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Identification of pigment cells during early amphibian development (*Triturus alpestris, Ambystoma mexicanum*)*

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Summary. The purpose of the present investigation was to provide and apply a methodological manual with which the distribution, patterning and relationship of melanophores and xanthophores can be analyzed during early amphibian development. For demonstration of the methods, which include ultrastructural, histochemical and biochemical approaches, *Triturus alpestris* and *Ambystoma mexicanum* (axolotl) embryos are used. These two species differ conspicuously in their larval pigment patterns, showing alternating melanophore bands in horizontal (*T. alpestris*) and vertical (axolotl) arrangements. With transmission- and scanning electron microscopy melanophores and xanthophores were distinguished by their different pigment organelles and surface structures. The presence of phenol oxidase (tyrosinase) was used to reveal externally invisible or faintly visible melanophores by applying an excess of 3,4 dihydroxy-phenylalanine (dopa). Xanthophores were made visible in fixed and living embryos by demonstrating their pterin fluorescence. In addition, pterins were analyzed by HPLC in embryos before and after pigmentation was visible.

Key words: Neural crest – Melanophores – Xanthophores – Pigments – *Triturus alpestris*, *Ambystoma mexicanum* (Amphibia, Urodela)

In amphibians the neural crest gives rise to three types of pigment cells: melanophores, xanthophores and iridophores, which, when fully differentiated, may be readily distinguished from each other by the presence of typical pigments. These are black to brown melanins in melanophores, yellow pterins in xanthophores and reflecting purin crystals in iridophores (Bagnara 1976).

With the light microscope, however, the different pigment cell types may be recognized with ease only in older larvae and in juveniles, in which they are externally visible under the epidermis and in which they can be demonstrated even more clearly by their ultrastructure (Dunson 1974; Frost et al. 1984). In earlier larval and in embryonic stages their identification is more difficult and requires special methods. This is because the pigment characteristics of chromatophores are still not or only with difficulty seen from outside, and because the epidermis contains many more maternal melanosomes, which hinder a clear recognition of chromatophores.

In the present investigation we demonstrate a new combination of electron-microscopical, histochemical and biochemical methods for the structural and functional analysis of melanophores and xanthophores during early amphibian development. The animals to which these methods have been applied are embryos and larvae of *Triturus alpestris* and *Ambystoma mexicanum* (axolotl) in stages of pigment pattern development (stages 28+ to 34 in *T. alpestris* and 30/31 to 41 in the axolotl; Figs. 1–4). These two species differ conspicuously in their larval pigment patterns, which consist of horizontal melanophore and xanthophore stripes in *T. alpestris* and of vertical melanophore and xanthophore bars in the axolotl (Epperlein and Claviez 1982a, b; Epperlein and Löfberg 1984). Applying our methods to various developmental stages of these two amphibian species, we demonstrate ways of discrimination between melanophores and xanthophores, and for studying their appearance, distribution and relationships.

Materials and methods

Embryos. Wild type and "albino-black" (matings of a/a females and D/− males) embryos of the Mexican axolotl (*Ambystoma mexicanum*) were obtained from spawnings in the Hubrecht Laboratory (Utrecht, Netherlands) and Department of Zoology, Uppsala, Sweden. *Triturus alpestris* eggs were collected from naturally spawning newts kept at the Anatomy Department in Freiburg. Embryos of both species were mechanically dejellied and stored in Niu-Twitty- (NT-) saline (Flickinger 1949), which contained antibiotics (25 mg/l streptomycin sulfate and 15 mg/l penicillin; Sigma). For grafting and tissue-culture experiments embryos were washed for 20 s in 70% ethanol and several times in sterile NT-saline before decapsulation. Axolotl embryos were staged according to the normal table of Bordzilovskaya and Dettlaff (1979), *T. alpestris* embryos according to the table of Epperlein and Junginger (1982). A stage is defined either as full stage (e.g., stage 25) or a stage intermediate between two stages (e.g., stage 37/38).

* Dedicated to the memory of Dr. Michael Claviez

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Abbreviations: DOPA dihydroxy-phenylalanine; *FCS* fetal calf serum; *FIF* formaldehyde-induced fluorescence; *FITC* fluorescein isothiocyanate; *HPLC* high performance liquid chromatography

Light microscopy (LM). Whole embryos or pieces of removed trunk epidermis were photographed under a dissecting microscope using cold-light beams and a dark field.

Transmission electron microscopy (TEM). Axolotl larvae were fixed in a trialdehyde fixative (3% glutaraldehyde (GA), 2% paraformaldehyde (PFA), 1% acrolein) in 0.1 M Na-cacodylate buffer containing 2.5% DMSO and 0.011% $CaCl_2$ (pH 7.8) for about 16 h at room temperature (Kalt and Tandler 1971). They were washed in 0.1 M Na-cacodylate + 7.5% sucrose for 90 min, postfixed in 2% OsO_4 in 0.1 M Na-cacodylate for 14 h, washed again in buffer and dehydrated through increasing concentrations of ethanol to propylene oxide and embedded in Epon. Ultrathin cross sections of the trunk were stained with 2% uranyl acetate in 96% ethanol for 13 min at 60° C and in 2% lead citrate in 1 N NaOH for 4 min. The sections were examined with a Siemens 102 electron microscope at 60 kV. When mixed melanophore-xanthophore aggregates had formed on the neural tube, cultures of tube and crest (see "Cultures") were fixed in Karnovsky's fixative (2% PFA and 2.5% GA in 0.1 M phosphate buffer, pH 7.4; Glauert 1975). After fixation, the cultures were washed in 0.1 M phosphate buffer, postfixed in OsO_4 in 0.1 M phosphate buffer and washed again. Then the tube explants containing the aggregates were dehydrated and embedded as indicated above for axolotl larvae. Ultrathin sections were cut through aggregate regions and stained and observed as indicated above.

Scanning electron microscopy (SEM). Pieces of dorsolateral trunk epidermis were excised from *T. alpestris* embryos (stages 28+ to 34), which were fixed previously for one to several days in 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer. After washing in buffer, the pieces of epidermis were dehydrated through increasing concentrations of acetone, critical-point dried (CO_2) and sputter-coated with gold-palladium. The specimens were then mounted with the inside facing outwards and observed with an Autoscan-ETEC scanning electron microscope at 10 or 20 kV.

Cultures. Trunk neural tubes with the neural crest were excised from stage 25 axolotl embryos after removal of the dorsal epidermis. They were cultured at room temperature for up to five days in plastic dishes (35 mm, Nunc) containing a modified Stearn's salt solution (Perris and Löfberg 1986) supplemented with 8% fetal calf serum (FCS). Antibiotics were added in the same amount as for the NT-solution (see above).

Grafting experiments: Transplantation of xanthophore regions. Pieces of trunk epidermis were excised from dorsolateral xanthophore band regions (for topography, see Epperlein and Löfberg 1984) of anesthetized (MS 222, Sandoz, Basel) stage 39/40 axolotl embryos in 4 × strength Steinberg solution (Steinberg 1957) containing antibiotics (same but fourfold as in NT-solution). They were grafted to a midflank position of stage 30 to 33 embryos from which a graft-size piece of epidermis had been previously removed. To assist healing, the graft was in some cases pressed against the somite surface by a small piece of glass. To avoid mixing of chromatophores leaving the graft with those of the host, the latter had been made "crest-free" at stage 15 by removing the trunk neural folds. The embryos bearing the graft were photographed with a color film before fixation for the dopa test (4–6 days after the operation).

