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PIGMENT PATTERNS OF ECTOTHERMIC VERTEBRATES: HETEROCHRONIC VS. NONHETEROCHRONIC MODELS FOR PIGMENT PATTERN EVOLUTION

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INTRODUCTION

A vast literature documents the prevalence of heterochronic changes during the evolution of morphology. Such heterochronies may be defined broadly as changes in the rate or timing of developmental events, compared with an ancestral ontogeny (Gould, 1977; Alberch et al., 1979; McKinney and McNamara, 1991). Typically, heterochronies have been identified at the whole organism level (so-called "global" heterochronies) or at the level of particular traits or organ systems ("local" heterochronies in the terminology of McKinney and McNamara, 1991). A classic example of a global heterochrony is the independently derived failure of metamorphosis in several species of salamander

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in the genus *Ambystoma*; this retention of a larva-like somatic morphology after sexual maturation has been considered a clear case of paedomorphosis (Shaffer and Voss, 1996; Voss and Shaffer, 1997; and see below). Another example, also from salamanders, is the extraordinary miniaturization observed in several lineages of plethodontid salamanders, which has resulted in radical changes in limb, skull, and central nervous system morphology, another example of paedomorphosis (Wake, 1991; Hanken and Wake, 1993).

The literature pertaining to heterochrony is vast (McKinney and McNamara, 1991), particularly in amphibians (Hanken, 1999). These studies have focused almost exclusively on patterns of morphological variation, as revealed by analyses of embryonic, larval, or adult morphology. Nevertheless, an explicitly hierarchical view of heterochrony has also been promoted by McKinney and McNamara (1991) in which organismal form and its evolutionary modification are seen as the product of underlying cellular events. In this framework, heterochrony can be considered an organizing principle at any biological level, as applicable to changes in the rate or timing of cellular events during development as to "global" changes in morphology. Pursuing this notion, McKinney and McNamara argued that heterochrony is both the cause of most developmental alterations and a source of major, morphological novelties (see p. 47). Despite these claims, relatively little attempt has been made to assess the frequency of heterochronic changes in cellular behaviors underlying the development of morphological traits or the morphological consequences such changes might produce. For example, we do not know whether a heterochrony identified at the whole organism level necessarily reflects a heterochrony at the underlying cellular or genetic levels. Likewise, there has been almost no effort to assess the extent to which seemingly nonheterochronic variation at a morphological level might nevertheless depend on heterochronic alterations to underlying cellular behaviors: Can continuous variation in a trait typically be explained by heterochronic cellular variation? Is discontinuous variation or morphological novelty often due to changes in the timing of cellular behaviors, as McKinney and McNamara postulated?

In this chapter, I seek to test the hypothesis that interspecific diversity in salamander pigment patterns is causally related to heterochronies at the cellular level. This hypothesis predicts that both continuous and discontinuous pigment pattern variation or novel pigment pattern variants can be related to changes in the rate or timing of underlying cellular mechanisms that are themselves conserved across taxa. Alternatively, if pigment pattern differences between species result from nonheterochronic changes in developmental mechanisms, for example, the appearance of a novel cellular behavior or a novel cue to which cells respond, this would call into question the utility of a heterochronic framework for understanding the mechanistic bases for pigment pattern variation.

To distinguish between these possibilities, I begin by reviewing some of the features of pigment patterns that make them a useful system for studying the evolution of form, as well as some of the cellular and developmental processes relevant to understanding pigment pattern evolution. I then briefly discuss some methodological issues relevant to studying the development of pigment patterns and other characters in an evolutionary context. Because heterochonic changes are only understandable with reference to an ancestral ontogeny, I then review our current understanding of phylogenetic relationships for several species in which pigment pattern development has been studied. These hypotheses of phylogeny then set the stage for examining how several features of pigment patterns develop and the extent to which phylogenetic transformations in these patterns may have resulted from heterochronic changes in developmental mechanisms. Finally, in the last part of this chapter, I ask whether a heterochronic framework at the cellular level is useful for understanding evolutionary changes in pigment patterns, and I suggest possible future directions for assessing heterochronies and other mechanistic changes relevant to pigment pattern evolution.

Pigment Patterns Are a Model System for Understanding Morphological Variation and Evolutionary Diversification in Vertebrates

Several features make pigment patterns a particularly useful system for studying the mechanistic bases for the evolution of form. Because pigment patterns are a classic and enduring system for studying development, there is a wealth of data on pigment cell morphogenesis and differentiation that helps guide studies of pigment pattern variation and evolution (DuShane, 1934; Bennett, 1993; Erickson, 1993; Reedy et al., 1998b). Some of these data are presented below.

Pigment patterns are also an attractive system because of their ecological and behavioral significance. For example, a host of studies have shown the importance of pigment patterns for thermoregulation, species recognition, locomotion, avoidance of predation, and mate choice (e.g., Endler, 1978; Houde, 1997). The selective factors that contribute to the evolution of specific pigment patterns are beyond the scope of this review. Nevertheless, the myriad functional roles served by pigment patterns offer the promise of truly integrative studies that span several levels of biological organization.

A final reason for using pigment patterns is the pigment cell lineage itself. Vertebrate pigment cells are derived from the neural crest, a transient population of embryonic precursor cells that arises during neurulation at the border between the neural plate and nonneural ectoderm and later are found dorsal to the neural tube (Le Douarin, 1982; Hall and Hörstadius, 1988; Erickson and Perris, 1993; Groves and Bronner-Fraser, 1999; Hall, 1999). In an evolutionary context, neural crest cells are of particular interest for at least two reasons: their differentiative potential and their epigenetic mode of development. Both of these features have implications for studying the potential roles heterochrony has played in phylogenetic transformations of morphology.

Neural crest cells give rise not only to pigment cells but also to a host of other cell types and tissues in the vertebrate embryo and adult (Hall and Hörstadius, 1988; Groves and Bronner-Fraser, 1999). For example, much of the peripheral nervous system, including both neurons and glia, originates from the neural crest. These cells also produce many of the dermal bones comprising

the craniofacial skeleton and contribute to teeth, fin mesenchyme, and bony fin rays of teleosts. Finally, neural crest cells are the progenitors of endocardial cushion cells in the heart, adrenal chromaffin cells, and some smooth muscle. The many organ systems that arise in whole or in part from neural crest cells suggest that much of vertebrate evolution can be understood in terms of changes in the patterning of these cells and their derivatives (Gans and Northcutt, 1983; Gerhart and Kirschner, 1997; Hall, 1999). In vitro clonal analyses show that some neural crest cells are pluripotent (i.e., can differentiate into more than one derivative), whereas other neural crest cells differentiate into just one or two cell types (Sieber-Blum and Cohen, 1980; Baroffio et al., 1988, 1991). Likewise, in vivo lineage tracing has demonstrated marked heterogeneity in the fates acquired by neural crest cells (Bronner-Fraser and Fraser, 1988; Frank and Sanes, 1991; Fraser and Bronner-Fraser, 1991). In the zebrafish *Danio rerio*, for example, Raible and Eisen (1994) showed that some neural crest cells produce progeny that ultimately differentiate into pigment cells, sensory and sympathetic neurons, as well as glia, whereas other neural crest cells produce type-restricted progeny differentiating as only one or another cell type. The developmental potential of neural crest cells becomes more restricted over time. Thus, early-appearing neural crest cells produce a more diverse array of fates than later-appearing neural crest cells. The factors responsible for this progressive restriction of cell fate remain a matter of investigation (Groves and Bronner-Fraser, 1999). Nevertheless, the existence of such a pattern and the observation that neural crest cells at different states of specification have different morphogenetic requirements and abilities (Erickson and Goins, 1995; Erickson and Reedy, 1998a) raise the possibility that heterochronic changes in mechanisms of neural crest specification could have downstream consequences for the allocation of these cells to particular fates. For example, the zebrafish *colourless* mutation specifically eliminates pigment cell, neuronal, and glial fates but does not affect fin mesenchyme or craniofacial derivatives, suggesting an early segregation of these cell lineages (Kelsh and Eisen, 1999). Thus, a naturally occurring temporal change in the activity of a gene required for some general aspect of neural crest development (e.g., proliferation) could conceivably have different effects across neural crest-derived lineages, depending on precisely when this change occurs. The notion of whether changes in timing affecting fate specification have roles in generating naturally occurring morphological variation remains almost completely unexplored, but a potential example in which changes in neural crest differentiation may have mediated the evolutionary loss of stripes will be presented below (also see Smith, 2000).

Besides the extraordinary range of derivatives to which neural crest cells contribute, this population of embryonic precursor cells is particularly intriguing because of the epigenetic nature of its development. Although cell movements characterize the morphogenesis of numerous cell types and organ systems in both vertebrate and invertebrate embryos (Bard, 1990), neural crest cells are truly exceptional in this regard. As noted above, neural crest cells arise at the border between the neural plate and epidermis and then are found along

the dorsal neural tube (or neural keel in teleosts; Moury and Jacobson, 1990; LaBonne and Bronner-Fraser, 1999; Nguyen et al., 2000). These cells then disperse from this location and migrate along stereotypical pathways throughout the embryo (Fig. 7.1; Löfberg et al., 1980; Loring and Erickson, 1987; Raible et al., 1992; Erickson and Perris, 1993). Most neural crest cells that will contribute to externally visible pigment patterns migrate along a dorsolateral migratory pathway, between the ectoderm and the somites (or, at later stages, the myotomes). In contrast, trunk neural crest cells that contribute to the peripheral nervous system take a ventromedial migratory pathway, between the somites and the neural tube or notochord. During this migration, the cells interact with one another and adjacent cell types, and these interactions can influence both the morphogenetic behavior of the cells and their state of differentiation. A large body of literature addresses the mechanisms by which migration may commence at the level of the neural tube, factors that contribute to the rate and pattern of cell movement as migration is underway, and the controls that determine when and where cells should cease their migration and take up residence (Erickson and Perris, 1993; Perris and Perissinotto, 2000; Sela-Donenfeld and Kalcheim, 2000). There is thus ample opportunity for changes in the patterning of neural crest derivatives as a result of changes in the timing or rate of migration or the interactions that occur during this migration. Several examples of such interactions will be presented in detail below. The epigenetic nature of neural crest development also suggests that phylogenetic changes could be either autonomous to the neural crest cells themselves or could arise as nonautonomous changes in the extracellular environment that the cells encounter. As discussed below, these different possibilities can have different evolutionary implications.

