# Pigment pattern evolution by differential deployment of neural crest and post-embryonic melanophore lineages in *Danio* fishes

**Ian K. Quigley, Jessica M. Turner, Richard J. Nuckels, Joan L. Manuel, Erine H. Budi, Erin L. MacDonald and David M. Parichy***

Section of Integrative Biology, Section of Molecular, Cell and Developmental Biology, Institute for Cellular and Molecular Biology, University of Texas at Austin, 1 University Station C0930, Austin, TX 78712, USA

*Author for correspondence (e-mail: dparichy@mail.utexas.edu)

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## Summary

**Latent precursors or stem cells of neural crest origin are present in a variety of post-embryonic tissues. Although these cells are of biomedical interest for roles in human health and disease, their potential evolutionary significance has been underappreciated. As a first step towards elucidating the contributions of such cells to the evolution of vertebrate form, we investigated the relative roles of neural crest cells and post-embryonic latent precursors during the evolutionary diversification of adult pigment patterns in *Danio* fishes. These pigment patterns result from the numbers and arrangements of embryonic melanophores that are derived from embryonic neural crest cells, as well as from post-embryonic metamorphic melanophores that are derived from latent precursors of presumptive neural crest origin. In the zebrafish *D. rerio*, a pattern of melanophore stripes arises during the larval-to-adult transformation by the recruitment of metamorphic melanophores from latent precursors. Using a comparative approach in the context of new phylogenetic data, we show that adult pigment patterns in five additional species also arise from metamorphic melanophores, identifying this as an ancestral mode of adult pigment pattern development. By contrast, superficially similar adult stripes of *D. nigrofasciatus* (a sister species to *D. rerio*) arise by the reorganization of melanophores that differentiated at embryonic stages, with a diminished contribution from metamorphic melanophores. Genetic mosaic and molecular marker analyses reveal evolutionary changes that are extrinsic to *D. nigrofasciatus* melanophore lineages, including a dramatic reduction of metamorphic melanophore precursors. Finally, interspecific complementation tests identify a candidate genetic pathway for contributing to the evolutionary reduction in metamorphic melanophores and the increased contribution of early larval melanophores to *D. nigrofasciatus* adult pigment pattern development. These results demonstrate an important role for latent precursors in the diversification of pigment patterns across danios. More generally, differences in the deployment of post-embryonic neural crest-derived stem cells or their specified progeny may contribute substantially to the evolutionary diversification of adult form in vertebrates, particularly in species that undergo a metamorphosis.**

Key words: Zebrafish, Pigment pattern, Evolution, Morphogenesis, Neural crest, Stem cell, Phylogeny

## Introduction

Neural crest cells give rise to many of the shared, derived traits of vertebrates (Gans and Northcutt, 1983; Hall, 1999). These cells arise along the dorsal neural tube during neurulation and then disperse widely throughout the embryo (Knecht and Bronner-Fraser, 2002; Halloran and Berndt, 2003). Among the cells and tissues derived from this transient, migratory population are pigment cells, glia and neurons of the peripheral nervous system, endocardial cushion cells, chromaffin cells of the adrenal gland, smooth muscle, and bone and cartilage of the craniofacial skeleton (Hörstadius, 1950; Le Douarin, 1999). Not surprisingly, in light of their many derivatives, neural crest cells are associated with a wide range of inherited and acquired disorders ranging from melanoma to neuroblastoma, Hirschsprung disease to Waardenburg syndrome, and Treacher Collins syndrome to craniofacial dysmorphogenesis following fetal ethanol exposure (Matthay, 1997; Amiel and Lyonnet, 2001; Ahlgren et al., 2002; Chin, 2003; Widlund and Fisher, 2003; Farlie et al., 2004). Changes in the patterning of neural crest cells and their derivatives are similarly thought to underlie much of vertebrate diversity, from variation in pigment pattern to variation in jaw morphology (Kelsh, 2004; Kulesa et al., 2004).

Given the biomedical and evolutionary significance of neural crest cells and their derivatives, it is of paramount importance to identify the mechanisms by which these cells are patterned to generate the particular forms expressed by juveniles and adults. Most studies have focused on the early patterning of neural crest cells during embryogenesis. Yet, recent studies have demonstrated post-embryonic neural crest-derived stem cells in peripheral nerves, gut and skin (Morrison et al., 1999; Bixby et al., 2002; Kruger et al., 2002; Nishimura et al., 2002; Iwashita et al., 2003; Sieber-Blum and Grim, 2004; Sieber-Blum et al., 2004; Joseph et al., 2004). These findings suggest

that the development and maintenance of adult traits, as well as the evolution of these traits, may depend on contributions from latent stem cells in addition to direct contributions from neural crest cells at embryonic stages.

A useful system for studying the development and evolution of neural crest-derived traits is the pigment pattern of teleost fishes (Quigley and Parichy, 2002; Parichy, 2003; Kelsh, 2004). In the zebrafish *Danio rerio*, an early larval pigment pattern develops during embryogenesis as neural crest cells differentiate into early larval melanophores and other pigment cell classes. This pattern is largely completed by 3 days post-fertilization (dpf), and includes melanophore stripes along the dorsal and ventral edges of the myotomes, and along the horizontal myoseptum (Milos and Dingle, 1978a; Kelsh et al., 2000). The early larval pigment pattern remains essentially unchanged for about two weeks, until the onset of pigment pattern metamorphosis. At this time, new melanophores appear over the flank in regions not previously occupied by these cells, and during the following two weeks, the pigment pattern is transformed into that of the adult (Fig. 1) (Kirschbaum, 1975; Johnson et al., 1995; Parichy et al., 2000b). Genetic and cellular analyses demonstrate that new melanophores arising at metamorphosis differentiate from latent precursors or stem cells of presumptive neural crest origin (Johnson et al., 1995; Parichy and Turner, 2003b); such melanophores also play a crucial role in pigment pattern regeneration (Goodrich and Nichols, 1931; Rawls and Johnson, 2000; Rawls and Johnson, 2001). Although previous studies provide compelling evidence that metamorphic and regenerative melanophores are derived from post-embryonic latent precursors, specific markers for these cells have not been demonstrated, and their locations, potencies and developmental requirements remain largely unknown. Given these caveats, we refer to these post-embryonic melanophores simply as 'metamorphic' melanophores.

The diversity of pigment patterns in species closely related to *D. rerio* allows the use of comparative and experimental approaches to understand pigment pattern development and evolution (Parichy and Johnson, 2001; Quigley and Parichy, 2002; Parichy, 2003). Besides horizontal stripes of varying width and number, these species exhibit vertical bars and uniform pigment patterns in which melanophores are dispersed evenly over the flank (Fig. 1). This interspecific variation provides an opportunity to dissect the genetic and cellular mechanisms underlying naturally occurring phenotypic variation in an adult trait of neural crest origin.

In this study, we ask whether adult melanophore stripes develop similarly across species, and in particular, whether the relative roles of neural crest-derived early larval melanophores and metamorphic melanophores have been maintained during evolution. To address this question, we first examine *D. nigrofasciatus* (Fig. 1), a species having fewer melanophores and stripes than *D. rerio*, but in which stripes that do develop are similar to those of *D. rerio*. Whereas stripes in *D. rerio* arise almost entirely from metamorphic melanophores, we show that stripes in *D. nigrofasciatus* arise from fewer metamorphic melanophores and an increased number of neural crest-derived early larval melanophores that persist into the adult. This interspecific variation led us to test the relative roles of these melanophore lineages during pigment pattern development in several additional species. These analyses demonstrate that a primary role for metamorphic melanophores in adult pigment pattern formation is likely to be ancestral for *Danio*, and that *D. nigrofasciatus* exhibits a unique, derived reduction in these cells, with a corresponding increased contribution of early larval melanophores to the adult pigment pattern. We further demonstrate that evolutionary changes within *D. nigrofasciatus* are extrinsic (non-autonomous) to the melanophore lineages, and we identify a candidate genetic pathway for mediating this change. These analyses highlight the potential for studies of *D. rerio* and its relatives to reveal basic mechanisms of post-embryonic neural crest development.

## Materials and methods

### Fish stocks, crosses and genotyping

Fish were reared at 28.5°C (14 hours light: 10 hours dark). Wild-type *D. rerio* were the inbred mapping strain $AB^{ut}$, or an outbred wild-type stock representing mixed $AB^{ut}$, $wik^{ut}$, commercially derived 'ekkwill',

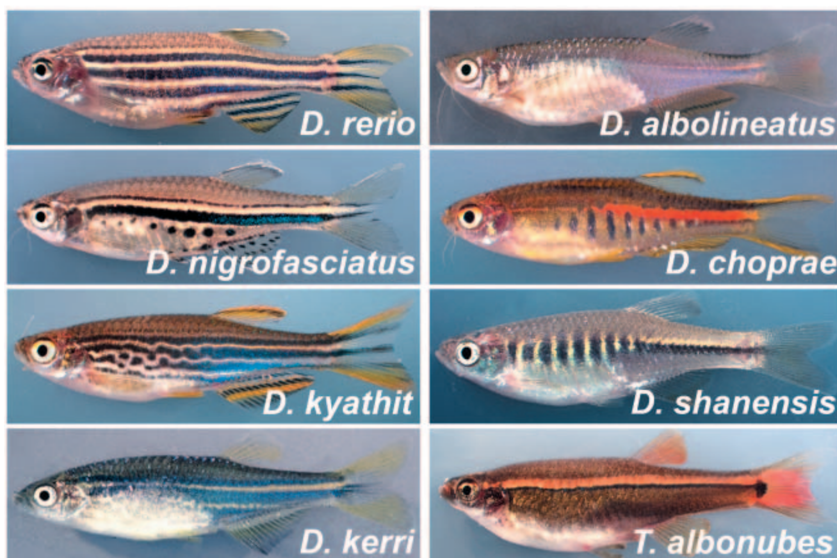


**Fig. 1.** Diverse pigment patterns of *Danio* fishes and their relatives. *D. rerio*, *D. nigrofasciatus* and *D. kyathit* all exhibit well-defined horizontal stripes of melanophores, although *D. kyathit* adults develop breaks and irregularities in the stripes both anteriorly (shown) and posteriorly. *D. kerri* exhibits a few broad and relatively diffuse melanophore stripes. *D. albolineatus* adults lack horizontal stripes and melanophores are evenly distributed, although larvae exhibit a transient pattern of stripes posteriorly. *D. choprae* have vertical bars of melanophores as adults and transient horizontal stripes at earlier stages. *Devario* (formerly *Danio*) *shanensis* adults have vertical bars of melanophores anteriorly, with horizontal stripes posteriorly. *Tanichthys albonubes* exhibit a narrow horizontal melanophore stripe with a broader pattern of evenly dispersed melanophores. Yellow coloration in fish derives from neural crest-derived xanthophores, whereas red pigment in some species derives from neural crest-derived erythrophores. Adult fish are 25-30 mm standard length.

and other backgrounds. No differences in development were observed between wild-type *D. rerio* strains. *D. nigrofasciatus*, *D. choprae*, *Devario shanensis* and *Tanichthys albonubes* were derived from stocks purchased originally from a commercial pet supplier (Transship Discounts, Jamaica, NY). *D. albolineatus* and *D. kerri* were derived from stocks originally provided by M. McClure (Cornell University). *D.* 'hikari' (used in phylogeny reconstruction) was obtained commercially and resembles *D. kerri* but has not been described formally. *D. kyathit* also was obtained commercially, but has more complete stripes than the type described (Fang, 1998), and may represent a variant or subspecies; for ease of presentation we refer to these fish simply as '*D. kyathit*' in the text, but to acknowledge the uncertainty of their precise taxonomic affinity, we refer to the fish as '*D.* aff. *kyathit*' in the phylogram. *D. rerio* mutants have already been described: *albino*$^{b4}$ (Kelsh et al., 2000), *sox10* (*colourless*) (Dutton et al., 2001), *endothelin receptor b1* (*ednrb1*, *rose*$^{b140}$) (Parichy et al., 2000a), *tfap2a* (*lockjaw*$^{ts213}$) (Knight et al., 2003; Knight et al., 2004), *mitfa* (*nacre*$^{w2}$) (Lister et al., 1999), and *puma*$^{j115e1}$ (Parichy and Turner, 2003b; Parichy et al., 2003). Additional *D. rerio* mutants were derived from on-going mutagenesis screens (D.M.P., E.H.B. and E.L.M., unpublished). Interspecific complementation tests were performed as previously described (Parichy and Johnson, 2001) by in vitro fertilization. Because of difficulties obtaining fertilizable eggs from heterospecific danios, most complementation tests were performed using *D. rerio* females and heterospecific males. When the identities or map positions of *D. rerio* mutant loci were known, heterozygotes were used for generating interspecific hybrids, to randomize effects across unlinked loci, and progeny were genotyped for the presence or absence of the mutant allele by PCR (primers and diagnostic single nucleotide polymorphisms available on request). Finally, *puma* mutant *D. rerio* are temperature-sensitive, with growth rate-dependent pigment pattern defects at 25°C, moderate pigment pattern defects at 28.5°C, and more severe defects at 33°C (Parichy et al., 2003); tester *puma* hybrids were reared at the intermediate temperature of 28.5°C to avoid mortality owing to stresses at the higher temperatures.

