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# Multidimensional Analysis of an Evolving Lineage

DAVID B. WAKE AND ALLAN LARSON

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To identify the forces directing organismal evolution, a general analytical system is developed to synthesize structuralist and Darwinian traditions in an explicitly historical framework. Morphological features of lungless salamanders are examined to identify hierarchical systems of developmental and functional constraint on evolution and their interactions with processes at the genic and populational levels. Characteristic patterns of change occur repeatedly in the evolutionary history of this group.

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TWO CONCEPTUAL AND EMPIRICAL TRADITIONS HAVE GUIDED investigations of the origin and diversification of biological form: structuralism (1), which derives from the rational morphology of the late 18th and early 19th centuries (2), and Darwinism (3–8). Structuralist investigations emphasize the importance of “epigenetic” rules of development that are emergent at the organismal level, whereas Darwinism emphasizes the properties of hereditary units and their sorting within populations. These approaches generate essential and complementary descriptions of morphological change in an evolving lineage (9), although a long history of opposition between them has prevented their unification (1, 10). We advocate a synthesis of these perspectives using a methodology that features historical analysis of evolutionary patterns and processes occurring at multiple levels of biological complexity.

## Structuralism and Neo-Darwinism

The central task of structuralism is to develop a theory of biological organization and to predict from the theory a finite set of possible forms that organismal systems can produce (1, 11, 12). Organismal ontogeny is viewed as a closed set of epigenetic transformations representing a time-independent property of organismal structure. The limitations on form imposed by this transformational system are called “developmental constraints” (13). Such constraints have been recognized empirically for many years. In comparative studies of natural variation, Vavilov observed that variants found in related species are not produced randomly but are limited to a discrete and categorizable set of alternative forms that he termed “homologous series” (14, 15). Early work on the homeotic transformations of *Drosophila melanogaster* also demonstrated that genetic mutation may elicit a discrete, nonrandom set of alternative

developmental patterns and that the same patterns may be elicited by environmental perturbation during development [phenocopies (16)]. Further study has revealed in detail the complex network of epigenetic transformations connecting the alternative homeotic states in *D. melanogaster* (17). The fact that biological systems maintain specific interactions among their parts when responding to internal and external perturbations is recognized by structuralists as the property of wholeness. The observation that possible morphologies form a closed set is termed self-regulation. “Wholeness,” “transformation,” and “self-regulation” are the defining properties of a biological “structure” (18).

Darwinian investigations of the evolution of form contrast with the main tenets of structuralism. Darwinism views form as being contingent on many unique events occurring sequentially through time, rather than on time-independent properties of organismal structure (3, 19, 20). Rather than emphasizing structuralist wholeness, the organism often is “atomized” to identify or quantify the genic-level basis for variation in particular features, and to measure the population-level forces, particularly selection, that govern evolution (4, 6, 7). The structuralist concept of epigenetic transformation is negated by presenting the nuclear genome as the “central directing agency” (1) of development; causal forces underlying the evolution of form are localized to the genic and populational levels, rather than to the structure of self-regulatory epigenetic systems. Organismal features generated by the time-dependent process of natural selection comprise the neo-Darwinian concept of adaptation (5, 21).

Our study of morphological evolution in the lungless salamanders (family Plethodontidae) assigns primacy to neither structuralist nor neo-Darwinian perspectives but emphasizes a synthesis of their opposing elements by incorporating hierarchy theory and historical analysis. Neo-Darwinian atomism and structuralist holism are combined by investigating the minimal organismal-level structures that demonstrate self-regulation, and their historical and hierarchical composition to form more inclusive structures (18). The variation observed at different levels of organismal complexity is superimposed on an independently generated phylogenetic topology to identify evolutionary transformations for different features and their historical superposition. We examine structures representing three different levels of organismal complexity: the premaxilla, the autopodium, and the feeding system. The systems of developmental transformation that generate these structures produce a discrete set of possible evolutionary transformations that are observed repeatedly during phylogenesis, as the structuralist position predicts (22, 23). Evolutionary transformations of these structures often are initiated at the level of organismal life history. Morphological evolutionary patterns are interpreted within a population genetic framework; comparative molecular data are used to examine the structure of natural populations and the implications of population structure for the organization of genetic variation that underlies both evolutionary stasis and evolutionary change.

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D. B. Wake is a professor in the Department of Zoology and director of the Museum of Vertebrate Zoology, University of California, Berkeley, CA 94720. A. Larson is an assistant professor in the Department of Biology, Washington University, St. Louis, MO 63130.

