

## MULTIPLE STRESSORS AND AMPHIBIAN DECLINES: DUAL IMPACTS OF PESTICIDES AND FISH ON YELLOW-LEGGED FROGS

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**Abstract.** More than 40% of Earth's 5700+ amphibian species have undergone recent declines. Despite the likely involvement of multiple factors in driving these declines, most studies continue to focus on single stressors. In California (USA), separate studies have implicated either introduced fish or pesticides as causal agents. To date, however, no study has simultaneously evaluated the respective roles of these two potential stressors nor attempted to assess their relative importance, information critical for the development of effective conservation efforts and environmental policies. We examined the role and relative effect of fish and pesticides on the mountain yellow-legged frog (*Rana muscosa*) using unusually detailed data sets for a large portion of *R. muscosa*'s historic range in California's Sierra Nevada. Habitat characteristics and presence/absence of *R. muscosa* and fish were quantified at each of 6831 sites during field surveys. Pesticide use upwind of each site was calculated from pesticide application records and predominant wind directions. Using generalized additive models, we found that, after accounting for habitat effects, the probability of *R. muscosa* presence was significantly reduced by both fish and pesticides, with the landscape-scale effect of pesticides much stronger than that of fish. The degree to which a site was sheltered from the predominant wind (and associated pesticides) was also a significant predictor of *R. muscosa* presence. Taken together, these results represent the strongest evidence to date that windborne pesticides are contributing to amphibian declines in pristine locations. Our results suggest that amphibian declines may have complex multi-factorial causes, and caution that single-factor studies that demonstrate the importance of one factor should not be used as evidence against the importance of other factors.

**Key words:** amphibian declines; generalized additive models; introduced fish; mountain yellow-legged frog; multiple stressors; pesticide drift; *Rana muscosa*; Sierra Nevada.

### INTRODUCTION

Amphibian populations have suffered alarming worldwide declines in recent decades. According to the first global assessment of the status of amphibian species (Stuart et al. 2004), more than 40% of the world's 5743 amphibian species have experienced recent declines, a situation far worse than that reported for mammals (IUCN 2003) or birds (BirdLife International 2004). It is widely believed that most declining amphibian species are being affected simultaneously by multiple stressors (Linder et al. 2003) and laboratory experiments have indeed identified a number of potentially interacting factors (e.g., Boone and Semlitsch 2001, 2002, Relyea and Mills 2001). However, few field studies have investigated the effects of more than a single factor in causing amphibian declines (but see Davidson et al. 2001, 2002, Kiesecker et al. 2001, Pounds et al. 2006). This focus on single factors can lead to erroneous conclusions when the demonstrated importance of one

factor is mistakenly taken as evidence that other factors are either unimportant or of lesser importance.

The mountain yellow-legged frog (*Rana muscosa*) is native to the Sierra Nevada of California and Nevada and to the Transverse Ranges of southern California (Stebbins 2003). In the Sierra Nevada, *R. muscosa* was once abundant in the extensive fishless habitats present at mid to high elevations (Grinnell and Storer 1924, Mullally and Cunningham 1956). Its unique life history, including a two-to-four-year larval stage and an adult stage that overwinters underwater (Bradford 1983, Matthews and Pope 1999, Vredenburg et al. 2005), restricts successful breeding to permanent water bodies (Knapp and Matthews 2000). During the past century, *R. muscosa* has disappeared from most of its historic range (Jennings and Hayes 1994, Drost and Fellers 1996). As a result of the severity of these declines, the southern California populations are now listed as "endangered" under the U.S. Endangered Species Act and the listing of the Sierra Nevada populations was recently determined to be "warranted" (Federal Register 2003).

