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What insights into the developmental traits of urodeles does the study of interspecific hybrids provide?

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ABSTRACT Natural and artificial hybrids represent an important source of material for developmental and evolutionary studies of urodeles. We review the available literature on hybrid salamanders, emphasizing the unique developmental insights that these organisms provide. Of particular interest is the application of new molecular tools to identify DNA markers for traditional characters in developmental research, and we discuss our own results using Bulk Segregant Analysis to identify RAPD markers for the white phenotype in the axolotl. We pay particular attention to the inferences that can be drawn from the many disparate crosses between ambystomatid salamanders that vary in their metamorphic response. These crossing experiments suggest that 1) metamorphosis is dominant to paedomorphosis, 2) that different ambystomatids use different genetic mechanisms to block metamorphosis and become sexually mature, larval paedomorphs, and 3) metamorphosis may be controlled by a few genetic loci. As increasingly sophisticated molecular approaches are applied to these and other hybrid crossing schemes, it should be possible to understand the mechanistic basis of a wide variety of developmental characters that differentiate urodele species.

KEY WORDS: *Ambystoma, Triturus, hybridization, salamander, paedomorphosis*

Introduction

An important aspect of urodele biology is the frequent lack of reproductive barriers among many species. This phenomena has allowed for the introgression of traits between species and subspecies in nature, as well as in the laboratory. The consequences of such hybridization "experiments" are the subject of this review. We emphasize how hybridization in nature and hybrid crosses in the laboratory provide both genetic and evolutionary insights into the developmental traits of urodeles. In the first section, we discuss evolutionary, developmental, and genetic consequences of hybridization as revealed by both population and laboratory studies. In the remaining three sections, we explore how hybrid crosses in the laboratory can provide important genetic insights into the developmental basis of species-specific differences. Also, we explain how this approach, in combination with phylogenetic data, can be used to examine the developmental basis of character convergence. Finally, we illustrate the potential insights that hybrid crosses may offer in the study of alternate modes of development in urodeles.

Population and laboratory studies of hybridization are complementary

Many different species of urodele have been crossed in the laboratory. Members of the salamandrid genera *Triturus* (reviewed by Macgregor *et al.*, 1990) and *Taricha* (reviewed by Twitty, 1966), and ambystomatids of the genus *Ambystoma* (reviewed by Nelson and Humphrey, 1972; Brandon, 1977; this paper) have been the most extensively analyzed, with additional crosses between species in the salamandrid genera *Notophthalmus* (Lipsett and Piatt, 1936) and *Pleurodeles* (Lacroix *et al.*, 1968; Gallien, 1969), the plethodontid genus *Plethodon* (reviewed by Arnold *et al.*, 1993), and the Asian hynobiid genus *Hynobius* (Kawamura, 1952, 1953). Results from hybrid crosses in the laboratory have allowed for a better understanding of the evolutionary relationships within some of these groups, as well as an understanding of the developmental basis of hybrid inviability and sterility. Here we will focus on the extensive database that exists for *Ambystoma* and *Triturus*, where results from field and laboratory studies provide complementary insights into evolutionary and developmental consequences of hybridization.

Hybridization studies of Triturus

Phylogenetic relationships within *Triturus* are consistently supported by several types of evidence, including chromosomal data from cytogenetic analyses and measures of viability and sterility from hybrid crosses (Macgregor *et al.*, 1990). This pattern of data congruence suggests, at least in part, that post-zygotic reproductive isolating mechanisms have evolved at roughly equal rates during the diversification of European *Triturus*. While one might expect reproductive isolating mechanisms to be roughly

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correlated with recency of common ancestry, the degree to which some measures of post-zygotic reproductive isolation provide a metric for determining taxonomic affinity is surprising. For example, male sterility is a general finding among hybrid crosses of *Triturus* (Spurwell and Callan, 1960; White, 1973; Herrero, 1991). Detailed studies of spermatogenesis indicate that the magnitude of some meiotic irregularities are proportionally correlated with taxonomic relatedness (White, 1946; Spurwell and Callan, 1960; Mancino, *et al.,* 1979). Other developmental anomalies are observed in hybrid crosses, including stage-specific measures of survival (Mancino *et al.,* 1978; Bucci-Innocenti *et al.,* 1983; Arntzen and Hedlund, 1990; Macgregor *et al.,* 1990), ploidy elevation (Lantz, 1947; Lantz and Callan, 1954), and sex-ratio distortion (Hamburger, 1935; Spurwell, 1953; Mancino *et al.,* 1978). Some of these measures are also informative for evaluating taxonomic affinities (Macgregor *et al.,* 1990).

