of LC in these regions and probably a subsequent migration of LC in the other regions of the body. Indirect evidence for migration of LC comes from regeneration experiments. After lesions number of LC increased in the regenerating area, but decreased in its near environment. The rate of mitoses, however, was similar at the wounded site and the opposite control site (*Triton* (=*Ichthyosaura*) *alpestris*: HADORN & CHEN, 1953). In larval *Salamandra salamandra* WEBER (1956), however, observed an increased mitotic activity of LC and "normal" epidermal cells during wound healings indicating stimulation of division. It was suggested that LC migrated into the regenerating epidermis, degenerate and new LC are believed to arise from the regenerating epithelium (PELC, 1965). In no case, evidence of dedifferentiation of LC was observed.

LC of *A. mexicanum* are fully developed at an age of six months. LC are considered herein as mature, when they are large, exhibit a strong Langerhans' net and when all or nearly all granules are intensely stained with toluidine blue. At the ultrastructural level this process involves a condensation of the large Golgi-derived vacuoles seen in immature LC (see FÄHRMANN, 1971 a, KANTOREK & CLEMEN, 1991; GERLING *et al*., in preparation).

When the epidermis becomes squamous and LC mature, replacement was suggested to take place by the mitotic activity of LC (GERSCH, 1942). Actually KELLY (1966) discussed a high capacity of LC for mitotic proliferation citing unpublished results on a rapid incorporation of tritiated thymidine.

However, mitoses of LC are described as being rare (e.g. *A. mexicanum*: PAULICKI, 1885; *Necturus maculosus*: DAWSON, 1920; *Salamandra salamandra*: DENNERT, 1923, WEBER 1956, KUHN & WEBER 1957; GREVEN, 1980; *Taricha torosa*: KELLY, 1966; *Triton* (=*Ichthyosaura*) *alpestris*: HADORN & CHEN, 1953) and occasionally authors were even unable to detect them (e.g. *Triton* (=*Lissotriton*) *vulgaris*: SEEGER, 1933; *S. salamandra*: ROSENBERG *et al*., (1982).

The larval urodele epidermis does not seem to have a true stratum germinativum at least in younger stages, as all epidermal constituents, i.e. basal cells, pavement cells and fully developed LC show mitotic stages (e.g. KELLY, 1966). In anuran tadpoles all epidermal cells were labelled after ^{3}H-thymidine exposition, but, in young tadpoles the apical pavement cells, which must be replaced frequently, had the highest labelling index, whereas in older tadpoles ^{3}H-thymidine is incorporated largely in the basal cells (e.g. ROBINSON & HEINTZELMAN, 1987). These newly formed basal cell layer functions as precursor cells of adult epidermal cells (KINOSHITA & SASAKI, 1994). Such data have not been published for larvae and paedomorphic urodeles, which obviously lack a defined moulting cycle. However, there is evidence that superficial layers of the epidermis including LC are shed more or less continuously (see also JARIAL, 1989).

Considering the rare mitoses observed in LC of urodeles and our counting, we think that numbers of mitoses are too small to account for the formation of new LC and replacement of old LC unless LC follow a hitherto unexplored circadian rhythm. Using ^{3}H-thymidine, such rhythms have been demonstrated in various urodele tissues including the larval epidermis of *Ambystoma tigrinum* and *Taricha torosa* (SCHEVING *et al*., 1959; SCHEVING & CHIAKULAS, 1965; CHIAKULAS & SCHEVING, 1966) and the adult epidermis of *Notophthalmus viridescens* (HOFFMANN & DENT, 1977) showing a bimodal pattern of mitotic rates. In one of the studies authors also counted mitoses in whole mount preparations, but did not differentiate between keratinocytes and LC in the larval epidermis (SCHEVING & CHIAKULAS, 1965). If, however, LC in juveniles and adults mainly arise from undifferentiated epidermal precursor cells, a maturation process from small vacuolized cells to highly differentiated

LC must be assumed as described herein, Currently, however, such young stages were rarely seen in our sections from juveniles and adults

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References

Alsleben, B. (2002): Analyse der Zusammenhänge zwischen der Entwicklung von Körper- und Klauenmerkmalen sowie der Druckverteilung unter den Klauen bei Jungrindern der Rassen Deutsche Holsteins und Deutsches Braunvieh. – Inauguraldissertation Tierärztlichen Hochschule Hannover.

