

Homology and the Evolution of Novelty During *Danio* Adult Pigment Pattern Development

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ABSTRACT Recent studies using zebrafish and its relatives have provided insights into the development and evolution of adult pigment patterns. In this review, I describe how an iterative approach using a biomedical model organism and its close relatives can be used to elucidate both mechanistic and organismal aspects of pigment pattern formation. Such analyses have revealed critical roles for post-embryonic latent precursors as well as interactions among different pigment cell classes during adult pigment pattern formation and diversification. These studies also have started to reveal homologous and novel features of the underlying developmental processes. *J. Exp. Zool. (Mol. Dev. Evol.)* 306B, 2006. © 2006 Wiley-Liss, Inc.

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The zebrafish *Danio rerio* has emerged as one of the major model organisms for biomedical research. Identified as a promising vertebrate for developmental genetics in the 1970s and 1980s, the zebrafish joined the ranks of premier biomedical models during the 1990s (Grunwald and Eisen, 2002). With this transition came genome sequencing, vast numbers of mutant lines, specialized techniques for cell labeling and transplantation, and methods for gene knockdown and transgenesis. Today, zebrafish is used for studies of basic developmental mechanisms, cancer and stem cell biology, disease syndromes, immunology and infection, hematopoiesis, physiology, addiction, regeneration, vision, and more. The zebrafish also is a powerful, and still underexploited, model system for studies of organismal evolution, ecology, and behavior (Engeszer et al., 2006).

Pigment pattern is one trait that lends itself especially well to both organismal and mechanistic studies using zebrafish. Animal pigment patterns long have fascinated biologists, professional, and amateur alike. In teleosts, pigment patterns serve in a variety of contexts, including shoaling, mate recognition, mate choice, and camouflage (Endler, '83; Houde, '97; Allender et al., 2003; Engeszer et al., 2004; Seehausen and Schluter, 2004). Pigment patterns also have been studied for many years at the genetic, cellular, and

developmental levels (Erickson, '93; Bennett and Lamoreux, 2003; Parichy et al., 2006). Given the wealth of knowledge on organismal and mechanistic aspects of pigment patterns, they constitute an outstanding model for integrative analyses bridging several levels of the biological hierarchy.

An additional reason to study pigment pattern in zebrafish is diversity, which presents itself in two ways. In a comparative sense, *Danio* and closely related genera such as *Devario* (together referred to here as "danios") include species with a variety of adult pigment patterns (Fig. 1A). For example, *D. rerio* has several well-defined horizontal stripes; *D. nigrofasciatus*, stripes and spots; *D. kerri*, broad relatively diffuse stripes; *D. dangila*, a reticulated pattern; *De. shanensis* and *D. choprae*, vertical bars; and *D. albolineatus*, a uniform pattern of evenly dispersed pigment cells. These are just a few

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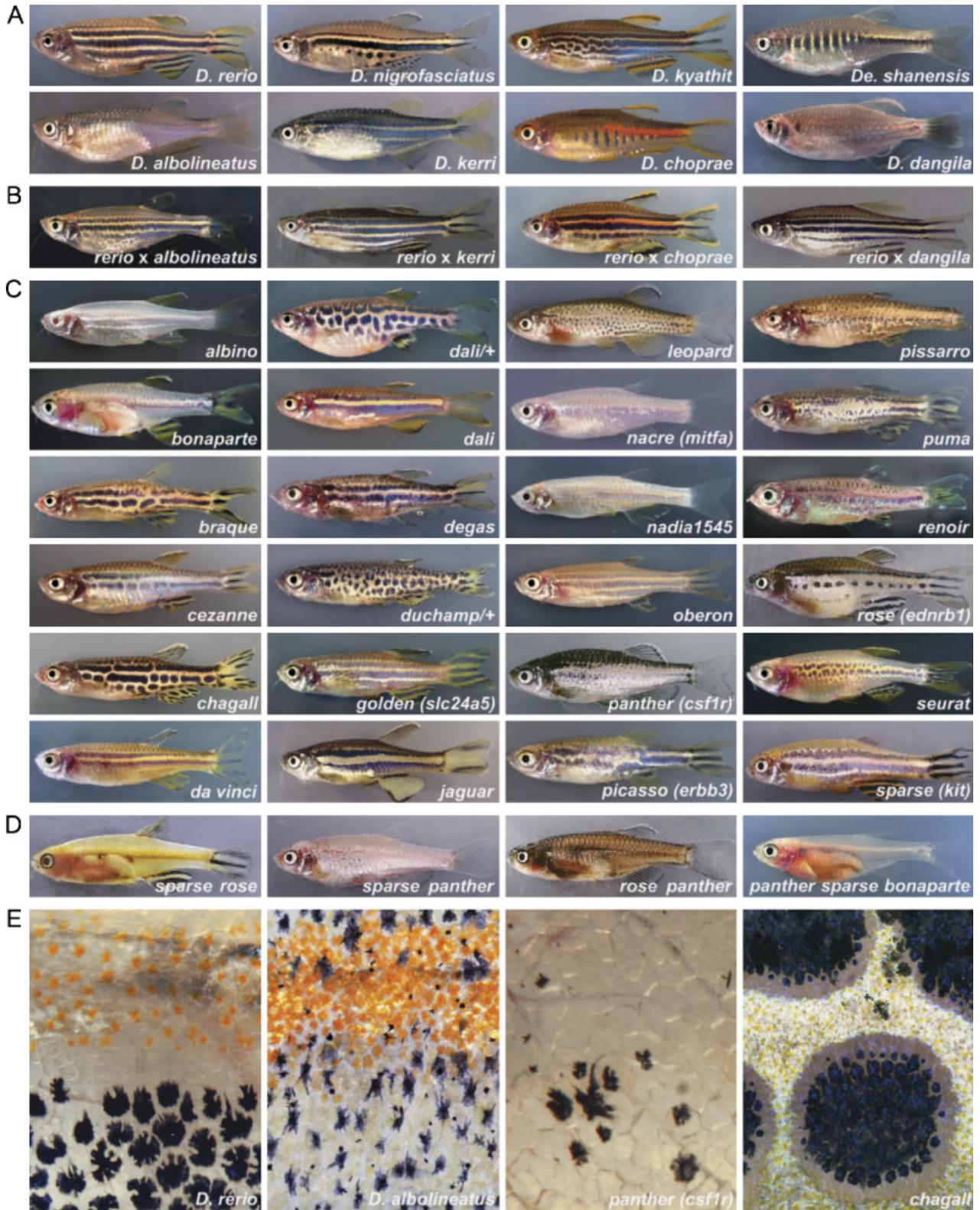


