

Experimental Analysis of Character Coupling Across a Complex Life Cycle: Pigment Pattern Metamorphosis in the Tiger Salamander, *Ambystoma tigrinum tigrinum*

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ABSTRACT Developmental relationships among characters are expected to bias patterns of morphological variation at the population level. Studies of character development thus can provide insights into processes of adaptation and the evolutionary diversification of morphologies. Here I use experimental manipulations to test whether larval and adult pigment patterns are coupled across metamorphosis in the tiger salamander, *Ambystoma tigrinum tigrinum* (Ambystomatidae). Previous investigations showed that the early larval pigment pattern depends on interactions between pigment cells and the lateral line sensory system. In contrast, the results of this study demonstrate that the major features of the adult pigment pattern develop largely independently of both the early larval pattern and the lateral lines. These results suggest that ontogenetic changes that occur across metamorphosis decouple larval and adult pigment patterns and could thereby facilitate independent evolutionary modifications to the patterns during different stages of the life cycle. *J. Morphol.* 237:53–67, 1998. © 1998 Wiley-Liss, Inc.

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Knowledge of the developmental relationships among characters is essential for a complete understanding of morphological variation. This is because the hierarchical and interactive nature of development makes some combinations of states across characters more likely than others. For example, when several characters depend on the same genes, mutations affecting those genes often have pleiotropic effects (Wright, '68). Similarly, when cells that give rise to different characters interact during development, mutations that influence the behavior of one cell population can have cascading, indirect effects on the others (Atchley and Hall, '91). Such biases on the generation of variant phenotypes (or "developmental constraints" [Maynard Smith et al., '85]) can contribute to genetic and phenotypic correlations at the population level (Riedl, '78; Cheverud, '84, '95; Riska, '86; Kingsolver and Wiernasz, '91; Arnold, '92; Cowley and Atchley, '92; but see Gromko, '95) and may influence the direction or rate of morphological evolution (Alberch, '80; Oster et al., '88; Wake, '91; Shubin et al., '95; also see Charlesworth et al.,

'82). For instance, selection on one character may lead to the correlated evolution of other developmentally related characters that are expressed at the same stage of ontogeny (Falconer, '89; Riska, '89; Price and Langen, '92; Hanken and Wake, '93; Price and Pavelka, '96).

Developmental relationships among characters also may affect the evolution of phenotypes across stages. Indeed, a character that is expressed at different stages can be viewed as a set of interdependent characters that share some or all of their developmental determinants (Atchley and Hall, '91; Cowley and Atchley, '92). Selection favoring a particular character state during one stage thus can result in the correlated evolution of the same character or developmentally related

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characters at other stages, such that phenotypes expressed in adults may reflect selection at preadult stages or the reverse (Riska and Atchley, '85; Slatkin, '87; Ebenman, '92). Alternatively, selection may favor developmentally incompatible character states across stages. For example, Price and Grant ('84) observed selection for small body size among juvenile finches but selection for large body size among adults (also see Clegg et al., '78; Roach, '86; Kaplan, '92; Chippindale et al., '96). In such instances, evolutionary change may be slow at both stages, or adaptive modifications during one stage may be accompanied by maladaptive, correlated responses at the other stage (Arnold, '92; Ebenman, '92; Price and Langen, '92; Kirkpatrick and Lofsvold, '92; Chippindale et al., '96).

A potential solution to the problem of differing and possibly conflicting selection across stages is the evolution of a complex life cycle, in which individuals undergo a metamorphosis one or more times during development. Complex life cycles and their associated metamorphoses are present in the majority of animals and are most often viewed as a means for decoupling traits that are expressed during different stages, thereby permitting independent adaptations to different environments and selective regimes (Haldane, '32; Szarski, '57; Istock, '67; Wassersug, '75; Wilbur, '80; Werner, '88; Ebenman '92; Moran, '94)¹. Support for this hypothesis of decoupling across life cycle stages comes from two principal sources.

First, closely related taxa often resemble each other at one life cycle stage but differ greatly at another (de Beer, '58; Strathman, '78; Williamson, '82; Wray, '92, '96; also see Wake, '89; Wake and Hanken, '96), suggesting that independent modifications have occurred within stages without affecting phenotypes across stages. Such observations provide retrospective evidence that pre- and postmetamorphic characters may be uncoupled from one another. Yet these studies provide relatively little insight into the extent of developmental coupling across life cycle stages for other characters in other extant taxa, particularly since the degree of morphological reorganization at metamor-

phosis varies among phylogenetic lineages (e.g., salamanders change relatively little at metamorphosis compared to frogs [Wassersug and Hoff, '82; Duellman and Trueb, '86; also see Shaffer and Lauder, '88; Ashley et al., '91]).

Second, intraspecific descriptive studies often reveal dramatic changes in morphology, physiology, and behavior at metamorphosis (Gilbert and Frieden, '81; Balls and Bownes, '83; Gilbert et al., '96). This suggests intuitively that pre- and postmetamorphic character states may be independent of one another. For example, metamorphosing amphibians undergo a host of changes in the craniofacial skeleton, skin, pigmentation, gastrointestinal tract, immune system, and other characters (Wilder, '25; Noble, '31; Gilbert and Frieden, '81; Fox, '84; Duellman and Trueb, '86; Reilly and Lauder, '90; Shaffer et al., '91; Rose and Reiss, '93; Hourdry et al., '96). Yet descriptive approaches often cannot provide strong evidence for or against developmental coupling. For instance, characters that look very different and thus might appear superficially to be uncoupled may nevertheless covary if they share common underlying developmental genetic mechanisms (e.g., Besmer et al., '93; Ingham, '95; van Eeden et al., '96). Indeed, many of the same genes involved in patterning during embryogenesis are reexpressed during the development of adult tissues, and results of laboratory studies suggest that naturally occurring mutations may affect both pre- and postmetamorphic characters (e.g., Shellenbarger and Mohler, '78; Brabant and Brower, '93; Patterson et al., '95; Ranganayakulu et al., '95; Rauskolb et al., '95; Stolow and Shi, '95).

