



# Lethal Effects of Water Quality on Threatened California Salamanders but Not on Co-Occurring Hybrid Salamanders

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**Abstract:** *Biological invasions and habitat alteration are often detrimental to native species, but their interactions are difficult to predict. Interbreeding between native and introduced species generates novel genotypes and phenotypes, and human land use alters habitat structure and chemistry. Both invasions and habitat alteration create new biological challenges and opportunities. In the intensively farmed Salinas Valley, California (U.S.A.), threatened California tiger salamanders (*Ambystoma californiense*) have been replaced by hybrids between California tiger salamander and introduced barred tiger salamanders (*Ambystoma tigrinum mavortium*). We conducted an enclosure experiment to examine the effects habitat modification and relative frequency of hybrid and native California tiger salamanders have on recruitment of salamanders and their prey, Pacific chorus frogs (*Pseudacris regilla*). We tested whether recruitment differed among genetic classes of tiger salamanders (hybrid or native) and pond hydroperiod (seasonal or perennial). Roughly 6 weeks into the experiment, 70% (of 378 total) of salamander larvae died in 4 out of 6 ponds. Native salamanders survived ( $n = 12$ ) in these ponds only if they had metamorphosed prior to the die-offs. During die-offs, all larvae of native salamanders died, whereas 56% of hybrid larvae died. We necropsied native and hybrid salamanders, tested water quality, and queried the California Department of Pesticide Regulation database to investigate possible causes of the die-offs. Salamander die-offs, changes in the abundance of other community members (invertebrates, algae, and cyanobacteria), shifts in salamander sex ratio, and patterns of pesticide application in adjacent fields suggest that pesticide use may have contributed to die-offs. That all survivors were hybrids suggests that environmental stress may promote rapid displacement of native genotypes.*

**Keywords:** agriculture, *Ambystoma*, *californiense*, die-off, pesticide, *tigrinum*

Efectos Letales de la Calidad del Agua sobre Salamandras de California Amenazadas pero no sobre Salamandras Híbridas Concurrentes

**Resumen:** *Las invasiones biológicas y la alteración del hábitat a menudo son perjudiciales para las especies nativas, pero es difícil predecir sus interacciones. El entrecruzamiento de especies nativas e introducidas genera genotipos y fenotipos nuevos, y el uso de tierras por humanos altera la estructura y química del hábitat. Tanto las invasiones como la alteración del hábitat crean nuevos retos y oportunidades biológicas.*

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En el intensivamente cultivado Valle Salinas, California (E.U.A.), la salamandra de California amenazada (*Ambystoma californiense*) ha sido reemplazada por híbridos de *A. californiense* y la salamandra introducida (*Ambystoma tigrinum mavortium*). Realizamos un experimento con encierros para examinar los efectos de la modificación del hábitat y de la frecuencia relativa de salamandras híbridas y nativas sobre el reclutamiento de salamandras y su presa, ranas (*Pseudacris regilla*). Probamos si el reclutamiento difirió entre clases genéticas de salamandras (híbridas o nativas) y el hidropertodo del cuerpo de agua (temporal o perenne). A seis semanas de iniciado el experimento, 70% (de un total de 378) de las larvas de salamandra murieron en 4 de 6 cuerpos de agua. Las salamandras nativas sobrevivieron ( $n = 12$ ) en estos cuerpos de agua solo si habían metamorfoseado antes de las declinaciones. Durante las declinaciones, murieron todas las larvas de salamandras nativas, mientras que 56% de las larvas híbridas murieron. Realizamos necropsias a las salamandras nativas e híbridas, probamos la calidad del agua y revisamos la base de datos del Departamento de Regulación de Pesticidas de California para investigar posibles causas de las declinaciones. Las declinaciones de salamandras, cambios en la abundancia de otros miembros de la comunidad (invertebrados, algas y cianobacterias), cambios en la proporción de sexos de las salamandras y patrones de aplicación de pesticidas en campos adyacentes sugieren que el uso de pesticidas pudo haber contribuido a las declinaciones. El hecho de que todos los sobrevivientes son híbridos sugiere que el estrés ambiental puede promover un rápido desplazamiento de los genotipos nativos.

