

Notes and Discussion

Possible Phenotypic Influence of Superinvasive Alleles on Larval California Tiger Salamanders (*Ambystoma californiense*)

ABSTRACT.—California tiger salamanders (*Ambystoma californiense*) exhibit an obligate metamorphic life history strategy in which aquatic larvae metamorphose into terrestrial juveniles early each summer. However, we have recently documented two occurrences of populations containing large gilled California tiger salamander larvae during the winter months. Large gilled larvae could be the result of genetic admixture between California tiger salamanders and an introduced congener, the barred tiger salamander (*Ambystoma tigrinum mavortium*), but the population occurrences we observed are located outside the presumed extent of the *A. californiense*/*A. t. mavortium*–hybrid swarm. We used single nucleotide polymorphisms (SNPs) to investigate the possibility the genomes of these atypical larvae have been introgressed by “superinvasive” loci that have escaped from the established hybrid swarm. We also discuss the possibility the overwintering larval phenotypes may be the result of increased prevalence of large, fishless, semipermanent ponds, or of a combination of both introgression and habitat modification.

INTRODUCTION

When confronted with variation in environmental conditions, some salamander species are able to respond with phenotypic plasticity in life history strategies by altering the timing or rate of their developmental morphologies (Wilbur and Collins, 1973; Wilbur, 1980; Whiteman, 1994; Schlichting and Pigliucci, 1998). For example when larval growth is slowed by poor forage, low water temperatures, a short growing season, and/or competition due to high larval densities, some pond breeding salamander species delay sexual maturity and remain in the larval stage for up to 2 y before undergoing metamorphosis (Zug, 1973; Sexton and Bizer, 1978; Stebbins, 1985; Stebbins and Cohen, 1995; Alvarez, 2004) and metamorphose at potentially larger than typical sizes. Alternatively, when conditions in the aquatic environment are good (*e.g.*, high prey abundance, low larval densities, high temperatures) and growth rates are high, some species can employ a paedomorphic life history strategy (Semlitsch, 1987; Ryan and Semlitsch, 1998; Denoel and Joly, 2001; Denoel *et al.*, 2005) in which larvae forego metamorphosis altogether and become reproductively mature adults that exhibit larval morphological characteristics, such as external gills and extensive tail fins. The ability to exhibit such heterochrony in life history processes has played an important role in the evolution of many groups of salamanders, including the members of the Ambystomatidae, and more specifically, certain members of the tiger salamander complex (Collins, 1981; Routman, 1993; Whiteman *et al.*, 1996). The California tiger salamander (*Ambystoma californiense*), however, has not been documented to use the paedomorphic strategy. Historically a vernal pool specialist, *A. californiense* has been obligated under selective pressure to metamorphose and assume a terrestrial morphology as vernal pools have dried up (Twitty, 1941; Petranka, 1998; Riley *et al.*, 2003; Shaffer *et al.*, 2004; Shaffer and Trenham, 2005).

In recent history two phenomena occurred in California that have the potential to influence life history strategies in *A. californiense*. (1) Large numbers of small reservoirs were constructed for livestock and to protect developing urban areas from floodwaters (Watershed Protection and Flood Prevention Act of 1954) even as, simultaneously, native vernal pool habitat was being lost to agriculture. As native vernal pool habitats were converted to agricultural fields, *A. californiense* began using the new, often perennial, constructed reservoirs (hereafter referred to as stock ponds) for breeding. (2) Hybridization between *A. californiense* and *A. tigrinum mavortium* began in California in the 1940s (Riley *et al.*, 2003), when *A. t. mavortium* was imported from Texas and New Mexico and released into ponds in the Salinas Valley (Monterey County; Fig. 1) (Johnson *et al.*, 2011). Until a few years ago, the Salinas Valley hybrid swarm appeared to be restricted to sites in close proximity to the original *A. t. mavortium* introductions (Fitzpatrick and Shaffer, 2007). Recent investigations, however, found three unlinked alleles of introduced *A. t. mavortium* ancestry have moved far (approximately 90 km) beyond the presumed northern limit of the