More definitive evidence concerning developmental coupling across stages of complex life cycles can be gained from quantitative genetic or experimental approaches. These strategies have been employed in studies of life history characters (e.g., Blouin, '92; Emlet and Hoegh-Guldberg, '97), but they typically have not been used to assess the extent of developmental coupling between discrete pre- and postmetamorphic morphological characters.

In the present study, I test experimentally whether pigment patterns are coupled across metamorphosis in the Eastern tiger salamander, *Ambystoma tigrinum tigrinum* (family Ambystomatidae), a species in which individuals hatch as aquatic larvae, grow

¹Developmental coupling of trait expression is an alternative and less frequently considered explanation for the evolutionary persistence of complex life cycles in some phylogenetic lineages (e.g., a larval stage might be maintained even in the face of selection for its loss if larval morphology is a necessary precursor to adult morphology [for a fuller discussion see Moran, '94]).

rapidly, and then metamorphose into terrestrial adults. Pigment patterns have been used to define species and subspecies comprising the geographically wide-ranging *Ambystoma tigrinum*—complex of salamanders (Dunn, '40; Gehlbach, '67; also see Shaffer, '93; Shaffer and McKnight, '96). These patterns are likely to be functionally important: they are distinctive at hatching, when salamanders are especially vulnerable to predation (Stine et al., '54; Anderson et al., '71; Kusano et al., '85), and pigment patterns of adults could be cryptic or aposematic depending on ecological context (Carpenter, '55; Norris and Lowe, '64; Hensel and Brodie, '76; Endler, '78; Collins et al., '80). Previous studies showed that a major element of the early larval pattern depends on interactions between migrating pigment cells and the lateral lines (see below), a bilateral sensory system that detects mechanical stimuli via periodically arranged neuromasts and functions in orientation, feeding, and predator avoidance (Wright, '51; Atema et al., '88; Blaxter and Fuiman, '90; Montgomery et al., '97). Here I use microsurgical manipulations to test whether larval and adult pigment patterns of *A. t. tigrinum* are coupled developmentally. If patterns are coupled across life cycle stages, an analysis of the evolutionary origins and adaptive significance of the adult pattern would have to consider not only selection occurring after metamorphosis but also selection occurring in the aquatic, larval environment.

DEVELOPMENTAL BACKGROUND

Ectothermic vertebrates possess three principal types of pigment cells, or chromatophores: black melanophores, yellow xanthophores, and silvery iridophores (DuShane, '43; Erickson, '93; Frost-Mason et al., '95). All are derived from neural crest cells, which arise along the dorsal neural tube shortly after neurulation and then disperse throughout the embryo. Neural crest cells also contribute to the teeth, craniofacial skeleton, peripheral nervous system, heart, endocrine glands, fin mesenchyme, and other characters (Hall and Hörstadius, '88; Selleck et al., '93).

Early larval pigment patterns

The pigment patterns of salamanders shortly after hatching consist primarily of melanophores and xanthophores. A prominent feature of the early larval pattern in

A. t. tigrinum (and many other salamanders in Ambystomatidae and Salamandridae) is a region over the lateral face of the myotomes where xanthophores are abundant but melanophores typically are not found (Parichy, '96a). Since melanophores occur further dorsally and ventrally, the overall pattern consists of a pale yellow horizontal stripe bordered by dark dorsal and ventral stripes. In *A. t. tigrinum*, formation of the early larval pattern begins with melanophores scattering evenly over the myotomes. Xanthophores are confined initially to aggregates dorsal to the neural tube, but these cells then disperse, and as they do so adjacent melanophores recede short distances to form a series of alternating vertical bars over the dorsal flank (Fig. 1) (also see Olsson and Löfberg, '92). Simultaneously, the trunk midbody lateral line begins to develop through the deployment of a migrating lateral line "primordium," which causes melanophores in its vicinity to retreat dorsally and ventrally, thereby establishing a subtle melanophore-free region. This region is then colonized by xanthophores and becomes progressively more distinctive. The lateral lines are responsible for initiating this pattern in *A. t. tigrinum*; a melanophore-free region does not form when lateral line development is prevented. Similar effects of the lateral lines have been demonstrated in several other ambystomatids and salamandrids (Parichy, '96b,c; for correlations in Plethodontidae and fishes, see Noble, '31; Mabee, '95).

Postmetamorphic pigment patterns

Pigment patterns of adult salamanders typically are composed of melanophores, xanthophores, and iridophores. Adult *A. t. tigrinum* display a black ground color of dermal melanophores and light spots of dermal xanthophores and iridophores (Bishop, '41; DuShane, '43). Epidermal melanophores are present but do not contribute to the overall appearance of the pigment pattern. Little is known of the developmental mechanisms underlying adult pigment patterns in amphibians (Frost-Mason et al., '95; Reedy et al., '98). Embryological grafting experiments have suggested roles for embryonic ectoderm and mesoderm in promoting the development of different classes of adult pigment cells in *A. maculatum* (Lehman, '53), but these experiments also may have perturbed lateral line development, complicating the interpretation of these results (see Parichy, '96B). Nevertheless, these and other studies