**Palabras Clave:** Agricultura, *Ambystoma californiense*, declinación, pesticida, *tigrinum*

## Introduction

Endangered species are often affected by multiple stressors, including introduced species and habitat modification (Czech & Krausman 1997). When endangered and introduced species interbreed, genetic interactions present an additional problem for endangered species that could potentially further decrease the probability of persistence (Allendorf et al. 2001). New genetic variation may increase fitness in a changing landscape (Anderson 1948), but the loss of genetic distinctiveness is considered by some to be a form of lineage extinction (Rhymer & Simberloff 1996; Allendorf et al. 2001).

Interactions between hybridization and habitat alteration are often difficult to predict. Habitat modification can affect the likelihood of hybridization and relative fitness of different genotypes (Arnold 1997; Ellstrand & Schierenbeck 2000). Hybridization can increase the invasiveness of introduced alleles (i.e., their ability to spread rapidly) through recombination into locally adapted genotypes or production of transgressive phenotypes (Arnold 1997; Rieseberg et al. 1999; Ellstrand & Schierenbeck 2000). Finally, hybrid organisms themselves may generate novel selection pressures on native or endangered species (Sakai et al. 2001; Ryan et al. 2009).

We were interested in the effects of native-hybrid ecological interactions and habitat modification on recruitment of the California tiger salamander (*Ambystoma californiense*) and hybrid descendants of California tiger salamanders and barred tiger salamanders (*Ambystoma tigrinum mavortium*), which were introduced approximately 60 years ago to the Salinas Valley of California (U.S.A.) (Riley et al. 2003; Fitzpatrick & Shaffer 2007). The California tiger salamander is listed as threatened

under the U.S. and California Endangered Species Acts. Admixed populations of hybrid salamanders in the Salinas Valley occupy roughly 25% of the original range of the California tiger salamander, hereafter referred to as the hybrid zone (Fitzpatrick & Shaffer 2007). Tiger salamander populations in the Salinas Valley have the greatest concentration of introduced genes and are subject to structural and chemical modifications of ponds associated with cattle ranching and industrial farming (dredging of ponds, agricultural chemical inputs, increased nitrogen from cattle feces) that may affect relative fitness of native and recombinant genotypes. In particular, seasonally drying, ephemeral ponds that historically characterized many wetlands in the California Central Valley and foothills landscapes have been converted to perennial ponds that hold water all year. Tiger salamander populations in perennial ponds within the hybrid zone have consistently higher frequencies of introduced alleles than those in seasonal ponds (Fitzpatrick & Shaffer 2004, 2007). However, environmental effects on fitness are complex, resulting in apparent retention of low-fitness hybrid genotypes and in potential advantages of native genotypes in some seasonal ponds (Fitzpatrick & Shaffer 2004; Johnson et al. 2010).

We conducted a field experiment to test whether larval fitness differed between 2 genetic classes of tiger salamanders (hybrid or native) and between pond hydroperiod (seasonal or perennial). During our experiment, we observed die-offs of aquatic invertebrates, increases in algal cover, and high mortality of salamander larvae that were associated with low water quality in 4 of 6 study ponds. Thus, we expanded our analyses to examine the cause of die-offs and changes in pond community via necropsy of dead salamander larvae, water-quality testing, and determination of pesticide use surrounding experimental ponds.

## Methods

### Experimental Design and Monitoring

We collected wild California tiger salamander embryos from populations near the hybrid zone (Monterey and Alameda Counties), wild hybrid embryos from within the hybrid zone (Monterey County), and eggs and tadpoles of salamander prey, Pacific chorus frogs (*Pseudacris regilla*), from Monterey County. We reared salamander embryos in the lab in individual containers of 10% Holtfreter's solution until they reached approximately 1 g and then placed them in cylindrical mesh field enclosures (fully enclosed, 145 cm wide × 70 cm tall). Initial lab rearing prevented escape of tiny larvae from mesh enclosures and largely eliminated early developmental mortality due to genetic disruptions not related to environmental conditions (Ryan et al. 2009).

