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Survival and Allozyme Expression in Laboratory Induced Hybrids Between Two Species of Salamander

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ABSTRACT

We examined hybrid survival and allozyme expression of five enzyme coding loci in laboratory induced hybrids between *Ambystoma texanum* and *Ambystoma tigrinum*. Survival of *A. tigrinum* x *A. texanum* hybrids was high (80%) compared to previous studies. Survival was much lower in *A. texanum* x *A. tigrinum* hybrids (32% and 43% for two different crosses). Electrophoretic patterns of ADA-1, LDH-1, LDH-2, and MDH-1 allozymes in all hybrids, and MDH-2 patterns in *A. tigrinum* x *A. texanum* hybrids, conform to patterns previously observed in diploid hybrids showing biparental allozyme expression. MDH-2 patterns in *A. texanum* x *A. tigrinum* hybrids are unusual, with weak expression of paternal allozymes.

Key Words: allozymes, hybrids

INTRODUCTION

Laboratory crosses between members of the salamander genus *Ambystoma* provide excellent opportunities to study patterns of allozyme expression in hybrid amphibians. Phylogenetic studies indicate that *Ambystoma* is an old lineage with large genetic distances among species (Kraus, 1988; Shaffer et al., 1991). In spite of this divergence, most attempts to produce interspecific hybrids through artificial insemination (over 40 interspecific combinations reported) produce viable F_1 hybrids (Nelson and Humphery, 1972; Brandon, 1972, 1977). Hybridization between at least four diploid species in

nature (*A. jeffersonianum*, *A. laterale*, *A. texanum*, and *A. tigrinum*) has produced populations with an interesting array of ploidy levels and genomic compositions (e.g., Uzzell, 1964; Downs, 1978; Morris and Brandon, 1984; Kraus, 1985a; Kraus, 1985b; Morris, 1985; Phillips et al. 1997).

Allozyme electrophoresis is one of the principle diagnostic tools used to determine ploidy levels and relative parental genomic doses in *Ambystoma jeffersonianum* complex salamanders (e.g. Bogart, 1982; Bogart et al., 1985; Licht and Bogart, 1989; Lowcock and Bogart, 1989; Spolsky et al., 1992). Some allozyme phenotypes of *in vitro* hybrids between triploid females and males of four different diploid species have been described (Bogart et al., 1989); however, allozyme expression of *in vitro* hybrids between diploid species of this group of salamanders has not been reported. The objectives of this study were to examine viability of F_1 hybrids between *Ambystoma texanum* and *Ambystoma tigrinum* produced in the laboratory and to describe patterns of allozyme expression in them.

MATERIALS AND METHODS

Laboratory Crosses

Crosses were made between *Ambystoma texanum* and *A. tigrinum* collected from western Kentucky and southern Illinois. Voucher specimens have been deposited in the Southern Illinois University Carbondale Fluid Vertebrate Collection (SIUC). Locality data and catalog numbers (H-numbers = alcoholic specimens and TC numbers = frozen tissues) of animals used in the hybridization experiment are as follows:

A. tigrinum (tig): SIUC TC 605. Female, Livingston Co., Kentucky, Route 137, 0.8 mi S jct. 1436, 1.4 mi N mile marker 9, 15 February 1989, R. Brandon and R. Weck; SIUC TC 612. Male, Jackson Co., Illinois, McGuire's Orchard pond, ca. 8 mi S Carbondale, 14 February 1989, R. Weck, G. Miller, G. Paleudis.

A. texanum: SIUC H-3891. Female (Ktex), Livingston Co., Kentucky, Route 137, S of jct. Route 133, 2.2 mi N mile marker 9, 15 February 1989, R. Brandon, R. Weck; SIUC H-3859 and H-3960. Females (ILtex), Williamson Co., Illinois, along Little Grassy Creek, sect. 4 T10S, R1E, 13 February 1989, R. Brandon, R. Weck, H. Moeller, S. Taylor; SIUC H-3861. Male (tex), Williamson Co., Illinois, along Little Grassy Creek, sect. 4 T10S, R1E, 13 February 1989, R. Brandon, R. Weck, H. Moeller.

