Developmental genetics of adaptation in fishes: the case for novelty

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Abstract

The last decade of study in evolutionary developmental biology has seen a shift in focus away from the stunning conservation of form and function between distantly related taxa, and towards the causal explanation of differences between closely related species. A number of fish models have emerged at the forefront of this effort to dissect the developmental genetic and molecular basis of evolutionary novelty and adaptation. We review the highlights of this research, concentrating our attention on skeletal morphology (cranial and postcranial), pigmentation patterning and sex determination. Thus far, the genes involved in adaptation among fishes belong to well-characterized molecular pathways. We synthesize the current state of knowledge to evaluate theories about the interplay between development and evolution. As of yet, general rules of evolutionary change have not materialized; however, the field is wide open, and fishes will likely continue to contribute insights to this central biological question.

Glossary

Reverse genetics: A “genotype to phenotype” approach to identify genotype-phenotype associations. In this approach, one starts by creating or identifying mutations in a gene of interest and then assays the phenotype of individuals carrying the mutation.

Forward genetics: A “phenotype to genotype” approach to identify genotype-phenotype associations. In this approach, one starts with a phenotype of interest and then tries to identify genetic variants that are associated with the phenotypic differences.

Quantitative trait locus (QTL): A genomic region that has been shown by linkage mapping studies to harbor genetic variation that contributes to segregating phenotypic variation.

Percent variance explained (PVE): The amount of segregating phenotypic variation explained by a particular QTL.

Neural crest: A pluripotent population of embryonic precursor cells that contributes to numerous vertebrate traits including, but not limited to pigment cells, neurons and glia of the peripheral nervous system, and craniofacial bone and cartilage.

Melanophore: Melanin-containing pigment cell of ectothermic verterbrates that is derived from the neural crest. Although melanophores are frequently called “melanocytes,” this term refers to the melanin-containing cells of endotherms, which transfer their melanin to keratinocytes for incorporation into hair or feathers. By contrast, melanophores retain their pigment granules intracellularly and can alter the location of these granules within the cell to effect lightening or darkening in response to the environment.

Cytogenetically visible sex chromosome: We use this term to mean that one sex possesses heteromorphic chromosomes that can be observed by examining chromosome squashes under a light microscope. However, when more sophisticated cytogenetic techniques have been applied to species previously believed to lack heteromorphic sex chromosomes, subtle differences in chromosome structure have been observed, suggesting that this is a relative term.

Sexually antagonistic gene: A gene that has a differential fitness effect in the sexes, such that expression in one sex is beneficial but expression in the other sex is detrimental.

Genetic data: An association between genotype and phenotype found by genetic linkage or genetic association analysis.

Transcriptional data: An association between genotype and phenotype found by showing that there is a correlation between a phenotypic difference and a difference in a gene's expression pattern. However, this type of data does not prove that the change in gene expression is the cause of the phenotypic change; the cause of the altered expression pattern could be genetic changes in an upstream regulator of the gene (*trans* effect) or genetic changes in the gene itself (*cis* effect).

Modularity: Generally, the evolutionary or developmental decoupling of components involved in form and/or function (see Klingenberg, this issue). Specifically, a gene may be ascribed modular function if it acts in multiple tissues of a multicellular organism (i.e., brain, heart, jaw, limb, kidney) and is targeted to these tissues by cis-regulatory "modules" in the gene's promoter. Expression and function in any of these tissues may be 'modulated' independently by changes to tissue-specific promoter elements.

Gene regulatory networks (GRNs): The sum total of genes and their connections that influence a biological output, often depicted or modeled as wiring diagrams or logic circuits. GRNs occupy a central place in the rediscovered field of "systems biology."

Future Issues

1. Adaptation to new environments involves a wide range of morphological, physiological and behavioral changes. In particular, the genetic basis of physiological and behavioral diversity has been relatively unexplored in any system. Because the fish models highlighted in this review display enormous morphological, physiological and behavioral diversity, it should be possible to use the genetic and genomic tools developed for these systems to identify the genetic and molecular basis of any trait of interest. It will be particularly interesting to determine whether the types of mutations, genes and pathways that are important for morphological adaptation are more generally involved in physiological and behavioral novelty.

2. As technical costs decrease, more fish lineages will become appropriate models to answer key biological questions. The richness and diversity found among teleost fishes is nearly limitless in this regard.

3. Many future research efforts will focus on traits expressed after embryogenesis or in adult life stages. New techniques and application of standard techniques to new situations (explant culture, tissue or stage specific gene knockdown) will be required to rigorously evaluate functional associations between genotype and phenotype.

Introduction

Fishes, Novelty and How Development Works

The publication in December 1996 of an entire issue of the journal *Development* dedicated to the zebrafish embryo and its embryogenesis changed the way that evolutionary biologists think about fishes. The description of mutants in pathways affecting most aspects of vertebrate morphology (brains, eyes, jaws, fins, pigment) provided resounding evidence of the interplay between genes and development on a comprehensive scale. The simple figures used to document phenotypes (e.g., cleared and stained embryos lacking jaw bones or with duplicated cartilages; fishes without melanophores) provided visual compendia of developmental diversity. Students with favorite traits now had favorite mutants. The landmark issue of *Development* was particularly inspirational to those interested in evolution. The zebrafish mutants, first the domain of biomedicine, contributed to an undercurrent of discovery that adaptation (when development works) was just the flip side of disease (when development fails). Comparative biologists recognized that understanding the key to complex phenotypes and evolutionary novelty, encoded in the genome and unveiled through the developing embryo, was a tractable research objective.

This mindset was accompanied by major challenges. Conceptually, mutant screens are an imperfect metaphor for the identification of genotype-phenotype associations in nature. First, the classical experimental paradigm of forward genetics has sought to minimize complexity by isolating the effects of single mutations. Second, most zebrafish mutants were embryonic lethals; they never

developed to function as adults. Subsequently, biologists have inferred how development works by studying *how development fails*. This approach has advanced our knowledge of gene function, but has also underscored the notion that genes do not operate in a vacuum, that environmental and genomic context matters. As such, a major and complementary objective of current research is to understand the molecular basis of natural diversity. Notably, understanding the origin of biological diversity was named one of the "25 Hard Questions" by *Science* magazine in July 2005 and "Evolution in Action" was *Science*'s 2005 Breakthrough of the Year.