We conducted the experiment in mesh enclosures (described above) in 6 ponds: 3 seasonal ponds that dry during summer (approximately July–November) in most years and 3 perennial cattle ponds that hold water all year long in most years. On 13 March 2008, we placed 7 enclosures in each pond. Enclosures were partially exposed to air. On 17 March, we added 9 tiger salamander larvae to each treatment enclosure (treatments randomized) and to a control enclosure including only California tiger salamanders. We added 2 replacement series (combinations with shifting ratios of native California salamanders:hybrids) of 3:6, 6:3, and 0:9 California tiger salamanders:hybrids (see Supporting Information for schematic of experimental design and map of pond locations). One series was populated with hybrids collected from perennial ponds and the other with hybrids collected from seasonal ponds to test whether salamanders were locally adapted to pond hydroperiod. We added 125 frog tadpoles to each enclosure as prey. Mesh gauge (1 mm) allowed passage of small invertebrate prey (zooplankton, worms) and oviposition by insects.

We conducted weekly salamander counts and on 23 April photographed and measured all larvae with ImageJ (Rasband 2008). On weekly visits to ponds we estimated invertebrate abundances and noted types of invertebrates (e.g., cladocerans, copepods, dipterans, zygopterans, anisopterans, annelids) in enclosures and determined percent cover of algae and cyanobacteria in the pond. We also noted general condition of the skin, gills (for salamanders), and body (robust or skinny) of experimental tadpoles and salamander larvae and free-living larvae naturally present in the pond. These qualitative observations allowed us to detect gross changes in the presence and abundance of classes of organisms and to track condition of experimental animals. Following salamander die-offs, we also monitored aquatic insect densities by lifting enclosures from the water and sampling

sections (20 cm × 20 cm quadrats) of the base of each enclosure.

We measured and weighed each salamander upon metamorphosis or death (when individual was retrievable) and counted frogs at metamorphosis. One pond (Chualar Vernal Pool [CVP]) dried before all larvae transformed. When pond depth dropped to <5 cm, we moved larvae to the lab and counted those that did not metamorphose within a week as drying mortalities. In 4 ponds (2 seasonal [BW1 & JC] and 2 perennial [RH, CT2]), we observed sudden die-offs and signs of illness among salamander larvae following sudden disappearance of most aquatic invertebrates and blooms of algae and cyanobacteria. We refer to these 4 ponds as unstable due to these changes and our determination that salamander mortality was caused by water-quality changes (described below). The remaining 2 ponds are referred to as stable because changes that occurred in the unstable ponds did not occur in these ponds. Values of environmental covariates for experimental ponds are in Supporting Information.

### Genetic Analyses

We genotyped each salamander from enclosures including more than one genetic class of salamanders (3:6 or 6:3 California tiger salamanders:hybrids) with 4–6 species-diagnostic single nucleotide polymorphism markers (one mitochondrial and 3–5 mapped nuclear genes) (Smith et al. 2005) to determine ancestry (California tiger salamander or hybrid) (Fitzpatrick & Shaffer 2007).

### Necropsy and Pathogen Testing

We tested for 2 pathogens implicated in amphibian die-offs (Boone & Bridges 2003): ranavirus (including *Ambystoma tigrinum virus*) and *Batrachochytrium dendrobatidis* (Bd). We tested for ranavirus by extracting DNA from tail muscle tissues from each dead individual and following methods of Picco and Collins (2008). A polymerase chain reaction (PCR) product of approximately 500 base pairs was interpreted as confirmation of ranavirus infection, whereas uninfected individuals produced no PCR bands (Picco & Collins 2008). To test for Bd and bacterial infections, we cultured swabs from necropsied animals (Berger et al. 1998).

We performed necropsies on 2–5 moribund or dead salamanders per pond plus control animals from unaffected (stable) ponds. Many larvae were too decomposed to necropsy when we discovered them. We did not remove animals that appeared potentially capable of recovery. During necropsy, we noted externally visible signs of illness and examined tissues under a dissection scope for irregularities and macroparasites. We then processed

tissues routinely for wax embedding and sectioned them at 5  $\mu$ . We stained sections with hematoxylin and eosin and examined them by light microscopy for microparasites, assessed histological responses and tissue damage, and looked for signs of the effects of toxins. To determine the sex of salamanders, we examined gonads under a dissecting microscope for presence of ova (Smith & Voss 2009). We classified salamanders with gonads consisting of a membrane surrounding translucent ova as females and those with gonads consisting of opaque lobed testes as males. To test deviation from a 1:1 sex ratio among surviving salamanders, we performed a Fisher's exact test.