Matings (Table 1) between these salamanders were made through artificial insemination following the procedures of Brandon (1977). Females were injected with follicle stimulating hormone to induce ovulation. Sperm suspensions from males were kept on ice to extend functional longevity as eggs were removed from the oviducts of females. Fertilization occurred in dechlorinated water at room temperature (ca. 20°C). The two Illinois *A. texanum* females individually produced too few eggs for all matings, so their eggs were pooled and treated as one source. These females were collected syntopically and had identical phenotypes at all allozyme loci examined. All parents were sacrificed during the artificial insemination procedure and were stored in plastic bags at -80°C until their tissues were processed for electrophoresis.

Embryos developed in 10.5-cm diameter glass bowls in dechlorinate water. Embryos were counted and scored daily to follow development. Dead embryos were removed and water was changed daily. Upon hatching, larvae were fed young brine shrimp nauplii (*Artemia* sp.) and, when larger, frozen bloodworms (*Chironomus* sp.). Larvae were raised until they were large enough (3-4 cm total length) to provide adequate electrophoresis tissue samples, after which most were anesthetized in MS-222 (tricaine methane sulfonate) and frozen for storage at -80°C.

Protein Electrophoresis

Horizontal starch gel techniques similar to those of May et al. (1979) were used to determine allozyme phenotypes from homogenates of skeletal muscle in two buffer systems: Amine Citrate (AC6.9) pH 6.9 as described by Clayton and Tretiak (1972), and Tris Citrate (TC8) pH 8.0 as described by Selander et al. (1971). Three enzyme systems (one monomeric, one dimeric, and one tetrameric), encoded by five presumptive gene loci, proved informative with two criteria: 1) the presence in the parents of diagnostic alleles (electromorphs) that allowed us to test for biparental inheritance and examine patterns of gene expression in the hybrids, and 2) loci that provided adequate enzymatic activity and electromorph resolution in the parents to allow us to detect dosage effects. Homogenates of tail and hind limb muscle tissue were examined from at least ten larvae from each interspecific cross for the enzymes ADA-1 (adenosine deaminase [enzyme commission number 3.5.4.4]), LDH-1 and LDH-2 (lactate dehydrogenase [enzyme commission number 1.1.1.27]), and MDH-1 and MDH-2 (malate dehydrogenase [enzyme commission number 1.1.1.37]) (International Union of Biochemistry, 1979). Samples of parental tissues were included on the gels as controls to verify parental bands in hybrids. Homogenates of five larvae from each intraspecific cross were analyzed for the LDH-1, LDH-2, MDH-1, and MDH-2 loci.

Alleles at each allozyme locus were coded by their relative mobility following the nomenclature of Shaklee et al. (1990). The most common allele in the Illinois *A. texanum* was used as the reference or 100 allele at each locus, and the electrophoretic mobilities of other alleles are reported as percentages of this allele.

RESULTS AND DISCUSSION

Hybrid Viability

Survival of the hybrids was higher (Table 1) compared to the same interspecific crosses reported by Brandon (1977). Brandon had commented that the *A. texanum* ova appeared to be "overripe." Even in the present crosses, embryos derived from *A. tigrinum* ova survived better than those derived from *A. texanum* ova; eighty percent of the 99 *A. tigrinum* eggs inseminated by *A. texanum* sperm developed to hatching. Reciprocal crosses involving two different *A. texanum* females and the same *A. tigrinum* male experienced significantly higher hybrid mortality (32 and 43 percent, respectively). Thus, the same relative nuclear combination in the interspecific hybrids (one *A. texanum* haplotype and one *A. tigrinum* haplotype) seems to have produced different degrees of embryonic mortality depending on the source of the egg. It is unclear, however, whether this was due to developmental differences in the interactions between the egg cytoplasm of each species and a hybrid nucleus (as has been described in other hybrid amphibians

[Elinson, 1977]) or, more simply, difficulty in handling *A. texanum* eggs. All crosses involving *A. texanum* females (including intraspecific controls) produced higher offspring mortality than did either interspecific or intraspecific crosses involving the *A. tigrinum* female. The eggs of gravid *A. texanum* females appear to become overripe quickly when females are held in captivity and their eggs seem more easily damaged during handling.