### Nomenclature for pigment pattern elements at larval and adult stages

Previous studies defined pigment pattern elements in *D. rerio* (Parichy and Johnson, 2001; Parichy and Turner, 2003b), including: *early larval* dorsal, lateral and ventral melanophore stripes (ELD, ELL, ELV); *adult* first-developing (primary) dorsal and ventral melanophore stripes (1D, 1V); and later-developing (secondary) dorsal and ventral melanophore stripes (2D, 2V). Additionally, between adult melanophore stripes are xanthophore-rich 'interstripe' regions. For simplicity, we use the term 'stripes' to refer exclusively to the adult primary melanophore stripes (1D, 1V), unless indicated otherwise.

### Microscopy, imaging and quantitative analyses

To examine melanophore behavior, we repeatedly imaged individual larvae during pigment pattern metamorphosis, allowing us to follow the appearance, disappearance and migration of individual melanophores (Parichy et al., 2000b; Parichy and Turner, 2003b). Individually reared fish were anesthetized with MS222 (Sigma) and imaged every 24 hours using an Olympus SZX-12 stereozoom microscope. To ensure that we could follow cells at the edges of the flank, all fish were imaged lying parallel to the camera, and also on a specially constructed stand providing an angle 30° from normal. Images were transferred to Adobe Photoshop CS for analysis, in some cases in conjunction with the FoveaPro 3.0 image processing and analysis package (Reindeer Graphics).

Individual melanophores were tracked as previously described (Parichy et al., 2000b; Parichy and Turner, 2003b), with newly differentiated melanophores clearly distinguishable from pre-existing melanophores by their initially lighter melanization (and in some instances different color, see below). We identified individual melanophores present in the early larval pigment patterns, then examined the fates of these cells by examining their positions in sequential images. In following early larval melanophores through metamorphosis, we could not formally observe cell division in static image series, so we tracked only one presumptive daughter following likely mitoses. Thus, our counts and estimated proportions of early larval melanophore contributions to later stages in *D. nigrofasciatus* are conservative and may underestimate true values to some degree. For comparisons of early larval and metamorphic melanophore numbers between species, we defined an area of interest bounded anteriorly by the anteriormost anal fin ray insertion and posteriorly at two myotomes anterior to the caudal peduncle. We counted individual early larval melanophores unilaterally within this region. We determined total adult melanophore numbers within this region, either within the adult ventral primary melanophore stripe, if present, or at an equivalent dorsoventral position as observed in *D. rerio*, with a height defined arbitrarily as one-quarter the flank height, as measured at the anterior boundary. In final images from each individual, all melanophores in the region of interest were marked and counted, either by eye or by the Count plug-in of FoveaPro 3.0. Numbers of metamorphic melanophores were thus calculated as the difference between the total numbers of melanophores identified in the final images, and the numbers of melanophores that had been followed into the region of interest from early larval stages. Statistical analyses were performed with JMP 5.0.1a Statistical Software (SAS Institute, Cary, NC). Additional information on quantitative image analyses is available on request.

### Cell transplantation and genetic mosaic analysis

We transplanted cells between mid-blastula stage [3.3-3.8 hours post-fertilization (hpf)] *D. rerio* and *D. nigrofasciatus* embryos, using a Narishige IM-9B micrometer-driven microinjection apparatus mounted on a Narishige micromanipulator. We placed embryos in agar-lined dishes containing 10% Hanks solution plus 1% penicillin/streptomycin, and dechorionated embryos with fine forceps. We transplanted 20-100 cells into each recipient and reared chimeric individuals through adult stages. To identify donor *D. rerio* cells in *D. nigrofasciatus* hosts, we used donors that were transgenic for EGFP driven by a ubiquitously expressed *D. rerio* β-actin promoter, kindly provided by Ken Poss (Parichy and Turner, 2003a; Parichy et al., 2003). To identify donor *D. nigrofasciatus* melanophores in *D. rerio* hosts, we used hosts mutant for *albino* or *nacre* (*mitfa*), which fail to develop melanin and melanophores, respectively. Both of these mutant loci normally act autonomously to the melanophore lineage, as revealed previously (Lin et al., 1992; Lister et al., 1999; Parichy and Turner, 2003a) and confirmed in control experiments performed for the present analyses (data not shown). Previous studies reveal minimal local correlation between the distributions of pigment cells and other tissues in genetic mosaics examined at metamorphic and adult stages (Maderspacher and Nusslein-Volhard, 2003; Parichy and Turner, 2003a; Parichy et al., 2003). We confirmed that donor melanophores typically develop independently of other local donor tissues in a subset of chimeras in which donor embryos were injected with rhodamine dextran prior to the four-cell stage, then were examined for the distribution of melanophores and other tissues at 4 dpf (data not shown). We sorted chimeras at 3 dpf for the presence or absence of donor melanophores, and as larvae approached metamorphosis, we repeatedly imaged individual larvae to follow the behavior of early larval melanophores and to assess the distribution of metamorphic melanophores. Survival rates for interspecific chimeras were typically 5-10% of that observed for comparable experiments involving only *D. rerio* (Parichy and Turner, 2003a; Parichy et al., 2003), suggesting some species incompatibilities; ~1% of chimeras were informative for analyses of pigment pattern formation (see Results).

### In situ hybridization and histology

We used in situ hybridization to detect transcripts for melanophore lineage markers, as described previously (Parichy et al., 2000a; Parichy et al., 2000b; Parichy et al., 2003). Larvae were fixed briefly in 4% paraformaldehyde, 1% DMSO in PBS, decapitated, and then fixed overnight at 4°C. Larvae were transferred to methanol, rehydrated to PBST (PBS with 0.2% Tween-20), then treated for 20 minutes at room temperature with 20 μg/ml proteinase-K in PBST containing 1% DMSO. Larvae were postfixed for 20 minutes at room temperature in 4% paraformaldehyde, 0.005% glutaraldehyde, washed in PBST, then washed three times in hybridization solution lacking tRNA and heparin. Prehybridizations were performed overnight at 60°C in hybridization solution (50% formamide, 5×SSC, 500 μg/ml yeast tRNA, 50 μg/ml heparin, 0.2% Tween-20, 9.2 mM citric acid). Hybridizations were performed at 60°C over two nights, in fresh hybridization solution containing digoxigenin-labeled riboprobes fractionated to ~300 nucleotides. Larvae were then washed twice, for 15 minutes each, in 2×SSCT, and three times, for 2 hours each, in 0.2×SSCT at 60°C. After graded changes to PBST, larvae were blocked overnight at 4°C in 2 mg/ml BSA, 5% heat-inactivated calf serum in PBST, then incubated at 4°C over two nights in fresh blocking reagent containing 1:5000 anti-digoxigenin alkaline phosphatase-conjugated Fab fragments (Roche). Larvae were washed over two nights in PBST, transferred to alkaline phosphatase buffer [100 mM Tris (pH 9.5), 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween-20], and the color developed with NBT/BCIP.

To assay for tyrosinase activity, larvae were fixed for 2 hours in 4% paraformaldehyde in PBS, rinsed three times in PBS, incubated in 0.1% L-dopa (Sigma) for 1 hour to overnight, rinsed in PBS, then stored in glycerol (Camp and Lardelli, 2001; McCauley et al., 2004). We verified the specificity of the assay for melanoblasts by the reduced staining on the flanks of metamorphosing *nacre* mutant *D. rerio*, which have defects in the melanophore lineage (Lister et al., 1999; Parichy et al., 2000b), and we verified that newly melanized (tyrosinase$^+$) cells are not macrophages by Neutral Red staining (Herbomel et al., 1999) (data not shown).

### Phylogenetic analysis

We reconstructed phylogenetic relationships based on mitochondrial 12S and 16S rDNA sequences, obtained using standard methods and universal primers (Kocher et al., 1989; Palumbi et al., 1991).

12S: H1478, 5′-TGA CTG CAG AGG GTG ACG GGC GGT GTG T-3′; L1091, 5′-AAA AAG CTT CAA ACT GGG ATT AGA TAC CCC ACT AT-3′.

16S: 16Sar-L, 5′-CGC CTG TTT ATC AAA AAC AT-3′; 16Sbr-H, 5′-CCG GTC TGA ACT CAG ATC ACG T-3′.

Sequences were aligned using CLUSTAL-W, inspected by eye and edited as necessary. We then analyzed combined 12S and 16S sequences (784 nucleotides) using maximum likelihood estimation in PAUP* 4.0b10 for Macintosh (Swofford, 2002). Maximum likelihood analyses used a general time-reversible plus gamma model. Substitution rate matrix, nucleotide frequencies, and among site rate variation were estimated from the data by preliminary parsimony analyses using a heuristic search strategy. Maximum likelihood, parsimony and distance methods produced trees with the same topology. To estimate confidence values for reconstructed nodes, we performed two independent analyses. First, we performed 100 nonparametric bootstrap replicates using PAUP*. Second, we performed a Bayesian analysis of the data using MrBayes (Larget and Simon, 1999; Huelsenbeck and Ronquist, 2001; Wilcox et al., 2002), with 3000 replicate trees from 300,000 generations following the approach to asymptotic likelihood values. Both approaches gave nearly identical confidence values, which we report as percentages of recovered trees in the phylogram (see Results).

## Results

### Different modes of pigment pattern metamorphosis in *D. rerio* and *D. nigrofasciatus*

To assess the generality of adult pigment pattern-forming mechanisms, we investigated whether stripes of different *Danio* species arise through similar underlying cellular behaviors. We chose to compare the closely related species *D. rerio* and *D. nigrofasciatus* (Parichy and Johnson, 2001), for which stripes on the flank are superficially similar (Fig. 1).

Closer inspection reveals about twice as many melanophores in *D. rerio* than in *D. nigrofasciatus* (Fig. 2A,C; see below).