One intraspecific example of a nonautonomous, heterochronic change in neural crest patterning comes from the axolotl *Ambystoma mexicanum* (itself a famous example of a global paedomorphosis). Nearly a century ago, the spontaneous white (*d*) mutant of *A. mexicanum* was first described (Häcker, 1907). Whereas neural crest-derived pigment cells give wild-type axolotls a dark color as both larvae and adults, white mutants are brilliant white (Fig. 7.2). A series of studies have since identified some of the factors that contribute to this striking phenotype and have shown that it first arises as a defect in neural crest migration. In contrast to wild-type axolotls, white mutants completely lack differentiated pigment cells in the skin as adults, and, in larvae, these cells are largely confined to the region immediately dorsal to the neural tube. Scanning electron microscopy shows that most neural crest cells fail to enter the dorsolateral migratory pathway, and a series of embryological grafting experiments revealed that the defect lies within the epidermis, rather than the neural crest cells themselves (DuShane, 1935, 1939; Keller et al., 1982; Spieth and Keller, 1984; Keller and Spieth, 1984). Subsequent histological, ultrastructural, and biochemical studies, as well as heterochronic grafting experiments, showed that white mutant embryos exhibit a retardation in the development of the subepidermal extracellular matrix (Löfberg et al., 1989; Perris et al., 1990), in-

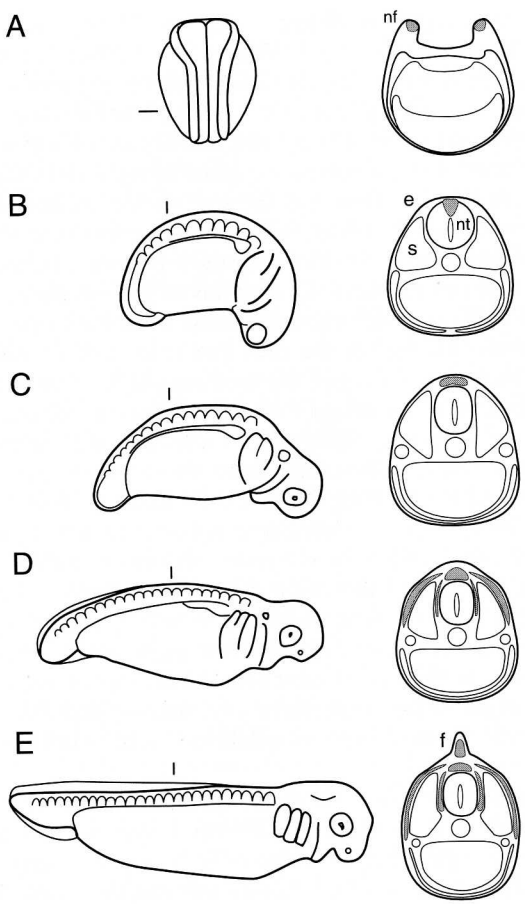

Figure 7.1. Development of neural crest cells in salamanders. Shown is a schematic of a salamander embryo at several stages of development. On the left are dorsal (A) and lateral (B–E) views; on the right are cross sections at the level indicated by the bar on the left. Positions of neural crest cells, their precursors, or derivatives are indicated in grey. A: at neurula stages, prospective neural crest cells are found within the rising neural folds (nf). B: subsequently, after the neural folds fuse, neural crest cells form a wedge within the dorsal neural tube (nt). Also shown is the position of bilateral somites (s) and the epidermis (e). The notochord is found immediately ventral to the neural tube. C: by early tailbud stages, neural crest cells have segregated from the neural tube, and, in salamanders, these cells form a distinctive cord running anterior to posterior along the dorsal midline immediately above the neural tube. Differentiation of neural crest cells into pigment cells can begin in this premigratory position. D: with further development, neural crest cells (or their derivatives) disperse from the neural tube and follow stereotypical pathways to destinations throughout the embryo. Cells that contribute to externally visible pigment patterns typically migrate between the somites and overlying epidermis. E: by hatching stages, neural crest cell migration is essentially complete, and derivatives of these cells are found throughout the body of the embryo as well as in the median fin fold (f).

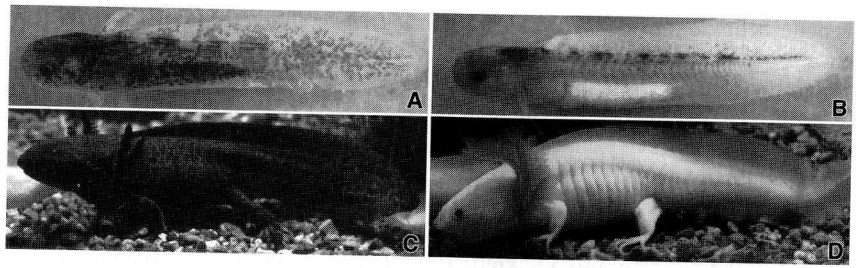

Figure 7.2. A mutant of the laboratory axolotl, *A. mexicanum*, provides a model for an intraspecific heterochrony affecting neural crest patterning. Wild-type axolotls exhibit a dark coloration as larvae (A) and adults (C). White mutant axolotls have a pigment cell deficiency as larvae (B) and an essentially complete absence of pigment cells as adults (D), owing to a heterochronic defect in extracellular matrix development that inhibits the normal dispersal of pigment cells from the neural crest. [From Parichy et al. (1999b).]

cluding reduced levels of a specific proteoglycan during the stages when neural crest cells normally disperse from the neural crest (Stigson et al., 1997a, 1997b). This delay in extracellular matrix development is postulated to result in an environment that is nonpermissive for neural crest cell migration when these cells are competent to disperse from their position above the neural tube; at a later stage, the environment becomes permissive, but neural crest cells are no longer competent to disperse. Thus, a heterochrony in the development of the environment through which neural crest cells migrate has been implicated in a change in neural crest patterning, in this instance, an essentially complete failure of pigment cells to colonize the flank. This interpretation can be tested further once the gene corresponding to the white mutation is identified (Parichy et al., 1999b). Although the phenotype of the white mutant is extreme, and probably not relevant to most naturally occurring variation, the underlying defect illustrates how even transient developmental changes that affect neural crest migration may have large phenotypic effects. A possible instance in which interspecific differences in the timing of cell migration determine whether a particular pigment pattern arises is presented below.

Approaches to Studying Heterochronic and Nonheterochronic Mechanisms in Pigment Pattern Development and Evolution

One advantage to pigment patterns for studying the developmental bases for evolutionary changes in form is that pigment cells are externally visible, and, in oviparous vertebrates like many amphibians and fishes, these cells may be followed essentially throughout the life of the organism. This has allowed for time lapse and imaging series in which the behaviors of individual cells are recorded and interspecific differences in cell behavior are identified by simply watching as the phenotypes develop (see below). In some instances, phylogenetic changes in morphology can thus be immediately interpreted as differences in the morpho-

genetic behaviors of cells, a major advantage over other traits in which differences in final form may provide little insight into the morphogenetic events by which the form was constructed.

Although the pigment in pigment cells serves as an autonomous marker of cell lineage, studies of neural crest cells and pigment cell precursors (i.e., before visible pigmentation has developed) typically require methods to distinguish these cells from surrounding cell types. A variety of approaches has been employed, including molecular markers for neural crest cells or their descendent cell types (Sadaghiani and Vielkind, 1990; Erickson and Goins, 1995; Lister et al., 1999; Parichy et al., 1999a, 2000a, 2000b), ultrastructural analyses to identify nascent pigment-containing subcellular organelles (Frost et al., 1984), neural crest grafts between embryos using species- or strain-specific characteristics (e.g., nuclear morphology; Sadaghiani and Thiébaud, 1987; Couly et al., 1993), and a variety of lineage tracers including radioactive isotopes (Weston, 1963; Chibon, 1967), retroviruses (Frank and Sanes, 1991), and fluorescent vital dyes (Bronner-Fraser and Fraser, 1988; Collazo et al., 1993; Raible and Eisen, 1994; Erickson and Goins, 1995; Olsson and Hanken, 1996). In addition, some pigment cell precursors are identifiable by their endogenous autofluorescence shortly before the development of externally visible pigmentation (Epperlein et al., 1988; Raible and Eisen, 1994). Thus, a number of different strategies may now be used to assess the development of pigment cell precursors even before they acquire visible pigment. By and large, however, these reagents have yet to be exploited for assessing the mechanistic bases for interspecific variation in the development of pigment patterns or other neural crest derivatives. Although a histological technique has been widely used for many years in which initially unmelanized melanoblasts darken after administration of the melanin synthesis intermediate DOPA (Epperlein et al., 1988), this technique is relatively nonspecific and also can stain a variety of other cell types that are not derived from the neural crest (Tucker and Erickson, 1986a). Moreover, because this method only reveals melanophore precursors that are already competent to produce melanin and the earliest time at which these cells are visible differs considerably among species (Epperlein and Löfberg, 1984; Olsson, 1993), an increased use of other, more specific markers will be required for further analyzing the distribution of pigment cell precursors during development and evolution.

Rigorous inferences concerning developmental mechanisms require not only careful descriptive analyses but manipulative experiments as well. This is particularly true in an interspecific context in which causality is even more difficult to demonstrate than during the ontogeny of a single species and more factors can contribute to confounding interpretations of developmental process (e.g., differences in overall development rate and morphology, failure of molecular probes to cross-react between species, unexpected differences in developmental genetic pathways). For instance, numerous studies of nonmodel organisms have documented the expression of genes thought to pattern various tissues, organs, or body axes (reviewed in Carroll et al., 2001). Nevertheless, these studies often have not demonstrated the relevance of the expression patterns in question for

the final form of the character. Indeed, there are many instances in which tissues express a gene yet do not require it for their development (e.g., Parichy et al., 2000a). Only through manipulative experiments or mutational analyses can this possibility be addressed. Likewise, descriptive analyses of cellular distributions during development may suggest cellular and tissue level controls, but perturbational approaches must be used to challenge these mechanistic hypotheses. Fortunately, both amphibians and fishes offer many opportunities for experimental approaches to dissecting the evolution of form, via transgenesis, mutational analyses, various kinds of microsurgical manipulations, and other approaches (e.g., Solnica-Krezel et al., 1994; Parichy, 1996a; Huang et al., 1999). In the long term, the use of such experimental methods, employed within the context of well-resolved hypotheses of phylogenetic relationships, will be essential for rigorous evolutionary inferences regarding changes in developmental mechanisms.

CASE STUDIES: SALAMANDER PIGMENT PATTERN DEVELOPMENT

Salamanders and Their Pigment Patterns

Salamanders exhibit a diverse array of pigment patterns. Moreover, many species of salamanders undergo a metamorphosis in which an aquatic larval morphology is transformed into a terrestrial adult morphology, and this often entails radical changes in externally visible pigment patterns. Figure 7.3 illustrates some of the diversity in pigment patterns at early larval stages, shortly after salamanders hatch and when they are especially vulnerable to predators (examples of adult pigment patterns are shown below). Recent work suggests that at least some of these patterns serve a role in crypsis (Storfer et al., 1999).