Teleost fishes represent a unique assemblage in which to study the genetics of adaptation and evolutionary novelty, or *how development works*. First, the group contains *bona fide* model organisms (*Danio*, *Takifugu*, *Tetraodon*, *Oryzias*), with research programs in forward and reverse genetics, molecular biology, and genomics providing information, hypotheses and technical insight. Second, the species richness and diversity of fishes is unrivaled among vertebrates. Closely related species differ in a wide range of traits, many of which are explored below. Numerous natural lineages are amenable to genetic and developmental analysis because barriers to hybridization are minimal or absent and embryos are easy to manipulate (e.g., danios, sticklebacks, cichlids). Understanding the genetics of development in natural lineages would theoretically provide novel insights into gene function because (i) new genes, not identified in mutant screens, might be involved and (ii) new mutations, compatible with adult viability, would likely play a role.

Here, we review recent advances in developmental genetics of adaptation in teleost fishes. We focus on three types of traits: skeletons (including craniofacial and post-cranial elements), pigmentation and sex (gender) determination. These traits have received considerable attention from researchers and fit together conceptually. Skeletal elements and pigment patterns have their cellular origin in the vertebrate cell type called the neural crest (Gans and Northcutt 1983; Hall 1999). Pigment patterns and skeletal variants are sometimes linked to sex chromosomes, and theoretical population genetic models of adaptive speciation predict linkage among these trait types (reviewed by Bolnick and Fitzpatrick, this issue). Some of the evolutionary lineages and the traits we highlight have been reviewed elsewhere in the last few years (Kocher 2004; Cresko *et al*. 2007; Kazianis *et al*. 2006). Our goal is to describe and summarize this vast primary literature to ask if diverse adaptations in different fish lineages share common developmental pathways or common gene regulatory logic. We integrate these data to address hypotheses that codify the rules of evolutionary development among closely related organisms.

Skeletons

Traveling Light: Adaptation via Loss

Recent work has yielded considerable insight into the developmental genetics of trait loss in fishes. Assorted lineages have lost features of the craniofacial (i.e., teeth) and postcranial skeletons (i.e., ribs and fins) as well as body armor, scales, eyes and pigmentation (see below; Table 1; Figure 1). Research to date

suggests that trait loss is controlled by a small number of genes of large effect and high penetrance; further study is required to determine if this is a general rule.

Understanding the developmental genetic basis of adaptation builds on decades of natural history, field ecology and evolutionary biology. For instance, Northern hemisphere stickleback fish have independently colonized freshwater habitats from marine ancestors soon after the last glacial maximum (~10,000 years ago). Riverine, lacustrine and stream populations have evolved numerous adaptations, including changes in body size, habitat use, gill raker number and the reduction of body armor (i.e., scales that are modified to form bony plates, as well as pelvic and dorsal spines, Bell and Foster 1994; Figure 1). Peichel *et al*. (2001) mapped the genetic basis of pelvic and armor reduction in backcross progeny of lacustrine benthic versus limnetic threespine sticklebacks from Priest Lake, British Columbia (BC). A single quantitative trait locus (QTL) for pelvic spine length was located on chromosome 8 and QTL for body armor (plates) were located on chromosomes 13 and 26. Each of these genomic regions explained a substantial portion of phenotypic variation in the focal trait (PVE = ~25%).

Subsequent to this study, numerous reports have refined the story for each trait. Colosimo *et al*. (2004) used F_2 fishes from an intercross of marine versus Paxton Lake, BC parents to document a QTL of major effect (PVE > 75%) for body armor on chromosome 4, with four additional minor effect loci on separate chromosomes. The major locus for armored plates on chromosome 4 also

segregated in a California stream population. This locus was later identified as *ectodysplasin* (*eda*) by positional cloning, linkage disequilibrium mapping and transgenesis (Colosimo *et al*. 2005). Notably, *eda* low plate alleles segregate at low frequency in marine high plated ancestral populations, explaining the parallel loss of armor in most freshwater lineages (Colosimo *et al*. 2005).

Shapiro *et al*. (2004) used a similar cross design to identify a major QTL for pelvic reduction on stickleback chromosome 7, with four additional minor effect loci on different chromosomes. Mapping of candidate genes and *in situ* hybridization strongly suggested that regulatory mutations in *pitx1* (paired-like homeodomain transcription factor 1) are responsible for this phenotype. Likewise, genetic complementation analysis implicated *pitx1* in pelvic reduction of other freshwater threespine stickleback populations (Shapiro *et al*. 2004) and distantly related (common ancestor at least 10 MYA) ninespine stickleback populations (Shapiro *et al*. 2006). Cresko *et al*. (2004) studied the genetics of bony armor loss among Alaskan freshwater threespine stickleback populations and demonstrated parallel Mendelian control of both pelvic and armor phenotypes. Alaskan sticklebacks segregated for a pelvic reduction gene on chromosome 7 (likely *pitx1*), and armor phenotypes mapped to the *eda* locus on chromosome 4 (Miller *et al.* 2007).

Other fish lineages show analogous loss of scale or pelvic structures; strikingly, these phenotypes are due to alterations in the same developmental pathways identified in stickleback. Kondo *et al*. (2001) reported that the spontaneous medaka mutant *rs-3*, which lacks scales, is encoded by the

receptor for *ectodysplasin* (*edar*). Pelvic fin loss in pufferfishes is accompanied by altered expression of the limb positioning marker *hoxd9a*, which is upstream of *pitx1* (Tanaka *et al*. 2005). Finally, additional fish groups are characterized by loss of morphological features, from eyes to oral jaw teeth. Blind cavefishes (*Astyanax*) possess eyes that degenerate during development (Figure 1). Cave populations are characterized by expanded sonic hedgehog (*shh*) and tiggy-winkle hedgehog (*twhh*) expression at the embryonic midline when compared to their surface-dwelling eyed ancestors (Yamamoto *et al*. 2004). Zebrafish and other cypriniform fishes lack teeth on their oral jaws. This may result from altered fibroblast growth factor (Fgf) signaling through *dlx2* in oral epithelium (Stock *et al*. 2006).

