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Pigment Patterns of Larval Salamanders (Ambystomatidae, Salamandridae): The Role of the Lateral Line Sensory System and the Evolution of Pattern-Forming Mechanisms

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In many species of salamanders, pigment cells derived from the neural crest give rise to a horizontal stripe pattern in hatchling larvae. A defining element of these horizontal stripe patterns is a region over the middle of the myotomes that is relatively free of melanophores. This study shows that formation of a ''melanophore-free region'' and horizontal stripe pattern in *Ambystoma tigrinum tigrinum* (family Ambystomatidae) correlates with the development of the trunk lateral line sensory system. Moreover, prevention of lateral line development results in greater densities of melanophores in the middle of the flank, essentially eliminating the melanophore-free region in this taxon. A phylogenetic survey also revealed that ablation of the lateral lines has qualitatively similar effects on melanophores in seven of eight additional taxa (Ambystomatidae: *A. barbouri, A. maculatum, A. talpoideum;* Salamandridae: *Notophthalmus viridescens, Pleurodeles waltl, Taricha granulosa, T. rivularis*). In *Taricha torosa,* however, a superficially similar melanophore-free region forms prior to lateral line development, and ablation of the lateral lines does not perturb the horizontal stripe pattern. Finally, heterospecific grafting experiments demonstrated that *T. torosa* lateral lines are competent to generate a melanophore-free region, and *T. torosa* melanophores are competent to respond to cues associated with the lateral lines. These results indicate that lateral line-dependent pattern-forming mechanisms are common and probably ancestral within the families Ambystomatidae and Salamandridae and suggest that these ancestral mechanisms have been retained in *T. torosa* as redundant, lateral line-independent mechanisms for stripe formation have evolved.

INTRODUCTION

The mechanisms of pattern formation and morphogenesis are becoming increasingly well understood, yet the details of how these processes evolve remain largely unexplored. Some progress has been made through recent comparative studies of gastrulation (Wray and Raff, 1991; Purcell and Keller, 1993; Collazo *et al.,* 1994a; de Robertis *et al.,* 1994), as well as limb (Warren *et al.,* 1994; Sordino *et al.,* 1995) and axial patterning (Dickinson *et al.,* 1993; Burke *et al.,* 1995). Nevertheless, we still know very little about evolutionary transformations in mechanisms that operate during later embryogenesis and organogenesis. This is particularly true for ecologically important larval or adult characters that are composed of multiple, functionally and developmentally integrated parts (e.g., the eye, limb, or skull). One approach to understanding the evolution of developmental processes underlying such complex characters is to examine pattern-forming and morphogenetic mechanisms within an explicitly phylogenetic framework (also see Hall, 1992; Wray, 1994). Salamander pigment patterns are convenient characters for this approach, because these patterns are diverse and accessible to both observation and experimentation, and because phylogenetic relationships among salamander taxa have been studied extensively (reviewed in Shaffer, 1993; Parichy, 1996a).

Salamanders exhibit three types of pigment cells, or chromatophores—black melanophores, yellow xanthophores, and silvery iridophores—and each of these is derived from the neural crest, a transient population of cells that also contributes to the peripheral nervous system, craniofacial skeleton, and many other characters (reviewed in DuShane, 1943; Le Douarin, 1982; Bagnara, 1983; Hall and Hörstadius, 1988; Selleck *et al.,* 1993; Epperlein and Löfberg, 1993; Erickson, 1993; Frost-Mason and Mason, 1996). Prospective neural crest cells are present initially within the neural

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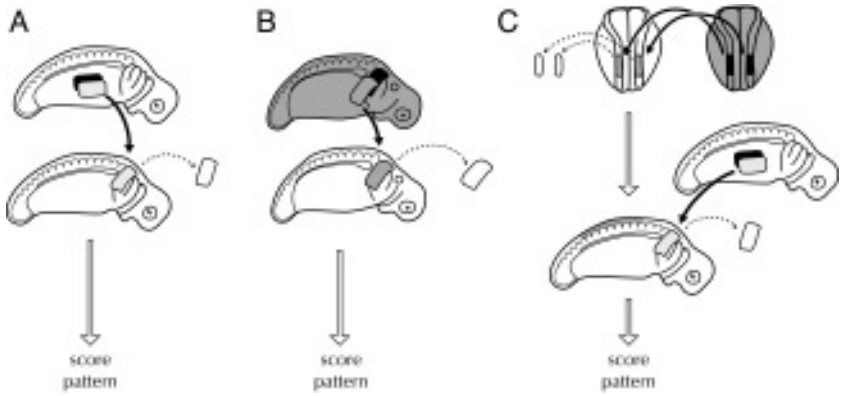


FIG. 1. Microsurgical procedures used in this study. (A) Ablation of the lateral lines by replacing lateral line placode-area ectoderm with belly epidermis (light shading). (B) Grafting of *T. torosa* lateral line placode-area ectoderm (dark shading) to *A. t. tigrinum* host. (C) Grafting of *T. torosa* neural folds containing prospective neural crest cells (dark shading) to *A. t. tigrinum* hosts, with subsequent unilateral ablation of the host lateral lines.

folds (Moury and Jacobson, 1990; Mayor *et al.*, 1995; Selleck and Bronner-Fraser, 1995), but these cells then segregate from the neuroepithelium and migrate from above the neural tube to various regions within the embryo (e.g., Löfberg *et al.*, 1980; Serbedzija *et al.*, 1990; Erickson *et al.*, 1992; Collazo *et al.*, 1993; Raible and Eisen, 1994; Tosney *et al.*, 1994; reviewed in Erickson and Perris, 1993). Although all three types of chromatophores can contribute to pigment patterns in larval and post-metamorphic salamanders, melanophores and xanthophores are primarily responsible for pigment patterns shortly after hatching.

A recent survey (Parichy, 1996a) categorized 38 salamander taxa representing 7 families according to the presence, absence, or variability of two prominent elements of the early larval patterns. First is a series of alternating vertical bars of melanophores and xanthophores that appears to be found only within the family Ambystomatidae (present in 14 of 20 ambystomatid taxa examined; also see Lehman, 1957; Epperlein and Löfberg, 1990; Olsson and Löfberg, 1992; Olsson, 1993, 1994). The second pattern element is a region over the lateral face of the myotomes in which melanophores are either absent or occur only sparingly. Since xanthophores are typically present in this ''melanophore-free region,'' the overall pattern consists of a yellow horizontal stripe bordered by dark dorsal and sometimes ventral concentrations of melanophores. Melanophore-free regions are widespread phylogenetically and are found within the families Ambystomatidae, Salamandridae, and Proteidae (present in 20 of 31 taxa examined within these families; variable or indistinct in an additional 7 of 31 taxa).

Several factors have been suggested to mediate the formation of a melanophore-free region (and hence horizontal stripe pattern), including: interactions among melanophores (Twitty, 1945); sorting-out of melanophores and xanthophores (Epperlein and Claviez, 1982a,b; Epperlein and Löfberg, 1990); passive movements of melanophores due to growth (Rosin, 1943); and cues for chromatophore localization associated with the myotomes, epidermis, dorsal fin, pronephric duct, or vasculature (Twitty, 1936, 1945; Tucker and Erickson, 1986a,b; Epperlein and Löfberg, 1990). Yet, visual inspection of early larvae reveals that the position of the melanophore-free region also correlates with that of the trunk midbody lateral line. Present in aquatic amphibians and fishes, the lateral lines comprise a bilateral sensory system for detecting mechanical (and sometimes electrical) stimuli, and probably function in orientation, feeding, and predator avoidance (Atema *et al.*, 1988; Winklbauer, 1989; Blaxter and Fuiman, 1990; Northcutt, 1992; Collazo *et al.*, 1994b; Smith, 1996). In salamanders, three trunk lateral lines (midbody or main, dorsal, and ventral) develop from postotic, ectodermal placodes in the head. These placodes give rise to lateral line ''primordia'' that migrate caudally within the epidermis, depositing clusters of cells at periodic intervals that subsequently erupt through the epidermis as mechanosensory neuromasts (e.g., Stone, 1933; Smith *et al.*, 1990; Northcutt *et al.*, 1994, 1995; Parichy, 1996b). Despite the location of the midbody lateral line, a role for the lateral lines in stripe formation has not been investigated previously.

In this study, I investigate the development and evolution of melanophore-free regions. I first compare pigment pattern formation in two distantly related taxa: in *Ambystoma tigrinum tigrinum* (Ambystomatidae), a melanophore-free region forms coincident with the migration of the midbody lateral line primordium, whereas in *Taricha torosa* (Salamandridae), this pattern element develops in advance of the primordium. I then demonstrate that preventing lateral line development eliminates the melanophore-free region in *A. t. tigrinum*, but does not perturb the melanophore-free region in *T. torosa*. Finally, I test for lateral line effects in seven additional taxa, and I use heterospecific chimeras to examine the evolution of pattern-forming mechanisms. These results suggest that lateral line effects on melanophores are a shared, ancestral feature of pigment pattern

development for the families Ambystomatidae and Salamandridae, whereas redundant, lateral line-independent stripe-forming mechanisms have evolved in *T. torosa*. An accompanying report investigates the details of melanophore–lateral line interactions (Parichy, 1996b).

