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ENVIRONMENT-DEPENDENT ADMIXTURE DYNAMICS IN A TIGER SALAMANDER HYBRID ZONE

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Abstract.—After an estimated five million years of independent evolution, the barred tiger salamander (*Ambystoma tigrinum mavortium*) was introduced by bait dealers into the native range of the California tiger salamander (*A. californiense*). Hybridization and backcrossing have been occurring in central California for 50–60 years, or an estimated 15–30 generations. We studied genetic and ecological factors influencing admixture of these two divergent gene pools by analyzing frequencies of hybrid genotypes in three kinds of breeding habitats: natural vernal pools, ephemeral man-made cattle ponds, and perennial man-made ponds. Perennial ponds tended to have higher frequencies of nonnative alleles than either type of seasonal pond, even in cases where perennial and seasonal ponds are within a few hundred meters. Thus, the hybrid zone has a mosaic structure that depends on pond hydrology or ecology. The presence of some broadly acting constraints on admixture is suggested by linkage disequilibria between physically unlinked molecular markers within ponds. In addition, we found several marker-specific deviations from Hardy-Weinberg equilibrium. One marker showed a consistent deficit of heterozygotes across pond types. Another showed heterozygote deficits only in vernal pools. A third was more likely to have heterozygote excess in ephemeral cattle ponds. These patterns indicate that admixture is influenced by complex genotype-by-environment interactions.

Key words.—*Ambystoma californiense*, *Ambystoma tigrinum mavortium*, heterozygote deficit, hybridization, invasive species, linkage disequilibria, mosaic hybrid zone.

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The evolution of distinct groups that can coexist in sympatry is one of the most interesting and difficult issues in evolutionary biology (Mayr 1942; Coyne and Orr 1998; Schluter 2000; Turelli et al. 2001). To coexist indefinitely, groups must be ecologically differentiated and, for sexual groups, reproductively differentiated (Dobzhansky 1937; Mayr 1963). Hybrid zones can occur between parapatric forms that are not completely reproductively isolated. When a hybrid zone is formed via secondary contact, differences that have accumulated over a period of isolation are effectively put to a test of compatibility (Orr 1995; Butlin 1998). This provides an important opportunity to explore the consequences of divergence. Here, we studied the distribution of multilocus hybrid genotypes among three habitat types to examine the influence of different ecological contexts and regions of the genome on the dynamics of admixture between native (*Ambystoma californiense*) and introduced (*A. tigrinum*) tiger salamanders.

One of the most fundamental problems in the study of hybrid zones is to understand the relative importance of ''exogenous'' and ''endogenous'' selection in determining the fitness of hybrids (Moore and Price 1993; Kruuk et al. 1999). Exogenous selection describes the maintenance of different alleles in different environments due to trade-offs in local adaptation. Endogenous selection describes the maintenance of different alleles in different genetic backgrounds due to within-genome interactions such as heterozygote disadvantage or epistasis. Exogenous selection is likely to result in a mosaic hybrid zone or step cline, in which differentiated populations segregate spatially according to the distribution of habitats (Endler 1977; Harrison and Rand 1989). On the other hand, endogenous selection should lead to the development of ''tension zones,'' in which clines are maintained by a balance between immigration of pure individuals and selection against their hybrid progeny (Barton 1979; Barton and Gale 1993). Hybrid fitness may be determined by combinations of endogenous and exogenous factors, as in the case of environment-dependent genetic incompatibilities (Nairn et al. 1996; Wade et al. 1999; Barbash et al. 2000). In addition, neither endogenous nor exogenous selection necessarily causes selection against hybrids; superiority of certain hybrid genotypes in certain environments is well documented (Anderson 1948; Moore 1977; Arnold 1997; Arnold and Emms 1998; Rieseberg et al. 1999a; Barton 2001).

In addition to variation among habitats, variation among different portions of the genome may have a dramatic effect on the dynamics of secondary contact. When several generations of hybridization and backcrossing have occurred, there may be thousands of recombinant hybrid genotypes, each with a different phenotype and fitness. Complex variation among parts of the genome in their propensity for admixture and recombination in hybrid populations has been illustrated in a few groups such as the sunflower genus, *Helianthus*, (Rieseberg et al. 1999b, 2000), *Drosophila* (True et al. 1996), and *Anopheles* mosquitoes (Garcia et al. 1996; della Torre et al. 2002). In other systems, hybrid zones exhibit remarkable concordance across traits and loci (Nürnburger et al. 1995; Dasmahapatra et al. 2002; Vines et al. 2003). Ultimately, the key question remains whether discrete species represent completely isolated and integrated coadapted gene complexes as proposed during the modern evolutionary synthesis (Dobzhansky 1937; Mayr 1963) or whether ecological and other phenotypic discontinuities typical of ''good species'' are maintained by a small set of genetic factors whereas the bulk of the genome is free to introgress across species boundaries (Barton and Hewitt 1985; Harrison 1990; Rieseberg and Burke 2001; Rundle et al. 2001; Schilthuizen 2001; Wu 2001). These issues will only be resolved by the accumulation of diverse case studies. Here we contribute one study illustrating complex locus-specific and environment-dependent patterns of genetic admixture.

The Ambystoma Study System

All members of the tiger salamander species complex court and breed in aquatic habitats. Most cannot coexist with predatory fish, restricting them to ephemeral or isolated ponds and lakes. Eggs are laid singly or in small strings, preferably on fresh vegetation. *Ambystoma* larvae are aquatic and remain in their natal ponds for several months, whereupon most metamorphose and disperse overland (Petranka 1998). Metamorphs spend months to years in underground mammal burrows before returning to breed (Trenham et al. 2000). All salamanders are predatory, and larvae feed on a wide variety of aquatic organisms, from plankton to other salamanders. Larvae are also subject to predation, primarily by aquatic insects such as dragonfly nymphs. Fish appear to relish larval tiger salamanders, and bass fishermen have long used salamander larvae, or ''waterdogs,'' as bait (Larsen 2000; Bean 2001).

Secondary contact between *A. californiense* (California tiger salamanders) and *A. tigrinum mavortium* (barred tiger salamanders, sometimes recognized as *A. mavortium* (Collins 1997)) was established in the 1940s by purposeful introduction (by bait dealers) of *A. tigrinum* from the southwestern United States into several areas, mostly in the inner coastal ranges of central California (Riley et al. 2003). Our previous work demonstrated that fertile hybrids are produced, and suggested that pond type may affect hybrid fitness (Riley et al. 2003). This system is a hybrid zone in the sense of being an area where hybridization occurs. However, it is not a cline between the ranges of two pure types. The nonnative salamanders were distributed into various parts of central California within the existing range of *A. californiense*. The geographical details of the hybrid zone are the subject of ongoing study.