Fish Jaws and Dentitions: Elaboration and Complexity

Detailed study of trait loss in fishes provided some of the first evidence that genetic mapping and assays of gene expression could be used to understand the molecular control of natural adaptations. Of course, trait loss may be a special case of adaptation. What of more complex morphologies where individuals differ in subtler aspects of shape, size and function? The natural history of fish feeding ecology, functional morphology and diversity provided a place to begin. Notable features of the fish craniofacial skeleton include (i) two sets of toothed jaws (oral and pharyngeal) elaborated to (sometimes) bizarre extremes (Figure 1), (ii) dentitions on jaws and numerous other bony elements replaced continuously

through development, and (iii) a long and perhaps dubious history of these traits as markers of evolutionary relationships.

Cichlid fishes have figured prominently in studies to identify the developmental genetic basis of craniofacial adaptation, largely because they represent closely related species with a wide range of trophic and dental morphologies (Albertson and Kocher 2006). Albertson *et al*. (2003) mapped QTL for craniofacial morphology in the F_2 of a cross between two Lake Malawi cichlids with divergent feeding strategies. Genes of large effect (10-25% PVE) for multiple craniofacial phenotypes mapped to common intervals of chromosomes 1, 2 and 16 (reassigned to chromosomes 7, 15 and 19 after comparison to the more extensive tilapia cichlid map; Lee *et al*. 2005; Streelman and Albertson 2006), leading to speculation that trait linkage on chromosomes might facilitate the rapid and replicative evolution of jaw design among rift lake cichlids (Figure 1). Using a test that compares the direction of QTL effects to a neutral expectation, the authors documented strong directional selection on the oral jaw apparatus and the dentition (Albertson *et al*. 2003). In 2005, Albertson and colleagues focused on functional aspects of lower jaw shape that represent a trade-off between speed and force of jaw opening and closing (Albertson *et al*. 2005; Hulsey *et al*. 2005). Importantly, they showed that opening and closing lever systems were genetically decoupled with QTL localized to different chromosomes. They observed that the gene *bmp4* mapped to the closing lever system QTL interval (on chromosome 19) and subsequently demonstrated greater *bmp4* expression in the parental species with more robust jaws (similar to

results in Darwin's finches, Abzhanov *et al*. 2004). Finally, they showed that *bmp4* injection into zebrafish embryos was sufficient to recapitulate the lower jaw shape phenotype observed in cichlids. This study provided a possible explanation for the observation that *bmp4* evolves rapidly and non-neutrally among East African cichlids (Terai *et al*. 2002). Given avid interest in modeling fish jaws as simple versus complex biomechanical systems (Alfaro *et al*. 2004; Hulsey *et al*. 2005; Wainwright, this issue), the cichlid system is ideal for further exploration in this context.

Recent work in fishes has demonstrated the complexity of dental patterning in vertebrates. Fraser *et al*. (2004) showed that first-generation teeth on the oral jaw of rainbow trout express *pitx2*, *shh* and *bmp4* in similar spatio-temporal patterns to the mouse, suggesting the conservation of these molecules in the initiation of odontogenesis since the common ancestor of fish and mammals (~450 million years ago). However, not all is conserved between mammals and fishes, or even between the oral and pharyngeal jaws of fishes. Notably, Fraser *et al*. (2004) described differences in *pitx2* expression during continued morphogenesis of trout teeth, with *pitx2* expression present in oral jaw teeth but absent from pharyngeal teeth. Working with zebrafish, Laurenti *et al*. (2004) likewise demonstrated differences between pharyngeal first-generation teeth and the oral teeth of mammals (zebrafish lack teeth on the oral jaw so no direct comparison is possible). Specifically, the gene *eve1,* a member of the homeobox-containing evx gene family, not expressed during tooth development in mammals, is expressed during tooth initiation and morphogenesis of the first pharyngeal tooth. Jackman

et al. (2004) used chemical knockdown of FGF signaling to show that FGFs are required for zebrafish first-generation tooth development. Furthermore, *fgf8* and *pax9* were not expressed under normal conditions in zebrafish tooth germs (unlike in mouse) and both Dlx and Lhx genes were expressed in dental mesenchyme (as in mouse molars).

In 2003, Streelman and colleagues demonstrated that tooth number was correlated with tooth cusp number in natural populations of cichlid fish from Lake Malawi, East Africa (Streelman *et al*. 2003a). Given simple genetic control of tooth shape in this system (Albertson *et al*. 2003a,b) and the iterative role of certain genes in the stages of tooth development (Peters and Balling 1999), these authors suggested that variation in the expression of a single activating or inhibitory molecule might integrate tooth and cusp number (Streelman *et al*. 2003a; also Plikus *et al*. 2005). Streelman and Albertson (2006) subsequently identified a QTL of major effect for tooth shape on cichlid chromosome 5, near genes for orange blotch (OB) color and sex, Streelman *et al*. 2003b and below). Furthermore, they demonstrated, using *bmp4* as a marker of tooth initiation, that tooth number and spacing is specified earlier than tooth shape.

Much is left to learn about fish dentitions. For instance, first-generation teeth are morphologically unlike replacement teeth (Sire *et al*. 2002), do not show species-specific adult shapes and exhibit unique gene expression programs (Fraser *et al*. 2006). There is great interest in tooth replacement and its molecular mechanisms because subsequent tooth generations may arise from stem-like cells (Huysseune and Thesleff 2004); yet only one study to date has examined

gene expression programs in replacement dentitions (Fraser *et al*. 2006). No study has investigated the molecular choreography of tooth replacement in species with adult teeth shaped differently than first-generation teeth and no study has examined how lingual rows of teeth are initiated and patterned (e.g., cichlid species can have more than 15 rows of teeth on the oral jaws). Understanding the molecules involved in the complexity of fish odontogenesis will shed light on general mechanisms of periodic patterning applicable not only to dentitions (Salazar-Ciudad and Jernvall 2002), but also to other organs such as hair and feathers (Houghton *et al*. 2005).

Pigmentation

Pigment patterns represent one of the most extraordinary illustrations of teleost adaptation (Figure 2). Famous examples include coral reef fishes, cichlids of east Africa, and aquarium favorites like guppies and loaches. The myriad pigment patterns of teleosts serve in a variety of roles including warning coloration, camouflage, schooling, mate recognition, and mate choice (Endler 1988; McMillan *et al.* 1999; Couldridge and Alexander 2002; Jordan *et al.* 2003; Engeszer *et al.* 2004; Rosenthal and Ryan 2005; Millar *et al.* 2006).