Separate studies have implicated introduced fish or pesticides as contributing to population declines of a number of California amphibians, including *R. muscosa*. The possibility that nonnative fish introduced into the

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historically extensive fishless portion of the Sierra Nevada are a cause of decline in *R. muscosa* was first suggested more than 80 years ago (Grinnell and Storer 1924), an assertion that has been repeatedly supported by quantitative analyses using both large-scale correlative studies (Bradford 1989, Bradford et al. 1998, Knapp and Matthews 2000, Knapp 2005) and experiments (Vredenburg 2004; R. A. Knapp, unpublished data). Although the results from these studies clearly indicate that fish have played an important role in the decline of *R. muscosa*, its continued decline (R. A. Knapp, unpublished data) despite many lakes reverting to a fishless condition following the termination of fish stocking (Knapp et al. 2001, 2005, Armstrong and Knapp 2004) and its disappearance from watersheds that have never been stocked indicates that additional factors are involved (Bradford 1991, Drost and Fellers 1996).

California contains only 3% of the agricultural acreage in the United States, but accounts for approximately 15% of all pesticide use (Census of Agriculture 2002). In 1998 alone, California farmers reported using over 90 million kilograms of pesticide active ingredients (Department of Pesticide Regulation 1999), the majority of which were applied to crops in the intensively agricultural Central Valley (Department of Pesticide Regulation 1999). The Sierra Nevada is located immediately downwind of the Central Valley, and airborne transport of pesticides to the Sierra Nevada (Zabik and Seiber 1993, Aston and Seiber 1997, McConnell et al. 1998, LeNoir et al. 1999) and their presence in the bodies of amphibians (Cory et al. 1970, Datta et al. 1998, Sparling et al. 2001, Fellers et al. 2004) is well documented. Recent reports indicate a strong association between the disappearance of four frog species (including *R. muscosa*) from historic sites in California, and either the amount of agricultural land use upwind (Davidson et al. 2002) or historic upwind pesticide use (Davidson 2004), suggesting that windborne agricultural chemicals may be contributing to declines.

Despite mounting evidence that introduced fish and airborne pesticides might both be important factors impacting amphibian populations, no study has yet attempted to examine the role and relative importance of both in influencing amphibian distributions. As a consequence, considerable debate continues over their respective roles in driving the decline of amphibians. The objective of the current study was to evaluate the importance of introduced fish and airborne pesticides, after accounting for habitat effects, in influencing the distribution of *R. muscosa* across a large portion of its historic range. Our study used two unusually large and detailed data sets describing habitat characteristics and presence/absence of *R. muscosa* and fish at more than 6800 sites, and the application amounts and locations for pesticides used in California between 1991 and 2000 (33.4 million records). Combining these two data sets into a single analysis allowed us to evaluate the roles and

relative importance of introduced fish and airborne agricultural contaminants on the decline of *R. muscosa* at an unprecedented spatial scale and level of detail.

## METHODS

### *Study area description*

The study area included all of Sequoia, Kings Canyon, and Yosemite National Parks, as well as a portion of the John Muir Wilderness. These areas encompass approximately 7373 km<sup>2</sup> of rugged mountainous terrain in the central and southern Sierra Nevada of California, USA (36°17'–38°11' N, 118°15'–119°53' E; Fig. 1). Lentic water bodies in the study area are located at elevations ranging from 1079 to 3930 m, are generally <10 ha in surface area, and all were historically fishless. Between 1870 and 1960, at least 62% of the larger water bodies (in the study area, there are 787 lakes >2 ha in surface area and >3 m deep) were stocked with one or more species of trout (primarily *Oncorhynchus mykiss*, *O. m. aguabonita*, and *Salvelinus fontinalis*) to create recreational fisheries. Additional details regarding the study area are provided in (Knapp and Matthews 2000) and (Knapp 2005).