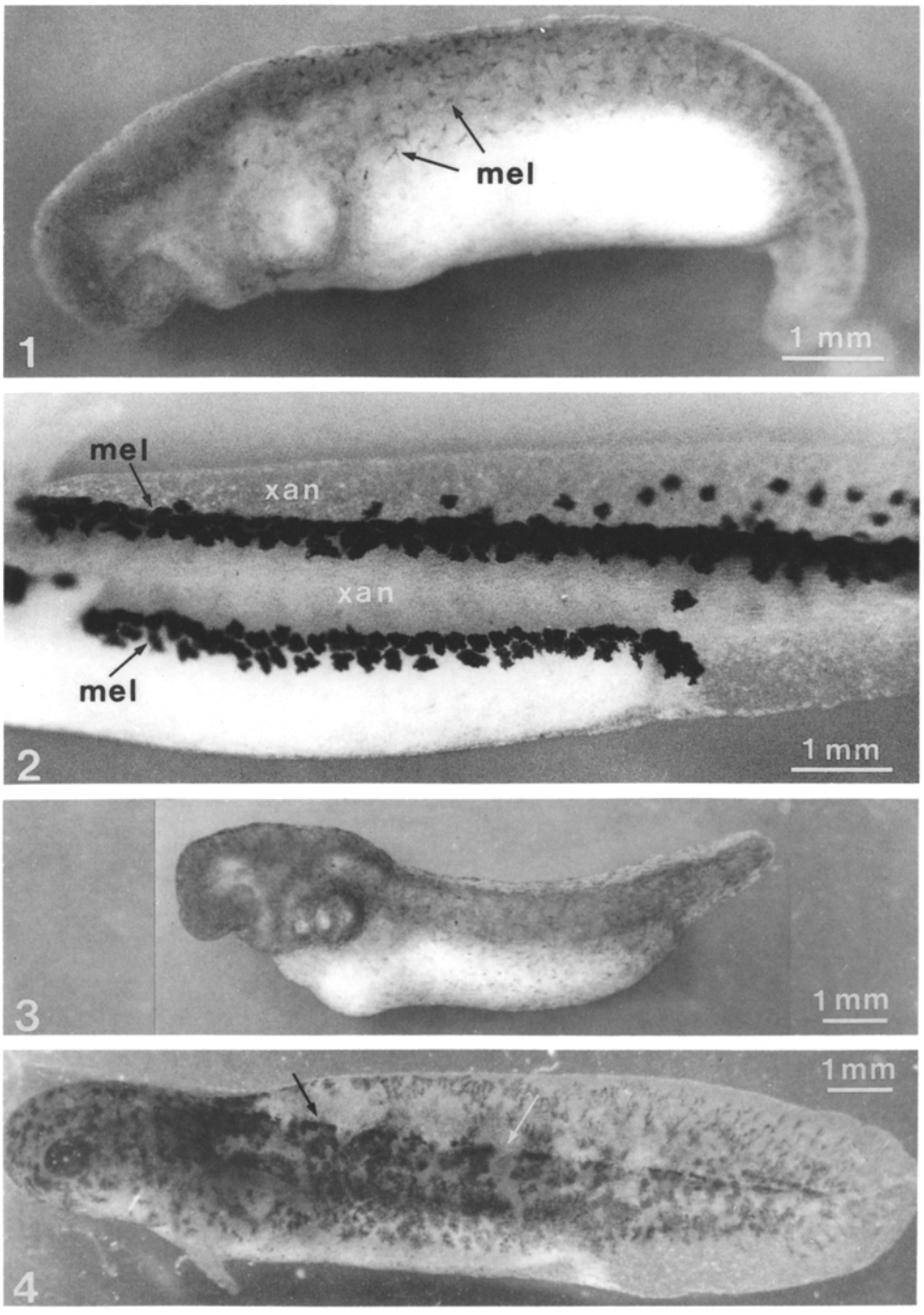
Removal of trunk epidermis. In stage 25–27 embryos kept in agar dishes with 4 × strength Steinberg solution (see above) large areas of the epidermis were removed leaving only the head, the posterior end of the trunk and the ventral trunk covered. The operated embryos were transferred to plastic dishes filled with Stearns' solution containing 8% FCS and observed for several days.

Dopa incubation. A. mexicanum and *T. alpestris* embryos of various stages as well as those axolotl embryos bearing xanthophore regions were anesthetized in NT-saline containing MS 222 and fixed for 10–15 min in 4% PFA in 0.1 M phosphate buffer (pH 7.4; at room temperature). After fixation, the embryos were stored for about 8 h to overnight in 0.1 M phosphate buffer. Their outer pigmented epidermal layer could then be easily removed. They were still covered by the inner transparent epidermal layer, which contains many fewer maternal melanosomes than the outer layer. Thereafter the dopa incubation was carried out (modified after Mishima 1964) to demonstrate the presence of phenol oxidase (formerly tyrosinase), EC 1.10.3.2. (Karlson 1984). The embryos were kept in the dark for at least 10 h or overnight in tightly sealed agar-coated plastic dishes containing N_2-gassed 0.1 DL−β−3,4 dihydroxy-phenylalanine (DL-dopa, Sigma) in 0.1 M phosphate buffer. Following the incubation, the embryos were transferred to agar dishes containing 0.1 M phosphate buffer and observed with a stereomicroscope.

Pterin fluorescence. Ammonia or other alkaline solutions with a pH of approximately 11 liberate pterins from their protein carrier in xanthophores. Consequently, these cells show a blue pterin fluorescence (Günder 1954; Ziegler 1965) under irradiation with long-wave UV-light. The excitation maximum for blue fluorescing pterins is in the range of 340–365 nm. To reveal this pterin fluorescence, we placed living anesthetized *T. alpestris* and axolotl embryos into a groove of an agar dish containing diluted ammonia (one drop 25% NH_3-solution in 10 ml NT-saline or distilled water, about pH 11; Epperlein and Claviez 1982a). For observing pterin fluorescence we used incident light fluorescence microscopes with the following filter combinations: Leitz: filter block A, BP 340–380, RKP 400, LP 430; Zeiss: filter BP 365, FT 395, LP 397. Photographs were taken on Kodak Tri-X-Pan and Ektachrome 400 and 1000 films.

Some axolotl embryos (stage 36) were also treated with diluted ammonia after the dopa test in order to reveal the presence of fluorescing xanthophores and their relation to melanophores.

The Falck-Hillarp histofluorescence technique (Falck et al. 1962; originally devised for detecting the presence of catecholamine-containing neurons) was used as an alternative for the ammonia-induced pterin fluorescence in xanthophores, since it allowed a stable visualization of these cells without quick blurring. Details concerning its application are as follows: Axolotl embryos were placed on slides, quickly frozen in isopentane precooled with liquid nitrogen, and freeze-dried at about −40° C for 2–3 days using a WKF L2 freeze-drying apparatus. The embryos were then exposed to PFA vapor of 50–70% relative humidity (Hamberger et al. 1965) for 2.5 h at 80° C. After the reaction



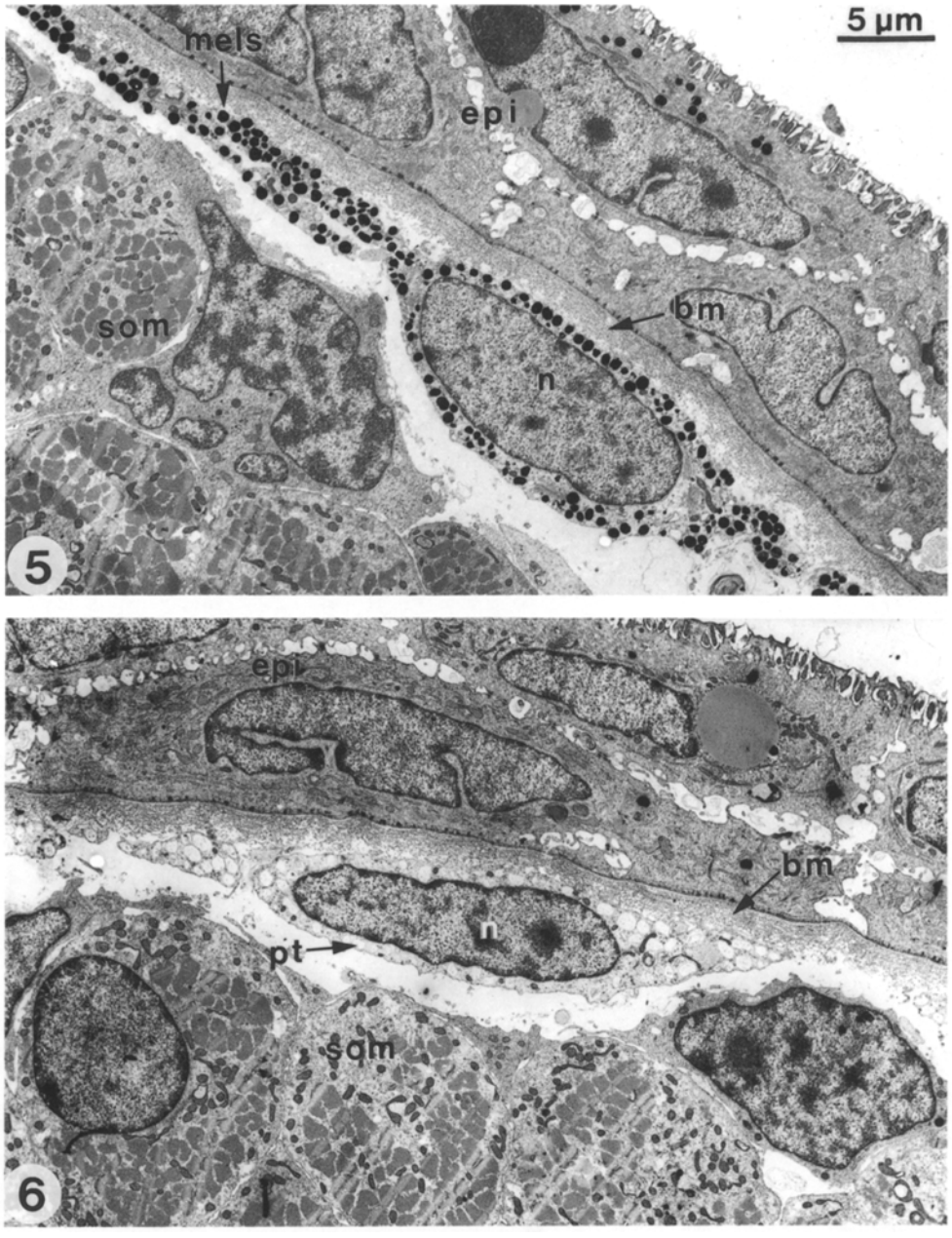
Figs. 1–4. Developmental stages of *Triturus alpestris* and *Ambystoma mexicanum* with the first emergence of pigment cells and the final larval pigment pattern. During pigment pattern formation in *T. alpestris* (stage 28+ to 34) evenly scattered melanophores (*mel*, **Fig. 1**; stage 28) and xanthophores (externally invisible in **Fig. 1**) become rearranged as alternating horizontal melanophore (*mel*) and xanthophore (*xan*) bands in the larva (stage 34, **Fig. 2**). Conversely, the pigment pattern of the axolotl larva consists of alternating vertical melanophore (*black arrow*) and xanthophore (*white arrow*) bars (stage 41, **Fig. 4**). This barred pattern is preceded by a dispersed distribution of melanophores, which may become weakly visible in the dorsolateral trunk of stage 35/36 embryos (**Fig. 3**; melanophores not discernible in this figure) and which start to appear around stage 30/31 in an externally invisible way. The barring of the pattern depends upon mixed chromatophore groups that start to form from stage 34 on along the dorsal trunk in an externally invisible way