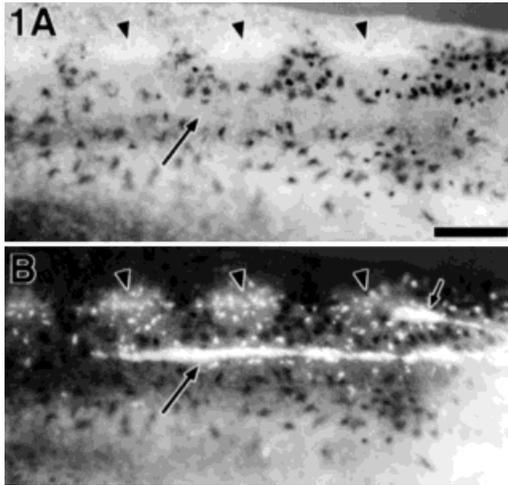


Fig. 1. Formation of a melanophore-free region and horizontal stripe pattern correlates with the development of the trunk midbody lateral line in *Ambystoma tigrinum tigrinum*. The trunk lateral lines develop from cranial ectodermal lateral line placodes that deploy migrating lateral line primordia. These primordia travel caudally within the epidermis and deposit clusters of mechanosensory cells at periodic intervals that later erupt through the epidermis as mature, mechanoreceptive neuromasts. Three trunk lateral lines develop in the sequence: midbody, dorsal, and ventral (Northcutt et al., '94). **A:** Brightfield micrograph showing the distribution of melanophores at stage 36/37 (Bordzilovskaya et al., '89) (prior to hatching) and the initially subtle melanophore-free region (arrow) as well as developing vertical bars of xanthophores (arrowheads). **B:** Corresponding fluorescence double exposure showing the migrating midbody lateral line primordium (large arrow) and dorsal lateral line primordium (small arrow), as well as xanthophores (autofluorescing cells) dispersing from premigratory aggregates (original positions indicated with arrowheads). The lateral lines are labeled with a fluorescent vital dye (for details see Parichy, '96b,c). In all photographs, anterior is to the right. Scale bar = 500 μ m.

(Smith-Gill, '74; Bagnara, '82) do suggest that some features of adult patterns are determined as early as the larval feeding stage, implying that larval and adult patterns might share some developmental determinants. Consistent with the hypothesis that pre- and postmetamorphic patterns are coupled in *A. t. tigrinum*, several related subspecies exhibit less distinctive melanophore-free regions in larvae and also display more variable patterns in adults (Dunn, '40; Bishop, '62; Stebbins, '85; Parichy, '96a, unpublished data). Likewise, the brightness of the late larval pattern correlates with the brightness of the postmetamorphic pattern in *A. t. nebulosum* (Fernandez and Collins, '88). Finally, iridophores are a major compo-

nent of the adult pattern, and some of the first of these cells to differentiate in larvae do so in association with lateral line neuromasts (see below), raising the possibility that the larval lateral lines may act as a patterning cue for cells contributing to adult spots. If larval and adult pigment patterns of *A. t. tigrinum* are coupled developmentally, such coupling could arise because 1) the adult pattern depends on the distribution of pigment cells constituting the early larval pattern, 2) the adult pattern shares with the early larval pattern a dependence on the lateral lines, or 3) the adult pattern depends on both the early larval pattern and the lateral lines. Under any of these scenarios, prevention of lateral line development should eliminate the larval melanophore-free region and also should perturb the adult pigment pattern.

MATERIALS AND METHODS

Embryos and manipulations

Ambystoma t. tigrinum embryos from a natural population (supplied by the Charles Sullivan Company, Nashville, TN) were maintained in 20% Hepes-buffered Steinberg's solution (HSS) (plus 37.5 IU/ml penicillin, 37.5 μ g/ml streptomycin [Asashima et al., '89]) at 9–18°C. For microsurgeries, embryos representing five clutches were decapulated with forceps, passed through four to five changes of sterile 100% HSS (plus 75 IU/ml penicillin, 75 μ g/ml streptomycin), and then placed into sterile agar-lined dishes containing 100% HSS. To prevent the development of trunk lateral line primordia, nerves, and neuromasts, ectoderm containing the lateral line placodes (Northcutt et al., '94) was removed unilaterally with tungsten needles and then replaced with a patch of donor belly epidermis (Fig. 2) (Parichy, '96b,c). Manipulations were performed on embryos at stages 28–31 (Bordzilovskaya et al., '89), which is prior to the onset of dispersal for most or all trunk neural crest cells (Olsson and Löfberg, '92). A series of embryos were sham-manipulated by removing and then replacing placode-area ectoderm unilaterally. For both lateral line-ablated and sham-manipulated embryos, operated sides were chosen at random. To test for nonspecific surgical effects, additional embryos were treated identically but were left unmanipulated.

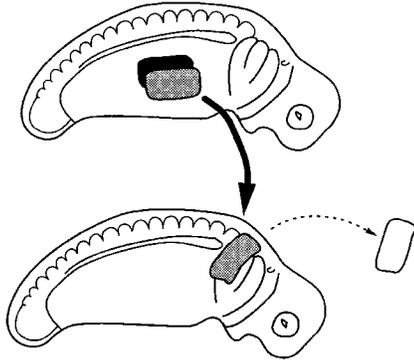


Fig. 2. Microsurgical procedure for ablating the trunk lateral lines in *Ambystoma tigrinum tigrinum*. To prevent lateral line development unilaterally, placode-area ectoderm was removed and replaced with belly epidermis from a similarly staged donor.

Rearing conditions

Salamanders were maintained individually (15°C; 14L:10D) throughout the experiment. Embryos and early larvae were reared in 60 mm Petri dishes containing 20% HSS. Shortly after the onset of feeding, larvae were transferred to plastic dishes (100 × 40 mm) containing 50% Holtfreter's solution (Asashima et al., '89). As individuals grew, they were transferred to plastic boxes (30 × 15 × 9 cm) containing aged tap water. Post-metamorphic salamanders were housed in plastic boxes lined with moist foam pads. Containers were rearranged at each cleaning to minimize position effects: dishes were rearranged daily; boxes containing larvae were rearranged every third day; boxes containing metamorphosed salamanders were rearranged once per week. Early larvae were fed newly hatched brine shrimp twice daily and then were acclimated to a diet of tubifex worms once per day. As larvae approached metamorphosis, they were fed tubifex worms and crickets. Metamorphosed salamanders were fed crickets dusted with calcium and vitamins three times per week. Days of development were calculated relative to completion of the early larval pattern (stage 41 [Parichy, '96b]); salamanders were considered metamorphosed when their gills and tail fin had regressed almost entirely and they could be transferred to foam pads.

Quantitative methods

Characterization of early larval pigment patterns has been described (Parichy, '96b).