Because of male sterility, the reproductive success of hybrids in nature is dependent upon backcrosses of hybrid female to 'pure' species males. Reproductive success for *T. marmoratus* x *T. cristatus* hybrid females is low, even though they may lay more eggs than either parental form (Arntzen and Hedlund, 1990). Apparently, very few hybrid larvae survive to hatching. Reproductive success for intraspecific crosses of both species is reduced in general, due to a balanced lethal heteromorphism on Chromosome 1 (Macgregor and Horner, 1980; Sims *et al.,* 1984; Sessions *et al.,* 1988). In addition, observations of interspecific pairs in the laboratory (Lantz and Callan, 1954) and in nature (Zuderwijk and Sparreboom, 1986) indicate that post-zygotic isolating mechanisms may be reinforced by effective pre-zygotic barriers to mating. These results predict low levels of introgression in natural populations, and this has been confirmed for the *T. marmoratus* x *T. cristatus* hybrid zone (Arntzen and Wallis, 1991). Two additional observations were made during the analysis of this natural hybrid zone. First, there is a differential introgression of nuclear and mitochondrial genetic material. Nuclear introgression of allozymes is bidirectional, but only the *T. cristatus* mtDNA haplotype has been found in F1 hybrids. This implies that the female *T. cristatus* - male *T. marmoratus* cross successfully occurs in nature, while the female *T. marmoratus* - male *T. cristatus* does not. Whether this is due to pre-zygotic (i.e. courtship) or post-zygotic (i.e. gamete incompatibility) mechanisms remains unknown at this time. Second, novel electrophoretic allozymes were observed among the hybrid offspring. The occurrence of such "hybrizymes" is well documented in other systems (Woodruff, 1989), and may represent variation that is expressed as a result of hybridization. Furthermore, the incidence of hybrizymes in hybrid populations was associated with an increase in developmental abnormalities of limbs, suggesting that morphological variability and novelty may also be a by-product of hybridization.

Hybridization studies in Ambystoma

The most intensively studied ambystomatid hybrids are those involving *A. laterale*, *A. jeffersonianum*, *A. texanum* and *A. tigrinum* in eastern North America. Hybrids of these species, as in *Triturus*, have reduced viability and exhibit a higher frequency of developmental abnormalities (Bogart and Licht, 1986; Bogart *et al.,* 1989; Lowcock *et al.,* 1991; Ellinson *et al.,* 1992). However, unlike *Triturus*, hybridization among these *Ambystoma* has been more extensive in nature. The resulting hybrids are primarily unisexual (all-female) salamanders that can be diploid to pentaploid (Hedges *et al.,* 1992), and are viable and abundant in natural populations. Recent allozyme and mitochondrial DNA studies have demonstrated that the initial hybridization events are generally old (4-5 million years; Hedges *et al.,* 1992; Spolsky *et al.,* 1992), although crosses with *A. texanum* as the maternal species are much younger (estimated at 10,000 yrs; Kraus, 1989). Originally, these various hybridization events were thought to produce purely gynogenetic salamanders, where female parthenogens require sperm from diploid sexual relatives to initiate development, but the sperm nucleus is rejected (Uzzell, 1964). However, this "simple" model of gynogenesis has been complicated by the discovery that the sperm nucleus may be incorporated at high temperature (Bogart *et al.,* 1989). Furthermore, at least for the *laterale-texanum* and *laterale-tigrinum* hybrids reported by Bogart (1989), it appears that nuclear reduction does occur in some female eggs, and male genomes occasionally introgress all-female lineages via hybridogenesis (Bogart *et al.,* 1989). This conclusion is consistent with observations by both Hedges *et al.* (1992) and Spolsky *et al.* (1992) for the other all-female species combinations, based on the dissociation of the evolutionary histories of mitochondrial and nuclear genomes in the polyploid lineages.

With our better understanding of ambystomatid evolutionary history and the tremendous variation in ploidy number within and among species combinations, the *Ambystoma* system provides an exceptional opportunity to analyze the developmental consequences of both hybridization and elevated ploidy levels in nature. The data suggest that pre- and post-metamorphic developmental rates are similar between diploid, triploid, or tetraploids and their parental forms (Licht and Bogart, 1987; Lowcock *et al.,* 1992). This result is consistent with earlier observations on artificially-induced autopolyploids (3N) of both salamandrids *(Notophthalmus viridescens, Cynops pyrrhogaster)* and Plethodontids *(Eurycea bislineata),* where no difference in early rates of development were found (Fankhauser, 1945). However, these triploids did show a delay in onset of metamorphosis (Fankhauser, 1945), and artificially-induced, autopolyploid (3N) axolotls *(A. mexicanum)* showed problems with the development and maintenance of their circulatory system, reduced gills, and microcephaly (Fankhauser and Humphrey, 1950). These variable results suggest that the developmental consequences of allopolyploidy are different from those of autopolyploidy (Lowcock and Murphy, 1991; Lowcock, 1994). However, a general delay in maturation rate and suppression of sexual maturation in 4N and higher ploidy forms has been observed for both allo- and autopolyploids (Humphrey and Fankhauser, 1956; Licht and Bogart, 1989; Lowcock and Murphy, 1991). Interestingly, these delays, especially with respect to tetraploids, appear to have important implications for the reproductive ecology of this ambystomatid community (Lowcock, 1994).