Electrophoretic Patterns

The genotypes of the parents at five allozyme loci are presented in Table 2. All electrophoretic patterns in the parents conform with the phenotypes expected from diploid organisms, based on the known quaternary structure of each allozyme and a codominant pattern of inheritance (Utter et al. 1987). Allozyme phenotypes of the hybrids at all loci other than MDH-2 also indicate diploidy although none of the hybrids was karyotyped. Following is a discussion of allozyme patterns of these five loci in the hybrids.

ADA-1 Patterns

ADA is a monomeric protein. Heterozygotes should show a two-banded phenotype, with each band equally intense. This pattern was observed in the hybrids of both tex/tig crosses. Figure 1 shows results of the Ktex/tig cross. All hybrids are 86/100 heterozygotes, showing maternal and paternal bands of equal intensity. A similar pattern was seen at a slower locus (ADA-2) on the same gels. The second locus was not scored because of poor resolution of the bands and does not appear on Figure 1. This cross confirms the heritability of ADA-1, which is important for our study of allozyme variation in the *A. texanum* complex where we use ADA-1 to detect gene flow between *A. texanum* and *A. barbouri* in an area of sympatry in western Kentucky. ADA was not examined in the reciprocal tig/tex hybrids. The tig female was a 68/75 heterozygote and hybrids would be expected to show a 1:1 Mendelian ratio of 68/100 and 75/100 phenotypes.

LDH Patterns

LDH is a tetrameric protein containing four polypeptide subunits. Two LDH loci were expressed clearly in muscle tissue of both *A. texanum* and *A. tigrinum*. Typical LDH patterns for each species are shown in lanes 1 (*texanum*) and 2 (*tigrinum*) of Figure 2. The fastest and slowest band in each lane represent the products of the two different LDH coding genes, LDH-1 and LDH-2, respectively. Three bands with intermediate mobilities are seen that represent heterotetrameric proteins (combinations of the polypeptide subunits produced by the two LDH loci). The *A. texanum* and *A. tigrinum* have a fixed difference at both loci, although the LDH-2 electromorph mobilities differ by a maximum of 1.5 mm under our electrophoretic conditions. LDH patterns typical of the tig/tex and tex/tig F_1 hybrids are shown in lanes 3-15 of Figure 2. The hybrids are heterozygous at both LDH loci.

Since the protein products of the two LDH loci interact in the parents to produce heterotetrameric bands, heterotetrameric proteins should be produced between each of the four homotetrameric products of the four alleles (LDH-1(100), LDH-1(78), LDH-2(100), LDH-2(82)) present in the hybrid offspring. If this were true, 22 distinct electromorphic bands (4 homotetrameric and 18 heterotetrameric) would appear on an ideal electrophoretic gel, as is illustrated in Figure 3. In our electrophoretic runs the maximum

difference in migration between electromorphs of LDH-1 and LDH-2 was 24 mm. Considering the proximity of the predicted bands and the intensity differences associated with heteromeric bands, there apparently is considerable comigration and blurring of bands. For example, the set of five bands resulting from the interactions between the two LDH-2 alleles would be confined to 1.5 mm of gel space and would certainly appear as one large band, as seen in Fig. 2. We, therefore, consider the zymogram (Fig. 3) a reasonable interpretation of the LDH patterns seen in the hybrids.

The interpretation of the LDH patterns is further complicated by a difference in the staining intensity of the products of the two loci, with LDH-2 electromorphs staining darker. We have observed similar patterns in LDH from muscle tissue of *A. barbouri*, *A. jeffersonianum*, and *A. texanum*/*A. jeffersonianum* F_1 hybrids (Weck and Brandon, unpublished data) and in published photographs of LDH of other *Ambystoma* (Bogart, 1982). During the initial electrophoretic screening of the parents in this study, LDH from heart and liver tissue, as well as skeletal muscle, were examined with both AC 6.9 and TC 8.0 buffer systems. More intense staining of LDH-2 was observed with muscle and liver tissue homogenates in both buffer systems. The AC 6.9 buffers provided superior resolution and electromorph separation. LDH from heart tissue, however, produced LDH-1 and LDH-2 electromorphs of equal staining intensity on AC 6.9 gels but more intense staining of LDH-1 electromorphs on TC 8.0 gels. It appears that the LDH-1 locus is expressed more strongly in muscle and liver tissue of *Ambystoma*, and optimal resolution of both LDH-1 and LHD-2 allozymes can be obtained by using heart tissue homogenates with the AC 6.9 buffer system.