they were observed under the fluorescence microscope using the FIF-filter combination (Leitz: filter block I 2, BP 355–425, RKP 455, LP 460; Zeiss: filter BP 390–440, FT 460, LP 470). Since residual salt crystals prevented a clear observation or photographic image of the embryos, drops of paraffin oil or glycerol were added. Photographs were taken with Kodak films as indicated.

To discover xanthophores in living embryos, which can survive the method for demonstration of pterins, the embryos were placed in agar dishes containing saline and observed under UV-light with the FIF-filter- (see above) or FITC-filter combination (Zeiss: BP 450–490, FT 510, LP 520) alone. Photographs were taken on Kodak films (see above).

Fluorescence emission measurements. The fluorescence emitted from FIF-treated cultured neural crest cells was measured with a Zeiss UMSP-100 microspectrophotometer with an HBO 100 W Osram lamp and filters BP 400–440, FT 460, LP 470. The diameter of the light-field diaphragm was 2 μm in the conjugated object plane, the measuring diaphragm 10 μm, and the spectral band width of the measuring monochromator 10 nm. We used the objective Ultrafluar 32/0.40 glycerol (Zeiss) and measured spectra in steps of 5 nm. The fluorescence spectra were corrected for the spectral sensitivity of the photomultiplier.

Pterin analysis by reverse-phase HPLC. Dorsal trunk fragments (consisting of the neural tube, neural crest, somites



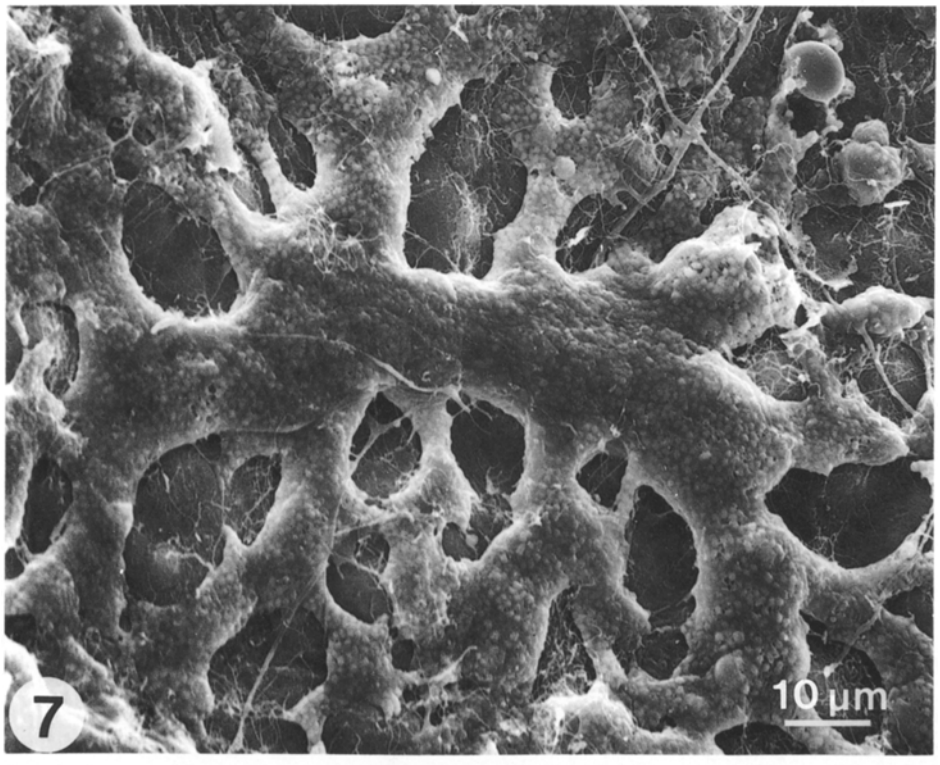
Figs. 5, 6. TEM cross sections through dorsolateral trunk regions of a 12-day-old axolotl larva (stage 41/42); same magnification. In **Fig. 5** portions of a dermal melanophore and in **Fig. 6** portions of a xanthophore are shown between epidermis (*epi*) and somites (*som*). Epidermal basement membrane (*bm*), melanosomes (*mels*), pterinosomes (*pt*), nuclei of chromatophores (*n*). The small spaces between chromatophores and somitic tissue are probably due to fixation artifacts

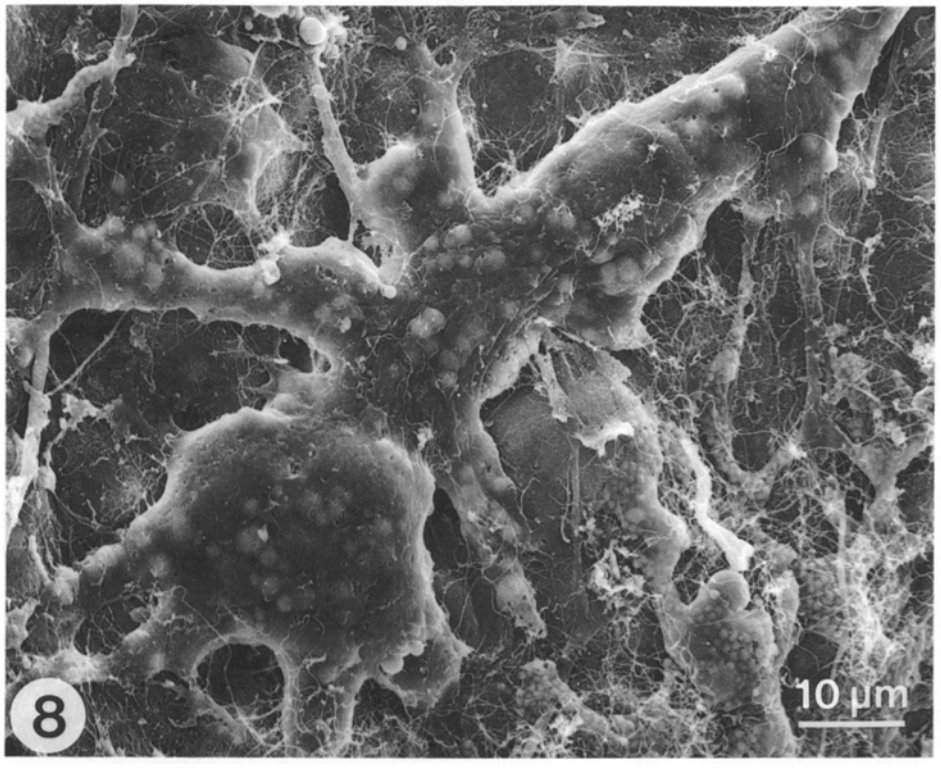
and covering epidermis; number of determinations in brackets behind the stage) from stage 29/30 (1), 33 (1), 34 (2), 35 (2) and 36 to 38 (4) axolotl embryos, the neural crest of stage 24/25 (3) embryos, complete late gastrulae, stage 13 (1), and ventral halves from stage 36 (2) embryos were frozen in isopentane precooled with liquid nitrogen, and freeze-dried at about −40° C for 2.5 days. The pterins were analyzed according to Fukushima and Nixon (1980). Briefly, after iodine oxidation at pH 1.0, they were prepurified by Dowex H^+ ion exchange chromatography, separated by HPLC and monitored by their blue fluorescence. Using this method, the pterin (6)-carbonic acid obtained results from unstable, reduced intermediates of the de novo biosynthetic pathway (see Nichol et al. 1985). After steam extraction (5–10 min at 121° C or in streaming water vapor at 100° C) sepiapterin was also separated by HPLC and monitored by its yellow fluorescence (excitation 425 nm, emission 530 nm). Details of the pterin analysis are described by Ziegler (1985).