Other aspects of the morphology of unisexual *Ambystoma* have received relatively little attention to date. Kraus (1985) examined 13 morphometric characters in pure *A. laterale*, *A. texanum*, and *A. laterale-texanum* and found that, for 10 characters the unisexuals were intermediate between the two parentals, whereas for prevomerine tooth number, the unisexuals were lower than either parental, for number of maxillary-premaxillary tooth rows they were identical to *A. laterale* parentals, and for relative tail length, all taxa were identical. These limited observations from field-collected

samples suggest that most of these characters are under polygenic control and show no hybrid breakdown: repeating these observations for other species combinations under more controlled laboratory conditions would be of interest.

Artificial hybrid crosses and species-specific differences

Urodeles offer a rich, natural source of genetic and phenotypic variation for the study of developmental phenomena. A number of functional and structural traits, that approximately span the spatial and temporal continuum of the urodele developmental program, are known to differ among closely related species. These range from regulatory gene differences (Etkin, 1978) to alternate modes of development in the juvenile and adult phases (Shaffer, 1993). In this section, we explore three motivations for using hybrid crosses to tap phenotypic and genotypic sources of variation. First, these differences are important to both evolutionary and developmental biologists because they reflect the potential for evolutionary divergence from a common ancestral developmental program. In this sense, hybrid crosses can be used to study evolutionarily relevant phenotypes and genotypes that are not normally expressed in a species. The primary insight offered by this approach is genetic, and we will therefore explore potential genetic interpretations for the inheritance patterns that are observed among hybrid offspring. Second, because species are variable with respect to the structural components of genes and proteins, species-specific molecular differences can be exploited as genetic markers in hybrid crosses. Here, hybrid crosses are an efficient methodological tool, and we discuss some applications for this approach. Third, if hybrids can be used to produce a second generation of offspring, then the segregation of species-specific differences can be used to test a hypothesis of simple Mendelian inheritance vs polygenic inheritance. Second-generation crosses may not be possible in all cases, especially if F1 hybrids are sterile or if an interpretation of segregation pattern in the second-generation is confounded by low survival. However, for many urodele groups, there is no significant mortality associated with second generation crosses (Twitty, 1961; Brandon, 1977; Voss, 1995). In fact, hybrid inviability from the handling of eggs may be the primary source of mortality in hybrid crosses (Twitty, 1964). In this section, we explore how all of these motivations can provide interesting insights into the developmental genetic basis of species-specific differences.

Hybridization makes it possible to study phenotypes that are not normally expressed in a species

When two species are crossed, genes underlying species-specific phenotypes assort and segregate among the offspring. The differential expression of these genes during development ultimately determine the hybrid phenotype. Three general types of hybrid offspring are possible in the F1 generation: hybrid offspring may express one of the parental phenotypes, an intermediate phenotype, or a non-parental phenotype.

The inheritance of a species-specific phenotype by all or most F1 offspring of a hybrid cross indicates either dominance for the manifestation of the expressed phenotype or a maternal "environmental" component of variation. If reciprocal crosses are produced, using males and females of the parental taxa to account for non-genetic maternal effects, the relative contribution of species-specific effects can be determined. This strategy has demonstrated that non-genetic maternal effects contribute to the expression of embryonic and larval traits of hybrid urodeles (Lipsett, 1938; Twitty, 1966).

Several different sets of crosses have demonstrated dominance or partial dominance for loci controlling species differences. For example, eye size (*Ambystoma*: Lipsett, 1938), pigment pattern (*Notophthalmus*: Lipsett and Piatt, 1936; *Taricha*: Twitty, 1961; *Triturus*: Spurwell, 1953; *Ambystoma*: Brandon, 1977), and metamorphosis (*Ambystoma*: Humphrey, 1967; this paper) follow this mode of inheritance. However, the genetic interpretation is less clear if there is variation among offspring in the inheritance of the two species phenotypes. For example, variation in the hybrid expression of the parental phenotypes can result if there is intraspecific variation for alleles at the gene(s) underlying the parental phenotypes. Usually, this pattern is restricted to qualitative traits for which there is no alternative other than that of the parental types. Interestingly, such variation can and does occur even when the parental species are phenotypically homogeneous (see below). Under this model, discrete phenotypes could be manifested if the developmental trait is controlled at a major locus, or if the alternate phenotypes are determined by many loci and expressed as threshold traits. Data from hybrid crosses of urodeles (see below) suggest that either of these genetic alternatives may underlie the control of discrete developmental transitions such as metamorphosis.