Fig. 1. Diversity of *Danio* pigment patterns. (A) Species within *Danio* and *Devario* exhibit a wide range of stripes, bars, uniform, and other pigment patterns. (B) Species within *Danio* can be hybridized to the zebrafish *D. rerio*, with hybrids typically developing horizontal stripes reminiscent of *D. rerio*. (C) Examples of single-locus mutant phenotypes isolated in *D. rerio* (see text for details). Corresponding genes, when known, are given in parentheses (Lister et al., '99; Parichy et al., '99, 2000a,b; Lamason et al., 2005) (Parichy, unpublished data). (D) Double and triple mutant phenotypes can be used to test mechanistic hypotheses and to explore the range of phenotype space. (E) Details of pigment patterns in different species and mutant backgrounds.

of the many species in the group, species whose phylogenetic relationships are becoming increasingly well understood (see below). This diversity draws attention to the hypothesis that pigment patterns may themselves have been involved in speciation in this group.

The analysis of mutants is a second way in which zebrafish pigment patterns are diverse (Fig. 1C). A major advantage to zebrafish is the ability to conduct forward genetic screens, in which mutants are isolated by their phenotypes with the subsequent goal of identifying the responsible loci.¹ Typically, mutations are induced chemically and alter one or a few nucleotides in single, affected genes. This approach allows the genetic dissection of traits, while facilitating gene discovery. Similar genetic approaches can be applied to other danios as well (discussed below).

Laboratory-recovered mutants also are valuable in an evolutionary context, as they describe a phenotype space that is one mutational step away from "wild-type". Fish bred to be mutant for two (or three) genes simultaneously describe additional mutational steps (Fig. 1D). The magnitude of these phenotypes is probably relevant to nature, as major effect single-locus mutant phenotypes can be recovered from natural population isolates (e.g., *nadia1545* in Fig. 1C), and many laboratory-recovered mutants resemble naturally occurring pigment pattern variants within and among species (Parichy, unpublished data). Thus, mutations induced in the laboratory identify candidate genes that can be tested for their association with standing variation. As just one example of this approach, interspecific complementation tests can be used to rapidly screen loci identified by zebrafish mutants for major differences in activity across species (see below).

Beyond diversity, another major advantage to zebrafish pigment patterns lies in their accessibility to observation and experimentation. Much of pigment pattern formation occurs under a mostly transparent skin and pigment cells carry

their own autonomous markers of cell lineage; i.e., their pigments. This means that behaviors of individual cells can be followed throughout pigment pattern formation even in the living fish, and differences in cell behaviors across species or mutant lines can be seen directly as the phenotypes develop (e.g., movies at: <http://protist.biology.washington.edu/dparichy/Movies.htm>).

One final advantage to studying pigment patterns is the developmental origins of vertebrate pigment cells: the neural crest. The neural crest is a transient population of embryonic precursor cells that arises along the neural tube (or in teleosts, the neural keel) shortly after neurulation. Neural crest cells disperse from this position and migrate throughout the embryo, contributing to a wide range of derivatives. These include pigment cells, but also much of the peripheral nervous system including neurons and glia, as well as bone, cartilage, and teeth of the craniofacial skeleton, adrenal chromaffin cells, fin mesenchyme, connective tissue of the heart and great vessels, and more (Hall, '99; Le Douarin, '99). So extensive is this anatomical contribution that the evolutionary appearance of a neural crest has been viewed as a key vertebrate innovation (Gans and Northcutt, '83). In turn, changes in the developmental patterning of these cells and their derivatives are associated with naturally occurring phylogenetic transformations, as well as normal phenotypic variation and disease states within populations. The diversity and accessibility of pigment patterns make them an outstanding system for studying features of neural crest development likely to be relevant also to other neural crest-derived traits.

PIGMENT CELL CLASSES AND OUTLINE OF PIGMENT PATTERN DEVELOPMENT

In contrast to mammals and birds, in which melanocytes are the only neural crest-derived skin pigment cell, teleosts have several types of pigment cells, or "chromatophores". These include black melanophores, yellow or orange xanthophores, red erythrophores, and iridescent iridophores (Fig. 1E) (Kelsh, 2004). All danios thus far examined have melanophores, xanthophores,

¹This approach differs from "reverse" genetic strategies, in which a gene of interest is manipulated, typically to generate a loss-of-function phenotype, for assaying what essential role that gene might have during development.

and iridophores; only a subset of species have erythrophores (McClure, '99; Quigley et al., 2005). Another difference from both mammals and birds is the ultimate fate of the pigments. Many melanocytes transfer melanin to keratinocytes for eventual incorporation into developing hair and feathers, whereas chromatophores retain their pigments intracellularly. Despite these differences, most studies have revealed an extraordinary conservation of genetic pathways, at least between melanocytes and melanophores among major vertebrate groups (Quigley and Parichy, 2002; Kelsh, 2004; Parichy et al., 2006).

In *D. rerio* and other danios, different pigment patterns are present during different phases of the life cycle. In early larvae, the pigment pattern consists of several stripes of melanophores along the dorsal myotomes, horizontal myoseptum, and ventral myotomes (Fig. 2A). A few iridophores occur within these stripes. By contrast, xanthophores are scattered over the flank, essentially everywhere that melanophores are not found. This pigment pattern is conserved in other danios (Quigley et al., 2004) and arises during embryogenesis as neural crest cells migrate and

differentiate into the various chromatophore lineages (Raible and Eisen, '94; Dutton et al., 2001).

