Pigment Pattern Formation During Embryogenesis in *Xiphophorus*

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INTRODUCTION

The multiplicity of vertebrate coloration and its morphological basis have attracted scientific interest since the second half of the last century. Initially considered as specializations of epithelial or connective tissue cells, pigment cells were recognized as cells *sui generis* by Ehrmann as early as 1890. At the beginning of the 20th century, Borcea (1909), Harrison (1910), and Weidenreich (1912) proposed that pigment cell precursors are originally located at the dorsolateral sites of the neural tube; they were, however, unable to prove this assumption (for reviews see Refs.[1,2,3]).

Thirty years later, evidence could be provided in amphibians by means of ortho- and heterotopic transplantations that pigment cells originate from a population of cells located dorsolateral to the neural folds. These cells undergo a transitory condensation and arrangement parallel to the longitudinal axis of the embryo; this morphological entity was early described as neural crest. Although the neural crest seems to be quite uniform at first sight, it gives rise to a variety of different tissue types. These have been studied extensively during the last two decades, especially in quail-chick chimera,[4,5] and this gave rise to a new concept of vertebrate evolution.[6]

In teleost fishes, transplantation experiments have been carried out by Humm and Young in *Xiphophorus,*[7] by Lopashov in *Perca fluviatilis,*[8] and by Lamers *et al.* in *Barbus conchonius* embryos.[9]

In fish, however, this experimental approach is more difficult, since neural crest cells are generally not as clearly distinguishable from the surrounding cells compared with the neural crest of amphibians and birds;[7,9,10] morphological investigations on this issue are rare.[10] The aim of the present study was to confirm in *Xiphophorus* the common origin of pigment cells from the neural crest, which has been demonstrated in other vertebrates,[11] and to investigate onset, development, and distribution of pigment cells during ontogeny. In addition, an attempt was made to study the possible influences and interactions of pigment cells with their cellular environment. This should enable better evaluation of pathological deviations in pigment cell development in the *Xiphophorus* melanoma system.

In *Xiphophorus,* the adult pigment pattern consists of melanophores, iridophores, and xanthophores, the latter containing a mixture of pteridine and carotenoid pigments.[12] Number and distribution of these cells are due to genotype- and species-specific modifications.[13]

MATERIALS AND METHODS

For our investigations, we used three genotypes of *Xiphophorus:* 1) The wild-type pigmented fish *X. helleri Heckel.* The pigment pattern consists of all three types of pigment cells. 2) The "golden" mutant (g/g) of *X. maculatus* (Günther). Melanophore differentiation is supposed to be blocked at an early stage of melanoblast differentiation.[14] Except for the melanin-containing pigment epithelium of the retina, the pigment pattern consists almost exclusively of xanthophores and iridophores (this mutant has also been designated as "non-stippled" by other authors.[15,16] 3) The albino mutant (a/a) of *X. helleri Heckel.* This mutant contains unpigmented melanophores; the pigment pattern of this genotype, however, consists of iridophores and xanthophores.

In vitro culture of embryos

Embryos of the appropriate stage of development (8–13 according to Tavolga[17]) were collected from the ovary of the female and washed four times for 5 min in sterile phosphate buffered saline (PBS: 5.82 g NaCl, 0.15 g KCl, 1.05 g Na_2HPO_4/1 l distilled water) containing antibiotics (500 IU penicillin/ml, 500 μg streptomycin/ml, and 7.5 μg amphotericin B/ml). They were cultured *in vitro* in an artificial culture medium (5.8 g NaCl, 0.15 g KCl, 1.05 g $Na_2HPO_4 \times 2H_2O$, 0.15 g KH_2PO_4, 0.08 g $CaCl_2$, 0.08 g $MgCl_2 \times 6H_2O$, 0.25 g glucose, 0.25 g casein dissolved in sterile aquarium water containing 0.2% chick embryo extract (GIBCO, Grand Island, USA), 100 IU penicillin/ml, 100 μg streptomycin/ml, and 2.5 μl amphotericin B/ml) and raised to fertile adults according to the method described by Haas-Andela.[18] In addition, controls were raised in culture medium containing no antibiotics. Development of embryos was documented by serial *in vivo* photography.

Transplantation procedure

After removing the embryos from the ovary, they were washed as described above. Donor tissue was taken from embryos of the same breed having the appropriate genotype. Transplantation was performed with the aid of a Leitz micromanipulator, glass needles, hooks, knives, rods, etc., being produced with a De Fonbrune microforge. The following transplantaions were carried out: a) Heterotopical transplantation of tissue containing pigment cell precursors from wild-type embryos into the prospective abdominal wall of g/g and a/a embryos, Tavolga stage 14–15. b) Heterotopical transplantation of tissue containing pigment cell precursors from the dorsal region of a wild-type embryo into the ventral region of another wild-type embryo, Tavolga stage 11–13. c) Homotopical transplantation of tissue containing pigment cell precursors from wild-type embryos into the dorsal transitional region between head and trunk of a g/g embryo, Tavolga stage 13–14. After transplantation the embryos were cultured as described above.

Light microscopy

Specimens were taken at intervals of two to twelve hr, fixed in Bouin's solution for 6–12 hr, and smoothly dehydrated and paraffinated (alcohol 50/70/80/90/96/99%, methylbenzoate, cedar wood oil, paraffin oil, soft paraffin), and embedded in paraffin (melting point 60–65°C).