One of the most common phenotypes seen among F1 offspring of a hybrid cross is an intermediate phenotype (Hamburger, 1935; Twitty, 1936; Lipsett, 1938; Spurwell, 1953; Twitty, 1961, 1966; Nelson and Humphrey, 1972). A possible genetic interpretation for an intermediate phenotype is that both parental phenotypes are co-dominantly expressed. Some pigment pattern components in newts (family Salamandridae) are simultaneously expressed in this fashion (Spurwell, 1953; Twitty, 1961). When the hybrid phenotype is quantitatively intermediate with respect to the parental phenotypes, it is likely that the phenotype is determined, at least in part, by the additive effects of several loci. However, more complex genetic interactions, possibly including epigenetic effects (Cowley and Atchley, 1992), may also underlie the expression of an intermediate phenotype. Hybrid crosses (Twitty, 1961, 1966) support the idea that epigenetic effects are involved in pigment pattern formation in *Taricha* (see Parichy in this journal issue), and intersexuality in *Pleurodeles* (Lacroix *et al.*, 1968; discussed in Collenot *et al.*, 1994).

The expression of novel phenotypes is a unique observation provided by hybridization studies. As was discussed briefly in the previous section, this phenomenon has been observed at both morphological and biochemical levels in natural populations of newts (Arntzen and Wallis, 1991) and ambystomatids (Kraus, 1985). Our experience with hybrid crosses in the laboratory also indicates a high incidence of developmental abnormalities for some crosses. These novel phenotypes are apparently caused by developmental instabilities that result from the incompatible expression of genes in the hybrid. This interpretation follows from the argument that species-specific differences evolve by modification of regulatory genes (Wilson, 1976). In hybrids, it is likely that regulatory gene interactions are disrupted, and that novel phenotypes are manifested as a result of epigenetic phenomena. However, the repeated occurrence of novel phenotypes, at least in our crosses, indicates a genetic basis and therefore a potential phenotypic source for the study of developmental phenomena.

Molecular polymorphisms in hybrid crosses can be used as methodological tools

In general, F1 hybrids have increased levels of polymorphism because they inherit species-specific differences at the DNA level and protein level. At the DNA level, Voss (1993) compared Randomly Amplified Polymorphic DNA (RAPD; see Williams *et al.*, 1990) variation between two *A. mexicanum* individuals versus interspecific RAPD variation between an *A. mexicanum* and an *A. t. tigrinum* individual. He found that 59% of the total number of loci scored were polymorphic between species, but only 11% polymorphism was observed within the two *A. mexicanum* individuals. These increased levels of DNA polymorphism were subsequently used to identify a molecular marker for a developmental trait (see below).

The regulation of protein coding genes can be studied in hybrid crosses when species exhibit tissue-specific and/or stage-specific patterns of expression. This strategy is possible because structural gene differences can be detected between interspecific parents. In this approach, parental effects can be identified by comparing species-specific protein expression patterns against expression patterns from reciprocal hybrid crosses. Etkin (1978) found that the temporal expression of ADH during embryonic development was maternally regulated in *A. mexicanum* x *A. texanum* hybrids. However, maternal and paternal alleles for other enzymes were expressed at the same stages of development. This methodology has been extended to examine the role of cis-acting regulatory genes in *Drosophila* development (Dickinson and Carson, 1979; Dickinson, 1980).

Second generation crosses make it possible to examine the segregation of species-specific phenotypes

Indirect methodologies have been developed for distinguishing between a single gene vs polygenic basis for character differences (Fain, 1978; Lande, 1981; Conner, 1993). However, the reliance of these methods upon large numbers of families often renders them of limited value for hybrid crosses. For testing a hypothesis of simple Mendelian inheritance vs polygenic inheritance, a few backcrosses and F2 hybrid crosses are often informative. Second generation crosses indicate that several urodele traits segregate into a few discrete phenotypic classes, suggesting that one or a relatively few genes underlie these traits (pigment phenotype: Lipsett and Piatt, 1936; Spurwell, 1953; Twitty, 1961, 1966; larval balancer and tail fin length: Twitty 1961, 1966; paedomorphosis: Humphrey, 1967; Voss, 1995; this paper). Traits such as these with relatively simple genetic bases may be of particular interest for developmental analyses because powerful molecular approaches (Michelmore *et al.*, 1991; Lisitsyn *et al.*, 1993) now make it possible to identify DNA markers for the underlying genes. These approaches are appropriate given that urodeles have large, relatively uncharacterized, genomes. Importantly, interspecific hybridization may complement these approaches. As was discussed in the previous section, interspecific hybridization introduces high levels of genetic polymorphism into F1 offspring (Voss, 1993). Higher levels of polymorphism increase the probability of identifying the DNA sequence differences that underlie, or are linked to, alternate alleles that underlie species-specific phenotypes.

