DECLINING DOWNWIND: AMPHIBIAN POPULATION DECLINES IN CALIFORNIA AND HISTORICAL PESTICIDE USE

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Abstract. Pesticides have long been proposed as a possible cause of amphibian population declines, but due to a number of challenges there has been relatively little ecotoxicological research on pesticides and declines in natural populations. My study examines the association between the spatial patterns of declines for five California amphibian species and historical patterns of pesticide use in California from 1974 to 1991 based on Department of Pesticide Regulation records. Information on declines was derived from maps of historical sites and current population status for the Yosemite toad (*Bufo canorus*), California red-legged frog (*Rana aurora draytonii*), foothill yellow-legged frog (*R. boylii*), Cascades frog (*R. cascadae*), and the mountain yellow-legged frog (*R. muscosa*). Multiple logistic-regression and generalized additive models were used to analyze the relationship between site status (present or absent) and total upwind pesticide use, 64 pesticide classes and groups, and covariates including precipitation, elevation, surrounding urban and agricultural land use, and spatial location. Total pesticide use was a strong, significant variable in logistic-regression models for all species, except *B. canorus*. Total pesticide use was a significant variable even when spatial autocorrelation was accounted for by inclusion of a spatial location covariate. Cholinesterase-inhibiting pesticides (most organophosphates and carbamates) stood out as more strongly associated with population declines than any other class of pesticides. This is the first study in which population declines of multiple declining species have been associated with historical pesticide applications.

Key words: amphibian decline; California (USA); carbamates; cholinesterase inhibitors; organophosphates; pesticides; spatial analysis; spatial autocorrelation.

INTRODUCTION

Assessing the role of pesticides in amphibian population declines presents scientists with difficult challenges. First, field studies of pesticide residues in the environment typically only analyze samples for a handful of different chemicals. Further, laboratory experiments on pesticide effects are usually done with only a single or a few pesticides. Yet several thousand different pesticides are currently in use or have been used in the recent past, overwhelming the ability of field studies to detect and laboratory experiments to assess the effects of even a small fraction of pesticides. Second, the phenomenon of amphibian population declines occurs on large temporal and spatial scales. However, laboratory and field experiments are necessarily restricted to effects on individual animals or small groups of animals, leaving a large inferential leap in extrapolating experimental results to population-level effects. Third, laboratory studies on pesticides and amphibians have mostly evaluated acute lethality. Yet many declines have occurred in remote areas where limited sampling to date suggests pesticide levels are well below known lethal levels based on laboratory studies. Therefore if pesticides are playing a role in amphibian declines, it is probably due to sublethal effects and

possible synergisms with other factors such as disease (Taylor et al. 1999, Gilbertson et al. 2003). Fourth, other factors such as introduced predators (e.g., Fisher and Shaffer 1996, Lawler et al. 1999, Knapp and Matthews 2000) and habitat destruction (Davidson et al. 2001, 2002) have certainly contributed to declines, making it difficult to disentangle the role of pesticides. Finally, although pesticides have long been suggested as a possible cause of amphibian declines (Carey and Bryant 1995, Stebbins and Cohen 1995, Drost and Fellers 1996, Lips 1998), there have been few toxicological studies on declines. This is the first study in which population declines of multiple declining species have been associated with historical pesticide applications.

Epidemiologists regularly deal with the problems of assessing the causes of events with multiple factors, operating on both large temporal and spatial scales. Observational studies are not a substitute for laboratory or field experiments, but they provide essential direction and insight for experimental work, and in turn support causal inferences on the population level from experimental work on individuals. My study uses an epidemiological approach to examine the role of pesticides in the decline of five California amphibians, the Yosemite toad (*Bufo canorus*), California red-legged frog (*Rana aurora draytonii*), foothill yellow-legged frog (*R. boylii*), Cascades frog (*R. cascadae*), and Sierra Nevada populations of the mountain yellow-legged

frog (*R. muscosa*). The species were selected based on two earlier studies (Davidson et al. 2001, 2002) indicating that declines for the species were associated with the amount of upwind agricultural land use, suggesting that windborne pesticides may be contributing to declines. My study builds upon those two earlier studies, but uses actual historical pesticide-application data, rather than just the amount of upwind agricultural land use.

Since 1970 the California Department of Pesticide Regulation (DPR) has required licensed agricultural pest-control operators to report all pesticide use, and farmers to report applications of all pesticides labeled by the state as restricted-use chemicals. Pesticide use reports contain information on the date of application, specific chemical product, weight of active ingredient, and location based on 2.6 km^2 (one square mile) Public Land System sections. In 1998 alone California farmers reported use of over 90×10^6 kg of pesticide active ingredients (Department of Pesticide Regulation 1998). California is an excellent place to study the role of pesticides in amphibian declines. In addition to high and well-documented pesticide use, California has extensive historical and current amphibian-location data (Shaffer et al. 1998). Furthermore, airborne transport of pesticides into remote locations is well documented (Zabik and Seiber 1993, Aston and Seiber 1997, McConnell et al. 1998, LeNoir et al. 1999) and pesticides have been found in the bodies of Sierra Nevada frogs (Cory et al. 1970, Datta et al. 1998, Sparling et al. 2001).