MATERIALS AND METHODS

Embryos, Culture Conditions and Staging

Field-collected *A. t. tigrinum* and *A. maculatum* embryos were purchased from the Charles D. Sullivan Co., Inc. (Nashville, TN) and also were a gift of K. Mierzwa (Cook County, IL). *A. talpoideum* and *A. barbouri* embryos were provided by J. Krenz (Savannah River Ecology Lab, SC) and A. Sih (University of Kentucky), respectively. *Pleurodeles waltl* embryos were obtained through natural spawnings and were provided by D. Glahn (University of California, Davis). Adult *Notophthalmus viridescens* were purchased from Charles Sullivan and *T. torosa, T. granulosa,* and *T. rivularis* embryos or adults were collected from local populations (Napa or Sonoma Counties, CA). *Notophthalmus* and *Taricha* adults were allowed to spawn naturally or were induced to do so with human chorionic gonadotropin (Sigma; see Armstrong and Duhon, 1989). Embryos were maintained in plastic dishes containing 20% Hepes-buffered Steinberg's solution (HSS, plus 37.5 IU/ml penicillin, 37.5 μg/ml streptomycin; Asashima *et al.*, 1989; 9–18°C, 12L:12D). Staging tables were: *A. t. tigrinum,* Bordzilovskaya *et al.* (1989); *A. maculatum, A. talpoideum, A. barbouri,* Harrison (1969); *T. torosa, T. rivularis, T. granulosa, N. viridescens,* Twitty and Bodenstein (1962); *P. waltl,* Vasseztky (1991). Stage numbers are preceded with "B," "H," "T," or "V" to indicate the table used.

Photographic Series

Using a Leitz Diaplan epifluorescence microscope, embryos were photographed repeatedly under brightfield illumination to reveal melanophores and FITC illumination to reveal autofluorescing xanthophores (Epperlein and Löfberg, 1990). *A. t. tigrinum* (representing 3 sibships) were photographed every 3 hr for the first 54 hr, then at 6- and 12-hr intervals through 110 hr (B34–42). *T. torosa* (representing 4 sibships) were photographed every 3 hr for the first 78 hr, then at 6-, 12-, and finally 24-hr intervals through 138 hr (T33–40). These schedules generally permitted reidentification of individual melanophores and xanthophores. The fluorescent, lipophilic dye, DiC_{18} (DiI; Molecular Probes, Eugene, OR) was used to enhance the visibility of the developing lateral lines in a subset of these embryos. DiI was made up as a 0.5% (w/v) stock solution in 100% ethanol and diluted to 0.05% in 0.3 *M* sucrose just before use. Prior to migration of the lateral line primordia (B33; T32), a small volume of dye was injected unilaterally in the vicinity of the postotic lateral line placodes (Northcutt *et al.*, 1994) using a mouth pipette fitted with a glass electrode. Embryos were thereafter maintained individually in 20% HSS (21–22°C, 0L:24D) and were anesthetized momentarily in 20% HSS/0.01% benzocaine for photographing. Images were recorded on Kodak P800/P1600 color transparency film (DiI-labeled embryos) or Kodak T-Max P3200 black and white negative film (unlabeled embryos). For the final photograph in each series, xanthophore autofluorescence was enhanced by fixing embryos 15 min in 4% paraformaldehyde, then immersing them in 20% HSS, pH 11 (Olsson and Löfberg, 1992).

Time-Lapse Videomicrography

For time-lapse videos, embryos were immobilized by placing them in wells cut into agar-lined dishes containing 20% HSS/0.01% benzocaine (21–22°C). Nonmotile landmarks were created by surgically implanting fine grains of charcoal within the epidermis, and the distinctiveness of the migrating lateral line primordium was enhanced in some embryos by staining the lateral line placodes with Nile blue sulfate (Stone, 1933). Videos were recorded at 1:140 hr using Sony CCD video cameras, Panasonic time-lapse video recorders, and Wild or Zeiss stereomicroscopes. Recordings were analyzed by tracing cell and landmark movements on acetate sheets placed over the video monitor. Because of a general expansion of the flank, acetate sheets were continually repositioned during tracing so that the location of the dorsal apex of the myotomes and the anterior edge of the region examined always coincided between traced and video images. This effectively constrained growth in the tracings to occur in ventral and posterior directions, thereby providing a conservative test for ventral-to-dorsal movements by melanophores (see below).

Microsurgical Procedures

For surgical manipulations, embryos were rinsed in sterile 20% HSS, decapsulated with fine forceps, and then passed through four to five changes of 100% HSS (plus 75 IU/ml penicillin, 75 μg/ml streptomycin). Manipulations were carried out in sterile agar-lined dishes containing 100% HSS. When necessary, embryos were anesthetized with benzocaine. Operations were performed with tungsten needles and grafted tissues were held in place (10–20 min) with fragments of glass coverslips. Embryos were then maintained in 20% HSS (15°C, 12L:12D).

To prevent lateral line development, ectoderm including the postotic lateral line placodes was removed unilaterally and replaced with belly epidermis from a similarly staged donor (Fig. 1A). Lateral line ablations were performed at a range of stages prior to primordium migration (B26–33; H30–33; T24–33; V24–25) without differences in results, and the side for ablation was chosen randomly for each embryo. Failure of lateral line development was confirmed by scanning electron microscopy (SEM), serial sectioning of paraffin- or plastic-embedded embryos, or visual inspection of the flank (see Parichy, 1996b). Additionally, to detect hair cells of lateral line neuromasts in *T. torosa,* larvae were viewed under FITC and RITC illumination after staining 5–10 min in 20% HSS/1 m*M* DASPEI [2-(4-dimethylaminostyryl)-*N*-ethylpyridinium iodide] (Molecular Probes; Balak *et al.*, 1990). To control for nonspecific effects of the surgery, sham manipulations were performed unilaterally by removing and then replacing placode-area ectoderm.

In two experiments, the behaviors of *T. torosa* lateral lines or melanophores were examined in heterospecific chimeras. First, *T. torosa* lateral line placode-area ectoderm (T28–33) was grafted unilaterally to *A. t. tigrinum* hosts (B28–29), from which corresponding regions of ectoderm had been removed (Fig. 1B). Second, *T. torosa* neural folds (containing prospective neural crest cells) were grafted bilaterally to *A. t. tigrinum* hosts from which the neural folds had been removed (T16, B15–16, respectively); the lateral lines of these chimeras were then ablated unilaterally (Fig. 1C). For both experiments, *T. torosa* tissues were labeled prior to grafting by injection of DiI, as described above.

Quantitative Methods

After early larval pigment patterns had formed (B41–42; H40–41; T39–40; V36–37), surgically manipulated larvae were photo-

graphed under brightfield (for melanophore distributions) or RITC illumination (for DiI-labeling). Photographs were then digitized and analyzed using the public domain NIH Image program (written by Wayne Rasban, NIH). To test for effects of lateral line ablation, *melanophore densities* at different positions along the dorsoventral axis were compared between unmanipulated and manipulated sides of larvae. Specifically, dorsoventral distances were measured between the ventral-most edge of each melanophore and the border between the dorsal fin and the dorsal apex of the myotomes. Using these distances, the absolute numbers of melanophores falling within successive intervals along the dorsoventral axis were computed. For each taxon, intervals were chosen to provide relatively smooth distributions of melanophores that best represented melanophore patterns on unmanipulated sides (without regard to patterns on manipulated sides). The absolute numbers of melanophores within each interval were then normalized according to the anteroposterior length of the region examined (typically 2–3 mm), yielding melanophore densities with units of cells/mm. For a given experimental treatment, mean densities of melanophores within each interval were then calculated by averaging across larvae. To calculate *total melanophore densities*, the total numbers of melanophores on the flank were divided by the length of the region examined, also yielding densities in cells/mm.

In *T. torosa*, individual melanophores contributing to the dorsal stripe could not be identified. For this reason, the average dorsoventral ''height'' of the dorsal stripe of melanophores, as well as the numbers of individual melanophores scattered over the flank, were compared between unmanipulated and manipulated sides. The height of the dorsal stripe was estimated by calculating the total area covered by dorsal stripe melanophores from digitized images, then dividing this area by the anteroposterior length of the region examined (ca. 3 mm/embryo). For *T. torosa* melanophores in *A. t. tigrinum* hosts (Fig. 1C) effects of lateral line ablation were scored by independent observers blind with respect to treatment. For each chimera, the side on which melanophores more completely colonized the middle of the flank received a rank of ''1,'' and the contralateral side received a rank of ''0;'' if no difference could be discerned, both sides were scored as ''0.'' After each chimera had been scored by five individuals, the ranks for each side were summed and compared between lateral line-intact and line-ablated sides.

RESULTS

To document the formation of the melanophore-free region, I photographed *A. t. tigrinum* repeatedly from the first appearance of chromatophores through formation of the definitive early larval pattern, and I tested for correlations with lateral line development by enhancing the visibility of the lateral lines with the fluorescent dye, DiI (n = 17 embryos total, 9 DiI-labeled). To investigate whether similar events occur in other taxa, I then repeated these procedures using *T. torosa* (n = 18 embryos total, 9 DiI-labeled).

