Evolutionary diversification of pigment pattern in *Danio* fishes: differential *fms* dependence and stripe loss in *D. albolineatus*

Ian K. Quigley*, Joan L. Manuel*, Reid A. Roberts*, Richard J. Nuckels, Emily R. Herrington, 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

*These authors contributed equally to this work
†Author for correspondence (e-mail: dparichy@mail.utexas.edu)

Accepted 28 October 2004

Development 132, 89-104
Published by The Company of Biologists 2005
doi:10.1242/dev.01547

Summary

The developmental bases for species differences in adult phenotypes remain largely unknown. An emerging system for studying such variation is the adult pigment pattern expressed by *Danio* fishes. These patterns result from several classes of pigment cells including black melanophores and yellow xanthophores, which differentiate during metamorphosis from latent stem cells of presumptive neural crest origin. In the zebrafish *D. rerio*, alternating light and dark horizontal stripes develop, in part, owing to interactions between melanophores and cells of the xanthophore lineage that depend on the *fms* receptor tyrosine kinase; zebrafish *fms* mutants lack xanthophores and have disrupted melanophore stripes. By contrast, the closely related species *D. albolineatus* exhibits a uniform pattern of melanophores, and previous interspecific complementation tests identified *fms* as a potential contributor to this difference between species. Here, we survey additional species and demonstrate marked variation in the *fms*-dependence of hybrid pigment patterns, suggesting interspecific variation in the *fms* pathway or *fms* requirements during pigment pattern formation. We next examine the cellular bases for the evolutionary loss of stripes in *D. albolineatus* and test the simplest model to explain this transformation, a loss of *fms* activity in *D. albolineatus* relative to *D. rerio*. Within *D. albolineatus*, we demonstrate increased rates of melanophore death and decreased melanophore migration, different from wild-type *D. rerio* but similar to *fms* mutant *D. rerio*. Yet, we also find persistent *fms* expression in *D. albolineatus* and enhanced xanthophore development compared with wild-type *D. rerio*, and in stark contrast to *fms* mutant *D. rerio*. These findings exclude the simplest model in which stripe loss in *D. albolineatus* results from a loss of *fms*-dependent xanthophores and their interactions with melanophores. Rather, our results suggest an alternative model in which evolutionary changes in pigment cell interactions themselves have contributed to stripe loss, and we test this model by manipulating melanophore numbers in interspecific hybrids. Together, these data suggest evolutionary changes in the *fms* pathway or *fms* requirements, and identify changes in cellular interactions as a likely mechanism of evolutionary change in *Danio* pigment patterns.

Key words: Zebrafish, Pigment pattern, Morphogenesis, Neural crest, *fms*, Csf1r, Xanthophore, Melanophore, Phylogeny, Evolution

Introduction

An outstanding challenge for developmental biology is to elucidate the mechanisms underlying adult form, and how changes in these mechanisms produce variation within and between species. Over the last few years, substantial progress has been made in identifying genes associated with major morphological differences (Galant and Carroll, 2002; Tanaka et al., 2002; Shapiro et al., 2004). Nevertheless, determining how genetic differences are translated into morphological differences will require a thorough understanding of how cellular behaviors are altered in different genetic backgrounds. Of particular interest are the mechanisms underlying variation in traits that have relevance to human health and disease, clear adaptive significance in nature, or both.

One useful system for studying the genetic and cellular bases for variation in adult form is the pigment pattern expressed by *Danio* fishes (Parichy, 2003; Kelsh, 2004; Quigley et al., 2004). These patterns differ dramatically across species, and include horizontal stripes, vertical bars, spots, and uniform patterns resulting from the arrangements of several classes of pigment cells, including black melanophores, yellow-orange xanthophores and reflective iridophores. Pigment cells in teleosts and other vertebrates are derived from neural crest cells, which also contribute to neurons and glia of the peripheral nervous system, bone and cartilage of the craniofacial skeleton, adrenal chromaffin cells, endocardial cushion cells, and other tissues (Hörstadius, 1950; Smith et al., 1994; Le Douarin, 1999). Neural crest-derived lineages are associated with a variety of human disease syndromes (Matthay, 1997; Amiel and Lyonnet, 2001; Ahlgren et al., 2002; Widlund and Fisher, 2003; Farlie et al., 2004) and have had major roles in the diversification of vertebrates (Gans and Northcutt, 1983; Hall, 1999). Besides serving as a potential model for development and evolution of other neural crest-

derived traits, pigment patterns are especially interesting because of their ecological and behavioral significance, with teleost pigment patterns having roles in shoaling, mate recognition, mate choice and predator avoidance (Endler, 1983; Houde, 1997; Couldridge and Alexander, 2002; Allender et al., 2003; Engeszer et al., 2004).

One approach to identifying the genetic and cellular bases for pigment pattern diversity in danios has used hybrids between zebrafish, *D. rerio*, and other danio species (Parichy and Johnson, 2001; Quigley et al., 2004). Wild-type *D. rerio* exhibit four to five melanophore stripes (Fig. 1A,D). When crossed with other danios, hybrid offspring develop pigment patterns that typically resemble *D. rerio* more closely than the heterospecific danio. This finding suggested that complementation tests could be used to screen loci identified as recessive *D. rerio* pigment pattern mutants for contributions to pigment pattern differences between species: mutants for which hybrids have pigment patterns different from controls identify genes that may differ between species and thus identify candidates for further analyses.

A previous study used interspecific hybrids to investigate the genetic bases for the evolutionary loss of stripes in *D. albolineatus*, in which pigment cells are nearly uniformly dispersed (Fig. 1B,E) (Parichy and Johnson, 2001). Hybrids between wild-type *D. rerio* and *D. albolineatus* develop stripes similar to *D. rerio* but unlike *D. albolineatus*. By contrast, one of ten mutant loci tested yielded a non-complementation phenotype in which hybrids lacked stripes like *D. albolineatus*. This locus was identified as *fms*, which encodes a type III receptor tyrosine kinase (Parichy et al., 2000b) known previously for roles in hematopoiesis and osteoclast development (Yoshida et al., 1990; Stanley et al., 1997; Dai et al., 2002; Scheijen and Griffin, 2002; Barreda et al., 2004). Analyses of *D. rerio fms* mutants (Fig. 1C) demonstrate that *fms* promotes the development of a late-appearing population of adult melanophores that differentiates from latent stem cells during the larval-to-adult transformation, or metamorphosis (Parichy et al., 2000b). *fms* also is essential for melanophore survival and migration into stripes, although melanophores themselves do not detectably express *fms*. Rather, *fms* is expressed by cells of the xanthophore lineage and is essential for recruiting xanthophores from latent precursors. In turn, interactions between melanophores and *fms*-dependent cells of the xanthophore lineage are required for melanophore stripe formation (Parichy and Turner, 2003a).

In this study, we test whether changes in *fms* or *fms*-dependent cell lineages underlie pigment pattern differences between *D. rerio* and *D. albolineatus*, as well as other danios (Parichy and Johnson, 2001). We first identify additional species for which pigment patterns of hybrids depend on *fms*, and show that stripe loss in *D. albolineatus* hybrids depends on *fms* and other modifier loci. We next ask whether pigment pattern development in *D. albolineatus* resembles that of *fms* mutant *D. rerio*, as would be predicted by the simplest model in which a loss of *fms* activity has contributed to the evolutionary loss of stripes in *D. albolineatus*. We find that melanophore deficits and behaviors in *D. albolineatus* are similar to *fms* mutant *D. rerio*, yet *D. albolineatus* exhibit a dramatic increase – rather than a decrease – in xanthophore numbers. These findings reject the simplest model in which stripe loss in *D. albolineatus* depends on a loss of *fms* activity and a corresponding loss of the xanthophore lineage. Finally, we use interspecific hybrids to test an alternative model in which evolutionary changes in pigment cell interactions are responsible for stripe loss. Together these results identify interspecific variation in the *fms* pathway or cellular requirements for *fms* activity, and support a model in which evolutionary changes in pigment patterns depend in part on alterations in melanophore interactions.

Materials and methods

Fish stocks and rearing conditions

Fish were reared at 28.5°C (14 hour light: 10 hour dark). *Danio rerio* were the inbred mapping strain AB^{ut} or a mixed genetic background comprising AB^{ut}, wik^{ut}, ekkwill and other stocks. *Danio* aff. *albolineatus*, *D. choprae*, *D.* aff. *dangila*, *D.* 'hikari' and *D.* aff. *kyathit* were from Transship Discounts (Jamaica, NY, USA). The precise taxonomic status of the stocks is uncertain. *Danio albolineatus* were derived from stocks provided by M. McClure (Cornell University).

Complementation tests between *D. rerio* and heterospecific danios were performed as described (Parichy and Johnson, 2001). When mutant loci were mapped, we examined hybrid phenotypes in crosses

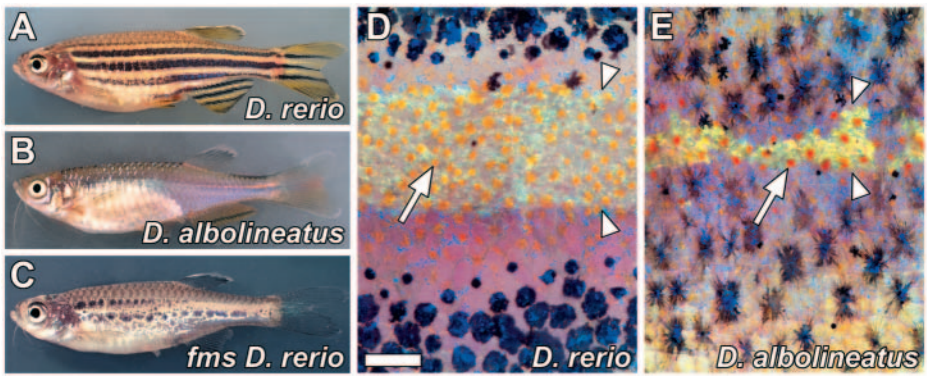

Fig. 1. Adult stripe development and loss in danios. (A) Adult *D. rerio* exhibit dark stripes of melanophores and iridophores (with few xanthophores), alternating with light stripes of xanthophores and iridophores (with few melanophores). (B) Adult *D. albolineatus* lack distinctive melanophore stripes and exhibit only a weak interstripe region posteriorly. (C) *fms* mutant *D. rerio* exhibit disrupted stripes in which metamorphic melanophores are reduced and xanthophores are absent. (D) Detail of stripes and interstripes in *D. rerio*. Dark cells are melanophores and yellow-orange cells are xanthophores (arrow). Reflective iridophores are found throughout, but are organized differently in interstripe regions (arrowheads); under this illumination, iridophores outside of the interstripe region are evident only by their blue iridescence over some melanophores. (E) Detail of pigment pattern in *D. albolineatus* adult. Melanophores are widely distributed over the flank. Adults exhibit reddish erythrophores (arrow) distributed widely over the flank and interstripe iridophores (arrowheads) form only a narrow, irregular band over part of the flank. Erythrophores are present in several other danios as well, although not in *D. rerio*. Adult fish are 25-30 mm in length. Scale bars in D: 200 μm for D,E.