The population data in my study are presence/absence information for five species at historical sites for the species. I define ''present'' sites as those that are currently occupied by a species, and ''absent'' sites as those that were previously occupied (based on museum or other historical records) but are currently not occupied. Absent sites therefore represent ''declines.'' I examined the spatial pattern of declines in relation to the pattern of historical pesticide use as documented by California DPR records from 1974 to 1991. The goal of the research was to determine whether total pesticide use upwind from a site was a significant predictor of amphibian site status (present or absent) once other covariates such as elevation, latitude, precipitation, and surrounding urban and agricultural land use were taken into account. A second goal was to analyze which specific classes of pesticides were most associated with the patterns of decline.

Materials and Methods

I used maps made by Jennings and Hayes (1994) to document the spatial patterns of decline for five California amphibian species. The individual species maps indicate whether populations are still present or not at historical sites at the time the maps were made in October 1991. The maps are based on verified museum records and extensive field surveys, and provide the most comprehensive evaluation of California amphibian declines available for a single point in time. A geographic information system was used to digitize the maps, producing a spatial data set of 1083 sites, for five species, spanning the entire state (Table 1). See Davidson et al. (2002) for more details on the creation of the data set and accuracy assessments.

I used U.S. Geologic Survey 1:250 000-scale digital elevation models for California to derive elevation for all sites, and estimated the 60-year (1900–1960) average annual precipitation for each site based on a digital precipitation map of California compiled by the state of California's Teale Data Center. Latitude and longitude for each location were determined directly from the coordinates for the site. To aid in assessing the contribution of habitat destruction to declines, I measured the percentage of urban and agricultural land use in a 5-km radius surrounding each site using USGS digital 1:250 000-scale land-use/land-cover maps. The land-use maps were primarily derived from aerial photos in the 1970s and 1980s.

Historical pesticide-use data for California came from annual Pesticide Use Reports produced by the California Department of Pesticide Regulation (DPR). The reports contain more than half a million records of pesticide application information per year. Because the maps of frog population status were based on information as of the end of 1991, I was interested in historical pesticide-use data up to and including 1991. I used Pesticide Use Report data for 1974, the earliest year of data without major errors, and then for every odd year through 1991, resulting in a data set with 10 years of data and 7.4 million records. I used every other year of data to reduce data cleanup work and the overall size of the final data set. Pesticide applications were highly correlated from year to year, with an average Spearman rank order correlation of 0.93 for total pesticide use upwind from sites for the five species across all consecutive every-other-year pairs (1975–1977, 1977–1979, etc.). Correlations for classes of pesticides were less than for total pesticide use, but still high. Given the high correlations, the every-other-year data set adequately represented the historical patterns of pesticide use.

The pesticide data were subjected to extensive cleanup and verification including removal of duplicate entries, checking the validity of all public land system locations and chemical codes, where possible fixing location information, and checking that the number of acres treated did not exceed the 700 maximum acres (284.9 ha) in a single Public Land System section, which is the maximum area covered by a single application record. The original pesticide data prior to 1984 contained errors in the conversion of liquid volumes to weights, which were corrected for the bulk of records based on current DPR formulas. Finally, extremely large applications (based on ''application rate,'' which is the weight of pesticide active ingre-

TABLE 1. Comparative mean characteristics of 1083 historic amphibian sites in California (USA) at which amphibian species were present (pres.) or absent (abs.).

Species	No. of sites			Sites absent (%)	Total pesticide use (kg/m)†			Latitude		
	Total	Pres.	Abs.		Pres. sites	Abs. sites	P	Pres. sites	Abs. sites	P
Bufo canorus	55	26	29	52	9.8	18.9	0.84	37.75	37.88	0.29
Rana aurora draytonii	279	88	191	68	60.0	137.5	<0.001	36.43	36.11	0.13
R. boylii	424	196	228	54	46.4	187.9	<0.001	39.35	37.68	<0.001
R. cancadae	70	24	46	66	5.7	71.4	<0.001	41.21	40.38	<0.001
R. muscosa	255	43	212	83	23.2	66.4	0.02	37.91	37.87	0.73
All species	1083	377	706	65	41.8	123.3	<0.001	38.5	37.5	<0.001

   Notes: Data on presence ("Pres.") or absence ("Abs.") of amphibians at a site are based on Jennings and Hayes (1994). *P* values are for Mann-Whitney test of difference of means between present and absent sites.
   † Total pesticide use is inverse distance-weighted total kilograms of active ingredients used upwind from a site.
   ‡ Percentage land use in a 5-km radius surrounding a site, based on land-use maps from aerial photos in the 1970s and 1980s.

dients applied per acre) that likely represented errors in the data were removed based on outlier criteria devised by DPR (Larry Wilhoit, *personal communication*).

   Historical (1974–1991) pesticide use upwind of each amphibian site was calculated by first estimating the predominant summer wind direction for each site from streamline surface wind maps for California and wind direction data from weather stations (Hayes et al. 1984). I used summer wind patterns because analysis of regional wind patterns in the San Francisco Bay Area, South Coast, Sacramento, and San Joaquin regions indicates that the predominant summer wind pattern in all regions is also the predominant annual wind direction (Hayes et al. 1984). Summer and spring, which have similar wind patterns, are also when roughly two thirds of California agricultural pesticides are applied (Department of Pesticide Regulation 1990, 1994). To define the area I considered to be upwind from a site, I used a geographic information system to construct an "upwind triangle," 22.5° wide (= 1 compass sector, where each sector equals one of the 16 standard compass directions), 100 km long, and facing upwind (Fig. 1). For each amphibian site, upwind pesticide use was calculated based on all the Public Land Systems sections (1-mile squares) that fell entirely or partially within the upwind triangle. I used an inverse distance-weighted measure of pesticide use to capture the joint effect of *weight* of upwind pesticide use, and the *proximity* of the application (Fig. 1). Total upwind pesticide use for a single site was calculated as $\Sigma_y\Sigma_c\Sigma_i (k_{icy}/d_i)$, where k_{icy} is the weight of pesticide active ingredient for pesticide c applied in year y in the ith public land system section within the upwind triangle, d_i is the distance from the centroid of the ith section to the amphibian site, and the summation is across all i sections within an upwind triangle, across all c individual pesticides, and across all y years of data. Similar measures were also calculated for pesticide classes and groups. Pesticide classes are mutually exclusive aggregations of individual pesticides based on simi-