An important step in analyzing the development or evolution of any character is to identify its component parts. At early larval stages, salamander pigment patterns comprise principally two classes of neural crest-derived pigment cell: black melanophores and yellow xanthophores, the latter of which can be visualized most easily by their autofluorescence under ultraviolet light of an appropriate wavelength (Epperlein et al., 1988; Fig. 7.4). As described in detail below, much of the variation in early larval pigment patterns across species can be understood in terms of the presence or absence of two pattern elements that reflect the spatial arrangements of melanophores and xanthophores. These elements are (1) alternating patches of melanophores and xanthophores extending ventrally from the dorsal flank so as to form a series of vertical bars and (2) a region running horizontally along the middle of the flank in which xanthophores are present but melanophores are not found, thereby forming a melanophore-free horizontal stripe. Both vertical bars and a melanophore-free region are shown in Figure 7.4. Although vertical bars and melanophore-free regions have sometimes been considered alternative states of a single character (i.e., a larval pigment pattern; Epperlein et al., 1996), these different elements

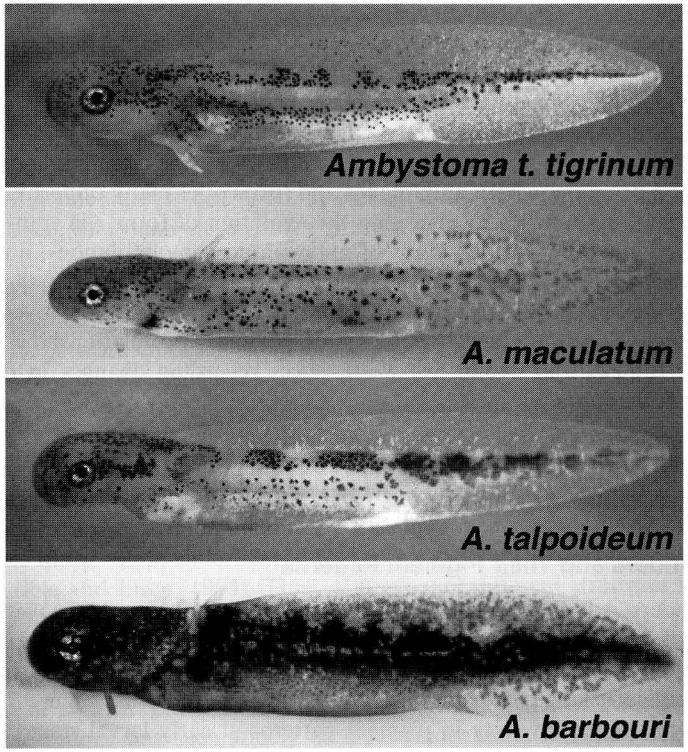

Figure 7.3. Salamanders exhibit diverse early larval pigment patterns. Shown are four species of *Ambystoma* that exhibit various combinations of vertical barring and a horizontal melanophore-free region in the middle of the flank.

depend at least partly on distinct developmental mechanisms, as described below (although the extent of such independence is an empirical question that can be addressed only using experimental approaches). Thus, I treat these pigment pattern elements as discrete characters for the purpose of assessing evolutionary changes in developmental mechanisms but also briefly discuss mechanisms that may be shared by these elements during their formation. How prevalent are these different pattern elements in salamanders, and what is the evolutionary history of the mechanisms that produce them? A complete answer to these questions requires a hypothesis of phylogenetic relationships.

Phylogenetic Relationships of Salamander Taxa

The evolutionary relationships of salamanders have been studied extensively. For the purpose of understanding the evolution of developmental mechanisms underlying pigment pattern formation, several different taxonomic levels must

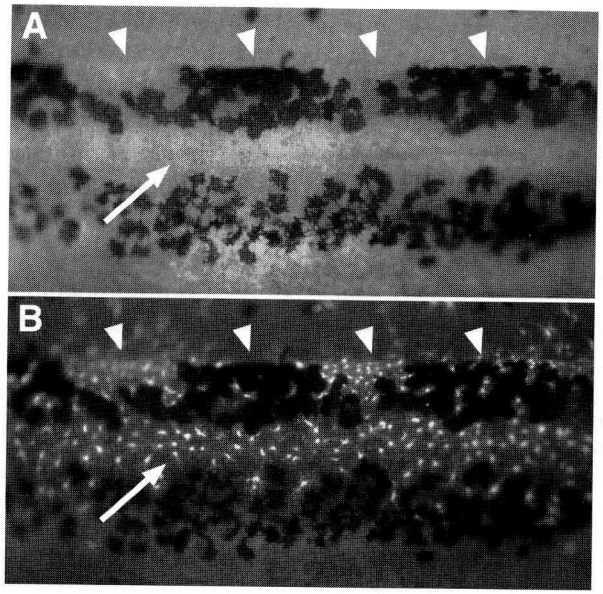

Figure 7.4. Pigment pattern elements in hatchling salamander larvae. Shown is the flank of a hatching stage *A. t. tigrinum* larva. Anterior is to the right. A: brightfield view shows the distribution of black melanophores. B: ultraviolet illumination of the same field reveals yellow xanthophores by their autofluorescence. Two distinctive pigment pattern elements are present in this species: a series of alternating vertical bars of melanophores and xanthophores (arrowheads) and a melanophore-free region in the middle of the flank (arrow) that defines a horizontal stripe pattern.

be considered. Here, I briefly review some of the current hypotheses for phylogenetic relationships among salamander families, as well as relationships within two families (Ambystomatidae, Salamandridae) that have figured prominently in studies of pigment pattern development.

Salamanders, Order Caudata, comprise 10 families of approximately 350 total species (Duellman and Trueb, 1986). Early studies of family-level relationships were based on morphological characters, which in salamanders are often plagued by homoplasies (Wake, 1991). More recently, molecular analyses by Larson (1991), which used large and small subunit rRNA sequence data, as well as by Larson and Dimmick (1993), which used these molecular data and additional morphological data, have suggested the hypothesis of phylogenetic relationships shown in Figure 7.5. The overall features of this hypothesis are well supported and are based on 209 phylogenetically informative characters comprising 177 rRNA nucleotide positions and 32 anatomical characters (derived from head, trunk, and cloacal morphology) from 20 species. Analyses of these characters as a combined data set support the monophyly of all salamander families, and examination of character state transformations revealed

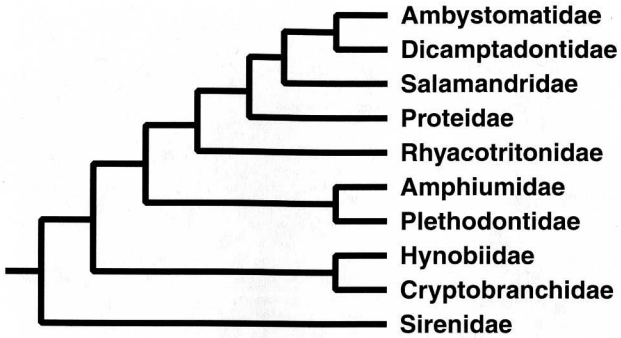

Figure 7.5. Phylogenetic relationships of the living salamander families. Shown is a recent hypothesis of relationships based on molecular and morphological data (Larson and Dimmick, 1993). Most studies of pigment pattern development have focused on species within Ambystomatidae and Salamandridae.

relatively little incongruence between molecular and morphological subsets of the data. With respect to pigment pattern analysis, this hypothesis differs in one important placement, compared with older hypotheses based strictly on morphological data. Specifically, phylogenies based on morphological criteria alone placed the speciose family Plethodontidae (~250 species; Wake and Hanken, 1996) in a relatively derived position as a sister taxon to Ambystomatidae (33 recognized species; Shaffer et al., 1991). In contrast, the phylogeny of Larson and Dimmick (1993) places Plethodontidae in a more basal position, with a terminal clade comprising Ambystomatidae, the three species of Dicamptadontidae, and Salamandridae. Thus, with the exception of three dicamptadontids that are close relatives of Ambystomatidae (and occupy a similar, unique larval habitat, discussed below), the two families in which pigment pattern development has been studied most extensively are sister taxa.

The phylogenetic relationships of taxa within Salamandridae and Ambystomatidae have been matters of contention. An abbreviated hypothesis of relationships is shown in Figure 7.6. Salamandridae comprise 15 genera and 53 recognized species in North America, Europe, and Asia (Titus and Larson, 1995). Studies of salamandrid phylogeny have employed a variety of characters, including anatomical traits, particularly of the hyobranchial apparatus (Wake and Özeti, 1969), courtship behavior (Salthe, 1967; Macgregor et al., 1990), and 12S and 16S rRNA sequences (Titus and Larson, 1995). A phylogenetic issue of debate has concerned the relationship between the genera of newts (e.g., *Pleurodeles*, *Taricha*, *Triturus*, and *Notophthalmus*) and the "true" salamanders (*Salamandra*, *Mertensiella*, *Chioglossa*) and specifically whether newts are monophyletic. A recent analysis (Titus and Larson, 1995) combining both molecular data (431 informative nucleotides) and morphological data (44 informative characters) indicates that newts are monophyletic and are a sister

taxon to true salamanders. To date, studies of pigment pattern development and evolution in salamandrids have been concerned solely with newts; thus it will ultimately be necessary to examine the "true" salamanders to rigorously infer mechanisms within the family as a whole. Within the newts, most studies of pigment pattern evolution have focused on species in the genera *Taricha* and *Triturus*. Relationships within the North American genus *Taricha* (formerly classified at *Triturus*) are well resolved (Riemer, 1958). Relationships within *Triturus* remain somewhat unclear, although currently pigment pattern development in only a single species, *Tr. alpestris*, has received extensive study (see below).

All species within Ambystomatidae are now generally considered to comprise a single genus, *Ambystoma* [reviewed by Shaffer (1993); "*Amblystoma*" in some older literature] and are found exclusively in North America. Disagreements over relationships within *Ambystoma* have centered on the appropriateness of combining morphological and molecular data sets vs. analyzing data sets separately, the extent and meaning of character correlations within morphological and molecular data sets, as well as the methods used for coding and analyzing these various data. Much of the controversy has revolved around the subgenus *Linguaelapsus* identified initially by Tihen (1958) and supported by Kraus (1988) as monophyletic based on morphological criteria and comprising *A. annulatum*, *A. cingulatum*, *A. barbouri*, *A. texanum*, and *A. mabeei*. In contrast, Shaffer et al. (1991) found only equivocal support for this clade, as allozyme-based phylogenies (data from 26 loci) suggested a polyphyletic grouping of these species, whereas a combined molecular and morphological data set (the allozyme loci plus 32 informative anatomical characters) supported monophyly. However, a more recent reanalysis of these combined molecular and morphological data supports the monophyly of *Linguaelapsus* (Jones et al., 1993; Fig. 7.6). In the present context, these alternative hypotheses suggest different reconstructions for the evolution of vertical barring patterns (see below). A similar conflict between morphological and molecular data sets concerns *A. maculatum* (formerly *A. punctatum*) and *A. gracile*, which are only distant relatives in morphologically based hypotheses but sister taxa in hypotheses from allozymes alone as well as from combined allozyme and morphological data sets (Kraus, 1988; Shaffer et al., 1991; Jones et al., 1993). Once again, these alternative hypotheses have different implications for the evolution of vertical barring patterns. The relatively large genetic distances among ambystomatids (excluding the *A. tigrinum* complex, see below) suggest an ancient divergence, and, given the disparities among current data sets, additional analyses using sequence data from sufficiently slowly evolving loci may be needed to fully resolve these lineages (Shaffer et al., 1991).