## Structural Transformations in the Evolution of Lungless Salamanders

The family Plethodontidae, a monophyletic group of salamanders, exhibits substantial diversity in morphology, behavior, and ecology (24). Its ancestor had a biphasic life cycle of aquatic larvae and metamorphosed semiterrestrial adults. This life cycle persists, but two additional, uniphasic life cycles have evolved, one in which sexual maturity is attained by aquatic forms that retain larval morphology, and another in which the larval phase is eliminated and replaced by completely terrestrial, directly developing embryos. The latter forms include species adapted to conditions ranging from subterranean burrows to arboreal habitats (25, 26). In addition to global developmental changes that affect the entire organism (loss of larvae), these adaptive transitions have featured localized changes in locomotor (autopodia) and trophic (tongue and jaws) structures.

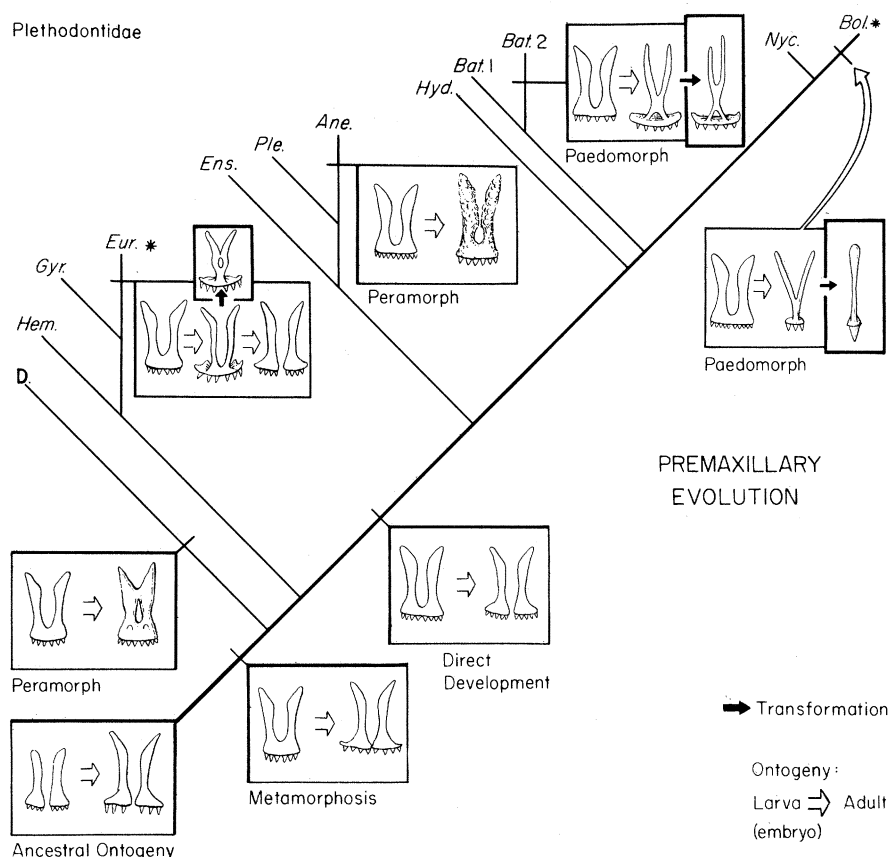
**The premaxilla.** Evolutionary changes in the plethodontid premaxilla are identified from the phylogenetic topology shown in Fig. 1. The premaxilla of salamanders is primitively bipartite, consisting of two laterally paired bones in larvae and adults. Plethodontid embryos and larvae demonstrate an evolutionarily derived, unipartite premaxilla, which serves as an adaptation for larval feeding by compensating the relatively late development of the maxillary bones in plethodontids [caenogenesis (24)]. The unipartite premaxilla is present in embryos even in plethodontid lineages that have replaced the larval stage with direct development, thereby eliminating the adaptive context of this feature. The ancestral plethodontid ontogeny restores the more primitive, bipartite condition at metamorphosis, as observed in some extant metamorphosing genera (for example, *Gyrinophilus*). Two separate evolutionary derivations feature unipartite premaxillae in adults. In one case, the premaxillary metamorphosis characteristic of the ancestral plethodontid ontogeny is lost, causing retention of the embryonic unipartite state

throughout life. In the other case, the unipartite premaxilla is inferred to have arisen by fusion of paired premaxillary bones.

The derivation of unipartite premaxillae in adults is often a manifestation of global changes in organismal development. "Paedomorphosis" describes the evolutionary derivation of new adult morphologies by retaining into the adult stage features found only in earlier developmental stages of ancestors (27). Evolutionary processes that produce paedomorphic transformations affecting the entire organism include neoteny and progenesis (27). Paedomorphic retention of the embryonic unipartite premaxilla throughout life has evolved several times as a result of these processes. Examples include most of the tropical genera, which demonstrate retention of the unipartite premaxilla throughout life (28) in the context of numerous other paedomorphic features (24, 29). Several intermediate stages in the paedomorphic evolution of the premaxilla are observed in *Batrachoseps*. All species of this genus demonstrate numerous paedomorphic features (24, 30, 31). The ancestral premaxillary metamorphosis is retained in *B. campi*, one of the least paedomorphic *Batrachoseps*. In *B. wrighti*, the unipartite premaxilla retained from the embryonic stage divides very late in life, at or beyond the age of sexual maturation. Further ontogenetic delay has removed premaxillary metamorphosis completely from the ontogenies of the remaining species of *Batrachoseps*, which retain unipartite premaxillae throughout life (24, 30, 31).