Formerly, the two lineages had evolved separately in allopatry, probably since about five million years ago when the ancestors of *A. californiense* are thought to have been isolated by the uplift of the Sierra Nevada Range and the subsequent formation of the Great Basin Desert (Shaffer and McKnight 1996). The sister lineage to *A. californiense*, which eventually gave rise to *A. tigrinum*, diversified extensively in the Mexican highlands, giving rise to at least 15 species (Shaffer and McKnight 1996). The tiger salamander complex is characterized by the repeated evolution of a paedomorphic life history in which adult animals remain in the fully aquatic larval form (Shaffer and Voss 1996). Paedomorphosis may be obligatory or facultative (Sprules 1974; Brandon 1989), and both conditions occur in the tiger salamander complex (Collins et al. 1980; Shaffer 1984). *Ambystoma tigrinum* exhibits facultative paedomorphosis, whereas pure *A. californiense* always metamorphose; this is the most obvious ecological difference between the two lineages (Twitty 1941; Rose and Armentrout 1976; Collins et al. 1980; Petranka 1998). In the hybrid zone, paedomorphs often occur in perennial ponds, and they always contain at least some *A. tigrinum* genes (B. Fitzpatrick and H. B. Shaffer, unpubl. data). Paedomorphosis is generally regarded as an adaptation for permanent aquatic habitats, because paedomorphs generally attain larger body size, larger clutches sizes for females, and possibly greater mating success for males (Rose and Armentrout 1976).

Within the hybrid zone, breeding ponds can be coarsely classified into one of three types: vernal pools, ephemeral cattle ponds, and perennial ponds. These types are distinguished by a series of potentially important ecological and hydrological factors.

Vernal pools.—Vernal pools, the natural breeding sites of *A. californiense*, fill with winter rain and dry out in late spring. They are usually shallow (rarely more than 1 m deep) and, in our study area, often contain emergent vegetation such as small sedges and rushes. Vernal pools are characterized by a rich invertebrate fauna including filter feeding fairy shrimp (*Branchinecta* and *Linderiella*) and clam shrimp (*Cyzicus californicus*) as well as important predators such as giant water bugs (Belostomatidae: *Lethoceras*), predaceous diving beetles (Dysticidae: *Dysticus*), and dragonfly larvae. Tree frogs (*Hyla regilla*), western toads (*Bufo boreas*), and spadefoot toads (*Spea hammondi*) also breed in vernal pools, and their tadpoles may be important prey items for growing salamander larvae. In our study, we have vernal pools that experience varying degrees of impact from livestock, which tend to make them muddy and nitrogen rich. Vernal pools are now rare in our study area and some fill with water only in exceptionally wet years.

Ephemeral cattle ponds.—These are artificial ponds scooped out of stream beds, drainages, or former vernal pools. They dry each summer but are usually designed to hold water a little longer than most vernal pools. Ephemeral cattle ponds are very muddy and contain cow excreta. There is little or no vegetation and the primary macrofauna—besides salamanders—we find are backswimmers (Notonectidae) and, rarely, waterscorpions (Nepidae: *Ranatra*). These hemipterans may prey on small salamander larvae. If cattle are excluded, shallower ephemeral cattle ponds become similar to vernal pools in their vegetation and fauna. Ephemeral cattle ponds are the most common breeding habitat for tiger salamanders in our study area and possibly throughout California.

Perennial ponds.—The unifying characteristic of this class of ponds is that they generally retain water throughout the year. We use the term ''perennial'' rather than ''permanent'' because these ponds do occasionally dry up during drought years or due to cattle or other water extraction activities. Perennial ponds with well-vegetated shallows will support large invertebrate predators, but salamander larvae are relatively safe if they avoid the shallows (Holomuzki 1986). The presence of large open-water refugia is a potentially important difference between these ponds and vernal pools. Perennial ponds in the hybrid zone often contain paedomorphic tiger salamanders, and even metamorphs may remain in the pond well into the summer.

METHODS

We obtained tissues from young-of-the-year larvae by seining ponds in the spring. This sampling strategy ensures that overlapping generations and immigration cannot explain any deviations from single-locus Hardy-Weinberg equilibrium. Table 1 gives details on each pond sampled. The 12 ponds include four vernal pools, four ephemeral cattle ponds, and four perennial ponds in the Salinas Valley and adjacent Gabilan Range (Table 1, Fig. 1). Data for three markers

TABLE 1. Information on the 12 ponds surveyed for hybrid tiger salamander genotypes.

	Pond	Date sampled	*N*	Comments
Vernal pools	F	4/8/98	65	Only fills in high-rain years; completely protected from cattle
	CVP	3/30/00	62	Fills most years; cattle excluded from one half
	GL	5/25–7/9/91	37	Cattle have full access
	A	4/8/98	49	Cattle have full access
Ephemeral cattle ponds	G	6/13/01	50	Cattle have full access
	H	6/5/00	51	Cattle have full access
	BW.2	6/5/00	55	Cattle have full access
	SYC	6/5/00	56	Cattle have full access
Perennial ponds	JCL.2	4/1/98	39	Landfill sedimentation pond
	BW.1	6/5/00	56	Cattle pond
	CT.2	3/21/01	49	Cattle pond
	CHL	3/30/00	58	Surrounded by crops

(mtDNA, *DLX3, Slc4a4*) from ponds F and A have been published previously (Riley et al. 2003).

Markers and Molecular Methods

We extracted genomic DNA from each individual using standard methods (Palumbi 1996). Each larva was genotyped for mtDNA and eight codominant nuclear markers that have been mapped in *Ambystoma* (Table 2; Voss et al. 2001). All markers were amplified via polymerase chain reaction (PCR), and PCR products from two to six pure *A. californiense* and pure *A. tigrinum* were sequenced at the University of California, Davis, Division of Biological Sciences DNA Sequencing Facility (http://dnaseq.ucdavis.edu/). We inspected sequences to identify diagnostically different restriction enzyme recognition sites for each marker (Riley et al. 2003). After digesting PCR products with restriction enzymes, fragments were separated by agarose gel electrophoresis and visualized with ethidium bromide. Homozygotes for the absence of a restriction site yielded a single band of unmodified PCR product and homozygotes for the presence of a restriction site yielded two smaller fragments. Samples showing both cut and uncut fragments were inferred to be heterozygous.