MDH Patterns

MDH is a dimeric protein. Two MDH loci are clearly expressed in muscle tissue of *A. texanum* and *A. tigrinum* (Figs. 4, 5). A third locus produced weak cathodal bands in some individuals. On all gels, MDH-1 electromorphs stained more intensely than MDH-2 electromorphs did. This difference in staining intensity was consistent with patterns we have seen in *A. barbouri* and *A. jeffersonianum*.

MDH-1. The ILtex/tig (Figure 5a) and tig/tex crosses both involved parents homozygous for either the MDH-1(100) or MDH-1(58) alleles. In both crosses, all F_1 hybrids were 58/100 heterozygotes at MDH-1 and all showed the three-banded phenotype with the staining intensity of 1:2:1 (homodimeric: heterodimeric: homodimeric expected for a heterozygous individual at a dimeric allozyme locus [Utter et al. 1987]). A similar pattern would be expected in the cross between the Ktex female and the ILtex male, although MDH was not examined in the offspring from this cross.

MDH-2. The *A. tigrinum* male was heterozygous at the MDH-2 locus (30/80), and crosses involving this male provided the opportunity to test the Mendelian segregation of the MDH-2(30) and MDH-2(80) alleles. MDH was examined in five offspring from the tig/tig control cross (80/80 X 30/80) revealing two 80/80 homozygotes and three 30/80 heterozygotes (Fig. 4). A chi square calculation shows no significant deviation from the expected 1 80/80: 1 30/80 ratio ($X^2 = 0.20$, $p = > 80\%$). The expected 1:2:1 staining intensity of the electromorphs also was observed.

MDH-2 patterns in three interspecific crosses were examined. Twenty F_1 hybrids from the tig/tex cross (100/100 X 80/80) all appear to be 100/80 heterozygotes at MDH-2. The MDH-2(100) and MDH-2(80) electromorphs were separated by only 1 mm on the gels and the three electromorphic bands produced by the hybrids were confined to this space, forming one large fuzzy band. Reciprocal crosses (tex/tig) produced weak MDH activity with unexpected patterns. Thirteen ILtex/tig and 13 Ktex/tig hybrids were analyzed (Figs. 5a, 5b). In both crosses, the MDH-2 alleles segregated in a Mendelian fashion (Table 3); however, the paternal electromorphs (*tigrinum*) were absent or were much weaker than either the maternal (*texanum*) or heterodimeric bands. The presence of the heterodimeric bands indicates that the paternal MDH-2 genes were expressed in the hybrids, but it is unclear why the allozyme products of these genes produced weaker electromorphs. MDH-2 in the tex/tig hybrids showed a pattern that would be expected in triploids. Dosage effects are predicted in triploid individuals where maternal:paternal allelic ratios are 2:1 (Seeb et al., 1988). MDH allozyme dosages have been found to correlate with the known ploidy level in *Ambystoma jeffersonianum* complex salamanders (Bogart, 1982; Bogart et al., 1989) and, in each case, both MDH-1 and MDH-2 patterns reflected the ploidy level of the individual. Alternatively, the hybrids may appear to be triploid because the maternal MDH-2 is expressed more strongly than paternal MDH-2. Wright (1975) reported expression of only maternal allozyme genes in early embryos (through neurula) of hybrids between *Rana pipiens* complex frogs, but paternal allozymes were expressed by the operculum development stage. Our hybrid larvae were relatively small (3-4 cm) when examined but well beyond limb formation. Ploidy of the hybrids remains to be confirmed by karyology.

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Table 1. Results of laboratory crosses involving *Ambystoma texanum* and *A. tigrinum*
* Indicates data from Brandon (1977) for comparison.