### *Amphibian, fish, and habitat surveys*

Within the study area, all lakes, ponds, and marshes shown on U.S. Geological Survey 1:24 000 topographic maps ( $n = 7084$ ) were visited once during June–September in 1995, 1996, 1997, 2000, 2001, or 2002. At those sites that were suitable for survey (i.e., not frozen or dry;  $n = 6109$ ), we quantified the presence/absence of *R. muscosa* and fish, and characterized physical habitats. Unmapped water bodies (typically small ponds) found while moving between mapped sites were also surveyed ( $n = 722$ ). The presence/absence of *R. muscosa* at all water bodies was determined using visual encounter surveys (Crump and Scott 1994) of the entire shoreline and the first 100 m of each inlet and outlet stream. *R. muscosa* was determined to be present at a site if one or more egg masses, larvae, subadults, or adults were detected. Previous studies have shown shoreline visual surveys to be an accurate method for determining *R. muscosa* presence/absence (Knapp and Matthews 2000, Vredenburg 2004). All surveyed sites are within the historic range of *R. muscosa*. Based on historical accounts (Grinnell and Storer 1924, Jennings and Hayes 1994), *R. muscosa* was once widespread and abundant in the Sierra Nevada and occupied a large proportion of lentic sites. Therefore, its current absence at sites provides a good indication of population declines.

All study water bodies were naturally fishless. The presence or absence of introduced fish was determined at each water body using visual encounter surveys or gill nets (Knapp and Matthews 2000). For shallow water bodies (<3 m deep) in which the entire bottom was visible, fish presence or absence was determined using visual encounter surveys conducted while walking the entire shoreline and the first 100 m of each inlet and