Putative diagnostic restriction-fragment-length polymorphism (RFLPs) were verified by genotyping population samples of 25 pure *A. californiense* from the Hastings Reserve (Monterey Co., CA) and 25 pure *A. tigrinum* from an introduced site in California but outside the native range of *A. californiense* (Clearlake Oaks, Lake Co., CA). According to a long-time bait dealer from Salinas, California, the Clearlake

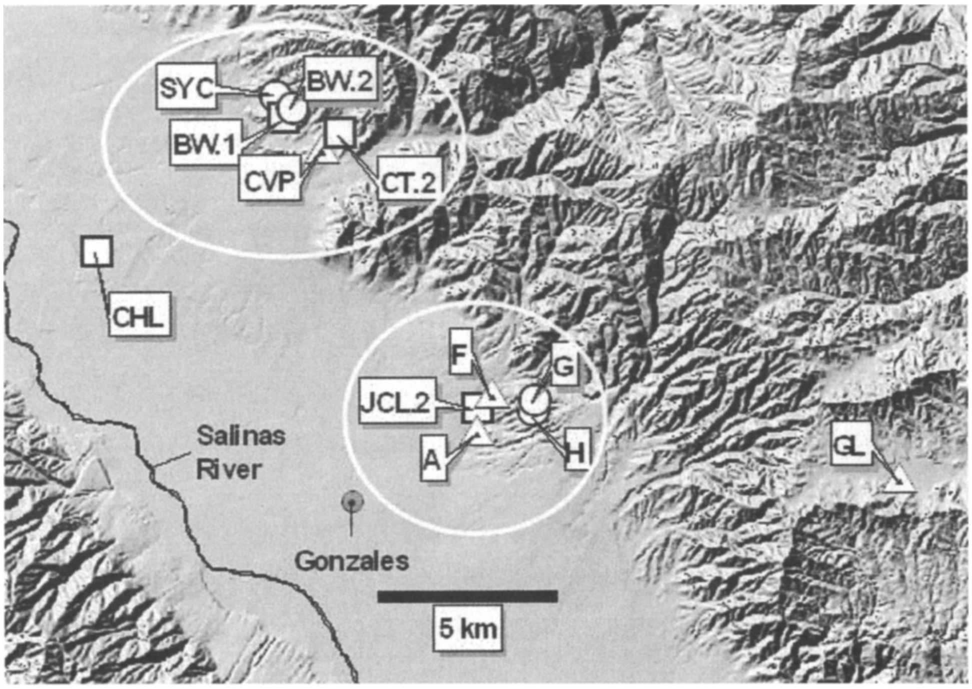

FIG. 1. Locations of 12 tiger salamander breeding ponds in the Salinas Valley hybrid zone. Triangles are vernal pools, circles are ephemeral cattle ponds, and squares are perennial ponds. White ellipses circumscribe the two spatial locales used in the logistic analysis (see Methods).

TABLE 2. Nine molecular markers used to characterize hybrid tiger salamanders. "Cuts cal" or "cuts mav" means that the enzyme cuts the *Ambystoma californiense* or the *A. tigrinum mavortium* allele, respectively.

Locus	Linkage group	Detection method	Primers	Primer source
D-loop	mtDNA	*Ssp*I cuts cal	THR-AAACATCGATCTTGTAAGTC DL1-AATATTGATAATTCAAGCTCCG	Shaffer and McKnight 1996
GNAT2	17	*Alu*I cuts mav	F6-AACCACATCCGCTTCTCAAC DR-CAGCCCTAG(C/T)(C/T)(A/G)AGGCCTTC	Designed from GenBank AF050654
SLC4A4	2	*Sau*96I cuts mav	F-TCCGCTTGCAGCAGTCTCCTTGCTCTC R-TAACGGCCTGATTGATGACCAGCGAAG	Voss et al. 2001
HOXD8	9	*Fnu*4HI cuts mav	F-CGGACTGTAAATCGTCCAGTGGTCAC R-GCTGGGAACTTGTCTTTGTTGTTCTC	Voss et al. 2001
GNAT1	39	*Fnu*4HI cuts cal	F2-GTAGAGGCAGCAACACTTTGG R-TTAGAGGGCGAAAAGTGTCATC	Designed from our sequences and Voss et al. 2001
DLX3	23	*Alu*I cuts mav	F-GGCGAGGCGCACCTCTCCAACTGGTGA R-AGGCTCCCACCTTCTGAGTTGGGAAGG	Voss et al. 2001
HOXB13	23	*Hpy*CH4V cuts mav	F-GTCTCCTTTTGCTTGATTTCCG R-TCACCAAAATCACAGAACACCTGC	Voss et al. 2001
FOXG1B	16	*Bsp*CNI cuts cal	F-GATGGACTGCTAATCTTTTGAGACCC R-AACAAGTGGGGCAAACAACCAACCAGC	Voss et al. 2001
WNT1	7	*Rsa*I cuts cal	F-CAGCGCCAAATCACACCTCA R-TCCATAAACGCTGCCAAAATAAAG	Voss et al. 2001

Oaks population was established from the same stock as those introduced into the Salinas Valley (Don Green, pers. comm. 2003). Additional genotypes were obtained from 25–35 individuals of each pure species sampled systematically from throughout their native geographic ranges (see Appendix online at http://dx.doi.org/10.1554/03-629.1.s1). One marker (*GNAT1*) was found to have a low level of polymorphism among nonnatives (frequency of the *californiense*-like allele was 0.02 in the Clearlake Oaks sample and 0.04 in the native range of *A. tigrinum*). Accordingly, it was excluded from any analyses that assumed markers were diagnostic of different ancestry. As in our previous work, heterozygote diagnosis was verified by cloning and sequencing PCR products from two heterozygotes for each marker to verify the presence of both *A. californiense*-derived and *A. tigrinum*-derived alleles (Riley et al. 2003). In addition, 10 F_1 hybrids from a laboratory cross served as heterozygote positive controls.

The nuclear markers used were all mapped to separate linkage groups except for *DLX3* and *HOXB13,* which show loose physical linkage (Voss et al. 2001). Voss et al. (2001) estimated their recombination distance as 25.7 centimorgans, whereas free recombination occurs at distances $\geq$ 50cM.

Statistical Analyses

This study involves several sets of statistical tests where the same statistical hypothesis is evaluated repeatedly for several markers or several ponds. We have used the sequential Bonferroni adjustment (Rice 1989) when assessing statistical significance unless otherwise stated. We report single test (unadjusted) P-values to aid in the interpretation of the strengths of various results, but these P-values are compared to adjusted critical values when declaring a test statistically significant ($\alpha = 0.05/[k + 1]$, where k is the number of single tests with P-values greater than the focal test, see Rice 1989).