Pigment Pattern Formation and Lateral Line Development in A. t. tigrinum

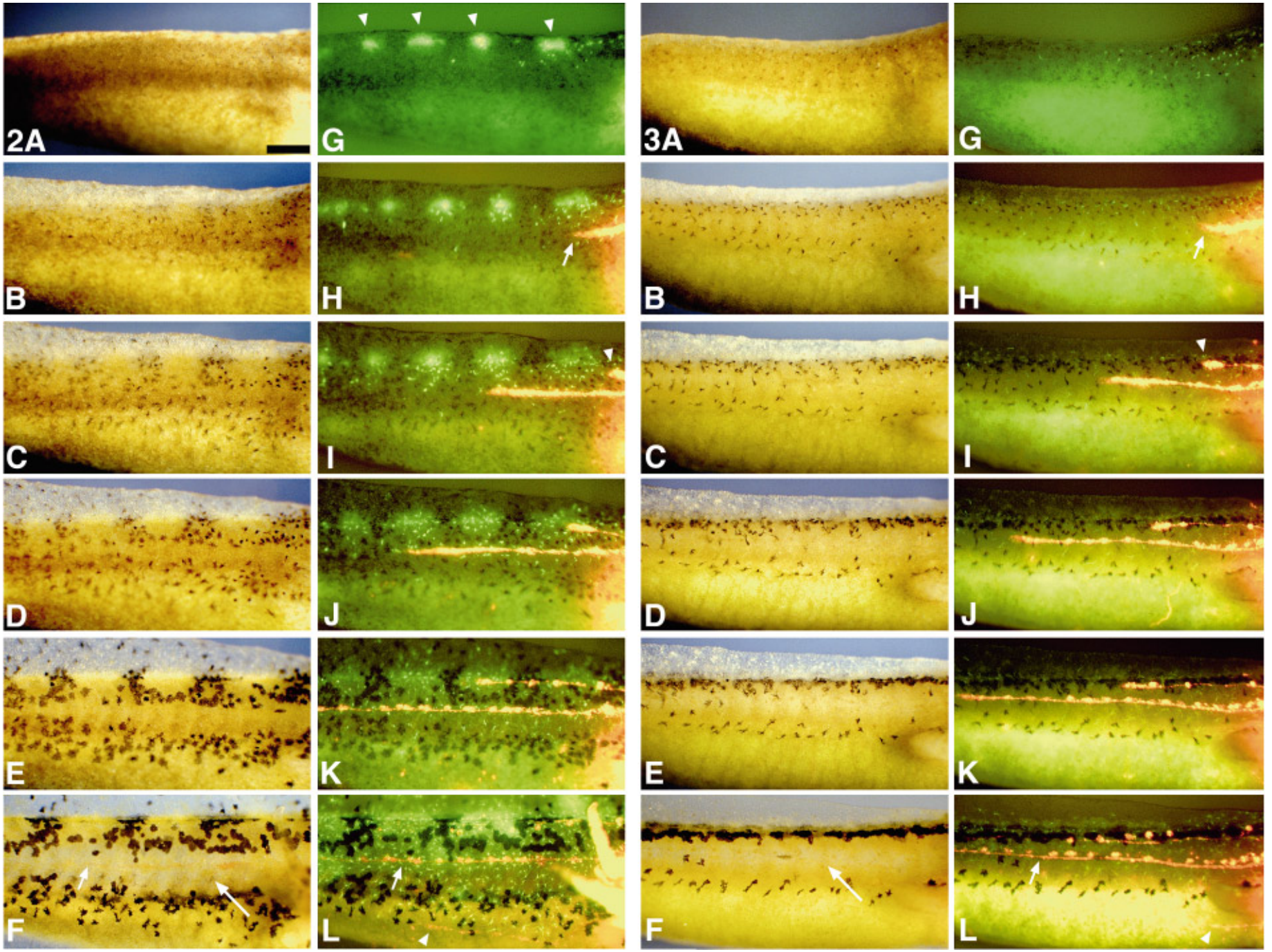
In *A. t. tigrinum*, lightly pigmented melanophores were already distributed relatively uniformly over the flank when first visible externally (B34–35; Fig. 2A). Many were elongated and could not be positively reidentified during the first 6–9 hr of the photographic series, suggesting that the cells were highly motile. In contrast, most xanthophores were found initially in aggregates dorsal to the neural tube (mean = 6.5 aggregates/embryo, SD = 1.01, range = 5–7, n = 17 embryos; Fig. 2G). Once xanthophores began to disperse (B35), the onset of dispersal from all aggregates occurred within 6–18 hr (mean = 10.3 hr, SD = 3.53, n = 17 embryos). Melanophores could sometimes be observed to recede short distances from the dispersing xanthophores to form alternating vertical bars of the two cell types (also see Epperlein and Löfberg, 1990; Olsson and Löfberg, 1992).

As the midbody lateral line primordium migrated onto the flank, melanophores were more heavily pigmented, less elongated, and apparently less motile. A subtle melanophore-free region was first evident in the anterior trunk (B35–37), and the position of this region was correlated temporally and spatially with that of the migrating, midbody lateral line primordium (Figs. 2B–2D, 2H–2J). Melanophores initially in the middle of the flank moved short distances dorsally or ventrally relative to the primordium (see Parichy, 1996b for details). A subtle melanophore-free region also was associated with the dorsal lateral line primordium (Figs. 2C, 2D, 2I, and 2J), but typically was obscured at later stages.

During later development (≥B37), the melanophore-free region was maintained and became increasingly distinct (Figs. 2E and 2K). Most dorsal melanophores failed to cross the lateral line primordium or nerve, which comigrates with the primordium (Smith *et al.*, 1990; Northcutt *et al.*, 1994).

FIG. 2. Development of the early larval pigment pattern in *A. t. tigrinum*. One representative embryo is shown at six stages of development. Anterior is to the right. Bright field micrographs (A–F) show the distributions of melanophores (black cells). The corresponding fluorescence double exposures (G–L) show the distributions of xanthophores (green cells) as well as the developing, DiI-labeled lateral lines (red). Times after the first appearance of melanophores are: (A, G) 3, (B, H) 12, (C, I) 21, (D, J) 27, (E, K) 45, and (F, L) 110 hr. The large arrow in (F) indicates the melanophore-free region in the definitive early larval pattern. Small arrows indicate the distal tip of the migrating midbody lateral line primordium (H), the midbody lateral line (L), or a blood vessel subjacent to the midbody lateral line (F). Arrowheads indicate aggregates of xanthophores dorsal to the neural tube (G), the distal tip of the dorsal lateral line primordium (I), or the ventral lateral line (L). Clumps of DiI-labeled cells (K,L) are lateral line neuromasts. Scale bar: A–L, 500 μm.

FIG. 3. Development of the early larval pigment pattern in *T. torosa*. One representative embryo is shown at six stages. Times after the first appearance of melanophores are: (A, G) 15, (B, H) 39, (C, I) 57, (D, J) 69, (E, K) 78, and (F, L) 138 hr. See text for details and Fig. 2 for symbols and scale.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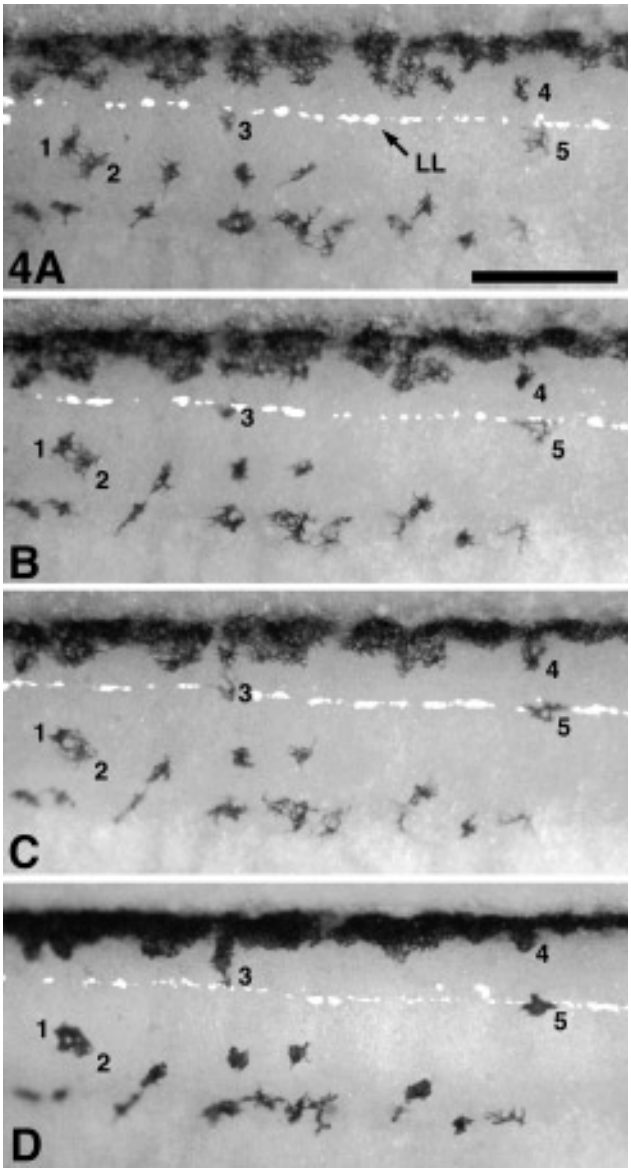


FIG. 4. Terminal stages of pigment pattern formation in a representative *T. torosa* larva. Times after the first appearance of melanophores are: (A) 90, (B) 102, (C) 114, and (D) 138 hr. In this individual, the midbody lateral line was only faintly labeled, and positions of DiI-labeled cells comprising the midbody lateral line nerve or neuromasts (LL) are superimposed in white on the brightfield image (originals recorded on color transparency film). Successive positions of five melanophores are indicated and anterior is to the right. Cells 1 and 2 became closely associated and remained in the middle of the flank, ventral to the midbody lateral line (A–D). Cell 3 was present initially in the path of the migrating primordium (not shown), but after contacting melanophores comprising the dorsal stripe (C), this cell moved further dorsally (D). Cell 4 was initially dorsal to the lateral line primordium (not shown), subsequently contacted cells further dorsally (B), and ultimately joined the dorsal stripe (C–D). Cell 5 moved slightly dorsally to associate with the lateral line nerve (A–D). Scale bar: 500 μm.

In regions of xanthophore dispersal, this often resulted in ''crossbars'' of melanophores immediately dorsal to the lateral line, spanning between adjacent anterior and posterior concentrations of melanophores (e.g., Figs. 2D and 2E). In contrast, xanthophores migrated ventrally between melanophores and traversed the lateral line to colonize the middle of the flank. During terminal formation of the early larval pattern (⩾B38), melanophores became increasingly arborized and rearrangements were not observed, though previously unidentified melanophores appeared (presumably due to proliferation of existing melanophores or *de novo* differentiation; Figs. 2E, 2F, 2K, and 2L). The ventral lateral line developed further ventrally than most melanophores (Fig. 2L). The definitive melanophore-free region (⩾B41; Figs. 2F and 2L) persists for as long as 3 months (Parichy, in preparation).