Armstrong, J.B. & Malacinski, G.M. (1989): Developmental Biology of the Axolotl. – Oxford University Press, Inc., New York.

Bizer, J.R. (1978): Growth rates and size at metamorphosis of high elevation populations of *Ambystoma tigrinum*. – Oecologia, **34**: 175–184.

Bordzilovskaya, N.P. & Dettlaff, T.A. (1979): Table of stages of the normal development of axolotl embryos and the prognostication of timing of successive developmental stages at various temperatures. – Axolotl Newsletter, **7**: 2–22.

Bordzilovskaya, N.P., Dettlaff T.A. & Malacinski, G.M. (1989): Developmental stage series of Axolotl embryos. – In: Armstrong, J.B. & Malacinski, G.M.: Developmental Biology of the Axolotl. – Oxford University Press Inc. New York: 201–219.

Bradford, D.F. (1990): Incubation time and rate of embryonic development in amphibians: The influence of ovum size, temperature and reproductive mode. – Physiological Zoology, **63**: 1157–1180.

Chiakulas, J.J. & Scheving L.E. (1966): Circadian phase relationships of ^{3}H-thymidine uptake, numbers of labelled nuclei, grain counts and cell division rate in larval urodele epidermis. – Experimental Cell Research, **44**: 256–262.

Dawson, A.B. (1920): The integument of *Necturus maculosus*. – Journal of Morphology, **34**: 487–589.

Delfino, G., Quagliata, S., Giachi, F. & Malentacchi, C. (2007): Clear cells in larval anuran epidermis: an ultrastructural study on tadpoles of *Phyllobates bicolor* (Dendrobatidae) and *Pelobates fuscus* (Pelobatidae). – Contributions to Zoology, **76**: 213–220.

Dennert, W. (1923): Über den Bau und die Rückbildung des Flossensaums bei den Urodelen. – Zeitschrift für Anatomie und Entwicklungsgeschichte, **72**: 407–462.

Dempster, W.T. (1930): The growth of Larvae of *Ambystoma maculatum* under natural conditions. – Biological Bulletin, **58**: 182–192.

Ebrahimi, M., Kami, H.G. & Stöck, M. (2004): First description of egg sacs and early larval development in hynobiid salamanders (Urodela, Hynobiidae, *Batrachuperus*). – Asiatic Herpetological Research, **10**: 168–175.

Epperlein, H.H. & Junginger, M. (1982): The normal development of the newt, *Triturus alpestris* (Daudin). – Amphibia-Reptilia, **2**: 295–308.

Fährmann, W. (1971a): Die Morphodynamik der Epidermis des Axolotl (*Siredon mexicanum* Shaw) unter dem Einfluss von exogen appliziertem Thyroxin. I. Die Epidermis des neotenen Axolotls. – Zeitschrift für mikroskopisch-anatomische Forschung, **83**: 472–506.

Fährmann, W. (1971b): Die Morphodynamik der Epidermis des Axolotl (*Siredon mexicanum* Shaw) unter dem Einfluss von exogen appliziertem Thyroxin. II. Die Epidermis während der Metamorphose. – Zeitschrift für mikroskopisch-anatomische Forschung, **83**: 53–568.

Fährmann, W. (1971c): Die Morphodynamik der Epidermis des Axolotl (*Siredon mexicanum* Shaw) unter Einfluß von exogen appliziertem Thyroxin. III. Die Epidermis des metamorphosierten Axolotls. – Zeitschrift für mikroskopisch-anatomische Forschung, **84**: 1–25.

Fox, A. (1986): The skin of the amphibia: Epidermis. – In: Bereiter-Hahn, J.; Matoltsy, A.C.; Richards, K.S. (eds.): Biology of the Integument: Vertebrates. – Springer Verlag, **2**: 78–108.