Do nonnative allele frequencies vary among habitat types?—Hierarchical analyses of F-statistics, or locus-by-locus analyses of molecular variance (AMOVAs), (Excoffier et al. 1992; Weir 1996) were performed for all markers to test for allele frequency variation among pond types and among ponds nested within pond type. A multilocus AMOVA was also evaluated for the nuclear markers. However, this is merely the weighted average of the single locus analyses and does not account for covariances (linkage disequilibria) among loci (Michalakis and Excoffier 1996). Further, the interpretation of a pooled test is clear when each locus provides an estimate of the same population parameter, but ambiguous when there are demonstrable locus-specific patterns. F-statistics can be defined as correlation coefficients and tested for heterogeneity among markers (Zar 1984; Weir 1996).

Our data demonstrate both linkage disequilibria (LD) and heterogeneity among F-statistics, violating the assumptions of multilocus AMOVA (see Results). Two other limitations of the AMOVA approach are (1) haploid mtDNA data cannot be included in the multilocus AMOVA (Schneider et al. 2000; Dyer 2001), and (2) the AMOVA framework does not allow us to account for the spatial structure of the samples. Ten of the 12 ponds fall into two spatial locales (Fig. 1). We analyzed genotype frequencies in these 10 ponds using a general loglinear model incorporating locus effects and the hierarchical structure of the sample (PROC GENMOD, SAS Institute 1999). We defined individual genotype as an ordinal dependent variable (0 for native homozygote, 1 for heterozygote, 2 for nonnative homozygote) and used maximum likelihood to fit an ordinal logistic model with pond type, locale, and locus as main factors and pond and individual as nested factors. The individual effect accounts for associations among loci (LD). Native and nonnative mtDNA haplotypes were coded as 0 and 2 to maintain a comparable ordinal scale with nuclear loci.

Is admixture within ponds random?—Within each pond, the distribution of genotypes was tested against the null hypothesis of random admixture at multilocus, single locus, and two locus levels. As a summary of the eight-locus (excluding *GNAT1*) genotype, we calculated an additive hybrid index, *I,* for each individual as the number of *A. tigrinum*-derived alleles across all diagnostic markers surveyed. With one haploid and seven diagnostic diploid markers, *I* can take on integer values from 0 to 15. We compared observed distributions of *I* to those expected after many generations of unimpeded hybridization as follows. For each pond, null distributions for *I,* assuming Hardy-Weinberg equilibrium and linkage equilibrium (HWLE), were estimated by permuting alleles among individuals for each locus 10,000 times and calculating the average number of individuals with each value of *I.* Observed and expected counts of each value of *I* were compared using the standard log-likelihood goodness-of-fit statistic, G^2 (with a constant of 1 added to each count to mitigate the influence of values with small expected counts). *P*-values were calculated as the fraction of the 10,000 Monte Carlo replicates that deviated from the null distribution as much or more than the observed data using the same statistic. Significant deviation from HWLE may be caused by a series of single-locus deviations from Hardy-Weinberg equilibrium (HWE), linkage disequilibria (LD) between loci, or both. A second set of null distributions and Monte Carlo tests were performed using genotypes rather than alleles, and therefore removing the HWE assumption. This tests for a between-marker component to the overall deviation from HWLE. Barton has described a framework for simultaneously estimating all the possible associations within and between loci (Barton 2000), however, the calculations with eight diploid markers (6561 possible multilocus genotypes) proved unmanageable.

Each individual marker was tested for deviation from HWE using standard likelihood ratio (G^2) tests and using the maximum-likelihood algorithms in Analyse 1.30 (Barton and Baird 2002) to evaluate the likelihood that F_{IS} is greater than zero. Results from these different analyses were entirely concordant, and we report results from Analyse.

Pairwise allelic LD for all pairs of these nine markers (including *GNAT1*) were estimated and tested for significant differences from equilibrium using Analyse and CND (Asmussen and Basten 1994). These estimates were corrected for nonzero F_{IS} (Asmussen and Basten 1994; MacCallum et al. 1998).

Do disequilibria vary among habitat types?—Hardy-Weinberg disequilibria and allelic linkage disequilibria were tested for variation among ponds and among pond types using the nested analysis of variance (ANOVA) framework described by Langley et al. (1978). This approach involves expressing disequilibria as correlation coefficients. $F_{IS} = [P_{AA} - p^2]/[p(1 - p)]$ is the correlation between *A* alleles at a single locus, where P_{AA} is the frequency of *AA* homozygotes and *p* is the frequency of the *A* allele; $r_{AB} = D_{AB}[p_A(1 - p_A)p_B(1 - p_B)]^{-1/2}$ is the correlation between alleles *A* and *B* at loci *a* and *b,* where D_{AB} is the coefficient of linkage disequilibrium (covariance between *A* and *B*). The formulas of Langley et al. (1978) can then be applied using sample sizes of 4 *n* for pairs of diploid markers, 2 *n* for cytonuclear comparisons, and *n* for single locus HWE analyses (where *n* is the number of sampled larvae).

RESULTS

Do nonnative allele frequencies vary among habitat types?—Distributions of the additive hybrid index indicate that perennial ponds tend to be inhabited by populations with more nonnative alleles than average for the area (Fig. 2). In keeping with this result, the multilocus hierarchical AMOVA showed a significant pond type effect (Table 3b). The χ^2 analyses in Table 3 indicate that the pond type effects (F_{CT}) do not vary significantly among the seven diagnostic nuclear markers (that is, excluding *GNAT1*). The ordinal logistic model also confirmed a significant relationship between pond type and occurrence of nonnative genotypes (Table 4). This analysis also showed evidence of significant associations among loci (the individuals within pond term), significant variation among loci, among ponds within a type, and between the two spatial locales shown in Fig. 1 (Table 4). Interaction terms between locus and pond, and between locus and pond type indicate that markers differ significantly in their degree of differentiation among sites and habitats.

Is admixture within ponds random?—Within pond tests of the fit between observed and expected distributions of the hybrid index indicated significant deviations from HWLE. Using a Bonferroni correction for 12 simultaneous tests, seven of 12 ponds deviated significantly from their expected distributions using all markers (Table 5). Ponds with highly skewed frequencies tended not to exhibit significant deviations. Because highly skewed ponds also tend to be perennial ponds, we cannot distinguish between the hypotheses that (1) perennial ponds are in HWLE, or (2) that we have low power to detect deviations from HWLE in ponds with skewed allele frequencies. However, the observation that one vernal pool (CVP) is also highly skewed toward nonnative alleles (Fig. 2), yet exhibits significant deviations from HWLE (Table 5), indicates that we do have sufficient statistical power, at least in some cases. The second null model, allowing single-locus deviations from HWE but still assuming linkage equilibrium, was rejected for six of the 12 ponds (Table 5). The skewed vernal pool (CVP) did not deviate significantly from this null model. These results were mildly sensitive to the inclusion/exclusion of *GNAT1* (the single nondiagnostic marker) and *HOXB13* and *DLX3* (the two weakly linked markers).