Pigment Pattern Formation and Lateral Line Development in T. torosa

In *T. torosa,* lightly pigmented melanophores were first visible in the anterior trunk and xanthophores were identified in this region 0–6 hr thereafter (T33; Figs. 3A and 3G). Both cell types were seen more posteriorly over the next 18–24 hr. Elongated melanophores and xanthophores moved in a dorsal-to-ventral direction and scattered relatively uniformly over the flank.

A subtle melanophore-free region was first apparent over the middle of the myotomes, but in contrast to *A. t. tigrinum,* a change in the distribution of melanophores was discernible at a given axial level 0–24 hr in advance of the midbody lateral line primordium (T35–36; Figs. 3B, 3C, 3H, and 3I). During initial formation of the melanophore-free region, melanophores either remained *in situ* at the dorsal apex of the myotomes or translocated from the middle of the flank to more dorsal regions. Melanophores typically were not observed contacting one another during these stages. A few melanophores became associated with the lateral line nerve (mean = 1.5 melanophores unilaterally/embryo, SD = 1.15, range = 0–4, n = 18 embryos; Fig. 4). Of 27 such melanophores, 25 moved in a ventral-to-dorsal direction to settle near the lateral line, and 2 were directly in the path of the primordium; melanophores did not approach the lateral line from dorsal regions.

During terminal formation of the melanophore-free region (T37–39; Figs. 3E and 3F), melanophores ventral to the apex of the myotomes frequently extended processes that contacted melanophores closer to the fin. These ventral cells then moved further dorsally (Fig. 4). Simultaneously, xanthophores interspersed among the melanophores shifted to more ventral regions over the myotomes (not shown). The dorsal concentration of melanophores gradually became more regular and compact, and individual melanophores could no longer be distinguished (Figs. 3F and 4D).

Dynamics of Melanophore Movements in A. t. tigrinum and T. torosa

I used low-magnification, time-lapse videos to compare further the overall dynamics of melanophore movements

in *A. t. tigrinum* and *T. torosa*. In *A. t. tigrinum*, gross melanophore movements were correlated with a dorsoventral and anteroposterior expansion of the flank (Fig. 5A). In *T. torosa*, however, melanophore movements often proceeded at rates and in directions that could not be accounted for by growth alone, and these movements were accompanied by active extension and retraction of cellular processes (Fig. 5B).

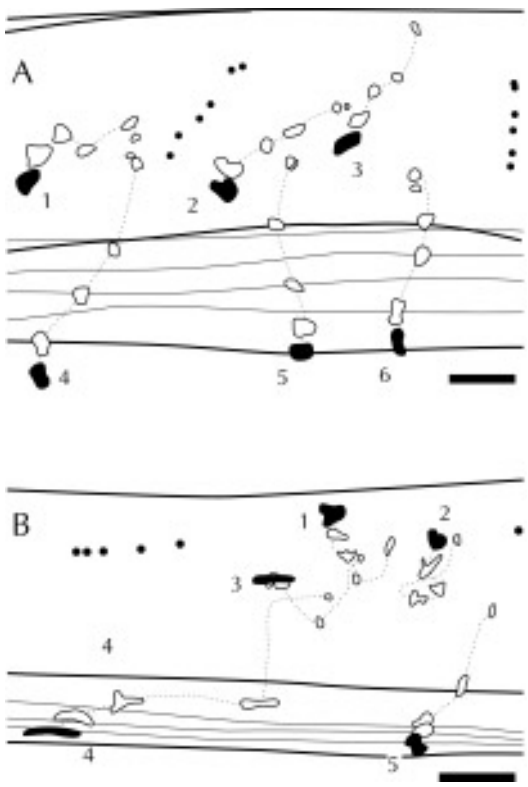


FIG. 5. Melanophore movements relative to growth of the flank during pigment pattern formation in *A. t. tigrinum* (A) and *T. torosa* (B). Final positions (closed cells) and intermediate positions (open cells) are shown for representative melanophores (additional melanophores are omitted for clarity). Thick lines indicate the base of the dorsal fin, or the ventral margin of the myotomes, at the beginning and end of recording. Light lines indicate successive positions of the ventral margin of the myotomes and black circles indicate successive positions of nonmotile landmarks implanted into the epidermis. Anterior is to the right. (A) In *A. t. tigrinum*, melanophore movements are generally correlated with growth of the flank over 54 hr (B36–40). Time intervals are 12 hr, except for the final time period, which is 6 hr. Cells 1–3 localized dorsal to the midbody lateral line (not shown), whereas cells 4–6 settled at the dorsal margin of the yolk mass. (B) In *T. torosa*, melanophore movements are not as well correlated with growth of the flank. Five cells are shown over 90 hr (T34–38). Time intervals are 24 hr, except for the final time period which is 18 hr. Cells 1 and 2 migrated ventrally from the sites at which they were first visible, then translocated actively in a ventral-to-dorsal direction to settle near the dorsal apex of the myotomes. Cell 3 moved ventrally then returned dorsally to localize in the middle of the flank along the midbody lateral line nerve. Cells 4 and 5 traveled ventrally and settled within the lateral stripe at the dorsal margin of the yolk mass. Scale bars: A,B, 200 μm.

Are the Lateral Lines Responsible for the Melanophore-Free Region in A. t. tigrinum?

To test whether the lateral lines are responsible for the melanophore-free region in *A. t. tigrinum*, I removed placode-area ectoderm unilaterally and grafted in its place belly epidermis (Fig. 1A). This procedure resulted in the absence of trunk lateral line primordia, nerves, and neuromasts (see below and Parichy, 1996b; in a few individuals, ventral lateral lines and a few scattered neuromasts developed, suggesting that not all placodal cells had been removed). Figure 6A shows that ablation of the lateral lines resulted in more uniform distributions of melanophores (sample sizes in Table 1). On lateral line-intact sides (Fig. 7A, upper panel), there were peaks in melanophore density dorsally (0.05–0.35 mm) near the base of the dorsal fin, as well as ventrally ($\geqslant$0.75 mm) near the yolk mass. A trough in melanophore density (0.45–0.65 mm) represented the melanophore-free region averaged across individuals. But on lateral line-ablated sides (Fig. 7A, lower panel), there were higher densities of melanophores in the middle of the flank and lower densities in dorsal regions. Lateral line ablation also resulted in a small but significant increase in total melanophore density (Table 1). Sham manipulations had only minor effects on melanophore distributions and did not affect total melanophore densities (Fig. 7I; Table 1). Thus, formation of the melanophore-free region in *A. t. tigrinum* depends on the lateral lines.

Are the Lateral Lines Responsible for the Melanophore-Free Region in T. torosa?

To test whether the lateral lines contribute to the melanophore-free region in *T. torosa*, I ablated the lateral lines in this taxon as well. Figures 6I and 6J show that ablation of the lateral lines did not perturb the horizontal stripe pattern. Prevention of lateral line development did not affect the height of the ''dorsal stripe,'' or the number of melanophores within the ''lateral stripe'' at the dorsal margin of the yolk mass, though there were slightly *fewer* melanophores between these stripes on sides without lateral lines (probably because cells that normally localize along the lateral line continued instead on a dorsal trajectory to join the dorsal stripe; Table 2). These results show that formation of the melanophore-free region in *T. torosa* does not depend on the lateral lines.

Phylogenetic Survey of Lateral Line Effects

The demonstration that the melanophore-free region in *A. t. tigrinum* depends on the lateral lines whereas the superficially similar melanophore-free region in *T. torosa* does not suggests several evolutionary scenarios. Melanophore-

TABLE 1
Effects of Lateral Line Ablation and Sham Manipulation on Total Melanophore Densities