Serial sections 5–15 μm in thickness were stained with a melanin-specific staining method described by Lillie[19] or with Masson-Goldner trichrome stain.

In addition, whole mounts of tissue were embedded with a drop of glycerine (87%), covered with a glass slide, and examined light microscopically.

Scanning electron microscopy

Specimens were fixed in 2.5% glutardialdehyde in 0.2 M Sörensen phosphate buffer for up to 24 hr and stored in 1% glutaraldehyde. After rinsing in phosphate buffer, the specimens were dissected, photographed, and either postfixed in 1% OsO_4 for 2–3 hr or processed directly without osmium fixation. Critical-point drying was executed with Freon 13; the specimens were then spattered with a gold layer 100–150 nm in thickness and investigated with a Philips SEM.

RESULTS

Onset of pigment cell development; the neural crest

During the fourth day of embryonic development (Tavolga stages 10–11) a thin and

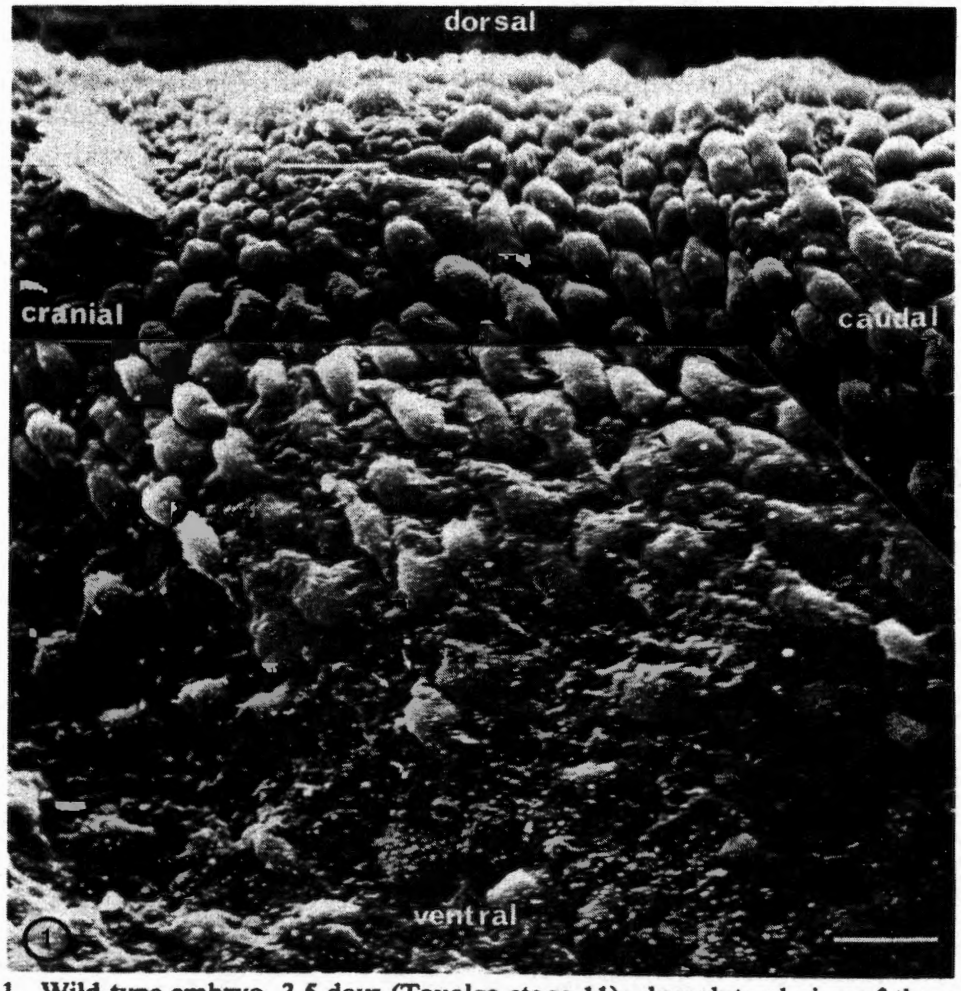

FIG. 1. Wild-type embryo, 3.5 days (Tavolga stage 11); dorsolateral view of the neural tube; vitelline membranes and ectoderm are removed. The ovoid-shaped neural crest cells that are visible dorsally assume a rounder shape ventrolaterally; in addition, surface protrusions and processes (arrows) appear. (SEM; bar = 10 μm)

unevenly distributed layer of ovoid-shaped cells becomes visible by scanning electron microscopy at the dorsolateral sites of the neural folds when the extraembryonic membranes and the ectoderm are removed. The surface morphology differs from that of the surrounding cells: they are larger, short, and have few protrusions, which have increased in size and number in the cells situated more peripherally (Fig. 1). In older stages, *i.e.*, in specimens taken out of culture 12–18 hr later, dendritic cells are visible in a position ventrolateral to the dorsal axis of the embryo. In even older embryos (Tavolga stages 14–15), cells of the described type are no longer visible. It is our strong belief that these cells are the neural crest cells of *Xiphophorus;* the relatively short time of appearance might be the reason for the fact that these cells have not yet been demonstrated.