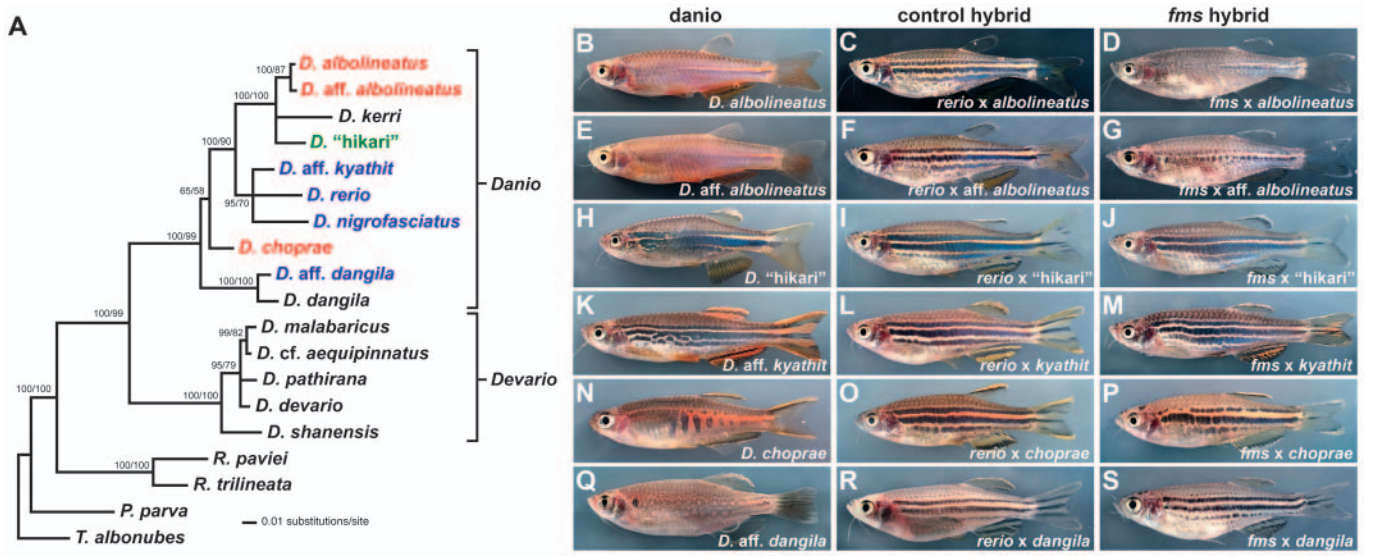

Fig. 2. Comparative analyses reveal *fms*-dependence of hybrid pigment pattern formation across *Danio* species. (A) Phylogenetic relationships. The traditional grouping of danios comprises at least two major clades, '*Danio*' and '*Devario*' (Kullander, 2001; Fang, 2003). Heterospecific danios with names in color were used for interspecific complementation tests with *fms*^{j4e1} mutant *D. rerio*. Colors of names indicate *fms*-dependence of hybrid (or heterozygous) pigment pattern: *red*, strong *fms*-dependence; *green*, mild *fms*-dependence; *blue*, no apparent *fms*-dependence (see text). (B,E,H,K,N,Q) *Danio* wild-type pigment patterns. (C,F,I,L,O,R) Control hybrids between wild-type *D. rerio* and heterospecific danios. (D,G,J,M,P,S) Hybrids between *fms*^{j4e1} mutant *D. rerio* and heterospecific danios. (B-D) *Danio albolineatus* lack distinctive stripes whereas control hybrids develop stripes with irregular borders; *fms* mutant hybrids lack stripes. (E-G) *Danio* aff. *albolineatus* resembles *D. albolineatus* but has a reduced interstripe region posteriorly and the anal fin lacks a melanophore stripe; these fish may represent a divergent population of *D. albolineatus* or a closely related species (Fang and Kottelat, 2000). Control hybrids develop stripes with irregular borders like *D. albolineatus* control hybrids; *fms* mutant hybrids lack distinctive stripes. (H-J) *Danio* 'hikari' resembles *D. kerri* (Parichy and Johnson, 2001): melanophore stripes are broad and diffuse and include very few xanthophores; interstripe regions are narrow and irregular by comparison with *D. rerio* (Fig. 1A). Control hybrids develop distinctive melanophore stripes and interstripes with regular borders. Tester *fms* mutants have fewer xanthophores than controls. (K-M) *Danio* aff. *kyathit* resembles *D. rerio* initially but develops fissures in melanophore stripes as the fish grows; *D.* aff. *kyathit* also exhibit red erythrophores, particularly in the fins. Control hybrids lack stripe fissures, and *fms* mutant hybrids are not discernibly different (minor differences between control and tester hybrids are within the range of variation exhibited by different families within genotypes). (N-P) *Danio choprae* transiently develop horizontal stripes, then lose these stripes as a uniform pattern of melanophores emerges; vertical bars of melanophores arise in adults. Control hybrids develop and maintain horizontal stripes resembling those of *D. rerio* whereas *fms* mutant hybrids have fewer melanophores and xanthophores and less organized patterns. (Q-S) *Danio* aff. *dangila* adults have melanophore stripes interrupted by lighter spots and interstripes, and are morphologically indistinguishable from *D. dangila* (Parichy and Johnson, 2001). Control hybrids resemble *D. rerio*, and *fms* mutant hybrids exhibit no clear difference from controls. Data not shown: *Danio nigrofasciatus* exhibit well-defined stripes and control hybrids are intermediate between *D. rerio* and *D. nigrofasciatus* parental species (Parichy and Johnson, 2001; Quigley et al., 2004); tester hybrids do not differ from controls for either *fms*^{j4blue} (Parichy and Johnson, 2001) or *fms*^{j4e1}. Tester *fms*^{j4e1} hybrids could not be obtained for *D. kerri*; tester *fms*^{j4blue} hybrids resemble control hybrids, similar to tester *fms*^{j4blue} hybrids with *D. albolineatus*, likely reflecting modifier loci in the *fms*^{j4blue} background (see text). Hybrids with species outside of the *Danio* clade (A) were not viable, consistent with previous observations (Parichy and Johnson, 2001). All fish are between 25-35 mm standard length, except *D. dangila* and its hybrids, which are 50-80 mm. GenBank accession numbers for 12S and 16S sequences used in phylogeny reconstruction were: AY707450, AY707456; U21372, U21381; AF322658, 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; AY37481, AY37482; AY37483, AY37484.

segregating the mutant allele to control for allelic variation at other unlinked loci, and we genotyped hybrid offspring by PCR. *Danio rerio* mutants used for interspecific complementation tests have been described: *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); *jaguar*^{c7} (Fisher et al., 1997); and *puma*^{j115e1} (Parichy and Turner, 2003b; Parichy et al., 2003). Additional mutants were derived from on-going mutagenesis screens (D.M.P., unpublished).

Nomenclature for pigment pattern elements

Previous studies defined elements of the adult pigment pattern in *D. rerio* (Parichy and Johnson, 2001; Parichy and Turner, 2003b), including the first developing or 'primary' melanophore stripes (1D, 1V) that develop dorsal and ventral to the horizontal myoseptum, as well as later-developing 'secondary' dorsal and ventral melanophore stripes (2D, 2V). We refer to the xanthophore-rich areas between melanophore stripes as 'interstripe' regions.

Genotyping

fms genotyping was accomplished by primer extension assays using conditions described (Parichy and Turner, 2003a). A 100 bp product was amplified from genomic DNA using forward and reverse primers flanking the *fms*^{j4e1} mutant lesion (fms-j1f, ACT CTT GGT GCT GGT GCG TTT G; fms-j1r, CTT TGA GCA TTT TCA CAG CC) (Parichy et al., 2000b). Wild-type *D. rerio* or *D. albolineatus fms* alleles result in the addition of two nucleotides (ddCA), whereas the *D. rerio fms*^{j4e1}

allele results in addition of four nucleotides (ddCTTA) to the extension primer (*fms*-j1r). Genotyping methods for other loci used in interspecific complementation tests are available on request.

In situ hybridization and histology

Methods for in situ hybridization, as well as tyrosinase assays and controls followed those described previously (Quigley et al., 2004).

Imaging and quantitative analysis

We examined melanophore behaviors by imaging individual larvae once-daily or twice-daily beginning when melanophores first appear outside of early larval melanophore stripes (~14 days post-fertilization, dpf) (Parichy et al., 2000b; Parichy and Turner, 2003b), through development of the adult pigment pattern (46 dpf; once-daily series) or middle stages of pigment pattern metamorphosis (35 dpf; twice-daily series). Images were acquired with a Zeiss Axiocam HRc digital camera mounted on an Olympus SZX12 stereozoom microscope then transferred to Adobe Photoshop for analysis with FoveaPro 3.0 (Reindeer Graphics).

To quantify melanophore numbers, three regions of the anterior flank in both *D. rerio* and *D. albolineatus* were defined to represent the location of the ventral primary melanophore stripe, the primary interstripe, and the region populated by dorsal and scale-associated melanophores in *D. rerio*. Regions were defined by measuring the height (h) of the flank at the anterior margin of the anal fin; the measurement areas were then placed 0.5 h anterior from this location, and extending 0.25 h further anteriorly. Regions were located dorsoventrally as functions of h, according to preliminary analyses of *D. rerio* and dorsoventral margins of each region were 0.1 h. All melanophores were counted within these regions for each day of imaging using semi-automated feature recognition. Melanophore densities were calculated according to the areas of each region. We examined three to six individuals of each species per image series.

To examine melanophore behaviors, we followed individual melanophores throughout pigment pattern metamorphosis (Parichy et al., 2000b; Parichy and Turner, 2003b). This approach allows quantification of new melanophores that arise by differentiation or proliferation (‘births’) and loss of melanophores by death or de-differentiation; we refer to losses as ‘deaths’ based on additional histological evidence, although we cannot formally exclude the possibility that some melanophores disappear by de-differentiation.

We assessed melanophore movements in twice-daily image series by determining the relative dorsal-ventral position of each melanophore followed, with the dorsal edge of the flank receiving a value of 0, and the ventral edge of the flank receiving a value of 1. We then examined the distances moved by melanophores relative to flank height, and calculated net dorsal-ventral changes in melanophore position as the difference between final and initial positions. Negative dorsal-ventral changes reflect dorsal movements, whereas positive dorsal-ventral changes reflect ventral movements. Total movements were calculated as the absolute values of these displacements. In once-daily image series, we overlayed sequential images that had been rescaled to correct for growth and aligned to minimize overall melanophore displacements and we calculated changes in melanophore position in any direction as proportions of flank height. In both approaches, using relative as opposed to absolute distances controls to some degree for passive movements due to growth, but cannot control entirely for potential differences in growth pattern between species. Thus, we further verified the magnitude of melanophore movements between species by examining relative changes in melanophore position that cannot be accounted for simply by passive movements. These rearrangements are consistent with quantitative analyses and are most easily viewed in animations (see below).

Statistical analyses were performed with JMP 5.0.1a for Macintosh (SAS Institute, Cary NC, USA). Residuals were examined for normality and homoscedasticity (Sokal and Rohlf, 1994). Total melanophore numbers, melanophore births and melanophore deaths were examined by nested analyses of variance, in which individuals were nested within species and day of development was treated as a categorical variable and main effect. Births and deaths were square-root transformed prior to analyses to normalize residuals. Melanophore movements were examined by nested analyses of variance or covariance in which species differences were tested after controlling for variation among individuals (nested within species). To assess species differences in absolute movements of melanophores, we calculated absolute values for net directional movements. To assess species differences in directional melanophore movements, we controlled additionally for variation among anteroposterior regions (anterior, middle, posterior; nested within individuals), and we treated melanophore starting position as a covariate; dorsal and ventral regions of the flank were analyzed separately owing to differences suggested by preliminary analyses. Absolute and directional movements were arcsine-transformed prior to analyses. Least squares means from these analyses are reported below. Alternative parameterizations of statistical models yielded qualitatively similar results.

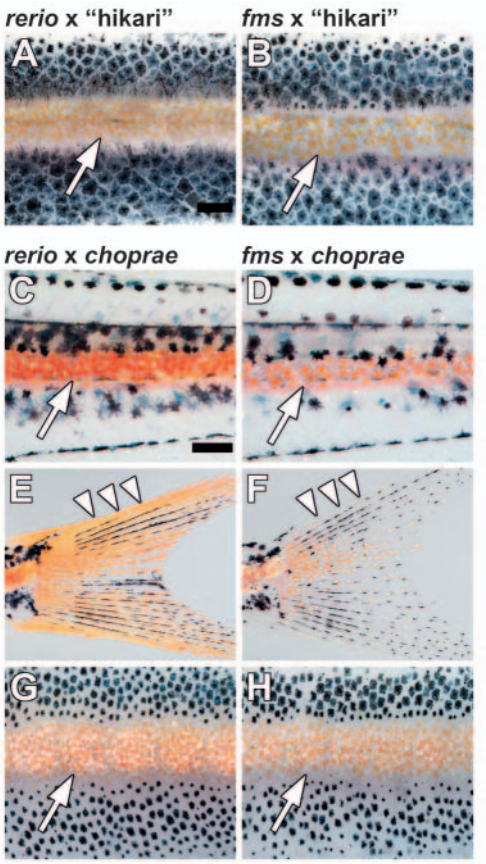

Fig. 3. Mild and strong *fms*-dependence of hybrid pigment patterns for *D.* ‘hikari’ and *D. choprae*. (A,B) *Danio* ‘hikari’ hybrids. (A) Wild-type control hybrids develop distinctive melanophore stripes and interstripe regions (arrow, A), whereas *fms* mutant hybrids have about half as many xanthophores as controls (B). (C-F) *Danio choprae* hybrids at larval stages. (C,E) Wild-type control hybrids have numerous xanthophores both on the body (arrow, C) and in the fins (arrowheads, E). (D,F) *fms* mutant hybrids have fewer xanthophores than controls. (G,H) Adult *D. choprae* control hybrids (G) have more xanthophores (arrows) than tester *fms* mutant hybrids (H). Scale bars: in A, 500 μm for A,B,G,H); in C, 150 μm for C-F.

Phylogenetic analysis

Phylogenetic relationships were reconstructed from mitochondrial 12S and 16S rDNA sequences (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′) (Kocher et al., 1989; Palumbi et al., 1991). Analyses were performed as described (Quigley et al., 2004) using PAUP* 4.0b10 and MrBayes (Huelsenbeck and Ronquist, 2001; Swofford, 2002).

Results

Comparative *fms* dependence of hybrid pigment patterns

Hybrids between wild-type *D. rerio* and *D. albolineatus* develop stripes, whereas hybrids between *fms* mutant *D. rerio* and *D. albolineatus* lack stripes (Parichy and Johnson, 2001). Given this association between *fms* and hybrid stripe loss in *D. albolineatus*, and the critical role for *fms* in melanophore stripe formation in *D. rerio*, we examined the *fms* dependence of hybrid pigment patterns for other danios. For each species, melanophore patterns of control hybrids with wild-type *D. rerio* resembled *D. rerio* more closely than the heterospecific danio (Fig. 2).

Our analyses reveal *fms*-dependent hybrid pigment patterns for additional taxa (Fig. 2A). For both *D. albolineatus* and *D.* aff. *albolineatus*, fms^{j4e1} hybrids lacked stripes (Fig. 2B-G). For *D.* ‘hikari’, fms^{j4e1} hybrids developed normal melanophore stripes but fewer xanthophores than controls (Fig. 2B-J, Fig. 3A,B). Within the clade that includes *D. rerio*, *D. kyathit* and *D. nigrofasciatus*, fms^{j4e1} hybrids did not differ consistently from controls (Fig. 2K-M) (Parichy and Johnson, 2001) (data not shown). Finally, *D. choprae* fms^{j4e1} hybrids exhibited disrupted adult stripes and a severe xanthophore deficit (Fig. 2N-P, Fig. 3C-H), but *D. dangila* fms^{j4e1} hybrids were not discernibly different from controls (Fig. 2Q-S).