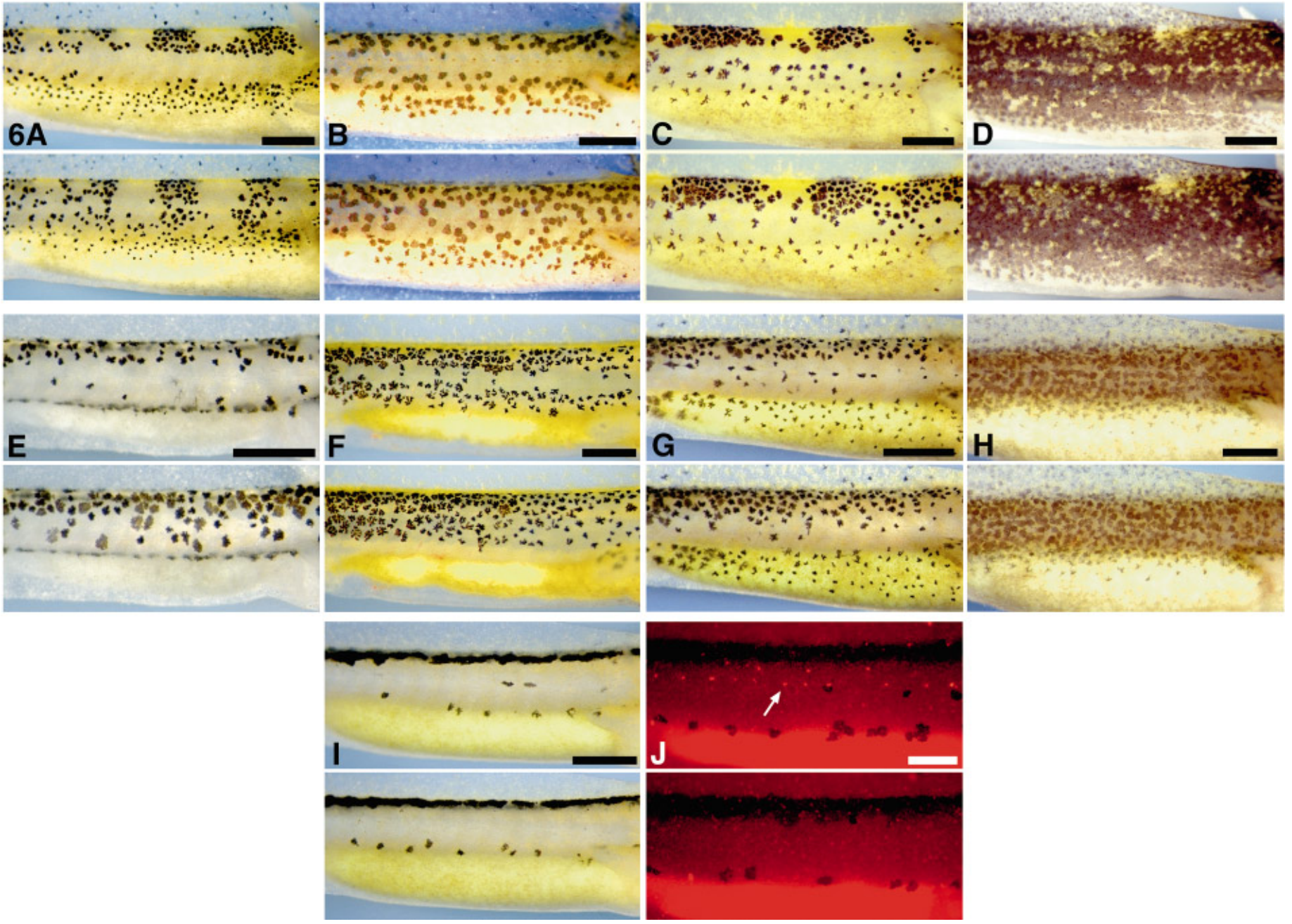
Experiment	Sample sizes: Embryos	Sample sizes: Cells	Total melanophore density (mean cells/mm + 95% CI)[a]: Unmanipulated sides	Total melanophore density (mean cells/mm + 95% CI)[a]: Manipulated sides	% Difference[b]	*t* (d.f.)[c]	*P*
Lateral line ablation[d]							
A. t. tigrinum	43	14,489	44.3 ± 2.97	47.6 ± 2.97	7.4	2.83 (42)	<0.01
A. maculatum	13	2,748	24.8 ± 5.69	28.3 ± 3.85	14.1	2.60 (12)	<0.05
A. talpoideum	15	2,854	43.1 ± 3.58	46.1 ± 3.79	7.0	1.93 (14)	0.08
A. barbouri	6[e]	1,562	196.0 ± 44.92	208.8 ± 26.45	6.5	0.58 (5)	0.59
P. waltl	12	1,759	28.2 ± 1.87	33.3 ± 2.26	18.1	5.79 (11)	<0.001
N. viridescens	5	1,034	55.9 ± 14.52	56.3 ± 18.92	0.7	0.14 (4)	0.90
T. granulosa	10	1,950	47.8 ± 4.15	48.7 ± 5.31	1.9	0.56 (9)	0.59
T. rivularis	8	2,112	76.6 ± 8.22	88.9 ± 10.59	16.1	3.78 (7)	<0.01
Sham manipulation							
A. t. tigrinum	15	4,021	42.2 ± 3.67	40.2 ± 3.36	−5.7	1.80 (14)	0.09
A. maculatum	9	1,130	19.1 ± 2.95	20.3 ± 3.56	6.3	1.16 (8)	0.28

[a] See Quantitative Methods.
[b] Calculated as: 100 × (density manipulated sides − density unmanipulated sides)/(density unmanipulated sides).
[c] Paired *t* test comparing unmanipulated and manipulated sides within individuals.
[d] Phylogenetic relationships in Fig. 10.
[e] Lateral line ablations were performed on 11 *A. barbouri* embryos, though positions of melanophores contributing to early larval pigment patterns could be quantitated for only 6 of these individuals.

free regions could have evolved convergently, via lateral line-dependent mechanisms in *A. t. tigrinum*, but via lateral line-independent mechanisms in *T. torosa*. Or, lateral line-dependent mechanisms could be ancestral for both taxa, but additional, lateral line-independent mechanisms have evolved in *T. torosa*. Finally, more complicated (and less parsimonious) scenarios also could be suggested: for example, lateral line-dependent mechanisms could be ancestral, but have been lost in *T. torosa* as lateral line-independent mechanisms evolved (see below). To evaluate these scenarios, I first tested whether lateral line effects could be a primitive feature of pigment pattern formation by ablating the lateral lines in seven additional taxa chosen from within the families Ambystomatidae and Salamandridae (Table 1; phylogenetic relationships in Fig. 10).

Among the ambystomatids, prevention of lateral line development in *A. maculatum* resulted in higher densities of melanophores in the middle of the flank (Figs. 6B and 7B), as in *A. t. tigrinum*. Sham manipulations in *A. maculatum* did not affect melanophore distributions (Fig. 7J). In *A. talpoideum*, ablation of the lateral lines similarly resulted in greater melanophore densities in the region normally subjacent to the midbody lateral line (ca. 0.35 mm; Figs. 6C and 7C). In *A. barbouri*, the melanophore-free region is very subtle. Nevertheless, melanophores more completely colonized the region normally occupied by the lateral line when the lateral lines were ablated (high melanophore densities typically precluded quantifying these effects; Figs. 6D and 7D). Among the salamandrids, prevention of lateral line development resulted in greater melanophore densities in the middle of the flank for both *P. waltl* (Figs. 6E and 7E) and *Notophthalmus viridescens* (Figs. 6F and 7F). Similarly in *T. granulosa*, ablation of the lateral lines yielded greater melanophore densities where the midbody lateral line normally is found (ca. 0.35 mm; Figs. 6G and 7G). Finally in *T. rivularis*, which again has a nearly uniform pattern, prevention of lateral line development nevertheless resulted in slightly greater densities of melanophores in the region

FIG. 6. Effects of lateral line ablation on early larval pigment patterns. For each taxon, opposite sides of a single representative embryo are shown, though one side has been reversed to facilitate comparison (anterior to the right). Upper panels are unmanipulated sides and lower panels are sides on which lateral line development has been prevented. (A) *A. t. tigrinum*. (B) *A. maculatum*. (C) *A. talpoideum*. (D) *A. barbouri*. (E) *P. waltl*. (F) *N. viridescens*. (G) *T. granulosa*. (H) *T. rivularis*. (I) *T. torosa*. (J) *T. torosa*, stained with DASPEI, which reveals hair cells of lateral line neuromasts on unoperated (upper) but not lateral line-ablated sides (lower). Subtle melanophore-free regions associated with the ventral lateral line are visible over the anterior yolk mass in *A. maculatum* and *A. barbouri* (top panels in B and D, respectively). Scale bars: A–I, 800 μm; J, 500 μm.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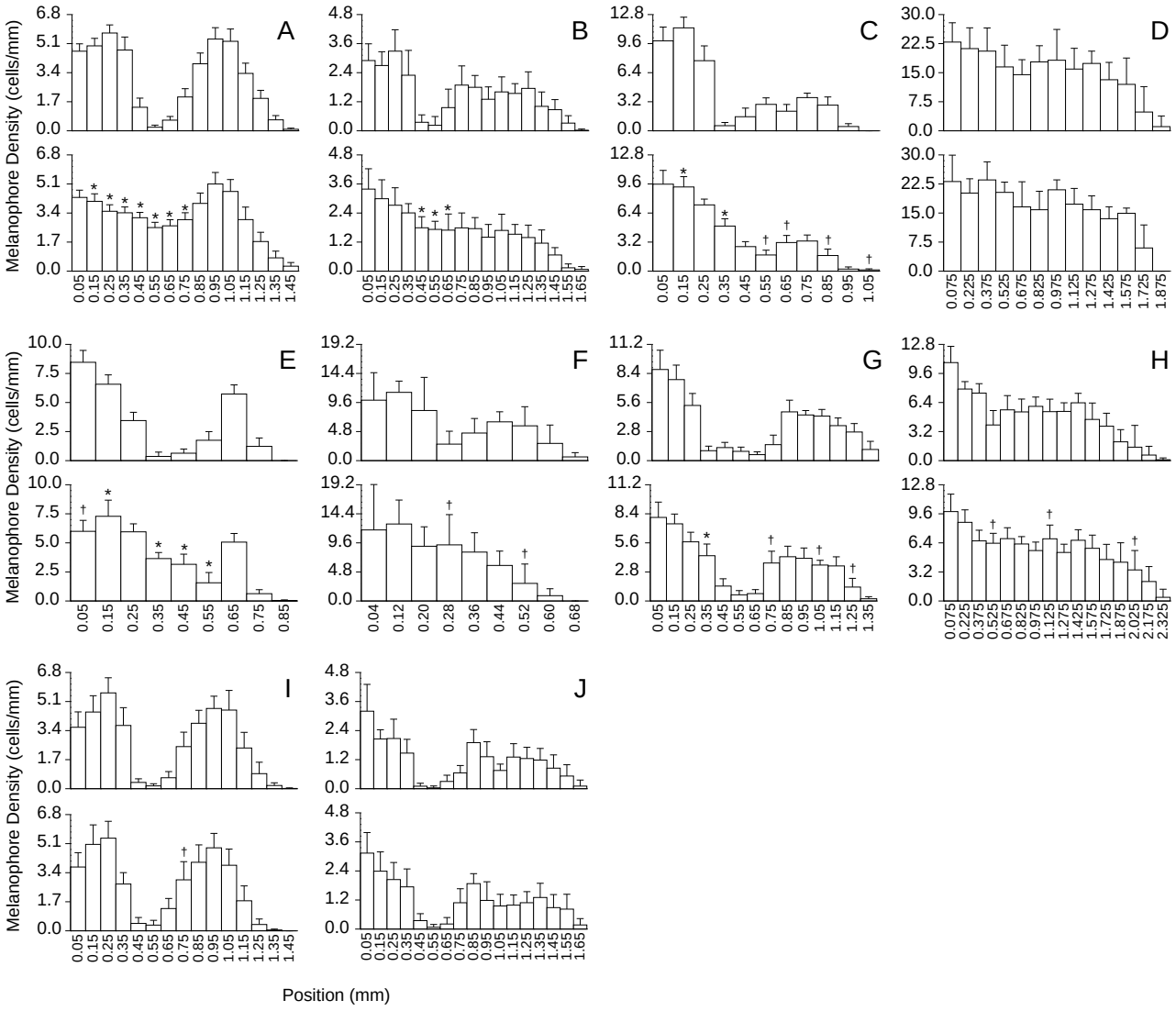