Appearance of pigment cells and pattern formation

The retinal pigment epithelium is visible at approximately 4½ days of development in wild-type pigmented embryos and at approximately five days in g/g embryos (between Tavolga stages 12 and 14). First, single scattered cells of the pigment epithelium become pigmented, and after 24 hr all cells are pigmented, forming a homogeneous layer. The pigment stains intensely green-black with Lillie's melanin stain (Fig. 2). This melanin pigment appears at the same time in embryos raised in culture media containing amphotericin B and in embryos raised in amphotericin-B-deficient media. About 20 hr later (Tavolga stage 14–15), the first Lillie-positive cells appear in the chorioidea and on the meninx primitiva in the rhombencephalic region (Fig. 2). These melanocytes are mostly bipolar and contain few pigment granules; they are visible in wild-type pigmented fish only.

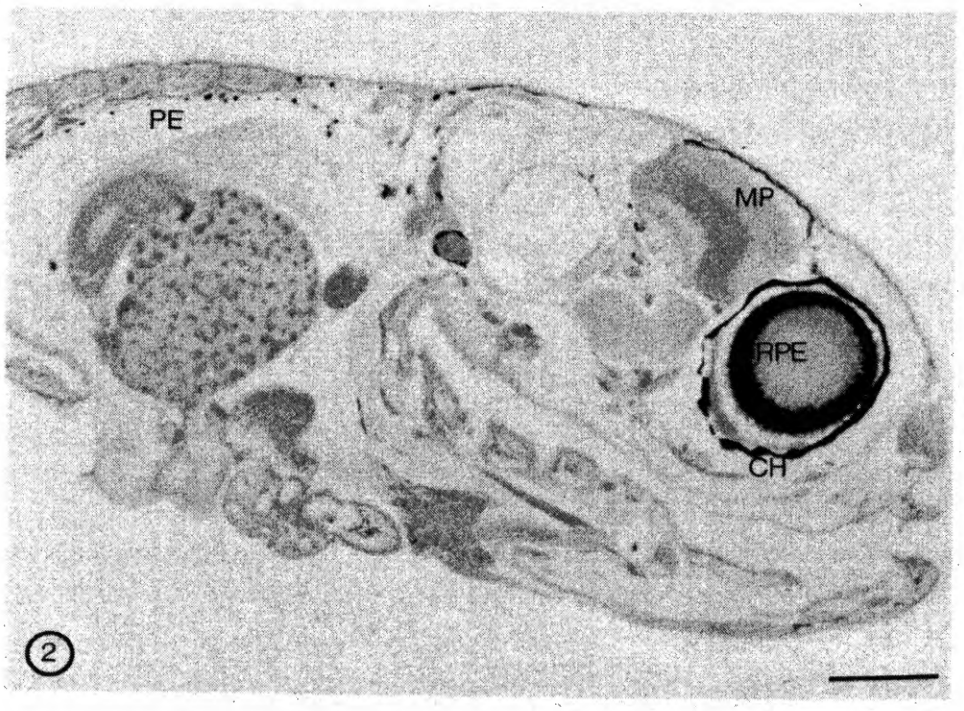

FIG. 2. Wild-type embryo, 12.0 days (Tav. st. 21), paramedian section. Retinal pigment epithelium (RPE) and melanocyte pigmentation of the chorioidea (CH), meninx primitiva (MP), and peritoneum (PE) are visible. (Lillie's stain, bar = 100 μm)

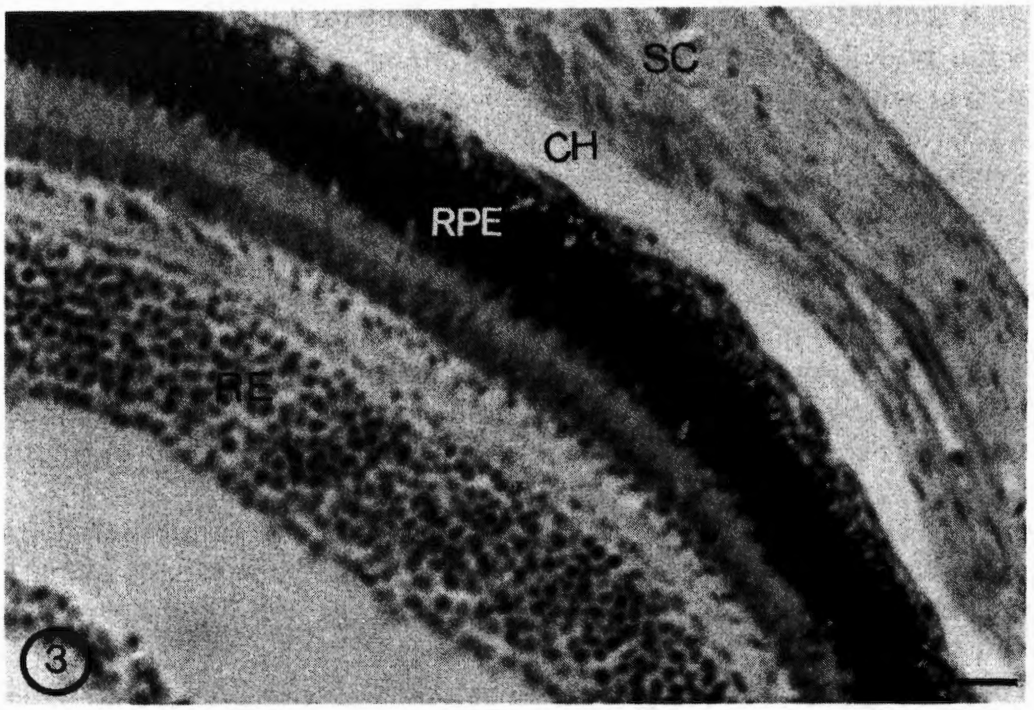

FIG. 3. g/g fry, 9 days postnatal age. No melanin is visible besides that of the retinal pigment epithelium (RPE). RE, retina; CH, chorioidea; SC, sclera. (Lillie's stain; bar = 10 μm)