larity of chemical structure. I used a classification scheme developed by Orme and Kegley (2002) because unlike other schemes it contains DPR chemical codes that match the Pesticide Use Report data, and its 96 classes included most of the pesticides in my data set (representing 95% of total pesticide use by weight). The remaining pesticides were lumped together in an "unclassified" class. In addition to the pesticide classes, I analyzed three pesticide groups based on shared biological effect rather than similarity of chemical structure: cholinesterase-inhibiting pesticides, suspected endocrine-disrupting pesticides, and developmental or reproductive toxins (Orme and Kegley 2002). I also constructed groups by dividing the organophosphate class into dimethyoxy, diethyoxy, and other organophosphates, combining five separate carbamate classes into a single carbamates group, and constructing an organophosphates plus carbamates group.

   In my 1974–1991 data set 821 different pesticide active ingredients were reported as used in California in 96 different pesticide classes, yet many of these pesticides were used in relatively small amounts, in only a few locations or only in a few years. If a pesticide was not used or only little used upwind of where a species declined, then it is not biologically plausible that the pesticide was contributing to declines. To identify pesticides classes plausibly associated with declines, pesticide use by class was calculated for all absent sites together, for each species. Only pesticide classes meeting the following minimum-use criteria were included in subsequent statistical analysis: total use > 10 000 pounds (9536 kg) active ingredients from 1974 to 1991, used at least once in at least 20% of the counties intersected by the combined upwind triangles of all the absent sites, and used at least for an average of two years across all the counties in which it was used. The resulting selected classes for each species were combined into a single set for all species combined. The minimum-use criteria reduced the number of pesticides classes considered for variable selection in multivariate modeling from 96 to 56, but included

Table 1. Extended.

Elevation (m)			Surrounding land use (%)‡						Precipitation (cm)		
			Urban			Agriculture					
Pres. sites	Abs. sites	P	Pres.	Abs.	P	Pres.	Abs.	P	Pres. sites	Abs. sites	P
2834	2420	0.10	0.00	0.01	0.12	0.00	0.00	0.34	44.3	44.3	0.73
276	445	<0.001	0.07	0.10	0.02	0.09	0.13	0.12	23.2	21.0	0.11
579	521	0.12	0.04	0.05	<0.001	0.04	0.15	<0.001	48.1	27.9	<0.001
1651	1542	0.54	0.01	0.01	0.04	0.00	0.00	0.20	54.5	56.5	0.65
2676	2253	<0.001	0.00	0.00	0.16	0.00	0.00	0.19	46.2	44.9	0.58
971	1165	<0.001	0.04	0.05	<0.001	0.04	0.08	<0.001	42.2	33.7	<0.001

pesticide classes that accounted for 99.86% of total statewide pesticide-use by weight.

Statistical analysis of pesticide variables was divided into three parts: (1) individual species models of total pesticide use and covariates, (2) analysis of total pesticide use for all five species combined and incorporating spatial autocorrelation, and (3) analysis of pesticide classes for all five species combined and incorporating spatial autocorrelation. I used univariate, nonparametric Mann-Whitney rank tests (Sokal and Rohlf 1995) and box plots to evaluate differences in the mean value of total pesticide use and covariates for present and absent sites for each species separately (Fig. 2, Table 1). The covariates were site elevation, precipi-
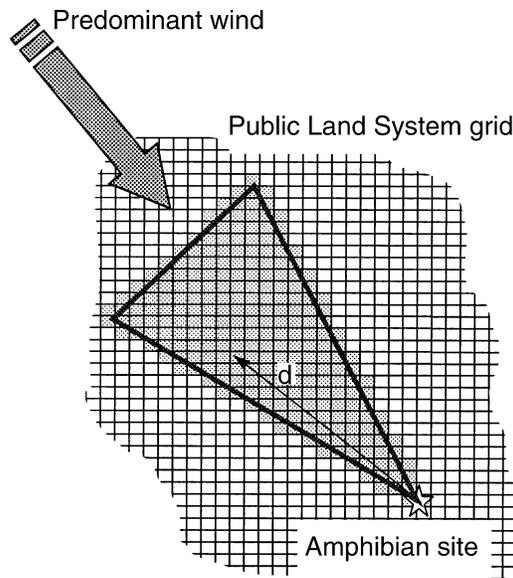

Fig. 1. Illustration of upwind pesticide-use measurements. For each amphibian site, a 22.5°, 100-km-long, "upwind triangle" was drawn facing into the direction of the predominant wind. Total pesticide use was calculated as the sum of the total mass of active ingredients of pesticides applied in a section from 1974 to 1991 for every other year, divided by the distance from the section centroid to the amphibian site (d) for all Public Land System sections falling at least partly within the shaded upwind triangle. Similar measures were calculated for pesticide classes and individual pesticides.

tation, latitude, and percent surrounding urban and agricultural land use. I built multiple logistic-regression models for each species to evaluate the multivariate relationship between declines, total pesticide use, and the covariates. For each species, I first built a full model with total upwind pesticide use and all covariates, and then one by one removed variables that did not significantly contribute to the model based on a likelihood-ratio test (Hosmer and Lemeshow 1989) to derive a reduced model with only significant variables. Based on the box plots and initial regression diagnostics, three amphibian sites with extreme high values for total upwind pesticide use were excluded from the statistical analysis because of their possible strong influence on regression coefficients.