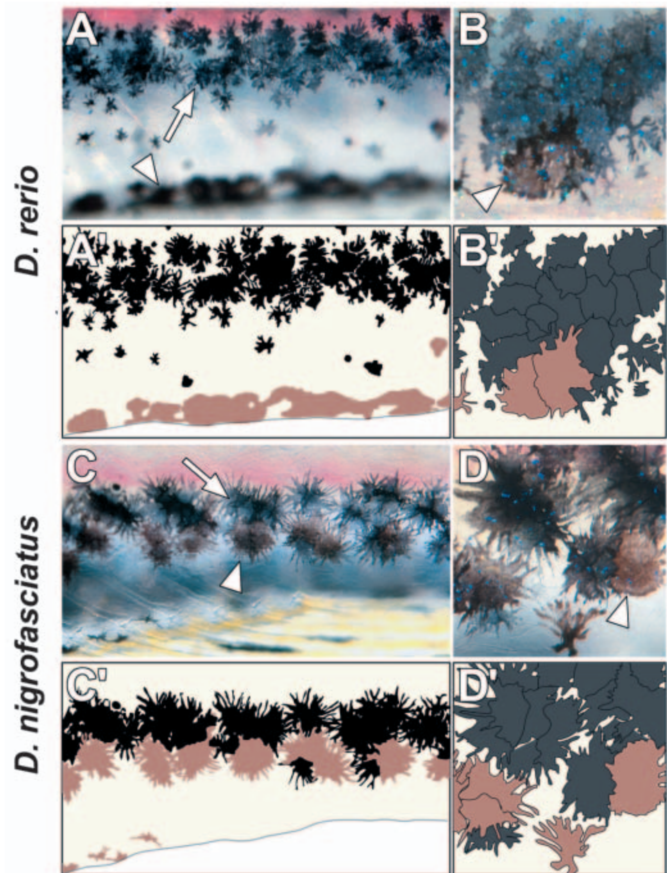


**Fig. 2.** Similar stripes with different melanophore numbers and colors in *D. rerio* and *D. nigrofasciatus* juveniles. (A,B) *D. rerio*; (C,D) *D. nigrofasciatus*. (A) The ventral primary melanophore stripe of *D. rerio* consists of numerous gray-black metamorphic melanophores (arrow). Melanophores persisting from embryonic stages at the site of the early larval ventral melanophore stripe (arrowhead) are browner than metamorphic melanophores. (B) In the *D. rerio* dorsal primary melanophore stripe, a few melanophores at the ventral edge are brown in color (arrowhead), where a few melanophores are derived from the early larval stripe pattern. (A′,B′) Schematics of fish shown in A and B, showing black and brown melanophores. No attempt is made to precisely delineate individual melanophore boundaries. (C) Unlike in *D. rerio*, the ventral primary melanophore stripe of *D. nigrofasciatus* includes numerous brown melanophores (arrowhead), in addition to black melanophores (arrow). (D) Detail showing *D. nigrofasciatus* brown melanophores (arrowhead) and black melanophores in the ventral primary melanophore stripe. (C′,D′) Schematics of fish shown in C and D.

Melanophore colors differ as well. In *D. rerio*, the dorsal and ventral stripes consist almost entirely of grey-black melanophores. Yet, occasional brownish melanophores occur at the ventral edge of the dorsal stripe (Fig. 2A,B), where a few melanophores derive not from latent precursors at metamorphosis, but from the rearrangement of embryo-derived melanophores originally present in the early larval lateral stripe along the horizontal myoseptum (Parichy and Turner, 2003b). In *D. nigrofasciatus*, however, both dorsal and ventral stripes contain numerous brown melanophores (Fig. 2C,D), and melanophores are not present along the ventral myotome edge (where the early larval ventral stripe had been). Melanophore color variation is apparent transiently after metamorphosis, and is not equally pronounced in all families; whether this variation reflects the age of the melanin contained within the cells or some other biochemical difference is not clear. Nevertheless, the differences in melanophore colors and their relative frequencies in the adult pigment patterns of *D. rerio* and *D. nigrofasciatus* led us to hypothesize that cryptic patterning variation might underlie the superficially similar stripes between these species.

To determine whether stripes develop differently in *D. rerio* and *D. nigrofasciatus*, we imaged larvae repeatedly through pigment pattern metamorphosis, allowing us to follow the appearance, disappearance and movements of individual melanophores (Parichy et al., 2000b; Parichy and Turner, 2003b). These analyses demonstrate that *D. rerio* stripes depend almost entirely on melanophores that differentiate from

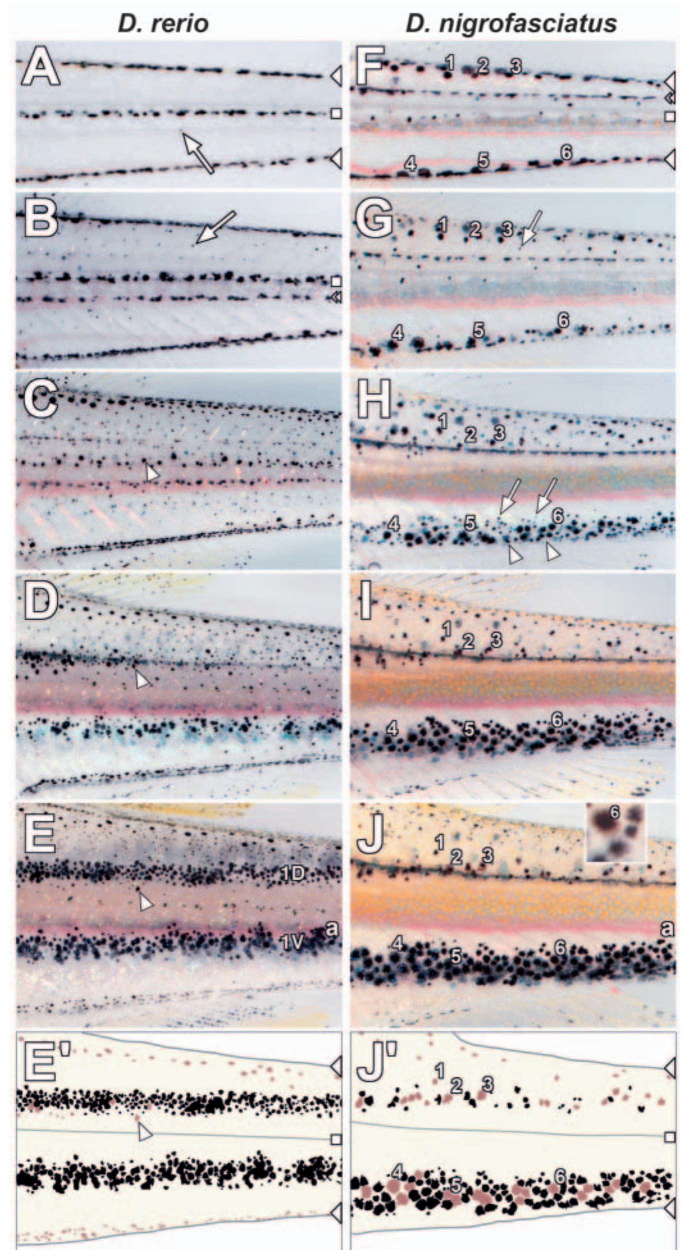


**Fig. 3.** Pigment pattern metamorphosis differs between *D. rerio* (A-E′) and *D. nigrofasciatus* (F-J′). Panels shown are of selected days from a complete image series for individual, representative larvae. In A and F, the sites of early larval melanophore stripes are indicated at the dorsal and ventral margins of the myotomes (horizontal arrowheads), and at the horizontal myoseptum (squares). In *D. rerio*, pigment pattern metamorphosis begins with the differentiation of pioneer metamorphic melanophores over the ventral myotomes (A, arrow), with additional metamorphic melanophores (B, arrow) appearing both dorsally and ventrally over a period of several days (B-D). Adult primary stripes become evident as dispersed melanophores migrate to sites of stripe formation and additional metamorphic melanophores differentiate within the stripes (D,E). A few early larval melanophores move from the horizontal myoseptum to join the adult dorsal primary melanophore stripe (C-E, arrowheads). Near the end of pigment pattern metamorphosis the larvae have developed an adult dorsal primary melanophore stripe and an adult ventral primary melanophore stripe (1D, 1V, respectively, in panel E). The adult ventral primary melanophore stripe develops just ventral to the level of the aorta (a, in panel E), about halfway between the horizontal myoseptum and the ventral margin of the myotomes (E′). In *D. nigrofasciatus*, pigment pattern metamorphosis begins with early larval melanophores becoming displaced from the larval stripes (F, melanophores 1-6). Whereas some metamorphic melanophores differentiate de novo (G,H, arrows), these are markedly fewer than in *D. rerio*. As metamorphosis proceeds, melanophores initially present in the ventral early larval stripe (H, arrowheads) become increasingly distant from the ventral margin of the myotomes. By late stages of pigment pattern metamorphosis, a complete adult ventral primary melanophore stripe has formed (J), and both dorsal and ventral stripes contain numerous early larval melanophores. The *D. nigrofasciatus* ventral primary melanophore stripe develops further ventrally relative to the level of the aorta (a, J), and closer to the ventral margin of the myotome (J′), compared to *D. rerio*. Inset (J) shows brownish cast of an adult stripe melanophore (6) that originated in the early larval stripe. (E′,J′) Schematics of fish shown in E and J, showing melanophores associated with the adult primary melanophore stripes, and residual melanophores from the early larval stripes dorsally and ventrally, as determined by following individual melanophores from early larval stages throughout the image series (i.e. by analyzing cell lineage rather than by examination of final melanophore colors). For consistency with Fig. 2, melanophores that originated in the early larval pattern are shown in brown, and melanophores that differentiated during metamorphosis are shown in black. Dorsal metamorphic melanophores that will cover the dorsum and dorsal scales are omitted for clarity. Double arrowheads in B and F indicate deep, internal melanophores that are ventral to the notochord, or dorsal to the neural tube, respectively, and that do not contribute to pigment patterns beneath the skin. Standard lengths of larvae (mm): A, 6.7; B, 7.4; C, 8.6; D, 10.3; E, 11.5; F, 6.7; G, 7.1; H, 8.6; I, 9.4; J, 10.3.

latent precursors at metamorphosis. By stark contrast, the superficially similar stripes of *D. nigrofasciatus* arise by the reorganization of early larval melanophores, with far fewer metamorphic melanophores.

In *D. rerio*, the onset of pigment pattern metamorphosis is marked by the differentiation of single 'pioneer' metamorphic melanophores over the middle of most ventral myotomes (Fig. 3A). Subsequently, metamorphic melanophores differentiate widely over the myotomes, between the early larval stripes (Fig. 3B,C). The adult primary stripes become increasingly apparent (Fig. 3D), as initially dispersed metamorphic melanophores migrate short distances to the sites of stripe formation, and as additional metamorphic melanophores differentiate within the stripes themselves (Fig. 3C,D). A few early larval melanophores migrate from the horizontal myoseptum to join the dorsal adult primary melanophore stripe (Fig. 3D,E), but most remain in place and eventually are lost (Parichy and Turner, 2003b). As fish approach the end of metamorphosis, a juvenile pattern emerges, with adult dorsal and ventral primary melanophore stripes consisting almost entirely of melanophores that have differentiated from latent precursors during metamorphosis (Fig. 3E,E′).