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

Gersch, H. (1942): Aufbau und Differenzierung des Integuments vom Axolotl auf Grund einer vergleichenden Untersuchung mit Hilfe vitaler und histologischer Färbemethoden. – Zeitschrift für mikroskopisch-anatomische Forschung, **51**: 513–554.

Greven, H. (1980): Ultrastructural investigation of the epidermis and the gill epithelium in the intrauterine larvae of *Salamandra salamandra* (Amphibia Urodela). – Zeitschrift für mikroskopisch-anatomische Forschung, **94**: 196–208.

Gould, S.J. (1971): Geometric similarity in allometric growth: A contribution to the problem of scaling in the evolution of size. – American Naturalist, **105**: 113–136.

Guex, G.D. & Greven, H. (1994): Structural and physiological aspects of viviparity in *Salamandra atra*. – Mertensiella, **4**: 161–208.

Hackford, A.W., Gilles, C.G., Eastwood, C. & Goldblatt, P.J. (1977): Thyroxine-induced gill resorption in the Axolotl. – Journal of Morphology, **153**: 479–504.

Hadorn, E. & Chen, P.S. (1953): Beobachtungen über die Wanderung der Leydigschen Drüsenzellen bei Regenerationsvorgängen in der Larvenhaut von *Triton alpestris*. – Roux' Archiv für Entwicklungsmechanik, **146**: 515–536.

HARRIS, R.N. (1999): The anuran tadpole. Evolution and maintenance. In: MC DIARMID, W. & ALTIG, R. (eds.): Tadpoles. The biology of anuran larvae. – University of Chicago Press, Chicago: 279–294.

HOFFMANN, C.W. & DENT, J.N. (1977): Hormonal effects on mitotic rhythm in the epidermis of the red-spotted newt. – General and Comparative Endocrinology, **32**: 512–521.

HOTA, A.K. (1994): Growth in amphibians. – Gerontology, **40**: 147–160.

HUI, F.W. & SMITH, A.A. (1976): Degeneration of Leydig cells in the skin of the salamander treated with cholinolytic drugs or surgical denervation. – Experimental Neurology, **53**: 610–619.

HUTCHERSON, J.E., PETERSON, C.L. & WILKINSON, R.F. (1989): Reproductive and larval biology of *Ambystoma annulatum*. – Journal of Herpetology, **23**: 181–183.

HUXLEY, J.S. (1950): Relative growth and form transformation. – Proceedings of the Royal Society London B, **137**: 465–469.

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

KANTOREK, S. & CLEMEN, G. (1991): The origin and development of Leydig cells in the epithelium of the oropharynx of *Ambystoma mexicanum* Shaw (Urodela: Ambystomatidae). – Zoologischer Anzeiger, **227**: 13–24.

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

KELLY, D.E. (1966): The Leydig cell in larval amphibian epidermis. Fine structure and function. – Anatomical Record, **154**: 685–700.

KINOSHITA, T. & SASAKI, F. (1994): Body-specific proliferation of adult precursor cells in *Xenopus* larval epidermis. – Histochemistry, **101**: 397–404.

KUHN, O. & WEBER, W. (1957): Mitosenverteilung in der larvalen Rumpfepidermis von *Salamandra maculosa* Laur. – Experientia, **18**: 25–27.

LEYDIG, F. (1853): Anatomisch-histologische Untersuchungen über Fische und Reptilien. – G. Reimer, Berlin.

LEYDIG, F. (1857): Lehrbuch der Histologie des Menschen und der Thiere. – Verlag von Meidinger Sohn & Comp., Frankfurt.

MULISCH, A. & WELSCH, U. (2010): Romeis Histologische Technik. – Spektrum Verlag, Heidelberg.

OHMURA, H. & WAKAHARA, M. (1998): Transformation of skin from larval to adult types in normally metamorphosing and metamorphosis-arrested salamander, *Hynobius retardatus*. – Differentiation, **63**: 237–246.