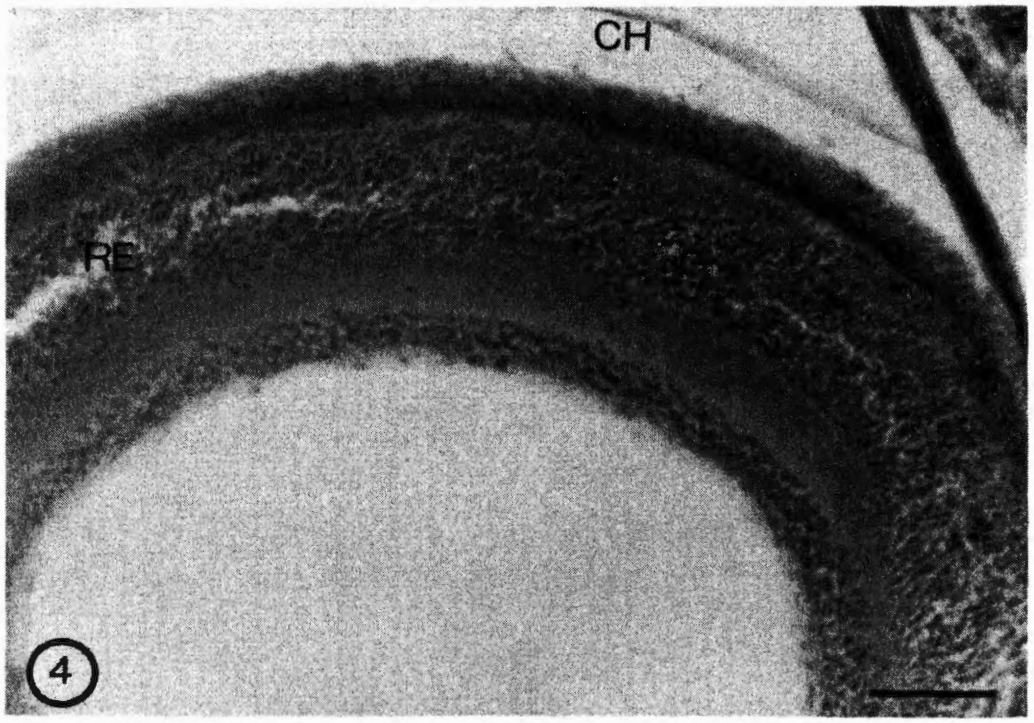

FIG. 4. a/a embryo 11 days (Tav. st. 20). Retina (RE) and chorioidea (CH); no positive stain indicating presence of melanin is detectable. (Lillie's stain; bar = 20 μm)

If paraffin sections of the head region are stained with Lillie's melanin stain, in wild-type fish the retinal and chorioideal pigment stains intensively green-black (Fig. 2); in g/g embryos and in g/g frys only the retinal pigment epithelium will exhibit a positive stain (Fig. 3); in a/a embryos the staining is completely negative (Fig. 4). This means that embryos of the three genotypes investigated can be clearly distinguished histologically, even at early stages of development.

The peritoneal melanocytes appear at the seventh day of embryonic development (Tavolga stage 16–17). Their pattern differs from the meningeal type from the onset: whereas the meningeal melanocytes start to become multidendritic and eventually form a round shape, the peritoneal melanocytes appear fusiform and tend to form a reticular pattern from the very beginning (Fig. 5). At this time, the meningeal melanocytes have increased in number and size and cover the dorsal surface of the meninx primitiva further caudally.

The vitelline veins are covered by small bipolar to oligodendritic melanocytes a half day later (Tavolga stage 18); the melanocytes distribute themselves along the major vitelline vessels to the yolk sac periphery before they abandon their close attachment to these major vessels (Fig. 6).

At the beginning of the second third of embryonic development a population of rosette-shaped, large, densely pigmented melanocytes is visible over the rhombencephalic area (Fig. 7). These "corolla melanophores," as designated by Tavolga,[15] cover the dorsal side of the meninx prinmitiva first, before similar melanocytes form in the remainder of the embryo.