"Peramorphosis" describes the evolutionary derivation of new adult morphologies produced by extending development beyond the point at which the ancestral ontogeny terminates. Evolutionary processes that produce this result include acceleration and hypermorphosis (27). These processes have produced fusion of the paired premaxillary bones in adults by increasing the extent of bone deposition in the whole organism. Examples include *Aneides*, which has extreme skeletal development relative to other plethodontids (24, 26, 32), and the subfamily Desmognathinae, which has greatly

**Fig. 1.** The evolution of the premaxilla of plethodontid salamanders displayed on a simplified cladogram (47). The ancestral ontogeny is indicated at the base, with a bipartite premaxilla persisting throughout ontogeny (hollow arrow). Synapomorphies are indicated in boxes. Evolutionary transformations (solid arrow) occur within some of the synapomorphous states. The unipartite premaxilla typically divides during metamorphosis or early ontogeny, but evolutionary changes causing unipartite premaxillae to appear in adults have occurred at least five times, two by peramorphosis and two by paedomorphosis. In Eur.\*, paedomorphosis seems likely because the genus *Typhlotriton* undergoes premaxillary separation near the end of life (24). Abbreviations: D, subfamily Desmognathinae; Hem., *Hemidactylium*; Gyr., *Gyrinophilus*; Eur.\*, all members of the tribe Hemidactyliini except the two preceding genera; Ens., *Ensatina*; Ple., *Plethodon*; Ane., *Aneides*; Hyd., *Hydromantes*; Bat. 1, *Batrachoseps campi* and *B. wrighti*; Bat. 2, all remaining species of *Batrachoseps*; Nyc., *Nyctanolis*; Bol.\*, all members of the supergenus *Bolitoglossa* except *Nyctanolis*.



strengthened skeletons and solidified skulls (24). Premaxillary fusion in both groups is a component of skull strengthening, and is a secondary derivation.

Evolutionary transformations observed in plethodontids suggest that the bipartite form of the premaxilla constitutes an ancestral epigenetic default state that can be produced whenever the system that generates the derived, unipartite condition is perturbed. This is evidenced by the presence of premaxillary metamorphosis, without apparent adaptive significance, in such plethodontid ontogenies as *Plethodon* and *Ensatina* (24), which lack a global organismal metamorphosis. The bipartite condition also is restored by evolutionary reversal in some lineages where the derived, unipartite condition has been fixed [for example, *Typhlotriton*, Fig. 1 (24)]. This latter observation demonstrates that the developmental system retains the ability to produce the bipartite premaxilla following genetic fixation of the unipartite condition in a lineage.

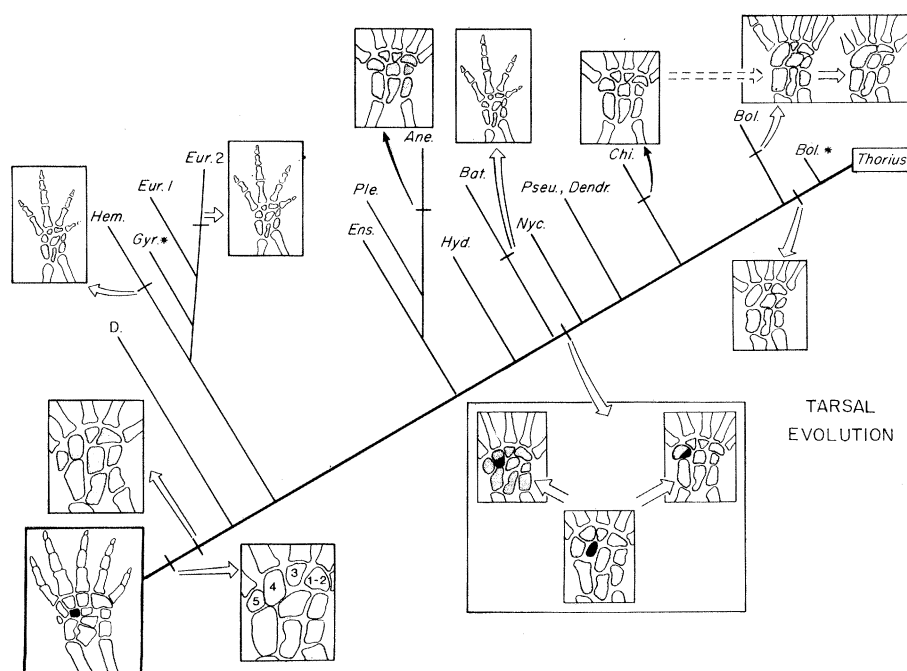
The premaxilla therefore constitutes a very localized structure, whose epigenetic transformational properties constrain its morphology to two basic alternative forms, both of which may appear during the course of an organismal ontogeny. Developmental and evolutionary transformations of the premaxilla constitute a series of switches between these two forms. The evolutionary forces responsible for the observed transitions may act directly on premaxillary morphology, as observed in the case of larval feeding; alternatively, they may be imposed indirectly through the dynamics of more complex structures (the skull, the entire organism) of which the premaxilla is a part, as observed in the paedomorphic and peramorphic transformations.