For example, we have recently taken advantage of these high levels of polymorphism to identify a molecular marker for the white color phenotype in *A. mexicanum*. The white color variant was the first developmental mutant to be discovered in *A. mexicanum*

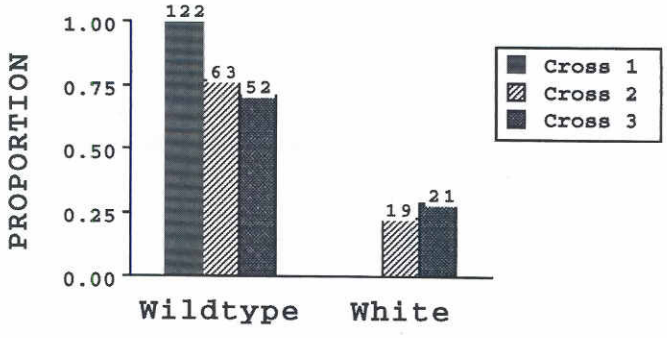

Fig. 1. Proportion of wildtype and white color phenotypes from Crosses 1-3. *All F1 offspring (Cross 1) inherited the dominant allele from A. t. tigrinum, and therefore exhibited the wildtype coloration. A 3:1 phenotypic ratio of wildtype to white coloration was observed among the backcrosses.*

(reviewed by Newth, 1960). This mutant is characterized by a lack of skin pigment after the first few weeks of larval development, with variable repigmentation after sexual maturity (Frost and Malacinski, 1980). Classical breeding techniques have provided limited mapping information for the white gene, including map distance from the centromere (Lindsley *et al.*, 1956) and linkage to the nucleolar organizer genes (Humphrey, 1975). However, as for other mutant phenotypes in *A. mexicanum*, molecular genetic techniques have not been used to identify specific genes or linked DNA sequences.

A cross (Cross 1) was made between *A. t. tigrinum* and an *A. mexicanum* that carried an allele for the white color phenotype (Fig. 1). Two male F1 offspring were then backcrossed (Crosses 2 and 3) to a different *A. mexicanum* that also carried the white allele. The white phenotype is known to be controlled by a single locus (Humphrey and Bagnara, 1967), and is recessive to wild-type coloration. The wildtype and white color phenotypes were inherited by F1 and F2 offspring according to expected Mendelian predictions (Fig. 2). Bulk-segregant analysis (Michelmore *et al.*, 1991) was then used to screen the F2 offspring for a diagnostic RAPD marker that co-segregated with the wildtype phenotype, and therefore the wildtype allele at the white locus. Although examples of codominant RAPDs are known (Williams *et al.*, 1990), for the majority of RAPDs, presence of the band is assumed to be the manifestation of a dominant allele and its absence (the null pattern) to signify a recessive allele. When 91 offspring from the two backcrosses were scored for the diagnostic RAPD, all of the white individuals scored for absence of the RAPD, and most of the wildtype individuals scored for presence of the RAPD (Table 1). This asymmetrical pattern suggested that the RAPD was informative for only the wildtype allele from *A. t. tigrinum*, and indeed the diagnostic RAPD was present only in the P1 *A. t. tigrinum* (data not shown). Therefore, assuming tight linkage, approximately 1/3 of the wildtype individuals (66 x 1/3 = 22) would be expected to score negative for this diagnostic RAPD because they would have a Dmex/d genotype (Fig. 1). When this correction is applied to the data, the number of wildtype recombinants (wildtype individuals scoring for absence of the RAPD) is reduced to 7 for this RAPD.

These results suggest that the combined strategy of using interspecific crosses and bulked segregant analysis is both feasible and efficient. The identification of molecular markers is an important

first step towards understanding the developmental basis of mutant phenotypes. For traits that are expressed early in ontogeny, this can allow for the examination of developmental events that occur prior to the manifestation of the phenotype. Moreover, heterozygotes and homozygotes can be identified if RAPDs (which are dominant markers) are converted to a co-dominant type of genetic marker. Although marker-assisted studies may not be informative for phenotypes that are expressed relatively late in development (i.e., color phenotype), the study of embryonic mutants in *A. mexicanum* may benefit from such an approach (Malacinski, 1989).