Pigment Patterns Through Development

Vertebrate skin pigment cells are derived embryologically from neural crest cells, which also contribute to craniofacial bone, cartilage, and teeth, and produce most of the peripheral nervous system (Hall 1999; Le Douarin 1999).

Neural crest cells have long been recognized as a key vertebrate innovation (Gans and Northcutt 1983) and pigment patterns, in addition to skeletons (above), have provided a valuable opportunity to study the developmental and genetic factors responsible for evolutionary changes in the patterning of neural crest-derived traits. In contrast to studies of skeletal diversification, which have focused largely on particular genes and tissues, studies of pigmentation have so far emphasized cellular mechanisms of pigment pattern development. The different emphasis reflects the notion that evolutionary changes in gene activity are only interpretable in a cellular context (e.g., Parichy 2005) and this cellular context has thus far been less explored for pigment patterning as compared to skeletogenesis.

Pigment patterns reflect the numbers and arrangements of several classes of pigment cells, or “chromatophores”. These include black melanophores, yellow or orange xanthophores, red erythrophores, blue cyanophores, white leucophores, and iridescent iridophores (Bagnara and Matsumoto 2006; Parichy *et al.* 2006). The color of each class of cell results from the particular pigments contained within specialized organelles. By combining different classes of cells, different spatial arrangements of cells, and different pigment concentrations within individual cells, a seemingly infinite range of patterns and colors can be produced.

Most fishes exhibit different pigment patterns during different life cycle phases. The first pattern to develop arises as embryonic neural crest cells disperse from above the neural tube, differentiating chromatophores during or

even prior to their migration, and subsequently colonizing specific locations to generate an embryonic/early larval pigment pattern (Raible and Eisen 1994; Kelsh 2004). Commonly this consists of stripes of melanophores dorsally, laterally, and ventrally, with xanthophores broadly scattered over the flank (Quigley *et al.* 2004; Lamoreux *et al.* 2005) though a variety of other patterns also occur. The functional significance of these pigment patterns remains unexplored.

The diversity of teleost pigmentation consists mostly of patterns expressed in the adult. In some species, the adult pigment patterns develop during metamorphosis, when the larval form is transformed into a juvenile by remodeling or initial appearance of a variety of traits [e.g., fins, skin, scales, skeleton, gut, kidney, and sensory systems (Webb 1999)]. Pigment pattern metamorphosis has been most studied in zebrafish, *Danio rerio* (Figure 2). In this species, metamorphic melanophores differentiate scattered over the flank, then melanophores coalesce at sites of adult stripe formation, with additional metamorphic melanophores differentiating already within the stripes; most embryonic/early larval melanophores die (Parichy and Turner 2003b).

Developmental changes in pigment pattern also can occur during later development, particularly with the onset of sexual maturation, and these may be either permanent, or transient, as is the case for nuptial coloration (Dickman *et al.* 1988; Mabee 1995; Beeching *et al.* 2002; Maan *et al.* 2006). To date, virtually nothing is known about the molecular and cellular bases of pigment pattern changes within the adult phases of the life cycle.

Genes Underlying Changes in Pigmentation

One way that teleost pigment patterns evolve is by modifying the quantity or quality of the pigments carried by chromatophores. Two recent studies provide nice examples of how genetic approaches can provide insights into the evolution of pigmentation in fishes and beyond.

In Mexican tetras, *Astyanax*, several cave-dwelling populations exhibit a suite of derived traits including albinism, reduced eyes, and enhancements of other sensory systems (Jeffery 2001; Yamamoto *et al.* 2004; Figure 1). The phylogeography of these populations is complex, though cave forms have clearly evolved repeatedly (Strecker *et al.* 2004). Despite their albinism, cavefish retain melanophores (McCauley *et al.* 2004) and genetic mapping identified a major effect QTL for melanin loss (Protas *et al.* 2006). By mapping candidate genes associated with mammalian albinism, a correspondence was found between the cavefish QTL and *oculocutaneous albinism-2* (*oca2*). Complementation tests showed that albinism in a second cavefish population is associated with the same locus, and molecular analyses revealed that each population harbors different small genomic deletions within *oca2*. The deletions are functionally significant as *oca2* cDNA from melanized, surface-dwelling *Astyanax* allows melanization of murine *oca2*-deficient melanocytes, whereas the two cavefish deletion cDNAs do not. This study nicely shows how pigmentation loss can result independently from changes at the same locus, and suggests such parallelism may reflect both an absence of pleiotropic effects and the large size of *oca2*,

making it a high frequency “target” for selection. These results are reminiscent of recent studies of *MC1R* in mammalian pigmentation (Hoekstra *et al.* 2006). The cavefish example also illustrates how knowledge of pigment cell genes and development in mammals can be applied to understanding pigment evolution in teleosts.

Knowledge of pigment development in teleosts also can inform us about the evolution of pigment in mammals, including humans. A striking example is the *D. rerio golden* mutant, which has reduced melanin but otherwise normal melanophores. Positional cloning identified *golden* as *slc24a5*, which encodes a sodium/calcium transporter localized to pigment granules within melanophores (Lamason *et al*. 2005). Mutations in *aim1*, also a transporter involved in melanin synthesis, explain a similar orange-red medaka variant called b (Fukamachi *et al*. 2001). Remarkably, a polymorphism within human *SLC24A5* is associated with different pigmentation between European and African populations and significantly reduced heterozygosity indicates past selection at this locus. Whether variation at *slc24a5* or *aim1* has contributed to pigment evolution in teleosts and other taxa remains to be determined.

Mechanistic Bases for Cellular Pattern Diversification

Beyond changes in pigment content, a major factor in teleost pigment pattern diversification has been changes to the numbers and arrangements of chromatophore classes. Such variation has received extensive theoretical attention (Asai *et al*. 1999; Painter *et al*. 1999; Miguez and Munuzuri 2006) and

recent studies have started to elucidate the underlying mechanisms, primarily using *D. rerio* and its relatives.