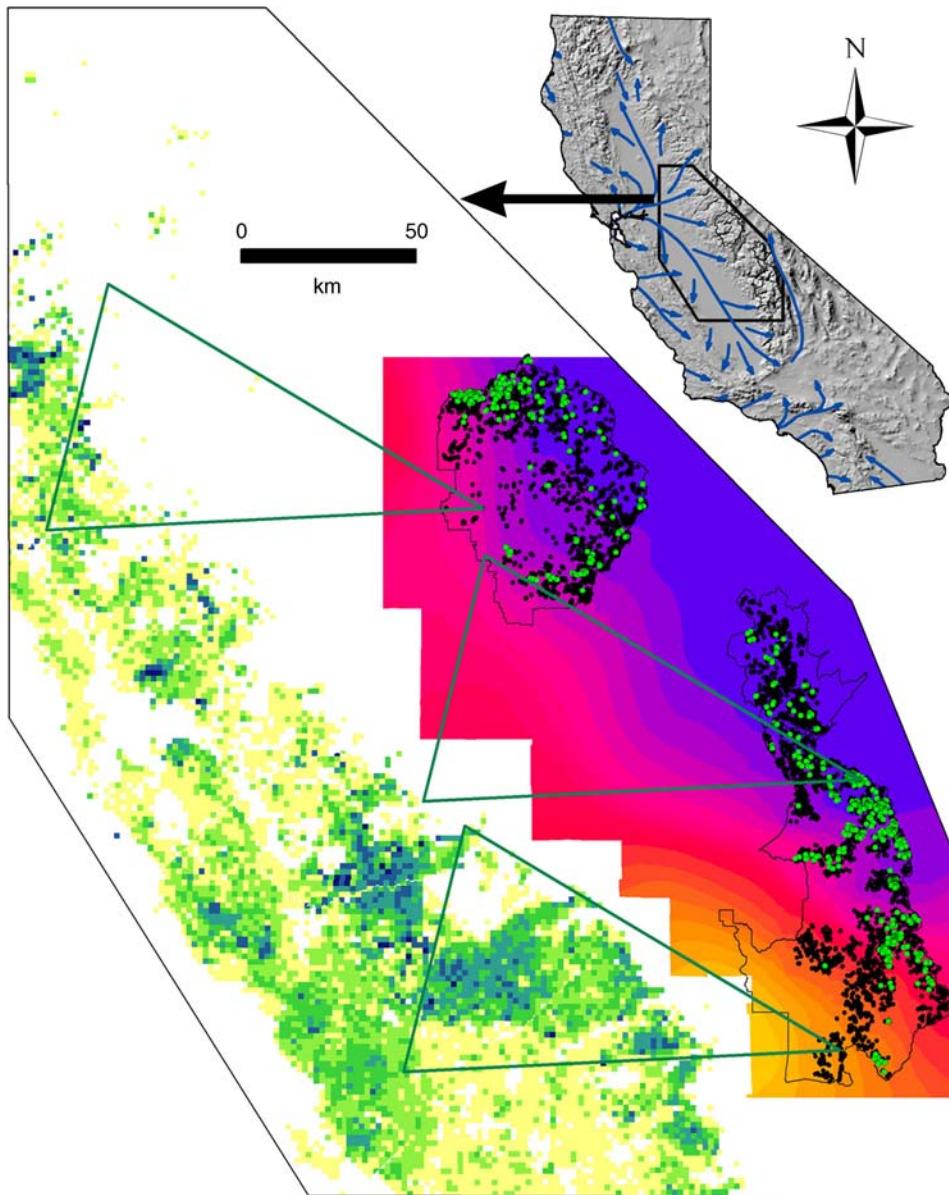


FIG. 1. Map showing the location of the Central Valley relative to the Sierra Nevada study area (Yosemite National Park is northernmost, John Muir Wilderness is in the middle, and Sequoia-Kings Canyon National Park is southernmost). In the portion of the figure representing the Sierra Nevada, *Rana muscosa* presence (green dots) or absence (black dots) is indicated for each of the 6831 surveyed water bodies. Pesticide use in the Central Valley (total mass of active ingredients applied between 1991 and 2000) ranged from  $<5000 \text{ kg/mile}^2$  [ $1 \text{ mile}^2 = 2.60 \text{ km}^2$ ] (not shown) to  $5000\text{--}550\,000 \text{ kg/mile}^2$  (shown as increasing from yellow to green to blue). Total pesticide use upwind of each of the surveyed water bodies (presented here as a kriged surface) ranged from  $<0.1 \text{ kg/m}$  (violet) up to  $1320 \text{ kg/m}$  (increasing from purple to pink to orange). Three example “upwind triangles” are shown extending from survey sites 125 km into the predominant wind. The inset map locates the study area within the state of California (USA), and the blue arrows describe the predominant wind pattern.

outlet stream. In deeper water bodies, fish presence or absence was determined using both visual surveys and a single monofilament gill net set for 8–12 hours (see Knapp and Matthews [1998] for additional gill netting details).

We described the physical attributes of each water body using elevation, maximum water depth, littoral zone (i.e., near-shore) substrate composition, the degree

of isolation from other water bodies, and annual average precipitation. Water body elevation was obtained from 1:24 000 topographic maps. Maximum water body depth was determined by sounding with a weighted line. Littoral zone silt was estimated as the percent of approximately 50 transects (evenly spaced around the water body perimeter and extending perpendicularly from shore 3 m into the water body) dominated by

substrates <0.5 mm in diameter. Water body isolation was characterized as the total number of other water bodies within 1 km of the water body shoreline. We 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.

#### *Estimating upwind pesticide use*

In 1990, the California Department of Pesticide Regulation instituted "full reporting," which required farmers and licensed agricultural pest control operators to report all pesticide use, including date of application, specific chemical product, weight of active ingredient applied, and location based on 1.6-km<sup>2</sup> (1-mile<sup>2</sup>) Public Land System (PLS) sections. This has resulted in one of the most extensive pesticide reporting systems in the world. To estimate upwind pesticide use for each surveyed water body, we used pesticide application data for 1991 through 2000 obtained from Department of Pesticide Regulation Annual Use Reports. These data were subjected to extensive error checking routines that included removal of duplicate entries, entries with invalid PLS locations or chemical codes, correction of formulas used to convert liquid measures into weights, and exclusion of extreme outliers.