The identification of molecular markers may also lead to the isolation of specific genes underlying mutant phenotypes. Previous studies of the white phenotype have not lead to a consensus hypothesis for the developmental basis of this mutation (Frost, 1989; Löfberg *et al.*, 1989). However, the developmental pathways that underlie the manifestation of the white phenotype are known to be associated with neural crest cell differentiation and migration. If candidate genes for the white phenotype are identified, probes for these genes can be developed for *A. mexicanum* and tested for linkage to the RAPD marker from this study. Alternatively, chromosome walking (Rommens *et al.*, 1989) or landing (Tanksley *et al.*, 1995) may also lead to the identification of the specific gene underlying the white phenotype. In theory, both of these approaches are feasible if flanking RAPDs in close proximity to the candidate gene are identified for the white locus.

Hybrid crosses can provide insights into the developmental basis of character evolution

Hybridization is also an efficient methodology for examining the mechanistic basis of evolutionary convergence among closely related species. During the evolutionary process, one frequently observes cases of convergent evolution, or the independent evolution of the same trait among unrelated species (Futuyma, 1986). Mechanistically, such cases are of particular interest: the key question hinges on whether the same or different developmental and/or genetic mechanisms are involved. A cross made between two species that have independently evolved the same developmental phenotype can provide an important insight. If the F1, F2, or backcross hybrid offspring also express the feature of the parental species, this would suggest that the feature was attained by modification of the same developmental (genetic and physiological) mechanisms in both parental species. However, if the offspring express the alternative condition, or a novel character state not seen in either parent, then it is likely that the derived condition was attained in each species by modification of different developmental mechanisms. (In this case, it may be that the same major-effect gene is involved in both species, but a different set of modifier loci produce the observed variation in the hybrids. In either case, if the entire set of loci, including modifiers, is considered, the cross correctly points to either identical or different control mechanisms.) Therefore, by determining whether the same or different mechanisms were altered in the evolution of a new character, hybridization allows one to determine the potential of various developmental mechanisms for truly convergent (independent evolution by modification of different developmental mechanisms) or parallel (independent evolution by modification of the same developmental mechanisms) evolution. This in turn provides unique information concerning the evolution of ontogenies within lineages.

In the following section we illustrate the potential insights that hybridization may offer to the study of alternate modes of development in urodeles.

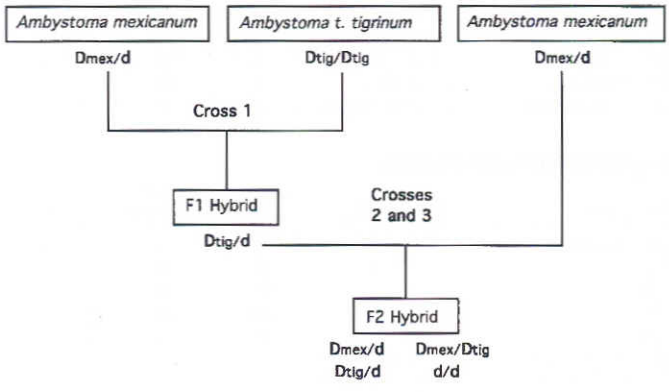

Fig. 2. White locus genotypes for all parental types in the crossing design, and the expected segregation of alleles among the backcross offspring. *The dominant wildtype allele is denoted 'D', and the recessive white allele is denoted 'd'. The segregation of alleles at the white locus would be expected to result in a 1:1:1:1 genotypic ratio (D_{tig}/D_{mex}, D_{tig}/d, D_{mex}/d, d/d) among backcross offspring, and assuming no intra- or interspecific variation among dominant alleles, a 3:1 phenotypic ratio of wildtype to white coloration.*

TABLE 1

PRESENCE OR ABSENCE OF THE DIAGNOSTIC RAPD MARKER AMONG BACKCROSS OFFSPRING

Phenotype	Presence	Absence
Wildtype	37	29
White	0	25

Artificial hybrid crosses and alternate modes of development in urodeles

Hybridization has proven to be useful in the study of life history evolution for those hybridizable species that exhibit alternate modes of development (Swalla and Jeffery, 1990; Jeffery and Swalla, 1991, 1992; Shaffer, 1993; Voss, 1995). In urodeles, many aspects of evolutionary change, from single bones in the hands and feet (Alberch and Alberch, 1981) to the entire morphology of adults (Gould, 1977; Shaffer, 1984a,b) have been characterized as changes in the rate and timing of development. Although we understand relatively little about the developmental mechanisms responsible for these shifts, some progress is being made, especially in ambystomatid salamanders, where inferences from the axolotl *(A. mexicanum)* can be applied to other, related species (Shaffer and Voss, 1996).