Subsequently the cutaneous and extracutaneous tissues (Fig. 2) and especially blood vessels become pigmented, the latter seeming to represent a preferential pathway for

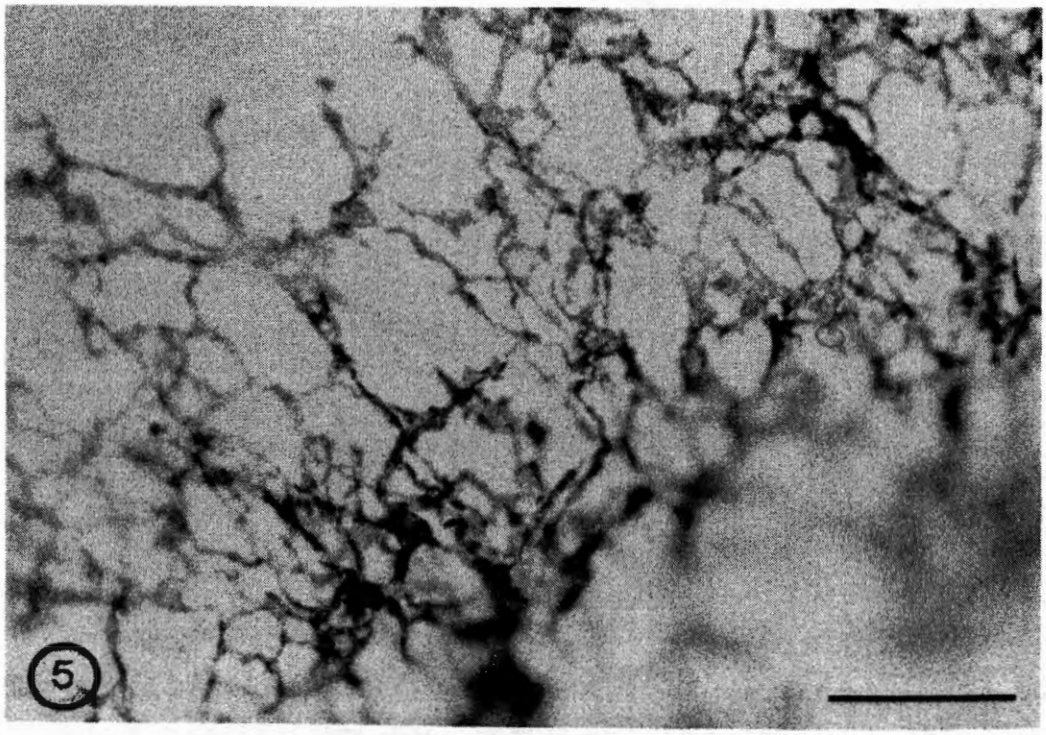

FIG. 5. Wild-type embryo, 8 days (Tav. st. 17), peritoneum; the peritoneal melanocytes form a reticulum. (whole mount; bar = 10 μm)

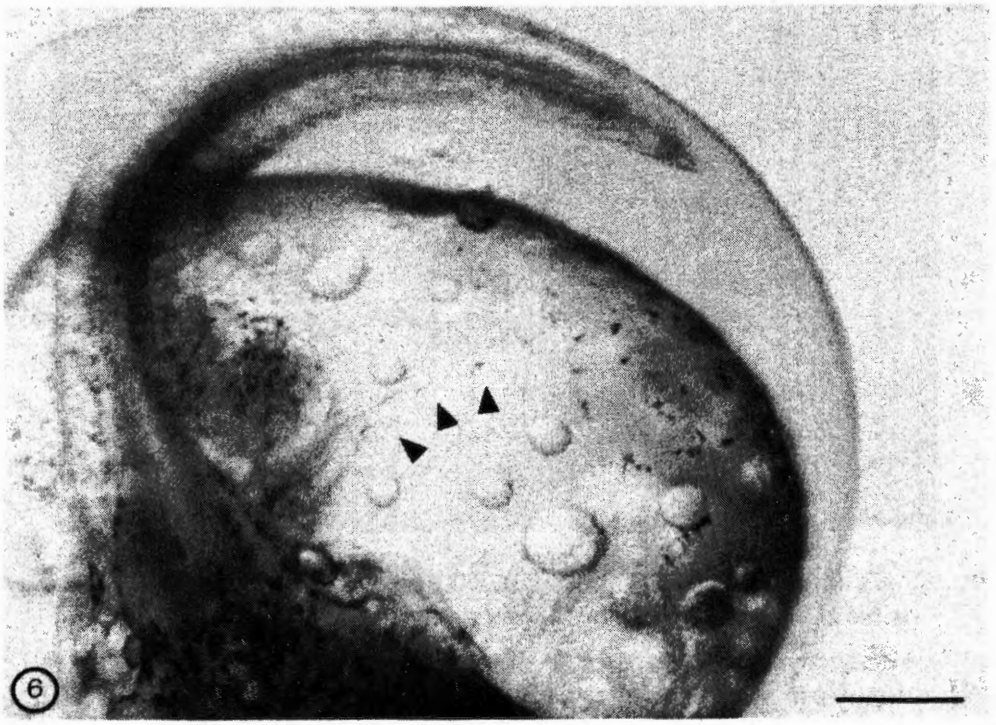

FIG. 6. Wild-type embryo, 9 days (Tav. st. 18); melanocytes on the left vitelline vein (arrows) and the yolk sac periphery. (*in vivo* photography; bar = 150 μm)