In *D. nigrofasciatus*, pioneer metamorphic melanophores are absent and the onset of pigment pattern metamorphosis is marked by a loss of cohesiveness within the early larval stripes (Fig. 3F). Gradually, early larval melanophores are visible more laterally over the myotomes; rearrangements of melanophores relative to one another indicate that movements result in part from migration (as opposed to passive movement due to growth; Fig. 4). A few metamorphic melanophores differentiate but these are drastically reduced in number compared with in *D. rerio* (Fig. 3G). As metamorphosis proceeds, early larval melanophores are increasingly observed at sites of adult stripe formation, and additional metamorphic melanophores differentiate at these sites (Fig. 3H). As compared with *D. rerio*, the *D. nigrofasciatus* dorsal stripe occurs at approximately the same dorsal-ventral location, whereas the ventral stripe lies closer to the ventral edge of the myotomes (Fig. 3E′,J′). By the end of pigment pattern metamorphosis, the pattern consists of dorsal and ventral stripes comprising a relatively high proportion of early larval melanophores (Fig. 3I,J). Differences in pigment pattern metamorphosis between species are particularly evident in animations compiled from multiple images of individual larvae (see Movies 1, 2 in supplementary material).

Our analyses demonstrate that superficially similar stripes in two closely related species develop in markedly different ways: in *D. rerio*, stripes arise almost entirely by the differentiation of melanophores from latent precursors during metamorphosis; in *D. nigrofasciatus*, stripes arise largely through the reorganization of early larval melanophores, with metamorphic melanophores playing a smaller role.

### Ancestral role for metamorphic melanophores in adult pigment pattern development and derived patterning mechanisms in *D. nigrofasciatus*

The relative contributions to adult pigment patterns of early larval melanophores and metamorphic melanophores could vary continuously across species. Alternatively, either the *D. rerio* or the *D. nigrofasciatus* mode could be typical. To distinguish between these possibilities, and to determine which, if either, mode is ancestral and which is derived, we sought to examine pigment pattern metamorphosis in additional species.

Because danio relationships remain poorly understood, we first sequenced 12S and 16S rDNA from additional taxa to infer phylogenetic relationships (Fig. 5). These analyses confirm the close relationship between *D. rerio* and *D. nigrofasciatus*, as well as *D. kyathit* (Fig. 1). The phylogeny also supports a split between *Danio* and *Devario* [formerly within *Danio* (Fang, 2003)]. Moreover, these data reveal additional pigment pattern diversity within *Danio* (as defined in Fig. 5): these fish have been known to have horizontal stripes, spots, uniform patterns, and more complex pigment patterns; *Danio choprae* adds vertical barring to the repertoire (Fig. 1).

Our analyses of additional species demonstrate that adult pigment patterns form primarily by the recruitment of metamorphic melanophores, rather than by the reorganization of early larval melanophores. Using inferred phylogenetic relationships as a guide (Fig. 5), we chose additional species representing *Danio* and more distant groups, as well as a range of adult pigment patterns, and we repeatedly imaged larvae through metamorphosis. Fig. 6 shows the ventral flank for representative larvae of *D. nigrofasciatus*, *D. rerio*, *D. kyathit*, *D. kerri*, *D. albolineatus*, *D. choprae*, and *Tanichthys albonubes*. In each species, the early larval pigment patterns are nearly identical (Fig. 6, row 1; see also Fig. S1 in supplementary material). Subsequently, melanophores differentiate from latent precursors during metamorphosis to generate the adult pigment pattern (Fig. 6, rows 2-8). Only in *D. nigrofasciatus* do large numbers of early larval melanophores become incorporated into adult stripes; a few early larval melanophores move small distances from their initial positions in *D. kerri*, *D. albolineatus*, *D. choprae*, *D. shanensis* (data not shown) and *T. albonubes*, but these do not contribute substantially to the adult pattern.

The greater persistence of early larval neural crest-derived melanophores into the adult pigment pattern of *D. nigrofasciatus* compared with other species is shown quantitatively in Fig. 7A. Total numbers of early larval melanophores do not differ dramatically across the species, yet

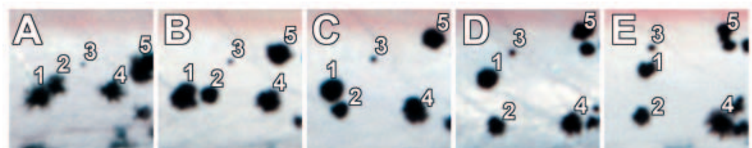


**Fig. 4.** Migration of melanophores during *D. nigrofasciatus* pigment pattern metamorphosis. Early larval melanophores (1, 2, 4, 5) and newly differentiating metamorphic melanophores (3) change positions as stripes form. Some changes in position are likely to reflect passive movements due to growth (e.g. increasing dorsal-ventral separation of melanophores 4 and 5), whereas others can be explained only by active rearrangements (e.g. relative dorsal-ventral positions of melanophores 1 and 2). Only selected days from the complete image series are shown. To maintain the same region of interest, images in this figure and in Figs 6, 9 and 12 are rescaled across days; exact sizes and stages are available on request.

many more of these cells contribute to the adult pigment pattern in *D. nigrofasciatus*. Variation in the total numbers of adult melanophores across species is shown in Fig. 7B. *D. nigrofasciatus* and *D. albolineatus* (and to a lesser extent *D. kerri*) each have reduced numbers of metamorphic melanophores compared with other species. However, only in *D. nigrofasciatus* is this deficit in metamorphic melanophores partly compensated by a significantly greater contribution from early larval neural crest-derived melanophores.

Adult pigment patterns of these species thus arise principally from metamorphic melanophores with little contribution from early larval melanophores. This finding supports a model in which *Danio* adult pigment patterns depend ancestrally on metamorphic melanophores, with *D. nigrofasciatus* exhibiting an evolutionarily derived reduction in metamorphic melanophores and a correspondingly greater role for neural crest-derived early larval melanophores in generating the adult pigment pattern.

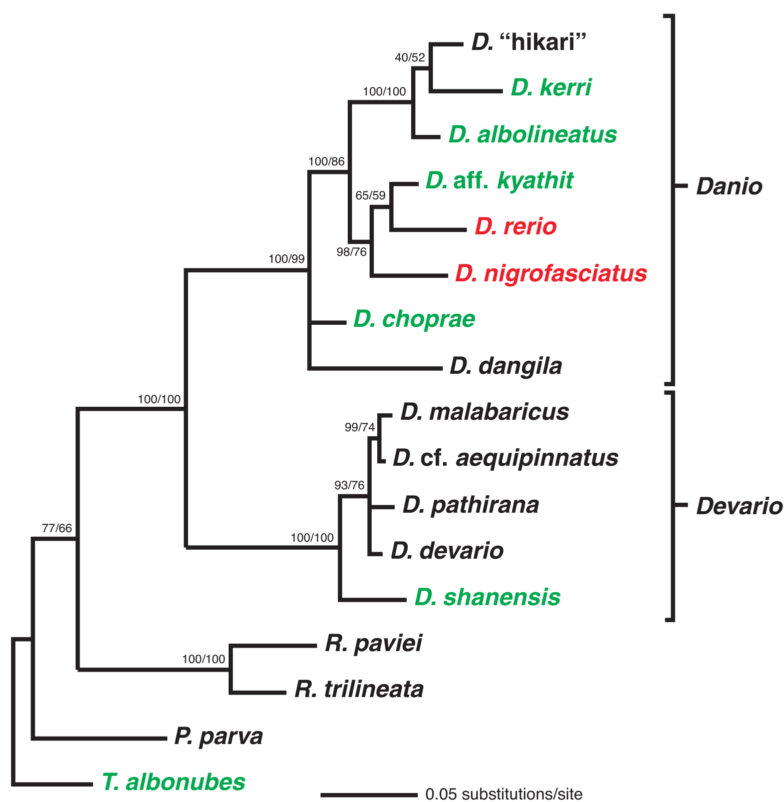


**Fig. 5.** Phylogenetic relationships of danios inferred from 12S and 16S rDNA sequences. Shown is a maximum-likelihood tree (branch lengths proportional to estimated divergence). Support values are percentages from Bayesian analysis followed by nonparametric bootstrapping. Taxa in red and green were chosen for analyses of pigment pattern metamorphosis based on phylogenetic position and embryo availability. The analysis supports the division of the danios into two genera, *Danio* and *Devario*, based on morphological criteria (Fang, 2003), and is in general agreement with previous molecular analyses of fewer taxa (Zardoya et al., 1996; Parichy and Johnson, 2001). GenBank Accession numbers for 12S and 16S sequences are (top to bottom): AY707450, AY707456; U21372, U21381; AF3226h58, AF322663; AY707446, AY707452; AF322663; AY707449, AY707455; AY707447, AY707453; AF322656, AF322661; U21376, U21384; AF322659, AF322664; U21377, U21377; U21375, U21370; AY707448, AY707454; U21553, U21554; AF322660, AF322665; U21378, U21386; AY707445, AY707451. Published sequences for *D.* aff. *tweediei* and *D. pulcher* were excluded owing to their limited length and the resulting loss of phylogenetic resolution.

### *D. rerio* mutants identify a candidate pathway for metamorphic melanophore reduction and early larval melanophore morphogenesis in *D. nigrofasciatus*

*D. rerio* mutants can identify genes and pathways that contribute to interspecific pigment pattern differences (Parichy and Johnson, 2001). Given the reduced number of metamorphic melanophores in *D. nigrofasciatus* compared to *D. rerio*, we investigated whether genes isolated as *D. rerio* mutants with defects in melanophore development also contribute to the difference between species. We used interspecific hybrids to test for complementation of *D. rerio* mutant alleles by crossing mutant *D. rerio* to *D. nigrofasciatus* and comparing these tester (mutant) hybrids to control (wild-type) hybrids. Tester hybrids exhibiting fewer melanophores than controls identify genes that may contribute to the interspecific difference, whereas tester hybrids that have similar melanophore numbers to controls identify genes less likely to have major effect roles.

Control hybrids between wild-type *D. rerio* and *D. nigrofasciatus* have phenotypes intermediate between species. Whereas melanophore numbers in primary adult stripes are increased over *D. nigrofasciatus* and are closer to *D. rerio*, melanophore numbers in secondary adult stripes, and the total numbers of stripes, are closer to *D. nigrofasciatus* than *D. rerio* (Fig. 8A) (Parichy and Johnson, 2001). Comparing adult hybrid phenotypes does not reveal gross non-complementation of the recessive melanophore mutants $sox10^{ut.r13e1}$, $tfap2a^{ts213}$, $bonaparte^{ut.r16e1}$, $cezanne^{ut.r17e1}$, $degas^{ut.r18e1}$, $oberon^{j198e1}$, $pissarro^{ut.r8e1}$, $picasso^{ut.r2e1}$, $primrose^{j199}$, $puma^{j115e1}$ or $seurat^{ut.r15e1}$ (e.g. Fig. 8), adding to the previously excluded loci *ednrb1*, *fms*, *kit*, *mitfa*, *leopard*, *fritz* and *jaguar* (Parichy and Johnson, 2001). Thus, genes contributing to the differences in the final numbers of adult melanophores between species either are not likely to be represented in this collection of 18 *D. rerio* pigment pattern mutants, or differences in allelic strengths are not sufficient to reveal non-complementation.

Although the preceding analyses tend to exclude loci from roles in reducing the total numbers of adult melanophores in *D. nigrofasciatus*, they did not explicitly address differences in early larval melanophore morphogenesis or contributions to the adult pigment pattern. To identify genetic pathways that might be responsible for interspecific differences in early larval melanophore fate and adult stripe composition, we further surveyed *D. rerio* mutants for morphogenetic similarities to *D. nigrofasciatus*.