A shortcoming of the individual-species logistic-regression models is their failure to account for spatial autocorrelation (Legendre 1993). One approach to modeling spatially correlated variables is to explicitly incorporate a spatial-location covariate into the regression model (Augustin et al. 1996). To accomplish this I used a generalized additive model (GAM) regression with a spatial covariate that was a function of longitude and latitude (Hobert et al. 1997, Knapp et al. 2003). In generalized additive models the linear predictor function of logistic regression is replaced by the sum of nonparametric smoothing functions for each predictor variable (Hastie and Tibshirani 1991). Because calculation of the spatial covariate requires a large sample size I combined all five species into a single data set, and incorporated a categorical variable for species into the model. The full total-pesticides GAM model consisted of a total-pesticides variable and all the covariates used in building the individual-species logistic-regression models, plus the categorical species variable and the spatial covariate. Thus the probability of site i having a present (extant) population was defined as $p_i = (e^{\theta_i})/(1 + e^{\theta_i})$ where

$$\theta_i = u + f(\text{species}_i) + g_1(\text{elevation}_i) + g_2(\text{urban}_i)$$
$$+ g_3(\text{precip}_i) + g_4(\text{agriculture}_i)$$
$$+ g_5(\text{total pesticides}_i) + g_6(\text{latitude}_i, \text{longitude}_i)$$

where u is the overall mean probability of presence, f gives how u changes by species, and $g_{1 \ldots 6}$ are loess
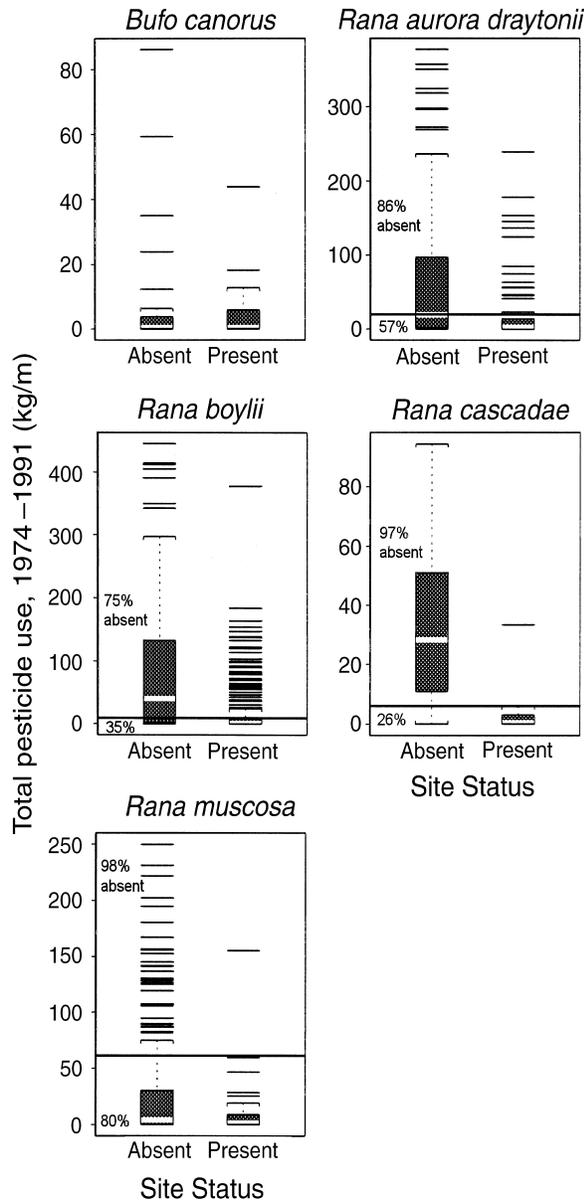
FIG. 2. Box plots of the distribution of total upwind pesticide use, 1974–1991, for present and absent sites for five species of declining amphibians. The white bar inside the box represents the median, the box top and bottom are the 75th and 25th percentiles, and the whiskers (dotted lines ending in horizontal line) indicate values 1.5 times the interquartile range above or below the 75th or 25th percentile. Short horizontal lines above the whiskers represent outlying values. The horizontal line across a plot indicates the pesticide level that maximized the difference between the percentage of total sites that are absent at higher pesticide levels vs. at lower pesticide levels. For example, for *R. a. draytonii* at sites with pesticide levels above the line, 84% are absent sites, while at sites with pesticide levels below the line, 58% of the sites are absent sites. The cutoff level for each species was determined by univariate tree regression with status as the dependent variable and total pesticide use as the independent variable.

smoothing functions that relate each of the covariates to the probability of presence.

As with the individual-species logistic models, nonsignificant variables (based on a likelihood-ratio test) were removed from the full model one by one to create a reduced model with only significant variables. Then all possible two-way interaction terms among the variables (except spatial location) were included in the model and significant terms retained to produce a final combined-species total-pesticides model. The species-interaction terms were included to account for possible different effects of pesticides or the covariates by species. I used S-PLUS version 6 statistical software (S-PLUS 2001) for the generalized additive modeling.