To document postmetamorphic patterns, salamanders were first anesthetized and then cradled in molds of modeling clay within a water-filled basin. A sheet of glass was rested on spacers and pressed against the salamander to flatten the upper surface of the integument. Images of left and right sides were captured with a Sony CCD video-camera and macro lens interfaced to an Apple Macintosh computer running the public domain NIH Image program (Wayne Rasband, NIH). Digital images were transferred to Adobe Photoshop, and a rectangular region between the second and ninth costal grooves and the dorsal and ventral margins of the flank was analyzed. Light spots of xanthophores and iridophores were traced manually and normalized to white; all dark ground color was normalized to black. Dorsoventral image heights were normalized to 100 pixels.

To describe patterns, I calculated the proportional *total area* covered by spots for each image as the number of white pixels divided by the total number of pixels. Proportional *regional areas* covered by spots were similarly quantified for five equally sized positions at different dorsoventral levels of the flank. The number of positions was chosen a priori based on examination of adult patterns on unmanipulated sides (without regard to patterns on manipulated sides) and represented a compromise between maximizing resolution and minimizing the impact of minor variation in the positioning of salamanders. A posteriori comparisons using different total numbers of positions yielded qualitatively similar results (data not shown). Also calculated were the numbers of spots and the mean perimeters and mean areas of spots (in pixels). Finally, the mean perimeter:area ratios of spots were calculated as a measure of shape since these ratios increase as spots of a given area depart from circularity. Differences between unmanipulated and manipulated sides (or left and right sides of unmanipulated controls) were assessed with paired *t*-tests, using arcsine transformations for ratio data (Sokal and Rohlf, '81). This allowed testing for effects on pigment patterns after controlling for interindividual variability.

RESULTS

To test whether early larval and adult pigment patterns are coupled developmentally, I prevented lateral line development unilaterally and reared the experimentally

manipulated and control embryos ($n = 131$) through metamorphosis.

Early larval pigment patterns

On unmanipulated sides of early larvae (stage 41, shortly after hatching normally would occur; approximately 15 mm total length), melanophores were found over the dorsal myotomes and further ventrally at the dorsal margin of the yolk mass, but relatively few melanophores were found over the lateral face of the myotomes. On lateral line-ablated sides, however, melanophores more extensively colonized the flank ($n = 84$) (Fig. 3). Figure 4 presents a reanalysis of melanophore distributions (from Parichy, '96b), in which melanophore densities are pooled within four dorsoventral regions of the flank (rather than the original 15 regions), to facilitate comparison with analyses of postmetamorphic patterns (below). On lateral line-ablated sides (Fig. 4A), melanophore densities were lower near the dorsal edge of the myotomes (0–400 μm from the base of the dorsal fin; paired $t_{42} = 6.84$, $P < 0.0001$) and higher over the lateral face of the myotomes (401–800 μm ; paired $t_{42} = 12.39$, $P < 0.0001$), in agreement with Par-

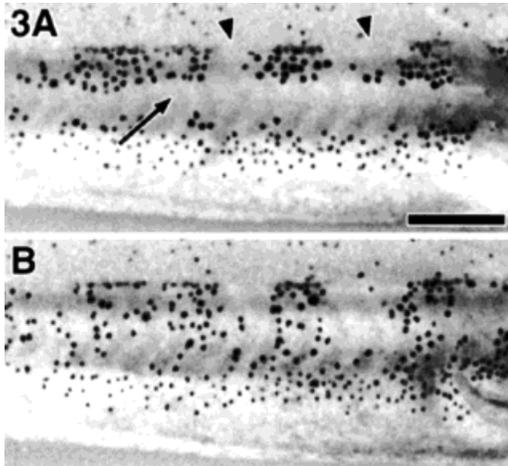


Fig. 3. Prevention of lateral line development eliminates the melanophore-free region and horizontal stripe pattern in early larval *Ambystoma tigrinum tigrinum*. Opposite sides of a single individual (stage 41) with the image in B flipped to facilitate comparison with A. **A:** On the unmanipulated side, a distinctive melanophore-free region is found over the lateral face of the myotomes at the level of the midbody lateral line (large arrow). Arrowheads indicate the positions of xanthophore bars. **B:** On the lateral line-ablated side, melanophores readily colonize this area. Scale bar = 1 mm.

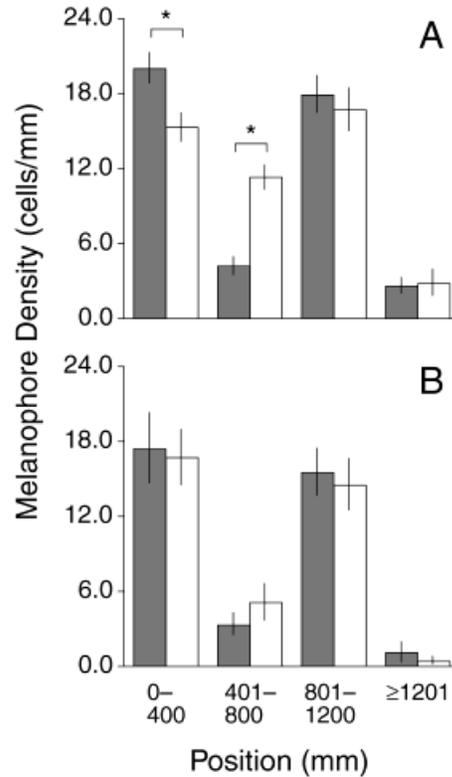


Fig. 4. Melanophore distributions in early larvae depend on the lateral lines in *Ambystoma tigrinum tigrinum*. Presented are mean melanophore densities ($\pm 95\%$ confidence intervals) from reanalyses of lateral line-ablated (A) and sham-manipulated (B) larvae of Parichy ('96b). Positions represent distances from the base of the dorsal fin and the dorsal apex of the myotomes, and only midpoints are shown. Filled bars, unmanipulated sides; open bars, manipulated sides. **A:** Lateral line ablation (43 larvae; 14,489 melanophores) resulted in more uniform distributions of melanophores, with lower densities dorsally and higher densities over the middle of the myotomes on lateral line-ablated sides (open bars) as compared to lateral line-intact sides (filled bars). **B:** Sham manipulation (15 larvae; 4,021 melanophores) typically did not affect melanophore densities on manipulated sides (open bars) as compared to unmanipulated sides (filled bars), though a subtle increase in melanophore density could sometimes be observed in the middle of the flank (401–800 μm) (see Parichy, '96b) probably due to minor damage inflicted on the lateral line placodes during removal and replacement. *Significantly different at $\alpha = 0.05$ level with sequential Bonferroni correction for four comparisons (Rice, '89).

ichy ('96b). Ablation of the lateral lines also resulted in a statistically significant 7% increase in total melanophore density and perturbed the distribution of xanthophores, which remained principally between vertical bars of melanophores (see Parichy, '96b,c).