### Water-Quality Testing

For 4 weeks following die-offs, we tested pond water for common contaminants: nitrates, nitrites, general organic contaminants (gas chromatograph/mass spectrometer [GS/MS] screen for multiple classes of pesticides including pyrethroids), organophosphate and carbamate pesticides, and microcystins (neuro- and hepatotoxic by-products of decomposing cyanobacteria). We measured pH and temperature on each visit to a pond following die-offs (5–7 d apart) and ammonium on 2 subsequent visits (immediately following die-offs) in all ponds except CVP, which dried in early May prior to the majority of die-offs. Die-offs occurred twice, in April and June, in pond CT2. Single-event die-offs occurred in all other unstable ponds later in May (see Supporting Information for dates of die-offs).

We searched the California Department of Pesticide Regulation database (<http://www.cdpr.ca.gov/>), which stores mandatory reporting data on annual pesticide use in California, for pesticide use in grid cells within 1.6 km of each pond from March through July 2008 (Supporting Information).

### Statistical Analyses

All statistical analyses were performed as linear mixed-effect models fitted with restricted maximum likelihood (REML) in the lme4 package of R (Bates et al. 2008). We modeled individual survival as a categorical variable with a logit link and binomial error term (Bates et al. 2008). To test whether probability of survival was associated with experimental and observed factors (pond stability), we used a nested set of candidate models. We selected a set of candidate models a priori (Johnson & Omland 2004) on the basis of factors known to influence tiger salamander larval survival and life-history traits and their mechanistic interactions. We added stability as a post hoc experimental factor following observations of pond community changes and larval salamander die-offs and salamander size in April prior to die-offs as a covariate. A full list of candidate models is in Supporting Informa-

tion. For each individual analysis, we report only Akaike information criterion (AIC) values for the best-fit model and each nested model that includes a subset of the same terms (Supporting Information).

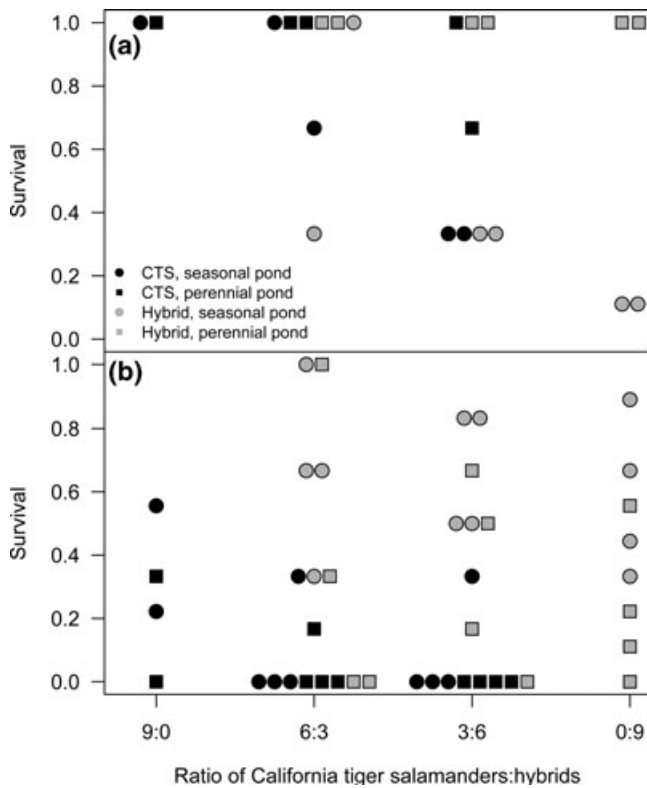
Hybrid frequency, pond hydroperiod (seasonal or perennial), natal pond type from which embryos were collected (seasonal or perennial), and pond stability (stable or unstable) were fixed effects. We analyzed hybrid frequency as a continuous variable to maintain information held in the ordered relation among frequencies. (Results were the same when hybrid frequency was treated as categorical.) Individual pond and cage within pond were random effects. We log-transformed mass and time to metamorphosis because linear models were a better fit to log-transformed data than to untransformed data. We modeled number of frogs surviving to metamorphosis per enclosure with a log link and Poisson errors in mixed models with fixed effects as above and individual pond as a random effect. Best-fit models were identified as those with minimum AIC. For models with similar AIC, we used likelihood ratio tests to test for significant differences among models. Results are presented as standard likelihood ratio tests of more complex models (including fixed and random effects) versus the simpler model including only random effects (Gotelli & Ellison 2004). Where appropriate, we used the Shapiro-Wilk test for normality and Levene test for homogeneity of variance.