Finally, I briefly consider relationships within the *A. tigrinum* complex of ambystomatid salamanders. This group comprises 5–7 subspecies of *A. tigrinum* distributed throughout the continental United States and southern Canada, as well as 15 species extending into central Mexico. Seven of the Mexican

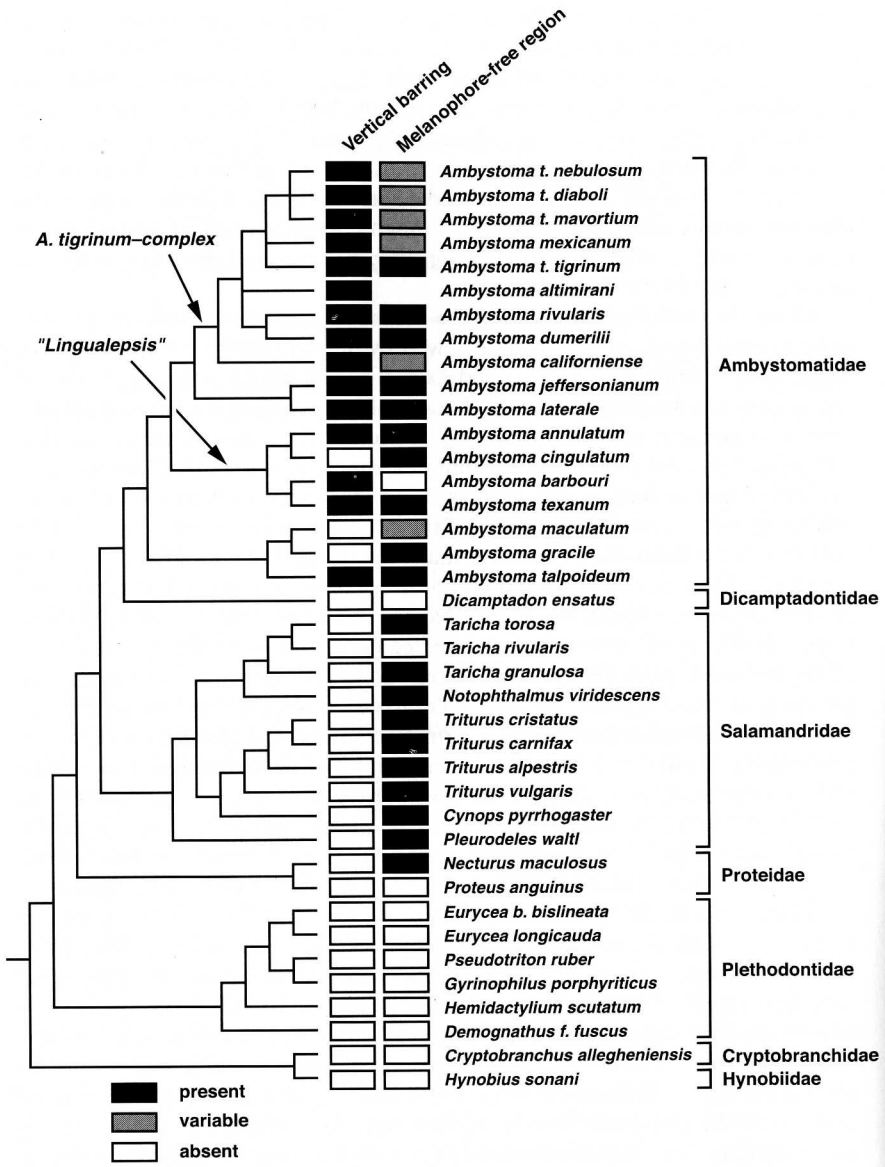

Figure 7.6. Phylogenetic distribution of salamanders in which early larval pigment patterns have been described. The phylogeny indicates the presence, absence, or variability of vertical bars and melanophore-free regions and is a composite of several hypotheses from different studies that used molecular or morphological characters other than pigment patterns. For interpretations of pigment pattern evolution under different hypotheses of phylogenetic relationships, see text. Vertical bars in salamanders are limited to Ambystomatidae and are most likely to have arisen in the common ancestor of *Ambystoma*, with a subsequent loss of this pattern element in the lineages leading to *A. maculatum* and *A. gracile*, as well as *A. cingulatum*. Melanophore-free regions are found in both Ambystomatidae and Salamandridae but have been lost independently in taxa within these clades. A lateral line-dependent stripe-forming mechanism is inferred to be ancestral for Ambystomatidae and Salamandridae, whereas evolutionarily derived stripe-forming mechanisms are present in *T. torosa* (see text). Data for pigment patterns are from Twitty, 1936; Henry and Twitty, 1940; Bishop, 1941; Orton, 1942; Fox, 1955; Riemer, 1958; Brandon, 1961; Durand and Vandel, 1968; Brandon, 1972; Brandon and Altig, 1973; Kakegawa et al., 1989; Pfingsten and Downs, 1989; Epperlein and Löfberg, 1990; Liozner and Dettlaff, 1991; Olsson, 1993; Olsson, 1994; Collazo and Marks, 1994; Parichy, 1996a, 1996b. Phylogenies are from Wake and Özeti, 1969; Shaffer, 1984; Macgregor et al., 1990; Larson, 1991; Shaffer et al., 1991; Jones et al., 1993; Larson and Dimmick, 1993; Titus and Larson, 1995; Shaffer and McKnight, 1996.

taxa exhibit an obligately paedomorphic (nonmetamorphosing) life history, including the Mexican axolotl *A. mexicanum* (Shaffer, 1993). An additional six Mexican taxa exhibit facultative paedomorphosis (i.e., some individuals metamorphose, whereas others do not). Although recognized as closely related to one another, the precise phylogenetic relationships of these taxa have remained obscure. In an attempt to resolve relationships within the *A. tigrinum* complex, Shaffer (1984) used allozyme electrophoresis and Shaffer and McKnight (1996) analyzed 840 nucleotides of the mitochondrial D loop as well as a mitochondrial intron from 83 individuals representing 77 localities. Despite analyzing rapidly evolving sequences of mitochondrial DNA, these authors found little divergence among taxa (0–8.5%). Although eight reasonably well-defined clades were identified, they were unable to further resolve relationships among these lineages. It is likely that most of these taxa split from one another in the relatively recent past (0.02–5 million years; Shaffer and McKnight, 1996; Shaffer, personal communication), which is striking from the perspective of pigment pattern evolution, as many of these taxa have markedly different larval and adult pigment patterns and these differences seem to have evolved very rapidly.

Larval Pigment Patterns. I. Evolutionary Novelty and the Development of Vertical Bars

Vertical barring is a distinctive pigment pattern in several salamanders and is especially apparent when larvae are viewed from above. This pattern may serve a cryptic function by allowing larvae to blend in with a mottled substrate, although this notion has not been tested. A survey of several species based on direct observations and data from the literature is summarized in Figure 7.6, which maps the presence or absence of vertical barring onto a phylogeny that includes representatives from 7 of the 10 recognized salamander families. This survey reveals that vertical barring within salamanders is limited to Ambysto-

matidae, although at least three species, *A. maculatum*, *A. gracile*, and *A. cingulatum*, lack vertical bars. This distribution of vertical barring suggests several equally parsimonious scenarios for the evolution of this pattern element. Vertical bars could have arisen independently in both *A. talpoideum* and the common ancestor of all other *Ambystoma*, excluding *A. maculatum* and *A. gracile*, and then have been lost in *A. cingulatum*. Consistent with the notion that barring might be independently derived multiple times within *Ambystoma* are the observations made in several species of frogs, which exhibit apparently similar barring patterns during the tadpole stage (Gosner and Black, 1957; Gaudin, 1965; Altig and Johnston, 1989). An alternative and probably more likely scenario is that vertical barring first arose in the common ancestor of all *Ambystoma* but was subsequently lost in the ancestor of *A. maculatum* and *A. gracile* and independently lost within *A. cingulatum*. In contrast, the morphologically based phylogeny of Kraus (1988) places *A. gracile* as the basal most ambystomatid and both *A. maculatum* and *A. cingulatum* deeply nested in different clades within *Ambystoma*, thereby raising the possibility that an absence of vertical barring in *A. gracile* (as in other salamander families) is plesiomorphic, with bars first arising in the ancestor to all other *Ambystoma* and then being lost independently in both *A. maculatum* and *A. cingulatum*. Under any of these phylogenetic hypotheses, however, vertical barring is a novelty for *Ambystoma* relative to other salamanders and has been either lost or gained in multiple lineages.

The developmental events of bar development were first described by Lehman (1954, 1957) for *A. mexicanum*. The photographic series in Figure 7.7 illustrates the development of vertical barring in *A. t. tigrinum*, which is similar to that seen in *A. mexicanum* and other species. At early stages, dispersing neural crest cells differentiate as melanophores that become relatively uniformly scattered over the flank. Meanwhile, xanthophores begin to differentiate in clusters within a premigratory cord of neural crest cells, immediately dorsal to