Sham manipulations (n = 27) did not affect melanophore distributions (Fig. 4B).

Later larval pigment patterns

No differences in behavior, growth, or vigor were observed among lateral line-ablated, sham-manipulated, and unmanipulated larvae. By the middle of the larval period (approximately 70 mm total length) in salamanders of all treatments, melanophores were apparent in dorsal regions of the flank previously occupied only by xanthophores (Fig. 5A). Nevertheless, a distinctive melanophore-free region was still evident over the lateral face of the myotomes on unmanipulated sides, centered on the midbody lateral line. Iridophores were present subjacent to midbody and ventral lateral line neuromasts, lining the peritoneum, and were also scattered irregularly over the trunk. On lat-

eral line-ablated sides, a melanophore-free region was not apparent (Fig. 5C).

At later larval stages (approximately 100 mm total length), the pattern gradually transformed to a mottled green and black in salamanders of all treatments (Fig. 5B). This change in coloration may reflect the invasion of the dermis by melanophores previously localized beneath the subepidermal basement membrane, which thickens gradually and is occupied by mesenchymal cells to form the definitive dermis (Stearner, '46). A distinctive melanophore-free region was no longer apparent over the middle of the myotomes on lateral line-intact sides. Nevertheless, a region of lighter pigmentation persisted in the vicinity of the midbody lateral line, and small spots of iridophores delineated the positions of neuromasts. These

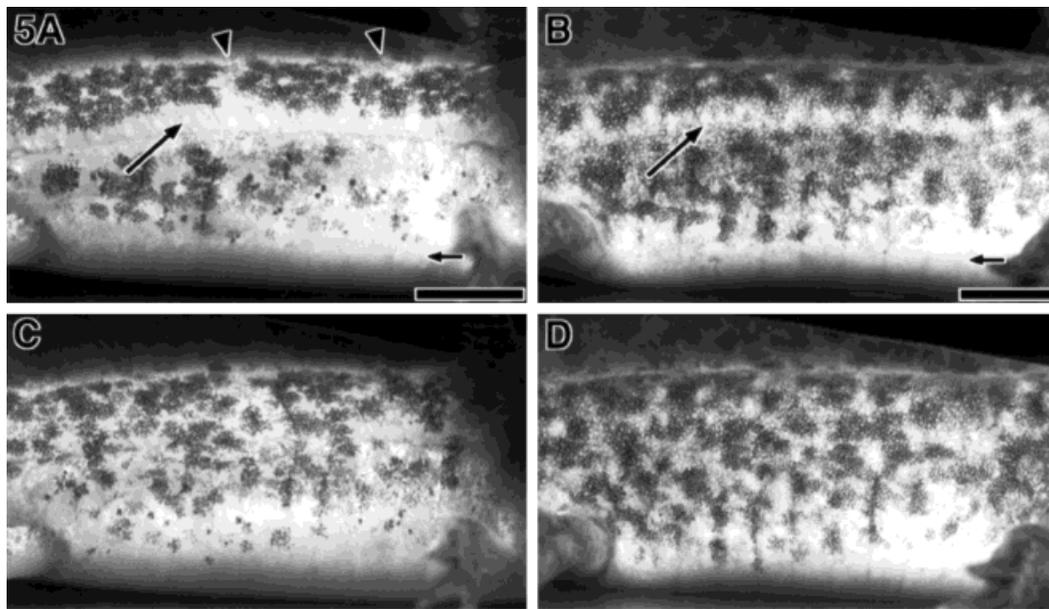


Fig. 5. Lateral line effects on the pigment pattern persist through the larval period of *Ambystoma tigrinum tigrinum*. Shown are opposite sides of the same larva presented in Figure 3, at day 47 (A,C) and day 95 (B,D). **A:** On the unmanipulated side, a distinctive melanophore-free region is still present in the vicinity of the midbody lateral line (large arrow) during the middle larval period, and the positions of lateral line neuromasts (one is indicated at the tip of the large arrow). Arrowheads indicate regions now occupied by melanophores that correspond to the positions of xanthophore bars at earlier stages. Small arrow, level of the ventral lateral

line. **B:** During the late larval period, a region of lighter pigmentation is still centered on the midbody lateral line (large arrow), but the distinctiveness of this region is considerably diminished. Small arrow, level of the ventral lateral line. **C:** On the lateral line-ablated side, melanophores are more uniformly distributed during the middle larval period as compared to the unmanipulated side in A. **D:** The consequences of lateral line ablation are still manifested during the late larval period, though the magnitude of the difference compared to the lateral line-intact side in B is reduced. Images in C and D are reversed to facilitate comparison with A and B, respectively. Scale bars = 5 mm.

features were not present on lateral line-ablated sides (Fig. 5D).

Postmetamorphic pigment patterns

Every salamander ($n = 131$) survived for scoring of the adult pigment pattern approximately 42 weeks after development of the early larval pattern and on average 29 weeks after metamorphosis. Times to metamorphosis did not differ among lateral line-ablated, sham-manipulated, and unmanipulated salamanders (pooled mean = 99 d, SD = 7.1, range = 80–119; $F_{2,128} = 0.15$, $P = 0.9$). Upon completion of the experiment, treatment groups did not differ in snout-to-vent lengths (pooled mean = 103 mm, SD = 4.1, range = 97–120; $F_{2,128} = 0.37$, $P = 0.7$) or total lengths (pooled mean = 204 mm, SD = 11.8, range = 190–243; $F_{2,128} = 0.64$, $P = 0.5$). Thus, manipulations did not adversely affect general growth and development under these conditions.