## Results

### Early Patterns and Pond Perturbations

By 23 April, hybrid larvae exceeded California tiger salamanders in size: mean California tiger salamander snout-vent length was 31.5 mm versus 37.4 mm for hybrids ( $\Delta$ AIC = 170.2 when genetic class was added to the random-effects model; likelihood ratio test versus null:  $\chi^2 = 172.12$ ,  $df = 1$ ,  $p < 0.001$ ; California tiger salamander  $n = 162$ , SE 2.41; hybrid  $n = 216$ , SE 0.42). The best-fit model included additive effects of genetic class and enclosure-level hybrid frequency (0, 3, 6, 9) and their interaction (AIC = 2030.5, test vs. null:  $\chi^2 = 188.85$ ,  $df = 3$ ,  $p < 0.001$ ). California tiger salamander size declined more than hybrid size as hybrid frequency increased. For this and all analyses below, see Supporting Information for candidate model results.

Between 17 April and 15 May, we began noticing changes in 2 seasonal (BW1, JC) and 2 perennial ponds (RH, CT2). Events progressed similarly in these ponds: aquatic invertebrates that previously were abundant ( $\gg 1000$ /enclosure) one week were suddenly absent the following week; salamander condition deteriorated (skin thinning, weight loss); and algal and cyanobacterial biomass increased dramatically (from thin film to thick mats). Generally 1 week after onset of these observed



**Figure 1.** Proportional survival of tiger salamanders in (a) stable ponds (Chualar Vernal Pool and pond LM2) and (b) unstable ponds (ponds BW1, JC, RH, and CT2) at different ratios of California tiger salamanders to hybrid salamanders in an enclosure (CTS, California tiger salamander; seasonal and perennial, hydroperiod of experimental ponds; points, values for individual enclosures). Unstable ponds are those where we observed salamander die-offs and signs of illness following algal blooms and disappearance of aquatic invertebrates. Stable ponds are those where we did not observe these changes. Effects table for best-fit model is in Table 1.

changes, a pulse of salamander mortality (large numbers of dead larvae following very minimal prior mortality) accompanied signs of illness (emaciation, skin lesions, gill abnormalities, impaired motor function) in surviving animals. Two to 3 weeks after onset of changes, surviving salamanders recovered to robust condition, invertebrate populations rebounded, and algal and cyanobacterial cover declined. All mortality in unstable ponds occurred during these die-offs. Except for one cannibalized animal, all mortality in stable ponds was related to drying.

**Tiger Salamander and Frog Responses**

The best-fit logistic regression model of individual salamander survival for all ponds included size (snout-vent

**Table 1.** Effects associated with best-fit logistic regression model for tiger salamander survival across all ponds.<sup>a</sup>

Term <sup>b</sup>	Coefficient	SE	Z	p
Intercept	-8.60	1.99	-4.32	<0.001
Genetic class (hybrid)	-3.56	0.85	-4.21	<0.001
Stability (unstable)	-6.10	1.33	-4.60	<0.001
Hybrid frequency	-0.25	0.08	-3.06	0.002
Size of larva	0.40	0.06	6.84	<0.001
Genetic class × stability	5.40	0.93	5.80	<0.001