Thus, four out of seven danios exhibited non-complementation phenotypes with fms^{j4e1} mutant *D. rerio* (Fig. 2A); one of these was mild (*D.* ‘hikari’) and three were severe (*D. albolineatus*, *D.* aff. *albolineatus*, *D. choprae*). Whether mild non-complementation for *D.* ‘hikari’ represents a unique derived change for this species, or a basal change within the *D.* ‘hikari’-*D. albolineatus* clade is uncertain without deeper taxonomic sampling. Given the close phylogenetic relationship of *D. albolineatus* and *D.* aff. *albolineatus*, these results imply at least two evolutionary changes resulting in severe non-complementation phenotypes, one separating *D. albolineatus*-

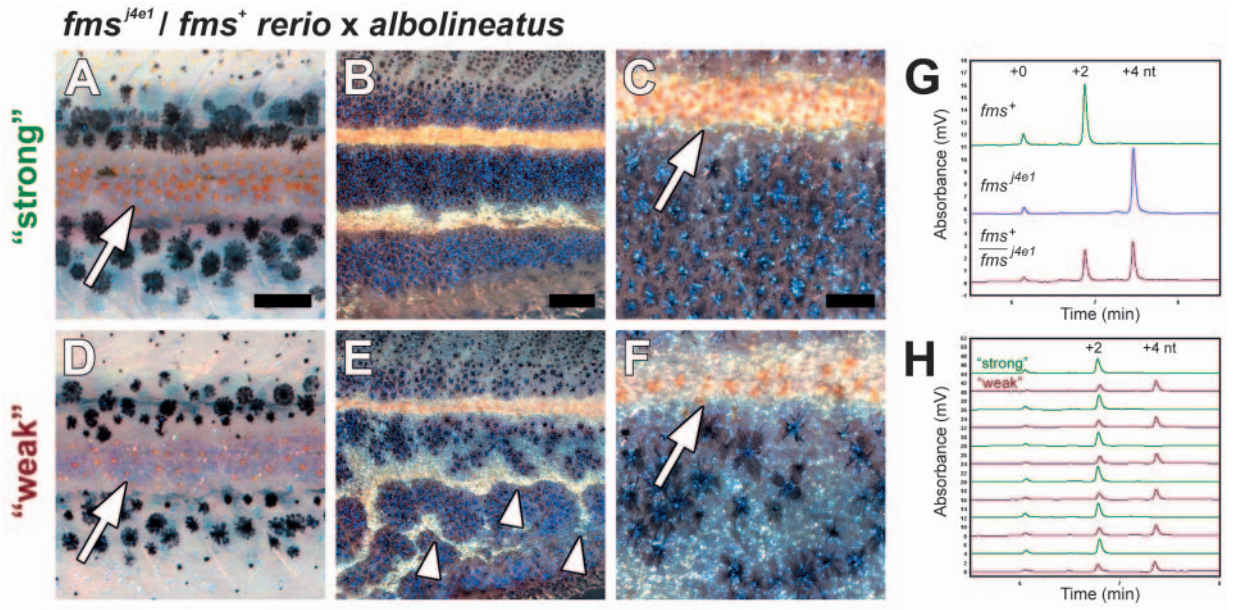

Fig. 4. Segregation analysis supports an essential role for *fms* in *D. albolineatus*×*D. rerio* hybrid stripe development. (A,B,D,E) Tester fms^{j4e1}/+ hybrids exhibit either of two easily distinguishable phenotypes, one in which stripes are well organized (A,B), and another in which stripes are weakly organized with irregular borders and fewer xanthophores (D,E). Differences are evident during pigment pattern metamorphosis (A,D) but are most apparent in adults (B,E). Details showing well-organized (C) and weakly organized (F) stripes in hybrid adults. Pigment cell complements in hybrids with weak stripes were significantly reduced compared with hybrids with strong stripes, with 84% of the number of melanophores ($F_{1,16}$=8.76, P<0.01) and only 14% of the number of xanthophores ($F_{1,16}$=20.85, P<0.0005). The reduction in melanophore numbers is comparable to that observed in homozygous fms^{j4e1} mutant *D. rerio* and fms^{174} mutant *D. rerio* at restrictive temperature; however, neither *D. rerio* mutant allele retains xanthophores (Parichy et al., 2000b; Parichy and Turner, 2003a). (G,H). Primer extension genotyping for *fms* alleles. (G) In *D. rerio*, an extension primer adjacent to the fms^{j4e1} lesion yields additions of two nucleotides in homozygous wild-type individuals, additions of four nucleotides in homozygous fms^{j4e1} individuals, and additions of both two and four nucleotides in heterozygous fms^{+}/fms^{j4e1} individuals. (H) Primer extension genotyping for tester fms^{j4e1}/+ hybrids that have been classified as having ‘strong’ or ‘weak’ stripes. All hybrids with strong stripes carry the *D. rerio* $fms^{+(wik)}$ wild-type allele and the *D. albolineatus fms* allele, resulting in two nucleotide additions to the extension primers. All hybrids with weak stripes carry the *D. rerio* fms^{j4e1} mutant allele and a *D. albolineatus fms* allele, resulting in the addition of four and two nucleotides to the extension primer, respectively. Scale bars: in A, 200 μm for A,D; in B, 500 μm for B,E; in C, 200 μm for C,F.

D. aff. *albolineatus* from other danios, and another separating *D. choprae* from other danios.

fms-dependent hybrid stripe disruption in *D. albolineatus*

Given the broader variation in *fms*-dependence across danios, we sought to further test evolutionary roles for *fms* and *fms*-dependent pathways, focusing on *D. albolineatus* because of the simplicity of its pattern. Previous analyses tested *D. albolineatus* hybrids for non-complementation of fms^{j4e1}, fms^{j4e3} and fms^{j4blue}, all of which are recessive in *D. rerio* and exhibit presumptive null phenotypes (Parichy et al., 2000b; Parichy and Johnson, 2001). Hybrids for fms^{j4e1} and fms^{j4e3} lacked stripes, whereas hybrids for fms^{j4blue} developed stripes. Since fms^{j4e1} and fms^{j4e3} were maintained in the inbred AB* (AB^{ut}) genetic background, whereas fms^{j4blue} was maintained in a different background, the formal possibility exists that other loci in the AB^{ut} background were responsible. Alternatively, modifier loci affecting the penetrance of a *fms* effect could differ across backgrounds. Thus, we asked whether pigment pattern variation in hybrids with *D. albolineatus* segregates with alleles at the *fms* locus.

Our analyses support a model in which stripe disruption in hybrids depends on *fms*, with the magnitude of this effect determined by additional modifier loci. We generated heterozygous *fms* mutant *D. rerio* by crossing fms^{j4e1}, maintained in the inbred background AB^{ut}, with another inbred mapping strain, wik^{ut}. We then crossed these $fms^{j4e1(AB)}/fms^{+(wik)}$ *D. rerio* to *D. albolineatus*. Hybrid offspring segregated two phenotypes in ~1:1 ratios: either well-organized, 'strong' melanophore stripes, or a poorly organized, 'weak' stripe pattern, with significantly fewer melanophores and xanthophores (Fig. 4A-F). We categorized fish into alternative 'strong' and 'weak' stripe classes, then asked whether individuals carried the $fms^{+(wik)}$ wild-type *D. rerio* allele or the $fms^{j4e1(AB)}$ mutant *D. rerio* allele. Primer extension genotyping for the fms^{j4e1} lesion demonstrates that, in every instance, hybrids with 'strong' stripes carried the wild-type allele whereas hybrids with 'weak' stripes carried the mutant allele (n=105; Fig. 4G,H).

These data confirm that a hybrid non-complementation phenotype segregates with *fms*. Yet, this phenotype is less severe than that of fms^{j4e1} (Fig. 2D) and fms^{j4e3} in the AB^{ut} background, and more severe than that for fms^{j4blue} in a different background (Parichy and Johnson, 2001). This suggests that modifier loci contribute to the phenotype, and that these modifiers differ across genetic backgrounds.

Segregation analyses thus place the non-complementing locus in the vicinity of *fms*, and suggest roles for modifier loci in determining hybrid pigment patterns.

Temperature-sensitive *fms* allele confirms role in hybrid stripe loss

We used a temperature-sensitive *fms* allele to further confirm the requirement for *fms* in *D. albolineatus* hybrid pigment pattern development. Segregation analyses placed the non-complementing locus within ~1 cM of *fms*, a region likely to include several other genes. We reasoned that a *fms* allele demonstrated previously to exhibit temperature sensitivity could be used to exclude roles for these neighboring loci: a *fms*-specific effect should be manifested as a complementation phenotype at the permissive temperature, and a non-complementation phenotype at the restrictive temperature. Thus, we used the temperature-sensitive allele $fms^{ut.r4e174A}$ (fms^{174}), which exhibits a wild-type phenotype at 24°C and a *fms* null phenotype at 33°C (Parichy and Turner, 2003a). We crossed homozygous fms^{174} mutant *D. rerio* to *D. albolineatus* and reared hybrid siblings at either 24°C or 33°C. Tester fms^{174} hybrids reared at 24°C were indistinguishable from control hybrids (Fig. 5A,B), as were wild-type hybrids reared at 33°C (data not shown). By contrast, fms^{174} hybrids reared at 33°C developed poorly organized melanophore stripes and fewer xanthophores (Fig. 5C,D). These results provide compelling additional evidence that hybrid non-complementation

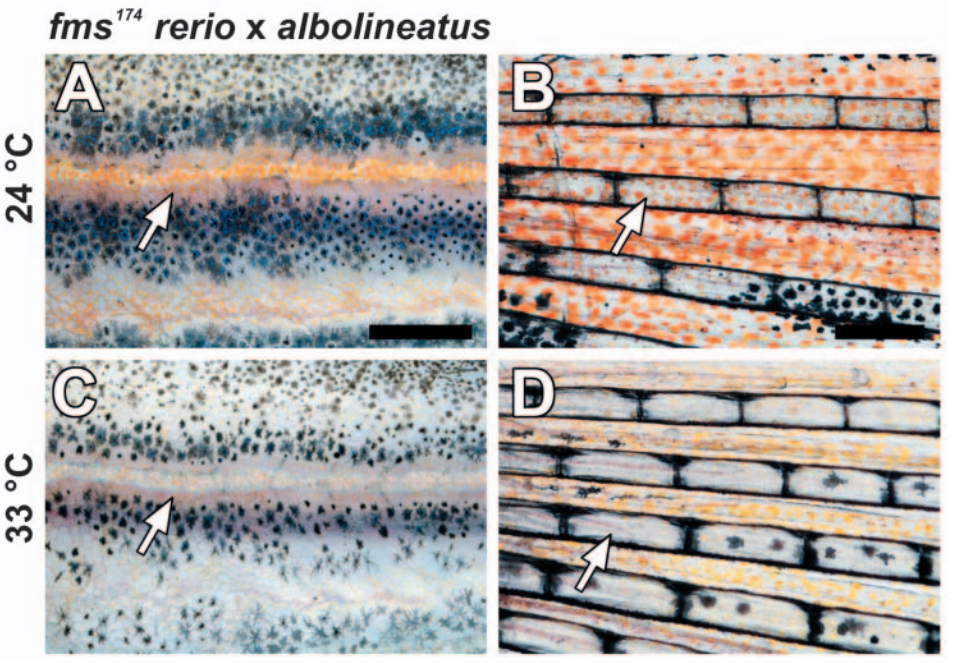

Fig. 5. A temperature-sensitive allele uniquely implicates *fms* in melanophore stripe disorganization and xanthophore reduction in hybrids. Tester fms^{174} mutant *D. rerio*×*D. albolineatus* hybrids were reared at 28.5°C through middle metamorphosis, then were transferred either to 24°C or 33°C until adult pigment patterns had formed. (A,B) fms^{174} hybrids reared at 24°C develop well-organized stripes and numerous xanthophores on the body (A) and fins (B, arrows). (C,D) fms^{174} hybrids reared at 33°C develop poorly organized melanophore stripes and have fewer xanthophores on the body (C) and fins (D, arrows). Pigment patterns of control wild-type hybrids do not differ between 24°C and 33°C (data not shown). Scale bars: in A, 1 mm for A,C; in B, 150 μm for B,D.

phenotypes depend on *fms*, rather than on other closely linked loci.

Finally, *D. albolineatus* hybrids did not reveal non-complementation phenotypes for any of 16 other recessive *D. rerio* mutants affecting pigment cell numbers and arrangements (*ednrb1*^{b140}, *kit*^{b5}, *mitfa*^{w2}, *sox10*$^{ut.r13e1}$, *tfap2a*^{ts213}, *cezanne*$^{ut.r17e1}$, *degas*$^{ut.r18e1}$, *jaguar*^{c7}, *leopard*^{t1}, *oberon*^{j198e1}, *pissarro*$^{ut.r8e1}$, *picasso*$^{ut.r2e1}$, *primrose*^{j199}, *puma*^{j115e1}, or *seurat*$^{ut.r15e1}$), including *bonaparte*$^{ut.r16e1}$, which has melanophore and xanthophore defects similar to *fms* mutants (D.M.P. and E.L.M., unpublished). Thus, *D. albolineatus* hybrid pigment patterns are uniquely *fms*-dependent within this broader collection of loci required for pigment pattern development.

Altered melanophore lineage development during *D. albolineatus* adult pigment pattern formation

Genetic analyses above reveal a strong *fms* dependence of hybrid pigment pattern development for *D. albolineatus* (as well as *D. choprae*) but do not indicate how this dependence