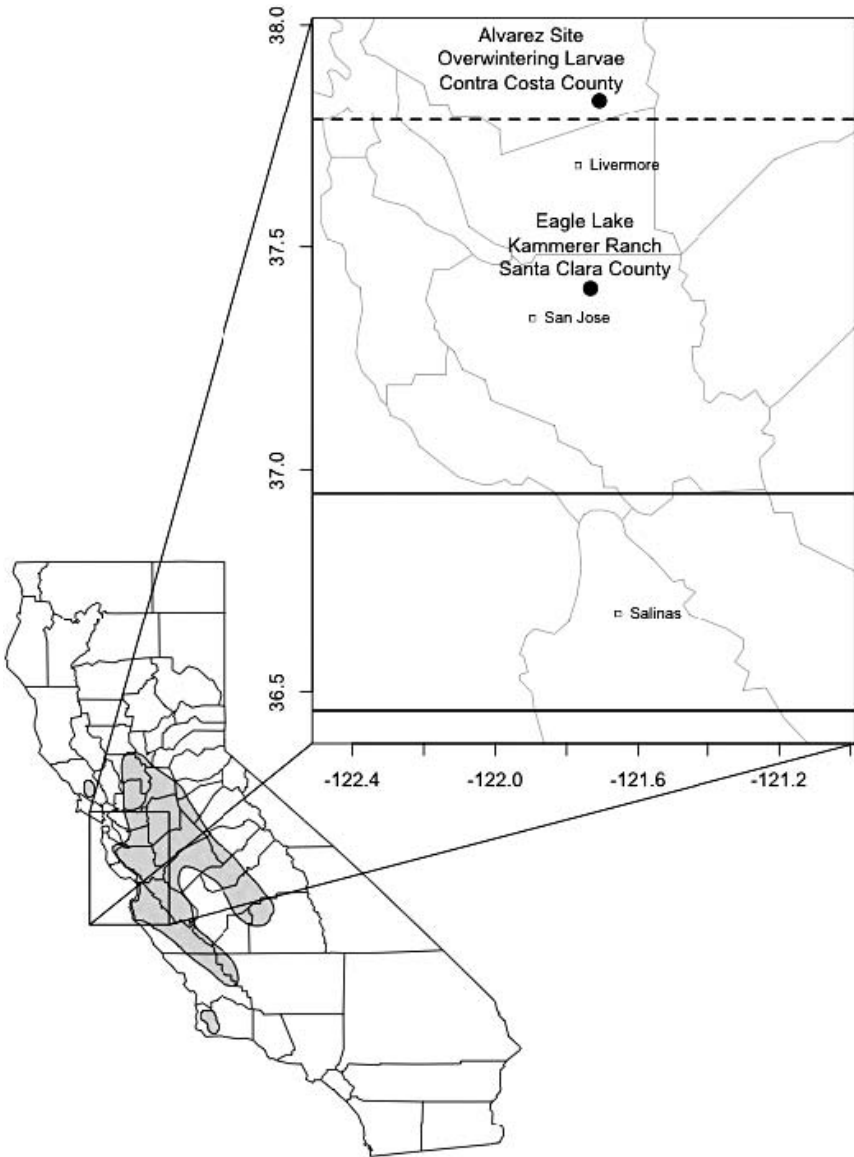


FIG. 1.—The position of the Contra Costa County, California (Alvarez, 2004) overwintering larvae and the Santa Clara County, California overwintering larvae. Shaded area depicts the known range of *A. californiense*. Inset: dashed line depicts northerly extent of “superinvasive” alleles; solid lines bracket the Salinas Valley hybrid swarm

hybrid swarm and occur at very high frequencies (fixed, or nearly so) in wild populations of *A. californiense* with no other evidence of hybridization (Fitzpatrick *et al.*, 2009; Fitzpatrick *et al.*, 2010).

Overwintering *A. californiense* larvae from three perennial stock ponds within the Los Vaqueros watershed in Contra Costa County, California have previously been reported (Alvarez, 2004). Here we

report on an additional population of overwintering *A. californiense* larvae discovered in a deep perennial stock pond while conducting a habitat restoration project for California red-legged frogs (*Rana draytonii*) in nearby Santa Clara County, California. The Contra Costa County population is 5 km north of the northerly extent of the superinvasive alleles. The Santa Clara County population is 94 km north of the hybrid zone but well within the area where three invasive alleles were previously mapped (Fitzpatrick *et al.*, 2010). At both locations *A. californiense* is part of the threatened Central Valley Distinct Population Segment (USFWS, 2004).