We first investigated whether any of several *D. rerio* mutants exhibiting stripes dorsally and spots ventrally, as in *D. nigrofasciatus*, might have similar modes of pigment pattern metamorphosis to *D. nigrofasciatus*. Examination of one of these mutants, *ednrb1* (Parichy et al., 2000a), revealed little contribution of early

larval melanophores to the adult pigment pattern, unlike in *D. nigrofasciatus* (Fig. 9A-D, and data not shown). Thus, a similarity of pigment pattern elements does not predict the underlying mode of pigment pattern metamorphosis.

We next examined additional *D. rerio* mutants for similarities to *D. nigrofasciatus*. We identified two recessive *D. rerio* mutants, *puma* and *picasso*, with severe deficits in metamorphic melanophores, as well as increased persistence of early larval melanophores into the adult (Fig. 9E-L) (Parichy and Turner, 2003b). To see whether *puma* or *picasso* contribute to the difference between species, we tested whether early larval melanophore fates and adult stripe compositions in tester hybrids differ from those of control hybrids. Repeated imaging of hybrid larvae reveals that early larval melanophores make little contribution to the adult stripes in control (wild-type) hybrids (Fig. 9M-P; *n*=9), tester *picasso* hybrids, or tester

hybrids for two additional mutants, *pissarro* and *seurat* (*n*=4,11 and 6, respectively; data not shown). In tester *puma* hybrids (*n*=9), however, a greater number of early larval melanophores leave their initial positions along the myotome edges and relocalize over the flank (Fig. 9Q-T), similar to *puma* mutants and *D. nigrofasciatus* (Fig. 9E-L, Fig. 3F-J). These hybrids have somewhat reduced numbers of metamorphic melanophores initially, but this deficit is regulated by adult stages so that adults have total melanophore complements that are indistinguishable from control hybrids. The developmental non-complementation phenotype of tester *puma* hybrids identifies *puma* or genes in its pathway as candidates for contributing to the evolutionary reduction of metamorphic melanophores, and increased contribution of early larval melanophores to the *D. nigrofasciatus* adult pigment pattern.

These results indicate that differences in total numbers of adult melanophores between *D. rerio* and *D. nigrofasciatus* are not likely to be explained by differences at loci already isolated as *D. rerio* melanophore mutants. Moreover, similarity of adult pigment pattern alone is not a good predictor for the underlying mode of pigment pattern metamorphosis. By contrast, interspecific complementation tests for melanophore morphogenesis suggest a role for *puma* or its pathway in determining the relative contributions of metamorphic melanophores and neural crest-derived early larval melanophores to the adult pigment patterns of *D. rerio* and *D. nigrofasciatus*.

### Reduction of metamorphic melanophore lineage in *D. nigrofasciatus*

The reduction in metamorphic melanophores in *D. nigrofasciatus* could reflect a failure to recruit committed melanophore precursors (melanoblasts) from uncommitted latent precursors or stem cells during metamorphosis. For example, *puma* mutant *D. rerio* exhibit severe reductions in metamorphic melanoblasts compared with wild-type *D. rerio* (Parichy et al., 2003). If the same pathway affected in *puma* mutant *D. rerio* has evolved between *D. rerio* and *D. nigrofasciatus*, then fewer melanoblasts should be observed in *D. nigrofasciatus* compared with wild-type *D. rerio*. Alternatively, fewer metamorphic melanophores in *D. nigrofasciatus* could reflect a later block in this lineage, with similar numbers of melanoblasts being recruited from latent precursors then failing to terminally differentiate as melanophores. To distinguish between these possibilities, we used molecular markers and histological assays to compare *D. rerio* and *D. nigrofasciatus* during metamorphosis.

Our examination of the melanophore lineage during metamorphosis reveals a severe reduction in the number of melanoblasts in *D. nigrofasciatus*, suggesting an early block in metamorphic melanophore development. We examined the distribution of cells expressing transcripts for two molecular markers, *dopachrome tautomerase* (*dct*) and *tyrosinase* (*tyr*), which encode enzymes required for melanin synthesis and thus identify committed melanophore precursors (as distinct from latent stem cells) (Kelsh et al., 2000; Camp and Lardelli, 2001). We observed fewer $dct^+$ and $tyr^+$ cells throughout metamorphosis in *D. nigrofasciatus* compared to *D. rerio* (Fig. 10). Importantly, however, we observed strong staining for each marker in fully differentiated melanophores, and in the more rare, unmelanized cells, in *D. nigrofasciatus*, demonstrating the efficacy of these probes in this cross-species comparison.

From molecular marker analyses, we predicted that metamorphosing *D. nigrofasciatus* would exhibit fewer cells that were competent to produce melanin when provided with exogenously supplied L-dopa, which is converted to melanin in cells expressing functional tyrosinase (McCauley et al., 2004). Comparing the same regions of the flank before and after L-dopa incubation, we observe newly melanized cells in *D. rerio* larvae, but only ~10% as many of these cells in *D. nigrofasciatus* larvae (Fig. 11). These findings support the conclusion that *D. nigrofasciatus* exhibit fewer melanoblasts during metamorphosis than *D. rerio* do.

Together, these analyses demonstrate that reduced numbers of metamorphic melanophores in *D. nigrofasciatus* result from an early block in the specification of these cells or their recruitment from latent precursors, rather than from a late block in their terminal differentiation as melanophores.

**Fig. 6.** Primary role for metamorphic melanophores in adult pigment pattern formation across species. Shown are repeated images of the same region of the ventral flanks in representative individuals of *D. nigrofasciatus*, *D. rerio*, *D. kyathit*, *D. kerri*, *D. albolineatus*, *D. choprae*, and *T. albonubes* (compare with Fig. 1). Only selected images are shown from the complete series for each individual. Row 1, shortly after the onset of pigment pattern metamorphosis in each species. Row 8, terminal stages of pigment pattern metamorphosis when the adult pigment patterns have formed; row 8′, schematics showing melanophores present at early larval stages (brown) and melanophores that differentiated during metamorphosis (black), as revealed by tracing individual melanophores throughout pigment pattern metamorphosis. Squares indicate the horizontal myoseptum; horizontal arrowheads indicate the ventral aspect of the myotome. In *D. nigrofasciatus*, numerous early larval melanophores relocate (arrowheads) from the early larval stripe along the ventral aspect of the myotome (horizontal arrowhead, row 1) to the adult ventral primary melanophore stripe on the flank (row 8,8′). In *D. rerio* and *D. kyathit*, early larval melanophores typically do not contribute to the compact stripes of the adult. In *D. kerri*, a more diffuse stripe pattern arises compared with in *D. nigrofasciatus*, *D. rerio* and *D. kyathit*; although a few early larval melanophores leave their initial positions (arrowheads, row 5), they typically do not enter into the adult stripes. In *D. albolineatus*, rare early larval melanophores leave the larval stripes (arrowhead, row 6) but do not contribute substantially to the uniformly dispersed anterior melanophores or weak melanophore stripes posteriorly. In *D. choprae*, a few early larval melanophores leave the larval stripes (arrowheads, row 7) but do not join the horizontal adult stripes that form during metamorphosis, or the vertical barring pattern that develops at later stages; the same early larval melanophore behaviors are seen in the vertically striped *D. shanensis* (I.K.Q. and D.M.P., unpublished). Finally, in *T. albonubes*, a few early larval melanophores (arrowheads, row 6) leave the larval stripes but do not move far onto the flank where diffuse horizontal adult stripes develop in the adult. In all panels, larvae were imaged at a 30° angle to better visualize the ventral-lateral margin of the flank and the early larval melanophores, and images are rescaled to show the same region of the flank. Slight differences in starting pigment patterns (row 1) principally reflect inter-individual variation and minor differences in developmental stage. *nigrof*, *D. nigrofasciatus*; *alb*, *D. albolineatus*; *T. alb*, *T. albonubes*. Number of larvae examined: *D. nigrofasciatus*, 10; *D. rerio,* 5; *D. kyathit*, 5; *D. kerri*, 2; *D. albolineatus*, 4; *D. choprae*, 2; *T. tanichthys*, 4. Overall contributions of embryonic neural crest-derived melanophores and metamorphic melanophores are similar in other regions of developing adult pigment patterns (data not shown).

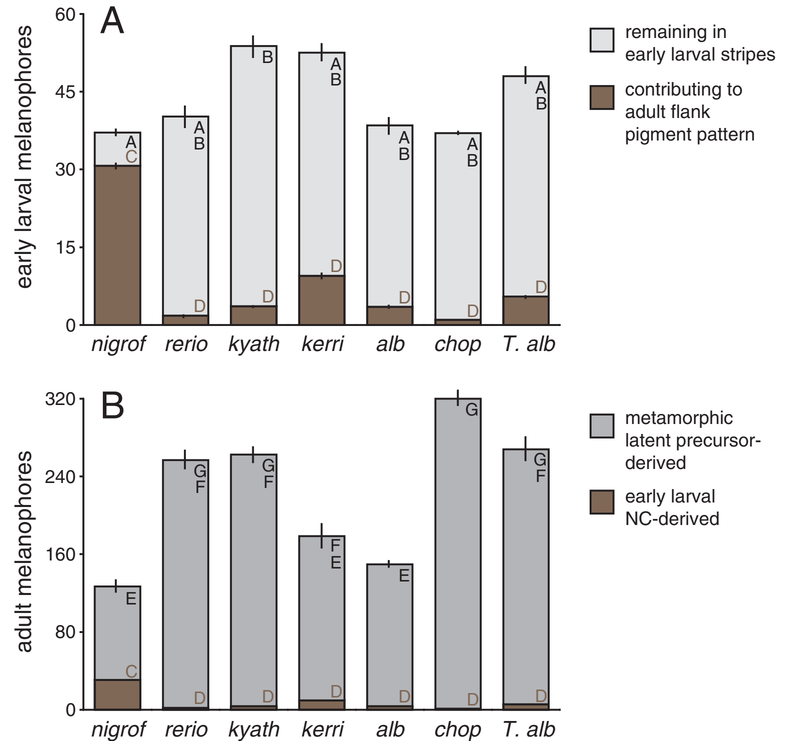


**Fig. 7.** Different fates of early larval melanophores, and variation in adult melanophore origins across species. All values are means±s.e.m. (A) Total numbers of early larval melanophores differ somewhat across species ($F_{6,25}$=4.44, $P$<0.005); black letters within bars indicate post-hoc Tukey comparisons of means and bars sharing the same letter do not differ significantly, thus only *D. nigrofasciatus* and *D. kyathit* differ significantly from one another. Total early larval melanophores for each species comprise melanophores that remain in the early larval pigment pattern during metamorphosis (light gray), and melanophores that leave the early larval stripes during metamorphosis and localize further laterally over the flank (brown). Different proportions of early larval melanophores leave the adult stripes in the different species (arcsine transformed proportions, $F_{6,25}$=41.88, $P$<0.0001). However, post-hoc means comparisons of numbers and proportions indicate that *D. nigrofasciatus* alone differs significantly from other species (brown letters within bars). (B) Pigment patterns after metamorphosis differ markedly in total melanophore numbers across species ($F_{6,25}$=18.93, $P$<0.0001). In all species, a majority of melanophores in the adult pigment pattern are metamorphic melanophores. Numbers of early larval neural crest-derived melanophores in the adult pattern are the same as in A. In adult pigment patterns, the proportions of early larval melanophores to metamorphic melanophores differ significantly among species (arcsine transformed proportions, $F_{6,25}$=54.56, $P$<0.0001), yet only *D. nigrofasciatus* differs significantly from other species in post-hoc means comparisons. *nigrof*, *D. nigrofasciatus*; *kyath*, *D. kyathit*; *alb*, *D. albolineatus*; *T. alb*, *T. albonubes*.