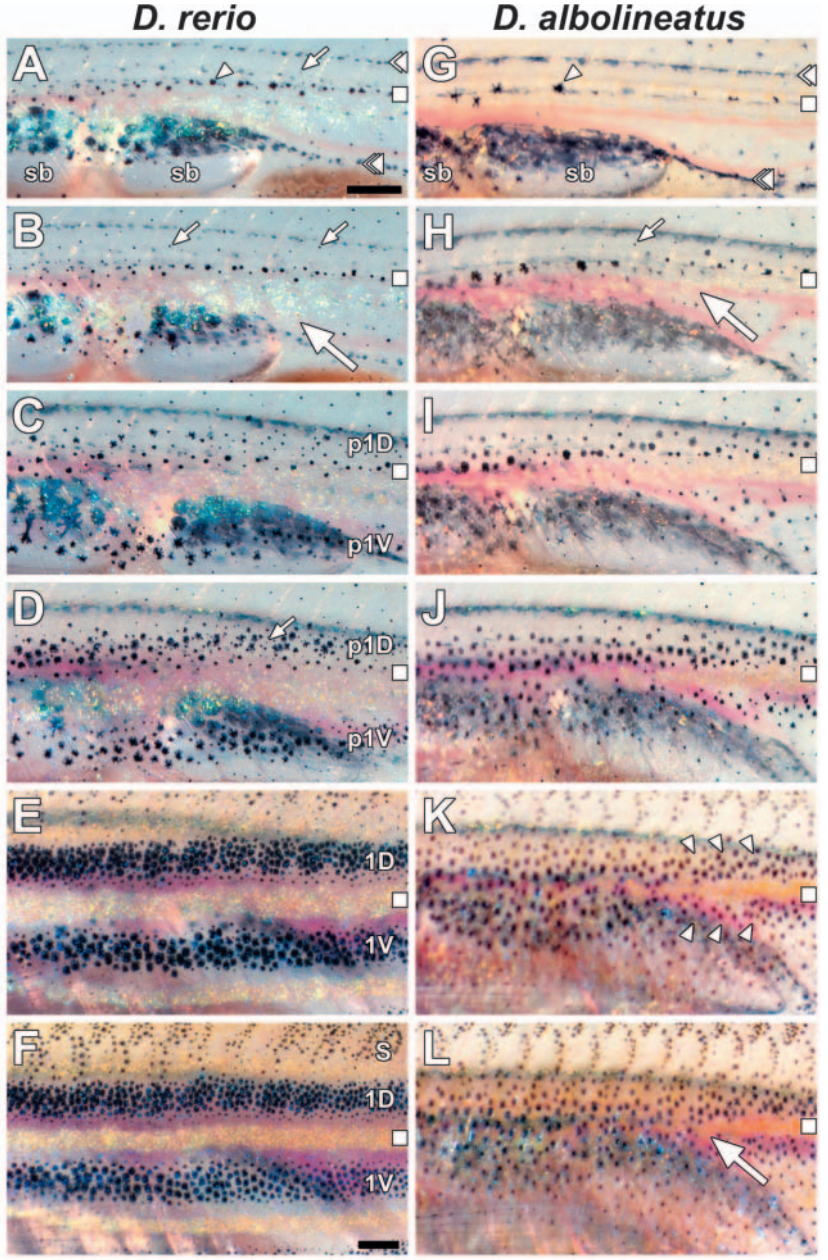

Fig. 6. Development of adult pigment patterns in *D. rerio* (A-F) and *D. albolineatus* (G-L). Panels shown are of selected days from a complete image series for individual, representative larvae. (A) In *D. rerio*, pigment pattern metamorphosis is marked by the differentiation of metamorphic melanophores (arrow, showing one of many) over the myotomes. The white box in each image delineates the horizontal myoseptum; the single arrowhead in A indicates one of several early larval melanophores that have persisted along the horizontal myoseptum through the beginning of metamorphosis. Double arrowhead indicates deep melanophores along the dorsal aspect of the neural tube; triple arrowhead, deep melanophores lining the dorsal surface of the peritoneum. sb, swim bladder. (B) As metamorphosis proceeds, additional metamorphic melanophores (small arrows) develop over the myotomes, and iridophores and xanthophores differentiate ventral to the horizontal myoseptum in the prospective primary interstripe region (large arrow). (C) By middle stages of pigment pattern metamorphosis, melanophores have started to organize into stripes in the region of the prospective dorsal primary melanophore stripe (p1D) and prospective ventral primary melanophore stripe (p1V). (D) As the dorsal and ventral melanophore stripes become increasingly distinctive, additional late metamorphic melanophores differentiate already within these stripes (e.g. arrow). (E) Near the completion of pigment pattern metamorphosis, distinctive dorsal and ventral primary melanophore stripes (1D, 1V) border a well-defined interstripe region. (F) Pigment pattern metamorphosis is completed with the development of scales and scale-associated melanophores (s). In *D. albolineatus*, melanophores typically do not persist along the horizontal myoseptum from earlier stages, and instead melanophores, initially deeper between the myotomes, migrate to the surface (arrowhead, data not shown). (H) Metamorphic melanophores (e.g. small arrow) differentiate widely scattered over the myotomes, as in *D. rerio*, but fewer iridophores (large arrow) develop ventral to the horizontal myoseptum. (I,J) As metamorphosis proceeds, additional metamorphic melanophores appear over the myotomes, yet these cells typically do not migrate far from their site of differentiation. Relatively few late metamorphic melanophores appear within the regions where stripes form in *D. rerio* (compare with D). (K) Near the completion of metamorphosis, melanophores remain relatively dispersed compared with *D. rerio*, and only a weak pattern of melanophore stripes (arrowheads) borders an irregular and narrow interstripe region on the posterior trunk. (L) At the end of metamorphosis, *D. albolineatus* have far fewer sub-dermal melanophores than *D. rerio*, and these cells are widely distributed where the interstripe region develops in *D. rerio*. The interstripe region extends only to the middle of the flank (arrow) and reddish erythrophores have started to differentiate within this region. Images shown have been rescaled across stages to maintain the same approximate field of view. Larval standard lengths in mm, (A-F) 7.1, 7.7, 8.3, 9.5, 12.0, 13.3, (G-L) 8.1, 9.3, 10.1, 11.0, 12.8, 13.4. Scale bars: in A, 500 μm for A,G; in F, 1 mm for F,L.

reflects natural variation between species. Given the non-complementation phenotype of *fms* hybrids, we reasoned initially that *D. albolineatus* might exhibit a loss of *fms* activity relative to *D. rerio*. This simple model predicts that pigment pattern metamorphosis in *D. albolineatus* should resemble that of *fms* mutant *D. rerio*. By comparison to wild-type *D. rerio*, *fms* mutants have fewer metamorphic melanophores, increased melanophore death, decreased melanophore movement into stripes, and an absence of xanthophores (Parichy et al., 2000b; Parichy and Turner, 2003a). We thus asked whether *D. albolineatus* pigment pattern metamorphosis entails some or all of these differences relative to wild-type *D. rerio*. Our

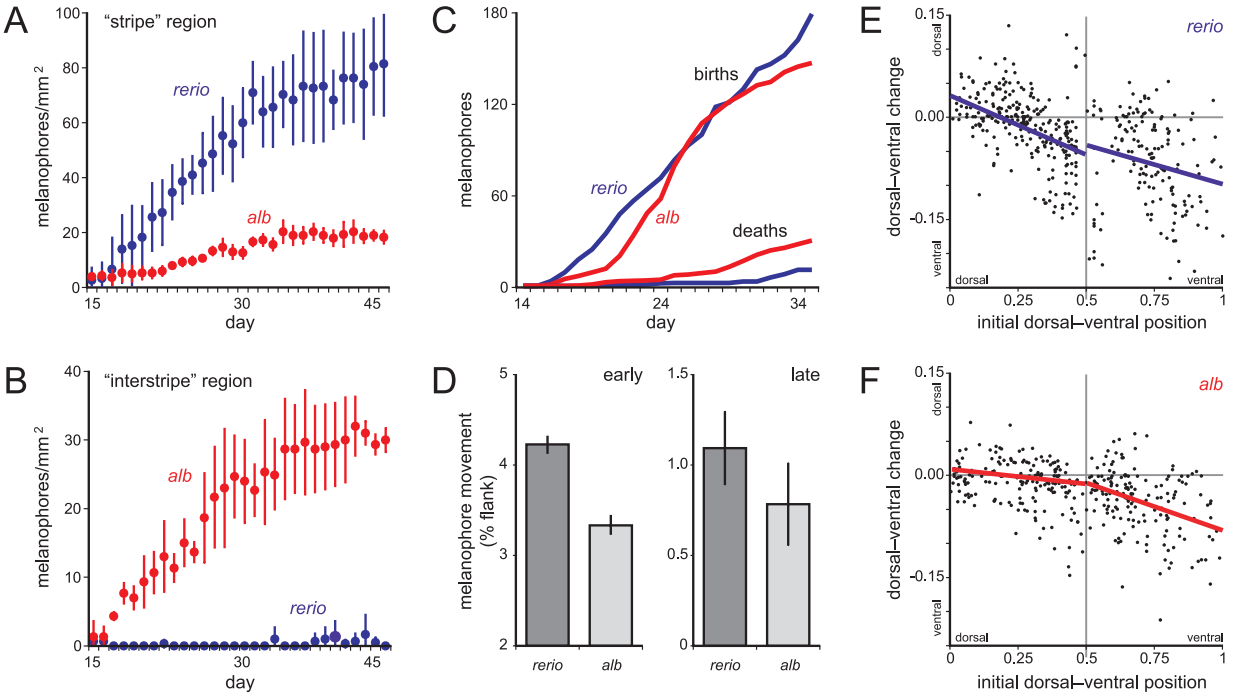

Fig. 7. Quantitative analyses of melanophore morphogenesis in *D. rerio* and *D. albolineatus*. (A) In regions where melanophore stripes develop in *D. rerio*, melanophore numbers are dramatically reduced in *D. albolineatus* and this deficit becomes more pronounced through pigment pattern metamorphosis. (B) In the middle of the flank, where the first interstripe region forms in *D. rerio*, melanophore numbers are increased in *D. albolineatus*, reflecting the absence of a distinctive and persistent melanophore-free region in the anterior of the flank. Despite the increased melanophore numbers, the overall complement of melanophores is dramatically reduced overall compared with *D. rerio*. (C) Analyses of twice daily image series reveal differential appearance ('births') and disappearance ('deaths') of melanophores between species. Shown are cumulative mean births and deaths recorded in each of three developing larvae through middle stages of pigment pattern metamorphosis, with 'births' defined as the appearance of new melanophores (either by differentiation or proliferation) and 'deaths' defined by the unambiguous loss of melanophores (see Materials and methods). Melanophore births were not significantly different between species ($F_{1,107}$=1.87, P=0.2) after controlling for variation among individuals ($F_{4,107}$=2.72, P<0.05) and across days ($F_{21,107}$=5.43, P<0.001). By contrast, melanophore deaths were significantly greater in *D. albolineatus* than *D. rerio* ($F_{1,81}$=3.04, P<0.005) after controlling for variation among individuals ($F_{4,81}$=2.10, P=0.09) and across days ($F_{29,81}$=2.47, P<0.001). Error bars are omitted for clarity. (D) Total melanophore movements are reduced in *D. albolineatus* compared with *D. rerio*, both during early and middle metamorphosis (left), and through later metamorphosis (right). Shown are mean (±s.e.m.) distances moved by individual melanophores, with distances expressed as percentages of the flank height. Left, species differences were significant ($F_{1,696}$=14.23, P<0.0005; n=368, 344 melanophores in *D. rerio* and *D. albolineatus*) after controlling for variation associated with individuals (nested within species: $F_{4,696}$=2.60, P<0.05), and anteroposterior region nested within individuals ($F_{10,696}$=5.19, P<0.0001). Right, species differences were significant ($F_{1,708}$=8.45, P<0.005; n=291, 419 melanophores in *D. rerio* and *D. albolineatus*) whereas inter-individual differences were not significant (P=0.2). (E,F) Directional movements of melanophores were significantly reduced in *D. albolineatus* compared with *D. rerio*. Each point represents a single melanophore followed from its first appearance to its final position at the end of the series or until it was lost (n=368, 344 melanophores in *D. rerio* and *D. albolineatus*, respectively). Plots show the initial dorsoventral positions at which melanophores first appeared, and the subsequent changes in dorsoventral positions by the end of the images series. The dorsal-most position on the flank is assigned a relative value of 0, and the ventral-most position on the flank is assigned a value of 1. Regression slopes are estimated separately for dorsal and ventral regions of the flank because of differences in shape and growth. (E) In *D. rerio*, melanophores that initially appear in more dorsal regions of the flank tend to move ventrally whereas melanophores that initially appear in more ventral regions of the flank tend to move dorsally (partial regression coefficients±s.e.m. for relationship between starting dorsoventral position and arcsine-transformed movements for dorsal and ventral regions of the flank, respectively: –0.17±0.02, –0.14±0.04). (F) In *D. albolineatus*, directional movements were significantly reduced compared with *D. rerio* in dorsal regions of the flank ($F_{1,399}$=6.43, P<0.05), although species differences were not detectable in ventral regions ($F_{1,399}$=1.40, P=0.2), as assessed by the magnitude of starting position×species interactions [partial regression coefficients for dorsal and ventral, respectively: –0.10±0.02, –0.10±0.02; after controlling in both dorsal and ventral analyses for variation among individuals (nested within species, both P<0.005), variation among the three examined anteroposterior regions of the flank nested within individuals (both P<0.0005), main effects of species (P=0.7, P<0.005, respectively), and starting position independent of species (both P<0.0001)].