**The autopodium.** The autopodium is a composite of two related structures, the mesopodial elements (wrist and ankle) and the digits. Analyses of evolutionary transitions (Fig. 2) and of naturally occurring variants of the autopodium demonstrate a finite set of transformations that recur throughout plethodontid evolution. The potentially attainable forms are constrained by chondrogenetic interactions that occur during development, and by other developmental processes (33). Despite these constraints, evolutionary transformations in autopodial morphology often appear to be adaptive.

Phylogenetic analysis shows that mesopodial diversity can be explained largely as a series of fusion and fission events involving

three cartilaginous elements, distal tarsals 3, 4, and 5 (d3, d4, and d5). The ancestral mesopodial arrangement for salamanders featured a total of 12 elements, with elements d3, d4, and d5 being distinct and separate (34). This inferred ancestral condition is observed in plethodontids only as an atavistic variant in *Pseudoeurycea anitae* (35). The atavistic element, m3 (black, Fig. 2), appears to be incorporated into d4 in the state inferred to be ancestral for plethodontids. It becomes part of d5 in two separate lineages (those ancestral to *Aneides* and *Chiropterotriton*), a rearrangement that is important for the evolution of grasping ability and arboreality (26). A mesopodial cartilage interpreted as a fusion of m3 and d5 appears also as a rare variant in *Bolitoglossa* (36), in which most animals have a composite m3-d4-d5. The latter arrangement evolved independently in the ancestor of several other related genera (29). An additional composite element (m3-d3-d4-d5) occurs in some species of *Bolitoglossa* (36, 37). Mesopodial rearrangements similar to those that distinguish many plethodontid taxa are observed as natural variants within certain populations (36, 38); these alternative states seem to be best explained as patterns of differential fusion and fission. The alternative states of the mesopodial cartilages constitute quantum outcomes of an epigenetic transformational system, rather than the products of selective discrimination of minor variants in the individual elements. The standard patterns and rules of mesopodial transformation are violated in two genera, *Oedipina* and *Thorius*, which are exceptional in having very small limb buds with few cells and, in some instances, ossified mesopodials (24, 39). We hypothesize that epigenetic rules may be suspended, in effect, when the number of cells at a given size and stage of morphogenesis is reduced below some threshold level.

Variation in the number of digits on the hind limb appears also to demonstrate production of discrete alternative phenotypes that are intrinsic to the generative system. A reduction in the number of digits from five to four has been fixed in three different plethodontid lineages (Fig. 2). Digital reduction similar to that observed in plethodontid evolution can be induced experimentally; soaking the limb bud in the mitotic inhibitor colchicine during a critical stage of pattern specification achieves this result by decreasing the number of cells and the size of the limb bud (33, 40, 41). The four-digit state has been observed as a rare variant in otherwise pentadactyl species



**Fig. 2.** The evolution of the tarsus in plethodontid salamanders. Dorsal views of the left foot are shown. Bone is outlined and cartilage is stippled. The first digit is to the right. The ancestral tarsus is shown in the box at the base of the cladogram. The distal row of tarsals (numbered 1 through 5) supports the five metatarsals. The blackened element is Schmalhausen's m3 (34). Derived tarsal arrangements are indicated as synapomorphies on the cladogram. The first is the joining of m3 with d4 to form a large d4. The second is the conversion of the bony tarsals to cartilage. The box containing three arrangements shows the postulated pattern of evolution by addition of the ancient m3 to one of two distal tarsals to form either a large d4 (plesiomorphic state in plethodontids) or a large d5 [a synapomorphy for *Aneides* and for *Chiropterotriton* (26, 29), and possibly in the ancestor of *Bolitoglossa* (36)]. The fifth toe has been lost in three different lineages. The genus *Thorius* is highly variable (39). Abbreviations as in Fig. 1, except Gyr.\*, all members of the tribe Hemidactyliini except *Eurycea* and *Hemidactylum*; Eur. 1, all species of *Eurycea* except *E. quadridigitata*; Eur. 2, *E. quadridigitata*; Pseu., *Pseudoeurycea*; Dendr., *Dendrotriton*; Chi., *Chiropterotriton*; Bol., *Bolitoglossa*; Bol.\*, *Bradytriton*, *Lincolnitriton*, *Nototriton*, *Oedipina*, and *Parvimolge*.