## Differences between *D. rerio* and *D. nigrofasciatus* are non-autonomous to melanophore lineages

The different modes of pigment pattern metamorphosis in *D. rerio* and *D. nigrofasciatus* could reflect evolutionary changes that are intrinsic (autonomous) or extrinsic (non-autonomous) to melanophore lineages. Although species differences have been attributed to intrinsic factors (Twitty and Bodenstein, 1939; Rawles, 1948; Schneider and Helms, 2003), the extensive migrations and cellular interactions during neural crest and melanophore development imply many opportunities for extrinsic factors to generate differences in form as well (Erickson and Perris, 1993; Parichy, 1996; Halloran and Berndt, 2003). To distinguish between these possibilities, we examined melanophore behaviors and patterns in genetic mosaics. These analyses demonstrate a primary role for extrinsic factors in determining early larval melanophore contributions to adult stripes, as well as the positions of adult stripes on the flank.

We transplanted cells from *D. nigrofasciatus* to *D. rerio*, and then reared chimeras through metamorphosis (Parichy and Turner, 2003a; Parichy et al., 2003). To identify donor *D. nigrofasciatus* melanophores, we used *D. rerio* hosts mutant for the *albino* locus, which acts autonomously to the melanophore lineage to promote melanization, but does not otherwise affect melanophore development or pigment pattern formation (Lin et al., 1992); *D. nigrofasciatus* melanophores thus appear as the only melanized cells in a field of unmelanized but otherwise normal melanophores (Lin et al., 1992; Parichy et al., 1999; Kelsh et al., 2000). To assess the mode of pigment pattern metamorphosis, we identified chimeras that developed *D. nigrofasciatus* early larval melanophores, then we repeatedly imaged these individuals through metamorphosis.

**Fig. 8.** Adult hybrid phenotypes exclude major-effect roles for genes isolated as *D. rerio* melanophore mutants. (A) Control (wild-type) *D. rerio*×*D. nigrofasciatus* hybrids develop adult dorsal and ventral primary melanophore stripes (large arrow) with melanophore numbers similar to those of *D. rerio*, but with fewer total stripes and fewer melanophores in the secondary melanophore stripes (small arrow) that develop as the fish grow (Parichy and Johnson, 2001). (B) Detail of dorsal primary melanophore stripe. (C) Tester hybrid for the *tfap2a* (*lockjaw*) mutant, a sibling to the hybrid in A. Despite the absence of melanophores in *tfap2a* mutant *D. rerio* (Knight et al., 2004), tester hybrids have as many melanophores as control hybrids, suggesting that *tfap2* does not contribute substantially to the different numbers of melanophores between wild-type *D. rerio* and *D. nigrofasciatus*. Minor individual variation in secondary melanophore and stripe numbers does not segregate with *tfap2* alleles (data not shown). (D) Detail of dorsal primary melanophore stripe in tester hybrid, showing a similar number of melanophores to the control in B.

We predicted that if species differences are autonomous to the melanophore lineages, then donor *D. nigrofasciatus* early larval melanophores should contribute to the adult ventral melanophore stripe (as in *D. nigrofasciatus*); if differences between species are non-autonomous to the melanophore lineages, then donor *D. nigrofasciatus* early larval melanophores should fail to contribute to this stripe (as in *D. rerio*). Fig. 12A-D shows a representative *D. nigrofasciatus*→*D. rerio* chimera. Donor *D. nigrofasciatus* early larval melanophores are present within the early larval stripe along the ventral myotomes but do not contribute to the adult ventral stripe. Thus, early larval melanophore morphogenesis resembles that of *D. rerio* rather than that of *D. nigrofasciatus* (compare with Fig. 3). Moreover, *D. nigrofasciatus* melanophores that differentiated during metamorphosis did so at the normal location of *D. rerio* stripes, rather than further ventrally as in *D. nigrofasciatus* (compare with Fig. 3). These findings indicate that factors non-autonomous to the melanophore lineages determine species differences in early larval melanophore contributions to adult stripes, as well as the positions of adult stripes. These results also obviate the identification of other donor *D. nigrofasciatus* cells in *D. rerio* hosts, as the final distributions of donor melanophores cannot easily be explained by a simple coincidence of *D. nigrofasciatus* melanophores and other *D. nigrofasciatus* donor tissues (which might have explained the alternative result, had donor melanophores behaved like their own, donor species, rather than the host species).

In reciprocal *D. rerio*→*D. nigrofasciatus* chimeras ($n$>40), we never observed donor *D. rerio* melanophores in *D.*

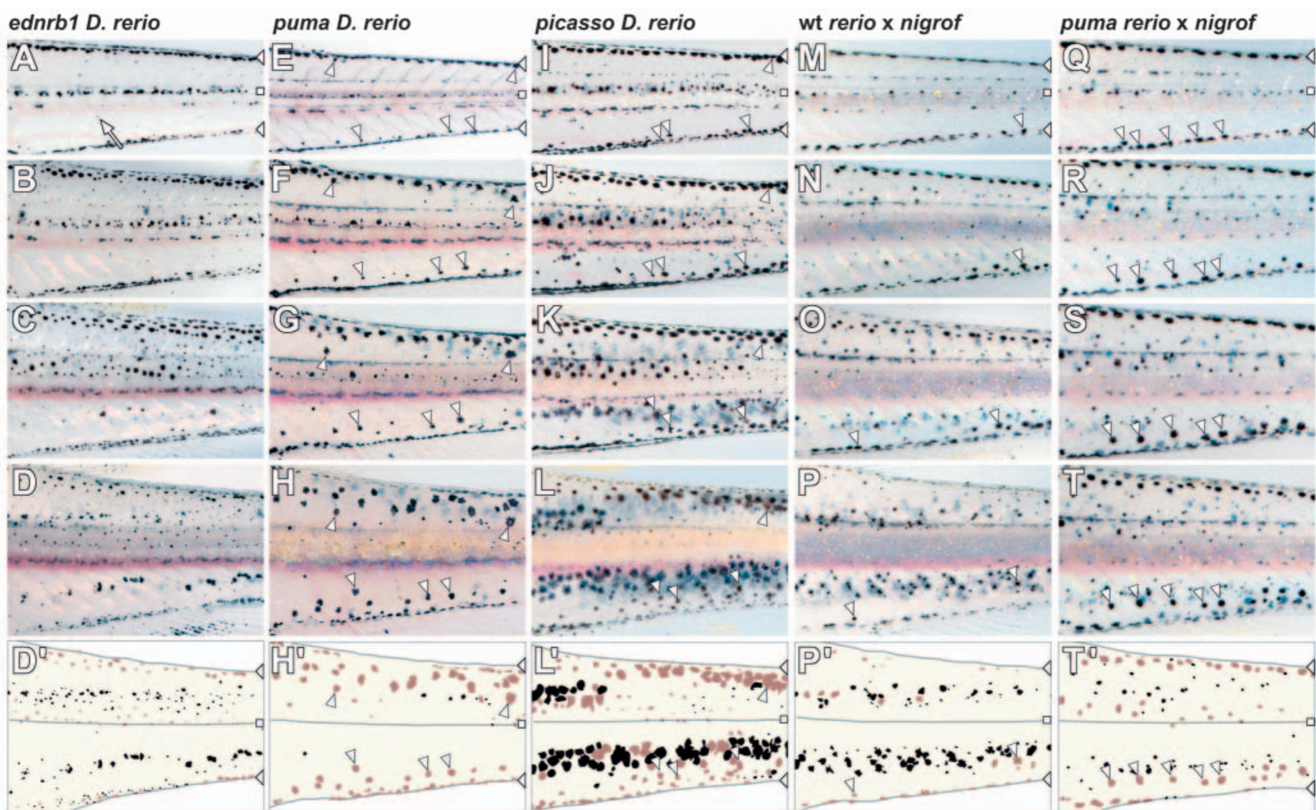


**Fig. 9.** *Danio rerio* mutants exclude and identify pathways for evolutionary changes in *D. nigrofasciatus*. Shown are selected images of representative larvae that were imaged throughout pigment pattern metamorphosis. Schematics (bottom row) illustrate the locations of early larval melanophores (brown) and metamorphic melanophores (black), as determined by the tracing of individual cells from the early larval pigment pattern into the adult pigment pattern (dorsal scale-associated melanophores are omitted for clarity). (A-D) *ednrb1* mutant *D. rerio* develop an adult pattern of stripe and spots, superficially similar to *D. nigrofasciatus* (Parichy and Johnson, 2001). Nevertheless, the underlying mode of pigment pattern metamorphosis differs from *D. nigrofasciatus*, as few early larval melanophores contribute to the developing adult stripes. Arrow in A indicates a newly differentiated metamorphic melanophore. (E-H) *puma* mutant *D. rerio* exhibit a severe reduction in metamorphic melanophore numbers, whereas early larval melanophores (arrowheads) spread laterally over the flank, similar to *D. nigrofasciatus*. (I,J) *picasso* mutant *D. rerio* also have fewer metamorphic melanophores, and increased persistence of early larval melanophores (arrowheads). (M-P) Hybrids between *D. rerio* and *D. nigrofasciatus* exhibit fewer metamorphic melanophores than *D. rerio*, yet early larval melanophores only rarely contribute to the adult stripes (arrowheads), similar to *D. rerio* but unlike *D. nigrofasciatus* (compare with Fig. 3E,J). A few early larval melanophores at the horizontal myoseptum persist into the adult pigment pattern (as in *D. rerio*), but early larval melanophores along the ventral myoseptum typically do not join the developing adult ventral primary melanophore stripe (as in *D. rerio*, but unlike *D. nigrofasciatus*). (Q-T) Hybrids between *puma* mutant *D. rerio* and *D. nigrofasciatus* exhibit early larval melanophore behaviors similar to those seen in *D. nigrofasciatus*. Although a few early larval melanophores leave their initial positions in control hybrids (P′), these cells are increased in number in *puma* tester hybrids, particularly among melanophores in the vicinity of the anal fin (arrowheads, T). Sites of early larval melanophore stripes are indicated at the dorsal and ventral margins of the myotomes (horizontal arrowheads), and at the horizontal myoseptum (squares) in A, E, I, M and Q.