One recent insight concerns the origins of chromatophores responsible for pattern diversification. Unlike embryonic/early larval melanophores that differentiate directly from neural crest cells, metamorphic melanophores in *D. rerio* differentiate from latent precursors of presumptive neural crest origin (Johnson *et al*. 1995; Parichy and Turner 2003b; Parichy *et al*. 2003). Mounting evidence suggests these precursors are stem cells, able to generate differentiated progeny while themselves remaining undifferentiated (Parichy and Turner 2003a; Yang and Johnson 2006). A sister species, *D. nigrofasciatus*, exhibits superficially similar adult stripes to *D. rerio*, yet cell lineage analyses reveal these stripes are formed largely by reorganizing embryonic/early larval, neural crest-derived melanophores, rather than differentiation of stem cell-derived metamorphic melanophores (Quigley *et al*. 2004). Thus, danios exhibit at least two different modes of pigment pattern metamorphosis.

Analyses of danios show that cryptic but genetically distinct populations of metamorphic melanophores differentially contribute to pigment pattern evolution (Johnson *et al*. 1995; Parichy *et al*. 1999; Parichy *et al*. 2000a,b). In *D. rerio*, early metamorphic melanophores that are initially dispersed and then migrate into stripes depend on the kit receptor tyrosine kinase, as they are ablated in *kit* mutants. By contrast, late metamorphic melanophores that develop already within stripes do so independently of *kit*; i.e., they persist—in stripes—in *kit* mutants. As distinct populations of *kit*-dependent and *kit*-independent

melanocytes have not been found in mammals, these cell populations might be unique to *D. rerio*. To test this idea, a recent study isolated a *kit* mutant in *D. albolineatus*, which normally exhibits uniformly dispersed melanophores. The mutant retained a population of *kit*-independent melanophores, showing conservation of these cellular populations in at least one other danio. Strikingly, and in contrast to the uniform wild-type *D. albolineatus* pattern (Figure 2), the *kit*-independent melanophores occurred in stripes. These and other data showed that *D. albolineatus* has latent stripe-forming potential, and that stripe loss in this species occurred in part by a failure of *kit*-dependent melanophores to migrate into stripes, thereby obscuring the stripes formed by *kit*-independent melanophores (Quigley *et al*. 2005; Mills *et al*. 2007). These studies show how a manipulative, genetic approach can be used to deconstruct the evolution of an adult phenotype.

Studies of danios also suggest that an important factor in pigment pattern diversification depends on chromatophore interactions. In *D. rerio*, stripes arise through interactions between melanophores and xanthophores, and between cells within each of these classes (Maderspacher and Nusslein-Volhard 2003; Parichy and Turner 2003a; Watanabe *et al*. 2006). Genetic analyses indicate that variation in danio pigment patterns likely reflect evolutionary modifications to the strength and timing of these interactions, which appear to serve as a pattern-generating mechanism that can be deployed at different times and in different places (Parichy and Turner 2003a; Quigley *et al*. 2005). Interspecific complementation testing of candidate genes identified as *D. rerio* mutants further

revealed that such interactions are likely to be perturbed in *D. albolineatus* – contributing to the uniform pigment pattern – owing to changes in *colony stimulating factor 1 receptor* (*csf1r*, *fms*), which encodes a receptor tyrosine kinase expressed by cells of the xanthophore lineage (Parichy and Johnson 2001; Quigley *et al*. 2005).

While danios are an especially tractable system for analyzing pigment pattern development and evolution, these species represent only a small fraction of teleost pigment pattern diversity. In this regard two additional groups are especially interesting—guppies and cichlids—both because of color pattern variation and because of the deep foundation of ecological and behavioral observations regarding these patterns (Seehausen *et al*. 1999; Lindholm *et al*. 2004; Genner and Turner 2005). For cichlids, a particularly exciting recent advance is the ability to map factors genetically using closely related species. For instance, a QTL associated with alternative barred and orange blotch (OB) post-metamorphic color patterns in *Metraclima zebra* maps to the vicinity of c-*ski1* on chromosome 5 (Streelman *et al*. 2003b; Figure 2). As representative cichlid genome sequences become available (Table 2), identification of this locus and other inferred genetic factors (Maan *et al*. 2006; Barson *et al*. 2007) will provide new and important insights into pigment pattern diversification. Moreover, mechanistic studies of danios and other model organisms should provide inroads to understanding the cellular bases for pattern diversification in these other species.

Pigmentation Genes Evolve Rapidly in Teleosts

A problem complementary to the evolution of pigment patterns is the evolution of pigment pattern genes, and several recent studies have assessed naturally occurring variation at such loci. For example, surveys of several cichlid species with diverse color patterns found differential rates of evolution among loci and between recently duplicated paralogous copies, including *csf1r* mentioned above (Sugie *et al*. 2004; Braasch *et al*. 2006). An especially intriguing example is *hagoromo*, which encodes an F-box/WD-40 repeat protein that is required for metamorphic melanophore development in *D. rerio* (Kawakami *et al*. 2000). Analyses of more than a dozen cichlid species reveals accelerated rates of amino acid evolution in specific domains and an extraordinary increase in the complexity of alternatively spliced *hagoromo* transcripts (Terai *et al*. 2002; Terai *et al*. 2003). It will be fascinating to learn how *hagoromo* functions in pigment pattern development and to test its causal involvement in generating species-specific pigment patterns.

Sex (Gender) Determination

Sex Determination Mechanisms in Fish are Diverse

Most developmental pathways, such as those discussed above, are well conserved across disparate taxa. By contrast, the developmental pathways that determine sex are strikingly variable and can even differ between closely related species. Teleost fishes present attractive models to understand the evolution of sex determination pathways, as the entire range of environmental and genetic

sex determining mechanisms is represented across lineages (Devlin and Nagahama 2002). For example, many fishes have environmentally determined sex, which can depend upon factors such as temperature or social interactions. Genetic mechanisms of sex determination in fishes may be polygenic or simple and associated either with no cytogenetically visible sex chromosomes or heteromorphic sex chromosomes in either males (XY systems) or females (ZW systems). This wide diversity of sex determination mechanisms can be found even in closely related fish species (Devlin and Nagahama 2002; Mank *et al*. 2006). Particularly apposite examples of this diversity are found within poeciliid fishes (guppies, mollies, swordtails and platyfish; Volff and Schartl 2001), salmonid fishes (Philips *et al*. 2001; Woram *et al*. 2003), the stickleback family Gastesteidae (Chen and Reisman 1970) and the tilapia genus *Oreochromis* (Lee *et al.* 2003, 2004). Diversity of sex determination mechanisms in closely related fish species supports the hypothesis that this developmental pathway is evolutionarily plastic and that sex determination mechanisms and sex chromosomes can evolve very rapidly.