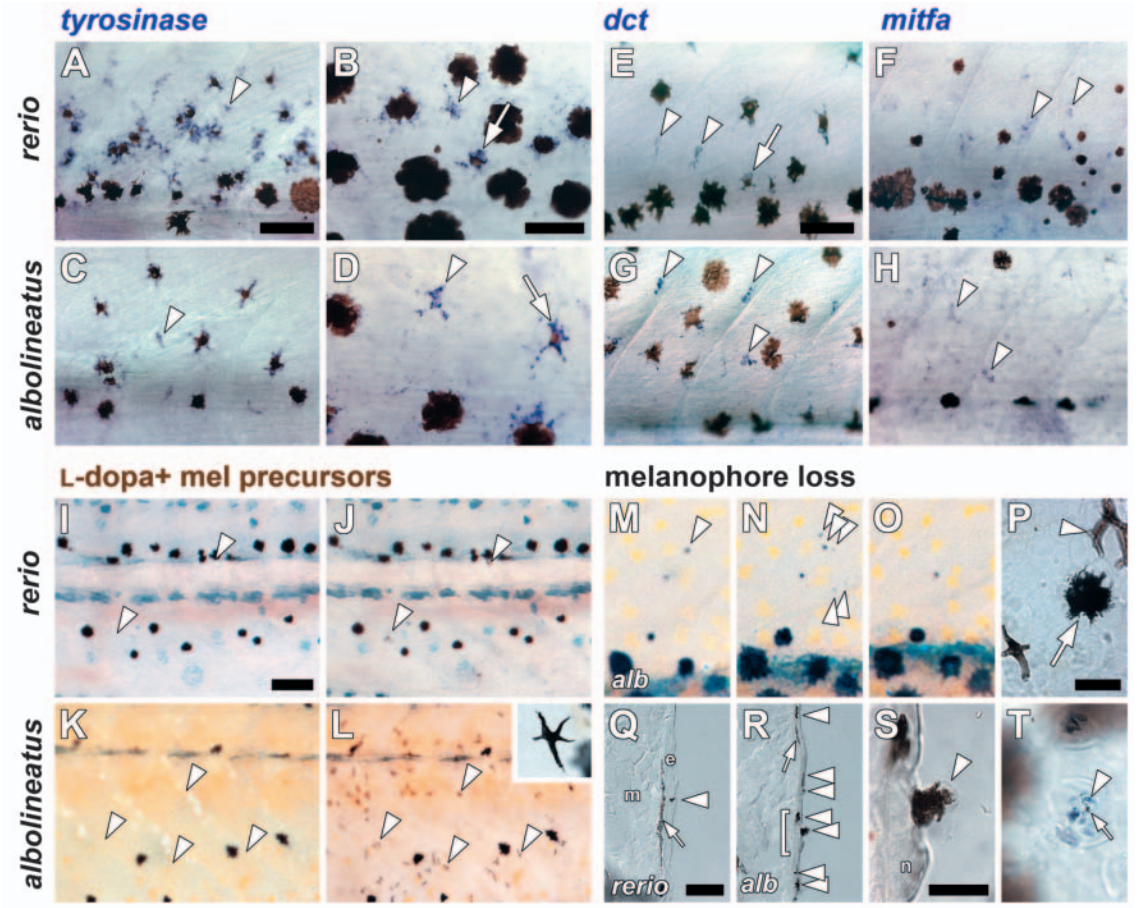

Fig. 8. Altered melanophore lineage development in *D. albolineatus*. (A-H) Melanophore precursor abundance does not differ dramatically between species as revealed by distributions of cells expressing *tyrosinase*, *dopachrome tautomerase* (*dct*) and *mitfa* (Lister et al., 1999; Kelsh et al., 2000; Camp and Lardelli, 2001). *tyrosinase* and *dct* encode enzymes required for melanin synthesis, whereas *mitfa* encodes a transcription factor essential for melanophore specification. (A-D) In situ hybridization for *tyrosinase* mRNA in *D. rerio* (A,B) and *D. albolineatus* (C,D) during middle stages of pigment pattern metamorphosis (equivalent to larvae shown in Fig. 6C,I). (A) In *D. rerio*, numerous *tyrosinase*$^{+}$ melanophores and unmelanized melanophore precursors (arrowhead) are observed in the region of the prospective dorsal primary melanophore stripe. (B) Higher magnification image of a different larvae showing *tyrosinase*$^{+}$ melanophores (arrow) and melanophore precursors (arrowhead). (C) In *D. albolineatus*, fewer melanophores are present but *tyrosinase*$^{+}$ melanophore precursors (arrowhead) are not obviously reduced in number. (D) Higher magnification of a different larva showing *tyrosinase*$^{+}$ melanophores (arrowhead) and unmelanized melanophore precursor (arrowhead). (E-H) Expression of other melanophore lineage markers also is similar between *D. rerio* and *D. albolineatus*. (E,F) *Danio rerio* exhibit unmelanized cells (arrowheads), and some melanized cells (arrow) expressing *dct* (E) and *mitfa* (F). (G,H) In *D. albolineatus*, the numbers of unmelanized cells expressing *dct* (G) and *mitfa* are similar to *D. rerio*. Results for *kit* and *sox10*-expressing cells were similar (data not shown) (Parichy et al., 1999; Dutton et al., 2001). (I-L) Tyrosinase-expressing melanophore precursors revealed by treating fixed larvae with the essential precursor for melanin synthesis, L-dopa (Camp and Lardelli, 2001; McCauley et al., 2004). Images in I and K are prior to L-dopa incubation, and images in J and L are the same fields of view after treatment with L-dopa for 5 hours. (I,J) In *D. rerio*, only a few tyrosinase^{+} melanophore precursors are revealed by L-dopa incubation (arrowheads). (K,L) In *D. albolineatus*, however, numerous tyrosinase^{+} melanophore precursors are revealed by L-dopa treatment (arrowheads show only a few of these cells). These cells exhibit morphologies typical of melanoblasts and recently differentiated melanophores (inset). The increased number of L-dopa stained, tyrosinase^{+} cells as compared with molecular markers in *D. albolineatus* may reflect perduring protein in the absence of transcriptional activity. (M-T). Melanophores and melanophore precursors frequently are lost in *D. albolineatus*. (M-O) The same region of a *D. albolineatus* larvae imaged at 12-hour intervals reveals the transient appearance of several melanophores (arrowheads). Yellow-orange cells are xanthophores; these and other melanophores do not change positions between images. (P) High magnification image of *D. albolineatus* reveals melanin-containing debris (arrow) typical of melanophore death. Arrowhead, melanophore precursor that acquired melanin following L-dopa incubation of this larva. (Q-S) Cross-sections reveal the locations of melanophores and tyrosinase^{+} melanophore precursors in *D. rerio* (Q) and *D. albolineatus* (R,S). (Q) In *D. rerio*, few melanophores or melanophore precursors are located within the plane of the epidermis; one such melanophore is indicated by the arrowhead. e, epidermis; m, myotome. Arrow indicates iridophores of the developing interstripe region. (R) In *D. albolineatus*, numerous melanophores and tyrosinase^{+} melanophore precursors occur within the plane of the epidermis (arrowheads), although some melanophores are found subdermally, as in *D. rerio* (arrow). (S) Higher magnification of bracketed region in R, showing a melanin-containing extrusion body typical of teleost melanophore death. Arrowhead indicates bounding membrane. n, neuromast. Melanin-containing debris in P also is superficially located, as revealed by hexagonal outlines of adjacent epidermal cells. (T) High magnification image of whole-mount larva, showing extrusion body containing melanin granule (arrow) and staining for *dct* mRNA (arrowhead). Scale bars: in A, 80 μm for A,C; in B, 40 μm for B,D; in E, 60 μm for E-H; in I, 80 μm for I-L; in P, 20 μm for P; in Q, 60 μm for Q,R; in S, 20 μm for S,T.

analyses reveal dramatic differences between wild-type *D. rerio* and *D. albolineatus* (Fig. 6; see Movies 1-4 in supplementary material). Although some similarities are seen between *D. albolineatus* and *fms* mutant *D. rerio*, there are major differences as well (next section).

We first examined the temporal accumulation of melanophores. Daily image series show that *D. albolineatus* exhibited only ~67% as many melanophores as *D. rerio* ($F_{1,142}$=245.56, P<0.0001) across the entire image series. The deficit was most evident where melanophore stripes form in *D. rerio*, and became increasingly severe at later stages (Fig. 6, Fig. 7A). Only where the primary interstripe forms in *D. rerio* were melanophore numbers greater in *D. albolineatus*, reflecting the more uniform pattern of melanophores (Fig. 7B).

Fewer metamorphic melanophores in *D. albolineatus* could reflect a change in melanophore specification, increased rates of melanophore or melanoblast death, or both. To distinguish among these possibilities, we first quantified the appearance and disappearance of melanophores in a temporally higher resolution series of images taken at 12-hour intervals. Averaged over entire series, the *D. albolineatus* larvae exhibited ~91% as many melanophore 'births' as the *D. rerio* larvae, but 376% as many 'deaths' (Fig. 7C); suggesting that fewer melanophores in *D. albolineatus* does not reflect a failure to recruit stem cells into the melanophore lineage, but to some extent a reduction in the subsequent survival of these cells. Consistent with this inference, numbers of melanophore precursors were not obviously reduced in *D. albolineatus*, as revealed by molecular markers (Fig. 8A-H) and tyrosinase activity (Fig. 8I-L). Moreover, *D. albolineatus* melanophores frequently appeared, then disappeared, over short time intervals (Fig. 8M-O) and these cells, as well as L-dopa-

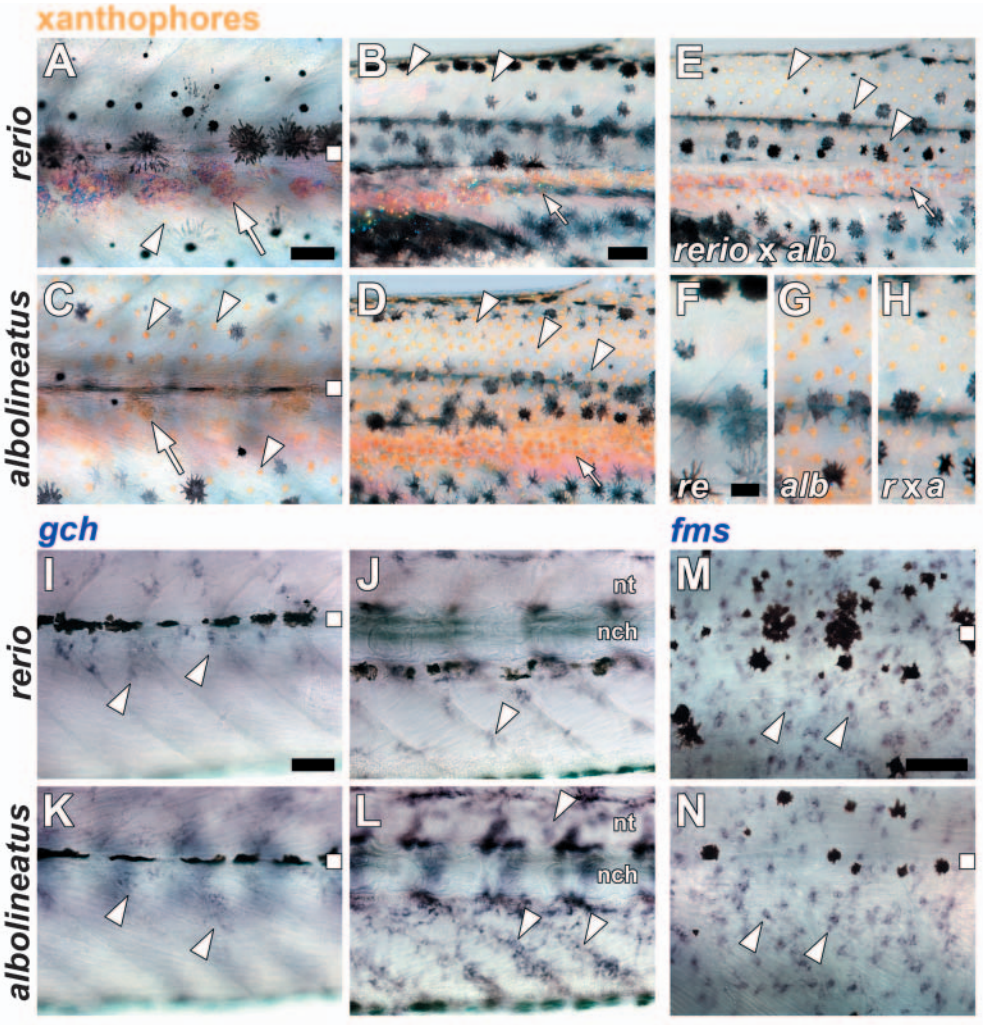

Fig. 9. Excess xanthophores in *D. albolineatus* compared with *D. rerio*. (A-H) Xanthophores in *D. rerio* (A,B,F), *D. albolineatus* (C,D,G), and wild-type *D. rerio*×*D. albolineatus* hybrids (E,H). (A) In *D. rerio*, few xanthophores (arrowhead) are visible during early stages of metamorphosis (stage equivalent to Fig. 6A) and most initially are found ventral to the horizontal myoseptum (box). Arrow indicates iridophores in the prospective primary interstripe region. (B) During middle stages of pigment pattern metamorphosis in *D. rerio* (e.g. Fig. 6C), xanthophores occur in the developing interstripe region (arrow) and a few faint xanthophores can be seen along the dorsal myotomes. (C) In *D. albolineatus*, xanthophores (arrowheads) are widely dispersed over the flank during early pigment pattern metamorphosis (e.g. Fig. 6G). (D) During later pigment pattern metamorphosis in *D. albolineatus* (e.g. Fig. 6I), xanthophores (arrowheads) persist widely scattered over the flank. Reddish erythrophores have started to develop in the interstripe region (arrow) and persist into the adult. Lineage relationships of erythrophores to xanthophores are unclear. (E) In hybrids between wild-type *D. rerio* and *D. albolineatus*, excess xanthophores (arrowheads) develop over the flank compared with *D. rerio*, and reddish erythrophores develop in the interstripe region (arrow). (F-H) Higher magnification images of larval *D. rerio*, *D. albolineatus*, and *D. rerio*×*D. albolineatus* hybrids shown in B-E. (I-L) In situ hybridization for early markers of the xanthophore lineage (Parichy et al., 2000b; Ziegler et al., 2000). Shown is *GTP cyclohydrolase I* (*gch*), which encodes an enzyme required for synthesizing pteridine pigments of xanthophores. Results for *xanthine dehydrogenase* (*xdh*), encoding a second pteridine synthesis enzyme were similar (data not shown). (I,J) *D. rerio* larvae; (K,L) *D. albolineatus* larvae. (I,K) In both species, gch^+ cells occur over the myotomes during early metamorphosis. (J,L) At an optical plane medially within the same larvae shown in I and K, relatively few gch^+ cells are observed in *D. rerio* (J), whereas many gch^+ cells are present in *D. albolineatus* (L). (M,N) In situ hybridization for *fms* mRNA does not reveal clear differences in the numbers or distributions of fms^+ cells between *D. rerio* (M) and *D. albolineatus* (N). Scale bars: in A, 100 μm for A,C; in B, 160 μm in B,D,E; in F, 60 μm for F-H; in I, 60 μm for I-L; in M, 80 μm for M,N.

stained, tyrosinase+ melanophore precursors, were common within the epidermis and in 'extrusion bodies' at the epidermal surface, characteristic of teleost melanophore death (Parichy et al., 1999; Sugimoto, 2002; Parichy and Turner, 2003a) (Fig. 8P,R-T); only ~10% as many epidermal, melanized cells occurred in *D. rerio* larvae (Fig. 8Q). These results indicate that fewer melanophores in *D. albolineatus* result, at least in part, from the death of these cells and their immediate precursors.

Finally, we examined melanophore migration. Total distances moved by melanophores were reduced in *D. albolineatus* larvae compared with *D. rerio* larvae (Fig. 7D). Moreover, stripes in *D. rerio* develop in part through the directional migration of initially more dispersed melanophores to sites of stripe formation, and such movements were significantly reduced in *D. albolineatus* (Fig. 7E,F). Animations of developing larvae further support the interpretation that *D. albolineatus* melanophores move less than *D. rerio* melanophores (see Movies 5, 6 in supplementary material).