The *D. rerio* adult pigment pattern consists of dark stripes, comprising melanophores, iridophores, and occasional xanthophores, alternating with light stripes, comprising xanthophores and iridophores (Figs. 1E and 2B) (Hirata et al., 2003; Parichy and Turner, 2003a). Recent studies have shown that different chromatophore classes occupy different strata beneath the skin: xanthophores occur most superficially, with a deeper layer consisting of melanophores in stripe regions or iridophores in interstripe regions, followed by another, deeper layer of iridophores beneath melanophores (Hirata et al., 2003). Pigment cells are similarly stratified in other tissues having distinct pigment patterns, but are not stratified in tissues lacking distinct pigment patterns (Hirata et al., 2005).

Development of the adult pigment pattern occurs during metamorphosis, defined in teleosts as the transition from the larval to the juvenile stage. Metamorphosis entails coordinated changes in a variety of other traits as well: the larval fin fold is resorbed and adult paired and unpaired fins develop; the skin becomes increasingly stratified and scales develop; the excretory, digestive, and peripheral nervous systems are modified; and fish exhibit changes in behavior and physiology. Pigment pattern metamorphosis has been described most extensively in *D. rerio* (Fig. 3) (Kirschbaum, '75; Milos and Dingle, '78; Johnson et al., '95; Parichy et al., 2000b; Maderspacher and Nusslein-Volhard, 2003; Parichy and Turner, 2003b; Quigley et al., 2004, 2005), and its onset is marked by the appearance of new melanophores, between the early larval melanophore stripes. These "early metamorphic" melanophores become more numerous over several days (Fig. 3A–C). The first metamorphic xanthophores and iridophores appear immediately ventral to the horizontal myoseptum, where the first interstripe region will form. Subsequently, "late metamorphic" melanophores appear already at the sites of adult stripe formation (Fig. 3C and D). Simultaneously, some of the initially dispersed early metamorphic melanophores migrate short distances to join the nascent adult stripes (Fig. 3E). As metamorphosis proceeds, gaps within the stripes are filled, the stripe borders become more regular, and many early larval melanophores are lost (Fig. 3E and F). The completion of pigment pattern metamorphosis can be defined by the presence of two "primary" adult melanophore stripes, and a

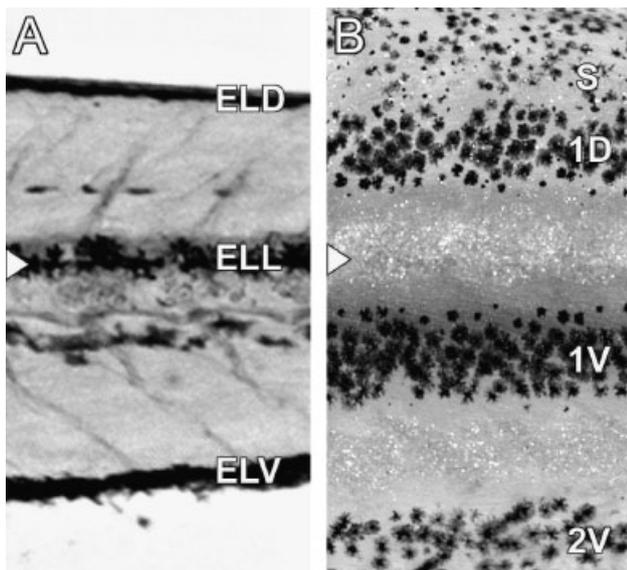


Fig. 2. Different pigment patterns across life cycle stages. Shown is a *D. rerio* larva near the onset of pigment pattern metamorphosis (A) and a juvenile (B). (A) Early larval melanophore stripes are present at the dorsal and ventral edges of the myotomes (ELD, ELV) and laterally, along the horizontal myoseptum (ELL). Metamorphic xanthophores and iridophores have just started to develop ventral to the horizontal myoseptum (arrowhead). (B) At the end of pigment pattern metamorphosis, two primary melanophore stripes (1D, 1V) border an interstripe region; a secondary stripe (2V) has started to form; and melanophore cover the scales (S).

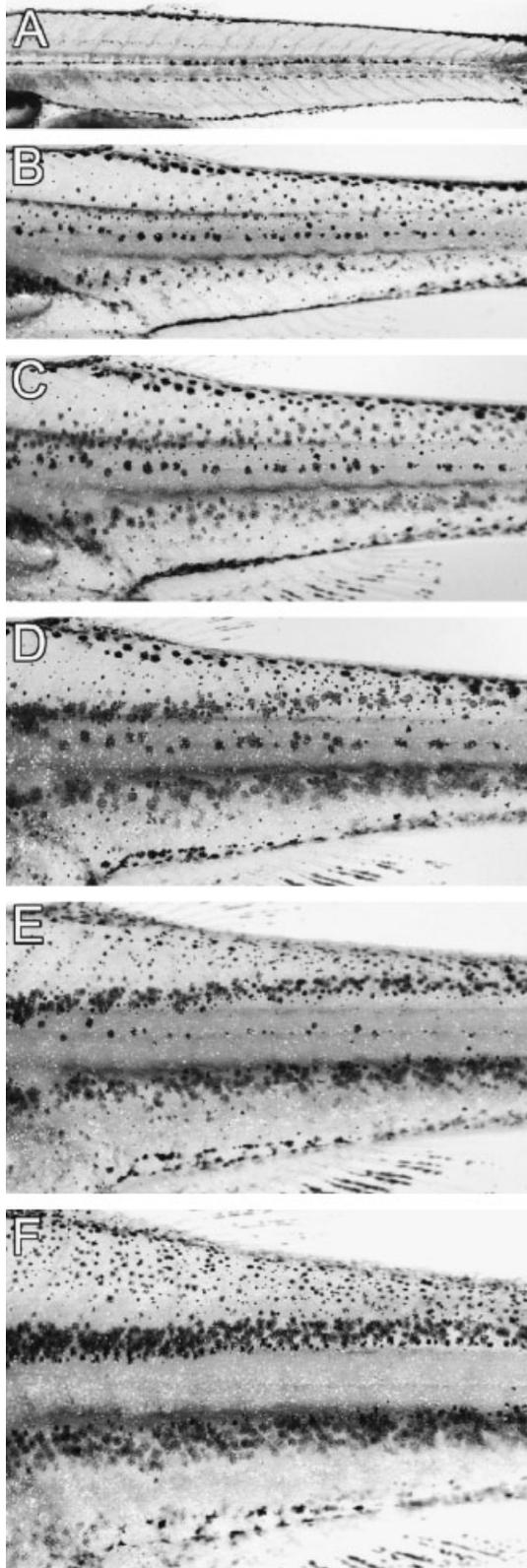


Fig. 3. Sequential images showing pigment pattern metamorphosis in an individual *D. rerio* larva from 14 days post-fertilization (dpf) through 29 dpf. See text for details as well as Parichy and Turner (2003b).

complete pattern of scales, which are themselves populated by melanophores (Fig. 2B). As the fish grows, additional “secondary” stripes are added dorsally and ventrally.