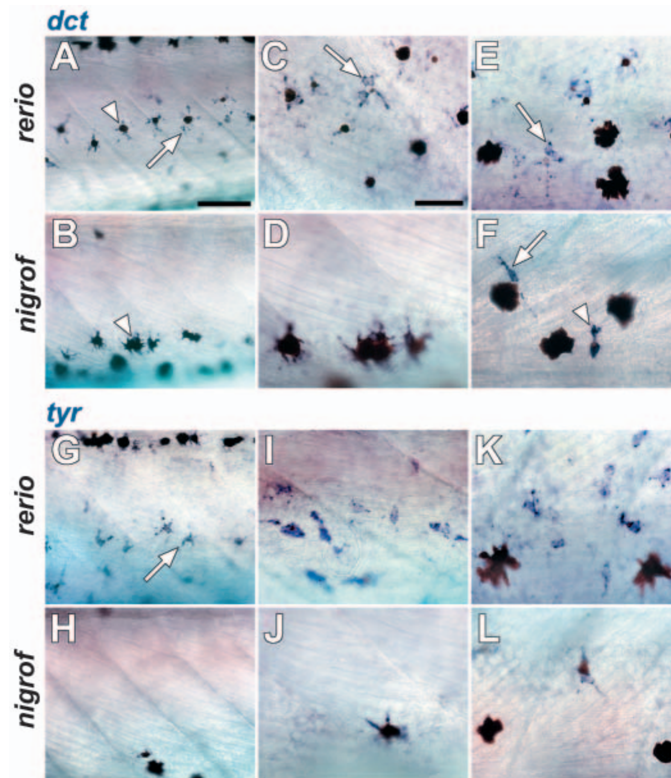


**Fig. 10.** Fewer metamorphic melanophore precursors in *D. nigrofasciatus* revealed by in situ hybridization for the melanoblast markers *dct* (A-F) and *tyr* (G-L). (A) During the early stages of pigment pattern metamorphosis in *D. rerio* (e.g. Fig. 3B), primary metamorphic melanophores (arrowhead) differentiate over the middle of each ventral myotome; these and a few unmelanized cells (arrow) stain for *dct*. (B) The corresponding region in *D. nigrofasciatus* is devoid of primary metamorphic melanophores and unmelanized *dct*$^+$ cells, although the early larval melanophores located further ventrally are *dct*$^+$ (arrowhead). (C) At middle metamorphic stages in *D. rerio* (e.g. Fig. 3C,D), unmelanized (arrow) and melanized *dct*$^+$ cells are abundant over the ventral myotome in the vicinity of the ventral primary melanophore stripe. (D) In the corresponding region of *D. nigrofasciatus*, only melanized cells express detectable levels of *dct*, even after overdevelopment (data not shown). (E) At middle metamorphic stages in *D. rerio*, unmelanized *dct*$^+$ melanoblasts (arrow) are abundant in the vicinity of the dorsal primary melanophore stripe. (F) In *D. nigrofasciatus*, unmelanized *dct*$^+$ cells (arrow) are infrequent compared with *D. rerio*, although a few are present and differentiate as melanophores (arrowhead), showing melanin in addition to *dct* staining. (G-L) Staining for *tyr* expression is similar to staining for *dct*. Shown are similar stages and positions to the corresponding panels in A-F. Scale bars: 100 µm for A,B,G,H; 40 µm for C-F,I-L.

*nigrofasciatus* hosts, although we frequently observed other donor tissues (e.g. myotomes, nerves, skin; data not shown). Similar studies in *D. rerio* yielded comparable frequencies of these tissues, but also melanophores (Parichy and Turner, 2003a; Parichy et al., 2003). These data suggest that *D. nigrofasciatus* hosts may be compromised in their ability to support *D. rerio* donor melanophores, supporting a model in which species differences in metamorphic melanophore differentiation are non-autonomous to this lineage as well.

Finally, we investigated whether evolutionary changes in interactions between melanophores themselves might contribute to the different metamorphic modes between species. We reasoned that a reduction in the numbers of metamorphic melanophores, and thus reduced contact inhibition of movement (Tucker and Erickson, 1986), might allow early larval melanophores to leave their initial positions during metamorphosis in *D. nigrofasciatus*. To test this possibility, we transplanted *D. nigrofasciatus* cells to *nacre* mutant *D. rerio* hosts. *nacre* mutants lack melanophores owing to an inactivating mutation in *mitfa*, which normally acts autonomously to the melanophore lineage (Lister et al., 1999). We predicted that if changes in melanophore-melanophore interactions alone are responsible for species differences, then *D. nigrofasciatus* early larval melanophores in *nacre* mutant hosts should contribute to the adult ventral stripe (as in *D. nigrofasciatus*). If other factors contribute to the species differences, the *D. nigrofasciatus* early larval melanophores should fail to contribute to this stripe (as in *D. rerio*). Fig. 12F-I shows a *D. nigrofasciatus*→*nacre* mutant *D. rerio* chimera. Repeated imaging demonstrates that donor *D. nigrofasciatus* early larval melanophores do not contribute to the adult ventral stripe, which forms at a position similar to that seen in *D. rerio*. These data demonstrate that factors extrinsic to melanophore lineages contribute to differences in pigment pattern metamorphosis between *D. rerio* and *D. nigrofasciatus*.

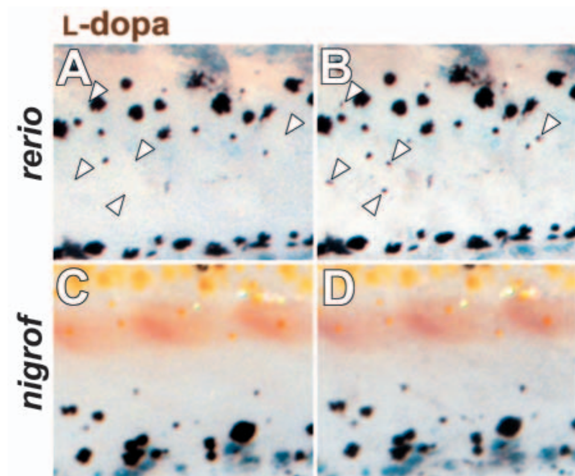


**Fig. 11.** L-dopa staining for tyrosinase activity reveals fewer melanoblasts in *D. nigrofasciatus* compared with in *D. rerio*. Shown are larvae during middle stages of pigment pattern metamorphosis before (A,C) and after (B,D) incubation with L-dopa. (A,B) In *D. rerio*, melanoblasts are revealed by new melanin deposition (arrowheads show locations of cells before and after incubation). (C,D) In *D. nigrofasciatus*, few melanoblasts are revealed in general, and no new cells are observed in the region shown.

## Discussion

Our analyses provide new insights into the generalized features of adult pigment pattern metamorphosis in danios and their relatives, how these patterns evolve, and the derived mode of pigment pattern metamorphosis in *D. nigrofasciatus*. These results suggest a model relating early larval and adult pigment pattern formation in *D. rerio* and other species, and how these processes have been modified in *D. nigrofasciatus* (Fig. 13).

## Ancestral role for post-embryonic latent precursors in pigment pattern formation

The cellular and genetic bases for adult form are largely unknown, but elucidating these mechanisms is crucial for a deeper understanding of human development and disease, as well as the evolution of morphology. Of particular interest are traits derived from the neural crest, given the many genetic disorders and cancers associated with these cells (Chin, 2003; Farlie et al., 2004), and their major role in vertebrate diversification (Gans and Northcutt, 1983; Hall, 1999). At least two extreme models can explain the forms taken by adult traits derived from the neural crest. First, the particular features of such traits could be determined entirely by patterning mechanisms during embryogenesis, while neural crest cells are migrating, or shortly after their arrival at target tissues. Second, forms taken by adult neural crest derivatives could depend not on neural crest cells themselves, but on the patterning of post-embryonic neural crest-derived latent precursors or stem cells resident in many tissues (Bixby et al., 2002; Kruger et al., 2002; Nishimura et al., 2002; Sieber-Blum and Grim, 2004; Sieber-Blum et al., 2004; Joseph et al., 2004).

Our results suggest a major role for latent precursors, presumptively of neural crest origin, during the development of adult pigment patterns in danios and their relatives (Fig. 13). Lineage analyses revealed that in each species (except *D. nigrofasciatus*), adult pigment patterns were formed principally by metamorphic melanophores derived from latent precursors, rather than by early larval melanophores derived from neural crest cells during embryogenesis. The prevalence of this mode of pigment pattern metamorphosis strongly suggests that this is a shared, ancestral trait for *Danio*. To our knowledge, this represents the first systematic survey across species to define the cellular origins for an adult neural crest-derived trait. Previous studies have demonstrated roles for melanocytes derived from stem cells in the development of mammalian pigmentation (Nishimura et al., 2002), and for melanophores derived from latent precursors in pigment pattern formation of some teleosts, including *D. rerio* (Johnson et al., 1995; Sugimoto, 2002; Parichy and Turner, 2003b). Latent precursors probably also generate adult pigment patterns of many amphibians (Parichy, 1998). Likewise, the adult epibranchial cartilage of the salamander *Eurycea bislineata* arises from a discrete population of cells in the perichondrium of the larval neural crest-derived epibranchial cartilage (Alberch and Gale, 1986). Given the presence of post-embryonic neural crest stem cells and specified latent precursors in a variety of tissues (see Introduction), it will be interesting to determine the extent to which other adult traits depend on these cells (as distinct from embryonic neural crest cells) for their initial patterning, maintenance, and repair after injury.

The comparative approach we have taken also implicates

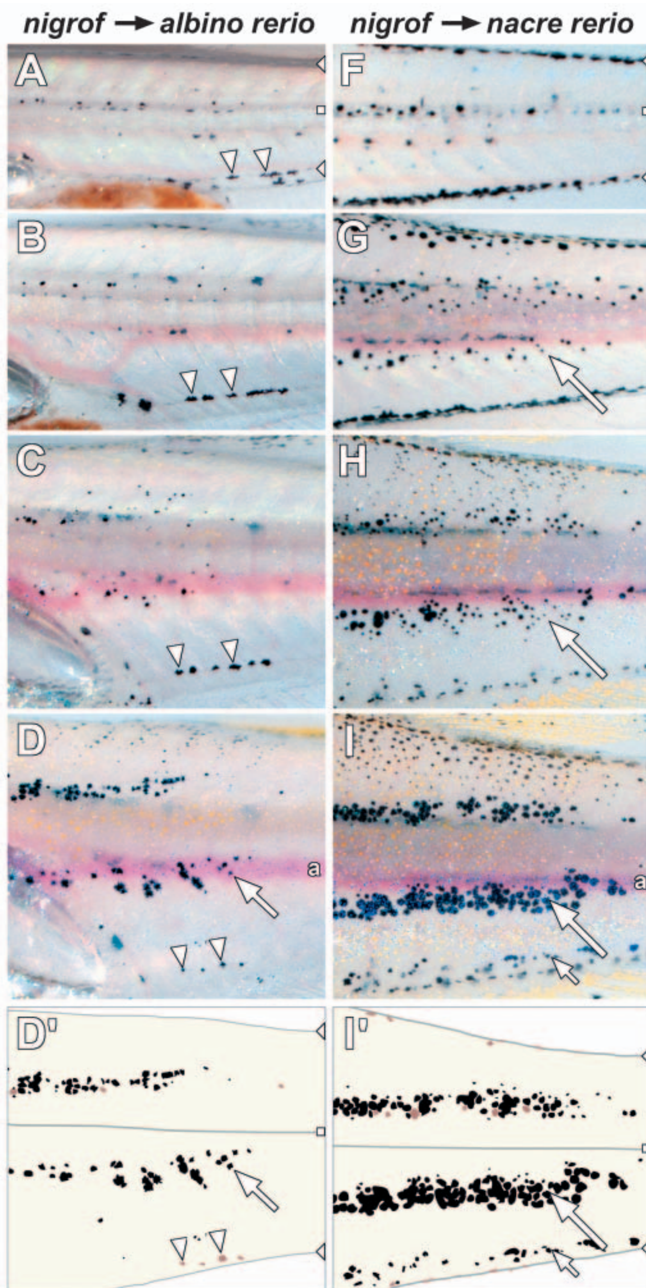


**Fig. 12.** Non-autonomous factors underlying the differences in pigment pattern metamorphosis between *D. rerio* and *D. nigrofasciatus*, revealed by interspecific genetic mosaic analyses. Shown are selected days in the development of two representative chimeras ($n$=10), taken from a complete image series through pigment pattern metamorphosis. (A-D) *D. nigrofasciatus* cells transplanted into *albino* mutant *D. rerio*. Melanized donor melanophores differentiate at embryonic stages within the early larval melanophore stripes (arrowheads, A). Yet these donor melanophores fail to contribute to the ventral primary melanophore stripe, as for host melanophores. Subsequently, donor metamorphic melanophores differentiate over the flank and contribute to adult primary melanophore stripes located at positions that are indistinguishable from host stripes. Arrow in D marks the primary ventral melanophore stripe (a, aorta; compare with Fig. 3E). (F-I) *D. nigrofasciatus* cells transplanted to *nacre* mutant *D. rerio*. Despite the absence of host melanophores, donor early larval melanophores still fail to contribute to the ventral primary melanophore stripe, which forms in the normal position for *D. rerio* (arrows, G-I). In this individual, a secondary adult melanophore stripe comprising late-appearing metamorphic melanophores has started to form ventrally (small arrow, I). Schematics (D′,I′) illustrate the locations of early larval melanophores (brown) and metamorphic melanophores (black), as revealed by following individual cells throughout development. Dorsal scale-associated melanophores are omitted for clarity.