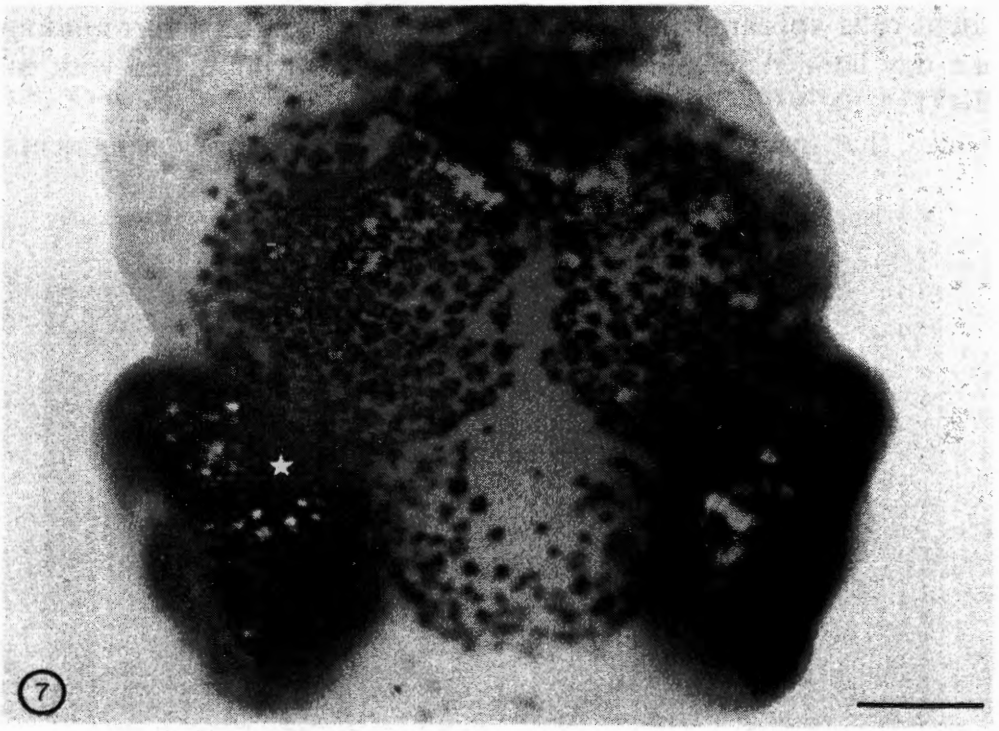

FIG. 7. Wild-type embryo, 12 days (Tav. st. 20); the tectal meninges are covered with rosette-shaped melanocytes; chorioideal iridophores (*) are visible. (*in vivo* photography; bar = 150 μm)

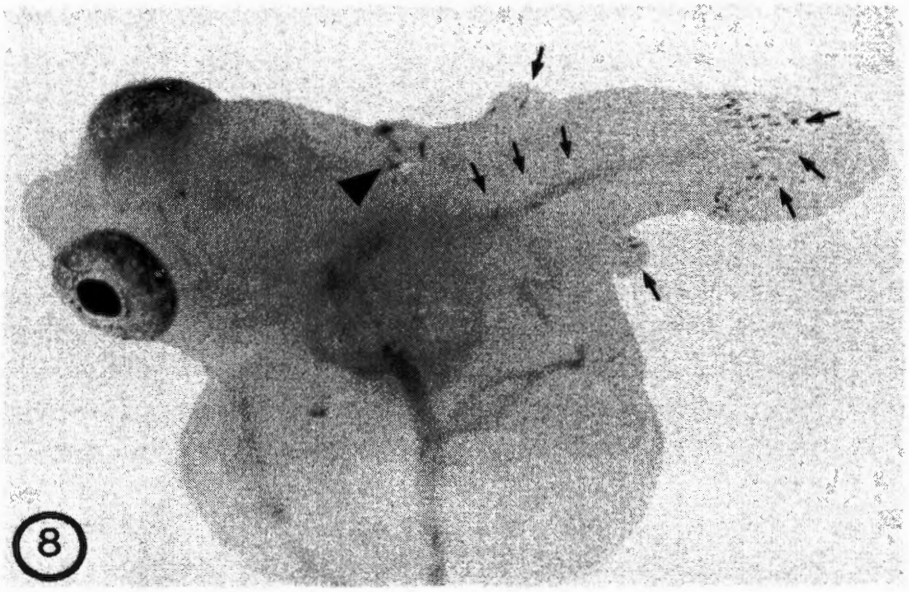

FIG. 8. g/g embryo 16 days (Tav. st. 20), wild-type graft (thick arrow); melanocytes are visible at various sites (arrows) several days later (see text).

pigment cell migration in late embryogenesis; this can be observed in the trunk (via dorsal aorta and segmental vessels) and the yolk sac (via vitelline vessels). This morphological observation could be confirmed by transplantation: tissue from wild-type embryos containing pigment cell precursors was homotopically transplanted into the dorsal transitional region between head and trunk of a g/g embryo stage 13–14 (the g/g mutant possesses melanin only in the retinal pigment epithelium). After 3–4 days, black pigment cells appeared in the close vicinity of the graft, surrounding the aorta dorsalis one day later. After ten days, the fins were also populated with melanin-pigmented cells (Fig. 8).