Melanophore morphogenesis in *D. albolineatus* thus resembles melanophore morphogenesis in *fms* mutant *D. rerio*, with fewer melanophores, increased death of cells in the melanophore lineage, and reduced melanophore migration as compared with wild-type *D. rerio*.

Enhanced xanthophore development in *D. albolineatus*

Melanophore morphogenesis in *D. albolineatus* is consistent with a model in which this species has evolved a loss of *fms* activity relative to wild-type *D. rerio*. This model also predicts that *D. albolineatus* should have fewer xanthophores, consistent with the reported absence of xanthophores in adult *D. albolineatus* (McClure, 1999). Yet, our analyses reject this

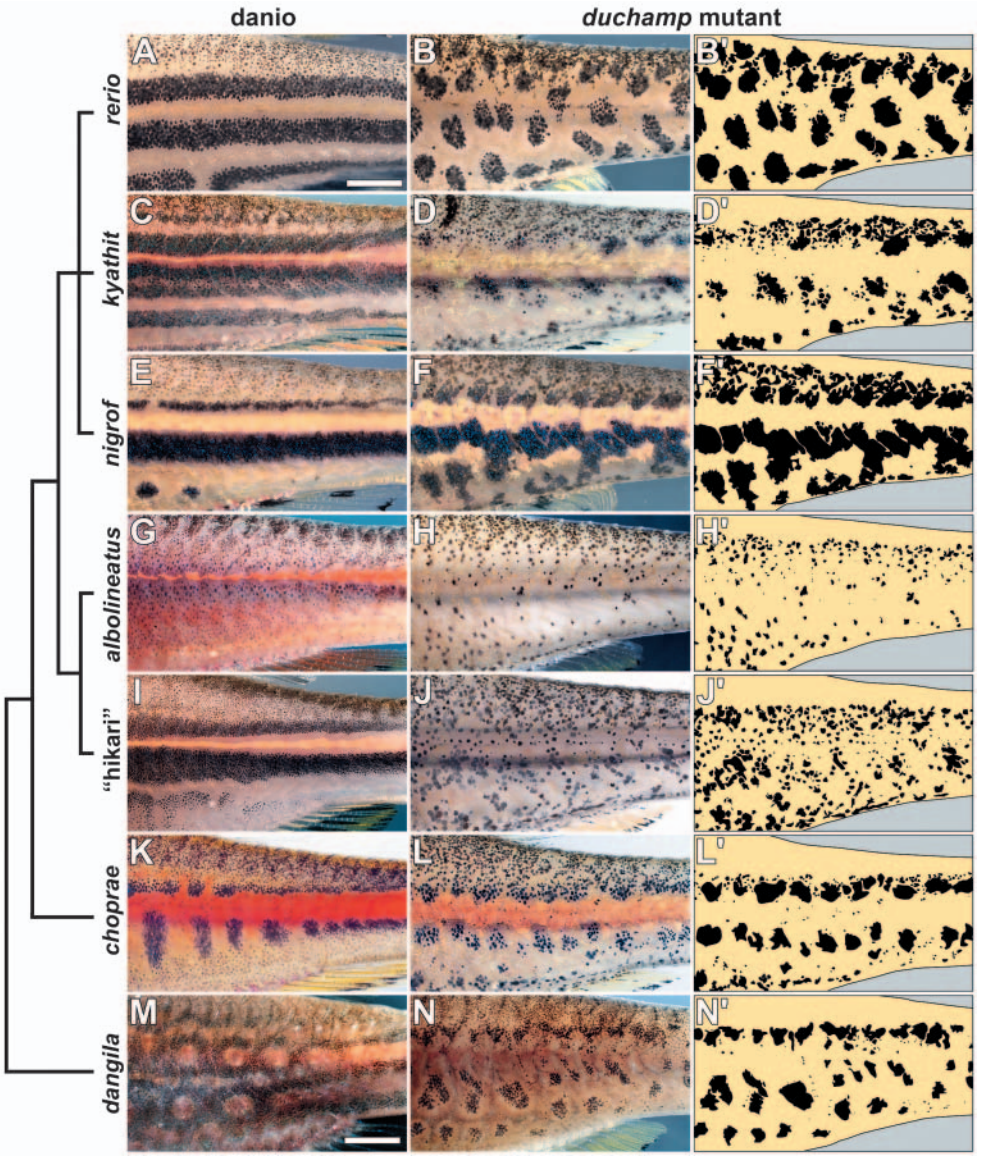

Fig. 10. Differential sensitivities across danios to melanophore reduction during hybrid pigment pattern development. An abbreviated phylogeny is shown on the left and wild-type danio pigment patterns are shown in A,C,E,G,I,K,M. Representative *duchamp* mutant hybrids are shown in B,D,F,H,J,L,N; schematics illustrate melanophore distributions (dorsal scale-associated melanophores are omitted for clarity). (A,B) Heterozygous *duchamp* mutant *D. rerio* (B) exhibit spots and fewer melanophores than wild type (A). Xanthophore and iridophore deficits are not apparent. (C,D) *duchamp* hybrids for *D. kyathit* develop spots of melanophores, although these are somewhat less regular than in *D. rerio*, as is the wild-type *D. kyathit* stripe pattern. (E,F) *duchamp* hybrids for *D. nigrofasciatus* develop well-organized spots or even complete stripes. (G,H) *duchamp* hybrids for *D. albolineatus* exhibit a more severe melanophore reduction than observed in heterozygous *duchamp* mutant *D. rerio* or other hybrids, and these melanophores remain widely dispersed over the caudal flank. As in *duchamp* mutant *D. rerio*, gross deficits in xanthophore numbers were not apparent during pigment pattern metamorphosis (data not shown). (I,J) *duchamp* hybrids for *D.* 'hikari' develop intermediate patterns, in which more melanophores are present than in *D. albolineatus* hybrids, but melanophore patterns range from weak clustering, to reticulation, to more uniform dispersion. (K-M) *duchamp* hybrids for the more distantly related *D. choprae* and *D. dangila* develop spots similar to the *D. rerio* species group. Scale bars: in A, 1 mm for A-L; in M, 0.5 mm for M,N.

notion: instead, we find that *D. albolineatus* actually have many more xanthophores than wild-type *D. rerio*.

During pigment pattern metamorphosis, *D. albolineatus* had greater numbers of xanthophores and these cells were distributed more widely than in *D. rerio*, in which xanthophores initially occur only near the horizontal myoseptum, and the dorsal and ventral margins of the flank (Fig. 9A-D,F,G). Xanthophores persist in older larvae and adult *D. albolineatus*, and are interspersed with melanophores (see Fig. S1 in supplementary material). Moreover, control hybrids between *D. albolineatus* and wild-type *D. rerio* had an intermediate number of xanthophores relative to parental species (Fig. 9E,H), in contrast to the severe xanthophore deficiency of *fms*^{j4e1} mutant hybrids (Fig. 4D). Thus, xanthophore development is enhanced in *D. albolineatus* and this trait is dominant in hybrids but highly sensitive to reduced *fms* activity. Interestingly, *D. choprae* similarly exhibit enhanced xanthophore development and a strong *fms* non-complementation phenotype (Fig. 2P; see Fig. S2 in supplementary material).

To further assess xanthophore development in *D. albolineatus*, we examined the distributions of cells expressing molecular markers of the xanthophore lineage. Between the epidermis and myotomes, where the adult pigment pattern develops, precursor distributions were similar between species (Fig. 9I,K). These data suggest that species differences in xanthophore development reflect differences in terminal differentiation of widely distributed precursors, rather than differences in the abundance or patterning of precursors themselves. In medial locations, however, precursors were more abundant in *D. albolineatus* than *D. rerio* (Fig. 9J,L).

The many xanthophores and xanthophore precursors in *D. albolineatus* suggest that fms continues to be functional in this species. Consistent with this possibility, we could not detect differences in *fms* expression between *D. rerio* and *D. albolineatus* during or after pigment pattern metamorphosis (Fig. 9M,N), and gross lesions are not apparent in the *fms* coding sequence (Parichy and Johnson, 2001).

These results show that *D. albolineatus* develop xanthophores in greater numbers and over a broader area than *D. rerio*, tending to exclude a model in which the evolutionary loss of stripes in *D. albolineatus* results simply from a loss of *fms* activity.

Evolutionary changes in cell–cell interactions during pigment pattern formation

The persistence of xanthophores in *D. albolineatus* led us to seek other explanations for the similarity of melanophore behaviors between this species and *fms* mutant *D. rerio*. In wild-type *D. rerio*, melanophore survival and organization into stripes depends on interactions between melanophores and *fms*-dependent cells of the xanthophore lineage (Parichy and Turner, 2003a), as well as interactions among melanophores. For example, the *D. rerio* leopard gene mediates both heterotypic interactions between melanophores and xanthophores, and homotypic interactions between melanophores (Maderspacher and Nusslein-Volhard, 2003), which we refer to collectively as ‘melanophore interactions’. The nature of these interactions is not yet known, but could include direct contacts between melanophores, xanthophores, or their precursors; alternatively, interactions could be indirect, involving secreted signaling molecules, trophic factors, or even intermediary cell types. Whatever their mechanism(s), the nearly uniform pigment pattern of *D. albolineaneatus* with interspersed melanophores and xanthophores (Fig. 1D; see Fig. S1C in supplementary material) and the irregular stripes of wild-type *D. rerio*×*D. albolineatus* hybrids (compared with other danios, Fig. 2) resemble different *D. rerio* mutant alleles of *leopard* (Asai et al., 1999), as well as *jaguar* (*obelix*), which contribute to homotypic interactions among melanophores (Maderspacher and Nusslein-Volhard, 2003). Thus, we hypothesized that instead of a loss of xanthophores, stripe absence in *D. albolineatus* might reflect changes in melanophore interactions. In principle, a species difference in melanophore interactions could be revealed with genetic mosaics (Parichy and Turner, 2003a; Quigley et al., 2004), but incompatibilities during early embryogenesis have so far precluded cell transplantations between *D. albolineatus* and *D. rerio* (D.M.P., unpublished). Thus, we used an alternative approach.

We reasoned that variation in melanophore interactions would be revealed if melanophore numbers were reduced (by analogy with reduced xanthophores in *fms* mutant *D. rerio* and hybrids with *D. albolineatus*): with fewer melanophores, strong interactions should allow the emergence of an organized pattern of stripes or spots, whereas weak interactions should result in a failure to organize such pattern elements. To achieve this, we used the *D. rerio* mutant, *duchamp*$^{ut.r19e1}$. A single mutant allele for *duchamp* reduces melanophores in heterozygous *D. rerio* to ~45% that of wild-type, yet the remaining melanophores form well-organized spots (Fig.

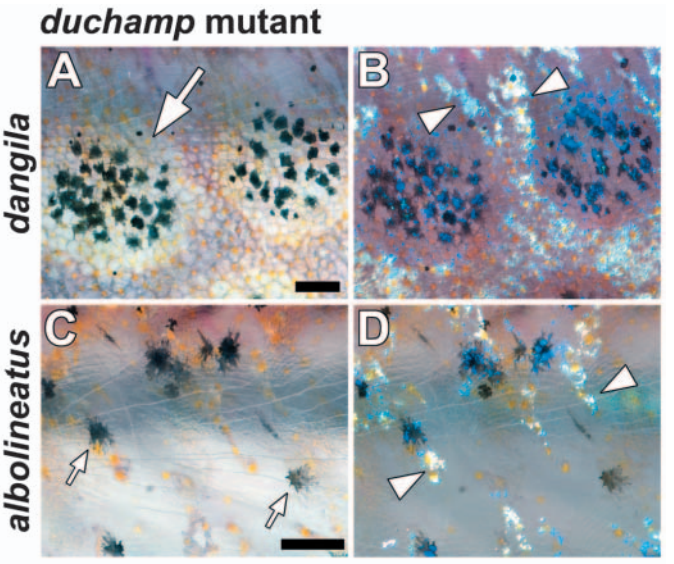

Fig. 11. Melanophore and iridophore patterns in tester *duchamp* mutant hybrids. (A,B) Different illumination of the same fields of view reveals melanophore organization (A,C) and iridophore organization (B,D). (A,B) Tester *duchamp* hybrids for *D. dangila* exhibit clusters of melanophores (arrows) with iridophores (arrowheads) organized around these clusters, as for hybrids of the *D. rerio* species group. (C,D) Tester *duchamp* hybrids for *D. albolineatus* typically do not form clusters of melanophores (arrows), and iridophores (arrowheads) are poorly organized. Scale bars: in A, 200 μm for A,B; in C, 200 μm for C,D.

10A,B); *D. rerio* homozygous for *duchamp* exhibit more dispersed melanophores (see Fig. S3 in supplementary material). We predicted that for species with melanophore interactions equivalent to *D. rerio*, *duchamp* hybrids should develop spots similar to heterozygous *duchamp* mutant *D. rerio*. For species with weaker melanophore interactions than *D. rerio*, tester *duchamp* hybrids should fail to generate organized pattern elements and could exhibit more severe melanophore deficiencies.

Phenotypes of tester *duchamp* hybrids support a model in which variation in melanophore interactions contributes to pigment pattern differences among danios. Within the clade that includes *D. rerio*, *duchamp* hybrids for both *D. kyathit* and *D. nigrofasciatus* developed spots or stripes of melanophores (Fig. 10C-F). By contrast, *duchamp* hybrids for *D. albolineatus* failed to develop organized spots or stripes and also had a reduction in melanophore numbers that became increasingly severe as fish grew (Fig. 10G,H, Fig. 11C,D). *duchamp* hybrids with *D.* 'hikari' developed an intermediate phenotype between *D. albolineatus* and other danio hybrids, in which melanophores failed to organize into spots and remained either dispersed or in a reticulated pattern (Fig. 10I,J). Finally, *duchamp* hybrids for *D. choprae* and *D. dangila* developed clusters of melanophores similar to *D. rerio* (Fig. 10K-N, Fig. 11A,B).

These analyses demonstrate that hybrid pigment patterns exhibit differential sensitivity across species to the *duchamp* mutant defect, with the greatest sensitivity in *D. albolineatus*. These findings are consistent with a model in which variation in melanophore interactions contribute to pigment pattern variation among danios.