The diversity of adult patterns across danios contrasts with the conservation of the early larval pigment pattern, and indicates that pattern evolution is to some extent uncoupled across life cycle stages. This observation raises the question of how such uncoupling is achieved. The normal developmental anatomy of zebrafish pigment pattern metamorphosis provides a critical benchmark for determining how and when pigment pattern differences arise in other danios, and in zebrafish mutants. In this way, the zebrafish represents not just a biomedical model organism, but one in which an iterative analytical approach can be used to sequentially address mechanistic questions about pigment pattern development, and evolutionary, ecological and behavioral questions at the population and species levels. In the following sections, I provide several examples of this iterative approach.

LATENT PRECURSORS AND THE GENERATION OF PIGMENT PATTERN DIVERSITY

Roles for neural crest cells in generating juvenile and adult pigment patterns have been studied for many years in many organisms (Lehman, '53; Rawles, '59; Mayer and Green, '68; Richardson et al., '91; Parichy, '98; Wilkie et al., 2002). In zebrafish, analyses of pigment pattern mutants have refined our understanding of the cellular origins for adult pigment patterns in danios, and have raised new questions relevant to other taxa. One such *D. rerio* mutant is *puma* (Fig. 1C), which exhibits a wild-type early larval pigment pattern but severe defects in the adult pigment pattern, with far fewer melanophores than wild-type (Parichy and Turner, 2003b; Parichy et al., 2003). This phenotype results from a failure of metamorphic melanophores to differentiate, with a concomitant persistence of early larval melanophores into the adult (Fig. 4A; see on-line movies cited above). Only during later adult development does a population of melanophores arise to generate a partially striped, regulative pigment pattern. A normal complement of early larval melanophores with fewer metamorphic melanophores is similarly found in the *picasso* mutant (Fig. 1C) (Quigley et al., 2004), which results from lesions in the *erbb3* receptor

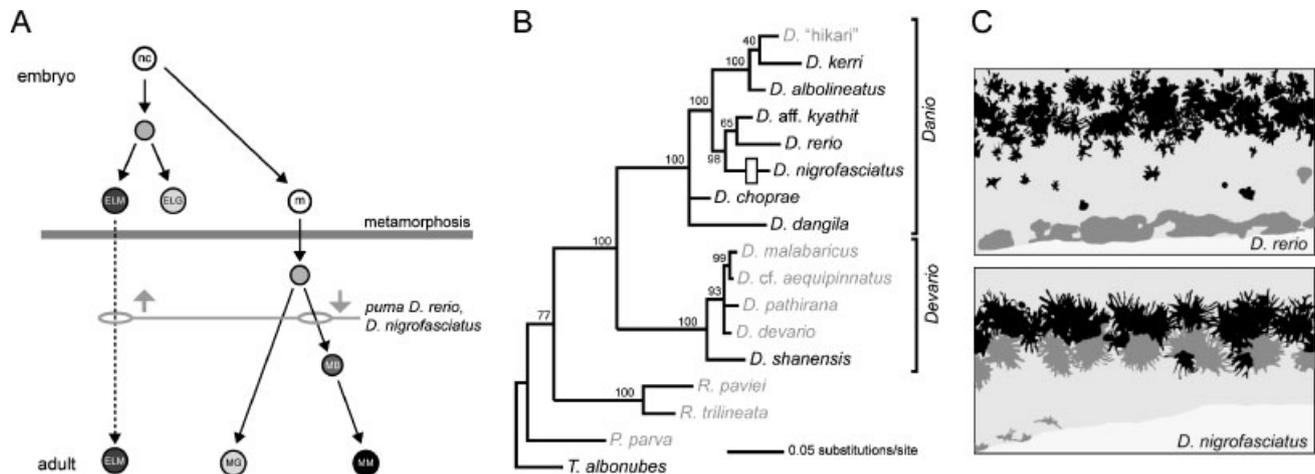


Fig. 4. Post-embryonic chromatophore precursors in pigment pattern development and evolution. (A) Analyses of wild-type and mutant *D. rerio* suggest a model for post-embryonic chromatophore lineage development. Neural crest cells (NC) in embryos give rise to early larval melanophores (ELM), other chromatophores, and other lineages, such as glia (ELG). Additional cells (m) are hypothesized to be set aside early, and recruited during later metamorphosis into post-embryonic lineages, including metamorphic melanophores (MM), metamorphic glia (MG), and others. mb, hypothetical lineage-restricted melanoblast. Most early larval melanophores fail to transit the metamorphic boundary in wild-type *D. rerio* (dotted line). In *puma* mutant *D. rerio*, as well as in *D. nigrofasciatus*, fewer metamorphic melanophores develop and early larval melanophores instead persist into the adult pigment pattern. (B) Phylogenetic analysis of selected *Danio* species and relatives, using mitochondrial 12S and 16S rDNA; numbers indicate Bayesian support levels (Quigley et al., 2004, 2005). Species in dark letters have been examined for mode of pigment pattern metamorphosis. The reduced contribution of metamorphic melanophores to the adult pigment pattern appears to be a unique, derived feature of *D. nigrofasciatus* pigment pattern formation (open bar). (C) Different contributions of early larval and metamorphic melanophores to stripes at the end of pigment pattern metamorphosis in *D. rerio* and *D. nigrofasciatus*. Shown are schematics of the ventral flank and the primary ventral melanophore stripe. Black cells are early larval melanophores, present only in remnants of the early larval ventral melanophore stripe in *D. rerio*, but redeployed into the primary ventral adult melanophore stripe in *D. nigrofasciatus*.

tyrosine kinase (Lyons et al., 2005) (D. Parichy and E. Budi, unpublished data). These and other mutant phenotypes illustrate how the zebrafish adult pigment pattern depends on the differentiation of new melanophores from latent precursors, rather than differentiation of melanophores directly from neural crest cells, as is the case during larval pigment pattern formation.