Examining which pesticide classes were most associated with declines required a different analytical approach due to the difficulty of variable selection with a large number of highly correlated variables. For the analysis of pesticide classes I retained all the significant covariates from the final combined-species total-pesticides model and constructed separate GAM models for the 54 pesticide classes meeting the minimum-use criteria described above, plus eight pesticide groups. The class and group models were examined to identify pesticide classes or groups that produced models with an Akaike Information Criteria (AIC) value less than that of the total pesticides model. AIC is calculated as the sum of the model deviance residuals plus 2 times the number of degrees of freedom used to calculate the model parameters. AIC is thus a measure of how well the model fits the data, with a penalty for model complexity (degrees of freedom used in calculating the model parameters) (Harrell 2001, Brunham and Anderson 2002). The goal was not to select the single pesticide class that produced the ''best'' model (lowest AIC value), but rather to identify the set of pesticides classes that best fit the data and produced a better fit than a total-pesticides model. Just identifying and reporting the single best model might obscure a situation in which a number of pesticide classes were all similarly associated with declines.

RESULTS

Clearly multiple factors are contributing to amphibian declines. However, in the results and discussion that follow I focus on the pesticide variables. For a full discussion of the multiple factors affecting these species as represented by the other model variables see Davidson et al. (2002). In the present study other model variables (e.g., elevation, surrounding urbanization) are treated solely as covariates necessary to properly assess possible pesticide effects.

Individual-species total-pesticides models

In univariate analyses, total pesticide use and a number of covariates showed significant differences between present and absent sites for all species except *Bufo canorus* (Table 1, Fig. 2). The box plots show a

TABLE 2.   Results for significant variables, by species, in the logistic regression models using total pesticides.

Significant variables	B	1 SE	P	Exp(B)
Bufo canorus (Model: $G = 71$, $P = 0.024$; $C = 12.5$, $P = 0.09$)				
Elevation	0.689	0.337	0.024	1.991
Rana a. draytonii (Model: $G = 297$, $P < 0.001$; $C = 9.35$, $P = 0.31$)				
Total pesticides	−0.946	0.229	<0.001	0.388
Elevation	−0.689	0.171	<0.001	0.502
Urban (%), 5-km circle	−0.568	0.173	<0.001	0.567
Rana boylii (Model: $G = 452$, $P < 0.001$; $C = 5.37$, $P = 0.72$)				
Total pesticides	−0.758	0.176	<0.001	0.469
Latitude	0.656	0.145	0.003	1.926
Precipitation	0.346	0.145	0.017	1.413
Rana cascadae (Model: $G = 43$, $P < 0.001$; $C = 8.86$, $P = 0.35$)				
Total pesticides	−5.124	1.449	<0.001	0.006
Elevation	1.055	0.476	0.010	2.873
Rana muscosa (Model: $G = 212$, $P < 0.001$; $C = 9.26$, $P = 0.32$)				
Total pesticides	−0.601	0.332	0.028	0.548
Elevation	0.607	0.189	0.001	1.835

Notes: The dependent variable for all models is frogs present ($=1$) or absent. *B* is the standardized regression coefficient, and Exp(*B*) is the odds ratio. *P* is variable significance based on likelihood-ratio tests. Statistics for the entire model are: *G*, likelihood-ratio test for overall model significance; *C*, Hosmer-Lemeshow goodness-of-fit test. *P* values for the *G* and *C* tests are reported following the species names.

striking pattern for all the ranid species. When upwind pesticide use is above a threshold value, sites are overwhelmingly absent sites. At low levels of upwind pesticide use there are still many absent sites, consistent with multiple causes of decline, but the percentage of absent sites is substantially less than at higher pesticide levels.

All four ranid frog species (*Rana aurora draytonii*, *R. boylii*, *R. cascadae*, and *R. muscosa*) showed a strong, statistically significant pattern of decline with greater amounts of total upwind pesticide use. Total pesticide use was a significant variable in the logistic-regression models for all species except *Bufo canorus* (Table 2). Total pesticide use had the largest standardized regression coefficient of all the variables in the model for *R. a. draytonii*, *R. boylii*, and *R. cascadae*, and for *R. muscosa* the magnitude of the pesticide coefficient was equal with that of elevation, the only other variable in the model. The magnitude of the standardized coefficient for a variable indicates how the probability of a site having a present population (as measured by the log of the odds ratio) changes with a 1 SD change of the variable, and is thus a way to compare the strength of association between the dependent variable (site population present/absent) and each of the independent variables.

In all five logistic-regression models the likelihood-ratio test for the overall model was significant, and the Hosmer-Lemeshow goodness-of-fit test (Hosmer and Lemeshow 1989) indicated the data fit the logistic model (i.e., $P > 0.05$). The model for *R. cascadae* was complicated by the fact that both latitude and upwind pesticide use were each highly significant in individual univariate tests, yet the two variables were fairly highly correlated for this species (Spearman correlation 0.74).

There is a relatively small latitudinal difference (40 km) between the Lassen area, where the species has largely disappeared, and the Trinity Alps area, where the species is still common. I therefore modeled declines for *R. cascadae* with the upwind pesticide use variable and not latitude. Otherwise, pair-wise Spearman correlations of variables within species were generally below 0.5, suggesting that multicollinearity was not a problem in the logistic models. The one other exception was a 0.7 correlation between latitude and precipitation in the *R. boylii* model. For the *R. boylii* model with both latitude and precipitation, variance inflation factors (Nash and Bradford 2001) indicated no multicollinearity problem.