One example of the plasticity of sex determination mechanisms in fish is highlighted by recent work in medaka (*Oryzias latipes*). With the identification of a duplicated copy of the *dmrt1* gene called *dmrt1bY* or *DMY* as the medaka master sex determination locus (Matsuda *et al*. 2002; Nanda *et al.* 2002), there was speculation that this gene would serve a similar role in all fish, just as *Sry* is the master sex determination switch in nearly all mammals (Marshall Graves 2002). Although the *Dmrt* gene family is widely present in fish (Volff *et al*. 2003a),

the *dmrt1bY*/*DMY* gene is absent from other fish species (Veith *et al.* 2003; Kondo *et al.* 2003). In fact, although *dmrt1bY*/*DMY* is present in a second species, *Oryzias curvinotus* (Matsuda *et al.* 2003; Kondo *et al.* 2004), other species within the *Oryzias* genus do not have this gene (Kondo *et al.* 2003; 2004), suggesting that *dmrt1bY*/*DMY* has arisen within the *Oryzias* lineage in the past 10 million years (Kondo *et al.* 2004).

The enormous variation in sex determination pathways in fish presents an opportunity to understand the mechanisms by which sex determination genes arise and sex determination pathways evolve. Remarkably, the mechanisms of sex determination remain unknown for *D. rerio*, although multiple loci and environmental influences are likely to be involved. Currently, efforts are underway to identify the master sex determination genes in platyfish, tilapia, salmonids, and stickleback. This work should identify whether there are common themes that connect the types of genes that are used as master sex determination genes as well as provide insights into the evolution of sex determination pathways.

Sex Chromosome Evolution in Teleosts

In addition to the diversity of sex determination mechanisms in fish, there is also great diversity in the presence of sex chromosomes. Approximately 10% of fish species have cytogenetically visible sex chromosomes (Devlin and Nagahama 2002). However, this is likely an underestimate of the number of fish species that have sex chromosome systems because young sex chromosome

systems that are in early stages of differentiation are unlikely to be observed by traditional cytogenetic analysis. Many closely related species of fish differ in sex chromosome complement, suggesting that sex chromosomes can arise rapidly in fish. Many fish sex chromosomes are therefore likely to be younger than the very stable XX-XY sex chromosome system in mammals, which is over 300 million years old (Graves 2006). Therefore, studying sex chromosomes in fish provides a unique opportunity to investigate the genetic and molecular events that accompany the earliest stages of sex chromosome evolution.

After the acquisition of a sex determination locus, one of the first steps in the evolution of a sex chromosome is suppression of recombination around a sex determination locus, which has been hypothesized to occur in order to reduce recombination between the sex determination locus and linked genes with sex-specific fitness effects (Fisher 1931; Bull 1983; Rice 1987a). This suppression of recombination leaves the heterogametic sex with one chromosome in a consistently heterozygous state, which ultimately results in the degeneration of sex-linked loci in the heterogametic sex (Bull 1983; Rice 1987b; Charlesworth 1991). Based on these models, it is predicted that a sex chromosome would show reduction of recombination near the sex determination region, resulting in the loss of homology between the X and the Y chromosome, particularly due to the accumulation of deleterious mutations, including an increase in transposable elements on the Y chromosome. Chromosome rearrangements may or may not accompany these early stages of sex chromosome evolution. Recent studies of the sex chromosomes of a number of different fish species have begun to

illuminate these processes on a molecular level, and have also begun to provide insight into the timing of events in sex chromosome evolution.

In particular, recent work in medaka fish (Kondo *et al.* 2006) has provided a detailed molecular view of the events that accompany the early stages of sex chromosome evolution, just after the evolution of a new sex determination gene. As described above, the sex determination gene in *O. latipes* was recently identified as the *dmrt1bY*/*DMY* gene, a duplicate copy of the *dmrt1* gene (Matsuda *et al.* 2002; Nanda *et al.* 2002). Kondo *et al*. (2006) cloned and sequenced the regions flanking *dmrt1bY* on both the X and the Y chromosome, as well as the *dmrt1* region. They found a completely Y-specific region that resulted from a duplication of a 43 kb region of chromosome 9 that includes the *dmrt1* gene. A number of repetitive elements have accumulated within the Y-specific region, accounting for an increase in its size to 258 kb. Thus, in this relatively young (less than 10 million years old) sex chromosome system (Kondo *et al.* 2004), there is evidence for both degeneration of Y-linked sequences and accumulation of repetitive DNA (Kondo *et al.* 2006).

It may be that the *dmrt1bY*/*DMY* locus in medaka represents a unique mechanism of sex chromosome evolution. To gain insights into the general mechanisms that underlie the evolution of sex chromosomes, it is important to analyze other sex chromosome systems of differing ages. In fishes, there are a number of other sex chromosome systems in species with the requisite genetic and genomic tools for this analysis. To date, the most well studied systems have been poeciliid fishes (guppies and platyfish), salmonid species, threespine

stickleback (*G. aculeatus*), and tilapiine cichlids (*Oreochromis spp*.). In most of these systems, genetic analysis has revealed a genetic basis for sex determination even in the absence of cytogenetically visible sex chromosomes.

There must be some differentiation between sex chromosomes in most of these sex chromosome systems, as reduction in recombination between the X and the Y-chromosomes near the sex determination region has been observed in threespine stickleback (Peichel *et al.* 2004), blue tilapia (Lee *et al.* 2004), and platyfish (Morizot *et al*. 1991; Gutbrod and Schartl 1999). Given the loss of recombination near sex determination regions of these fish, it is not surprising that there is also evidence that many of these systems have accumulated repetitive DNA. In tilapia, there are subtle differences in the amount of heterochromatin, which consists of repetitive DNA elements that has accumulated on the Y chromosome relative to the X (Harvey *et al.* 2002; Griffin *et al.* 2002). Similarly, the sex determination region of lake trout, brown trout, and Atlantic salmon are all next to a large heterochromatic block (Philips and Ihssen 1985; Philips *et al.* 2002; Artieri *et al.* 2006). Sequencing of X and Y-specific BAC clones in threespine stickleback (*G. aculeatus*) and platyfish (*X. maculatus*) revealed that the Y chromosomes in both species had significantly more repetitive and transposable elements than the X chromosomes (Froschauer *et al*. 2002; Peichel *et al*. 2004; Schultheis *et al*. 2006).