To test whether adult pigment patterns of other danios also are derived from post-embryonic latent precursors rather than directly from embryonic neural crest cells, Quigley et al. (2004) examined the *D. rerio* sister species, *D. nigrofasciatus*. Both species have similar horizontal stripes, though *D. nigrofasciatus* has fewer stripes than *D. rerio* (Figs. 1A and 4B). Despite the apparent similarity, lineage analyses in which individual melanophores were followed through development revealed a very different mode of stripe formation: in *D. nigrofasciatus*, far fewer metamorphic melanophores develop and adult stripes instead comprise large numbers of embryonic neural crest-derived melanophores that are redeployed from their original positions in the early larval stripes to new positions in the adult stripes (Fig. 4C).

Interspecies cell transplants further revealed this species difference to lie extrinsic to the melanophores themselves, perhaps indicating differences in growth factor availabilities, hormonal influences, or other aspects of the extracellular environment. Thus, two sister species with seemingly similar stripes actually develop these stripes in very different ways.

Genetic analysis of differences in pigment pattern metamorphosis between *D. rerio* and *D. nigrofasciatus* were possible because *D. rerio* can be hybridized to many species within *Danio*, including *D. nigrofasciatus*. In general, phenotypes of hybrids between other *Danio* species and wild-type *D. rerio* resemble *D. rerio* more closely than the other species (Fig. 1B); hybrids between wild-type *D. rerio* and *D. nigrofasciatus* are phenotypically intermediate between the two. By comparing phenotypes of control hybrids (using wild-type *D. rerio*) to phenotypes of tester hybrids (using recessive mutant *D. rerio*), one can screen for interspecific differences in the activity of loci affected in the zebrafish mutants [(Parichy and Johnson, 2001; Quigley et al., 2004, 2005); also see: (Long et al., '96; Sucena et al., 2003)].

If phenotypes of control and tester hybrids are indistinguishable, there is no evidence for an interspecific difference at the locus in question. On the other hand, if phenotypes differ between control and tester hybrids, the *D. rerio* mutant locus or pathway may differ between species. This approach has revealed that hybrids between wild-type *D. rerio* and *D. nigrofasciatus* principally exhibit the *D. rerio* mode of pigment pattern metamorphosis, with a major contribution from latent precursor-derived metamorphic melanophores. Similar phenotypes were observed for hybrids between *picasso* (*erbb3*) or other mutant *D. rerio* and *D. nigrofasciatus*. By contrast, *puma* mutant *D. rerio* hybrids with *D. nigrofasciatus* had fewer metamorphic melanophores and more early larval melanophores in their adult stripes. Since *puma* acts autonomously to the metamorphic melanophore lineage (Parichy et al., 2003), rather than non-autonomously, as the species difference is predicted to be, these data raise the possibility that another gene in the *puma* pathway is responsible for the difference in pigment patterns between *D. rerio* and *D. nigrofasciatus*.

Given the different modes of pigment pattern metamorphosis between *D. rerio* and *D. nigrofasciatus*, the question arises as to which mode is ancestral. Cell lineage analyses were used to examine additional species representing different clades and a spectrum of pigment pattern diversity (Figs. 1A and 4B). In each species, the adult pigment pattern arises principally by differentiation of metamorphic melanophores, and not early larval melanophores (Quigley et al., 2004) (D. Parichy and L. Patterson, unpublished data). Thus, pigment pattern metamorphosis by recruitment of metamorphic melanophores from latent precursors is common and likely ancestral for this group. This mode of pigment pattern metamorphosis suggests one way in which early larval and adult pigment pattern evolution may be partly uncoupled. Compartmentalization of cell lineages and gene activities may allow for phenotypic variation and responses to selection that are independent across life cycle stages. By contrast, the reduced contribution of metamorphic melanophores and concomitant redeployment of early larval, neural crest-derived melanophores appears to be a unique, derived mode for *D. nigrofasciatus*.

That danio adult pigment patterns typically arise from latent precursors, rather than directly from neural crest cells, suggests that a complete

understanding of pigment pattern evolutionary diversification will require the identification of factors responsible for establishing and maintaining such precursors, as well as recruiting them at particular times and places to particular chromatophore lineages. Identifying critical periods for gene activity within *D. rerio* can provide insights into these issues. For example, studies using temperature-sensitive alleles show that both *puma* and *panther* gene products are essential during adult pigment pattern metamorphosis, but not before, for the normal development of adult chromatophores (Parichy and Turner, 2003a,b), as might be expected for genes needed to recruit cells from latent precursors. By contrast, analyses using a pharmacological inhibitor of ErbB receptors show that *picasso* (*erbb3*) is required during embryogenesis, but not afterwards, for the normal development of adult chromatophores (E. Budi and D. Parichy, unpublished data), perhaps indicating a role for this gene in the establishment of the latent precursors themselves. It will be interesting to see if pigment pattern variation within and among species arises primarily through late-acting genes, or whether early-acting genes are contributors as well.