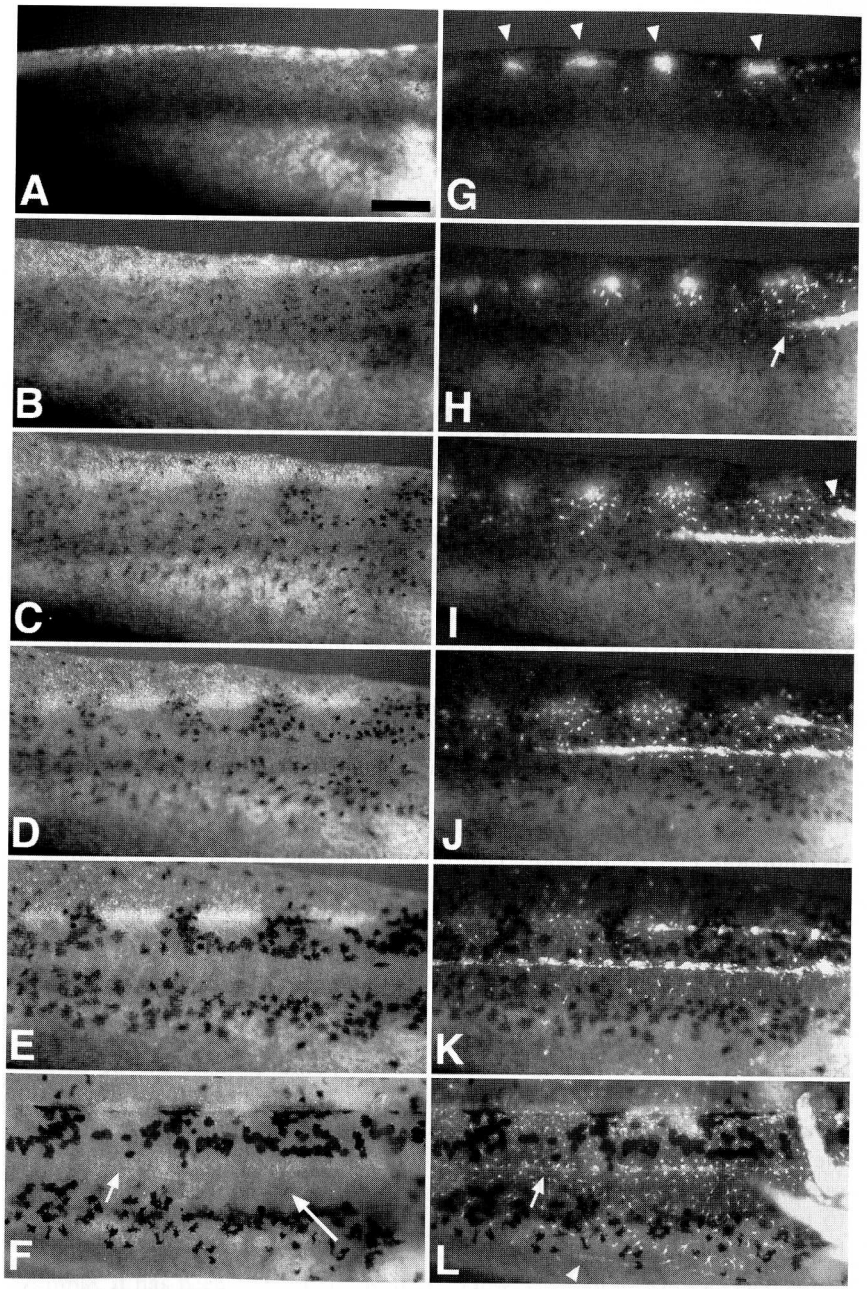

Figure 7.7. Image series reveal cellular events of pigment pattern development in *A. t. tigrinum*. Shown is a single *A. t. tigrinum* embryo during 110 h of pigment pattern development. Anterior is to the right. Images on the left are under brightfield illumination and reveal melanophores. Corresponding images on the right are under epifluorescent illumination and reveal the positions of xanthophores and the developing lateral line sensory system (see text). Vertical bar development is associated with the formation of aggregates of cells containing xanthophores in the premigratory neural crest (arrowheads, Fig. 7.7G), at stages when melanophores already have dispersed over the flank. Subsequent emigration of xanthophores from these aggregates and interactions between xanthophores and melanophores already on the flank generate the vertical barring pattern seen at hatching stages (bottom). Formation of a melanophore-free region occurs as initially uniformly dispersed melanophores retreat from the advancing lateral line primordium (arrow, Fig. 7.7H). Subsequently a distinctive stripe forms (large arrow, Fig. 7.7F), probably in response to persistent effects of the lateral line, interactions between melanophores and xanthophores, and passive movements of melanophores due to growth of the flank. Anterior is to the right. [From Parichy (1996a).]

the neural tube. These clusters are not externally visible; however, with techniques that were not available to Lehman, xanthophores within the clusters can be readily identified using epifluorescent illumination even in living embryos. These xanthophore groups represent a prepattern that is subsequently translated into the definitive vertical barring pattern as xanthophores disperse from these groups and melanophores in their path recede short distances.

The mechanisms underlying vertical bar development are poorly understood. Early scanning electron microscopy revealed that xanthophores occur in morphologically distinctive groups of cells within the premigratory neural crest cord of *A. mexicanum*, and these "aggregates" of cells were further shown to contain not only autofluorescing xanthophore precursors but also cells containing organelles typical of both melanophores and xanthophores, the fates of which remain unresolved (Epperlein and Löfberg, 1984). Morphologically similar aggregates have since been identified in *A. t. tigrinum*, *A. barbouri*, *A. talpoideum*, and *A. annulatum* (Olsson and Löfberg, 1992; Olsson, 1993). Other taxa with vertical barring patterns presumably also develop aggregates at early stages, although this inference must be made cautiously because even superficially similar patterns sometimes depend on different underlying mechanisms (see below). In contrast, the two species lacking bars that have been examined, *Tr. alpestris* and *A. maculatum*, also lack cellular aggregates within the premigratory neural crest. Thus, an understanding of how cellular aggregates form should provide insights into the mechanisms underlying the phylogenetic appearance of vertical barring and what mechanisms could have changed to eliminate this evolutionary novelty in the lineages leading to *A. maculatum* and *A. gracile*, or *A. cingulatum*, or both.

Might an underlying heterochrony in cellular behaviors determine whether vertical bars develop? Perhaps. Lehman (1957) proposed a model in which the presence or absence of vertical barring depends on the relative timing of melanophore and xanthophore emigration from the neural crest. Specifically, Lehman's (1954, 1957) observations of *A. mexicanum* as well as subsequent studies of barred species (Epperlein and Löfberg, 1984; Olsson and Löfberg, 1992; Olsson, 1993; Parichy, 1996a) indicate that melanophores are visible on the flank before xanthophores, which remain initially at the level of the premigratory neural crest. In contrast, Lehman suggested that melanophores and xanthophores disperse simultaneously from the neural crest in species that fail to form vertical bars. Lehman was unaware of cellular aggregates in the premigratory neural crest of barred species, and his mechanism for translating delayed xanthophore migration into a barring pattern probably is incorrect. Nevertheless, with the finding of xanthophore aggregates in the neural crest, one may postulate either that delayed migration allows xanthophore precursors to form aggregates (e.g., by directed cell motility), or that xanthophores differentiate initially in the position of future aggregates, then proliferate until visible aggregates are formed. Consistent with a relationship between the timing of xanthophore dispersal and bar formation, Lehman (1957) found that outgrowths from explanted trunk neural crest of *A. mexicanum* embryos differentiate principally as melanophores after brief periods in culture, whereas increasing proportions of xanthophores were found after a longer time in culture. In contrast, outgrowths from *A. maculatum* neural crest explants produced relatively large proportions of xanthophores from the earliest times in culture. If the in vitro data reflect events in the embryo, these results could indicate a staggered emigration of melanophores and xanthophores in *A. mexicanum* (supported by direct observation) and simultaneous emigration of these cell types in *A. maculatum*.

An identical interpretation of the evolutionary change in cellular behavior was advanced independently in a more recent comparison of pigment pattern development between *A. maculatum* and barred species (Olsson, 1993). In this study, melanophores and xanthophores were observed already dispersed over the flank when each cell type was first visible, and this was inferred to indicate a simultaneous dispersal of these cells from the neural crest. It was further suggested (Olsson, 1993, 1994) that melanophores and xanthophores disperse simultaneously from the neural crest in *Tr. alpestris*, in which these cells are first visible already dispersed together over the flank (Epperlein and Claviez, 1982; Epperlein, 1982). Despite these inferences, no direct evidence has been gathered to indicate precisely when melanophores and xanthophores (or their precursors) actually leave the neural crest in species that lack bars. Thus, the relationship between timing of xanthophore emigration and subsequent patterning remains unresolved; Lehman's (1957) in vitro data remain the most compelling evidence for an underlying heterochrony in cellular behavior. Future studies using molecular markers for melanophore and xanthophore precursors (e.g., Wehrle-Haller and Weston, 1995; Reedy et al., 1998a; Parichy et al., 1999a, 2000a, 2000b) or other methods of lineage analysis (e.g., Raible and Eisen, 1994) may be able to resolve these issues. Clearly, multiple taxa with and without bars need to be examined to statistically support or refute the hypothesized phylogenetic relationship between differential timing of xanthophore emigration and vertical bar development.

An assessment of heterochronies in the dispersal of xanthophores and melanophores will provide insights into the evolutionary acquisition and loss of vertical barring patterns. So would other data. Grafting of neural folds containing prospective neural crest cells between species that either form bars or fail to form bars indicates that the ability to generate a barring pattern lies within either the neural crest or the epidermis, which also is found within the neural folds (DuShane, 1943; Lehman, 1957; Hirano and Shirai, 1984; Epperlein and Löfberg, 1990; Parichy, unpublished data). What this property reflects is unclear. As suggested above, an intrinsic difference in the timing of xanthophore dispersal may have a causal relationship in determining whether aggregates form. At the level of molecular mechanisms, several different heterochronic and nonheterochronic hypotheses could account for this behavior. For example, it has been suggested repeatedly (Epperlein and Löfberg, 1990, 1996; Epperlein et al., 1996) that the formation of cellular aggregates reflects a sorting out of differentially adhesive cell types (sensu Steinberg, 1970). No direct evi-

dence has been gathered to assess the strength of adhesive interactions between xanthophores compared with interactions between melanophores or between melanophores and xanthophores. Nevertheless, such a differential adhesion hypothesis is plausible based on studies of other systems and the finding that, in *A. mexicanum* embryos denuded of epidermis (which prevents all neural crest dispersal), xanthophores occur in the middle of aggregates, adjacent to the neural tube, whereas melanophores occur on the periphery (Epperlein and Löfberg, 1990). Thus, it is conceivable that similar cell-cell adhesion molecules might be expressed by melanophores and xanthophores, with a delayed down-regulation of these molecules in xanthophores that prevents their early dispersal into the periphery. Such a finding would constitute a molecular heterochrony resulting in a corresponding heterochrony at the cellular level. Alternatively, different classes of pigment cells may express different adhesion molecules at the cell surface, thereby conferring different cellular behaviors. Consistent with this possibility, melanophores and xanthophores of an anuran and a teleost express different cell-cell adhesion molecules (Fukuzawa and Obika, 1995), and different neural crest derivatives in other species exhibit a broad spectrum of adhesion receptors (Erickson and Perris, 1993; Parichy, 1996b). An additional possibility is that very generalized differences in the dynamics of neural crest cell dispersal or the number of cells within the premigratory neural crest cord (i.e., not limited to melanophore or xanthophore lineages) results in aggregate formation and a microenvironment that is favorable for xanthophore differentiation. Indeed, morphologically similar aggregates form in the avian premigratory neural crest (which does not produce xanthophores) when neural tubes are explanted in culture, and prolonged proximity to the neural tube can affect the specification of these cells (Vogel and Weston, 1988; Rogers et al., 1990). Examination of the few salamander taxa that have been studied thus far suggests that aggregate formation correlates with the total number of neural crest cells in the premigratory cord (Detwiler, 1937; Epperlein and Löfberg, 1990; Olsson, 1993, 1994). Thus, several different nonheterochronic mechanisms could account for aggregate and vertical bar formation. It will be interesting to see how many mechanisms have changed phylogenetically to enable aggregation to occur and whether some or all of these mechanisms have reverted to a pleisiomorphic state during the evolutionary loss of a vertical barring pattern. In a broader context, identification of molecular and cellular bases for bar development in anurans should provide insights into whether this superficially similar phenotype has evolved via the same (parallel) or different (convergent) underlying mechanisms.