Results

(1) Characterization of early differentiated chromatophores with the electron microscope

For revealing the presence and distribution of melanophores and xanthophores in early stages of pigment-pattern formation of *T. alpestris* and *A. mexicanum* embryos (developmental stages with first emergence of pigment cells and final pigment pattern, see Figs. 1–4), various methods will be presented. Using light microscopy as the easiest approach, melanophores and xanthophores can be recognized only in stages when they are already visible as differentiated black and yellow cells. Therefore we will start with TEM



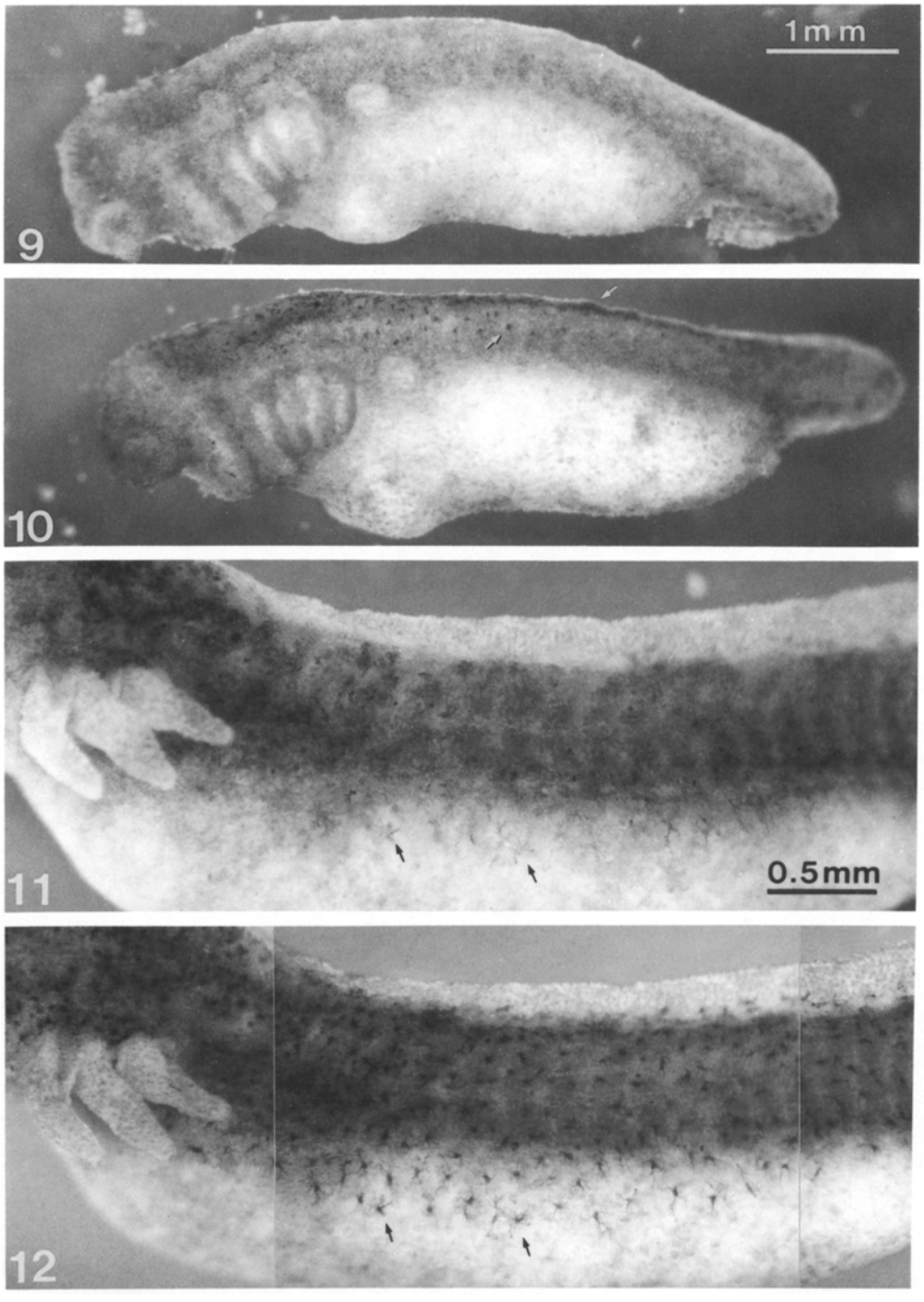


Figs. 7, 8. SEM micrographs of a melanophore (**Fig. 7**) and a xanthophore (**Fig. 8**) on the undersurface of a piece of detached dorsolateral epidermis from a stage 31+ *T. alpestris* embryo. Both cell types extend several processes and are recognized by their different surface morphology. Original: Dr. M. Claviez

and SEM investigations, since with these methods melanophores and xanthophores may be distinguished by their internal and external differences, even before they become outwardly visible.

With TEM melanophores can be recognized in trunk cross sections of axolotl larvae because of their abundant content of melanosomes (Fig. 5), which are distributed around the cell nucleus and within cell processes. Xanthophores are easily distinguished from melanophores by possessing electron-lucent pigment organelles (pterinosomes) with a flocculent, amorphous content, which are about three times the volume of melanosomes and occur in a similar distribution within the cell (Fig. 6).

Both pigment cell types may contain a few larger melanosomes in addition to their specific pigment organelles. These "granular melanosomes" are of maternal origin and are also distributed in the cells of other embryonic tissues.



Figs. 9, 10. Visualization of melanophores after dopa treatment in a stage-34 axolotl embryo. **Fig. 9** before, **Fig. 10** after dopa treatment. Melanophores (*arrows*) become visible along the trunk neural crest and in dorsolateral parts of the anterior trunk

Figs. 11, 12. Enhancement of faintly visible, grey melanophores by dopa incubation in a stage-36/37 axolotl embryo (left side, head to the left outside the figure). Melanophores (*arrows*), which are faintly visible before the reaction (**Fig. 11**), stain deeply black, particularly in the nucleus, following dopa treatment (**Fig. 12**)

With the SEM, melanophores and xanthophores may be distinguished by their surface structures. On the undersurface of dorsolateral epidermis from stage 31+ *T. alpestris* embryos, which contain stellate melanophores and xanthophores, melanophores show a regular granular surface caused by melanosomes of 0.3–0.5 μm diameter (Fig. 7). Xanthophores have such a granular surface (caused by pterinosomes) only from stage 34 on. At earlier stages their surface is irregular, although numerous pterinosomes are present (revealed in TEM sections of the Epon-embedded specimens; Epperlein and Claviez 1982a). The irregularity results from yolk platelets of different sizes and from granular melanosomes (Fig. 8). Both organelles are also present in melanophores, but masked by the predominating melanosomes. In ambiguous cases, in which the type of cell cannot be judged from the surface, a comparison between the SEM skin preparation and its corresponding area on a LM-photograph from the embryo before its removal will make the distinction clearer: Xanthophores are not visible on the LM-micrograph.

(2) Early recognition of melanophores with the dopa reaction

In contrast to *T. alpestris* embryos, in which the dopa reaction did not lead to an enhancement of melanophores (Fig. 14), axolotl embryos stained positively after dopa

treatment. Melanophores become visible as early as at stage 30/31 (premigratory neural crest stage). At this stage the melanophores appear as rounded or slightly elongated black cells sparsely scattered along the dorsal neural tube. In Figs. 9 and 10 an axolotl embryo (stage 34) is shown in which melanophores have started to migrate. Melanophores become visible after dopa treatment along the trunk crest and in dorsolateral parts of the anterior trunk.