Two other important issues concerning latent precursors to adult chromatophores are their self-renewal capability and developmental potential. Precursors to adult chromatophores may be bona fide stem cells, able to self-renew while producing differentiated progeny (Potten and Loeffler, '90; Weissman et al., 2001). In the regenerating zebrafish fin, new chromatophores differentiate de novo from putative stem cells (Goodrich and Nichols, '31; Rawls and Johnson, 2000, 2001), and a corresponding population of precursors may be present on the body. Consistent with this idea, neural crest-derived, post-embryonic melanocyte stem cells have been identified in mammals (Nishimura et al., 2002, 2005; Osawa et al., 2005), and are presumed to exist in amphibians (Parichy, '98). Alternatively, latent precursors could have more limited self-renewal abilities, perhaps acting simply as a transit-amplifying population. Finally, it remains unclear whether normal adult chromatophore precursors are lineage-restricted, and able to generate just single differentiated cell types, or are multipotent, and able to produce multiple chromatophore classes and perhaps other lineages. The stem cell properties and developmental potential of these precursors are now being

elucidated (M. Mills, E. Budi, E. Herrington, and D. Parichy, unpublished data). It will also be interesting to determine how these or analogous cells contribute to the development and maintenance of other adult neural crest-derived traits (Alberch and Gale, '86).

NOVELTY THROUGH MODULARITY: CHROMATOPHORE INTERACTIONS AND THE ORIGINS OF ADULT PIGMENT PATTERN VARIATION

Beyond questions of cell lineage, zebrafish pigment pattern mutants and other species also are beginning to reveal morphogenetic mechanisms of adult pigment pattern development and evolution. Among such mechanisms are interactions between chromatophores, as revealed by studies of three zebrafish mutants: *panther*, *jaguar* (*obelix*), and *leopard* (Fig. 1C).

Mutants for *panther* have disorganized adult melanophore patterns, fewer adult melanophores overall, and a complete absence of xanthophores in both embryos and adults (Fig. 1E). Interspecific complementation tests identified *panther* as a candidate to explain the difference between *D. rerio* (stripes) and *D. albolineatus* (no stripes; Fig. 1A) (Parichy and Johnson, 2001). Molecular cloning subsequently identified the *panther* locus as *colony stimulating factor-1 receptor* (*csf1r*; also known as *fms*) (Parichy et al., 2000b), which is also allelic to the independently isolated mutants *pfeffer* and *salz* (Haffter et al., '96; Odenthal et al., '96; Maderspacher and Nusslein-Volhard, 2003).

The simultaneous melanophore and xanthophore defects in *panther* (*csf1r*) mutants raised the possibility that interactions between these chromatophore classes might be required for stripe development in wild-type *D. rerio*. Interactions between melanophores and xanthophores, as well as among melanophores themselves, have previously been implicated in bar and stripe formation in salamander larvae (Twitty, '45; Epperlein and Lofberg, '90; Parichy, '96a,b). To see if analogous interactions might occur in danios, a cell transplantation approach was used (Parichy and Turner, 2003a). If interactions between melanophores and xanthophores are required for melanophore stripe organization, then "reintroduction" of the missing chromatophore class should restore stripes. This hypothesis was tested using wild-type fish that produce both melanophores and xanthophores; *panther* (*csf1r*)

mutant fish that produce only melanophores; and *nacre* mutant fish that produce only xanthophores (Fig. 1C), owing to a mutation in *mitfa*, which encodes a transcription factor essential for melanophore specification (Lister et al., '99). When xanthophores and melanophores (of different genotypes) were experimentally brought together in the same fish, organized stripes developed consisting of donor and host cells (Fig. 5).

A requirement for pigment cell interactions was further supported by analyses of a conditional *panther* (*csf1r*) allele, *csf1r*¹⁷⁴ (Parichy and Turner, 2003a). A major advantage to genetic analyses with zebrafish lies in the ability to isolate temperature-sensitive mutant alleles, typically corresponding to mis sense substitutions that presumably destabilize the affected protein at high temperature (Johnson and Weston, '95; Rawls and Johnson, 2001). Such alleles allow one to turn on or to turn off protein activity, simply by shifting fish between permissive and restrictive temperatures. For *panther* (*csf1r*¹⁷⁴) mutants reared at restrictive temperature (and so lacking xanthophores and melanophore stripes), shifting to permissive temperature allows the recovery of xanthophores and the organization of melanophore stripes at all stages examined. Likewise, for mutants reared at permissive temperature (and thereby having a wild-type complement of xanthophores and wild-type stripes), shifting to restrictive temperature results in the loss of xanthophores and the degeneration of melanophore stripes at all stages (see on-line movies at: <http://protist.biology.washington.edu/dparichy/movies.htm>). Molecular analyses further revealed that *csf1r* is expressed by cells of the xanthophore lineage, rather than fully differentiated melanophores. Together, these various lines of evidence show that interactions between melanophores and *csf1r*-dependent xanthophores are essential for stripe formation and maintenance in *D. rerio*.

Similar cell transplantation experiments involving *jaguar* (*obelix*) and *leopard* mutants (Fig. 1C) have identified additional cell-cell interactions (Maderspacher and Nusslein-Volhard, 2003). Mutants for *jaguar* have fewer and broader melanophore stripes and also more xanthophores within melanophore stripes compared to wild-type. An allelic series of *leopard* mutants has phenotypes ranging from irregular stripes, to spots, to a nearly uniform pattern of intermingled melanophores and xanthophores (Haffter et al., '96; Asai et al., '99). Genetic mosaic analyses suggest a model in which normal stripe formation depends on *jaguar*-dependent interactions between mela-