FIG. 7. Melanophore distributions following lateral line ablation (A–H) or sham manipulation (I–J). Each plot shows the mean densities of melanophores (±95% confidence intervals) at different dorsoventral positions on the flank. Positions are measured relative to the base of the dorsal fin and distances along the abscissas are midpoints of each position (see Quantitative Methods and Results). Upper plots are unmanipulated sides and lower plots are the corresponding, manipulated sides of the same individuals. See Table 1 for sample sizes. Lateral line ablations: (A) *A. t. tigrinum*, (B) *A. maculatum*, (C) *A. talpoideum*, (D) *A. barbouri*, (E) *P. waltl*, (F) *N. viridescens*, (G) *T. granulosa*, (H) *T. rivularis*. Sham manipulations: (I) *A. t. tigrinum*, (J) *A. maculatum*. At each position, melanophore densities are compared between unoperated and operated sides of individuals using paired *t* tests. To ensure conservative hypothesis tests given that multiple tests were performed within each taxon, significance levels were reduced according to the sequential Bonferonni procedure (with the number of tests for each species defined as the number of positions compared; see Rice, 1989). Asterisks denote positions in which densities on unoperated and operated sides differ at a significance level equivalent to $P < 0.05$ using the Bonferonni procedure; daggers indicate positions in which densities differ at the $P < 0.05$ level only without correcting for multiple comparisons (note however that such differences were found consistently at the level normally occupied by the lateral line; see text). Digitized images of *P. waltl* typically did not permit distinguishing between melanophores that were either immediately beneath the epidermis or over the peritoneum. Consequently, all visible melanophores were included and calculated melanophore densities at the border between the ventral edge of the myotomes and the dorsal edge of the yolk mass (ca. 0.65 mm) in E slightly overestimate actual densities beneath the epidermis on both unoperated and operated sides.

TABLE 2
Effects of Lateral Line Ablation on Melanophore Distributions in *T. torosa*[a]

	Treatment			
	Unmanipulated sides (mean ± 95% CI)	Lateral line-ablated sides (mean ± 95% CI)	t_{28}[b]	*P*
Height of dorsal melanophore stripe (μm)[c]	122 ± 6.1	124 ± 9.6	0.55	0.6
Melanophores within lateral stripe (No. cells)	6.3 ± 1.12	6.5 ± 1.47	0.50	0.6
Melanophores between dorsal and lateral stripes (No. cells)	3.7 ± 0.93	2.6 ± 0.58	2.90	<0.01[d]

[a] Lateral line ablations were performed unilaterally on 29 *T. torosa* embryos.
[b] Paired *t* test comparing unmanipulated and lateral line-ablated sides within individuals.
[c] See Quantitative Methods.
[d] Sham manipulations did not affect the number of melanophores between the dorsal and lateral stripes. (paired $t = 0.63$, d.f. = 9, $P = 0.5$; $n = 10$ larvae).

normally subjacent to the midbody lateral line (ca. 0.525 mm; Figs. 6H and 7H). Ablation of the lateral lines also resulted in greater total melanophore densities in *A. maculatum*, *P. waltl*, and *T. rivularis* (Table 1).

These experiments demonstrate that the lateral lines can influence melanophore distributions in both ambystomatids and salamandrids, even in taxa that lack distinctive melanophore-free regions and horizontal stripe patterns. This suggests that lateral line-dependent pattern-forming mechanisms are primitive within the families Ambystomatidae and Salamandridae and probably were present in the common ancestor of these taxa.

Does T. torosa Retain the Potential for Lateral Line Effects on Melanophores?

The inference that lateral line effects are primitive suggests two scenarios for the evolution of pattern-forming mechanisms in *T. torosa:* ancestral lateral line-dependent mechanisms could have been retained as redundant, lateral line-independent mechanisms evolved; or, ancestral mechanisms could have been lost and replaced by new mechanisms. To distinguish between these scenarios I tested the behavior of *T. torosa* cells in *A. t. tigrinum* hosts. If *T. torosa* retains the potential for generating a melanophore-free region through lateral line-dependent mechanisms, this implies that: (i) *T. torosa* lateral lines must be competent to generate a melanophore-free region; and (ii) *T. torosa* melanophores must be competent to respond to cues provided by the lateral lines. If either or both of these behaviors is not observed, this would be consistent with the loss of the ancestral, lateral line-dependent mechanisms.

T. torosa lateral line placodes grafted to *A. t. tigrinum* hosts (Fig. 1B; $n = 17$) produced lateral line primordia that migrated ca. one stage later than normal (presumably due to the slower developmental rate of *T. torosa*). Nevertheless, melanophore-free regions were observed in the immediate vicinity of the donor primordia (Fig. 8A). These regions subsequently remained free of melanophores and were populated by xanthophores (Figs. 8B and 8C). Melanophore-free regions generated by *T. torosa* lateral lines resembled those that form when lateral line development is delayed experimentally in *A. t. tigrinum* (not shown).

T. torosa melanophores grafted to *A. t. tigrinum* hosts (Fig. 1C) were found principally over the dorsal flank on sides with intact lateral lines (Figs. 9A, 9C, and 9D). These cells failed to organize into the compact, dorsal stripes seen normally in *T. torosa*, and instead formed a loose meshwork in which processes of adjacent melanophores frequently contacted one another (Figs. 9G and 9H). Moreover, *T. torosa* melanophores behaved in a manner parallel to melanophores of other taxa when the lateral lines were ablated: on sides without lateral lines, donor melanophores more completely colonized the middle of the flank (Wilcoxon matched pairs signed ranks test, $P < 0.0001$; $n = 42$ chimeras; Figs. 9B, 9E, and 9F).

These two experiments demonstrate that *T. torosa* lateral lines are competent to produce a melanophore-free region, and *T. torosa* melanophores are competent to respond to cues provided by the lateral lines. Thus, *T. torosa* retains the potential for lateral line effects on melanophores.

DISCUSSION

A distinctive element of the early larval pigment pattern in many salamanders is a region over the middle of the myotomes that is relatively free of melanophores (Parichy, 1996a). Because melanophores typically are found dorsally and ventrally, the presence of a ''melanophore-free region'' results in a horizontal stripe pattern. This study shows that: (i) the lateral line sensory system can influence melano-

phore distributions and can contribute to melanophore-free regions (and hence horizontal stripes) in both the family Ambystomatidae and the family Salamandridae; (ii) lateral line-dependent pattern-forming mechanisms are probably ancestral for both of these families; and (iii) the melanophore-free region in *T. torosa* arises independently of the lateral lines, though this taxon exhibits the potential for lateral line effects, suggesting that ancestral mechanisms have been retained as redundant, lateral line-independent mechanisms have evolved (Fig. 10).

A Lateral Line-Dependent Pattern-Forming Mechanism

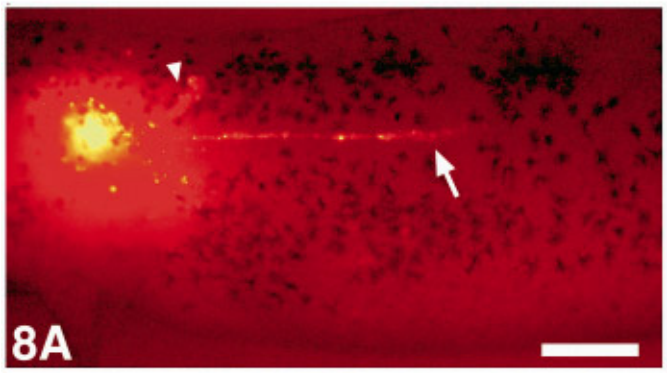
Photographic series showed that in *A. t. tigrinum*, melanophores disperse evenly over the somites initially and a subtle melanophore-free region subsequently appears in the vicinity of the migrating midbody lateral line primordium. During later development, the melanophore-free region is maintained and becomes increasingly distinctive. When the lateral lines were ablated, however, more uniform distributions of melanophores were found, essentially eliminating the melanophore-free region in *A. t. tigrinum*, and having similar or more subtle effects in *A. maculatum*, *A. talpoideum*, *A. barbouri*, *P. waltl*, *N. viridescens*, *T. granulosa*, and *T. rivularis*. These results demonstrate that lateral line-dependent mechanisms can contribute to melanophore-free regions, and in some taxa, lateral line effects can be the primary determinants of a horizontal stripe pattern.