Our goal was to take advantage of recently developed single nucleotide polymorphisms (SNPs; Fitzpatrick *et al.*, 2009) to examine the extent of genomic admixture between *A. californiense* and *A. t. mavortium* in these populations containing large gilled individuals and to ascertain the prevalence of the “superinvasive” alleles at each site.

METHODS

SANTA CLARA COUNTY

In mid Nov., 2008 we drained a large perennial stock pond (Eagle Lake, 37.406726; -121.732979; max depth = 11.8 m; surface area = 1.1 ha) on the Kammerer Ranch in northern Santa Clara County, California, for the purpose of eradicating mosquitofish (*Gambusia affinis*). The pond draining was timed to avoid *A. californiense* mortality and to occur after the observed metamorphosis of *A. californiense* in nearby ponds, but prior to the *R. draytonii* breeding season. We used a siphon made of joined sections of 15 cm PVC pipe to drain the bulk of the water over 5 d. On the final day, we used three 10 cm trash pumps to drain the last 60 cm of standing water in the basin. The mud slurry of the pond was then treated with swimming pool “shock” (KIK Pool Additives, Inc., 5160 East Airport Drive, Ontario, California, 91761) to eliminate any remaining *G. affinis*. With the introduction of shock, 28 gilled salamanders fled the slurry. We captured them immediately and placed them in a 20 L bucket with fresh water. Eleven of the smallest larvae died within a few minutes. We preserved tail clippings from these 11 larvae in 95% ethanol for genetic analysis and released the surviving larvae in a perennial stock pond located 100 m away.

CONTRA COSTA COUNTY

Overwintering *A. californiense* larvae were discovered during the winter of 1998–1999 in Contra Costa County, California (Fig. 1; Alvarez 2004) while conducting pond maintenance in three perennial stock ponds: G1 (37.790002; -121.718764; max. depth = 2.4 m; surface area = 0.24 ha); M2 (37.845715; -121.740525; max. depth = 1.5 m; surface area = 0.07 ha); and I10 (37.788766; -121.759346; max. depth = 5.1 m; surface area = 0.39 ha). One cm clips from the distal tips of the tail from the larvae in two of the stock ponds (n = 20) were preserved in 95% ethyl alcohol and analyzed genetically using the methods of Shaffer and McKnight (1996). These larvae were determined to be purely native *A. californiense*. For this study 10 of the Contra Costa County larvae from 1998–1999 were re-analyzed.

GENOTYPING

We chose single nucleotide polymorphism (SNP) markers that have been determined to be ‘ancestry informative,’ meaning that each SNP genotype can indicate the taxon of origin (*A. californiense* or *A. t. mavortium*) for the segment of the genome containing the SNP. Five of these loci (mtDNA, Ctg325, Gnat1, FoxG1b, HoxD8, and WNT1) were among the first to be developed for investigations of genetic admixture in the main body of the hybrid swarm in the Salinas Valley and had been successfully used repeatedly in previous studies (*e.g.*, Fitzpatrick and Shaffer, 2007). The remaining three loci (E23C6, E6E11, and E12C11) had recently been determined to have rapidly introgressed into populations farther north of the primary hybrid swarm (Fitzpatrick *et al.*, 2009, 2010), and we predicted they might show some non-native ancestry in the study ponds.

We extracted DNA from all tissue samples using a DNEasy extraction kit (Qiagen, Inc.) and amplified the loci of interest via a standard polymerase chain reaction (PCR). A second ‘single base extension’ PCR was performed to attach a fluorescently labeled terminator nucleotide to an oligonucleotide primer adjacent to the SNP site. Genotyping was accomplished using the Victor3 plate reader (Perkin-Elmer, Inc.), which measures the fluorescence of the terminator molecules to ascertain the incorporated nucleotide (A, G, C, or T) and reveal the genotypes.