Postmetamorphic salamanders displayed pigment patterns typical of *A. t. tigrinum*: a black ground color with dark yellow-brown spots dorsally and lighter yellow spots ventrally. After pooling unmanipulated sides of lateral line-ablated and sham-manipulated salamanders, and arbitrarily chosen sides of unmanipulated controls total proportional areas covered by spots did not differ among clutches ($F_{4,126} = 1.63$, $P = 0.2$) and were not correlated with times to metamorphosis ($r = 0.03$, $P = 0.8$) but tended to increase with body size (correlations with snout-to-vent length and total length: $r = 0.28$, 0.30 ; $P < 0.05$). At metamorphosis, neuromasts are covered by epidermis and presumably regress to a less-differentiated state as in other salamanders (Noble, '31; Dawson, '36; Wright, '51; Frittsch et al., '88); well-differentiated neuromasts were not observed in histological sections of integument at the end of the experiment (not shown).

Effects of lateral line ablation on early larval pigment patterns (Figs. 3, 4) suggested the hypothesis that correlated effects in adults might be manifested at dorsolateral levels of the flank. Nevertheless, no major effects of lateral line ablation on the adult pattern were evident ($n = 84$) (Fig. 6), and comparisons of areas covered by spots at corresponding dorsoventral positions did not reveal effects of lateral line ablation in the most dorsal four of five regions (Fig. 7A). In the most ventral region, however, lateral line-ablated sides had slightly but significantly less area covered by spots (paired

$t_{83} = 4.54$, $P < 0.0001$), and these spots tended not to form as orderly or continuous a row as compared to lateral line-intact sides (Fig. 6A,C). This region corresponds to the position of the ventral lateral line in larvae; most melanophores and xanthophores constituting the early larval pattern are not localized this far ventrally, and larval melanophore density is not perturbed at this level following lateral line ablation (Figs. 3, 4A, 5) (Parichy, '96b). Total areas covered by spots across the entire flank did not differ significantly due to lateral line ablation (1% less on lateral line-ablated sides; paired $t_{83} = 1.80$, $P = 0.08$). Among control salamanders, total and regional areas covered by spots did not differ between unmanipulated and sham-manipulated sides (total areas: paired $t_{26} = 0.52$, $P = 0.6$, $n = 27$; regional areas: see Fig. 7B) or between left and right sides of unmanipulated individuals (total areas: paired $t_{19} = 0.47$, $P = 0.6$, $n = 19$; regional areas: data not shown).

Spot numbers as well as mean spot areas, perimeters, and perimeter-to-area ratios did not differ between lateral line-ablated and lateral line-intact sides (mean perimeters: 8% less on lateral line-ablated sides; paired $t_{83} = 1.79$, $P = 0.08$; all others: $P > 0.2$) (data not shown). Visual inspections by observers blind with respect to treatment failed to identify other lateral line effects.

DISCUSSION

Interactions between pigment cells and the lateral line sensory system contribute to a distinctive horizontal stripe pattern in larval salamanders. This study demonstrates that in *A. t. tigrinum* lateral line effects on chromatophores persist through middle larval stages but diminish as metamorphosis approaches, and the postmetamorphic pigment pattern arises largely independently of both the lateral lines and the distribution of pigment cells that contribute to larval stripes. These findings are consistent with a model in which ontogenetic changes that occur across metamorphosis decouple larval and adult pigment patterns, and could thereby facilitate independent evolutionary modifications to the patterns during different phases of the life history.

Pigment pattern development

Prevention of lateral line development profoundly altered the distributions of melanophores and xanthophores over the dorsal

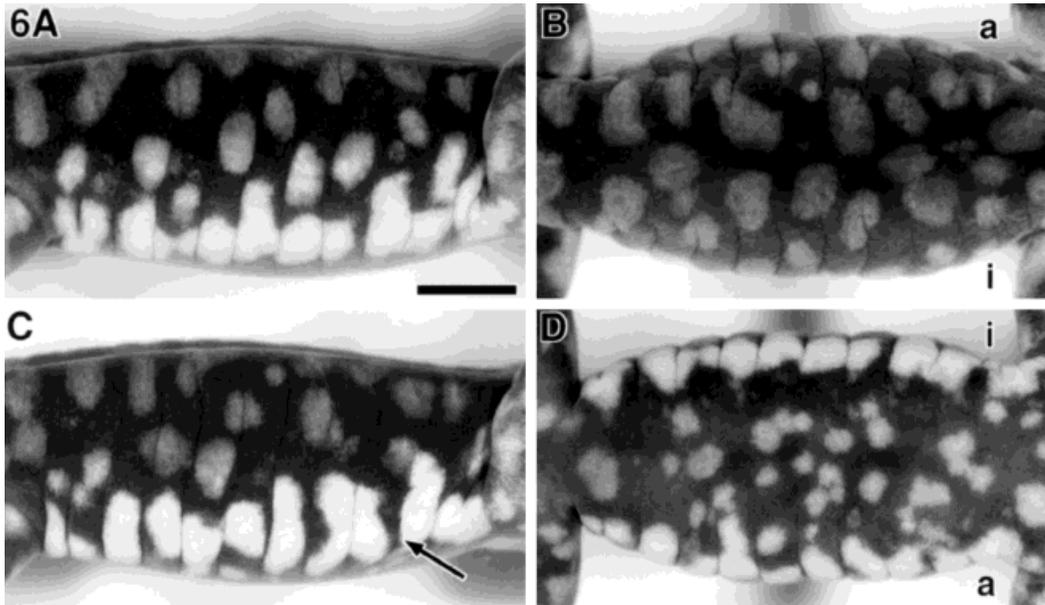


Fig. 6. Major features of the adult pigment pattern do not depend on the lateral lines or the distribution of pigment cells comprising the early larval pattern in *Ambystoma tigrinum tigrinum*. Shown is the same individual presented in Figs. 3 and 5 after metamorphosis on day 299. **A:** On the unoperated side, light yellow and brown spots are typical of the normal adult pigment pattern in *A. t. tigrinum*. **B:** Dorsal view of the same salamander. a, lateral line-ablated side; i, lateral line-

intact side. **C:** On the side without lateral lines, spots in dorsal and lateral regions of the flank (corresponding to the position of the midbody lateral line in larvae) are not obviously perturbed, though bright spots along the ventrolateral flank (arrow) are somewhat less regular and continuous as compared to the unmanipulated side in A. This image is reversed to facilitate comparison with A. **D:** Ventral view. Scale bar = 10 mm.