Pesticide use upwind of each amphibian site was calculated using methods nearly identical to those used previously (Davidson 2004). Briefly, we first estimated 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) (Fig. 1). We used summer wind patterns because analysis of regional wind patterns in the San Joaquin Valley area indicated that the predominant spring, summer, and fall wind patterns are very similar (predominately from the Central Valley up into the Sierra Nevada) and the summer pattern is also the predominant annual wind direction (Hayes et al. 1984). Because winter wind patterns are markedly different, our measures of upwind pesticide use excluded applications made December through February. To define the area we considered to be upwind from a site, we used a geographic information system to construct an "upwind triangle," 33.75° wide (=1.5 compass sectors, where each sector equals one of the 16 standard compass directions), 125 km long, and facing upwind (Fig. 1). We used a 125 km long triangle instead of the 100 km long version used previously (Davidson et al. 2002, Davidson 2004) because the longer triangle produced an upwind pesticide use variable which, while similar to that resulting from the 100 km triangle, was less spatially autocorrelated and as a result produced a more stable regression model. For each water body, upwind pesticide use was calculated based on all the PLS sections that fell at least partially within the upwind triangle. We 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 (see Fig. 1 in Davidson [2004] for additional details). Total upwind pesticide use for a single site was calculated as  $\sum_y \sum_c \sum_i (k_{icy}/d_i)$ , where  $k_{icy}$  is the weight of pesticide active ingredient for pesticide  $c$  applied in year  $y$  in the  $i$ th PLS section within the upwind triangle,  $d_i$  is the distance from the centroid of the  $i$ th 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. We also conducted analyses based on several different definitions of upwind pesticide use, all of which are described in the Appendix.

Drainage orientation may influence patterns of pesticide deposition by altering fine-scale patterns of air circulation. For example, water bodies in drainages oriented into the predominant wind may be more exposed to windborne contaminants than those in drainages oriented at other angles (Angermann et al. 2002). To examine this possibility, we calculated the angle between the predominant wind direction and drainage orientation for all water bodies ("topographic sheltering"). For example, if the predominant wind direction was due east and the drainage faced due west, the topographic sheltering angle is 0°. Similarly, drainages facing due north, south, or east would have sheltering angles of 90°, 90°, and 180°, respectively. Drainage orientation was based on the direction of a straight line from the water body to a point 5 km downstream as derived from a 30 m digital elevation model.

#### *Statistical analysis*

We used multivariate generalized additive models (GAMs) to evaluate the importance of total upwind pesticide use and fish presence on *R. muscosa* presence/absence (after accounting for the effects of habitat variables and location). GAMs are similar to generalized linear models, but relax the assumption that the relationships between the dependent variable (when transformed to a logit scale) and predictor variables are linear. Relaxation of this assumption is accomplished by estimating a nonparametric smooth function to describe the relationships between the dependent and predictor variables (Hastie and Tibshirani 1991). All analyses were conducted using S-Plus 6.2 (S-PLUS 2001).

Statistical models that use landscape-scale data are complicated by the fact that species distributions and associated habitat data are often spatially autocorrelated (Legendre 1993), violating the assumption of independence of error terms inherent in most statistical tests (Augustin et al. 1998). As a result, correct inference from spatially autocorrelated data is possible only by first extracting spatial dependencies or by explicitly modeling spatial autocorrelation (Legendre and Fortin 1989). In this study, we extracted spatial dependencies by including a locational covariate ( $X$  and  $Y$  coordinates for each water body) as a predictor variable (Hobert et al. 1997, Knapp et al. 2003, Davidson 2004). All

predictor variables were included in the model because correlations between all variables were sufficiently low (range  $-0.58$ – $0.18$ ) to indicate only a minimal level of multicollinearity (Berry and Felman 1985, Hair et al. 1998).

Regression methods were similar to those described in Knapp et al. (2003) and Davidson (2004). Briefly, we first built a full model with all variables, 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 final model containing only significant variables. The relationships between the significant predictor variables and the probability of *R. muscosa* occurrence are shown graphically in separate plots (“response curves”) in which the  $y$ -axis represents the log odds of *R. muscosa* occurrence, standardized to have an average value at zero. Odds are the probability of an event occurring divided by the probability of it not occurring. Therefore, log odds values indicate the direction and strength of the effect of each predictor variable on the likelihood of *R. muscosa* presence at a single site.

Prior to regression analysis, we set aside 1000 randomly chosen water bodies as a “test” data set, and used the remaining 5831 water bodies for all model development. To ensure that water bodies in the test data set were an unbiased subset of the full data set and were geographically stratified, we randomly selected a fixed percentage of water bodies from each of the 224 drainages in the study area. To validate the final GAM, we used goodness-of-fit measures to check if the model performed substantially worse on the test data set versus the model development data set, which would indicate a lack of model validity beyond the data used in model development (see Appendix for details).