Larval Pigment Patterns. II. Evolutionary Elaboration of Developmental Mechanisms and the Ontogeny of Horizontal Stripes

The second distinctive pattern element in several species of salamanders is a horizontal melanophore-free region in the middle of the flank. Because this region is bordered by melanophores dorsally, and usually ventrally as well, the

overall pattern consists of a horizontal stripe. Figure 7.6 maps the presence, absence, or variability of melanophore-free regions and reveals that this pattern element often is present in both ambystomatids and salamandrids. However, some taxa exhibit variable or indistinctive melanophore-free regions (e.g., *A. maculatum* and several taxa within the *A. tigrinum* complex), and other taxa within the clade [Ambystomatidae, Dicamptadontidae, Salamandridae, Proteidae (ADSP)] essentially lack melanophore-free regions (i.e., *A. barbouri*, *D. ensatus*, *T. rivularis*, *Proteus anguinus*). A strict parsimony reconstruction of the evolution of melanophore-free regions suggests that this pattern element may have arisen independently across families or just once in the common ancestor of ADSP. As detailed below, this latter hypothesis is reasonable, based on an understanding of how this pattern element develops. In turn, if melanophore-free regions first arose in the ancestor of ADSP, the phylogeny in Figure 7.6 further suggests that a melanophore-free region has been lost independently in *A. barbouri*, *D. ensatus*, *T. rivularis*, and *P. anguinus*, and variation in melanophore-free regions has arisen independently in *A. maculatum* and within the *A. tigrinum* complex. Thus, melanophore-free regions appear to be evolutionary labile pattern elements. Superficially similar melanophore-free regions are also found in tadpoles of the anuran *Xenopus laevis*, as well as in many fish larvae, although the mechanisms underlying the development of melanophore-free regions in these taxa appear to differ from those in salamanders (see below).

The developmental mechanisms underlying horizontal stripe development in salamander larvae have been studied for nearly three-quarters of a century. Early studies by Twitty and colleagues focused on stripe formation (and loss) in newts of the genus *Taricha* (Twitty and Bodenstein, 1939; Twitty, 1945; Twitty and Niu, 1948). Because more recent analyses indicate that some mechanisms in *Taricha* are likely to be derived evolutionarily, I first describe what appears to be a more general, plesiomorphic, mechanism for stripe development in several ambystomatids and other salamandrids. I use *A. t. tigrinum* as an example of this mode of stripe development. I then describe two types of evolutionary transition involving melanophore-free regions: an elaboration of developmental mechanisms in *T. torosa* and the loss of stripes in *T. rivularis* and *A. barbouri*. For both sorts of transition, I assess the explanatory and heuristic power of heterochronic vs. nonheterochronic models of developmental evolution. Finally, I describe briefly what appear to be convergent mechanisms of stripe development in larval *X. laevis* and teleosts.

Recent studies reveal that melanophore-free regions in several ambystomatids and salamandrids depend on interactions between pigment cells and the developing lateral lines (Parichy, 1996a, 1996c). In fishes and aquatic amphibian larvae, the lateral lines comprise a bilateral sensory system for detecting mechanical stimuli and function in orientation, feeding, and predator avoidance (Stone, 1933; Blaxter and Fuiman, 1990; Northcutt et al., 1994; Montgomery et al., 1997; Higgs and Fuiman, 1998). A potential role for the lateral lines in stripe development was suggested by the position of the midbody lateral line, which lies approximately in the middle of the melanophore-free region. As

a first step in testing this possibility, the developing lateral lines were labeled with a fluorescent vital dye to distinguish them from surrounding tissues. Lateral lines arise from ectodermal placodes in the head of the embryo. Each placode that contributes to a lateral line on the trunk produces a missile-shaped primordium of cells that subsequently migrates along the inner epidermis toward the tail. Vital dye labeling in *A. t. tigrinum* showed that the midbody lateral line primordium enters onto the trunk after melanophores have already dispersed uniformly over the flank, and, as the primordium advances, melanophores in its path retreat dorsally and ventrally (Fig. 7.7). These movements inferred from static image series were subsequently confirmed by time-lapse analyses of lateral line and melanophore behaviors. To test whether the correlation between lateral line development and stripe development reflects a causal, epigenetic interaction between cell types, lateral line development was prevented by microsurgically ablating the lateral line placodes on one side of *A. t. tigrinum* embryos. Analyses of resulting melanophore distributions showed that melanophores more completely colonized sides without lateral lines and that most of this effect was attributable to changes in melanophore positions rather than overall changes in melanophore number (Fig. 7.8). Thus, lateral line development is essential for stripe development in *A. t. tigrinum* (a fuller treatment of the mechanisms underlying these interactions, as well as contributions of melanophore-xanthophore interactions and passive movements due to growth, can be found in Parichy, 1996c).

To further test the generality of lateral line-dependent mechanisms, lateral

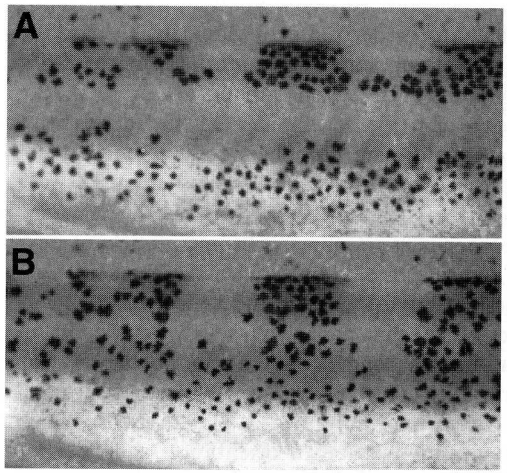

Figure 7.8. Lateral line ablation eliminates the melanophore-free region in *A. t. tigrinum*. Shown are opposite sides of a single larva. One image is reversed so that anterior is to the right in both A and B. A: a distinctive melanophore-free region is present on the lateral line-intact side. B: on the side without a lateral line, melanophores readily colonize the middle of the flank, implicating lateral line-dependent mechanisms in stripe development.

line ablations were repeated in several taxa chosen to represent a wide range of stripe morphologies, as well as distinct phylogenetic lineages within Ambystomatidae and Salamandridae: *A. mexicanum*, *A. barbouri*, *A. maculatum*, *A. talpoideum*, *T. torosa*, *T. rivularis*, *T. granulosa*, *Nothophthalmus viridescens*, and *Pleurodeles waltl* (Parichy, 1996a, and unpublished data). With the exception of *T. torosa* (see below), similar lateral line effects on melanophore distributions were found in each species, even though some lack distinctive melanophore-free regions. These findings strongly suggested that lateral line effects on melanophores are an ancestral way to make a stripe, and presumably arose in the common ancestor of Ambystomatidae and Salamandridae (and perhaps the ancestor to ADSP). In an ecological context, the presence of melanophore-lateral line interactions raises the possibility that horizontal stripes in many of these species might even be regarded as side effects of lateral line development. As such, they might or might not contribute to some overall function of the pigment pattern (like predator avoidance). This possibility highlights the importance of understanding the mechanistic bases for character development, as different interdependencies among traits can sometimes suggest very different hypotheses for the selective factors responsible for evolutionary changes in morphology. Here, stripes could have arisen as a direct response to selection on the pigment pattern or as a correlated response to selection on attributes of the lateral line sensory system (e.g., selection for more mechanoreceptive cells would result in a larger lateral line primordium with a bigger effect on the pigment pattern). These hypotheses await empirical challenge but would not have been apparent without an experimental approach to understanding trait development. Nor do these developmental-evolutionary insights depend on a heterochronic framework of trait evolution.

Stripes do not depend on lateral lines in all species, however. In *T. torosa* (Fig. 7.9), melanophores initially scatter uniformly over the flank and then segregate to form stripes, like *A. t. tigrinum*. However, in contrast to other ambystomatids and salamandrids tested, lateral line ablations do not perturb the horizontal stripe pattern in *T. torosa*. This in turn suggested two alternative hypotheses: (1) ancestral lateral line-dependent mechanisms could have been lost in *T. torosa* as different, lateral line-independent mechanisms evolved or (2) the ancestral mechanisms could have been retained in *T. torosa* as novel and redundant lateral line-independent mechanisms were layered over them. Interspecific chimeras were used to distinguish between these hypotheses (Parichy, 1996a). Specifically, if lateral line-dependent mechanisms have been retained in *T. torosa*, then its lateral lines should be competent to make a stripe, and its melanophores should be competent to respond to cues provided by lateral lines. If either or both of these conditions are not met, this would be consistent with the loss of the ancestral lateral line-dependent mechanisms. Transplanting *T. torosa* lateral line placodes in place of *A. t. tigrinum* lateral line placodes demonstrated that *T. torosa* lateral lines can generate a melanophore-free region. Moreover, transplanting *T. torosa* neural folds (containing prospective neural crest cells) in place of *A. t. tigrinum* neural folds, then ablating the lateral lines on one side of each chimera, showed that *T. torosa* melanophores more com-

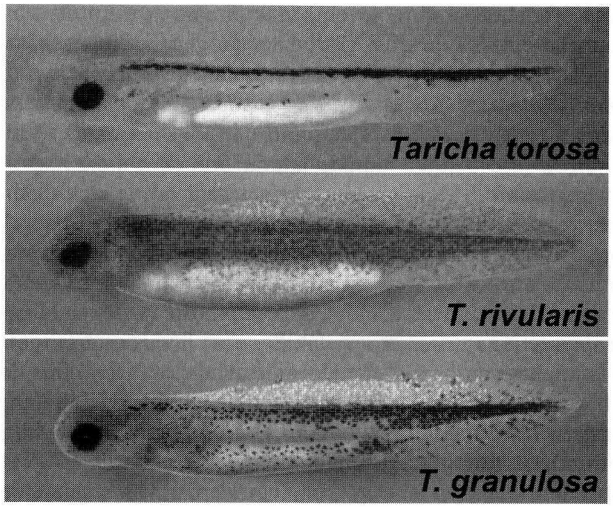

Figure 7.9. Pigment patterns of larvae in the genus *Taricha*. Top: *T. torosa*. Middle: *T. rivularis*. Bottom: *T. granulosa*.

pletely colonized sides without lateral lines. Thus, *T. torosa* melanophores are competent to respond to cues associated with the lateral lines. Together, these experiments suggest that *T. torosa* has retained ancestral lateral line-dependent mechanisms as novel and redundant lateral line-independent mechanisms for stripe formation evolved. Consistent with this idea, studies of taxa in which the lateral lines are required for stripe development show that the anti-adhesive extracellular matrix molecule tenascin is transiently deposited in a lateral line-dependent manner in the middle of the flank (i.e., in the developing melanophore-free region). These and other data suggest a model in which the future melanophore-free region arises in part because the local extracellular matrix is made unfavorable for melanophore localization (also see Epperlein and Löfberg, 1990; Parichy, 1996b, 1996c). Microsurgical manipulations of *T. torosa* show that transient lateral line-dependent deposition of tenascin still occurs, although it is not required for stripe development in this species (Parichy, 2001).