(40), and the pentadactyl condition appears as a rare variant in some species that normally have four digits (42); the potential to produce either state persists even when one of the alternatives appears to become fixed.

Another repeated feature of autopodial evolution observed in *Bolitoglossa* and *Chiropterotriton* is increased interdigital webbing which produces a pad (25). This trend is often accompanied by reduction in the length and number of phalanges. Pad formation may be induced strictly at a local level, where it functions as an adaptation for climbing by increasing the suction and adhesion produced at the foot surface; alternatively, it may be part of a paedomorphic transformation affecting the entire organism (43), in which case the pad looks like a suction-producing mechanism but lacks that function (44). Such transformations occurred three to five times within the beta lineage of *Bolitoglossa* alone (45). There are some common features in the transformations, but there are also some major differences. For example, although phalangeal reduction recurs, different phalanges are affected in the separate transformations and the degree of reduction follows a continuous scale rather than producing discrete morphs. Thus, structural transformations provide a bias to the direction of change, but the degree of change achieved and the exact mechanisms demonstrate quantitative variation.

**Feeding systems.** Tongues of all metamorphosed plethodontids are protruded to capture prey, generally terrestrial arthropods. Lunglessness is the critical initial factor in the evolution of tongue specialization. The skeleton of the tongue is part of a force pump that fills the lungs in lunged salamanders; loss of lungs in ancestral plethodontids freed the skeleton and muscles of this functional constraint (46). Three kinds of tongues have evolved: attached protrusible tongues (thought to be ancestral for plethodontids), attached projectile tongues (capable of long-distance projection but attached anteriorly by genioglossal muscles), and freely projectile tongues (no anterior attachment) (Fig. 3). The two kinds of projectile tongues each have evolved three times with extensive parallelism and convergence (47).

The tongue skeleton is formed by articulated cartilaginous rods (an unpaired median basibranchial, and paired lateral first and second ceratobranchials and epibranchials) that are folded during projection. Mechanical efficiency is the product of many local factors associated with their function and development (48). The muscles controlling protraction and retraction fire nearly simultaneously, and both the patterns of muscle firing and the spatiotemporal arrangement of the motor nuclei of the brainstem and cervical spinal cord have been identified (49, 50). Dimensions of the muscles and cartilages evolve in conjunction with degree of anterior freedom of the tongue. Free tongues have the longest epibranchials and the shortest ceratobranchials; basibranchials are nearly the same relative length in all species (46).

Evolution of attached projectile tongues was accompanied by the elongation of genioglossal muscles (46). These muscles have elongated independently in three genera by a narrowing of the site of origin of the muscle accompanied by posterior migration along the mandible. The extreme limit at the posterior end of the mandible is reached in *Batrachoseps*. This constitutes an evolutionary stopping point—the limit imposed by the mechanical structure of an attached tongue.

Freely projectile tongues lack genioglossal muscles and represent extreme states of specialization. In theory there are two options for folding the skeleton during projection, and both are encountered (46). Option 1 is based on force transmission through the first ceratobranchial, and the alternative (option 2) through the second ceratobranchial. Both function effectively, but there are different stopping points (46, 51, 52). The skeleton in option 2 is much

lighter and more versatile; the epibranchials are greatly elongated and consequently can be projected further.

The particular option is determined by the history of the lineage (51). Plethodontids with freely projectile tongues and aquatic larvae exercised option 1. Strongly cephalized development results in an early size bias that causes the first ceratobranchial to be larger than the second in larvae. This arrangement is functionally significant, both in gill ventilation and in feeding. Metamorphosis in salamanders is not sufficiently profound to reverse this situation. Thus in the *Eurycea* group (Fig. 3) one expects, and finds, freely projectile tongues based on option 1.

In the tribes Plethodontini and Bolitoglossini (53) the larval stage has been lost and early development takes place in the egg. The Plethodontini (modes II and III in Fig. 3) did not evolve freely projectile tongues. Such tongues evolved twice in the Bolitoglossini (modes VI and VII), in the context of relatively profound ontogenetic repatterning. Three epibranchials are present in embryos of the Plethodontini; only one is found in the Bolitoglossini (24, 54). The first ceratobranchial of bolitoglossines develops late and is never very large, but the early developing second ceratobranchial is relatively stout. Lunglessness and direct development are necessary preconditions for the evolution of option 2 tongues, and a larval phase constitutes a developmental constraint that imposes option 1 on the evolution of freely projectile tongues by plethodontids that lack the derived life history trait.