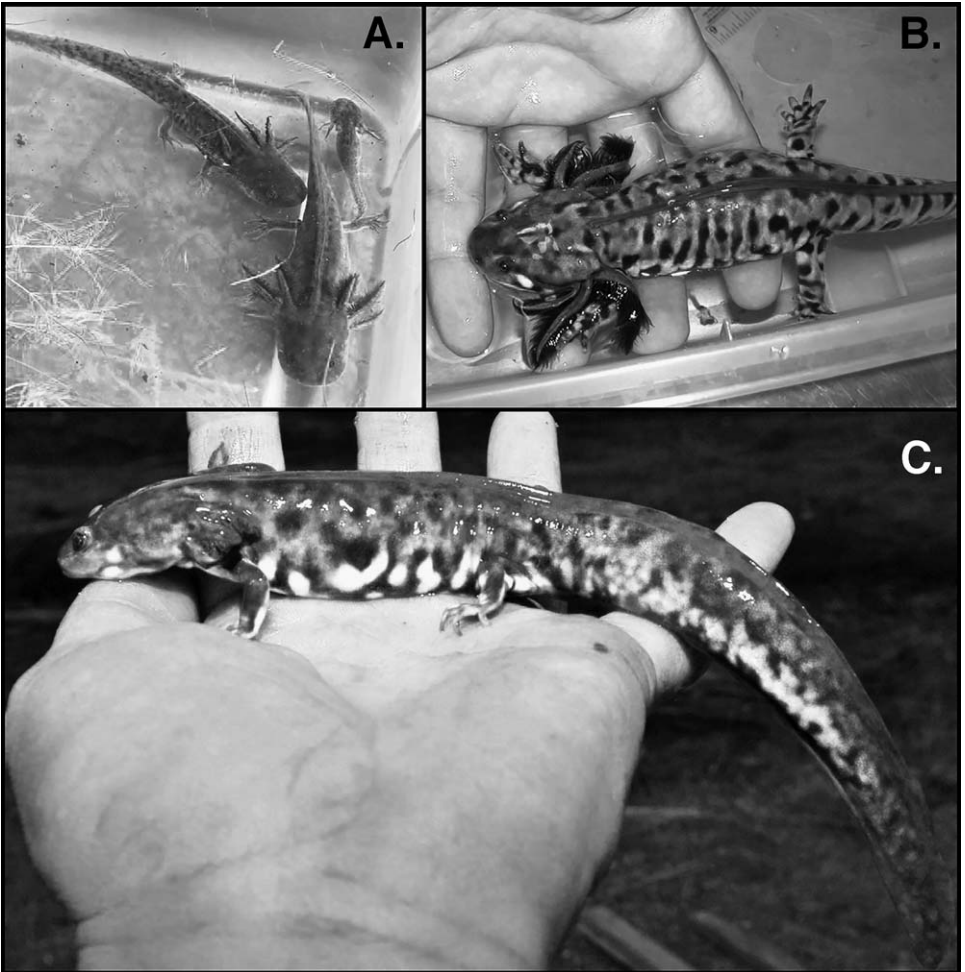


FIG. 2.—(A) Typical California tiger salamander larvae from Santa Clara County, California (~3.5 km S. of Eagle Lake), captured 16 May 2008; (B) Example of Salinas Valley hybrid swarm admixed individual larva, Monterey County, California; (C) Large larval tiger salamander (atypical in appearance) from Eagle Lake in northern Santa Clara County, California (15 Nov. 2008)

RESULTS

LARVAL PHENOTYPE

The 28 larvae we encountered in Santa Clara County fell into two distinct groups based on physical size and coloration. The more numerous ($n = 23$) and physically smaller (49–70 mm, SVL; maximum total length = 158 mm) group, from which we took the 11 tail clip samples, was characterized by deep gray green coloration with a pale yellow venter. With the exception of some unusual diffuse mottling, these individuals appeared typical of *A. californiense* approaching metamorphosis when observed in early summer at Grant Vernal Pool (Fig. 2A; hereafter we refer to these smaller larvae as “typical”). We did not sample larvae in the second group ($n = 5$), which were substantially larger in size (95–122 mm SVL; maximum total length = 241 mm) and khaki in color with a bright yellow venter mottled with black (Fig. 3; hereafter we refer to these larger larvae as “atypical”). Despite their large individual sizes, these

atypical individuals exhibited no external morphological characteristics (such as swollen cloacae) that would indicate they were in a sexually reproductive physiological state (*i.e.*, paedomorphic). They greatly resembled the highly admixed hybrid larvae normally encountered within the Monterey County hybrid swarm (JRJ, pers. obs.; Fig. 2B).