Cross (f/m)	Number of Eggs Inseminated	Damaged	% Hatched	Remarks
tig/ILtex	99	0	80	20% mortality during gastrula and neurula
ILtex/tig	57	3	32	68% embryonic mortality; 1 egg unfertilized
Ktex/tig	71	3	43	57% mortality during gastrula; 1 egg unfertilized
tig/tig	84	0	77	23% mortality during gastrula; a few survivors deformed
ILtex/ILtex	33	2	52	48% mortality during gastrula; a few survivors abnormal, 2 eggs unfertilized
Ktex/ILtex	59	6	74	26% embryonic mortality; 2 eggs unfertilized
tig/ILtex*	27	0	23	many abnormal
ILtex/tig*	108	?	3	5 reached neurula; none fed
tig/tig*	21	0	2	4 died as gastrulae, 8 as neurulae, 7 died later
Iltex/ILtex*	57	57	0	none survived cleavage

Table 2. Allozyme genotypes of the parents at five loci.

	Locus				
Specimen (sex)	ADA-1	LDH-1	LDH-2	MDH-1	MDH-2
ILtex (F-1)	100/100	100/100	100/100	100/100	100/100
ILtex (F-2)	100/100	100/100	100/100	100/100	100/100
ILtex (M)	100/100	100/100	100/100	100/100	100/100
Ktex (F)	100/100	100/100	100/100	58/58	100/100
tig (F)	68/75	78/78	82/82	58/58	80/80
tig (M)	68/68	78/78	82/82	58/58	30/80

Table 3. Observed and expected MDH-2 genotype frequencies for the ILtex/tig cross (100/100 x 30/80) and the Ktex/tig cross (100/100 x 30/80).

	ILtex/tig (n = 14)		Ktex/tig (n = 13)	
Genotype	Observed	Expected	Observed	Expected
30/100	42.9%	50%	53.8%	50%
80/100	57.1%	50%	46.2%	50%

Figure 1. ADA-1 phenotypes of an *A. texanum* female (lane 1, genotype 100/100), an *A. tigrinum* male (lane 2, genotype 68/68) and 13 of their F_1 hybrids (lanes 3-15). All hybrids are 68/100 heterozygotes.

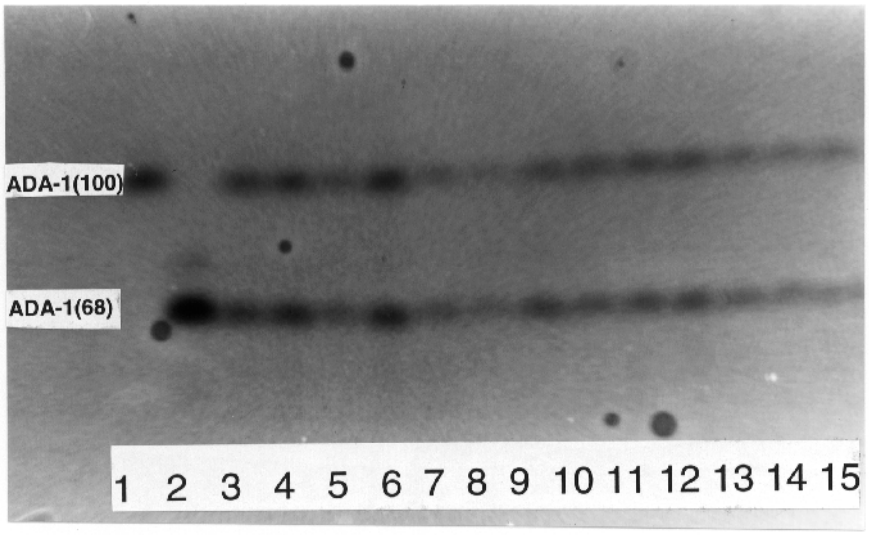

Figure 2. LDH phenotypes of an *A. texanum* female (lane 1), an *A. tigrinum* male (lane 2) and 13 of their F_1 hybrids (lanes 3-15). A diagrammatic interpretation of these phenotypes is shown in Figure 3.