The lateral lines appear to generate a melanophore-free region principally by affecting the localization of melanophores that are already present over the myotomes. Photographic series revealed that *A. t. tigrinum* melanophores moved short distances dorsally or ventrally when approached by the midbody lateral line primordium, and typically failed to cross the primordium or lateral line nerve during later development (also see Parichy, 1996b). Similar events are observed in *A. maculatum*, *A. talpoideum*, *P. waltl*, *N. viridescens*, and *T. granulosa*, and time-lapse videos of *A. t. tigrinum* show dorsal melanophores migrating further ventrally when the lateral lines are ablated (unpublished data). The exclusion of melanophores from the middle of the flank is hypothesized to result initially from physical effects of the lateral lines on melanophores and the subepidermal basement membrane (Parichy, 1996b).

In addition to affecting melanophore localization, failure of lateral line development significantly increased the total densities of melanophores in *A. t. tigrinum*, *A. maculatum*, *P. waltl*, and *T. rivularis*. This suggests that the lateral lines also can repress the absolute numbers of melanophores that contribute to the early larval pattern, perhaps by releasing inhibitory factors (e.g., Stocker *et al.*, 1991; Thibaudeau and Frost-Mason, 1992; Fukuzawa *et al.*, 1995) or exhausting local supplies of trophic factors or mitogens (e.g., Frost-Mason *et al.*, 1992; Sherman *et al.*, 1993). For example, murine melanoblasts express the growth factor receptor c-*kit* and ectopic expression of c-*kit* inhibits melanoblast proliferation and/or survival, presumably because the c-kit ligand is sequestered competitively (Duttlinger *et al.*, 1993; Wehrle-Haller and Weston, 1995). Since *Xenopus laevis* lateral lines express a c-*kit*-like receptor (Baker *et al.*, 1995), melanoblasts might compete with the lateral lines for c-kit ligand in salamanders with lateral line-dependent patterns. Alternatively, effects of the lateral lines could be less direct. For instance, if the lateral lines reduce the area over the myotomes suitable for melanophore colonization, higher local melanophore densities (e.g., in dorsal regions), could inhibit further proliferation or the continued entry of cells onto the flank. Additional studies will be needed to distinguish among these possibilities. Taken together, however, these findings and those presented in Parichy (1996b) indicate that the lateral lines must be counted among other tissues that influence the morphogenesis or differentiation of neural crest cells and their derivatives (e.g., Twitty, 1936; Tucker and Erickson,1986a,b; Epperlein and Löfberg, 1990; Bronner-Fraser and Stern, 1991; Oakley *et al.*, 1994; Spence and Poole, 1994; Tosney *et al.*, 1994). These results also suggest caution when inferring patterning roles for somitic mesoderm or epidermis, since Smith *et al.* (1990) have shown that surgical manipulations of these tissues can perturb lateral line development, and grafts of epidermis could include lateral line primordia, nerves, or neuromasts.

FIG. 8. *T. torosa* lateral line placodes grafted to *A. t. tigrinum* hosts (anterior to the left). (A) Migration of the DiI-labeled *T. torosa* dorsal (arrowhead) and midbody (arrow) lateral line primordia, and establishment of a subtle melanophore-free region in the middle of the flank. (B) Melanophore-free region generated by *T. torosa* lateral lines in the early larval pattern. Outlined area shown in C. (C) Epifluorescence illumination reveals xanthophores and the donor, DiI-labeled midbody lateral line (arrow). Scale bars: A, B, 500 μm; C, 200 μm.

FIG. 9. Behavior of *T. torosa* melanophores in *A. t. tigrinum* hosts. (A,C,D) Lateral line-intact sides. (B,E,F) Lateral line-ablated sides. (A) *T. torosa* melanophores do not form normal compact stripes even on unmanipulated sides of *A. t. tigrinum* hosts. Rounder *A. t. tigrinum* melanophores are visible anteriorly (left side of panel). (B) On the opposite side of the same individual, *T. torosa* melanophores more completely colonize the middle of the flank in the absence of lateral lines. (C,D) Higher magnification brightfield and epifluorescence views of *T. torosa* melanophores on the lateral line-intact side of a different chimera. (E,F) Opposite, lateral line-ablated side of the same individual. Arrow indicates unlabeled *A. t. tigrinum* melanophores. (G) Detail of *T. torosa* melanophores in close contact with one another. (H) Epifluorescence double exposure revealing differential labeling of melanophores as well as a previously cryptic, DiI-labeled xanthophore. Scale bars: A, B, 500 μm; C–F, 200 μm; G, H, 200 μm.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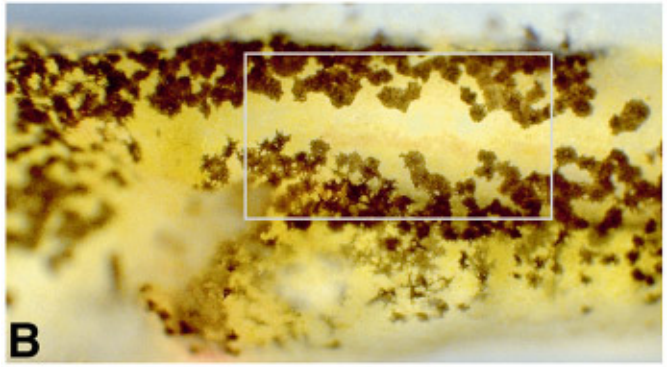
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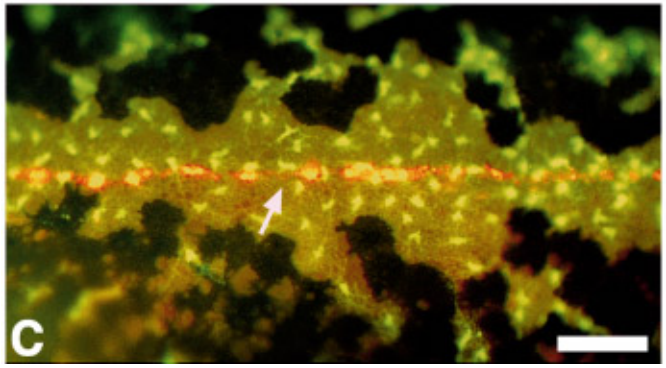
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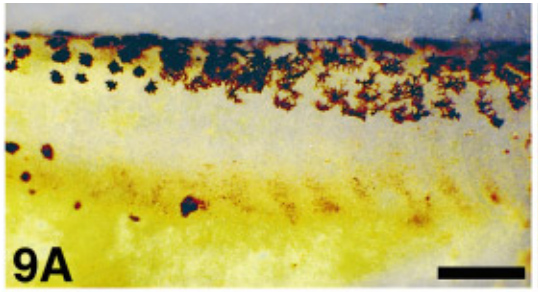
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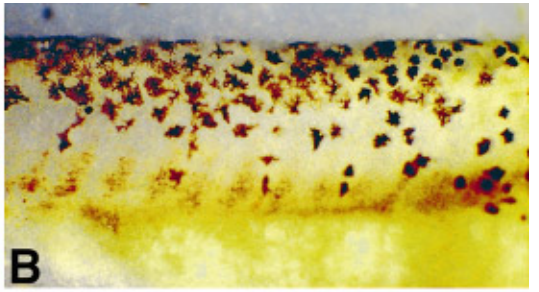
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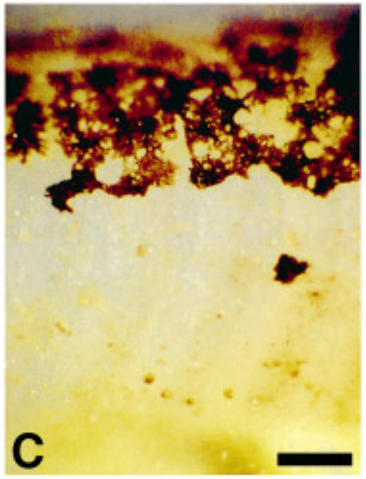
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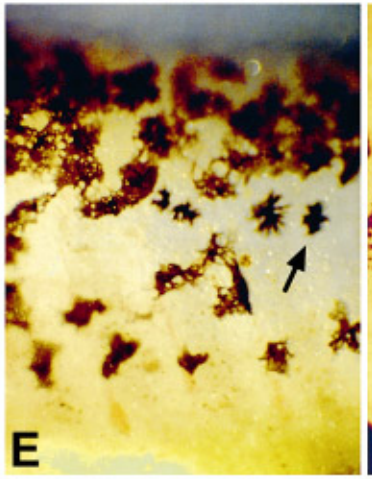
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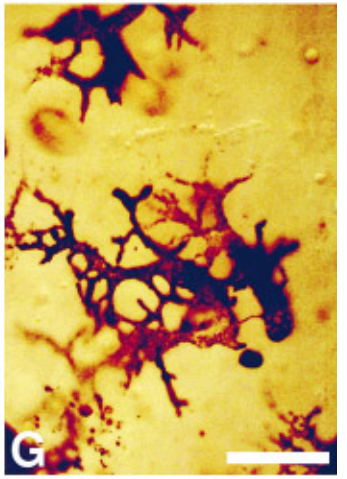
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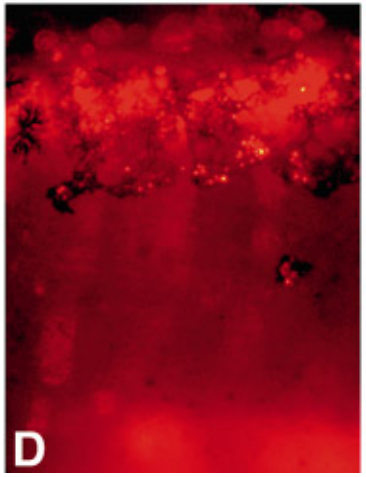
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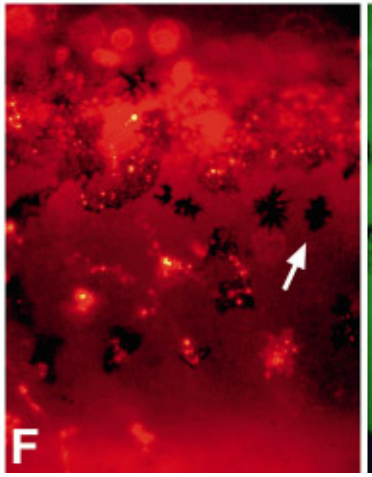
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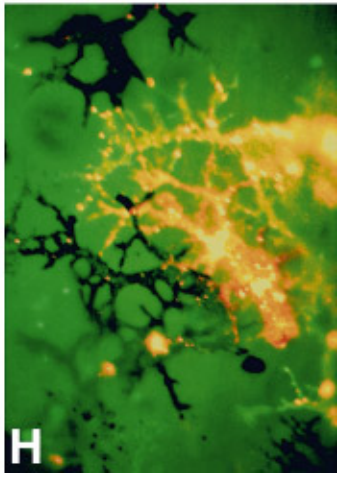
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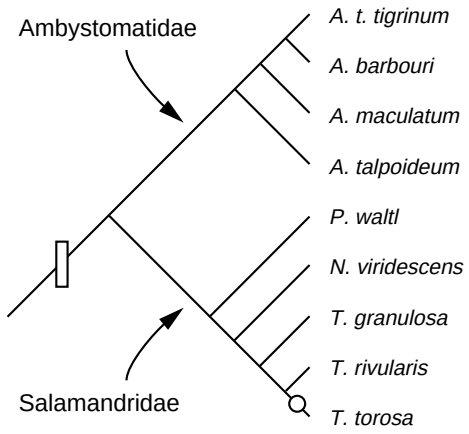