All larvae collected in Contra Costa County were phenotypically similar irrespective of pool location and microhabitat conditions. Coloration included a gray green to olive green dorsal surface with a pale yellow to gray yellow ventral surface, typical of *A. californiense* approaching late summer metamorphosis. Additionally, the larvae were within the size range (58–97 mm SVL; 126–144 mm maximum total length; Alvarez, 2004) of larvae observed prior to metamorphosis in the summer (Johnson *et al.*, 2010).

GENETIC ANALYSIS

Genetic analysis of the Santa Clara County larvae showed our 11 typical larvae contained three introduced nonnative alleles at multiple loci (Table 1). Eight of the 11 samples were homozygous for all three superinvasive alleles and all individuals were homozygous for at least two of the three superinvasive alleles (*sensu* Fitzpatrick *et al.*, 2009, 2010). Five individuals were heterozygous for a nonnative allele at one additional locus (WNT1).

We found the Contra Costa County larvae were completely native at the nine independent markers, with the exception of a single individual, which was heterozygous at a single superinvasive locus (Table 1). These data validate the original genetic results of Alvarez (2004) in which three molecular markers used by Shaffer and McKnight (1996) demonstrated that the larvae were likely not hybrids.

DISCUSSION

We found 28 gilled *A. californiense* in a deep (11.5 m) stock pond, Eagle Lake in Santa Clara County, during the month of Nov.—a period when terrestrial adults might be returning to ponds to breed but by which time all larvae from the previous winter breeding event should already have metamorphosed into terrestrial juveniles. We concluded that these 28 gilled salamanders were likely to overwinter as larvae, a behavior previously reported by Alvarez (2004).

The atypical larvae found in Santa Clara County could be: (1) overwintering larvae representing a more rapidly growing cohort relative to the typical larvae; (2) larvae that overwintered for more than 1 y; (3) a cohort that had hatched earlier than the typical larvae in the same season but developed at a similar rate; or (4) the offspring of admixed hybrids. Multiple year overwintering is not unknown for amphibians (Bruce, 1972; Collins, 1979) and is commonly associated with environmental conditions such as increased competition, reduced food availability, and low water temperature (Collins, 1979). Low temperature conditions apply to the Eagle Lake population, due to the pond's depth, but poor larval diet and intraspecific competition do not; the stomachs of the larvae we collected were full of *G. affinis* (JTW, pers. obs.), which had existed in very high numbers before we drained the pond.

The atypical larvae from Eagle Lake shared many physical characteristics of larvae from the Salinas Valley hybrids, including their large size, blotching, and color patterns (Fig. 2). It is possible that this coloration is environmentally induced, simply a function of the shift to using deep perennial pools and adopting an uncommon overwintering strategy. Alternatively, larval coloration may be more variable than has been reported.

The genetic analyses indicate that the genomes of the Santa Clara County typical larvae are introgressed by the superinvasive alleles described by Fitzpatrick *et al.* (2010). This is not surprising, given Eagle Lake falls within the area north of the hybrid swarm that is predicted to contain the superinvasive pattern of introgression (Fitzpatrick *et al.*, 2009, 2010). Furthermore, the typical larval appearance of the individuals is common among the superinvasive haplotypes (JRJ, pers. obs.). This raises the question, however, of whether the presence of the superinvasive alleles contributes to the expression of an overwintering larval strategy. The outcome of genetic analysis of the Contra Costa County larvae using new genetic markers validates the original genetic results of Alvarez (2004), in which three molecular markers demonstrated that the larvae were almost certainly not hybrids. From these data we must conclude that the overwintering strategy is likely not dependent upon high levels of introgression at the superinvasive genes.

The genotyped typical larvae at Eagle Lake show clear molecular evidence introgression has occurred there (Table 1). These individuals have the genotype of hybrid larvae from admixed populations in the

TABLE 1.—Genotype data at nine markers for Eagle Lake samples (Kammerer Ranch, Nov. 2008) and Contra Costa County samples (Los Vaqueros Watershed, Oct., Nov., and Jan., 2004). Daggers denote “superinvasive” loci (SI); ‘a’ denotes native ancestry and ‘g’ denotes introduced ancestry for each diploid genotype. Hybrid index scores (HIS) are calculated as the proportion of non-indigenous alleles sampled from an individual’s genome. HIS values of 0 indicate pure native ancestry and values of 1 indicate pure nonnative ancestry. HIS has been calculated here two ways: “with” the SI loci and “without”.