It is obvious that in the absence of melanocytes or melanocyte pigmentation irido-

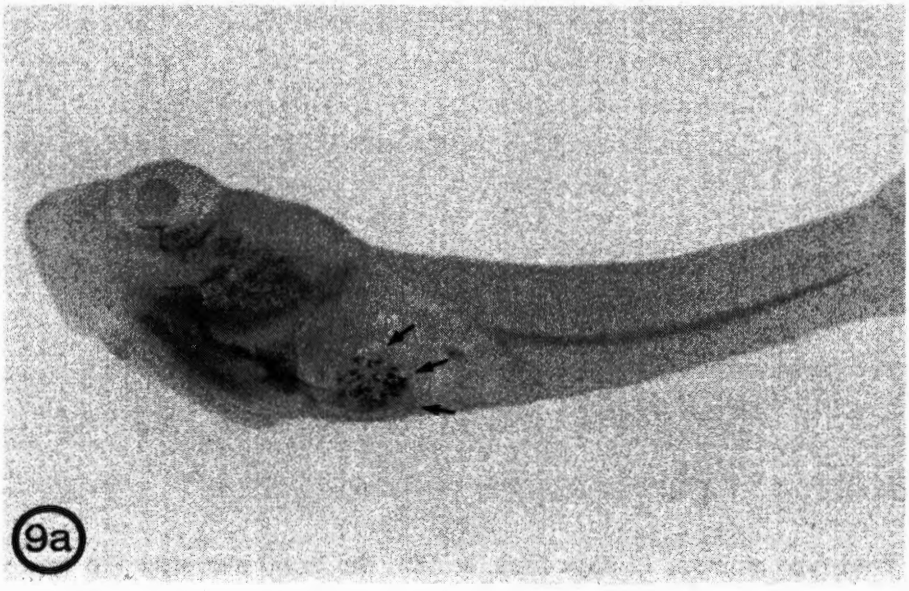

FIG. 9a. a/a fry, 6 days; melanocytes appear in the graft tissue (arrows) only.

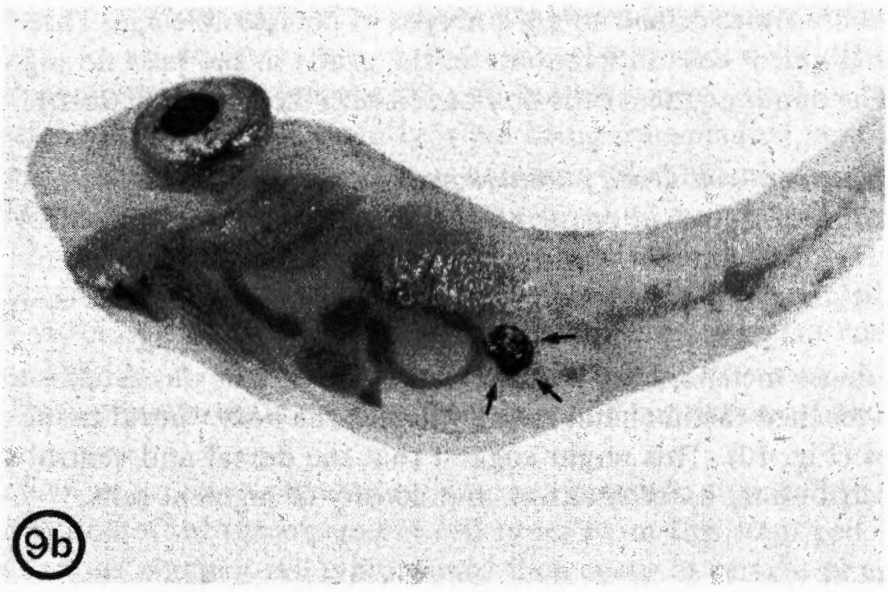

FIG. 9b. g/g fry, 10 days; no melanocytes are visible in the host tissue (see text). Arrows show graft tissue.

phores are the predominant pigment cell type. Xanthophores appear very late (18–21 days, Tavolga stage 25–26), and even then inconstantly, and become visible mainly during the postnatal period.

Iridophores appear at the same sites as melanocytes and in the same sequence (rhombencephalomeningeal/chorioideal, peritoneal, yolk sac, cutaneous). There is always a gap of at least 6–12 hr between the appearance of the first melanocytes and the iridophores.

Cell autonomy of pigment cell differentiation and pigment formation

Tissue from wild-type embryos containing pigment cell precursors was heterotopically