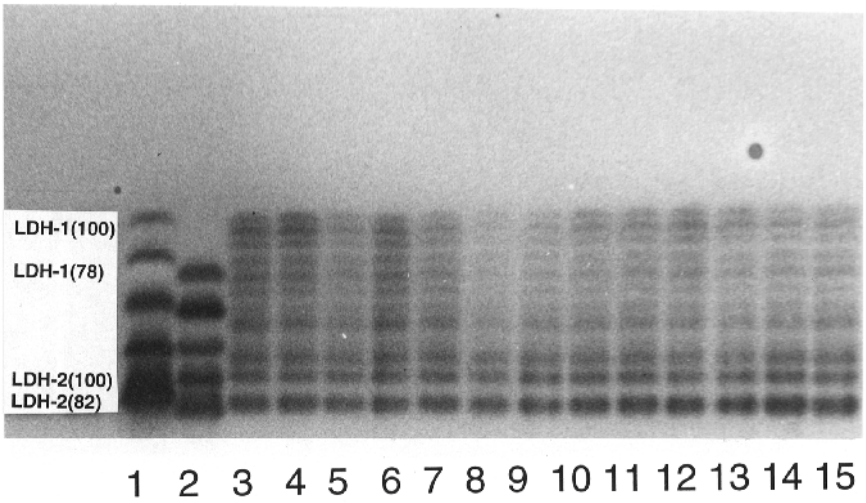

Figure 3. Zymogram of the LDH allozyme phenotypes expected in an F_1 hybrid (lane 3) between the *Ambystoma texanum* female (lane 1) and the *A. tigrinum* male (lane 2). The LDH-1 (100) allele produces subunit B, the LDH-1 (78) allele produces subunit b, the LDH-2 (100) allele produces subunit A, and the LDH-2 (82) allele produces subunit a. The expected hybrid phenotype contains 22 electromorphs representing all possible tetrameric combinations of the 4 subunits.

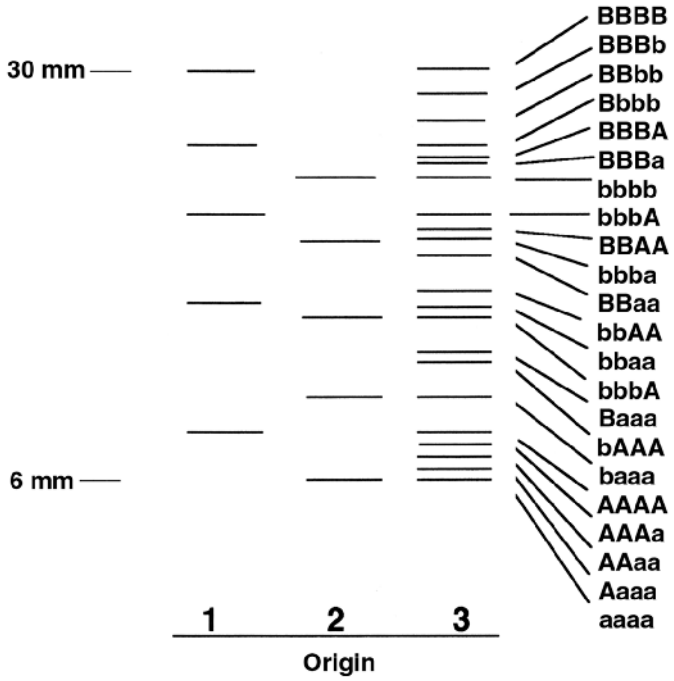

Figure 4. MDH phenotypes for an *A. tigrinum* female (lane 1), an *A. tigrinum* male (lane 2), and 5 of their F_1 offspring (lanes 3-7). All individuals are homozygous for MDH-1. The male is heterozygous at MDH-2, as are the offspring in lanes 5-7.

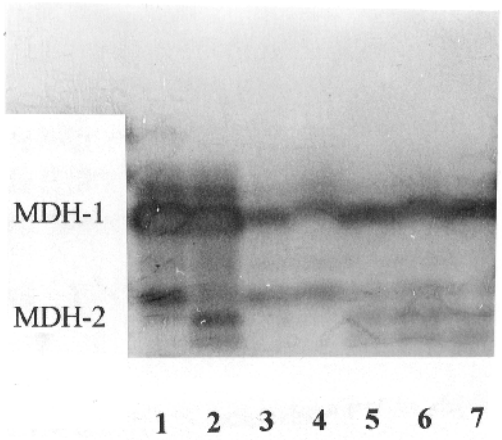

Figure 5. MDH phenotypes from the ILtex/tig cross (A) and Ktex/tig cross (B). Females are in lane 1, males in lane 2, followed by F_1 hybrids. MDH-1 (more anodal) shows more intense staining activity. Paternal bands at MDH-2 show reduced activity or may be absent. MDH-3 is evident in the tig male and in the Ktex female.