Can a heterochronic framework shed light on the phylogenetic transformation in patterning mechanisms in *T. torosa* relative to ambystomatids and other salamandrids? Image series and time-lapse videos of pigment pattern formation in *T. torosa* revealed, unlike in *A. t. tigrinum*, a melanophore-free region that begins to form several hours in advance of the migrating lateral line primordium. Conceivably, this timing difference could reflect a novel mechanism for stripe development. For example, in a series of elegant embryological manipulations, Twitty (1936, 1945) showed that, at stages before neural crest migration, the somites are able to specify the future site of stripe formation. This potential later shifts to the epidermis (Tucker and Erickson, 1986a). Together,

these findings suggest the hypothesis that a cue for melanophore localization is produced by somitic mesoderm and associates with the epidermis or that mesoderm induces the epidermis to produce such a cue. Although several candidates for such a cue can be suggested (Tucker and Erickson, 1986b; Epperlein and Löfberg, 1990; Wehrle-Haller and Weston, 1995), its identity remains unknown. It also remains unclear whether this phylogenetic transformation reflects the novel production of a cue by the extracellular environment or a novel ability of melanophores to respond to a preexisting cue that they encounter during their migration. The ability of *T. torosa* melanophores (and those of other species) to generate stripes in species that normally lack distinctive melanophore-free regions has been interpreted to mean that an autonomous change in the melanophore lineage has occurred and stripe-forming cues are present in other species (Twitty, 1936, 1945; Epperlein and Löfberg, 1990). These conclusions are premature for two reasons. First, these experiments sometimes have relied on neural fold transplantation and thus cannot distinguish effects that are autonomous to the neural crest cells from effects that are associated with epidermis also contained within the neural folds. Second, the nature of the stripes that form in heterospecific hosts remains unresolved. These stripes have been inferred to be homologous to endogenous stripes in *T. torosa*. Nevertheless, our current understanding of stripe-forming mechanisms, the morphology of the stripes themselves (Twitty, 1945), and results of *T. torosa-A. t. tigrinum* chimeras (Parichy, 1996a) suggests that these stripes may actually reflect an ability of *T. torosa* cells to respond to the lateral lines of a heterospecific host, not a latent cue to which only *T. torosa* cells are competent to respond. Likewise, inferences regarding the role of somitic mesoderm in producing stripe-forming cues in *Tr. alpestris* (Epperlein and Löfberg, 1990) may need to be reexamined in light of lateral line-dependent mechanisms, since manipulations of epidermis and somites can perturb lateral line development (Smith et al., 1990), resulting in a potential confounding of effects due to different tissues.

A second heterochrony is evident in *T. torosa* during a terminal phase of stripe development, and this may also reflect a novel mechanism for melanophore patterning. Specifically, time-lapse videos and static image series indicate that *T. torosa* melanophores remain motile at a later stage than melanophores of other taxa (Parichy, 1996a). During this time, *T. torosa* melanophores migrate in a ventral-to-dorsal direction and eventually form persistent contacts with melanophores already at the site of dorsal stripe formation (Fig. 7.10). Similar contacts have been observed in *T. torosa* melanophores in culture, and a melanophore-autonomous reaggregation was suggested by Twitty (1945) to be responsible for stripe development in *T. torosa*. Nevertheless, the failure of *T. torosa* melanophores to form normal stripes in lateral line-ablated *A. t. tigrinum* argues that, although melanophore-melanophore interactions probably contribute to stripe development, they are not sufficient to ensure that stripes will form. The nature of this heterochrony in the persistence of melanophore motility remains unresolved. For example, differences in the expression

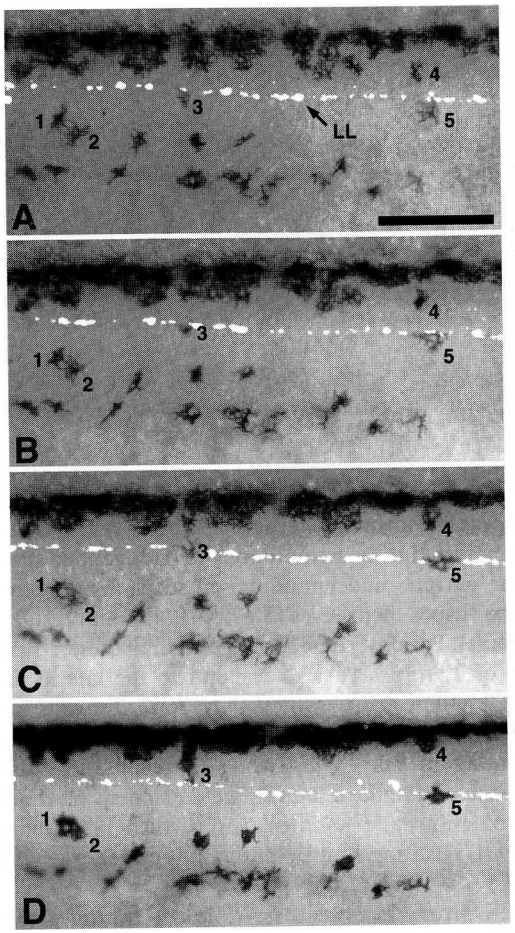

Figure 7.10. Melanophore behaviors differ in *T. torosa* compared with other taxa. Shown are representative images recorded over 48 h during the terminal stages of larval pigment pattern development. The position of the vital dye-labeled midbody lateral line is indicated in white (LL). Unlike other species, *T. torosa* melanophores remain motile during relatively late stages of development. For example, cell 3 travels in a ventral-to-dorsal direction, contacts melanophores further dorsally, and ultimately joins the dorsal stripe Anterior is to the right. [From Parichy (1996a).]

of cell adhesion molecules or other receptors (e.g., Parichy et al., 1999a) may enable these cells to form melanophore-melanophore contacts prohibited in other species because melanophores already have settled. Or, novel cues in the extracellular environment, or an absence of arresting extracellular matrix molecules (Parichy, 2001) may allow melanophores to wander and interact at those late stages. Additional experimental manipulations will be needed to distinguish

between these possibilities. Finally, the relationships between heterochronic changes, an elaboration of developmental mechanisms, and possible selective factors contributing to the evolution of redundancy remain wholly unexplored but should be exciting to uncover.

The other interesting evolutionary transformation in melanophore-free regions is the independent loss of this pattern element in *T. rivularis* (Fig. 7.9), *A. barbouri*, and possibly *Dicamptadon* and *P. anguinus*. Intriguingly, larvae of *T. rivularis*, *A. barbouri*, and *Dicamptadon* all occur in rapidly moving streams (an autapomorphic life style at least for *T. rivularis* and *A. barbouri*), and each shares several other correlated traits in addition to the absence of a stripe: all lack or have reduced balancer organs, all have reduced dorsal fin folds, and all develop slowly from relatively large eggs. This convergent stream-type morphology suggests that selection may have occurred similarly across lineages to modify these various characters (Duellman and Trueb, 1986). Can a change in the rate or timing of cellular behaviors explain the loss of stripes in each of these species? Virtually nothing is known of pigment pattern development in dicamptadontids (Henry and Twitty, 1940). In *T. rivularis* and *A. barbouri*, however, the most noticeable feature of these pigment patterns is the apparent increase in total melanophore number relative to close relatives with distinctive melanophore-free regions. Compared with *T. torosa*, melanophores of *T. rivularis* differentiate more slowly and may stop their differentiation prematurely, at an early stage (Twitty, 1945). *Taricha rivularis* melanophores also have a higher overall rate of proliferation than *T. torosa* melanophores (Youngs, 1957). Conceivably, both of these factors could contribute to the development of a relatively uniform distribution of these cells (e.g., if less differentiated cells proliferate more extensively and essentially "fill" even unfavorable regions of the flank or if these cells never reach a state of differentiation at which they are competent to recognize cues for stripe formation). Although the mechanisms of pigment pattern development in *A. barbouri* are not known, the relatively uniform pattern in this species may have arisen via convergent developmental mechanisms: in *T. rivularis*, the increased number of melanophores is matched by a similarly increased number of xanthophores interspersed among them; in *A. barbouri*, the proportion of melanophores appears to have increased at the expense of xanthophores (Parichy, 1996b, unpublished data). The change in the proportions of different classes of pigment cells presumably also explains a less distinctive vertical barring pattern in *A. barbouri* and suggests a partial coupling between stripe development and vertical bar formation that may be present more generally (MacMillan, 1976; Parichy, 1996a). Thus, a variety of mechanisms may have contributed to the evolutionary loss of a horizontal stripe pattern. A heterochronic framework provides a context for understanding some of the relevant changes, but their mechanistic underpinnings remain unknown.

A final example of convergent stripe-forming mechanisms may be found in other species. For example, larvae of the anuran *X. laevis* exhibit a melanophore-free region that is superficially similar to that of salamanders. It

arises by very different mechanisms. Specifically, neural crest cells in *X. laevis* exhibit an evolutionarily derived pattern of migration (Andres, 1963; Tucker, 1986; Collazo et al., 1993). In contrast to other species that have been studied, these cells typically do not travel between the somites and epidermis. Instead, melanophores that comprise a dorsal stripe represent cells that failed to disperse over the flank, whereas melanophores comprising a ventrolateral stripe arrive there only after emerging from more medial regions of the embryo. Thus, stripe formation in *X. laevis* appears to result from a heterotopy (McKinney and McNamara, 1991) in the pattern of neural crest cell migration. Likewise, stripes in larvae of several teleosts resemble stripes in salamanders, particularly *T. torosa*, and melanophores exhibit somewhat similar behaviors during stripe development (Orton, 1953; Raible and Eisen, 1994; Kimmel et al., 1995; Parichy et al., 1999a). The lateral lines probably are not required for stripe formation in teleost larvae (Parichy, unpublished data), but a role for somitic mesoderm is suggested by mutants in which somite patterning is perturbed, and melanophores localize ectopically in regions where stripes do not form normally (Kelsh et al., 1996). Lastly, analyses of zebrafish mutants are also beginning to reveal mechanisms of stripe development in adults of this species (Kawakani et al., 2000; Parichy et al., 2000b), though it is still too soon to know the extent to which these stripe-forming mechanisms are shared with amphibians.

Adult Pigment Patterns: Morphological Reorganization Across Metamorphosis

Salamanders exhibit a wide variety of adult pigment patterns (Fig. 7.11). At a cellular level, these patterns typically include melanophores and xanthophores, as well as a third class of neural crest-derived pigment cell, the silvery iridophore. To date, very little is known about how these patterns develop, and concerted efforts have not been made to either identify the component elements of adult pigment patterns or reconstruct the evolution of these elements phylogenetically. Early studies revealed roles for epidermis and somitic mesoderm in specifying where spots will form in some species (Lehman, 1953; Lehman and Youngs, 1959), and a few studies have documented the cellular changes that occur during pigment pattern metamorphosis (Stearner, 1946; Niu and Twitty, 1950). A more recent study showed that, in *A. t. tigrinum*, the adult pigment pattern arises largely independently of both the distribution of pigment cells comprising the early larval pattern and the larval lateral line sensory system (Parichy, 1998). Thus, larval and adult pigment patterns, at least in this species, appear to be relatively uncoupled from one another. Despite this paucity of information, the diversity of adult pigment patterns, particularly within the context of life history variation across species, suggests a fruitful area of inquiry.