Discussion

Danio pigment patterns are emerging as a useful system for understanding both the development and evolution of adult form in vertebrates. Our study suggests a model for how evolutionary changes in pigment cell development have generated naturally occurring variation in this ecologically and behaviorally significant trait.

Evolution of melanophore patterning in *Danio*

Melanophore patterns vary markedly among danios, and our analyses demonstrate dramatic differences in melanophore morphogenesis underlying the uniform pattern of *D. albolineatus* and the striped pattern of *D. rerio*. Fewer melanophores accumulate during pigment pattern metamorphosis in *D. albolineatus*, largely due to increased death of melanophores and their immediate precursors. This late block in melanophore development resembles that seen in *Astyanax* cavefish (McCauley et al., 2004), but contrasts with an early block affecting melanophore specification in *D. nigrofasciatus*, which accounts for an equivalent total melanophore deficit as compared with *D. rerio* (Quigley et al., 2004). Interestingly, the mode of melanophore loss in *D. albolineatus* resembles that of *kit* mutant *D. rerio* (Parichy et al., 1999), raising the possibility of a difference in kit signaling between species. Finally, we also demonstrate that *D. albolineatus* melanophores move little and thus do not coalesce into distinctive stripes as in *D. rerio*. In these respects, pigment pattern metamorphosis of *D. albolineatus* resembles that of *fms* mutant *D. rerio*. However, this is where the similarities end, as *D. albolineatus* retain large numbers of xanthophores, in stark contrast to *fms* mutant *D. rerio*.

We propose a model in which changes in melanophore interactions underlie the evolutionary loss of stripes in *D. albolineatus*. In *D. rerio*, stripe formation depends on interactions between melanophores and *fms*-dependent cells of the xanthophore lineage, as well as on interactions among melanophores; in the absence of such interactions, initially dispersed melanophores fail to migrate into stripes and melanophore death is increased (Maderspacher and Nusslein-Volhard, 2003; Parichy and Turner, 2003a). Genetic analyses of *D. albolineatus* initially suggested that changes in melanophore behaviors might result from a *fms*-dependent loss of xanthophores. Yet, the persistence of xanthophores excludes this model. Rather, we favor an alternative scenario involving changes in interactions between melanophores and xanthophores, or between melanophores themselves. This model does not exclude the possibility that differences outside of pigment cell lineages also influence species differences in melanophore morphogenesis, either directly, or by modulating the competence of pigment cells to interact with one another.

Several lines of evidence support a model in which the loss of stripes in *D. albolineatus* results at least partly from changes in melanophore interactions. First, we find an interspersed arrangement of *D. albolineatus* melanophores and xanthophores, which resembles *leopard* mutant *D. rerio* that are defective for melanophore-melanophore and melanophore-xanthophore interactions, as well as *jaguar* (*obelix*) mutant *D. rerio* that are defective for melanophore-melanophore interactions (Maderspacher and Nusslein-Volhard, 2003). Moreover, hybrids between *D. albolineatus* and semi-dominant *jaguar*^{c5} mutant *D. rerio* develop uniform pigment patterns like *D. albolineatus* that are qualitatively more severe than the heterozygous *jaguar*^{c5} pigment pattern (Parichy and Johnson, 2001), suggesting a difference between species in the *jaguar* pathway or in *jaguar*-dependent cellular interactions. However, hybrids between *D. rerio* carrying a recessive *jaguar*^{c7} deficiency and *D. albolineatus* are indistinguishable from control hybrids, suggesting the *jaguar* gene of *D. albolineatus* is not grossly hypomorphic compared to wild-type *D. rerio* (this study, data not shown).

Second, phenotypes of hybrids between *fms* mutant *D. rerio* and *D. albolineatus* are consistent with evolutionary changes in melanophore interactions. In these hybrids, melanophore patterns more closely resemble the uniform pattern of *D. albolineatus*. *fms* acts autonomously to the xanthophore lineage in promoting melanophore stripe organization in *D. rerio* (Parichy and Turner, 2003a). Thus, the dramatically fewer xanthophores in hybrids between *D. albolineatus* and *fms* mutant *D. rerio* may phenocopy the actual difference between species; i.e. melanophore interactions likely to have been lost evolutionarily are restored in the control hybrid, but abrogated by the reduced xanthophore number of the *fms* mutant hybrid (14% of control; Fig. 4).

Third, phenotypes of hybrids between *D. albolineatus* and *duchamp* mutant *D. rerio* are consistent with interspecific changes in melanophore interactions. When we use the *duchamp* mutant allele of *D. rerio* to reduce melanophore numbers in hybrids, clusters of melanophores form on the flank of *D. rerio* and hybrids of four other species, but not *D.*

albolineatus. The failure of *D. albolineatus* hybrids to organize melanophore spots does not reflect phylogenetic distance from *D. rerio*, as spots were formed in hybrids of the more distantly related *D. dangila* and *D. choprae*. Nor does the absence of spots result merely from a low starting number of melanophores in *D. albolineatus*, as the adult melanophore number is indistinguishable between this species and *D. nigrofasciatus* (Quigley et al., 2004). Finally, the lack of spots does not result from a higher growth rate that might carry melanophores passively away from one another, as *D. dangila* hybrids develop spots, despite growing more rapidly than *D. albolineatus* hybrids (D.M.P., unpublished). Our finding that *duchamp* hybrids for *D.* 'hikari' have a phenotype intermediate to *D. albolineatus* and the other danios suggests that changes in melanophore interactions may have contributed to overall differences between the *albolineatus*-'hikari' clade and other danios, with a more extreme difference in *D. albolineatus* conceivably responsible for the absence of stripes in this species. Identification of the *duchamp* gene product will allow a fuller analysis of roles for this gene and pathway in melanophore interactions and their evolution.

Our analyses and previous studies of *D. rerio* highlight the potentially important role that intercellular interactions are likely to play in the development and evolution of neural crest derivatives. Such interactions have started to be characterized within melanocyte, neurogenic and rhombomeric lineages as well (Aubin-Houzelstein et al., 1998; Hagedorn et al., 1999; Trainor and Krumlauf, 2000; Paratore et al., 2002; Hou et al., 2004). In principle, melanophore interactions could involve factors secreted or presented at the cell surface (Wehrle-Haller, 2003; Hou et al., 2004), or adhesive or junctional contacts among pigment cells or their precursors (Twitty, 1945; Tucker and Erickson, 1986; Parichy, 1996), although increased stratification of skin and pigment cell locations may preclude some direct contacts in adults (Hirata et al., 2003). By extension, evolutionary changes could reflect modifications to the interactions, if the competence to provide or receive signals is altered. Or, the same outcome could be effected by changes to the cellular context in which these interactions occur. For instance, simply the increased number of xanthophores in *D. albolineatus* may interfere with melanophore-melanophore interactions. In support of this notion, melanophores that become isolated within xanthophore-rich interstripe regions typically are lost in *D. rerio* (Goodrich et al., 1954; Parichy and Turner, 2003b), whereas in the anal fin of *D. albolineatus*, melanophores and xanthophores appear in temporally and spatially distinct waves, and a narrow melanophore stripe develops (Goodrich and Greene, 1959). Whatever their mechanisms, interactions within and among pigment cell classes suggest a rich source of variation for the evolutionary diversification of pigment patterns, without the necessity of correlated changes in other cell and tissue types. Such an independence of pigment pattern variation from other traits may in turn explain rapid and extensive pigment pattern evolution across species that are otherwise relatively similar in form.

Evolution of *fms* activity and function during xanthophore development

Interspecific complementation tests initially suggested that *D. albolineatus* could have reduced *fms* activity compared with *D. rerio*, as hybrids between wild-type *D. rerio* develop well-organized stripes, whereas hybrids with fms^{j4e1} mutant *D. rerio* either lack stripes or have poorly formed stripes, depending on genetic background (this study) (Parichy and Johnson, 2001). Despite this non-complementation phenotype, our analyses do not support a model in which *D. albolineatus* have evolved a loss of *fms* activity. Rather, these data suggest that species differ in their dosage sensitivity for *fms* during xanthophore development, or that evolutionary changes have occurred that affect molecular interactions within the *fms* pathway.

Despite a reported absence of xanthophores in adult *D. albolineatus* (McClure, 1999), we find that larvae develop more xanthophores, these cells and their precursors arise in a broader range of locations than in *D. rerio*, and also persist into the adult. These observations contrast with the simplest loss-of-function model, in which xanthophores should be absent or reduced.

The excess of xanthophores in wild-type control *D. rerio*×*D. albolineatus* hybrids, and the reduction of xanthophores in tester *fms* mutant *D. rerio*×*D. albolineatus* hybrids reveals a genetic interaction not predicted by the recessive *fms* mutant phenotype in *D. rerio*. We envisage at least two complementary explanations for this interaction. First, xanthophore development may differ between species in its sensitivity to changes in Fms signaling. Genetic background effects are well known for mouse melanocyte mutants, including the structurally and functionally similar *Kit* locus, and variable degrees of haploinsufficiency have been associated with modifier loci for mutants affecting neural crest derivatives more generally (Lamoreux, 1999; Ingram et al., 2000; Rhim et al., 2000; Nadeau, 2001; Cantrell et al., 2004). Conceivably, the more rapid and extensive development of xanthophores in *D. albolineatus* (and *D. choprae*) could entail a greater, continuous requirement for *fms*, such that any deficit results in pigment pattern defects.

A second explanation for *fms* hybrid non-complementation phenotypes lies in interspecific structural differences in fms, its ligand, csf1, or both. As fms and csf1 each act as dimers (Li and Stanley, 1991; Carlberg and Rohrschneider, 1994; Ingram et al., 2000), signaling could be reduced if interspecific receptors or ligands dimerize less efficiently, or if structures of receptors and ligands co-evolve so that mismatched receptor-ligand pairings function less efficiently. To illustrate this point, we can imagine an extreme model in which any species mismatch in a receptor-ligand pairing ablates signaling. In a wild-type hybrid, functional receptor-ligand pairings would drop to one-eighth that of parental species (i.e. each species' pairing would comprise one-sixteenth of all combinations in the hybrid individual). In the tester *fms* mutant hybrid, functional receptor-ligand interactions would drop to one-sixteenth that of parental species. Given a fixed threshold of dosage sensitivity (here, between one-eighth and one-sixteenth of maximal), this model can easily account for the non-complementation phenotype of tester *fms* mutant hybrids.

By extension, our analyses suggest evolution of cellular requirements for *fms* or rapid evolution of genes within the *fms* pathway. In *fms* hybrids, we observed strong non-complementation phenotypes for *D. albolineatus*, *D.* aff. *albolineatus* and *D. choprae*, a weak non-complementation phenotype for *D.* 'hikari', but complementation indistinguishable from wild-type *D. rerio* for *D. kyathit*, *D.*

nigrofasciatus and *D. dangila*. These findings suggest most parsimoniously that: (1) changes have occurred that differentiate the *D. albolineatus-D.* 'hikari' clade from the *D. rerio-D. kyathit-D. nigrofasciatus* clade; and (2) additional changes have occurred in the lineages leading either to *D. choprae* or *D. dangila*. This interspecific variation is striking, as similar tests across danios have failed to reveal non-complementation for more than a dozen other *D. rerio* pigment pattern mutants, including the structurally and functionally similar kit locus (Parichy and Johnson, 2001) (D.M.P., unpublished). Thus, the *fms* pathway may be particularly useful for investigating the evolution of genetic dominance and developmental robustness, as well as the co-evolution of gene products within molecular pathways (Meir et al., 2002; Nijhout, 2002; Kondrashov and Koonin, 2004).

Finally, this study reveals enhanced xanthophore development in both *D. albolineatus* and *D. choprae* compared with wild-type *D. rerio*. These findings raise the possibilities of differences in the distribution or abundance of csf1, or quantitative (as distinct from constitutive) gains of fms function in *D. albolineatus* and *D. choprae* compared with other danios. The latter possibility is opposite to initial predictions of allelic strengths (Parichy and Johnson, 2001), but is not inconsistent with the models proposed above. Immunohistochemical, transgenic, and other approaches should allow distinguishing between these models.

Thanks to C. Lee for help rearing fish, as well as M. Reedy, J. Wallingford and two anonymous reviewers for insightful discussions or comments on the manuscript. T. Wilcox provided valuable advice on phylogenetic methods. Mutant stocks were generously provided by M. Halpern, R. Knight, J. Lister, D. Raible, T. Schilling, S. Johnson, K. Poss and the Zebrafish International Resources Center at the University of Oregon. Supported by NIH R01 GM62182 to D.M.P.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/1/89/DC1

References

Ahlgren, S. C., Thakur, V. and Bronner-Fraser, M. (2002). Sonic hedgehog rescues cranial neural crest from cell death induced by ethanol exposure. *Proc. Natl. Acad. Sci. USA* **99**, 10476-10481.

Allender, C. J., Seehausen, O., Knight, M. E., Turner, G. F. and Maclean, N. (2003). Divergent selection during speciation of Lake Malawi cichlid fishes inferred from parallel radiations in nuptial coloration. *Proc. Natl. Acad. Sci. USA* **100**, 14074-14079.

Amiel, J. and Lyonnet, S. (2001). Hirschsprung disease, associated syndromes, and genetics: a review. *J. Med. Genet.* **38**, 729-739.

Asai, R., Taguchi, E., Kume, Y., Saito, M. and Kondo, S. (1999). Zebrafish leopard gene as a component of the putative reaction-diffusion system. *Mech. Dev.* **89**, 87-92.

Aubin-Houzelstein, G., Bernex, F., Elbaz, C. and Panthier, J.-J. (1998). Survival of patchwork melanoblasts is dependent upon their number in the hair follicle at the end of embryogenesis. *Dev. Biol.* **198**, 266-276.

Barreda, D. R., Hanington, P. C. and Belosevic, M. (2004). Regulation of myeloid development and function by colony stimulating factors. *Dev. Comp. Immunol.* **28**, 509-554.

Camp, E. and Lardelli, M. (2001). Tyrosinase gene expression in zebrafish embryos. *Dev Genes. Evol.* **211**, 150-153.