Beyond examining the accumulation of transposable elements, relatively little has been done to explore the effects of loss of recombination at the sequence level. The fact that viable and fertile YY salmonids (Chevassus 1988), tilapia

(Penman and McAndrew 2000), and platyfish (Kallman 1984) males can be generated suggests that genes required for viability and fertility on the Y chromosome have not yet been rendered nonfunctional. Some sex-linked genes in platyfish appear to be pseudogenes; however, there are a number of duplicate copies of these genes, such that at least one functional copy might remain (Volff *et al*. 2003b). There are a number of sequence differences between the X and the Y chromosome in the threespine stickleback (Peichel *et al.* 2004); however, it is not known whether genes on the stickleback Y have become nonfunctional or whether YY individuals can be generated in stickleback. In the future, it will be important to compare the levels of cytogenetic differentiation with levels of sequence divergence and to explore in more detail the molecular changes that have occurred in the regions around a sex determination locus.

Pigmentation and Skeletal Traits are Linked on Sex Chromosomes

Reduction of recombination around a sex determination locus appears to be a general phenomenon in sex chromosome evolution. Theoretical work suggests that this may result from linkage of a sexually antagonistic gene to the sex determination locus, which would select for the loss of recombination to prevent detrimental alleles from being expressed in the wrong sex (Fisher 1931; Bull 1983; Rice 1987a). Thus, we might expect that there would be an excess of sexually antagonistic genes linked to the sex chromosomes. In particular, male display traits, such as color, can be considered sexually antagonistic traits because expression in males is beneficial, but expression in females would be

deleterious, as it might expose females to predation and incur production costs (Fisher 1931; Endler 1980; Bull 1983). This model does not exclude species with female display traits; in this case we might simply expect to see linkage of female display traits to a female determining locus. In support of this model, there is good evidence for linkage of (fe)male display traits to sex chromosomes in a number of fish species (Lindholm and Breden 2002).

The poeciliid fish provide some of the most spectacular examples of sex linkage of male display traits (Lindholm and Breden 2002). In guppies, which have an XY sex determination system, pigmentation, fin size and shape, courtship behavior and male “attractiveness” are linked to the Y chromosome (Brooks 2000; Brooks and Endler 2001). The Y-linked color patterns are extremely polymorphic in natural populations, and differ in their attractiveness to females (Lindholm *et al*. 2004). Different Y-linked color alleles are associated with increased predation (Endler 1983) and mortality (Brooks 2000), suggesting a balance between natural and sexual selection contributes to maintenance of color polymorphisms in guppy populations (Endler 1980).

In another poeciliid fish genus, *Xiphophorus*, there are a number of traits involved in male attractiveness that are closely linked to the sex determination locus on the Y chromosome (Basolo 2006; Rosenthal *et al*. 2006; Cummings *et al*. 2006). As in guppies, pigmentation loci are tightly linked to the sex determination locus and are highly polymorphic within and between *Xiphophorus* populations (Kallman 1975). In addition, the puberty or pituitary (P) locus is tightly linked to the sex determination locus and determines both the onset of sexual

maturity (Kallman *et al*. 1973; Kallman and Borkowski 1978) and reproductive tactics (Zimmerer *et al.* 1989). This locus is also highly polymorphic, leading to alternative mating strategies within populations. Males that mature later are robust, ornamented and have elaborate courtship behaviors, while the males that mature early are small, and have little ornamentation, and perform sneaker copulations. As for color, this polymorphism is likely to be maintained within populations due to a balance of natural and sexual selection (Ryan *et al.* 1992). Although large males are favored by sexual selection and are preferred by females (Ryan *et al*. 1990), they are not favored by natural selection and are more heavily preyed upon (Rosenthal *et al*. 2001), providing an advantage for smaller and less conspicuous males.

Traits important for adaptation have been found linked to sex chromosomes in several other fishes. Among Malawi cichlids of the genus *Metriaclima*, sex is determined by a locus on chromosome 7, unless the orange blotch trait (OB) is segregating in the family, in which case sex is under the control of a dominant female determiner linked to OB on chromosome 5 (TD Kocher, personal communication; Streelman *et al.* 2003b). Notably, genes for jaw shape and function map to cichlid chromosome 7 (Albertson *et al*. 2003; Albertson *et al*. 2005; above) and a QTL of major effect for tooth shape maps to chromosome 5 near OB, sex and an opsin gene cluster (Carleton and Kocher 2001; Streelman and Albertson 2006). In tilapiine cichlids, a red color mutant maps close to the sex-determining locus of female heterogametic species on chromosome 3 (Lee *et al*. 2005). Finally, at least one skeletal trait, the size of the opercle bone, has

been mapped to the stickleback sex chromosome (Kimmel *et al*. 2005). These latter data provide empirical evidence for quantitative genetic models of adaptive speciation that predict gametic association between ecological, marker and preference traits (Dieckmann and Doebeli 1999; Kondrashov and Kondrashov 1999), on incipient sex chromosomes with reduced recombination.

Synthesis and Perspective

The studies reviewed here have engendered novel insights into the developmental genetic basis of adaptation. Conceptually, this has shifted focus towards studying *how development works* in diverse and highly complex natural systems. Much has been learned about how genes with manifold pleiotropic functions (e.g., *pitx1*, *bmp4*, *shh*) can be employed specifically in an organ- or tissue-specific manor (Shapiro *et al*. 2004; Yamamoto *et al*. 2004; Albertson *et al*. 2005). Less satisfying however, is that new genes or new gene functions have not been discovered; the genes involved in the traits we highlight might have been predicted in the context of traditional developmental genetics research. This is either because forward and reverse genetic screens are so thorough as to be redundant, or because investigators have thus far studied a biased set of natural mutations (i.e., genes of large effect; Orr 1998). The next 5-10 years of research will address this question as new techniques (e.g., Miller *et al*. 2007) and improved genomic resources (Table 2) are used to investigate new traits in more teleost lineages. In summation, we consider a major question in evolutionary biology addressed by the studies reviewed here.