<sup>a</sup>Model includes random effects of pond and cage within pond.  
<sup>b</sup>In the stability term, unstable refers to ponds where we observed salamander die-offs and signs of illness following algal blooms and disappearance of aquatic invertebrates. Stable ponds are those where we did not observe these changes.

length on 23 April), hybrid frequency (0, 3, 6, or 9), individual genetic class, pond stability, and a genetic class × stability interaction (AIC = 243.74, test versus null:  $\chi^2 = 167.23$ , df = 5,  $p < 0.001$ ) (Fig. 1 & Table 1). Overall, 36.4% of California tiger salamanders survived, the majority of which were from stable ponds, versus 51.4% of hybrids. Larger individuals were more likely to survive (Table 1), and survival was lower in unstable ponds (29.9% in unstable ponds vs. 74.8% in stable ponds) and in cages with higher frequencies of hybrids (Fig. 1). Also, the relative difference in survival between California tiger salamander and hybrids was reversed by pond instability (Table 1), largely due to the ability of greater numbers of California tiger salamanders to successfully metamorphose in the fast-drying, stable CVP relative to hybrids. The significant genetic class by instability interaction term supports the inference that pulses of mortality in unstable ponds disproportionately affected California tiger salamander larvae. Increased mortality of California tiger salamander larvae thereby enhanced the relative fitness of hybrids (Table 2).

Considering only unstable ponds, the best-fit model included genetic class, hybrid frequency, and size and

**Table 2.** Estimates of relative fitness of native California tiger salamanders in stable versus unstable ponds.<sup>a</sup>

	Relative fitness	
	stable <sup>a</sup>	unstable <sup>a</sup>
CTS only <sup>b</sup>	1.286	0.761
Mixed (6 CTS:3 hybrids) <sup>c</sup>	1.031	0.205
Mixed (3 CTS:6 hybrids) <sup>c</sup>	0.913	0.136

<sup>a</sup>Unstable refers to ponds where we observed salamander die-offs and signs of illness following algal blooms and disappearance of aquatic invertebrates. Stable refers to ponds where we did not observe these changes.

<sup>b</sup>California tiger salamander (CTS) survival in enclosures with only CTSs divided by average survival in enclosures with only CTSs and enclosures with only hybrids.

<sup>c</sup>California tiger salamander (CTS) survival in enclosures with both CTSs and hybrids, divided by average survival in enclosures containing both genetic classes. Relative fitness of 1.0 indicates equal fitness of native CTSs and hybrid salamanders.

included positive coefficients for both size and hybrid genotype (AIC = 195.09, test vs. null:  $\chi^2 = 100.35$ ,  $df = 3$ ,  $p < 0.001$ ). Size was strongly associated with survival for both genetic classes, but for different reasons. Large hybrid larvae tended to survive die-offs and associated changes in pond conditions, whereas large California tiger salamanders, mainly from California tiger salamander-only enclosures, metamorphosed prior to these events. There was no support for including hybrid natal source type in survival models.

Effects of experimental factors on California tiger salamander life-history traits were difficult to assess due to extremely low (11%) survival of California tiger salamanders in unstable ponds. Hybrids emerged substantially larger than California tiger salamander from all ponds (Fig. 2). The best-fit model for salamander mass at metamorphosis included all additive and interactive effects of genetic class, hydroperiod, and pond stability (AIC = 114.13, test vs. null:  $\chi^2 = 134.68$ ,  $df = 6$ ,  $p < 0.001$ ).

Free-living and experimental frog tadpoles retained robust physical and behavioral condition throughout the experiment and survival did not appear to be directly affected by pond perturbations. The full model including all interactions was the best fit (AIC = 113.38, test vs. null:  $\chi^2 = 32.712$ ,  $df = 7$ ,  $p < 0.001$ ) and was not improved by including final tiger salamander density. Hybrid frequency had the greatest magnitude of effect: very few (mean < 3) frogs emerged from enclosures containing any hybrid larvae, whereas significant numbers (mean > 26) survived in treatments with only California tiger salamanders (Supporting Information).