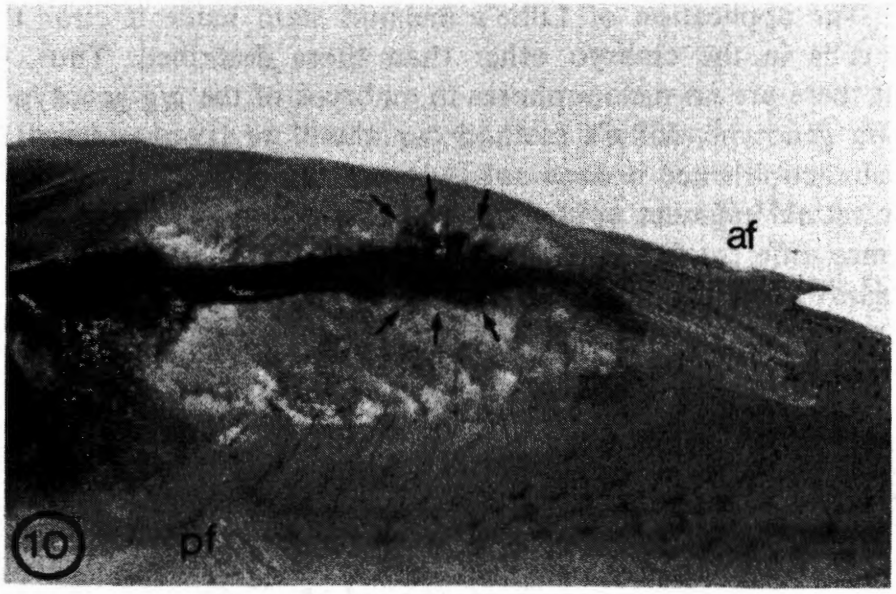

FIG. 10. Wild-type fry, prospective dorsal integument grafted into prospective abdominal wall (arrows); graft-type melanocytes are not visible in the host tissue. af: anal fin; pf: pectoral fin.

transplanted to a/a embryos and to g/g embryos of comparable age. Three to seven days later wild-type pigment cells differentiate in the graft; in the host no pigment becomes visible at all. The donor pigment cells do not colonize the host (Fig. 9a, b).

Dependency of pigment cell density on surrounding tissue

Transplantation of dorsal integument of a wild-type pigmented embryo was grafted to the ventral region of another wild-type embryo; the dorsal integument is densely pigmented with melanocytes in older embryos and in adult fish, whereas the ventral integument is not.

The typical dense melanophore pattern of the dorsal skin was established in the graft when the host reached the neonatal stage, whereas the host ventral tissue remained free of melanocytes (Fig. 10). This might suggest that the dorsal and ventral skin tissue influences the distribution, differentiation, and density of pigment cells.

DISCUSSION

No information exists to date in the literature concerning the morphology of neural crest cells in the genus *Xiphophorus*. Except for an investigation of neural crest cells of *Fundulus heteroclitus* and some other species,[9,10] knowledge about the structure of neural crest cells is rare in teleost fishes. The surface morphology of the cells described by the present authors and their regional distribution resemble descriptions of the neural crest cells of other vertebrates.[4]

Melanophore development of wild-type pigmented fish has been described frequently;[14,15,17] however, a combination of transplantation methods, histology, and scanning electron microscopy—as performed in this study—allows a more precise analysis of this process. The onset of pigment cell appearance, as described in this study, appears to be slightly earlier than that reported by Tavolga.[17] This might be due to the application of a melanin-specific stain that enables detection of small amounts of melanin, not readily visible so far. The application of Lillie's melanin stain made it clear that there are no melanized cells in the embryo other than those described. Thus, we could demonstrate that there are no melanophores in embryos of the g/g genotype and no melanin in the a/a genotype. Lillie's method can therefore be considered more specific than the dopa reaction since it does not induce melanization of cells other than melanophores or retinal pigment epithelium cells, which may result from oxidation of dopa by enzymes different from tyrosinase.[20] This fact is important when studying a developing organism consisting of a heterogeneity of different tissues.

In embryo cultures, apparently no precocious melanogenesis is caused by amphotericin B as reported elsewhere.[21] This might indicate that the applied culture method resembles physiological conditions. Cell autonomy of normal pigment cell differentiation and pigment formation could be confirmed for *Xiphophorus*, as it has been shown for other vertebrates.[1,4] Interestingly, the differentiation of neoplastically transformed pigment cells in *Xiphophorus* seems to be influenced by a diffusible factor from normal tissue containing the differentiation gene "diff." This process is therefore not considered to be cell autonomous.[22]

The preservation of the dorsal integument-type pigment pattern in grafts transferred to the ventral abdominal wall suggests an influence of the surrounding tissue on de-

veloping pigment cells, which cannot merely be explained by topographical distribution and relationships. As we described above, pigment cells are not only distributed along blood vessels during late embryogenesis (Fig. 6) but also move actively towards blood vessels to migrate along them subsequently when being transplanted near a large vessel (Fig. 8). Pigmentation of blood vessels is a common phenomenon in vertebrates: in Poeciliid fish, blood vessels are densely pigmented by all three types of pigment cells in adult fish (R. U. Peter, unpublished data, Ref. 16), and with melanocytes in lizards.[3)]

This study has shown that the heterogeneity of pigment patterns in different tissues, which becomes visible by investigation of the extracutaneous pigment cells (Peter, unpublished data, Ref. 3), is already present from the very first onset of pigment cell development. This supports the view of a tissue-specific regulation of the melanoblast population.[3)]

Our data confirm an origin of pigment cells from a common stem cell.[11)] They suggest a sequential derivation of the three pigment cell types from this stem cell. The differentiation into the various pigment-cell types would then occur at certain phases. If such a time sequence of differentiation is established in a species, it takes place at a defined phase, even when a pigment cell type is lacking by mutation. This would explain why, even in the absence of melanophores, iridophores and xanthophores still do not appear earlier than they would in wild-type pigmented fish possessing melanophores. The differentiational sequence melanophore-iridophore-xanthophore might not be a general one; in white axolotls, xanthophores apparently appear before the iridophores.[23)]

However, the fact remains that not all three types appear at one time, as it might theoretically be possible, and, if once there is a time sequence of appearance, it is constant and not accidental.

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