How Does Evolution Happen?

Many authors have discussed whether there are general rules governing evolutionary developmental biology (Gerhart and Kirschner 1997; Wilkins 2002; Carroll 2005). Davidson and Erwin (2006) have codified such rules in terms of gene regulatory networks (GRNs) and the evolutionary scale of change among the components of such networks. At one extreme are 'kernels,' or sets of genes at the core of GRNs that may be conserved over long periods of evolutionary time. At the other extreme are differentiation gene batteries (DGB), genes involved in terminal differentiation of tissues or structures; DGBs reside at the periphery of GRNs and might be employed to distinguish among closely related species. In fact, Davidson and Erwin propose a "relation between the network-component class in which changes might occur and the taxonomic level of morphogenetic effects." According to Davidson and Erwin's hierarchical scheme (their Figure 3), all of the genes responsible for adaptive differences among closely related fish species (Table 1; *pitx1*, *shh*, *twhh*, *oca2*, *eda*, *bmp4, dmy*) should belong in DGBs. However, 6 of the 7 are better characterized as input/output (I/O) switches or plug-ins, both of which are classes of evolutionarily conserved components of multiple developmental networks. Davidson and Erwin hypothesize that changes in I/O switches and plug-ins explain differences at the taxonomic level of class, order or family. Only *oca2* fits the definition of a DGB. So why don't the data from fish adaptations fit the schema of Davidson and Erwin? The answer seems to lie in the degree of modular function for these I/O

switch and plug-in genes. I/O switches and plug-ins can elicit major morphological change (because they regulate other genes through morphogenesis, unlike DGB genes), but the modularity of their regulation allows other pleiotropic functions of the encoded protein to remain unchanged (e.g., fin vs. jaw function of *pitx1*; Shapiro *et al*. 2004). Perhaps a better prediction is that the genes involved in adaptation among closely related species will be those genes central to key morphogenetic processes (e.g., cell proliferation, differentiation, death, and migration) whose regulation across tissue- and cell-type is highly modular. In the language of GRNs, these are well-connected 'hubs,' but the genes to which they are connected may vary across tissues, and from species to species. The developmental and evolutionary flexibility of GRNs has not yet been examined among closely related species, but the approach is tractable in vertebrates (Tsaparas *et al.* 2006).

In summary, the next decade of research, highlighting these and other fish models, will surely contribute important data regarding the developmental genetic basis of adaptation. Further study fusing the power of molecular biology and genomics in fish groups of tremendous morphological, functional, physiological and behavioral diversity will shape our understanding of how development works.

Table 1. Summary of genes involved in adaptation among different fish lineages. The column “data” specifies the type of data [genetic (G), transcriptional (T) or both] used to demonstrate the relationship between genotype and phenotype. The column “gene type” assigns genes according to the terminology of Davidson and Erwin 2006 (see text for abbreviations).

Trait	**Lineage**	**Gene**	**Data**	**Gene type**
Pelvic fin loss	pufferfishes	*hoxd9a*	T	I/O
Pelvic fin loss	stickleback	*pitx1*	both	I/O
Eye loss	cavefish	*shh, twhh*	T	Plug in
Pigment loss	cavefish	*oca2*	both	DGB
Armor loss	stickleback	*eda*	G	Plug in
Tooth loss	cypriniforms	Fgf, *dlx2*	T	Plug in
Jaw function	cichlid	*bmp4*	both	Plug in
Sex determ.	medaka	*dmy*	G	I/O

Table 2. Genomic resources for model teleosts.

Resource	Species	Website
Cichlid Genome Consortium	cichlids	http://hcgs.unh.edu/cichlid/
Ensembl	zebrafish, stickleback pufferfish, medaka	http://www.ensembl.org
JGI	Pufferfish - *Takifugu*	http://genome.jgi-psf.org/Takru4/Takru4.home.html
Medaka Homepage	medaka	http://biol1.bio.nagoya-u.ac.jp:8000
Genoscope	Pufferfish - *Tetraodon*	http://www.genoscope.cns.fr/externe/tetranew/
Sanger Institute	zebrafish	http://www.sanger.ac.uk/Projects/D_rerio/
Stanford Genome Evolution Center	zebrafish, stickleback	http://cegs.stanford.edu/index.jsp
Xiphophorus Home Page	*Xiphophorus*	http://xiphophorus.org
Zebrafish Info. Network	zebrafish	http://zfin.org

Acknowledgements

We thank members of our laboratories (especially Darrin Hulsey, Gareth Fraser), and our colleagues (Craig Albertson, William Cresko, Yoshiyuki Yamamoto, Tom Kocher, Felix Breden) for discussion and comments on the manuscript. Our research is funded by the NIH (JTS, CLP, DMP), the NSF (JTS, DMP), the Burroughs Welcome Fund (CLP) and the Alfred P. Sloan Foundation (JTS).

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Figure Legends

Figure 1. Variation in skeletal morphology and anatomy among model teleosts. Panels a and b are *Placidochromis milomo* (Lake Malawi) and *Lobochilotes labiatus* (Lake Tanganyika), demonstrating parallel evolution of cartilaginous fleshy lips, function unknown. Panel c is *Rhamphochromis esox*, a piscivore from Lake Malawi with a highly kinematic jaw and unicuspid teeth; d is an oral view of *Pseudotropheus elongatus*, an algae eater from Lake Malawi with multiple rows of multicuspid teeth. Panels e and f represent eyed and pigmented (e) versus eyeless and albino tetras, *Astyanax* (f). Panels g and h demonstrate variation in body armor and pelvic spines among Alaskan sticklebacks. Photos of tetras and sticklebacks are courtesy of Yoshiyuki Yamamoto and William Cresko, respectively.

Figure 2. Pigment pattern variation and pigment cells of teleosts. Shown are several species within *Danio* (a-g) as well as the cichlid *Labeotropheus fuelleborni* (h-j), illustrating differing color patterns associated with the absence or presence of the orange blotch polymorphism (BB [blue-black], OB, respectively). Panel c shows melanophores, xanthophores, and iridophores in the *D. rerio* adult pigment pattern. Iridescent iidophores are present throughout but can be seen here only where they catch the light.