and lateral flank in early larvae (also see Parichy, '96b,c) but did not yield pigment pattern defects in the corresponding regions of adults. This failure to detect significant effects of lateral line ablation in dorsolateral regions of the flank after metamorphosis probably is not due to insufficient statistical power. Estimates of error were low (see Fig. 7), and even a subtle effect of the lateral lines was detectable ventrolaterally, where lateral line-ablated sides had a 3% deficit in the area covered by spots (presumably contributing to marginal effects of lateral line ablation on total areas covered by spots and mean spot perimeters). Since early larval patterns in this region do not depend on the lateral lines, this difference could indicate that the ventral lateral line has a direct (albeit minor) influence on cells that contribute to adult ventral spots, rather than acting indirectly via some effect on early larval chromatophores. For example, the ventral lateral line might stimulate the proliferation or differentiation of dermal iridophores

that appear during later larval stages or might act as a patterning cue that contributes to the localization of these cells. The present observations also cannot exclude the possibility that larval and adult patterns might be coupled through other shared patterning mechanisms that have yet to be identified. For instance, chromatophores of both larvae and adults are derived from neural crest cells (Twitty and Bodenstein, '39; DuShane, '43), so mutations affecting general properties of these cells (e.g., morphogenetic behaviors or specification of chromatophore lineages) might yield pattern defects at both stages. Despite these caveats, however, the general conclusion suggested by this study is that the major features of the adult pigment pattern in *A. t. tigrinum* do not depend on either the distribution of pigment cells contributing to larval stripes, or the midbody lateral line, despite the latter's pivotal role in establishing the early larval pattern.

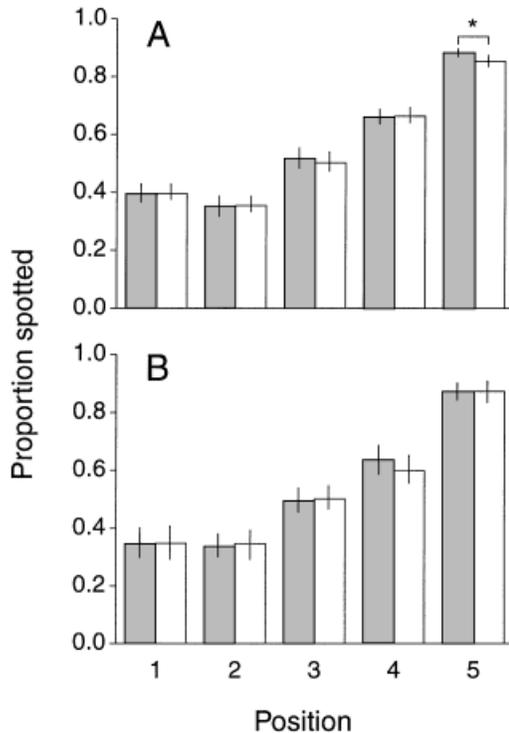


Fig. 7. Lateral line ablation has only subtle effects on the adult pigment pattern in *Ambystoma tigrinum tigrinum*. Mean areas covered by spots at different dorso-ventral positions of the flank are presented for lateral line-ablated (A) and sham-manipulated (B) individuals. Filled bars, unmanipulated sides; open bars, manipulated sides. **A:** Areas covered by spots did not differ between lateral line-intact sides (filled bars) and lateral line-ablated sides (open bars) at dorsal and lateral regions (positions 1–4) but differed significantly in the most ventral region (position 5). **B:** Areas covered by spots did not differ between unmanipulated (filled bars) and sham-manipulated sides (open bars). Error bars are 95% confidence intervals converted back to ratio scale after arcsine transformation. *Significantly different at $\alpha = 0.05$ level with sequential Bonferroni correction for five comparisons (Rice, '89).

The relative independence of larval and adult pigment patterns in *A. t. tigrinum* could be achieved through either of two developmental strategies (also see Alberch, '87; Moran, '94). If the same population of melanophores and xanthophores in larvae gives rise to the pattern in adults, then morphogenetic remodeling of this population through differential proliferation or spatial rearrangements could contribute to decoupling larval and adult phenotypes. Several studies are consistent with these possibilities (Berweger, '26; Woronzowa, '32; Stearner,

'46; Lehman, '53; Yasutomi, '87; Lechaire and Deneffe, '91), though direct evidence is lacking. Or, different populations of pigment cells could contribute to patterns across stages. For example, chromatophores constituting the adult pattern might arise from precursor cells that are set aside at embryonic stages and recruited to differentiate only at metamorphosis. Consistent with such compartmentalization of larval and adult cell lineages, adult melanophores differentiate de novo at metamorphosis in the newt *Taricha torosa* (family Salamandridae) (see below), and distinct populations of melanophores contribute to larval and adult pigment patterns in the zebrafish, *Danio rerio* (Johnson et al., '95). Similarly, the adult epibranchial cartilage of the salamander *Eurycea bislineata* arises from just a few cells in the perichondrium of the larval, neural crest-derived epibranchial cartilage (Alberch and Gale, '86), and different populations of cells contribute to larval and adult epidermis, intestine, and musculature in anuran amphibians (Alley, '89; Kinoshita and Sasaki, '94; Hourdry et al., '96; Furlow et al., '97). Nevertheless, the presence of "latent" pigment cell precursors in the skin of *A. t. tigrinum* has yet to be demonstrated. If such a population does exist, the results of this study indicate that it must be under sufficiently different control so as to be unaffected by defects in the arrangements of larval chromatophores.