Do disequilibria vary among habitat types?—Deviations from HWE (F_{IS}) by pond type and locus are shown in Table 6 and Fig. 3. Significant deficits of heterozygotes (positive F_{IS}) were observed for the marker *GNAT1* in nine of 12 ponds, and the heterogeneity among ponds for this locus was not significant ($\alpha = 0.0125$ after sequential Bonferroni adjustment). F_{IS} estimates for other markers were variable among ponds (Table 6). However, pond type could account for a significant component of among-pond heterogeneity in only two markers: *HOXD8* for which ephemeral cattle tanks tended to have exceptionally high heterozygote frequencies, and *DLX3* for which vernal pools tended to have heterozygote deficits relative to HWE (Fig. 3). Heterogeneity at other loci is more idiosyncratic.

Linkage disequilibria between alleles derived from the same species are common (Figs. 2 and 4). Partitioning the pairwise associations showed that on average, all were, significantly positive (mean effect in the ANOVA framework of Langley et al. 1978). All were significantly heterogeneous in magnitude among ponds, but habitat type did not account for a significant portion of that heterogeneity in any single case. Skewed allele frequencies will tend to cause a down-

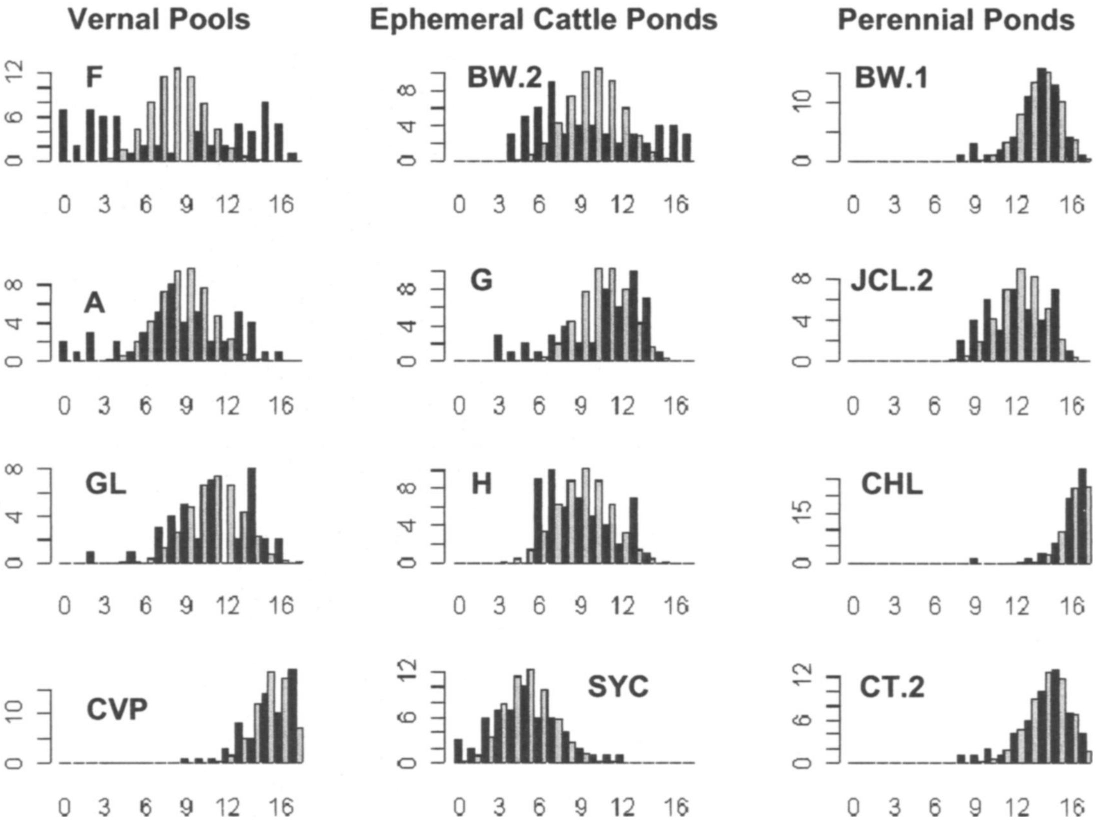

FIG. 2. Frequency distributions of the hybrid index (number of an individual's alleles derived from *Ambystoma tigrinum*). Black bars are observed and grey bars are expected counts for each value of the index under Hardy-Weinberg and linkage equilibrium (HWLE).

ward bias in estimates of LD (Lewontin 1988). Because perennial ponds tend to have primarily nonnative allele frequencies, these tests of variation among pond types are somewhat biased toward finding a pond-type effect (Fig. 4). Even so, we detected no significant pond type effects in LD. Detailed statistical results for any or all of the 36 pairwise disequilibria are available upon request from the senior author.

TABLE 3. Hierarchical AMOVA analyses of F-statistics for mtDNA and eight nuclear markers in 12 hybrid tiger salamander populations. F_{SC} summarizes variation among ponds within pond types, F_{ST} summarizes variation among ponds without regard to hierarchical structure, and F_{CT} summarizes variation among pond types. P-values for the AMOVAs were obtained from 10,000 permutation tests: * $P < 0.05$, *** $P < 0.001$. The heterogeneity test evaluates variation among nuclear markers by transforming F-statistics into χ^2-distributed test statistics as described in the text.

A. Single locus AMOVAs

Locus	F_{ST}	F_{SC}	F_{CT}
mtDNA	0.103***	0.116***	−0.015
HOXB13	0.322***	0.164***	0.189*
DLX3	0.390***	0.156***	0.277*
SLC4a	0.389***	0.280***	0.151
WNT1	0.302***	0.233***	0.09
FOXG1b	0.428***	0.361***	0.105
GNAT1	0.249***	0.260***	−0.014
GNAT2	0.415***	0.247***	0.223*
HOXD8	0.425***	0.408***	0.029

B. Multilocus analyses (nuclear markers only):

	F_{ST}	F_{SC}	F_{CT}
Pooled F	0.272***	0.192***	0.099*
Heterogeneity χ^2 (7 df)	577.1***	294.3***	105.9***
Excluding *GNAT1*			
Pooled F	0.290***	0.197***	0.116*
Heterogeneity χ^2 (6 df)	509.8***	252.8***	10.1

TABLE 4. Mixed effect model fitted by ordinal logistic regression with genotype (0, 1, or 2) as the dependent variable; mtDNA haplotypes were coded 0 for native and 2 for nonnative. The whole-model test compares the likelihood of the fitted model to a null model with no effects and to a saturated model (the latter tests whether the fitted model is adequate to explain the data). Locale refers to the two spatial blocks illustrated in Figure 1.