The typical mode of urodele development is metamorphosis; an aquatic larva metamorphoses into a terrestrial adult. However, a few species have an alternate developmental mode, which we refer to as paedomorphosis. [Gould (1977) provides an account of the numerous terms associated with this phenomenon. In the

axolotl literature, it is frequently referred to as neoteny, although Gould makes a convincing case that this more restrictive term should be applied only when the evolutionary history of the character's development is well-understood. A variety of other terms have been applied (Reilly, 1987)]. In relation to metamorphosis, paedomorphosis is characterized by metamorphic failure; paedomorphs remain in the aquatic habitat and reproduce while retaining the larval morphology (Gould, 1977). A third developmental mode, called facultative metamorphosis, may link the obligate extremes of metamorphosis and paedomorphosis. In this case, either the expression of the metamorphic or paedomorphic developmental mode is possible, and this variation exists within populations, families, or even individuals (Sprules, 1974; Harris *et al.*, 1990; Licht, 1992).

All three modes of development are represented among salamanders of the genus *Ambystoma*. The ancestral mode of development is obligate metamorphosis (Shaffer 1984a, 1993). However, in a few lineages, and particularly in the speciose tiger salamander complex of the U.S. and Mexico, paedomorphosis is common. Paedomorphic populations of ambystomatids in Mexico appear to be recently, independently derived from metamorphic or facultative ancestors. This suggests that the paedomorphic developmental mode evolved by modification of physiological mechanisms underlying metamorphic regulation. Diversity of developmental mode in this group thus raises an important mechanistic question: during the independent evolution of paedomorphosis, did different species achieve paedomorphosis by modifying similar or different developmental mechanisms?

Many interspecific crosses have been made among ambystomatid salamanders. Although different research questions motivated these crosses, many studies did score survival and expression of developmental mode. Table 2 summarizes both published and unpublished data from interspecific crosses in which life cycle response (metamorphosis or paedomorphosis) was scored. Crosses were included if the experiments were of sufficient duration to evaluate the paedomorphic life cycle response (>12 mos). For each cross, percent metamorphosis is the percentage of surviving offspring that were scored for life cycle response.

With respect to the developmental mode of the crossed species, there are three types of interspecific cross: (1) metamorph x metamorph; (2) metamorph x paedomorph; (3) paedomorph x paedomorph. Although sample sizes are low for most crosses, the results provide several insights into the genetic basis of developmental mode. First, metamorphosis appears to be regulated in the same manner among the different species. When two obligate metamorphic species are crossed, all surviving offspring metamorphose. This result is expected given that metamorphosis is the ancestral developmental mode in *Ambystoma*, and probably for urodeles in general.

A second insight from Table 1 is that metamorphosis is dominant to paedomorphosis. In all but two metamorph x paedomorph crosses (crosses 20, 21), all offspring metamorphosed. Nelson and Humphrey (1972) also observed metamorphosis among offspring from hybrid crosses between the obligate paedomorph *A. mexicanum* and various obligate metamorphic species. These results suggests that the segregation of alleles from the metamorphic parent 'rescue' the metamorphic developmental mode in hybrid offspring. If we assume no intraspecific allelic variation at metamorphic control loci for species that exhibit obligate life history

TABLE 2

PERCENT METAMORPHOSIS AMONG HYBRID CROSSES OF AMBYSTOMATID SALAMANDERS

METAMORPH x METAMORPH CROSSES

Cross	Male		Female	N	Percent Metamorphs	Reference
1	**texanum**	x	**talpoideum**	3	100	4
2	**talpoideum**	x	**texanum**	2	100	4
3	**texanum**	x	**t. tigrinum**	4	100	4
4	**t. tigrinum**	x	**talpoideum**	3	100	4

METAMORPH x PAEDOMORPH CROSSES

Cross	Male		Female	N	Percent Metamorphs	Reference
5	**t. tigrinum**	x	dumerilii	10	100	4
6	**R. rivularis**	x	dumerilii	4	100	4
7	**R. rivularis**	x	t. (Zac)	2	100	4
8	**talpoideum**	x	dumerilii	4	100	4
9	dumerilii	x	**talpoideum**	8	100	4
10	**t. (Col.)**	x	mexicanum	31	100	1
11	mexicanum	x	**t. (Col.)**	11	100	1
12	mexicanum	x	**t. tigrinum**	122	100	5
13				82	100	1
14				?	100	2
15	**t. tigrinum**	x	mexicanum	5	100	4
16	**t. (Xoch.)**	x	mexicanum	?	100	4
17	mexicanum	x	**t. (Xoch.)**	?	100	4
18	**opacum**	x	mexicanum	15	100	6
19				4	100	4
20	mexicanum	x	**t. nebulosum**	22	55	6
21	t. mavortium	x	**t. nebulosum**	13	31	6