Whichever developmental strategy or combination of strategies is responsible for the transition from a larval to adult pigment pattern in *A. t. tigrinum*, the extent of this transformation can be contrasted with other taxa. For example, *Taricha torosa* larvae exhibit horizontal stripes of melanophores, but, unlike other ambystomatids and salamandrids, stripes in *T. torosa* do not depend on the lateral lines because redundant, evolutionarily derived patterning mechanisms have been layered over the primitive lateral line-dependent mechanisms (Parichy, '96b, in preparation). This species also may exhibit derived mechanisms for the development of the uniform orange-brown adult pattern since, unlike *Ambystoma*, dermal melanophores that constitute the larval pattern degenerate at metamorphosis and are replaced by a second population of melanophores that differentiates de novo in the epidermis (Stearner, '46; Niu and Twitty, '50; McCurdy, '71). Thus, mechanisms under-

lying larval pattern formation appear to be more complex, and the larval-to-adult transformation appears to be more extensive in *T. torosa* than in *A. t. tigrinum*. At a deeper phylogenetic level, pigment pattern metamorphosis in many frogs involves the organization of chromatophores into functionally and physiologically integrated dermal chromatophore units (Bagnara et al., '68), in contrast to the much simpler arrangements of chromatophores in adult salamanders (Stearner, '46). Increased complexity of patterning mechanisms within stages and greater disparity of mechanisms across stages (i.e., increased pattern modularity) may reflect conflicting selection in larval and adult environments (Riska, '86; Raff, '96; Wagner and Altenberg, '96), although the fitness consequences of variation in morphological characters expressed both before and after metamorphosis remain largely unexplored.

Consequences of metamorphosis

What are the consequences of metamorphosis for the development and evolution of pigment patterns and other characters? Complex life cycles and their associated metamorphoses are generally thought to decouple characters that are expressed at different stages and in different selective regimes, thereby allowing stage-specific adaptations for resource acquisition, dispersal, reproduction, or other activities (Istock, '67; Bryant, '69; Wassersug, '75; Wilbur, '80; Werner, '88; Ebenman, '92). This "adaptive decoupling" hypothesis (Moran, '94) predicts that traits expressed before and after metamorphosis should depend on different developmental mechanisms and gene activities (Haldane, '32; Alberch, '87; Ebenman, '92). The finding that the adult pattern of *A. t. tigrinum* arises largely independently of the lateral lines and the larval pattern is thus consistent with the adaptive decoupling hypothesis and a role for metamorphosis in dissociating trait expression across life cycle stages. Moreover, the same mechanisms that govern other events at metamorphosis also may regulate the transition from the larval to the adult pigment pattern. Thyroid hormone (TH) triggers metamorphic changes in a variety of characters (Gilbert and Frieden, '81; Rose and Reiss, '93; Gilbert et al., '96), and results of several studies suggest a possible role for TH in pigment pattern transformation via direct effects on chromatophore motility, proliferation, or dif-

ferentiation, or indirect effects on surrounding tissues (Woronzowa, '32; Bagnara et al., '79; Yasutomi, '87; Frost-Mason et al., '95; Brown, '97). Nevertheless, the results of this study do not test directly the role of metamorphosis per se in decoupling larval and adult patterns. Indeed, characters may exhibit reduced interdependencies as more disparate stages are compared even in taxa with simple life cycles (Arnold, '92; Kirkpatrick and Lofsvold, '92; Cheverud et al., '96). For example, this study showed that midbody lateral line effects on chromatophores were very distinctive during the first half of the larval period, but were less apparent during later larval development and were not detectable after metamorphosis. Thus, alterations in the pigment pattern were not confined to the period of metamorphic climax when other remodeling was most apparent (also see Bishop, '41; Stearner, '46; Lehman, '53), raising the possibility that pattern changes might not be regulated by the same mechanisms that govern other metamorphic transformations. If so, a TH-dependent metamorphosis might not itself be responsible for decoupling patterns across life cycle stages. Consistent with the idea that pattern transformation does not depend on a metamorphic climax, several subspecies of *A. tigrinum* and the closely related *A. rosaceum*, *A. andersoni*, and *A. taylori* can develop adult markings even as larvae or paedomorphs (Taylor, '41; Anderson, '61; Shaffer and McKnight, '96; Shaffer and Voss, personal communication). Alternatively, these observations could simply indicate that pigment patterns (or their determinants) differ from other characters in their sensitivity to TH (e.g., Hanken et al., '89; Rose and Reiss, '93; Rose, '95). Experimental perturbation of TH levels should illuminate the extent to which pigment pattern transformation is developmentally and hormonally integrated with other events at metamorphosis (Shaffer and Voss, '96), and would provide a more direct test of the adaptive decoupling hypothesis for the role of metamorphosis in dissociating the expression of pigment patterns and other characters (e.g., Shaffer et al., '91; Berger-Bishop and Harris, '96; Vaglia et al., '97) across life cycle stages.

Manipulative experiments for studying character evolution

Experimental studies of the mechanisms underlying character development can complement studies of character evolution

at the population level. Although manipulative approaches such as the one presented here are not likely to provide insights into subtle effects of alleles segregating in natural populations, they should reveal major developmental interdependencies within and among characters and can suggest predictions regarding morphological evolution. For example, these data suggest that selection on features of the lateral line sensory system (such as receptor number) might yield correlated responses in larval but not adult patterns. Conversely, selection on the adult pattern would seem unlikely to result in the correlated evolution of either the larval pattern or the lateral lines. These hypotheses could be further tested by estimating quantitative genetic correlations across stages or measuring correlated responses to artificial selection (but see Gromko, '95). More generally, a mechanistic understanding of character development will be essential for assessing the biological bases for character correlations as well as long-term constraints on their evolution (Riska, '89; Arnold, '92; Shaw et al., '95).

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