Although both options appeared in different groups of plethodontids as freely projectile tongues evolved, limits to specialization and evolutionary potential differ. The limit of specialization under option 2 occurs in *Thorius*, which contains miniaturized species with many morphological specializations (24, 55, 56). Here reduction in the size of the feeding apparatus permits the attainment of a condition not possible under option 1. The cartilages of the tongue skeleton are so small that only one or two cartilage cells are found in a given cross section. The tiny first ceratobranchial has partially disappeared, to be replaced by a perichondrial remnant that acts as a ligament (46). The hyobranchial apparatus disarticulates during projection and does not fold but collapses into a coplanar structure that is the most biomechanically efficient arrangement attainable; this could not occur with option 1 tongues, for the element that detaches in option 2 is the main force-transmitting member in the option 1 system and imposes a functional constraint.

The tongue is more complex than either the premaxillary bone or the autopodium, and once again an understanding of its evolution is best attained by combining structuralist and historical approaches.

## Genetic Variation and Population Structure

Morphological evolutionary patterns are placed within a population genetic perspective by analyzing patterns of protein variation in natural populations. Nearly all plethodontid species demonstrate high levels of geographical fragmentation, even where morphological features are uniform or continuous (57, 58); conspecific populations are often so distinct at the protein level that cessation of genetic exchange among them must have occurred millions of years ago. Phenotypic uniformity reflects the resilience of stabilized developmental pathways that are retained from a distant common ancestor (59); despite the considerable diversity of plethodontid morphologies, morphological stasis predominates, and periods of morphological change account for a relatively minor portion of plethodontid evolutionary history (26, 45, 57).

Speciation is distinct and often disjunct from morphological evolution. The distribution of protein variants shows that genetically closed populations occur within the boundaries of geographically

continuous and morphologically homogeneous sets of populations (57). Furthermore, genetically homogeneous populations may be polymorphic for morphological traits whose alternative forms resemble different morphological states that distinguish higher taxa (35, 39, 55, 60). We view speciation as a special case of Pattee's principle of statistical closure (61), whereby the collective reproductive dynamics of a population generate constraints on mating patterns. Because the units thus formed do not necessarily differ in ecological or functional morphological ways from sister populations, the primary evolutionary effect of reproductive closure is to limit the potential exchange of genes among populations. The origin of reproductive closure is a time-dependent, historical process occasionally observed in intermediate stages of completion (57, 62); once completed, however, reproductive closure represents an irreversible, time-independent system of constraints.

Although plethodontids demonstrate a punctuated pattern of morphological change through time, the causal process cannot be viewed as punctuated equilibrium, which postulates that morphological change is concentrated in the same historical events that produce reproductively distinct species (63). We seek the causal basis of morphological evolutionary stasis in the interactive genic, organismal, and population-level forces characterizing Schmalhausen's theory of stabilizing natural selection (64), Wright's theory of shifting balance (65) and the emergent, organizational properties of the organismal level (59). Epigenetic transformational systems, such as those illustrated in the previous section, constrain development and morphology to a limited set of alternative pathways whose penetrance and expressivity in a particular population presumably are subject to genic-level regulation.

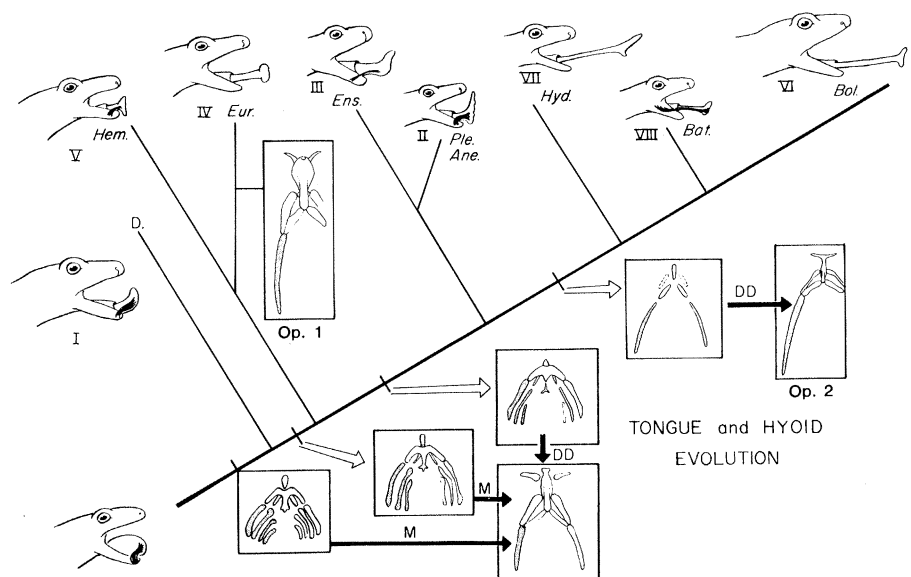
Natural selection may generate complex genetic mechanisms whose function is to stabilize the penetrance and expression of a particular epigenetic pathway in a particular lineage (66, 67). The hierarchical nature of organismal development predisposes a hierarchically organized genetic structure (68); genetic variants influencing threshold conditions at early developmental bifurcations would produce relatively large phenotypic effects, and genes acting later would produce more restricted modifications of phenotype that are contingent upon the directions taken earlier in development. A predicted consequence of this hypothesized relation between genic