An intriguing question related to the role of heterochrony in adult pigment pattern evolution is whether the mechanisms of adult pattern development are

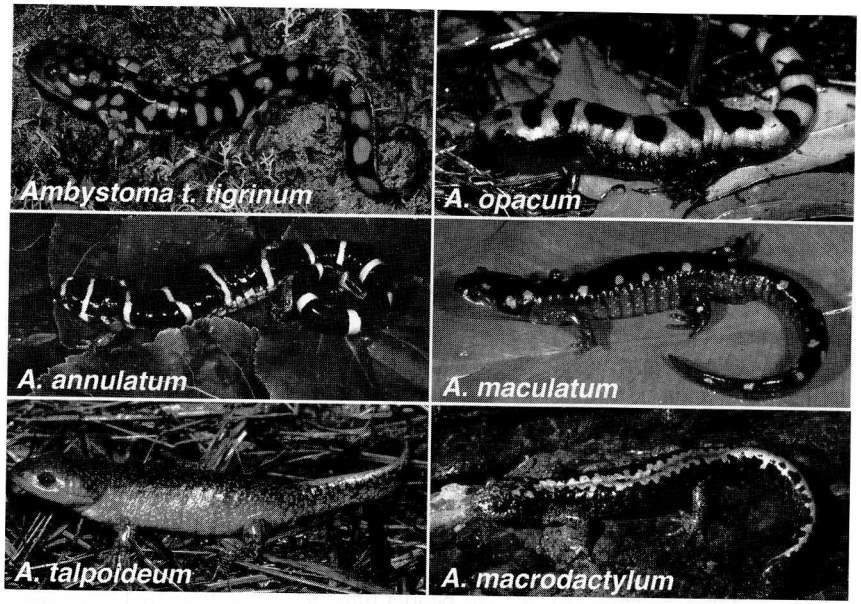

Figure 7.11. Adult pigment pattern diversity in salamanders. Shown are several species of adult *Ambystoma* that exhibit various spots, stripes, and mottled patterns.

integrated with the mechanisms controlling the metamorphosis of other characters (e.g., resorption of the gills and fin fold, as well as changes in the craniofacial skeleton and integument). More specifically, metamorphosis in these animals depends on tissue-level responses to circulating thyroid hormone (Gilbert et al., 1996; Shaffer and Voss, 1996; Brown, 1997). Although thyroid hormone can influence pigment cell behaviors (Reedy et al., 1998b), an in vivo role in pigment pattern metamorphosis remains unresolved. In support of a role for thyroid hormone in determining whether an adult pattern develops, when *A. mexicanum* are induced to metamorphose by exogenously administering thyroid hormone (or pituitary extract), these salamanders develop a pigment pattern of bright spots on a dark background, somewhat similar to that seen in other ambystomatids (Woronzowa, 1932; Brown, 1997). Whether this pigment pattern represents a true atavism remains unclear. In contrast, an independence of adult pigment pattern development from other aspects of somatic metamorphosis is suggested by several facultatively or obligately paedomorphic taxa within the *A. tigrinum* complex (e.g., *A. t. diaboli*, *A. ordinarium*) in which an apparently adult pattern is expressed despite the retention of an otherwise larval-like morphology (S. R. Voss, personal communication; Parichy, unpublished data). Different thresholds of sensitivity to thyroid hormone across tissue types might account for this apparent heterochrony in pigment pattern development, but this idea has yet to be tested.

IMPLICATIONS

Utility of a Heterochronic Framework in Understanding the Evolution of Pigment Pattern Development

The studies reviewed above suggest that evolutionary changes in pattern-forming mechanisms may be understandable, at least in part, as changes in the rate or timing of developmental events and cellular behaviors. Nevertheless, in no instance is there conclusive evidence that heterochrony provides the definitive change resulting in a phylogenetic transformation. In the vertical barring example, a heterochrony in cell migration associated with the presence or absence of this pattern element is suspected but not yet proven. In the elaboration of developmental mechanisms in *T. torosa*, at least two heterochronies are identifiable in melanophore behavior, but their causal significance for stripe development remains unclear. Thus, despite many years of study, we still do not have a definitive answer as to whether heterochronies at the cellular level are decisively involved in salamander pigment pattern evolution.

Does a heterochronic framework then provide a useful device for understanding the evolution of patterning mechanisms? Given the many ways in which cell behaviors and molecular mechanisms can change, it is unlikely that broad patterns of heterochrony will be identified as causally related to pigment pattern evolution. Even where heterochronies are suspected, they are not universally paedomorphic or peramorphic in nature. Indeed, the literature reviewed above made almost no use of the many and varied terms that have been introduced to describe different sorts of heterochronic change (Alberch et al., 1979; McKinney and McNamara, 1991; Reilly et al., 1997). These studies did not explicitly search for heterochronies either: where they are suspected, they were stumbled on in more general investigations of developmental and cellular mechanisms. These observations suggest that a heterochonic framework is not essential for understand evolutionary changes in developmental mechanisms. In some instances, it can be positively misleading (e.g., the early evacuation of *T. torosa* melanophores from the middle of the flank does not indicate an inability of the lateral lines to influence their behavior). Clearly, a heterochronic framework can be a useful heuristic device as it ensures consideration of various possibilities for rate and timing changes that otherwise might be overlooked. Nevertheless, investigations directed solely toward testing for heterochronies may provide relatively little insight on their own: heterochronic patterns are easy to identify, but establishing their significance for trait development or evolution requires a rigorous experimental approach to understanding the mechanisms themselves.

Future Directions for Studying Pigment Pattern Evolution and Development

Pigment patterns offer considerable opportunities to identify the mechanistic bases for the evolution of form in vertebrates. The studies reviewed above have

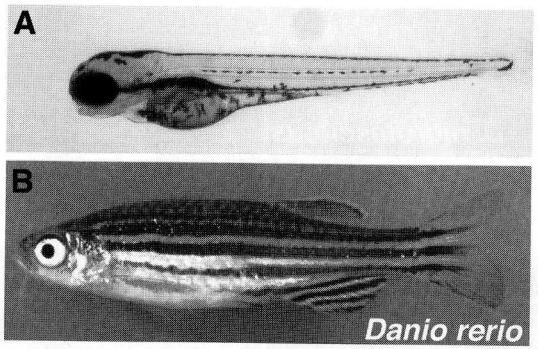

Figure 7.12. The zebrafish, *D. rerio*, undergoes a pigment pattern metamorphosis. An embryonic/early larval pigment pattern (top, 72 h, 3 mm) is transformed into an adult pigment pattern (bottom, >28 days, 4 cm).

focused principally on events at the cellular level, but an area for future studies is the identification of the molecular mechanisms that control these cellular behaviors. Although salamanders and other amphibians pose some difficulties for molecular and genetic analyses (but see Parichy et al., 1999b; Voss et al., 2001), investigations in teleosts may help to shed light on these matters. Specifically, the zebrafish, *D. rerio*, has become an increasingly important model organism for developmental and genetic studies on a variety of vertebrate traits, including pigment patterns. Similar to salamanders, pigment patterns in *D. rerio* comprise principally three classes of neural crest-derived pigment cell, and these fish undergo a pigment pattern metamorphosis in which a relatively simple early larval pattern is transformed into a more complex adult pigment pattern (Fig. 7.12; Haffter et al., 1996; Parichy et al., 2000a, 2000b). Also, as in salamanders, fishes in the genus *Danio* exhibit considerable adult pigment pattern variation (Fig. 7.13; Fang, 1997a, 1997b, 1998; McClure, 1999). Although the existing hypothesis of phylogenetic relationships (Meyer et al., 1995) is taxonomically depauperate, more comprehensive phylogenies will ultimately allow rigorous inferences regarding the frequency and polarity of changes in developmental mechanisms. Moreover, because one of these danios is a developmental genetic model organism, a host of resources are available that can be applied to understanding pigment pattern evolution within this genus. For example, a large number of mutants affecting pigment pattern development have been isolated in *D. rerio*, and several of these have pigment patterns that resemble the naturally occurring patterns of other danios, making them candidate genes for pigment pattern diversification (Fig. 7.13). Indeed, recent genetic analyses identify the *panther/fms* gene as a good candidate for contributing to an evolutionary loss of stripes in *D. albolineatus* (Parichy et al., 2000b; Parichy and Johnson, 2001). Some of these changes may also be interpretable within a heterochronic framework. For example, *endothelin receptor b1/rose* mutant *D. rerio* lack a

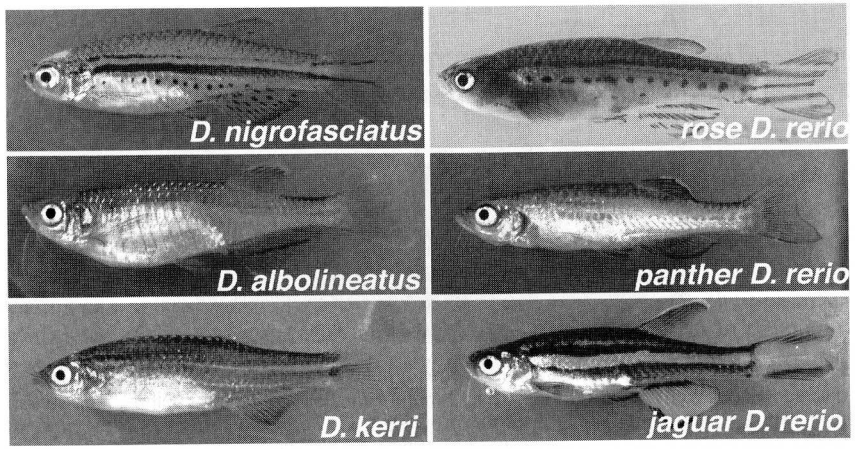

Figure 7.13. 1Fishes in the genus *Danio* offer an opportunity to investigate the genetic bases for pigment pattern evolution. Top left: zebrafish *D. rerio* has a relatively simple early larval pigment pattern comprised principally of melanophores and xanthophores. Top right: wild-type adult *D. rerio* exhibits a series of alternating light and dark horizontal stripes. Bottom left: adult pigment patterns of danios differ from *D. rerio* (although larval patterns are indistinguishable). Bottom right: pigment pattern mutants of *D. rerio* resemble other danios and identify candidate pigment pattern diversification genes.

late-developing subpopulation of adult melanophores (Johnson et al., 1995; Parichy et al., 2000a, 2000b). Although *endothelin receptor b1* probably is not responsible for the superficially similar phenotype in *D. nigrofasciatus* (Parichy and Johnson, 2001; Parichy, unpublished data), a similar cellular change may have occurred (essentially a local paedomorphosis, limited to the pigment pattern). Analyses of these mechanisms in danios should ultimately provide insights that can be applied to understanding pigment pattern development and evolution in salamanders, as well as other nonmodel organisms. Some of these insights may involve heterochrony.

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