Whole-model test:

R^2	log likelihood	P vs. null (562 df)	P saturated vs. fitted (9556 df)
0.3574	−1825.46	<0.000001	0.999

Effect tests:

Source	DF	Wald χ^2	P
Pond type	2	34.12	<0.0001
Locus	8	652.24	<0.0001
Locale	1	7.85	0.0051
Pond within type, locale	4	16.17	0.0028
Individual within pond	521	1212.69	<0.0001
Locus × type	16	272.45	<0.0001
Locale × type	2	18.72	0.0001
Locus × locale	8	73.05	<0.0001

TABLE 5. Tests for deviation of the distributions of the additive hybrid index from two null distributions: Hardy-Weinberg and linkage equilibrium (HWLE) or linkage equilibrium only (LE). Summary statistics are G^2, tested via 10,000 Monte Carlo permutations.

	Pond	All markers	Excluding *GNAT1*	Excluding *HOXB13*	Excluding *DLX3*
Null model assuming HWLE					
Vernal	F	158.85***†	174.81***†	146.73***†	142.52***†
Pools	GL	27.36***†	15.81**†	18.52***†	16.31**†
	A	30.82***†	26.76***†	23.58***†	25.33***†
	CVP	24.47***†	16.58***†	21.10***†	20.69***†
Ephemeral	BW2	56.71***†	48.70***†	52.98***†	55.94***†
Cattle	G	34.35***†	21.50***†	35.08***†	29.89***†
Ponds	H	21.60***†	14.89*	26.51***†	18.00***†
	SYC	12.58*	11.96*	12.08*†	14.16*†
Perennial	BW1	8.42	10.97*	17.75***†	9.78*
Cattle	Jcl2	12.64*	5.16	11.98*†	9.18
Ponds	CHL	4.10	5.39*	3.02	5.76
	CT2	6.00	9.69*	6.20	6.67
Null model assuming LE					
Vernal	F	134.40***†	145.06***†	123.93***†	115.05***†
Pools	GL	26.63***†	16.31***†	16.93**†	17.33***†
	A	31.25***†	27.78***†	23.30***†	25.50***†
	CVP	11.06*	8.99*	7.43	8.97*
Ephemeral	BW2	61.45***†	55.09***†	60.42***†	60.48***†
Cattle	G	29.97***†	20.09***†	31.13***†	24.23***†
Ponds	H	28.13***†	22.38***†	36.18***†	25.11***†
	SYC	11.91*	12.36*	11.69*	14.00*
Perennial	BW1	9.39	11.42*	18.83***†	10.30*
Cattle	Jcl2	12.76*	6.25	11.02*	9.19
Ponds	CHL	4.10	5.78*	3.08	5.67*
	CT2	5.62	9.27*	6.05	6.45

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, † Significant after sequential Bonferroni correction.

DISCUSSION

Our analysis of multilocus hybrid genotypes in three kinds of breeding habitat emphasizes the importance of heterogeneous genotype × environment interactions in determining the dynamics of admixture in the tiger salamander system. The hybrid zone appears to be taking on a mosaic configuration, suggesting that exogenous selection causes habitat-dependent invasion success of nonnative alleles. Marker-specific deviations from Hardy-Weinberg expectations within ponds illustrate discordance in admixture dynamics across the limited part of the genome examined to date. A role for exogenous selection in generating some of this heterogeneity is suggested by the fact that two of the three marker-specific deviations from HWE are restricted to single habitat types.

The patterns described here are based on single samples from a single life-history stage (aquatic larvae). Therefore, our ability to generalize across the life cycle and across generations is limited. However, preliminary analysis of newer samples from three ponds (JCL2, $n = 47$; Pond G, $n = 32$; Pond H, $n = 44$) revealed no detectable changes in genotype frequencies for mtDNA and DLX3 over one to three years of sampling (B. M. Fitzpatrick and H. B. Shaffer, unpubl. data). Further, allele frequencies in a sample of adults taken from JCL2 in 1997 are consistent with allele frequencies in larvae reported here, suggesting that allele frequencies are relatively stable across generations and life-history stages. In the following discussion we explore several of the strongest patterns in our data and propose possible explanations for these patterns. Future studies will expand and test these ideas.

Mosaic Hybrid Zone Structure

The most straightforward of our results is the preponderance of nonnative alleles in perennial ponds (Fig. 2, Tables 3 and 4). This differentiation between perennial and ephemeral ponds exists despite very short physical distances between ponds of the different types, for example, about 300 m between BW1 and BW2 and about 600 m between JCL2 and pond F (Fig. 1). Both of these are well within observed

TABLE 6. Deviations from Hardy-Weinberg expectations (F_{IS}) by pond and by locus.

Pond	Marker							
	HOXB13	*DLX3*	*SLC4a*	*WNT1*	*FOXG1b*	*GNAT1*	*GNAT2*	*HOXD8*
Pooled F_{IS}	0.129**	0.121**	0.057	0.017	−0.097	0.372***	−0.029	−0.123**
ANOVA F-ratios (Langley et al. 1978)								
Pond type	2.35	15.61***	1.43	0.56	0.34	4.39	3.07	9.73***
Pond within type	9.37***	39.75***	6.05	2.92	2.16	6.55	11.1***	20.51***

Statistical significance: ** $P < 0.0125$ (Bonferroni critical value), *** $P < 0.001$.