and epigenetic systems is that the phenotypic response to genic introgression between populations will be nonlinear if the populations differ in genetic architecture. Consistent with this prediction, introgression at the genic and morphological levels is highly incongruent for hybridizing populations of *Bolitoglossa* (69), although other plethodontid hybrid zones demonstrate more linear relation between genic and phenotypic introgression (70). Destabilization of normal development and of resistance to disease is observed extensively in hybrids of other species (71). Additional studies with natural populations of plethodontids are now feasible because comparative biochemical methods permit localization of genetic contacts among populations, even where organismal morphology has not diverged (72).

Evolutionary alteration of a hierarchically organized genetic system is unlikely to occur in populations that are either large and panmictic or very small and isolated. The features most conducive to genetic restructuring include spatial subdivision of a large population into small, partially isolated demes (65), and temporal fluctuations that induce periodic inbreeding while maintaining a large variance effective population size (73, 74). The history of plethodontid populations seems to feature gradual expansion and contraction of densely populated demes, rather than the structures conducive to disruption of established genetic systems (57). This may explain why plethodontids (and salamanders in general) evolve slowly at the organismal morphological level relative to many birds and mammals whose populations appear to be structured in ways that promote frequent disruption of the genetic systems underlying phenotypic stabilization (75).

The "adaptive zone" identifies boundaries on organismal-environmental interactions that characterize evolving lineages (76). Several distinct adaptive zones have been established during plethodontid evolution (24, 57). The adaptive zone inferred to be ancestral for plethodontids featured habitation of mountain streams by both larval and metamorphosed adult salamanders. The replacement of lungs with cutaneous respiration is believed to have been a key feature permitting utilization of this environment; reduction and loss of lungs are observed in other lineages of salamanders that use stream habitats (24, 77). The directly developing plethodontids (*Bolitoglossini* and *Plethodontini*) never use stream habitats and

**Fig. 3.** Evolution of the feeding system of plethodontid salamanders. Roman numerals indicate the eight modes of tongue projection (46). In the ancestral stock and in modes I and II (attached protrusible tongues), a short, stout genioglossus muscle attaches the tongue to the mandible. In modes III, V, and VIII (attached projectile tongues) the muscle is moderately to greatly elongated and is slenderer and more stretchable. The muscle is absent in freely projectile tongues (modes IV, VI, and VII). A distinctive hyobranchial apparatus in larvae, with four cartilaginous epibranchials (stippled), is a synapomorphy for the family and is indicated in the box to the lower left. A synapomorphy for the subfamily Plethodontinae is the reduction to three larval epibranchials. Direct development is a synapomorphy for the tribes Plethodontini and *Bolitoglossini*. An apparent synapomorphy for the family is the replacement of the larval epibranchials by a new adult epibranchial (M, by metamorphosis, or DD, through direct development) (54). An extreme situation is found in the tribe *Bolitoglossini* (boxes to upper right), in which only the replacement epibranchial is present and the larval epibranchials do not form. The medial element is the basibranchial, and paired first and second ceratobranchials extend laterally from it and articulate distally with the epibranchial. Option 1 and option 2 pathways result in freely projectile tongues having different biomechanical properties and different evolutionary stopping points (46, 51). Abbreviations as in Fig. 1, except Eur., all members of the tribe Hemidactyliini except *Hemidactylium*; Bol., all members of the supergenus *Bolitoglossa*.



may be viewed as occupying a novel, terrestrial adaptive zone. Subsets of both terrestrial lineages evolved adaptations for arboreality and subsets of the bolitoglossines also have evolved adaptations for fossorial existence. In each case, these adaptive transitions featured the fixation of novel features ["key innovations" (26)] that altered the boundaries on organismal-environmental interaction and stimulated subsequent speciation and morphological diversification. Key innovative features simultaneously place constraints on the environments that an organism may occupy, and confer the opportunity for morphological and adaptive diversity. The previously discussed rearrangement of mesopodial cartilages in *Aneides* and *Chiropterotriton*, and the altered structure of the digits and interdigital webbing in *Bolitoglossa*, constitute key innovations that permitted the evolution of arboreality.

The concept of the adaptive zone is most meaningful in the context of a synthesis of structuralist and population genetic perspectives. Inherited, genetically stabilized structures can confer properties of unity and constraint on an evolving lineage that transcend the species level of organization; these properties may be manifested as characteristic rates or patterns of speciation and extinction (78). Without the populationist perspective, the notion of adaptive zone lacks meaning because it contains as an essential element the generation and fixation of novel forms by selective processes. The structuralist perspective introduces the notion of inherent bounds or constraints (on form and consequently on organismal-environmental interactions) without which there would be no unity to supraspecific taxa; novel features can be studied as historically generated composites of structures having empirically identifiable properties of wholeness and self-regulation, rather than as indefinitely continuous and therefore uncharacterizable forms.