PAGE, R.B., MONAGHAN, J.R., WALKER, J.A. & VOSS, S.R. (2009): A model of transcriptional and morphological changes during thyroid hormone-induced metamorphosis of the axolotl. – General and Comparative Endocrinology, **162**: 219–232.

PAULICKI, D. (1885): Über die Haut des Axolotls. – Archiv für mikroskopische Anatomie, **24**: 120–174.

PELC, Z. (1965): Appearance of Leydig cells in the limb regenerate of the axolotls (*Ambystoma mexicanum* Cope). – Acta Biologica Cracoviensia, **8**: 225–233.

PETRANKA, J.W. (1984): Sources of interpopulation variation in growth responses of larval salamanders. – Ecology, **65**: 1857–1865.

POSKA-THEISS, L. (1930): Über die larvale Amphibienepidermis. – Zeitschrift für Zellforschung und mikroskopische Anatomie, **11**: 445–483.

ROBINSON, D.H. & HEINTZELMAN, N.B. (1987): Morphology of ventral epidermis of *Rana catesbeiana* during metamorphosis. – The Anatomical Record, **217**: 305–317.

ROSENBERG, M., LEWINSON, D. & WARBURG, M.R. (1982): Ultrastructural studies of the epidermal Leydig cell in larvae of *Salamandra salamandra* (Caudata, Salamandridae). – Journal of Morphology, **174**: 275–281.

SCHEVING, L.E., CHIAKULAS, J.J. & ABZUG, H.I. (1959): A basic rhythmicity in the mitotic rate of urodele epidermis and the difference in mitotic rate between larvae reared in the laboratory and in a pond. – Journal of Cellular and Comparative Physiology, **54**: 109–114.

SCHEVING, L.E. & CHIAKULAS, J.J. (1965): Twentyfour-hour periodicity in the uptake of tritiated thymidine and its relation to mitotic rate in urodele larval epidermis. – Experimental Cell Research, **39**: 161–169.

SCOTT, D.E. (1990): Effects of larval density in *Ambystoma opacum*: An experiment in large-scale field enclosures. – Ecology, **71**: 296–306.

SCOTT, D.E. (1996): The effect of larval density on adult demographic traits in *Ambystoma opacum*. – Ecology, **75**: 1383–1396.

SEEGER, P.G. (1933): Untersuchungen an den Leydigschen Zellen der Urodelenhaut. – Zeitschrift für Zellforschung und mikroskopische Anatomie, **19**: 441–487.

SEMLITSCH, R.D. (1983): Growth and metamorphosis of larval red salamanders (*Pseudotriton ruber*) on the Coastal Plain of South Carolina. – Herpetologica, **39**: 48–52.

SPURR, A.R. (1969): A low-viscosity epoxy resin embedding medium for electron microscopy. – Journal of Ultrastructure Research, **26**: 31–43.

STENHOUSE, S.L., HAIRSTON, N.G. & COBEY, A.E. (1983): Predation and competition in *Ambystoma* larvae: Field and laboratory experiments. – Journal of Herpetology, **17**: 210–220.

THEIS, A. (1932): Histologische Untersuchungen über die Epidermis im Individualcyclus von *Salamandra maculosa* Laur. – Zeitschrift für wissenschaftliche Zoologie, **14**: 356–420.

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**: 483–488.

WARBURG, M.R. & LEWINSON, D. (1977): Ultrastructure of epidermis of *Salamandra salamandra* followed throughout ontogenesis. – Cell and Tissue Research, **181**: 369–393.

WELLS, K.D. (2007): The ecology and behavior of amphibians. – The University of Chicago Press, Chicago.

Weber, W. (1956): Experimentelle Untersuchungen über das Problem der Wundheilung bei *Salamandra maculosa* Laur. – Roux Archiv für Entwicklungsmechanik, **149**: 528–563.

Wilbur, H.M. (1976): Density dependent aspects of metamorphosis in *Ambystoma* and *Rana sylvatica*. – Ecology, **57**: 1289–1296.

Wilbur, H.M. & Collins, J.P. (1973): Ecological aspects of amphibian metamorphosis. – Science, **182**: 1304–1314.