The effect of the dopa reaction may still be better demonstrated in grey melanophores of stage 36/37 axolotl embryos (Fig. 11). After treatment, the visibility of faintly visible melanophores is markedly enhanced, particularly in the nucleus (Fig. 12).

(3) Early recognition of xanthophores by analyzing their pterins

We wanted to determine which pteridines are present in axolotl embryos at stages when xanthophores become visible as yellow cells. We were further interested to see whether pteridines are possibly present in stages or embryonic regions without any visible pigment cells. Analyzing freeze-dried samples with HPLC (Fig. 13), we found that axolotl gastrulae and early tailbuds (premigratory crest stage, stage 24/25) already contain biopterin. Even ventral halves of stage-36 embryos (consisting predominantly of yolk) contain biopterin and neopterin (not indicated in Fig. 13). In dorsal trunk fragments of stage 34 embryos neopterin appears (not indicated in Fig. 13) and is increased at stage 35. From stage-36 onwards the levels of neopterin and biopterin increase by five- to tenfold as compared to stages 34 or 35. The appearance of neopterin may correspond to the emergence of mixed chromatophore groups (stage 33 to 35), its sudden rise at stage 36 to 38 to the visible yellow color in xanthophores. Sepiapterin, the detection of which is less than one tenth as sensitive, was found only in several trunk fragments from stage-38 embryos, which were analyzed together. In samples from stage 34 and 36–38 pterin carbonic acid was present, probably due to the degradation of unstable reduced biopterins. For the appearance and relation of the pteridines analyzed, which are all 2-amino-4-hydroxy-pteridines = pterins, see the biosynthetic pathway in Table 1.

In living embryos individual xanthophores may be recognized by eliciting the fluorescence of their pterins. Using diluted ammonia the specificity of the pterin fluorescence for xanthophores is best demonstrated in differentiated yellow xanthophores as they appear in the transparent dorsal fin of larvae (Fig. 27). After addition of ammonia these cells fluoresce blue under UV-light (Fig. 28).

Externally invisible but fluorescing xanthophores could be observed for the first time in axolotl embryos after ammonia treatment at stage 35/36. They were uniformly scattered in the dorsal and lateral head regions and occurred as small condensed groups at intervals along the trunk neural crest (Fig. 23). In *T. alpestris* embryos, fluorescing xanthophores were first observed from stage 28+ onwards. They occurred mixed with melanophores along the dorsal trunk. A few stages later xanthophores and melanophores became arranged in different zones (stage 31, Figs. 14, 15). Due to their fluorescence, xanthophores were recognized above the somites, while black melanophores became loosely arranged as horizontal bands along the dorsal somite edges and at the yolk border.

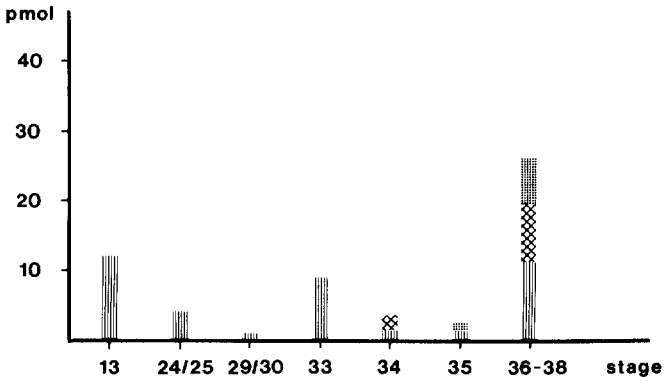


Fig. 13. HPLC-analysis of intermediates of pteridine biosynthesis, neopterin (▦) and biopterin (▥), and the degradation product of biopterins, pterin carbonic acid (6) (⊗), determined in pmol amounts during development of axolotl embryos. In case of multiple determinations the mean value is indicated. At stage 34 the value for neopterin is 0.22 and too low for indication. At stage 13 a whole gastrula, at stage 24/25 neural crests, and at all other stages dorsal trunk fragments (consisting of neural tube, neural crest, somites and covering epidermis) were analyzed

Using the FIF procedure we attempted to stabilize the pterin fluorescence in xanthophores in order to allow repeated observations. The specificity of this method for xanthophores was tested in older larvae with externally visible xanthophores and melanophores. After FIF-treatment only xanthophores showed an intense, light-green fluorescence that was stable and did not fade considerably following repeated observations. In addition to xanthophores the yolk also showed a strong green autofluorescence.

Based on the specific reaction of xanthophores after FIF-treatment, this method was also applied to younger axolotl embryos in which xanthophores were not yet externally visible (e.g., stage 35/36). In these embryos green-fluorescing cells became visible (Fig. 24) and showed a distribution similar to what was observed before with ammonia treatment (Fig. 23). However, the green fluorescence was relatively stable.

To characterize the green fluorescence, an emission spectrum was recorded from FIF-treated, green-fluorescing xanthophores in culture (measurements in the embryo were not possible for technical reasons). The maximum of this spectrum was at 480–495 nm.

For the observation of xanthophores in living embryos neither the ammonia nor the FIF method are suitable, since the embryos have to be killed and therefore only one state of xanthophore distribution can be observed. However, by using UV-light and the FIF- or FITC-filter combination alone, xanthophores can be recognized for a short while (10–20 s) in the living embryo (Figs. 25, 26). Since the fluorescence reappears in a few hours, observations can be repeated at intervals. In comparison with the FIF-filter, under which green-fluorescing xanthophores and a high green autofluorescence of the yolk are observed, the FITC-filter gives a yellow-green fluorescence in xanthophores and reduces the autofluorescence of the yolk considerably. Which of the two filters gives a more satisfying result, is, however, dependent on the species used and on the state of differentiation of xanthophores. Generally, xanthophores of older axolotl larvae (from stage 41 onwards) cannot be recognized with filters at all. In younger larvae the FITC-filter