FIG. 10. Phylogenetic relationships and the inferred evolution of pattern-forming mechanisms. Only taxa examined in the present study are shown. The open rectangle at the base of the tree represents primitive lateral line-dependent pattern-forming mechanisms present in the common ancestor of the families Ambystomatidae and Salamandridae. The open circle along the branch leading to *T. torosa* indicates the evolution of redundant, lateral line-independent mechanisms for stripe formation. The hypothesis of phylogenetic relationships is based on several independent studies that used characters other than pigment patterns (Wake and Özeti, 1969; Shaffer *et al.*, 1991; Larson and Dimmick, 1993; Titus and Larson, 1995; Shaffer, personal communication).

Comparative and Evolutionary Aspects of Lateral Line Effects and Melanophore-Free Regions

Ablation of the lateral lines had qualitatively similar effects in all of the taxa with lateral line-dependent melanophore-free regions, but the magnitude of these effects varied considerably. For instance, failure of lateral line development drastically perturbed the pigment pattern in *A. t. tigrinum*, but had only subtle effects in *A. talpoideum* and *A. barbouri*. Such differences across taxa are probably explained in part by corresponding differences in the total numbers or ratios of melanophores and xanthophores. For example, lower densities of melanophores in *A. talpoideum* could be insufficient to drive melanophores further ventrally even in the absence of lateral lines; higher densities in *A. barbouri* could force melanophores to settle in close proximity to the lateral lines (Rovasio *et al.*, 1983; Erickson, 1985; Tucker and Erickson, 1986a). In either instance, only subtle effects of lateral line ablation would be apparent. Unfortunately, direct comparisons of cell densities and proportions across taxa are not readily interpretable because of interspecific differences in embryo and chromatophore sizes, and the difficulty of precisely homologizing stages across taxa (unpublished data). Other factors that could modulate lateral line effects are the sizes of lateral line primordia relative to melanophores and the height of the flank; differences in the timing or extent of flank growth (Rosin, 1943; Parichy, 1996b); and the presence of additional cues for chromatophore localization (Twitty, 1945; Tucker and Erickson, 1986a,b; Epperlein and Löfberg, 1990; see below).

The results of this study suggest that lateral line-dependent pattern-forming mechanisms are a shared, ancestral feature of pigment pattern development for the families Ambystomatidae and Salamandridae. The lateral lines had similar effects on melanophore distributions in eight of nine ambystomatid and salamandrid taxa examined, and the potential for such effects was demonstrated in the remaining taxon (*T. torosa;* see below). Moreover, the taxa chosen for this study are a broad phylogenetic spectrum: the ambystomatids represent several distinct monophyletic groups within the single genus *Ambystoma* that comprises the Ambystomatidae (Kraus, 1988; Shaffer *et al.*, 1991; Jones *et al.*, 1993; Reilly and Brandon, 1994); and the split between the European genus *Pleurodeles* and the North American genera *Taricha* and *Notophthalmus* was probably an ancient divergence within the Salamandridae (Wake and Özeti, 1969; Titus and Larson, 1995). Since recent investigations (Larson, 1991; Larson and Dimmick, 1993) reveal a monophyletic group consisting of the families ((Salamandridae) (Dicamptadontidae, Ambystomatidae)), the data presented here imply most parsimoniously that lateral line effects on melanophores were present in the common ancestor of these families, 65–200 Myr BP (Larson, 1991). Once lateral line-dependent mechanisms had appeared, the distinctiveness of melanophore-free regions presumably could have been modified by evolutionary adjustments to additional factors that contribute to pigment pattern formation (e.g., chromatophore numbers). Such alterations apparently can occur rather rapidly since *A. tigrinum tigrinum* displays a distinctive melanophore-free region, yet several other subspecies of *A. tigrinum* do not (Parichy, 1996a), and all probably diverged within the last 0.02–5 Myr (Shaffer and McKnight, 1996; Shaffer, personal communication). Likewise, different taxa (and patterns) within *Taricha* are believed to have evolved during the Pliocene, 3–6 Myr BP (see Riemer, 1958). The demonstration of lateral line effects on melanophores also suggests that lateral line-dependent melanophore-free regions could be viewed simply as epiphenomena of lateral line development, which might or might not contribute to functions of the overall patterns, like predator avoidance (Endler, 1978). Thus, distinctive lateral line-dependent melanophore-free regions (and hence horizontal stripes) could be products of selection on the pigment patterns, or could represent correlated responses to selection (Price and Langen, 1992) on characters associated with the lateral lines.

Pattern-Forming Mechanisms in T. torosa

In *T. torosa*, melanophores first scatter widely over the somites, but most of these cells then segregate to form a distinctive melanophore-free region, superficially similar to that of *A. t. tigrinum* (also see Twitty, 1936, 1944, 1945; Twitty and Bodenstein, 1939; Twitty and Niu, 1948; Tucker and Erickson, 1986a). Nevertheless, the melanophore-free

region in *T. torosa* develops in advance of the midbody lateral line primordium, and—unlike the other taxa examined—ablation of the lateral lines failed to perturb the melanophore-free region or horizontal stripe pattern. These results indicate that not all stripes are equivalent developmentally and add to a growing list of examples in which different underlying mechanisms lead to similar or identical phenotypes (Hall, 1992; Wagner and Misof, 1993).

If lateral line effects on melanophores are primitive, how have pattern-forming mechanisms evolved in *T. torosa*? Heterospecific grafting experiments suggest that the ancestral mechanisms have been retained in a latent form: when grafted to *A. t. tigrinum* hosts, *T. torosa* lateral lines were competent to generate a melanophore-free region, whereas *T. torosa* melanophores were competent to respond to cues provided by the lateral lines. *T. torosa* thus exhibits the potential for lateral line effects on melanophores. In turn, the failure of lateral line ablation to perturb the melanophore-free region *in situ* suggests that redundant lateral line-independent mechanisms have been layered over primitive lateral line-dependent mechanisms. By effectively decoupling the melanophore-free region from the lateral lines, redundant mechanisms could provide a more canalized (i.e., developmentally buffered; Waddington, 1952) horizontal stripe pattern, perhaps reflecting the evolutionary acquisition of novel functional and selective advantages (see Riska, 1986). Moreover, a preexisting lateral line-dependent melanophore-free region might have biased available phenotypic variation so as to favor the evolution of present-day, lateral line-independent stripes over other selectively equivalent patterns.

Finally, these results also provide insights into lateral line-independent mechanisms in *T. torosa*. Stripes have been suggested to form because melanophores translocate in a ventral-to-dorsal direction toward the dorsal apex of the myotomes (Tucker and Erickson, 1986a) or because melanophores are pulled passively as processes connecting these cells are retracted (Twitty, 1945). Yet, these earlier studies did not control for growth, which could result in passive displacements of melanophores away from the middle of the flank. Here, analyses of melanophore movements using native and surgically implanted landmarks confirmed the existence of active ventral-to-dorsal melanophore movements, but neither photographic series nor time-lapse videos revealed stable interconnections between these cells during establishment of the melanophore-free region (though only melanin-containing processes would have been visible). This suggests that melanophores could translocate dorsally initially in response to chemotactic or haptotactic cues. During terminal stages of pigment pattern formation, however, these analyses showed that *T. torosa* melanophores often form persistent contacts with one another. If these contacts reflect adhesive interactions between ventral melanophores and melanophores already ''anchored'' dorsally, they could be an additional factor contributing to melanophore localization. Adhesive interactions among migrating cells have been suggested to contribute to the morphogenesis of primordial germ cells (Gomperts *et al.*, 1994) and neural crest cells contributing to the peripheral nervous system (Nakagawa and Takeichi, 1995; also see Krull *et al.*, 1995). Consistent with this idea in *T. torosa*, cultured melanophores can aggregate (Twitty, 1945) and can exhibit intercellular junctions (Tucker and Erickson, 1986b), and chromatophores of other taxa express a variety of adhesion molecules that could mediate such interactions (Qian *et al.*, 1994; Tang *et al.*, 1994; Fukuzawa and Obika, 1995).

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