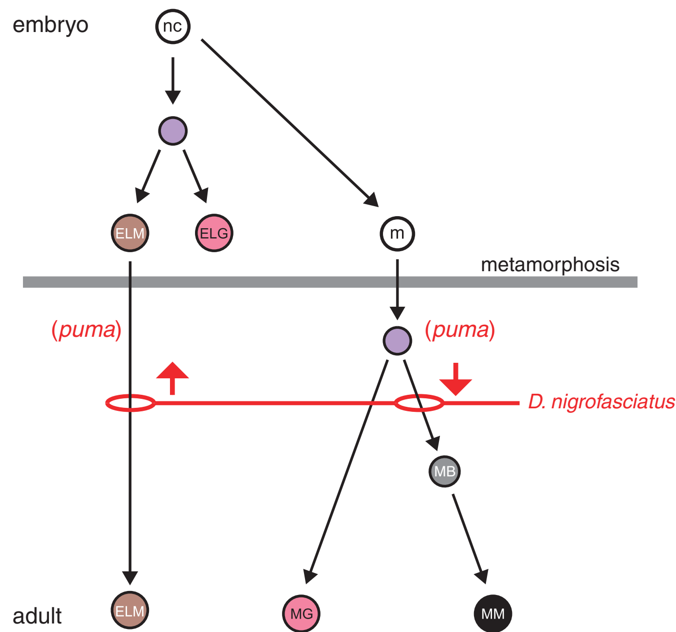


**Fig. 13.** Model for the development of early larval and adult neural crest derivatives. In embryos, neural crest (nc) cells develop into a variety of derivatives, including early larval glial cells (ELG) and early larval melanophores (ELM). Some cells are set aside as post-embryonic stem cells or specified precursors (m) that will be recruited to differentiate during metamorphosis. During the larval-to-adult transformation, metamorphic glia (MG) (Parichy et al., 2003) and metamorphic melanophores (MM) differentiate from latent precursors, with melanophores passing through a melanoblast intermediate (MB, expressing the melanophore lineage markers *dct* and *tyr*; gray circle). Additionally some early larval melanophores can transit the metamorphic boundary and persist into the adult pigment pattern, although relatively few do so in *D. rerio* or in most of the other species examined in this study. In *D. nigrofasciatus*, there has been a reduction (right red arrow) in metamorphic melanophores owing to an early block in this lineage, possibly associated with a *puma*-dependent pathway; concomitantly, there has been an increase (left red arrow) in the number of early larval melanophores persisting into the adult pigment pattern.

post-embryonic, latent precursors of presumptive neural crest origin in the generation of organismal diversity. We examined species exhibiting a variety of adult pigment patterns, including horizontal stripes that are compact (*D. rerio*, *D. kyathit*) or diffuse (*D. kerri*, *T. albonubes*), as well as vertical bars (*D. choprae*) and uniform patterns (*D. albolineatus*). Despite this variation in adults, the larvae of these species exhibit melanophore patterns that are indistinguishable from one another, except for small differences in melanophore numbers (see Fig. S1 in supplementary material; Fig. 7A). Our results demonstrate that much of the pigment pattern diversity of adults reflects interspecific variation in the differentiation and morphogenesis of metamorphic melanophores that are derived from latent precursors or stem cells, rather than the reorganization of embryonic neural crest-derived melanophores. That embryonic/early larval pigment patterns and adult pigment patterns depend on different melanophore lineages suggests a mechanism by which these pigment patterns may be relatively uncoupled across life-cycle stages. Thus, evolutionary responses to selection on the adult pigment pattern may be relatively unconstrained by features of the earlier developing embryonic/early larval pigment pattern, if genetic controls differ to some extent between neural crest-derived and metamorphic melanophore lineages (Haldane, 1932; Ebenman, 1992; Parichy, 1998). Indeed, several *D. rerio* pigment pattern mutants have defects limited to particular embryonic or metamorphic melanophore lineages (Johnson et al., 1995; Parichy et al., 1999; Parichy et al., 2000a; Parichy et al., 2000b; Parichy and Turner, 2003). Nevertheless, the extent of genetic independence across life-cycle stages – and its evolutionary consequences – remains an empirical question that deserves further analysis.

## Evolution of pigment pattern metamorphosis in *D. nigrofasciatus*

A central problem in evolutionary developmental biology is the extent to which similar phenotypes depend on the same or different underlying mechanisms. Several recent analyses have demonstrated the repeated, independent evolution of traits via common underlying genetic changes (Sucena et al., 2003; Mundy et al., 2004; Shapiro et al., 2004). Such cases of evolutionary parallelism suggest that pathways of evolutionary change in development may be more limited than classical evolutionary theory might suggest (Barton and Turelli, 1989). By contrast, other analyses reveal divergent mechanisms underlying repeated trait evolution (Hoekstra and Nachman, 2003; Wittkopp et al., 2003). Despite this recent focus on traits that have evolved independently, we still know little about developmental variation underlying traits having a common evolutionary origin.

Our analyses reveal substantial differences in stripe development between *D. rerio* and *D. nigrofasciatus*, despite the superficial similarity of the final stripes that form, and the close phylogenetic relationship of these species. Cryptic patterning variation has been observed for other traits (Hall, 1984; Minsuk and Keller, 1996; Jungblut and Sommer, 2000), and argues for the importance of a comparative approach in validating conclusions gleaned from studies of model organisms (Parichy, 2005; Bolker, 1995; Metscher and Ahlberg, 1999). Such variation may reflect selection to maintain a particular adult phenotype, in the absence of selection for precisely how this phenotype is achieved. The roles of teleost pigment patterns in predation avoidance, mate recognition, mate choice and shoaling behavior suggests strong selection on adult phenotypes (Endler, 1983; Houde, 1997; Couldridge and Alexander, 2002; Engeszer et al., 2004; Allender et al., 2003); the behavioral roles and selective consequences of early larval and metamorphic pigment patterns remain wholly unexplored.

Adult pigment pattern formation in *D. nigrofasciatus* differs from that of *D. rerio* in having a lesser contribution from metamorphic melanophores, and a correspondingly greater contribution from persisting early larval melanophores (Fig. 13). Thus, *D. nigrofasciatus* may be viewed as exhibiting a heterochronic change in pigment pattern development, with a relatively paedomorphic (or juvenilized) mode when compared with the inferred ancestral condition. This uncoupling of pigment pattern and somatic metamorphosis is somewhat similar to several species and subspecies of salamanders, in which adult spots and stripes appear during the larval stage, prior to somatic metamorphosis (Anderson, 1961; Anderson

and Worthington, 1971; Parichy, 1998). Dissociability of pigment pattern and somatic metamorphosis may be a generalized feature of post-embryonic development in these ectothermic vertebrates.

The reduction of metamorphic melanophores and the persistence of early larval melanophores in *D. nigrofasciatus* also is reminiscent of several *D. rerio* mutants. This concordance highlights the utility of *D. rerio* mutants both for understanding developmental mechanisms within zebrafish, and for framing hypotheses that can be tested across species to dissect mechanisms of evolutionary change. In this study, we examined roles for several of these mutant loci in pigment pattern diversification using interspecific complementation tests (Parichy and Johnson, 2001). Only hybrids between *D. nigrofasciatus* and *puma* mutant *D. rerio* exhibited non-complementation phenotypes, with fewer metamorphic melanophores and increased early larval melanophores persisting into the adult pattern, when compared with control hybrids. This observation raises the possibility that *puma* activity differs between species, and might therefore explain the derived mode of pigment pattern metamorphosis in *D. nigrofasciatus*. Nevertheless, *puma* acts autonomously to the metamorphic melanophore lineage (Parichy et al., 2003), whereas interspecific genetic mosaics constructed in this study reveal differences that are non-autonomous to melanophore lineages (see below). Thus, it seems unlikely that variation at the *puma* locus itself contributes to these species differences. Rather, the non-complementation phenotype may reflect interspecific variation in a sensitized *puma*-dependent pathway. Consistent with this idea, our analyses demonstrate that both *puma* mutant *D. rerio* (Parichy et al., 2003) and *D. nigrofasciatus* exhibit far fewer metamorphic melanophore precursors than wild-type *D. rerio*. These findings imply a change in the early development or specification of the metamorphic melanophore lineage in *D. nigrofasciatus*. This early block differs from the situation found in several species of *Astyanax* cave fish (McCauley et al., 2004) and *D. albolineatus* (Quigley et al., 2005), in which melanophore numbers are reduced owing to a later block in this lineage, such that melanoblasts develop but then fail to differentiate or survive. Examination of additional species should allow a more complete reconstruction of the evolutionary history of melanophore patterns and melanophore lineage modifications across taxa.

Besides the reduction in metamorphic melanophores, *D. nigrofasciatus* exhibit a dramatic increase in the contribution of early larval melanophores to the adult pigment pattern. By examining melanophore behaviors and patterns in interspecific genetic mosaics, we demonstrate that factors non-autonomous to melanophore lineages determine the different behaviors of these cells between species. This contrasts with several studies that have identified changes autonomous to neural crest or melanophore lineages in determining species differences (Twitty and Bodenstein, 1939; Twitty, 1945; Rawles, 1948; Epperlein and Löfberg, 1990; Schneider and Helms, 2003), although some of these results are open to alternative interpretations (Parichy, 1996; Parichy, 2001). Our findings suggest that the relative roles of intrinsic and extrinsic factors differ both across species and across traits, and it is too simplistic to ascribe evolutionary changes to intrinsic factors alone.

At least two models can be suggested for the non-autonomous factors contributing to the differences in early larval melanophore morphogenesis between *D. rerio* and *D. nigrofasciatus*. First, early larval melanophores could reorganize in *D. nigrofasciatus* owing to the reduced numbers of metamorphic melanophores, which might otherwise prevent the cells from leaving their original positions within the early larval stripes by contact inhibition of movement (Tucker and Erickson, 1986). This explanation would suggest a relatively simple pattern regulatory process, such that early larval melanophores fill gaps resulting from reduced numbers of metamorphic melanophores (Milos and Dingle, 1978b). Our examination of genetic mosaics between *D. nigrofasciatus* and *nacre* mutant *D. rerio*, which lack melanophores, excludes the loss of interactions between metamorphic melanophores and early larval melanophores as being the sole factor underlying the difference between species. Second, early larval melanophores could reorganize owing to changes in other factors in the extracellular environment. Consistent with this idea, the adult ventral melanophore stripe develops closer to the myotome edge in *D. nigrofasciatus* than in *D. rerio*. Conceivably, *D. nigrofasciatus* early larval melanophores may be close enough to respond to stripe-forming cues that *D. rerio* early larval melanophores do not encounter because these cues are situated further dorsally on the flank. The difference between species also may reflect multiple partially redundant changes in factors extrinsic to the melanophore lineage. These possibilities are now being addressed by additional cell transplantation studies and by seeking the nature of the stripe-forming cues themselves.

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### Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/131/24/6053/DC1

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