Combined-species total-pesticides model

The reduced combined-species total-pesticides model had four significant variables: species categorical variable, spatial location, percent surrounding urban land use, and total upwind pesticide use. Spatial location was the strongest variable, as measured by AIC value (Table 3a), with the three other variables all of about equal importance. The spatial-location variable showed lower probability of presence in the southern part of the state, and higher probability of presence in the north (Fig. 3). The categorical species variable indicated lower probability of presence for *R. muscosa* sites than for those of the other four species (Fig. 3). The urbanization variable indicated a decline in probability of presence for sites with any urbanization compared to those with no surrounding urbanization. For total pesticides there was a continuous linear decline in probability of presence with increasing levels of total pesticides. There was no significant interaction between urbanization and total pesticides, or between the

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TABLE 3. Generalized additive models for site presence/absence for all five amphibian species combined.

Variable	Deviance†	df	P‡	AIC§
a) Reduced total pesticides model				
Model	997.48	1045		1066
Spatial location	152.91	18.23	<0.00001	1183
Species	23.40	3.97	0.00010	1082
Urbanization	25.13	5.80	0.00027	1080
Total pesticides	24.32	5.46	0.00028	1080
b) Final total pesticides model with species × pesticides interaction				
Model	977.00	1041		1054
Spatial location	150.91	18.30	<0.00001	1168
Species	24.50	4.02	0.00007	1070
Urbanization	24.99	5.79	0.00029	1067
Total pesticides	8.76	4.39	0.08549	1053
Species × Pesticide	20.48	4.03	0.00041	1066
c) Cholinesterase-inhibiting pesticides (CIP) model				
Model	964.14	1041		1041
Spatial location	171.27	18.25	<0.00001	1176
Species	28.97	3.99	0.00007	1062
Urbanization	21.00	5.80	0.00158	1051
CIP	12.43	4.59	0.02230	1045
Species × CIP	25.78	4.01	0.00041	1059

† For model rows, deviance is residual model deviance. For variable rows, deviance is the test deviance, the change in model deviance when the variable is removed from the model (always negative, sign not shown). The null model, with only a constant term for all three models, has a residual deviance of 1396, on 1079 degrees of freedom.

‡ P is for a deviance test of variable significance.

§ For variable rows, values for Akaike Information Criteria (AIC) correspond to the model with the variable removed; thus, larger variable AIC values indicate greater importance of a variable.

species categorical variable and urbanization. Consistent with the individual-species models, the interaction between species and total pesticides was significant, indicating that the pesticide effect varied by species. A total-pesticides × species interaction term was there-fore added to the reduced model to form the final combined-species total-pesticides model (Table 3b).

Accounting for spatial autocorrelation may change regression results, often by indicating that previously apparently significant variables are not truly statisti-
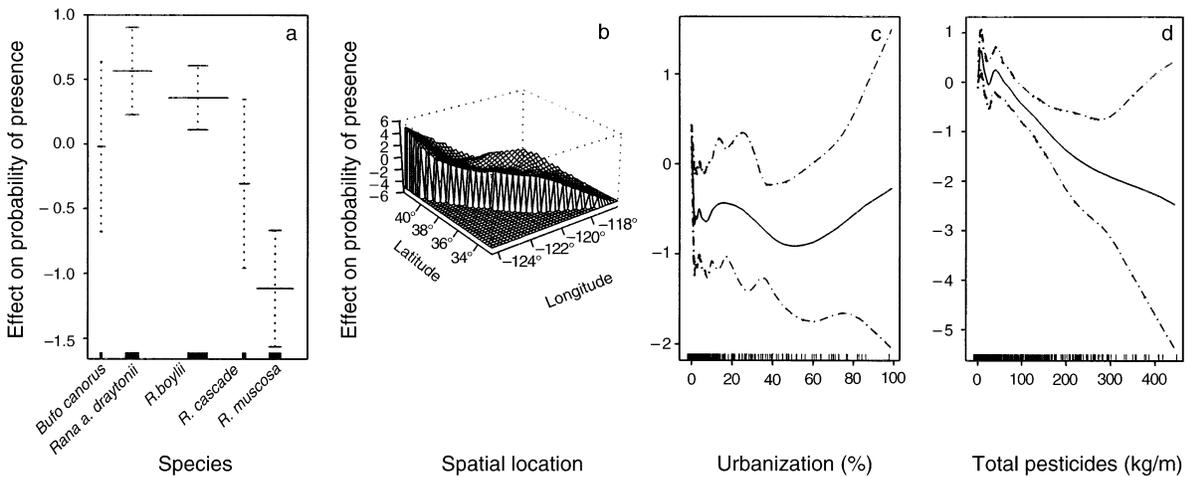

FIG. 3. Plots of variables for the reduced combined-species total-pesticides generalized additive regression model. The plots indicate the relationship between individual model variables (x-axis) and the probability of a site having a present (extant) population [y-axis in panels (a, c, and d), z-axis in panel (b)], once other variables are taken into account. In (a) the solid rectangles just above the x-axis indicate the relative number of data points for each species; in (c) and (d) the hatch marks just above the x-axis indicate the distribution of variable values. In (a) the vertical dotted lines ending in a horizontal line represent 95% confidence intervals around the center horizontal line, which is the effect of each species value on probability of presence; in (c) and (d) the dashed lines are the approximate 95% confidence intervals. The y-axis is log scale, and for each panel is standardized so the mean value is zero. Note that each panel has a unique y-axis range [or, for (b), z-axis range], indicating the relative impact of the variable on probability of presence for a site.