Locality	ID #	mtDNA	Cig325	Gnat1	FoxG1b	HoxD8	WNT1	Marker		HIS		
								E29C6†	E6E11†	E12C11†	With SI	Without SI
Eagle Lake, Kammerer Ranch, Santa Clara County, CA	117949	a	aa	aa	aa	aa	ag	gg	aa	gg	0.294	0.059
	117950	a	aa	aa	aa	aa	ag	gg	gg	gg	0.412	0.059
	117951	a	aa	aa	aa	aa	aa	gg	gg	gg	0.353	0.000
	117952	a	aa	aa	aa	aa	ag	gg	gg	gg	0.412	0.059
	117953	a	aa	aa	aa	aa	aa	gg	gg	gg	0.353	0.000
	117954	a	aa	aa	aa	aa	aa	gg	aa	gg	0.235	0.000
	117955	a	aa	aa	aa	aa	ag	gg	gg	gg	0.412	0.059
	117956	a	aa	aa	aa	aa	aa	gg	gg	gg	0.353	0.000
	117957	a	aa	aa	aa	aa	aa	gg	gg	gg	0.353	0.000
	117958	a	aa	aa	aa	aa	aa	gg	aa	gg	0.235	0.000
	117959	a	aa	aa	aa	aa	ag	gg	gg	gg	0.412	0.059
	Los Vaqueros Watershed, Contra Costa County, CA	100361	a	aa	aa	aa	aa	aa	aa	aa	aa	0.000
100362	a	aa	aa	aa	aa	aa	aa	aa	aa	aa	0.000	0.000
100363	a	aa	aa	aa	aa	aa	aa	aa	aa	aa	0.000	0.000
100364	a	aa	aa	aa	aa	aa	aa	aa	aa	aa	0.000	0.000
100365	a	aa	aa	aa	aa	aa	aa	aa	aa	aa	0.000	0.000
100366	a	aa	aa	aa	aa	aa	aa	aa	aa	aa	0.000	0.000
100367	a	aa	aa	aa	aa	aa	aa	ag	aa	aa	0.059	0.000
100368	a	aa	aa	aa	aa	aa	aa	aa	aa	aa	0.000	0.000
100369	a	aa	aa	aa	aa	aa	aa	aa	aa	aa	0.000	0.000
1E + 05	a	aa	aa	aa	aa	aa	aa	aa	aa	aa	0.000	0.000

“superinvaded” region north of the hybrid swarm, but the phenotype of individuals that more closely resemble larvae of the pure native *A. californiense*. This may indicate the different morphology exhibited by the atypical larvae (not sampled) has been induced by additional nonnative alleles, or by native alleles already present in populations of *A. californiense*.

The abundance of stock ponds in this area of California may provide a release from selective pressures that obligated *A. californiense* to metamorphose to a terrestrial morphology as vernal pools dried each year. Therefore, our discovery of overwintering larvae in multiple localities indicates that *A. californiense* may be exhibiting an inherent phenotypic plasticity that was suppressed by habitat constraints, or that a recent genetic mutation has occurred which favors perennial pond use. The spillover of *A. californiense* from converted vernal pool habitats to constructed perennial stock ponds has favored exploitation of conditions suitable for overwintering. It is possible overwintering larvae exist in many deep, fishless, perennial ponds but have gone undetected because investigators’ surveys are biased toward searching ephemeral ponds and vernal pools, or the shallower edges of deeper ponds.

Our findings indicate *A. californiense* may be inherently more flexible in adapting to novel environmental changes than previously thought. We may be witnessing a behavioral change in life history that allows *A. californiense* to exploit a previously unused aquatic habitat type, a change that favors species survival as historic vernal pool habitat disappears. Managers and agency personnel should require protocol surveys of deep, fishless, perennial stock ponds when such aquatic habitats are located within known historic *A. californiense* range.

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