PAEDOMORPH x PAEDOMORPH CROSSES

Cross	Male		Female	N	Percent Metamorphs	Reference
22	dumerilii	x	t. (LP)	29	41	7
23	t. (LP)	x	dumerilii	29	62	7
24	t. (LP)	x	mexicanum	40	75	7
25	mexicanum	x	t. (LP)	41	49	7
26	dumerilii	x	mexicanum	12	17	7
27				5	100	4
28	mexicanum	x	dumerilii	34	15	7
29				11	73	4
30	mexicanum	x	t. (Zac)	11	82	4
31	t. (Zac)	x	mexicanum	3	67	4
32	t. (Zac)	x	dumerilii	9	100	4
33	dumerilii	x	t. (Zac)	11	64	4
34	t. mavortium	x	mexicanum	22	0	6

SECOND GENERATION CROSSES

Cross	Male		Female	N	Percent Metamorphs	Reference
35	**mex/t. tig**	x	mexicanum	29	52	3
36a				42	38	5
36b				36	44	5
36c				35	37	5
36d				40	70	5
37	dum/t. (Zac)	x	t. (Zac)/dum	10	0	4
38	t. (Zac)/dum	x	t. (Zac)/dum	1	0	4

1) We define a metamorph as an individual that completely absorbs its external gills during metamorphosis. We define a paedomorph as an individual that does not undergo complete gill absorption. 2) Bold case indicates that a metamorphic individual was used for the cross, regular case indicates a paedomorph. 3) Reference Key: 1, Lipsett, 1938; 2, Humphrey, 1944; 3, Humphrey, 1967; 4, Brandon, 1977; 5, Voss, 1995; 6, Voss, unpublished data; 7, Shaffer, unpublished data.

responses, this further suggests complete dominance at metamorphic control loci for heterozygous F1 hybrids. In the two crosses that did not follow the pattern, an obligate paedomorphic species *(A. mexicanum)* was crossed to a facultative metamorphic species *(A. t. nebulosum)* in Cross 20, and two facultative metamorphic species were crossed in Cross 21. In these crosses, intraspecific allelic variation at metamorphic loci in the facultative metamorphic species presumably led to the observed response. Interestingly, the lack of complete dominance by alleles from *A. t. nebulosum* suggests that obligate paedomorphosis in *A. mexicanum* and facultative metamorphosis in *A. t. nebulosum* and *A. t. mavortium* are manifested as a result of allelic variation at some of the same loci. This interpretation is also supported by results from Cross 34 (see below). However, the possibility that the expression of developmental mode also involves environmental factors cannot be eliminated for crosses between morphs of facultative species (Semlitsch and Gibbons, 1985; Semlitsch, 1987; Licht, 1992).

A third insight from Table 1 is that paedomorphosis has evolved by modification of different genetic architectures, at least in some species. This interpretation follows from an analysis of the paedomorph x paedomorph crosses. If offspring from a cross between two paedomorphs also exhibit the paedomorphic developmental mode, then one cannot reject the interpretation that the two parents share similar alleles at loci controlling metamorphosis (Cross 34). However, if there is variation in developmental mode among the offspring such that some or all metamorphose, then some combination of different loci and/or alleles in the two parental species must be combining to allow the completion of metamorphosis. If we assume no intraspecific variation at metamorphic control loci for obligate paedomorphic species, this would suggest that different loci have been altered (Crosses 26-29). For crosses between an obligate paedomorph species and facultative metamorphic species, a distinction between different loci and alleles is potentially confounded by interspecific and intraspecific allelic variation at segregating metamorphic control loci (Crosses 22-24; 30-33).

Finally, several backcrosses and F1 x F1 hybrid crosses have been made, thereby making it possible to examine the segregation of developmental mode among second generation offspring. The single gene hypothesis for paedomorphosis in *A. mexicanum* was based partially upon the result of Cross 35 (Tompkins, 1978). The numbers of metamorphs and paedomorphs in this backcross were consistent with an expected 1:1, simple Mendelian segregation. Voss (1995) recently tested the single gene hypothesis by rearing backcross offspring among four environmental treatments (Cross 36a- low food, low temp; Cross 36b - low food, high temp; Cross 36c high food, low temp; Cross 36d high food, high temp). Results from three of the treatments were consistent with a hypothesis of 1:1 segregation (36a-c), however the proportions of metamorphs and paedomorphs in 36d were not consistent with a single gene model. These data suggest that more than one gene underlies paedomorphosis in *A. mexicanum*, with a two-gene model as the most parsimonious genetic interpretation.

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