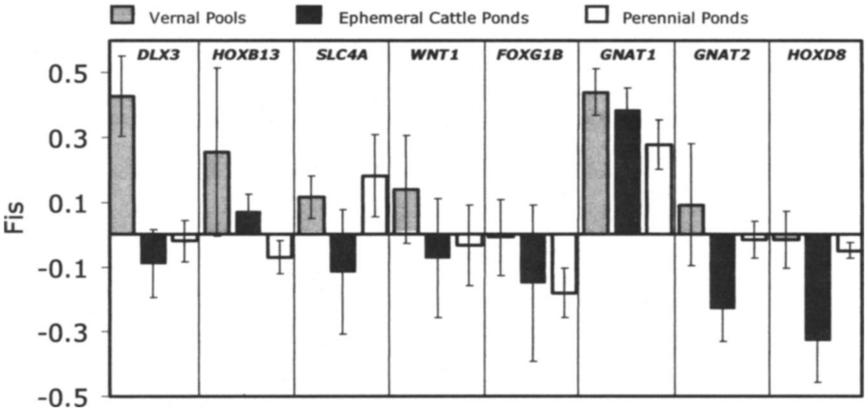

FIG. 3. Deviations from Hardy-Weinberg (F_{IS}) by marker and pond type. Means and standard error bars are shown for each.

movement distances for individual *A. californiense* (Trenham et al. 2001; Trenham and Shaffer, unpubl. ms.). Two features of perennial ponds provide plausible explanations for a nonnative advantage: (1) potential for a temporally extended breeding season, and (2) opportunity to engage the paedomorphic life-history strategy.

In their native range, *A. tigrinum* from the Great Plains and Rocky Mountains typically hibernate during the cold winter months and breed in late spring and early summer. However, in California's Mediterranean climate, *A. californiense* breed during the winter and early spring (Nov–March) when seasonal rains fill ephemeral pools (Trenham et al. 2000). The introduced *A. tigrinum* have adjusted their breeding phenology to match California's seasons. For example, we observed egg laying in the pure nonnative population near Clearlake Oaks in mid-February of 2002. Rose and Armentrout (1976) reported breeding by *A. tigrinum* in west Texas at any time of year when rainfall was sufficient to fill temporary pools, and this pattern appears typical of populations from the southern Plains (Petranka 1998). If their breeding phenology is more flexible than that of *A. californiense*, the introduced barred tiger salamanders may be able to take better advantage of perennial ponds by breeding earlier in the fall, thereby giving their larvae a competitive head start over later breeding native genotypes.

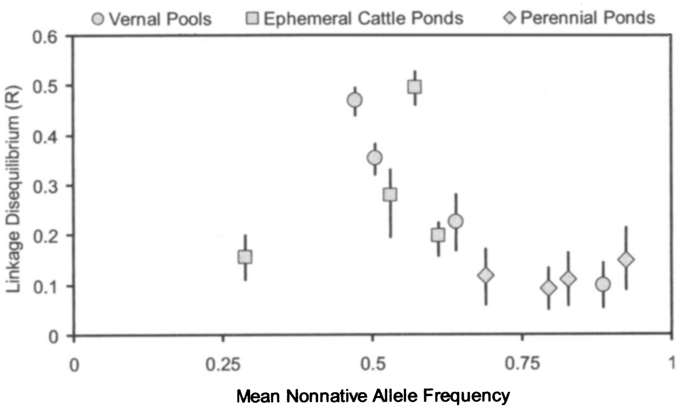

FIG. 4. Estimated linkage disequilibria (*R*) for each pond plotted against the mean nonnative allele frequency in each pond. Vertical lines span the likelihood support limits supplied by Analyse (Barton and Baird 2002). Circles are vernal pools, squares are ephemeral cattle ponds, and diamonds are perennial ponds.

Facultative paedomorphosis, a nonnative trait, is also likely to provide an evolutionary advantage in perennial ponds (Wilbur and Collins 1973; Whiteman 1994). Paedomorphs grow larger than metamorphs, and as a consequence paedomorphic females produce more eggs (Rose and Armentrout 1976; Petranka 1998). Paedomorphs may also tend to breed earlier in the fall than the incoming metamorphs, as in *A. talpoideum* (Scott 1993; Krenz and Sever 1995). This may provide larvae derived from paedomorphs with a competitive advantage (due to age and size) over larvae produced later in the season by metamorphic adults (Scott 1993).

Either or both of these mechanisms (extended breeding phenology and facultative paedomorphosis) would initially increase the relative fitness of nonnative alleles in perennial ponds, although only alleles in strong linkage disequilibria with selected loci would continue to increase in frequency via genetic hitchhiking over many generations (Maynard Smith and Haigh 1974). Paedomorphosis in the related *A. mexicanum* appears to be polygenic and largely recessive (Voss 1995; Voss and Shaffer 2000a,b). If true for facultative paedomorphosis in *A. tigrinum*, these factors may increase the efficiency of hitchhiking in the early generations of admixture because the benefits of paedomorphosis would have been limited to individuals with a high proportion of loci homozygous for nonnative alleles. It is noteworthy that the markers on linkage group 23, *DLX3* and *HOXB13,* seem to show the strongest differentiation between perennial and ephemeral habitats (Table 3a); this linkage group also includes a thyroid hormone receptor gene, the product of which is involved in the hormonal control of metamorphosis (Voss et al. 2000, 2001).

Without some opposing selection in neighboring ephemeral ponds, nonnative advantage in perennial ponds should result in high frequencies of nonnative alleles throughout the hybrid zone, particularly because perennial ponds tend to be larger and may have more consistent breeding and recruitment across years. In low rainfall years, paedomorphs in perennial ponds may be the only tiger salamanders that reproduce, as we observed for JCL.2 in 2001–2002. Immigrants from these more productive breeding sites should eventually swamp resident populations in ephemeral ponds. At estimated dispersal rates of 20–30% per generation (Trenham et al. 2001), a pure native ephemeral pond receiving immigrants from a pure nonnative perennial pond should become over 90% nonnative within 10–15 generations of one-way dispersal and neutral admixture (Long 1991). More symmetrical dispersal hastens the process of homogenization. Nonnative and native tiger salamanders have been interbreeding in the Salinas Valley for at least 50 years, corresponding to 12–25 generations (Petranka 1998; Trenham et al. 2000). This is ample time for allele frequencies to have become homogeneous if admixture were neutral. Thus, the disparities that we observe between ephemeral and perennial ponds in very close physical proximity are almost certainly maintained by opposing exogenous selection. In this respect, the hybrid zone between *A. californiense* and *A. tigrinum* resembles the mosaic hybrid zone model (Harrison and Rand 1989).