### Potential Causes of Die-Offs

Necropsy results were consistent with effects of poor water quality, on the basis of known causal associations between water-quality changes and salamander signs of illness, and suggested a chemical stressor. We found no support for a causal role of disease, parasitism, or natural pond turnover in die-offs (Supporting Information). Compared with control specimens from stable ponds, sick and dying larvae exhibited common signs of illness consistent with stress and starvation. All individuals exhibited premature gill involution, which can be induced by stress or exogenous chemicals (Armstrong & Malacinski 1989). Premature gill involution created hypoxic animals with underdeveloped lungs that could not respire properly. All specimens were starving; had empty stomachs, depleted liver glycogen, no fat bodies (anatomical structures that store fat); exhibited signs of metabolic dysfunction; and showed cystic degeneration of Hassall's corpuscles typically associated with stress. Salamander sex ratios also shifted following die-offs. Early metamorphs and individuals from stable ponds did not significantly deviate from 1:1 sex ratio. The post-die-off sex ratio was significantly skewed toward morphological females (6 males: 37 fe-

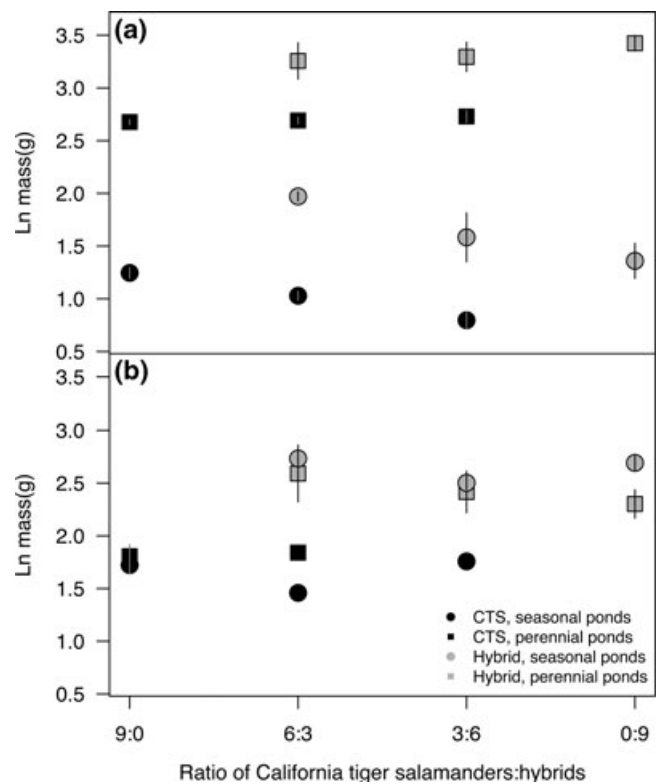


Figure 2. Tiger salamander mass at metamorphosis in (a) stable ponds (Chualar Vernal Pool, pond LM2) and (b) unstable ponds (ponds BW1, JC, RH, CT2) at different ratios of California tiger salamanders to hybrid salamanders in an enclosure (CTS, California tiger salamander; points, mean values of mass at metamorphosis in enclosures averaged across survivors; lines, SE; points with no line, one survivor; no symbol for a treatment type, no animals survived across all replicates). Unstable ponds are those where we observed salamander die-offs and signs of illness following algal blooms and disappearance of aquatic invertebrates. Stable ponds are those where we did not observe these changes.

males; Fisher's exact test:  $p = 0.02$ , odds ratio = 3.56). All necropsied individuals were morphological females, which indicated skewed sex ratios were not caused by sex-biased mortality.

Water-quality tests did not indicate high levels of nitrates or nitrites, with the exception of one sample from pond CT2 that showed high nitrate levels (14 mg/L). Water-quality tests also failed to detect pyrethroid, carbamate, organophosphate pesticides, or microcystins. The total number of registered applications of bioactive chemicals within 1.6 km of unstable ponds from March–July 2008 ranged from 1150 to 3067; 646–1626 applications were made prior to die-offs. In contrast, 48 applications were made around stable perennial pond LM2 (located

on an organic ranch), and 169 were made around stable CVP (in a residential subdivision) prior to its drying in early May. The most frequently used chemicals at 5 sites were carbamates (carbaryl, maneb, methomyl, propamocarb hydrochloride), organophosphates (chlorpyrifos, diazinon, malathion, oxydemeton-methyl), and pyrethroids (lambda-cyhalothrin, permethrin). None of these chemicals were used near LM2, where an herbicide (copper hydroxide) was the most commonly used chemical.