## Conclusion

Morphological evolution in the plethodontid salamanders demonstrates characteristic historical transformations that recur throughout phylogeny. These transformations are used in different combinations and ecological contexts to generate morphological and adaptive diversity. The epigenetic rules of development underlying these transformations are identified from structuralist analysis, whereas the fixation of different structures and their combination to construct complex novelties are investigated as Darwinian evolutionary processes. We suggest that multidimensional analyses combining the elements of structuralism and Darwinism be used to test the generality of these evolutionary patterns and processes in other groups of organisms.

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## Research Articles

# A Portable Signal Causing Faithful DNA Methylation de Novo in *Neurospora crassa*

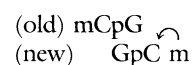
ERIC U. SELKER, BRYAN C. JENSEN, GLADYS A. RICHARDSON

Methylation of cytosine residues in eukaryotic DNA is common, but poorly understood. Typically several percent of the cytosines are methylated; however, it is unclear what governs which sequences eventually become modified. *Neurospora crassa* DNA containing the “zeta-eta” ( $\zeta$ - $\eta$ ) region, which is a region of unusually heavy methylation, was tested for its ability to direct DNA methylation de novo. DNA stripped of its methylation by propagation in *Escherichia coli* was reintroduced into *Neurospora crassa* by transformation. The  $\zeta$ - $\eta$  region reproducibly became “properly” methylated whether inserted at its native chromosomal position or at ectopic sites. Adjacent *Neurospora* and bacterial sequences in the transforming DNA rarely became methylated. A model is presented that accounts for position-independent faithful methylation as observed in the  $\zeta$ - $\eta$  region, as well as position-dependent methylation, as occasionally observed, especially with sequences not native to *Neurospora*.

IN MANY AND POSSIBLY ALL ORGANISMS, DNA IS MODIFIED after synthesis by methylation of cytosine or adenine residues. Methylation alters physical properties of DNA, affects DNA-protein interactions, and has important biological consequences. In prokaryotes, DNA methylation is central to restriction-modification systems (1), provides for discrimination between new and old strands for DNA repair (2), and can be involved in gene regulation (3). In eukaryotes, a number of observations suggest connections between methylation and gene expression, but no clear causative relationships have been determined (4). The primary role of DNA methylation in eukaryotes remains to be established.

A serious gap in our understanding of DNA methylation in eukaryotes concerns what determines which sequences are destined to become methylated. In contrast to the situation in prokaryotes, eukaryotic DNA methylation does not appear limited to particular oligonucleotide sequences recognized by corresponding methyltransferases. Nevertheless, 5-methylcytosines are not randomly dis-

tributed in the eukaryotic genome. In animal DNA, for example, most of the cytosines located immediately preceding guanines (5' CpG) are methylated, and methylated cytosines at other positions are rare. This is thought to reflect a process that acts to perpetuate methylation patterns by methylating cytosines in newly replicated DNA at positions diagonally opposed to 5-methylcytosines (5):



Indeed, in several instances it has been found that when DNA that was methylated at every cytosine of one strand is introduced into animal cells and allowed to replicate, methylation was maintained, but only at CpG sites (6). Unmethylated transfecting DNA only infrequently became methylated (7). It has been suggested that de novo methylation of cellular DNA is also rare (8). Thus the distribution of 5-methylcytosine in eukaryotic DNA has been regarded as the sum of two, potentially independent, processes: de novo and maintenance methylation. In the most extreme scenario, the de novo methylase activity would make the “intelligent” decision concerning which sequences should become methylated, but only those sites recognized by the maintenance activity (possibly a subset of those methylated de novo) would remain methylated. Although this model is attractive, the distinction between de novo and maintenance methylation may be artificial; there is evidence that a purified eukaryotic methyltransferase can act on both unmethylated and methylated substrates, albeit at different rates (9). Whether or not most methylation found in eukaryotes is simply due to obedient maintenance, methylation remains to be established. In either case, a mechanism that depends on recognition must exist to set up the observed methylation patterns. A major unanswered question is what determines specificity of DNA methylation in eukaryotes.

We discovered a case of methylation in *Neurospora crassa* in which methylation is not limited to CpG sites or other sites of obvious

E. U. Selker is an assistant professor in the Institute of Molecular Biology and Department of Biology, University of Oregon, Eugene, OR 97403. B. C. Jensen is a student in the Department of Biology, University of Oregon, Eugene, and G. A. Richardson is now a student at the Washington University School of Medicine, St. Louis, Missouri 63130.