To evaluate the relative effects of fish and pesticides on *R. muscosa* at the landscape scale requires consideration of the three components of a stressor’s impact on a population. One component is the strength of the effect of the stressor at a single site. This is analogous to a “dose-response” in a toxicological study; it is the relationship between different levels of the stressor and the resulting magnitude of the effect on a single population. A second component is the frequency or intensity of the stressor across the study landscape. For example, if fish are present in only a small number of sites, their influence on frogs will be relatively weak even if fish have a very strong effect where they are present. A third component is the distribution of the stressor with respect to other factors that affect frog presence. For example, across the study landscape, fish will have a stronger negative effect on frog populations if the fish are in high quality frog habitat than if they are in poor quality frog habitat.

We calculated odds ratios to assess the relative magnitude of the effect of the pesticide and fish variables on the likelihood of *R. muscosa* presence at a single site (given the distribution of fish and pesticides relative to

all other factors that affect *R. muscosa* presence—components 1 and 3 of stressor impacts combined). In the context of our study, the odds ratio is the ratio between the odds of an event under two situations: exposed or not exposed to a stressor (for binomial variables), or exposure to the actual level of a stressor vs. some comparison level (for continuous variables). Thus, to evaluate the magnitude of the effect of fish on the likelihood of frog presence at a single site we calculated the odds ratio for fish as the odds of *R. muscosa* being present at a water body with fish divided by the odds of frogs being present at a water body without fish. For pesticides, we calculated the odds ratio as the odds of frogs being present at a given pesticide level divided by the odds of frogs being present when pesticides were absent.

To compare the overall effects of fish and upwind pesticide use on *R. muscosa* presence or absence across the study area (all three components of stressor impacts combined), we compared the predicted probabilities produced by the final GAM based on observed variable values versus those produced under “no fish” and “no pesticide” simulations. For the “no fish” simulation, we set the fish variable values at all water bodies to “no fish,” and then used the regression model to predict the probability of *R. muscosa* presence at each water body. In the “no pesticide” simulation, we generated predicted probabilities for all water bodies with all pesticide values set to zero.

To examine which pesticide classes (pesticides with similar chemical structures), pesticide groups (pesticides with similar biological effects, i.e., cholinesterase inhibition), or individual pesticides were most strongly associated with *R. muscosa* occurrence, we followed the methods of Davidson (2004). Briefly, we constructed 230 separate GAMs by replacing the total pesticides variable in our final GAM with one of 63 pesticide classes, one of seven groups, or one of 160 individual pesticides. All other model variables were the same as in the final GAM. All pesticide classes, groups, and individual pesticides were widely used upwind from our study area (see Appendix for details). To identify the pesticide classes, groups, and individual pesticides that produced models with a better fit than a model based on total pesticides, we calculated Akaike Information Criteria (AIC) values for all models and identified those with an AIC value less than that of the total pesticides model.

## RESULTS

Of the 6831 water bodies surveyed, *R. muscosa* was present at 13% (Fig. 1) and introduced fish were present at 16%. Habitat characteristics of sites at which *R. muscosa* was present versus absent are provided in Table 1. Introduced fish were all salmonids and for the water bodies that were gill netted, 26% contained rainbow trout (*O. mykiss*), 46% contained golden trout (*O. m. aguabonita*) or golden trout  $\times$  rainbow trout hybrids,

TABLE 1. Mean and interquartile range of each predictor variable at sites with and without *Rana muscosa*.