## Discussion

Our findings illustrate the severe costs to recruitment resulting from reductions in California tiger salamander growth rates in the presence of hybrids. Results of previous mesocosm experiments show the negative effects of cohabitation with hybrid tiger salamanders on California tiger salamander growth, time to metamorphosis, and survival (Ryan et al. 2009). In our study, reduced growth rates in the presence of hybrids led to increased exposure to adverse conditions and mortality in unstable ponds in particular.

Across all pond types, California tiger salamanders that shared enclosures with hybrids had lower survival than those that shared enclosures only with other California tiger salamanders. In stable pond CVP, California tiger salamanders were far more likely to metamorphose before the pond dried. However, both classes had low survival in mixed enclosures. In unstable ponds, a few large California tiger salamanders metamorphosed prior to pond perturbations, nearly all from enclosures with only California tiger salamanders; all others died. Hybrids were far more likely to survive. Although many hybrids died, larger hybrid individuals were more resilient to apparent changes in water-quality conditions and to loss of food because they were able to feed on anuran tadpoles during invertebrate die-offs. Hybrids also suffered lower mortality in response to food and water-quality perturbations regardless of size. This result suggests that other aspects of hybrid genotype contribute to their survival advantage over California tiger salamanders. Native California tiger salamanders had greater fitness than hybrids in stable ponds, but lower fitness than hybrids in unstable ponds (Table 2).

The outcome of these differences in survival was rapid conversion of experimental salamander populations in unstable ponds from nearly 50% California tiger salamanders to nearly 100% hybrids. In the wild, populations at the edge of the hybrid zone tend to be either 100% California tiger salamanders (or populations carrying a few superinvasive markers, see Fitzpatrick et al. [2010]) or highly admixed (Fitzpatrick & Shaffer 2004, 2007). This steep genetic gradient implies that California tiger salamander populations may be rapidly

converted to majority-hybrid populations with high frequencies of introduced alleles. In combination with phenotypic dominance of introduced alleles affecting growth and metamorphosis (Ryan et al. 2009; Johnson et al. 2010), our observations suggest a mechanism for rapid, episodic genetic change. Advantages of introduced alleles in agricultural regions may also help to explain the sharp drop in introduced allele frequencies as land-use shifts away from farming (Fitzpatrick & Shaffer 2007).

The mechanism underlying pond perturbations and associated die-offs is unknown. We suspect agricultural contamination. Signs of salamander illness were consistent with poor water quality, chemical exposure, and indirect effects of invertebrate prey die-offs. The lack of contaminant detection is inconclusive because nitrogen levels cycle rapidly, modern pesticides can break down within hours to days of application and do not bioaccumulate, and pesticide-induced trophic cascades can occur weeks after pesticides have degraded (Boone & Bridges 2003; Relyea & Diecks 2008). Pesticides reduce the abundance of invertebrate prey for obligate predators, including salamander larvae, and can interact with other stressors. Lack of signs of illness among Pacific chorus frogs is consistent with previous work on herbivorous tadpole response to pesticides (Boone & Bridges 2003; Fleeger et al. 2003; Relyea & Diecks 2008). Other indications that agricultural contamination may have led to illness include the observed shift in salamander sex ratio (some pesticides can feminize genetic male amphibians [Supporting Information] [Hayes et al. 2002]) and our observation that the only pond supporting robust salamander recruitment was on an isolated organic ranch exposed to chemicals considered less toxic to wild animals (mostly herbicides; no cholinesterase inhibitors).

Our unexpected findings suggest a possible mechanism for reduced fitness of a threatened species and highlight the potential importance of environmental pollutants and short-term ecological dynamics in the long-term outcome of hybrid invasion dynamics.

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## Supporting Information

Additional information on experimental methods, necropsy results and interpretation, and additional experimental findings (Appendix S1) are available online. The authors are solely responsible for the content and functionality of these materials. Queries (other than absence of material) should be directed to the corresponding author.

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