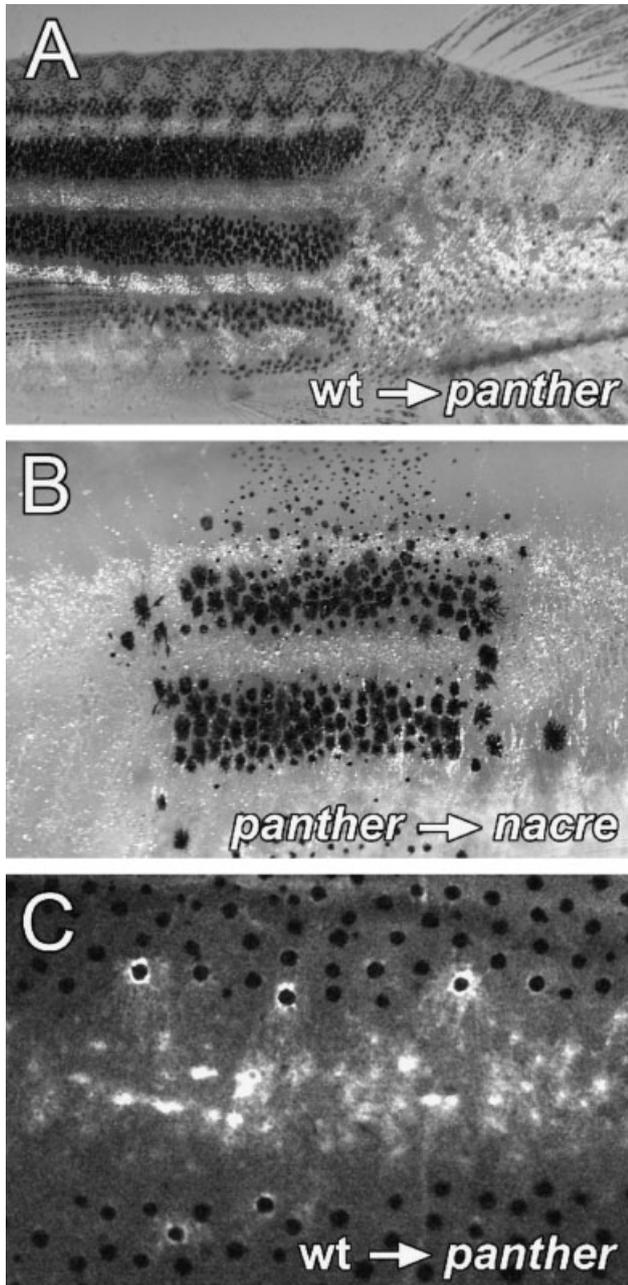


Fig. 5. Genetic mosaic analyses indicate requirements for chromatophore interactions during *D. rerio* stripe development. (A) Wild-type cells (melanophore⁺, xanthophore⁺) transplanted to *panther* mutant (melanophore⁺, xanthophore⁻) hosts result in wild-type stripe formation. (B) *panther* mutant cells (melanophore⁺, xanthophore⁻) transplanted to *nacre* mutant hosts (melanophore⁻, xanthophore⁺) generate stripes in regions containing both melanophores and xanthophores. (C) A ubiquitously expressed GFP can be used to identify donor cells in a host background, here illustrating wild-type, GFP⁺ melanophores and xanthophores, adjacent to GFP⁻ host melanophores.

nophores, as well as *leopard*-dependent interactions between melanophores, between xanthophores, and between these two chromatophore classes (Maderspacher and Nusslein-Volhard, 2003).

The mechanisms for interactions within and among chromatophore classes have yet to be determined, but could include adhesive or repellent physical contacts between cells, secreted signaling molecules or trophic factors, or even intermediary cell types. *Leopard* was recently identified as the gap junction gene, *connexin41.8*, consistent with a role for cell–cell interactions through gap junction-mediated communication or adhesion (Watanabe et al., 2006). Whatever their precise nature, such interactions suggest that pigment cells and the patterns they form can be considered in some respects as a modular trait, partially, though not completely, independent of other tissues (von Dassow and Munro, '99; Beldade et al., 2002; Klingenberg, 2005). Such modularity could, in turn, explain much of the pigment pattern diversity in this group, as pigment pattern changes could arise without requiring correlated changes in other traits.

Although direct evidence is lacking, several observations suggest changes in pigment cell interactions as a possible mechanism of evolutionary change. For example, analyses of temperature-sensitive *panther* (*csf1r*¹⁷⁴) mutant *D. rerio* hint at the range of phenotypes that pigment cell interactions could generate among species: when *csf1r* activity is allowed for the first time only late in development, stripes in the caudal fin can be either horizontal (normal), or vertical (Parichy and Turner, 2003a). This result suggests that cues normally required to set the orientation of these stripes are no longer present, or are not recognized by chromatophores, at late stages. Thus, if pigment cell interactions serve to paint a pattern, the features of the canvas may differ depending on when and where the process occurs. For a species like *De. shanensis*, vertical bars instead of horizontal stripes could form simply owing to the particular cues that happen to be present during the stage of pigment pattern formation (which occurs much later in *De. shanensis* compared to *D. rerio*; Parichy, unpublished data). Alterations in the strength of pigment cell interactions also could effect pigment pattern change, by modulating the degree to which pigment cell classes sort out from one another and what sorts of boundaries are formed. For example, the *D. kerri* pigment pattern of fewer and broader stripes resembles

jaguar (*obelix*) mutant *D. rerio*, whereas the *D. albolineatus* pattern of intermingled chromatophore classes resembles *leopard* mutants (Fig. 1A, C, and E). Phenotypes of hybrids between either *D. kerri* or *D. albolineatus* and *jaguar* (*obelix*) mutants also are consistent with the notion of weaker pigment cell interactions in these species with broader or missing stripes (for details, see Quigley et al., 2005). By iterative analyses between *D. rerio* mutants and other species, identification of the mechanisms and evolutionary consequences of such interactions should be achievable.

STRIPES AND INTERSTRIPES: A GENETIC AND CELLULAR GROUNDPLAN FOR DANIO ADULT PIGMENT PATTERNS

While *D. rerio* mutants like *panther* (*csf1r*), *jaguar* (*obelix*), and *leopard* suggest the importance of pigment cell interactions, and mutants like *puma* highlight the distinction between embryonic and metamorphic chromatophores, other mutants have provided a window into the genetics and evolution of the metamorphic melanophores themselves. One such mutant is *sparse*, which results arising from mutations in *kit*, which encodes a receptor tyrosine kinase related to *csf1r* (Fig. 1C) (Johnson et al., '95; Parichy et al., '99; Rawls and Johnson, 2003; Grassot et al., 2006). *sparse* (*kit*) mutant *D. rerio* have fewer embryonic melanophores than wild-type and those melanophores that do develop die during the first week of development so that mutants completely lack melanophores by the onset of pigment pattern metamorphosis. During late stages of pigment pattern metamorphosis, however, a new population of *kit*-independent melanophores develops already in the position of adult stripes, where no melanophores had been present, nicely demonstrating again the dependence of the adult pigment pattern on latent precursors.