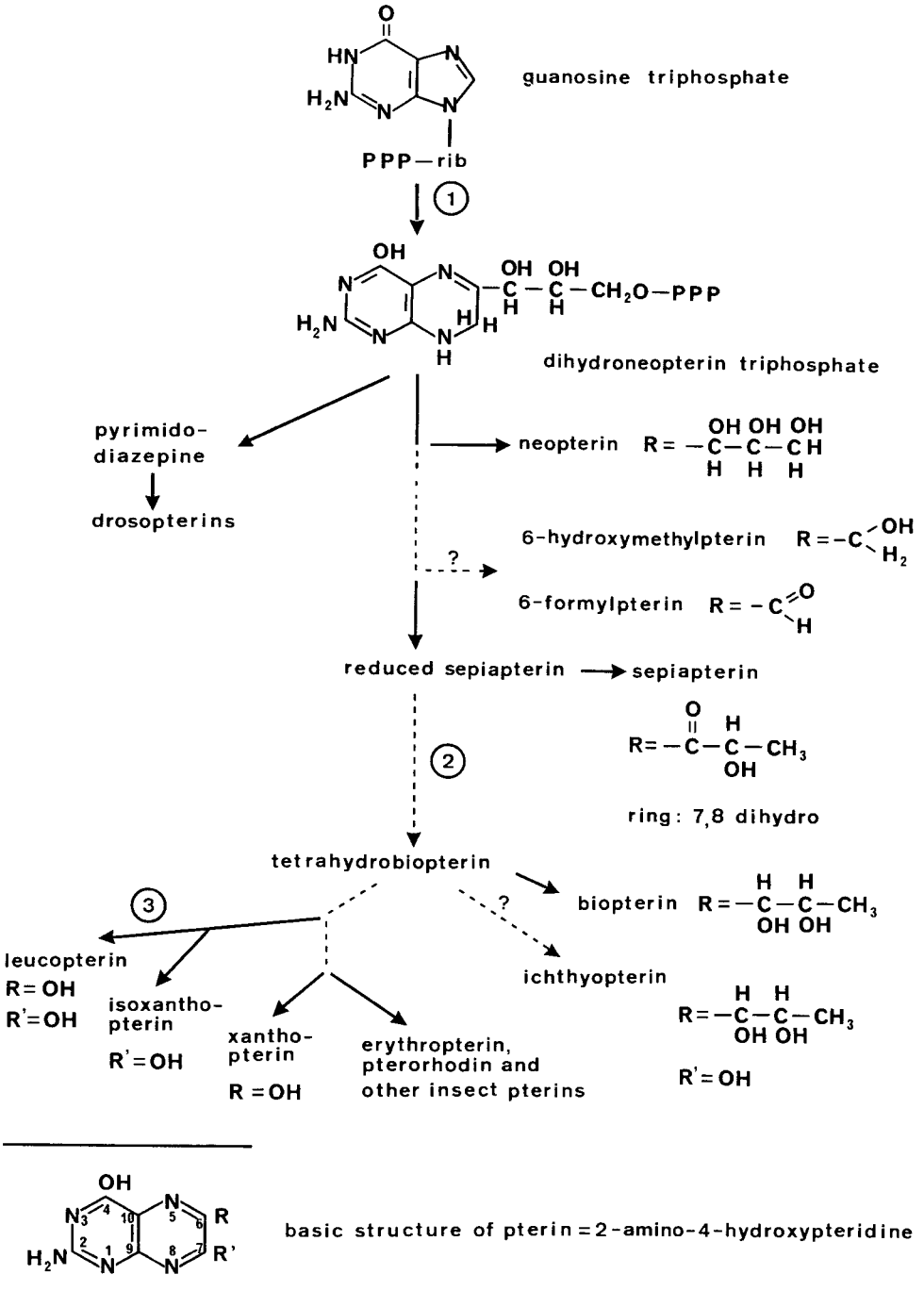


Table 1. Biosynthesis of pigment pterins (unconjugated pterins). Simplified scheme based on Nichol et al. (1985), Wiederrecht et al. (1984) and Rembold (1985). *1* GTP-cyclohydrolase; *2* sepiapterin reductase; *3* xanthine dehydrogenase or xanthine oxidase; ----- intermediate products omitted; ? uncertain pathway, not yet clarified

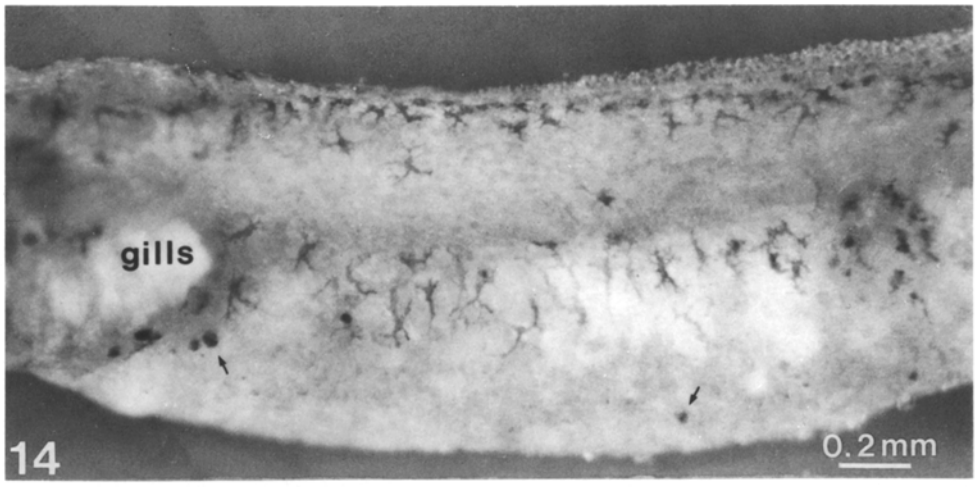
gives better results in *T. alpestris* and the FIF-combination in the axolotl.

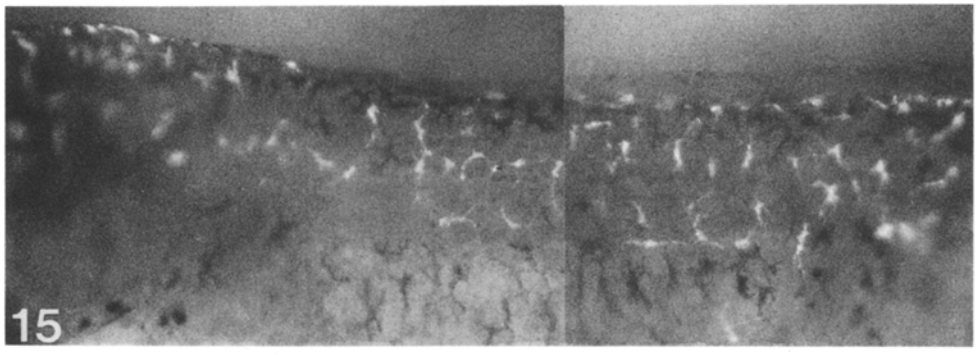
If xanthophores, which fluoresce blue after ammonia treatment, are observed with the FIF- (Fig. 29) or FITC-filters, they emit a light green and yellow-green fluorescence, respectively. This shows that pterins in their bound state in the living cell also emit light if excited by longer wavelengths. This property is the basis for using the FIF- and FITC-filters for identification of xanthophores in addition to the ammonia method.

(4) Dual characteristics of melanophores and xanthophores

In the axolotl embryo, melanophores and xanthophores share common sites in early stages of pigment pattern development forming mixed, rounded humps in the trunk alongside the dorsal neural tube (Epperlein and Löfberg 1984). Although this arrangement is less stable in dark (D/−) axolotl embryos in which some neural crest cells have already left the crest before the onset of hump formation, it becomes very obvious in dark embryos from which the trunk epidermis has been removed and in which the migration of neural crest cells is hindered. In these epidermis-deprived embryos (grown in the presence of FCS), mixed chromatophore aggregates (with melanophores mostly in the periphery) and, occasionally, pure yellow xanthophore humps, may develop on the neural tube (Fig. 30).

Mixed melanophore-xanthophore aggregates may form also on axolotl neural tube cultured together with neural crest. In one of these aggregates TEM investigation revealed





Figs. 14, 15. Different arrangement of melanophores and xanthophores in a stage-31 *Triturus alpestris* embryo (head to the left outside). Combination of dopa treatment and pterin fluorescence. **Fig. 14** After dopa treatment melanophores of the horizontal bands are not enhanced. Instead, a few black, rounded cells (*arrow*), which are probably blood cells, appeared in ventrolateral areas of the trunk.

Fig. 15 Blue pterin fluorescence in xanthophores that were externally invisible before and appeared mainly between the melanophore bands. The black and white print was prepared from a color negative