TABLE 4. Akaike Information Criteria (AIC) values for pesticide class/group general additive regression models that fit the data better than the final combined-species total-pesticides model.

Pesticide class/group	AIC
Cholinesterase inhibitors	1041.39
Organophosphates and carbamates	1044.90
Carbamates	1045.81
N-methyl carbamates	1046.62
Bipyridyium	1049.51
2,6-Dinitroaniline	1050.63
Diethyoxy organophosphates	1051.65
Organophosphates	1052.59
Guanidine	1052.64
Phosphonoglycine	1053.14
Combined-species total-pesticides model	1053.90

Note: In addition to the specific pesticide class or group term, all models have the same set of covariates as the final combined-species total-pesticides model: species, urbanization, a species × pesticide class/group interaction term, and the spatial covariate.

cally significant (e.g., see Knapp et al. 2003). If a spatial covariate is not included in the combined species model, then both elevation and precipitation variables appear as significant in a reduced model. Furthermore, in the absence of the spatial covariate, total pesticides is the strongest term in the model, with an AIC value (1212) even greater than that of the spatial covariate in the reduced model. Although inclusion of a spatial covariate and accounting for spatial autocorrelation is necessary for the proper interpretation of the significance of regression results, spatial location does not in itself have biological meaning. Spatial location may represent factors not included in the model, or in this case it may represent spatially correlated pesticides (and to a lesser extent elevation and precipitation).

Combined-species pesticide-class models

Application of the minimum-use criteria for considering a pesticide class for multivariate modeling resulted in selection of 33 plausible pesticide classes for *B. canorus*, 55 for *R. a. draytonii*, 56 for *R. boylii*, 33 for *R. cascadae*, and 52 for *R. muscosa*, and a union set of 56 classes for all species combined. When separate generalized additive models were constructed with each of these pesticide classes and the 9 pesticide groups, 10 pesticide classes or groups produced a regression model with a lower AIC value than the final combined-species total-pesticides model (Table 4). The cholinesterase-inhibiting pesticides group produced the model with the lowest AIC value (best fit to the data). Furthermore, 6 of the 10 models with a lower AIC value than the total-pesticides model were based on some grouping of cholinesterase-inhibiting pesticides, such as organophosphates or carbamates classes. In my 1974–1991 data set, 85% of applied weight of active ingredients of these two classes were cholinesterase inhibitors. Four other classes also produced better models than total pesticides: Bipyridyium (mainly the her-

bicide paraquat dichloride), 2,6-Dinitroaniline (mainly trifluralin, pendimethalin, and oryzalin), Guanidine (the single fungicide dodine), and Phosphonoglycine (the glyphosate herbicides). It was not possible to build a model for the pesticide class dinitrophenol derivatives, likely due to an extremely skewed distribution of amounts used, and therefore there are no results for this class.

The cholinesterase-inhibiting pesticides model was significantly better than the total-pesticides model. Since the two models are not nested, they cannot be compared to each other directly. However by embedding the models in a combined model that contains all the terms from each model, each model is then a subset of the combined model and can be compared using a standard deviance test (Harrell 2001). The total-pesticides terms (main effect and interaction with species) do not significantly contribute to a model based on cholinesterase-inhibiting pesticides (deviance test $P = 0.32$). Conversely, the cholinesterase-inhibiting pesticides terms were a significant addition to a model based on total pesticides (deviance test $P = 0.007$). The plot for the cholinesterase-inhibiting pesticides term (not shown) has a similar linear form as the total-pesticides term (Fig. 3), but is steeper, indicating a greater effect on probability of site presence. Finally, in the cholinesterase-inhibiting pesticides model both the main pesticide effect and pesticide × species terms are significant, while in the total-pesticides model the main pesticide effect is only marginally significant once a pesticide × species interaction term is added (Table 3c).

The strong association between cholinesterase inhibitors and amphibian declines is not just the result of the amount of use, or high correlations between classes or groups of pesticides. Although organophosphates and carbamates are widely used, together they make up just 12% of the reported statewide use in my 1974–1991 data set. There was little correlation between total weight applied by class or group and how well a model based on that class or group fits the data as measured by AIC values (Pearson correlation = -0.2). The amount of pesticide use in a large area such as an upwind triangle is largely driven by the amount of agricultural land use. Therefore the amounts of any particular class of pesticide applied upwind from a site are highly correlated with each other and with total pesticides applied. However, model AIC values show little association with the correlations between amount applied by class or group. For example, if we look at the correlation across sites between the amount of cholinesterase-inhibiting pesticides and each of the other classes or groups, and compare this with AIC values, the Pearson correlation is -0.2. In other words pesticides classes that show correlated applications with cholinesterase-inhibiting pesticides, nonetheless produce models with a wide range of AIC values.

Discussion

There is a strong association between amphibian declines and total upwind pesticide use for the four ranid frogs. In the individual-species total-pesticides models, pesticides were the single strongest explanatory variable across the four frog species. These results represent, at a minimum, three independent tests of the association between total upwind pesticides and amphibian declines. The range of *Rana aurora draytonii* and *R. boylii* overlap by roughly two thirds, and therefore could be considered a test for a single geographical area. However, there is virtually no overlap between the ranges of these two species and the ranges of *R. cascadae* and *R. muscosa*. The combined-species total-pesticides model indicates that the relationship between declines and upwind pesticide use holds even when spatial autocorrelation is taken into account.