In *sparse* mutants, the temporal and spatial distribution of *kit*-independent metamorphic melanophores contrast with the condition in several other mutants, including *panther* (*csf1r*) and *rose* (*ednrb1*), which exhibits lesions in the gene encoding the seven-pass transmembrane receptor, endothelin b receptor (Parichy et al., 2000a,b; Rawls et al., 2001) (Fig. 1C). In these latter two mutants, metamorphic melanophores are present in normal numbers during early stages of pigment pattern metamorphosis, and arise widely dispersed over the flank, rather than at sites of stripe

formation. These contrasting mutant phenotypes suggest that early-appearing, dispersed metamorphic melanophores and late-appearing, stripe metamorphic melanophores may be genetically distinct populations. If so, then fish doubly mutant for these loci should have predictable phenotypes: metamorphic melanophores should be absent in fish mutant simultaneously for *sparse* (*kit*) and *rose* (*ednrb1*), or for *sparse* (*kit*) and *panther* (*csf1r*), because both early and late populations should be ablated; however, metamorphic melanophores should persist in fish mutant for both *rose* (*ednrb1*) and *panther* (*csf1r*), because *kit*-dependent early metamorphic melanophores should still be present. These predictions are borne out by analyses of actual double mutant phenotypes (Fig. 1D). These results imply two melanophore populations: an early population that requires *kit*, but not *ednrb1* or *csf1r*, and migrates from a dispersed arrangement into stripes; and a late *kit*-independent population that requires *ednrb1* and *csf1r*, and appears already within stripes.

The presence of two metamorphic melanophore populations in *D. rerio* raises the question of whether or not similar populations occur in other species, and if so, whether evolutionary changes reside principally in one or the other population. Since the different melanophore populations of *D. rerio* are defined by mutant phenotypes, the generation of corresponding mutants in other species provides one opportunity to investigate these issues. As a first step towards this goal, a null allele of *kit* was isolated in *D. albolineatus* (R. Nuckels, M. Mills, and D. Parichy, unpublished data), a species chosen in part because of its

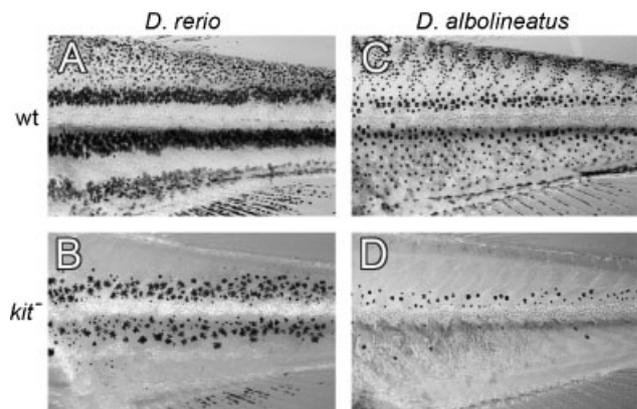


Fig. 6. Mutational analyses in *D. albolineatus* reveal conserved and novel features of pigment pattern formation. Shown are wild-type (A, C) and *kit* mutants (B, D) for both *D. rerio* and *D. albolineatus*. See text for details.

dramatically different pigment pattern compared to *D. rerio* (Fig. 1A). Similar to *D. rerio*, *kit* mutant *D. albolineatus* completely lack melanophores during early metamorphosis (not shown). Also similar to *D. rerio*, *kit* mutant *D. albolineatus* develop new melanophores from latent precursors during late metamorphosis (Fig. 6). These phenotypes demonstrate that temporally distinct populations of *kit*-dependent and *kit*-independent melanophores occur in at least one other species, and might be present among *Danio* species more generally.

This mutational approach with *D. albolineatus* prompts at least two additional observations. First, most of the late, *kit*-independent melanophores that develop in *kit* mutant *D. albolineatus* do so in a striped arrangement, despite an evolutionarily derived absence of stripes in adult *D. albolineatus* (Quigley et al., 2005). Thus, *D. albolineatus* retains stripe-forming potential. This finding is reminiscent of studies that demonstrated cryptic cues for stripe formation in salamander larvae (Parichy, '96a). Second, the similarity of residual melanophore patterns in *D. rerio* and *D. albolineatus kit* mutants suggest that differences between species have involved changes to the *kit*-dependent population (which is essentially "subtracted away" in the mutants, revealing the underlying similarity). Consistent with this interpretation, initially uniformly distributed (*kit*-dependent) melanophores fail to migrate in *D. albolineatus*, unlike *D. rerio* (Quigley et al., 2005), perhaps owing to changes in pigment cell interactions (discussed above).

Cryptic stripe-forming abilities in *D. albolineatus* are not entirely unexpected, as larval *D. albolineatus* display larger and darker melanophores adjacent to a narrow and irregular inter-stripe region, giving the impression of weak stripes, despite an otherwise uniform distribution of metamorphic melanophores. Conceivably, a pattern of two stripes bordering an inter-stripe region constitutes a ground plan from which patterns diverge across species. Such stripes are formed not only in *D. rerio* and *D. albolineatus*, but also *D. nigrofasciatus*, *D. kyathit*, and *D. kerri*. Even *D. choprae* and *D. dangila*, which have very different mature adult pigment patterns (Fig. 1A), develop transient horizontal stripe patterns similar to *D. rerio* (Parichy, unpublished data; see online movies at: <http://protist.biology.washington.edu/dparichy/movies.htm>). It will be especially interesting to discover the cues that determine when and where these first stripes and inter-

stripes form, and how chromatophores of different species respond to such cues and to each other during pigment pattern formation.

CONCLUSIONS

In this review I have illustrated how an iterative approach using studies focused on developmental mechanisms and studies focused on evolutionary change can provide a more complete picture of how these adult phenotypes develop and evolve. Such analyses also can suggest testable hypotheses that are applicable to other species groups and other traits. Given the recent methodological and conceptual strides in genomics and systematics, as well as in molecular, cellular, and developmental biology, this is truly an exciting time for integrative research spanning multiple levels of biological organization.

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