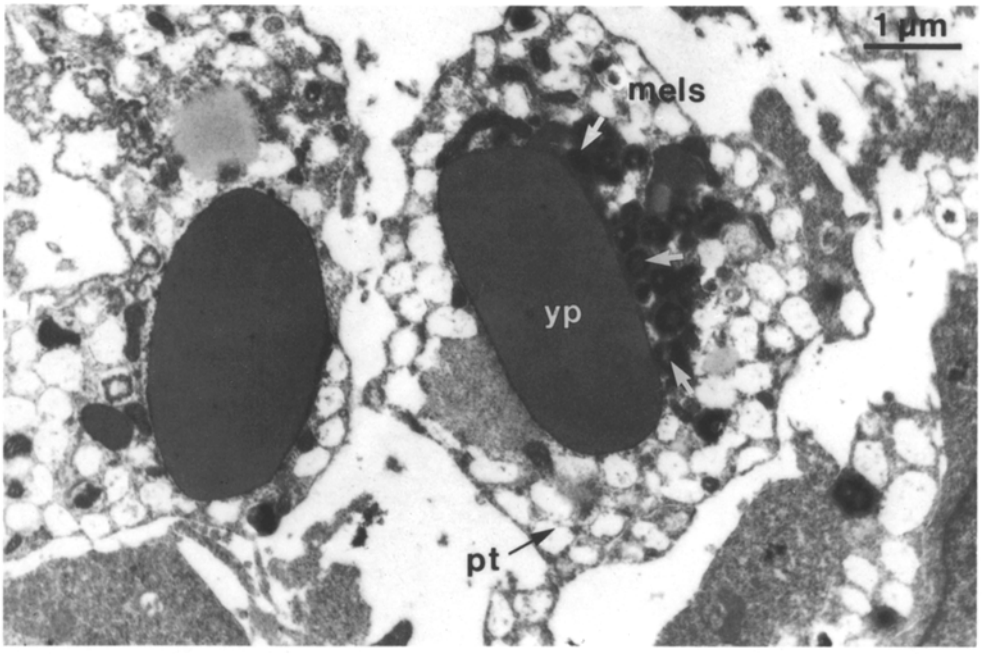
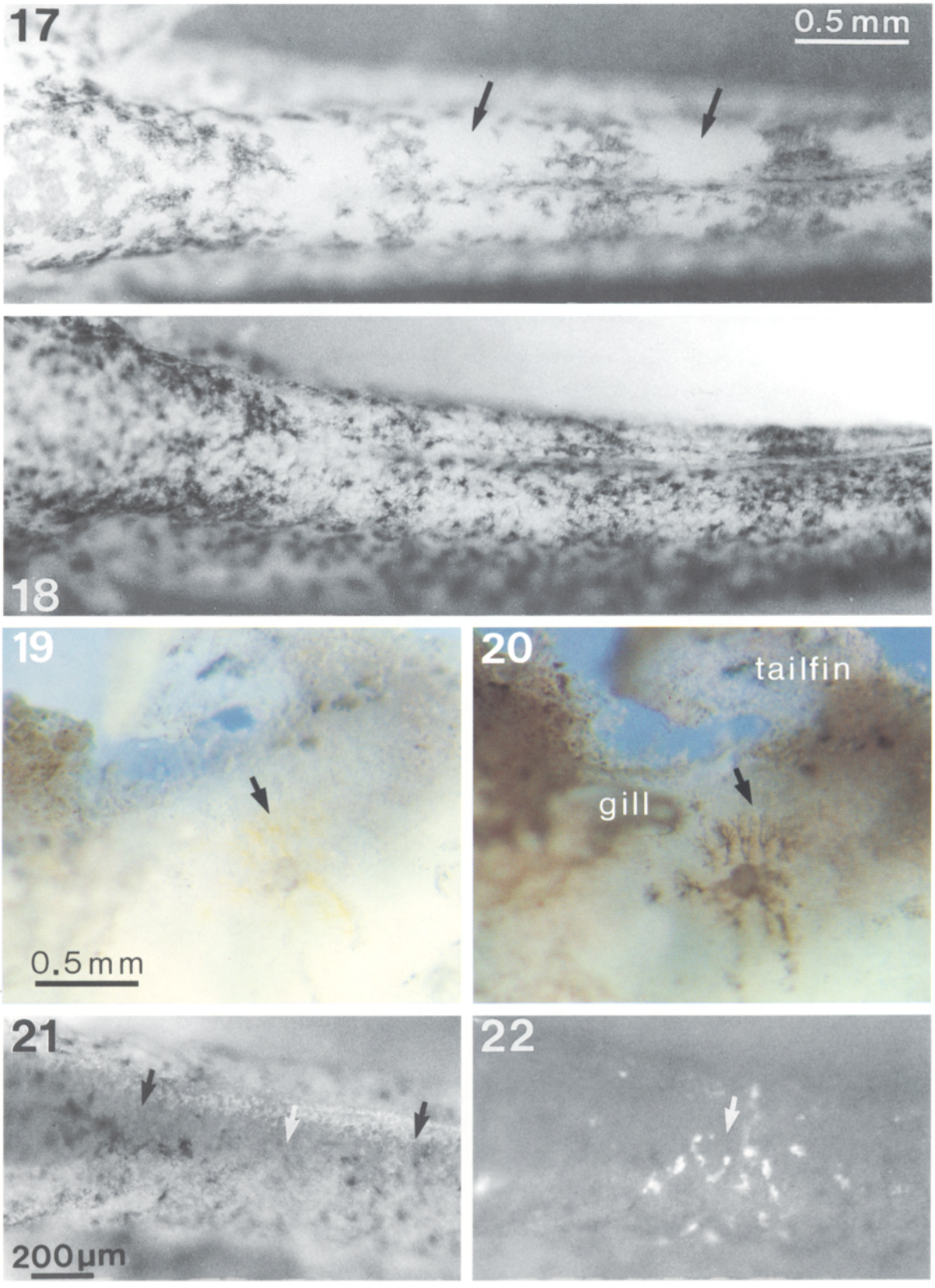


Fig. 16. Mosaic chromatophore containing both melanosomes (*mels, white arrows*) and pterinosomes (*pt*) in a culture of trunk neural tube and crest. This cell was found in an aggregate of melanophores and xanthophores, which developed from neural crest cells on top of the neural tube; *yp* yolk platelets

cells containing both melanosomes and pterinosomes (Fig. 16).

Xanthophores may also react positively to dopa treatment. This is demonstrated in an "albino-black" axolotl larva (Figs. 17, 18), which lacks maternal melanosomes, and in which the larval pigment pattern can be observed particularly clearly. After dopa incubation the xanthophore areas on the dorsal neural tube show many grey, stellate cells (Fig. 18), which were not visible before (Fig. 17). These cells are probably xanthophores. In contrast to melanophores, xanthophores seem to lack the deep black staining of the nucleus and are grey throughout.

Since it might be argued that "silent" or "faded" melanophores or even mesenchymal cells might also have stained positively in xanthophore areas, in another experiment epidermal pieces containing an undeterminable



Figs. 17, 18. Positive dopa reaction in dorsal xanthophore bands (or bars) of a stage-40/41 "albino-black" axolotl larva (head to the left outside, view from above). This larva shows a definite "barred" pigment pattern with alternating vertical bars of melanophores and xanthophores. The yellow bands (*arrows*) in which no individual cells can be recognized before the reaction (**Fig. 17**) show many grey, stellate cells after dopa treatment (**Fig. 18**) that are probably xanthophores

number of visibly differentiated yellow xanthophores were transplanted to the flank of younger crest-free hosts. In these embryos xanthophores migrated out from the graft after a few days and could be counted (Fig. 19). After dopa treatment all these xanthophores stained black to slightly brown (Fig. 20), which suggests the presence of phenol oxidase. The slightly brownish tone was probably due to the concomitant presence of still yellow pterins. No particular staining was observed in the nucleus as in dopa-treated melanophores (Fig. 12).

To investigate whether externally invisible xanthophores show a positive dopa reaction (e.g., at stage 36), a combination of dopa and ammonia treatment was applied to the same embryo. Black cells appeared after dopa treatment only in future melanophore, but not in xanthophore areas. In the latter, no black but some pale grey cells may occasionally become visible (Fig. 21). After ammonia treatment, however, several fluorescing xanthophores appeared in future xanthophore zones (Fig. 22). A few seemed to be identical with grey cells, although this was difficult to judge. This result indicates that at early stages xanthophores do not possess phenol oxidase in amounts sufficient or in a state suitable for staining as darkly as do melanophores after dopa treatment.

Discussion

In this investigation we demonstrate several methods by which the appearance and interrelationships of melanophores and xanthophores can be characterized in developing *T. alpestris* and axolotl embryos. Essentially, these methods are optimal only for a special purpose, e.g., for the structural characterization of an early differentiated pigment cell phenotype, for the detection of externally invisible melanophores, or for an overall mapping of xanthophores in living embryos. In the discussion we will try to make some remarks on advantages or limitations of particular methods for investigating chromatophore differentiation and distribution during pigment pattern formation.

Melanophores and xanthophores do not become externally visible in axolotl and *T. alpestris* embryos until later on in pigment pattern development. In embryos in which they are not yet or only vaguely discernible from outside, they can be made visible by the dopa and pterin fluorescence techniques, respectively. Cells reacting positively to the dopa reaction are, however, clearly observed only in axolotl embryos. At first they stain deeply in the nucleus and weakly in the cell processes. In later stages the cell processes also become black. This confirms the similar finding of McCurdy (1969). In melanophores of *T. alpestris* embryos the dopa reaction is negative. The reason may be that in externally invisible cells the enzyme phenol oxidase is present in much smaller and less easily detectable amounts. The pterin fluorescence technique, in contrast, can be applied equally well to xanthophores of either *T. alpestris* or the axolotl. Both the dopa reaction and pterin fluorescence have recently proved very useful for studying the differentiation and pattern formation, not only in early *Triturus* and axolotl embryos but also in embryos of other amphibian species (Epperlein and Claviez 1982a, b; Ohsugi and Ide 1983; Epperlein and Löfberg 1984; Perris and Löfberg 1986; Tucker 1986; Tucker and Erickson 1986a–c).

The dopa reaction is not entirely specific for melanophores, since the pterinosomes in xanthophores may also show phenol oxidase activity (Yasutomi and Hama 1976). Even erythrocytes and mucous glands of the skin may give positive results (discussed in McCurdy 1969; Model 1973; Tucker and Erickson 1986c; see also Fig. 14 this paper). The axolotl embryos that we investigated seemed to possess no or only very weak phenol oxidase activity in their xanthophores during early development; whereas in later stages, when the cells were yellow, they gave a clear positive result like that of the grafting experiments.