Disequilibria among Markers

The general pattern of association between conspecific alleles is consistent with (1) a polygenic model of hybrid dysfunction, (2) consistent immigration of pure genotypes into the zone, and (3) assortative mating based on highly polygenic traits. Polygenic hybrid dysfunction is expected to maintain genome-wide disequilibria because every marker is likely to be partially linked to a number of genetic factors, each contributing a very small amount to the decreased fitness of hybrids (Barton and Gale 1993; Naviera and Maside 1998). Immigration of parental (or more parental-like) genotypes into mixed populations maintains disequilibria by renewing admixture in each generation. Natural immigration of pure nonnatives is unlikely unless a pure nonnative population was established somewhere in central California. Based on extensive fieldwork, we believe that no such population exists (B. M. Fitzpatrick and H. B. Shaffer, unpubl. ms.). Repeated introductions continuing into the 1980s or 1990s would mimic waves of immigration, however, the bait dealer we interviewed confirmed that introductions of *A. tigrinum* ceased by the end of the 1950s (Don Green, pers. comm. 2003). Perennial ponds with high levels of recruitment of nonnative-like individuals could act like local sources of nearly pure nonnative genotypes. This, in addition to immigration of pure natives from elsewhere in their natural range, would help renew disequilibria each generation. Thus, disequilibria may be maintained by the interplay of gene flow and local adaptation (exogenous selection) in the mosaic of different pond types, or by endogenous selection on recombinant genotypes within ponds. However, although such selection will slow the decay of linkage disequilibria, it will not do so indefinitely. Disequilibria are expected to decay and allele frequencies to gradually homogenize as neutral markers become increasingly independent of the true foci of selection. Our goal is to monitor these processes over next few decades with periodic resampling of the hybrid zone.

Tiger salamanders in California may have small and variable breeding populations in some years (Trenham et al. 2001). Therefore, genetic drift may have a substantial influence on variation in allele frequencies and disequilibria. This underscores the importance of replication for making defensible inferences in this system. The large amount of variation among ponds within pond types (Table 4) probably reflects the importance of drift and the idiosyncratic history of nonnative tiger salamander translocations. Nevertheless, if drift were the sole cause of linkage disequilibrium, then we should observe negative disequilibria as often as we see associations between conspecific alleles. Average linkage disequilibria are significantly greater than zero in all of our 12 ponds (Fig. 4), suggesting that drift has not overwhelmed deterministic causes of disequilibrium in this hybrid zone.

Heterogeneity in F_{IS} among Markers

We have clearly demonstrated strong heterogeneity of Hardy-Weinberg disequilibria, F_{IS} (Table 6, Fig. 3). These dramatic marker-specific patterns suggest that there are genomic regions with large, disparate effects on hybridization dynamics. Because our samples consist of young-of-the-year larvae from landlocked ponds, we can eliminate immigration as a cause of deviations from HWE. Other potential causes of Hardy-Weinberg disequilibria within ponds include: (1) temporal differences in breeding times of adults with alternative genotypes (that is, temporal assortative mating); (2) nonrandom mating at any given time; (3) assortative fertilization (e.g., via gamete interaction or maternal ''sperm choice''); (4) heterozygote disadvantage; and (5) low mean fitness of heterozygotes as a byproduct of complex epistatic interactions between loci.

The habitat-dependence of F_{IS} for *DLX3* and *HOXD8* makes variation in breeding phenology and nonrandom fertilization seem unlikely explanations. The cues used by amphibians to emerge from their underground retreats in a Mediterranean climate are poorly understood. It is probably determined in part by physiological clocks and in part by how individuals experience the weather and other seasonal stimuli in their subterranean terrestrial habitat, rather than by the pond in which they will breed. Nonrandom fertilization seems unlikely to be affected much by the pond environment, although spermatophores may spend several minutes in the open water prior to internal fertilization (Arnold 1976; Howard et al. 1997). Other factors are more likely to have strong environment dependence. For example, visual or olfactory mate choice systems may be disrupted in the turbid, eutrophic water of artificial ponds, potentially explaining why there is a deficit of *DLX3* heterozygotes only in the less turbid vernal pools. Alternatively, components of the physical or biotic environment may cause stronger viability selection on embryos and young larvae in vernal pools. The habitat-dependent heterozygote excess at *HOXD8* could arise because cattle ponds may present immunological challenges that would be unusual in cleaner vernal pools, leading to balancing selection or heterosis in gene regions involved in pathogen response.

These environment dependent deviations from HWE illustrate two different views of the relationship between hybridization dynamics and environmental disturbance or habitat degradation. The deficit of *DLX3* heterozygotes in natural vernal pools but not in artificial ponds is consistent with the idea that reproductive isolation often breaks down in novel or degraded environments, perhaps because ecological differentiation between the hybridizing taxa is only relevant in natural habitats (Anderson 1948; Mayr 1963; Moore 1977; Kruuk and Gilchrist 1997; Orr and Smith 1998; Schluter 1998). The excess of *HOXD8* heterozygotes in ephemeral cattle ponds is consistent with the perspective that hybridization is an important source of genetic variability from which new selection pressures may distill novel adaptive phenotypes (Anderson 1948; Anderson and Stebbins 1954; Lewontin and Birch 1966; Grant and Grant 1996; Arnold 1997; Arnold and Emms 1998; Rieseberg et al. 1999a). These are not mutually exclusive views. The former emphasizes mechanisms that break down barriers to hybridization, while the latter focuses on potentially creative consequences of admixture.

The deficit of heterozygotes at the *GNAT1* marker was consistent across ponds, further emphasizing that endogenous forces may select against admixture in some gene regions. This leaves open any explanation that does not vary significantly with habitat. The only effect of polymorphism for the *GNAT1* marker in nonnative populations would be to decrease the expected deviation from Hardy-Weinberg proportions at the marker by decreasing linkage disequilibrium between the marker and the selected site. A preliminary laboratory experiment suggested that the deficit of *GNAT1* heterozygotes is caused by hybrid dysfunction during embryogenesis (B. Fitzpatrick, unpubl. data).

Conclusions and Future Directions

We analyzed the distributions of genotypes of mtDNA and eight nuclear DNA markers within and between ponds of varying ecological types in a tiger salamander hybrid zone. We found abundant evidence of constraints on admixture between *A. californiense* and *A. tigrinum mavortium*. However, the overall pattern seems to be one of differential success of various hybrid genotypes rather than overall low hybrid fitness. Potentially pure genotypes (hybrid index of 0 or 15) were rare except in those ponds with extremely skewed allele frequencies (Fig. 2). Patterns vary strikingly among markers and among ponds. Our results suggest that ecology plays a major role in the dynamics of secondary contact between these salamanders and that admixture dynamics vary substantially among different regions of the genome. These conclusions emphasize that the evolutionary consequences of admixture are best understood by studying hybrid genotypes rather than pedigrees (Rieseberg and Linder 1999). As the tiger salamander genome becomes increasingly well understood (http://salamander.uky.edu/), our work with a handful of genes hints at the potential for true genome-level population biology, in which a significant fraction of the genome can be evaluated for evolutionary trends across variable environments. Stay tuned.

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