Cantrell, V. A., Owens, S. E., Chandler, R. L., Airey, D. C., Bradley, K. M., Smith, J. R. and Southard-Smith, E. M. (2004). Interactions between Sox10 and EdnrB modulate penetrance and severity of aganglionosis in the Sox10Dom mouse model of Hirschsprung disease. *Hum. Mol. Genet.* **13**, 2289-2301.

Carlberg, K. and Rohrschneider, L. (1994). The effect of activating mutations on dimerization, tyrosine phosphorylation and internalization of the macrophage colony stimulating factor receptor. *Mol. Biol. Cell* **5**, 81-95.

Couldridge, V. C. K. and Alexander, G. J. (2002). Color patterns and species recognition in four closely related species of Lake Malawi cichlid. *Behav. Ecol.* **13**, 59-64.

Dai, X. M., Ryan, G. R., Hapel, A. J., Dominguez, M. G., Russell, R. G., Kapp, S., Sylvestre, V. and Stanley, E. R. (2002). Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. *Blood* **99**, 111-120.

Dutton, K. A., Pauliny, A., Lopes, S. S., Elworthy, S., Carney, T. J., Rauch, J., Geisler, R., Haffter, P. and Kelsh, R. N. (2001). Zebrafish *colourless* encodes sox10 and specifies non-ectomesenchymal neural crest fates. *Development* **128**, 4113-4125.

Endler, J. A. (1983). Natural and sexual selection on color patterns in Poeciliid fishes. *Env. Biol. Fishes* **9**, 173-190.

Engeszer, R. E., Ryan, M. J. and Parichy, D. M. (2004). Learned social preference in zebrafish. *Curr. Biol.* **14**, 881-884.

Fang, F. (2003). Phylogenetic analysis of the Asian cyprinid genus *Danio* (Teleostei, Cyprinidae). *Copeia* **2003**, 714-728.

Fang, F. and Kottelat, M. (2000). *Danio roseus*, a new species from the Mekong basin in northeastern Thailand and northwestern Laos (Teleostei: Cyprinidae). *Ichthyol. Explor. Freshw.* **11**, 149-154.

Farlie, P. G., McKeown, S. J. and Newgreen, D. F. (2004). The neural crest: basic biology and clinical relationships in the craniofacial and enteric nervous systems. *Birth Defects Res. Part C. Embryo Today* **72**, 173-189.

Fisher, S., Amacher, S. L. and Halpern, M. E. (1997). Loss of *cerebum* function ventralizes the zebrafish embryo. *Development* **124**, 1301-1311.

Galant, R. and Carroll, S. B. (2002). Evolution of a transcriptional repression domain in an insect Hox protein. *Nature* **415**, 910-913.

Gans, C. and Northcutt, R. G. (1983). Neural crest and the origin of vertebrates: a new head. *Science* **220**, 268-274.

Goodrich, H. B. and Greene, J. M. (1959). An experimental analysis of the development of a color pattern in the fish *Brachydanio albolineatus* Blyth. *J. Exp. Zool.* **141**, 15-45.

Goodrich, H. B., Marzullo, C. M. and Bronson, W. R. (1954). An analysis of the formation of color patterns in two fresh-water fish. *J. Exp. Zool.* **125**, 487-505.

Hagedorn, L., Suter, U. and Sommer, L. (1999). P0 and PMP22 mark a multipotent neural crest-derived cell type that displays community effects in response to TGF-beta family factors. *Development* **126**, 3781-3794.

Hall, B. K. (1999). *The Neural Crest in Development and Evolution*. New York, NY: Springer-Verlag.

Hirata, M., Nakamura, K., Kanemaru, T., Shibata, Y. and Kondo, S. (2003). Pigment cell organization in the hypodermis of zebrafish. *Dev. Dyn.* **227**, 497-503.

Hörstadius, S. (1950). *The Neural Crest: Its Properties and Derivatives in Light of Experimental Research*. London, UK: Oxford University Press.

Hou, L., Pavan, W. J., Shin, M. K. and Arnheiter, H. (2004). Cell-autonomous and cell non-autonomous signaling through endothelin receptor B during melanocyte development. *Development* **131**, 3239-3247.

Houde, A. E. (1997). *Sex, Color, and Mate Choice in Guppies*. Princeton, NJ: Princeton University Press.

Huelsenbeck, J. P. and Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* **17**, 754-755.

Ingram, D. A., Yang, F. C., Travers, J. B., Wenning, M. J., Hiatt, K., New, S., Hood, A., Shannon, K., Williams, D. A. and Clapp, D. W. (2000). Genetic and biochemical evidence that haploinsufficiency of the Nf1 tumor suppressor gene modulates melanocyte and mast cell fates in vivo. *J. Exp. Med.* **191**, 181-188.

Kelsh, R. N. (2004). Genetics and evolution of pigment patterns in fish. *Pigment Cell Res.* **17**, 326-336.

Kelsh, R. N., Schmid, B. and Eisen, J. S. (2000). Genetic analysis of melanophore development in zebrafish embryos. *Dev. Biol.* **225**, 277-293.

Knight, R. D., Nair, S., Nelson, S. S., Afshar, A., Javidan, Y., Geisler, R., Rauch, G. J. and Schilling, T. F. (2003). lockjaw encodes a zebrafish tfap2a required for early neural crest development. *Development* **130**, 5755-5768.

Knight, R. D., Javidan, Y., Nelson, S., Zhang, T. and Schilling, T. (2004). Skeletal and pigment cell defects in the lockjaw mutant reveal multiple roles for zebrafish tfap2a in neural crest development. *Dev. Dyn.* **229**, 87-98.

Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S. V., Paabo, S., Villablance, F. X. and Wilson, A. C. (1989). Dynamics of mitochondrial DNA evolution in animals. *Proc. Natl. Acad. Sci. USA* **86**, 6196-6200.

Kondrashov, F. A. and Koonin, E. V. (2004). A common framework for

understanding the origin of genetic dominance and evolutionary fates of gene duplications. *Trends Genet.* **20**, 287-90.
Kullander, F. F. (2001). *Phylogeny and Species Diversity of the South and Southeast Asian Cyprinid Genus Danio Hamilton (Teleostei, Cyprinidae)*. Stockholm, Sweden: Stockhold University.
Lamoreux, M. L. (1999). Strain-specific white-spotting patterns in laboratory mice. *Pigment Cell Res.* **12**, 383-390.
Le Douarin, N. M. (1999). *The Neural Crest*. Cambridge, UK: Cambridge University Press.
Li, W. and Stanley, E. R. (1991). Role of dimerization and modification of the CSF-1 receptor in its activation and internalization during the CSF-1 response. *EMBO J.* **10**, 277-288.
Lister, J. A., Robertson, C. P., Lepage, T., Johnson, S. L. and Raible, D. W. (1999). *nacre* encodes a zebrafish microphthalmia-related protein that regulates neural-crest-derived pigment cell fate. *Development* **126**, 3757-3767.
Maderspacher, F. and Nusslein-Volhard, C. (2003). Formation of the adult pigment pattern in zebrafish requires leopard and obelix dependent cell interactions. *Development* **130**, 3447-3457.
Matthay, K. K. (1997). Neuroblastoma: biology and therapy. *Oncology* **11**, 1857-1866.
McCauley, D. W., Hixon, E. and Jeffery, W. R. (2004). Evolution of pigment cell regression in the cavefish Astyanax: a late step in melanogenesis. *Evol. Dev.* **6**, 209-218.
McClure, M. (1999). Development and evolution of melanophore patterns in fishes of the genus Danio (Teleostei: Cyprinidae). *J. Morphol.* **241**, 83-105.
Meir, E., von Dassow, G., Munro, E., Odell, G. M., Eldar, A., Dorfman, R., Weiss, D., Ashe, H., Shilo, B. Z., Barkai, N. et al. (2002). Robustness, flexibility, and the role of lateral inhibition in the neurogenic network. *Curr. Biol.* **12**, 778-786.
Nadeau, J. H. (2001). Modifier genes in mice and humans. *Nat. Rev. Genet.* **2**, 165-174.
Nijhout, H. F. (2002). The nature of robustness in development. *BioEssays* **24**, 553-563.
Palumbi, S. R., Martin, A., Romano, S., McMillan, W. O., Stice, L. and Grabowski, G. (1991). *The Simple Fool's Guide to PCR*. Honolulu, Hawaii: University of Hawaii Press.
Paratore, C., Hagedorn, L., Floris, J., Hari, L., Kleber, M., Suter, U. and Sommer, L. (2002). Cell-intrinsic and cell-extrinsic cues regulating lineage decisions in multipotent neural crest-derived progenitor cells. *Int. J. Dev. Biol.* **46**, 193-200.
Parichy, D. M. (1996). Pigment patterns of larval salamanders (Ambystomatidae, Salamandridae): the role of the lateral line sensory system and the evolution of pattern-forming mechanisms. *Dev. Biol.* **175**, 265-282.
Parichy, D. M. (2003). Pigment patterns: fish in stripes and spots. *Curr. Biol.* **13**, R947-R950.
Parichy, D. M. and Johnson, S. L. (2001). Zebrafish hybrids suggest genetic mechanisms for pigment pattern diversification in *Danio*. *Dev. Genes Evol.* **211**, 319-328.
Parichy, D. M. and Turner, J. M. (2003a). Temporal and cellular requirements for Fms signaling during zebrafish adult pigment pattern development. *Development* **130**, 817-833.
Parichy, D. M. and Turner, J. M. (2003b). Zebrafish *puma* mutant decouples pigment pattern and somatic metamorphosis. *Dev. Biol.* **256**, 242-257.
Parichy, D. M., Rawls, J. F., Pratt, S. J., Whitfield, T. T. and Johnson, S. L. (1999). Zebrafish sparse corresponds to an orthologue of *c-kit* and is required for the morphogenesis of a subpopulation of melanocytes, but is not essential for hematopoiesis or primordial germ cell development. *Development* **126**, 3425-3436.
Parichy, D. M., Mellgren, E. M., Rawls, J. F., Lopes, S. S., Kelsh, R. N. and Johnson, S. L. (2000a). Mutational analysis of *endothelin receptor b1* (*rose*) during neural crest and pigment pattern development in the zebrafish *Danio rerio*. *Dev. Biol.* **227**, 294-306.
Parichy, D. M., Ransom, D. G., Paw, B., Zon, L. I. and Johnson, S. L. (2000b). An orthologue of the *kit*-related gene *fms* is required for development of neural crest-derived xanthophores and a subpopulation of adult melanocytes in the zebrafish, *Danio rerio*. *Development* **127**, 3031-3044.
Parichy, D. M., Turner, J. M. and Parker, N. B. (2003). Essential role for *puma* in development of postembryonic neural crest-derived cell lineages in zebrafish. *Dev. Biol.* **256**, 221-241.
Quigley, I. K., Turner, J. M., Nuckels, R. N., Manuel, J. L., Budi, E., MacDonald, E. L. and Parichy, D. M. (2004). Pigment pattern evolution by differential deployment of neural crest and post-embryonic melanophore lineages in *Danio* fishes. *Development* **131**, 6053-6069.
Rhim, H., Dunn, K. J., Aronzon, A., Mac, S., Cheng, M., Lamoreux, M. L., Tilghman, S. M. and Pavan, W. J. (2000). Spatially restricted hypopigmentation associated with an *Ednrb*^{s}-modifying locus on mouse chromosome 10. *Genome Res.* **10**, 17-29.
Rohlf, F. J. and Sokal, R. R. (1994). *Biometry*, 3rd edn. New York, NY: W. H. Freeman.
Scheijen, B. and Griffin, J. D. (2002). Tyrosine kinase oncogenes in normal hematopoiesis and hematological disease. *Oncogene* **21**, 3314-3333.
Shapiro, M. D., Marks, M. E., Peichel, C. L., Blackman, B. K., Nereng, K. S., Jonsson, B., Schluter, D. and Kingsley, D. M. (2004). Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks. *Nature* **428**, 717-723.
Smith, M., Hickman, A., Amanze, D., Lumsden, A. and Thorogood, P. (1994). Trunk neural crest origin of caudal fin mesenchyme in the zebrafish *Brachydanio rerio*. *Proc. R. Soc. Lond., B, Biol. Sci.*. **256**, 137-145.
Stanley, E. R., Berg, K. L., Einstein, D. B., Lee, P. S., Pixley, F. J., Wang, Y. and Yeung, Y. G. (1997). Biology and action of colony-stimulating factor-1. *Mol. Reprod. Dev.* **46**, 4-10.
Sugimoto, M. (2002). Morphological color changes in fish: regulation of pigment cell density and morphology. *Microsc. Res. Tech.* **58**, 496-503.
Swofford, D. L. (2002). *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4*. Sunderland, MA: Sinauer Associates.
Tanaka, M., Munsterberg, A., Anderson, W. G., Prescott, A. R., Hazon, N. and Tickle, C. (2002). Fin development in a cartilaginous fish and the origin of vertebrate limbs. *Nature* **416**, 527-531.
Trainor, P. and Krumlauf, R. (2000). Plasticity in mouse neural crest cells reveals a new patterning role for cranial mesoderm. *Nat. Cell. Biol.* **2**, 96-102.
Tucker, R. P. and Erickson, C. A. (1986). Pigment cell pattern formation in *Taricha torosa*: the role of the extracellular matrix in controlling pigment cell migration and differentiation. *Dev. Biol.* **118**, 268-285.
Twitty, V. C. (1945). The developmental analysis of specific pigment patterns. *J. Exp. Zool.* **100**, 141-178.
Wehrle-Haller, B. (2003). The role of Kit-ligand in melanocyte development and epidermal homeostasis. *Pigment Cell Res.* **16**, 287-296.
Widlund, H. R. and Fisher, D. E. (2003). Microphthalamia-associated transcription factor: a critical regulator of pigment cell development and survival. *Oncogene* **22**, 3035-3041.
Yoshida, H., Hayashi, S., Kunisada, T., Ogawa, M., Nishikawa, S., Okamura, H., Sudo, T., Shultz, L. D. and Nishikawa, S. (1990). The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature* **345**, 442-444.
Ziegler, I., McDonaldo, T., Hesslinger, C., Pelletier, I. and Boyle, P. (2000). Development of the pteridine pathway in the zebrafish, *Danio rerio*. *J. Biol. Chem.* **275**, 18926-18932.