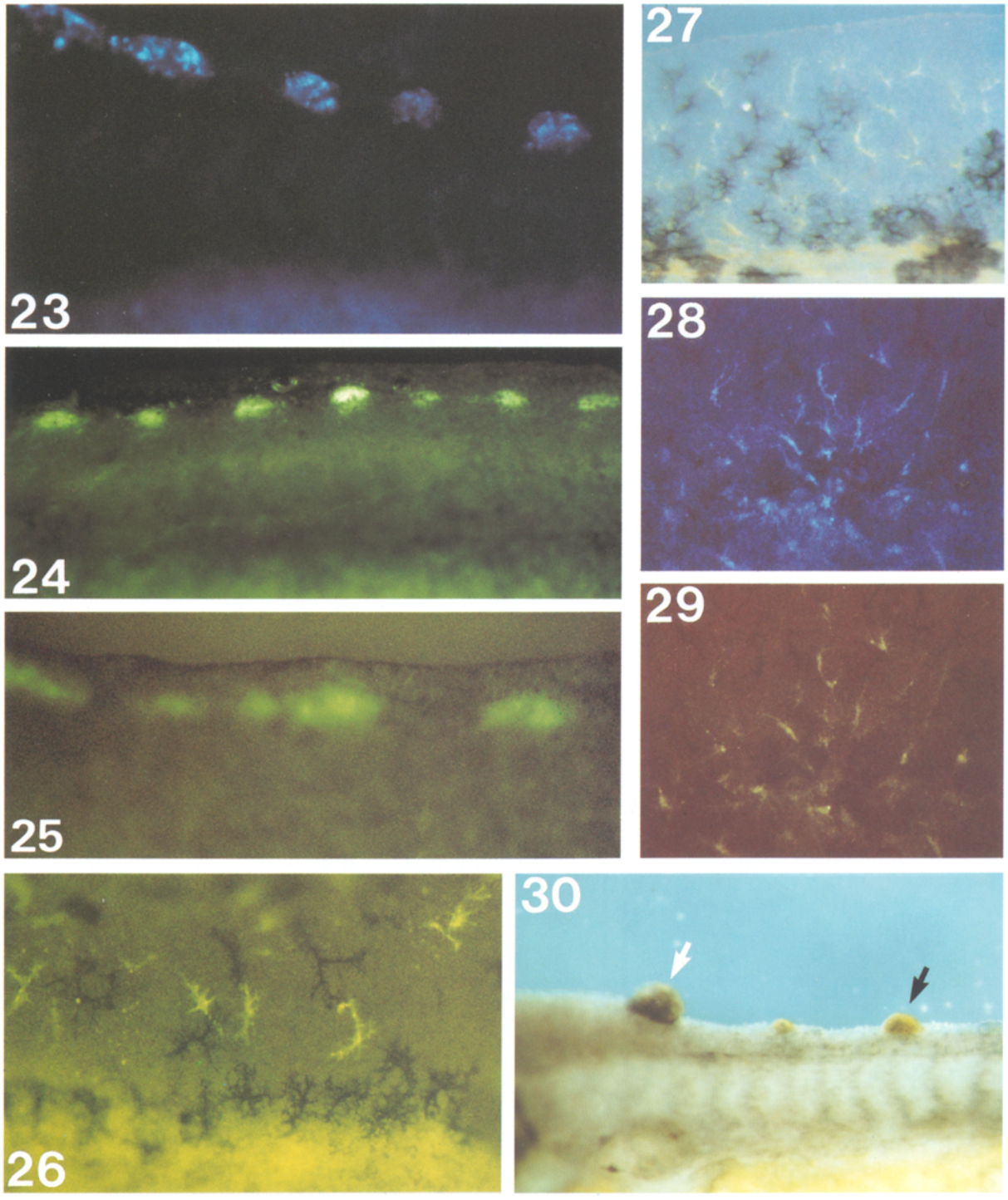
Xanthophores can be specifically identified by their content of blue-fluorescing pterins, which are set free from pterinosomes by ammonia or other alkaline solutions with a pH of approximately 11. The fluorescing xanthophores can be observed for some seconds to a few min depending on the stage of differentiation, before the pterinosomes burst and the fluorescing pterins become blurred. The ammonia method is the most sensitive, but less useful for mapping all xanthophores in whole embryos.

Following FIF treatment, xanthophores emitted a green fluorescence, which was relatively stable and allowed repeated observations to be made. Mapping of xanthophores in whole embryos is possible, although the freeze-drying that precedes FIF treatment is rather time consuming. The staining of xanthophores after FIF treatment indicates that this procedure, which according to Corrodi and Jonsson (1967) is highly sensitive and specific for biogenic monoamines (including catecholamines and dopa), is unsuitable for detecting catecholamine- (CA-) containing cells in early amphibian embryos and in cultures of differentiating neural crest cells, if these cells do not show additional characteristics typical of neuronal cells. The reason is that the fluorescence of pterins present in xanthophores, in the yolk (?) and in the FCS (Ziegler 1985) of the culture medium, interferes with fluorophore formation by any CA that may be present. This is also evident from our microspectrophotometric measurements on the fluorescence emission of xanthophores in culture. The maximum of these spectra, at 480–495 nm, partially overlaps with that typical for catecholamines (480 nm).

For the demonstration of xanthophores in living embryos we used the FIF- and FITC-filters alone with the UV-light source. To obtain optimal results, the choice of filters used must depend upon the age and species of the embryo to be investigated. The use of the filters alone

Figs. 19, 20. Positive dopa reaction in older axolotl xanthophores. Xanthophores transplanted with a piece of epidermis from a stage-39/40 donor larva to the left flank of a stage-30 neural crest-free host are shown as yellow cells (*arrow*) at an age corresponding to stage 44/45 before (**Fig. 19**) and as black cells (*arrow*) after dopa treatment (**Fig. 20**)

Figs. 21, 22. Negative dopa reaction in younger axolotl xanthophores. Corresponding micrographs after dopa reaction (**Fig. 21**) and pterin fluorescence (**Fig. 22**). In a stage-36 axolotl embryo (shown is part of the dorsal midtrunk) melanophores were only faintly visible. After dopa treatment (**Fig. 21**) melanophores stained black only in future melanophore (*black arrow*) but not in xanthophore areas (*white arrow*), where only a few pale grey cells occasionally appeared. In the latter areas several blue-fluorescing xanthophores (*arrow*) appeared after ammonia treatment (**Fig. 22**), a few of which seemed to be identical with the grey cells



Figs. 23–25. Blue-or green-fluorescing xanthophore groups in the dorsal trunk regions of stage-35/36 axolotl embryos (head to the left outside) revealed by ammonia treatment (**Fig. 23**; ×45), the FIF procedure (**Fig. 24**; ×40) and in a living specimen with the FIF filter set (**Fig. 25**; ×60)

Fig. 26. Yellow-green pterin fluorescence in xanthophores on the lateral trunk of a living *T. alpestris* larva (stage 34) observed with the FITC filter. In this larva no dense horizontal melanophore and xanthophore bands have developed since most of the trunk crest was removed at the neurula stage. ×55

Figs. 27–29. Differentiated yellow xanthophores in the dorsal fin of axolotl larvae. **Fig. 27**, untreated larva (stage somewhat older than last stage (=44) in the normal table; ×30). **Fig. 28**, xanthophores show blue pterin fluorescence under UV-light following treatment with diluted ammonia (stage 42; ×60). **Fig. 29**, xanthophores fluoresce green under the FIF filter combination (same area as in Fig. 28; ×60)

Fig. 30. Formation of mixed chromatophore (*white arrow*) or pure yellow xanthophore aggregates (*black arrow*) on the dorsal trunk neural tube of an epidermis-deprived dark axolotl larva (left side, head to the left outside). ×30

proves particularly helpful for repeated observations of xanthophores during differentiation and pattern formation in developing embryos. This method is superior to the ammonia method.

With HPLC-analysis we found 2-amino-4-hydroxypteridines (=pterins) in axolotl embryos at and prior to stages of pigment pattern formation. Surprisingly, pterins are present much earlier in development than when they become accumulated in xanthophores as visible yellow pigment. During early stages tetrahydrobiopterin, for example, serves as cofactor for phenylalanine hydroxylation (Kaufman 1963) and thus, for melanin biosynthesis. Moreover, both the modulating effect of tetrahydrobiopterin on interleukin 2 activity (Ziegler 1987) and the ubiquitous occurrence of pteridines in trace amounts support the idea that pteridines take part in and regulate the interaction of cellular growth hormones and their target cell receptors. Later in development, when xanthophores emerge, the substance that acted previously as cofactor and regulator in growth and development, may well appear as the pigment pterin observed in xanthophores. The regulatory steps that "tune up" pteridine synthesis and induce divergence of the pathway are still unknown and were not the subject of the present study.

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