It is unclear if these results represent a taxonomic pattern: ranid frog declines associated with pesticide use, non-ranid declines not associated with pesticides. This pattern could easily be due to chance alone. However in an earlier study on these same species, plus three additional ones, Davidson et al. (2002) found that only declines of the same four ranid species were significantly associated with upwind agricultural land use, a taxonomic pattern unlikely due to chance. The lack of association between pesticide use and declines for the Yosemite toad in this study may reflect low power to detect an association. The sample size for the Yosemite toad was the smallest of any of the species in the study, and the geography of the species' range (all downwind from the Central Valley, all high elevation, and a narrow north-to-south range) likely made it the most difficult species in which to detect a pesticide pattern.

In the analysis of pesticide classes, cholinesterase-inhibiting pesticides emerged as most strongly associated with declines. At several sites in the Sierra Nevada (downwind from heavy pesticide use in California's (USA) Central Valley, Sparling et al. (2001) found that cholinesterase levels in the non-declining Pacific treefrog (*Hyla regilla*) were lower than at sites in the Coast Range (with low pesticide exposure). The lower cholinesterase levels in Sierra Nevada treefrogs may have been due to exposure to cholinesterase-inhibiting pesticides. Given that there have been sharp declines of a number of ranid frog species in the Sierra Nevada, but not in the Coast Range, Sparling et al. (2001) suggested that the ranid frog declines may be due to exposure to cholinesterase-inhibiting pesticides.

In the United States, organophosphate and carbamate pesticide use increased dramatically in the 1960s and early 1970s, partly as a replacement for organochlorine pesticides that were phased out due to environmental concerns (Aspelin 2003). Unfortunately pesticide-use data for California began only in the early 1970s, too late to provide a picture of when organophosphate and carbamate use became widespread in the state. The rise of organophosphate and carbamate use in the United States immediately proceeded or coincided with amphibian declines, many of which are believed to have begun in the early to mid-1970s (Jennings 1996). While lack of coincidence of timing is strong evidence to rule out a causal link between two events, coincidence of timing is at most weak evidence for a link between two events, because there are a near-infinite number of events with similar timing (Susser 1986, Fox 1991). Based on the existing temporal data alone, the best we can say is that the temporal pattern of organophosphate and carbamate use (unlike that of organochlorines) does not rule out these pesticides as contributors to amphibian declines.

A challenge for future field-residue studies and laboratory study of pesticide effects will be to bridge the large gap between pesticide levels found to have effects in the laboratory and the much lower levels currently observed in the field at locations with amphibian declines. I do not know of field-concentration and laboratory-effects studies on the same pesticide for California amphibians. However a comparison of California field data with laboratory studies for non-California species is illustrative. For example, at low elevations (100–800 m) in the Sierra Nevada, LeNoir et al. (1999) found concentrations of 67 ng/L of diazanon, and 20 ng/L of malathion in surface water. Laboratory tests found 48- or 96-h median lethal concentrations (LC50) for diazanon of 0.05 mg/L for *Rana clamitans* and 4.5 mg/L for R. limnocharis (EPA 2004). Similarly LC50 levels for malathion were 0.4 mg/L for *Bufo woodhousei* and 0.2 mg/L for *Psuedacris t. triseriata*. Thus for all four species, measured field concentrations were 3–5 orders of magnitude below concentrations found to be lethal in the laboratory. The large gap between field concentrations and laboratory-measured lethality suggests that if pesticides are contributing to declines, it may not be due to direct lethal effects, but rather sublethal effects and interactions with other factors.

The perception that field levels are extremely low may be partly an artifact of the current limitations in field sampling. Measurements of pesticide concentrations in water taken at a single point in time may be deceptive, because at any one location the bulk of pesticide applications occurs in a few short time periods. For example, in 2000 at four randomly selected sites in my data set, the peak application week for the pesticide carbaryl accounted for about 20% of the annual total upwind use. Thus animals could easily experience much higher short-term exposures than a typical one-time sampling would indicate. Furthermore, short-term exposures may have longer term impacts. For example, Gilbertson et al. (2003) found that a one-time sublethal injection of malathion almost completely suppressed part of the immune function of leopard frogs for as long as eight weeks.

Due to its large temporal and spatial scale the actual phenomenon of amphibian declines can never be directly subjected to experiments. For complex phenomena that cannot be experimentally manipulated, causality is not proven by a single study but rather inferred from the weight of evidence from multiple studies, both observational and experimental. Therefore observational studies will be key pieces of evidence in assessing the causes of declines. Epidemiologists have long struggled with how to infer causality from observational studies and multiple strands of evidence (Susser 1986, Fox 1991). Two of the key epidemiological criteria for inferring causality are strength of association and consistency of association. The results here show a strong association between upwind pesticide use and amphibian declines and the relationship is consistent across a number of different species representing at least three independent ranges.

The strong association between declines and upwind pesticide use clearly points to the urgent need for additional research on the role of pesticides in amphibian declines. Field studies are needed to assess the types and amount of wind-borne pesticide exposure for declining species. Several recent studies in the Sierra Nevada (Datta et al. 1998, Sparling et al. 2001) have documented current-use pesticide residues in the non-declining Pacific treefrog (*Hyla regilla*). This work needs to be extended to current-use pesticide residues in declining species, and with better geographic coverage to allow for an analysis of the relationship between declines and pesticide residues in frogs. In addition, laboratory experiments are needed to assess possible causal mechanisms of pesticide impacts at field-relevant doses. Given the findings here, examination of the impact of cholinesterase-inhibiting pesticides on immune response, hibernation, and other life functions could be especially illuminating.

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