Variable name	<i>R. muscosa</i>		Interquartile range
	presence/absence	Mean	
Fish presence (%)	present	17	
	absent	16	
Upwind pesticide use (kg/m)	present	48.5	1.7–50.2
	absent	89.3	2.2–65.8
Topographic sheltering (degrees)	present	68	33–95
	absent	71	33–100
Maximum water depth (m)	present	4.2	0.8–5.0
	absent	2.9	0.4–3.0
Water body elevation (m)	present	3165	2969–3447
	absent	3093	2841–3406
Littoral zone silt (%)	present	42	7–73
	absent	45	4–89
No. water bodies within 1 km	present	18	9–25
	absent	14	5–18
Precipitation (cm)	present	103	89–114
	absent	106	89–114

Note: For the categorical variable, fish presence, the value indicates the percentage of sites containing fish.

0.6% contained cutthroat trout (*Oncorhynchus clarkii*), 2% contained brown trout (*Salmo trutta*), and 38% contained brook trout (*S. fontinalis*; percentages don't add to 100% because more than one species of trout were present in some water bodies). In the generalized additive model that included total pesticides used upwind, the presence/absence of introduced fish, amount of upwind pesticide use, and topographic sheltering, as well as all covariates except precipitation (location, water depth, elevation, littoral zone silt, water body isolation), had highly significant effects on the presence/absence of *R. muscosa* (Table 2). After accounting for the effects of all other predictor variables, the presence of fish reduced the odds of *R. muscosa* occurrence (Fig. 2a). Furthermore, the odds of *R. muscosa* occurrence declined steeply with increasing upwind pesticide use (Fig. 2b). The relationship between *R. muscosa* occurrence and topographic sheltering was somewhat complex, but in general supported our hypothesis that the odds of *R. muscosa* occurrence

would be lowest at low angles, and highest at angles of 90°–180° (Fig. 2c). Response curves for all other covariates (Fig. 3) were similar to those reported in earlier studies (Knapp et al. 2003, Knapp 2005). In addition, these results were unchanged when unmapped water bodies were excluded from the analysis. Model validation indicated that the model was not overfit and had generality beyond the data used to develop the model (see Appendix for details).

The odds ratio for the effect of fish on *R. muscosa* was 0.51, indicating that at a single water body, the presence of fish reduces the odds of frog presence by twofold. Upwind pesticide use is a continuous variable, and the odds ratio therefore varies depending on what upwind pesticide levels are being compared. At the median upwind pesticide level (13.2 kg/m), the odds ratio was 0.32 (i.e., this level of upwind pesticides reduces the odds of frog presence at a single site by threefold). At the highest pesticide levels, the odds ratio was 0.000023, indicating that at this level the strength of the pesticide effect on *R. muscosa* at a single site is four orders of magnitude stronger than the fish effect (0.000023 vs. 0.51). In 63% of the water bodies, the upwind pesticide odds ratio was less than the 0.51 fish odds ratio.

Using the observed values for fish presence/absence, the mean predicted probability of *R. muscosa* presence was 0.134 (i.e., the actual proportion of sites occupied by *R. muscosa* in the study area). In the “no fish” simulation, the mean predicted probability increased to 0.144, a 7.4% increase (Fig. 4). In the “no pesticides” simulation, the mean predicted probability more than doubled to 0.287, indicating that upwind pesticide use is associated with a much greater overall effect on *R. muscosa* presence/absence than are fish. The overall stronger effect of upwind pesticide use reflects the combination of a stronger single-site effect of upwind pesticide use (as described by the odds ratios) and the wider distribution of upwind pesticides than fish. Introduced trout are present in 16% of the water bodies in our study area, while pesticides are used upwind, at

TABLE 2. Analysis of deviance table showing the statistical significance of the predictor variables in the final generalized additive model.

Model	Model		Test		P
	Deviance	df	Deviance†	df‡	
Null model	4566	5830			
Full model	3221	5767			
Full model minus:					
Water body location	4037	5805	816	38	$<1 \times 10^{-10}$
Water body depth	3364	5771	143	4	$<1 \times 10^{-10}$
No. water bodies within 1 km	3323	5770	102	3	$<1 \times 10^{-10}$
Elevation	3286	5770	65	3	$<1 \times 10^{-10}$
Fish presence/absence	3247	5768	26	1	$2.8 \times 10^{-7}$
Topographic sheltering	3238	5771	18	4	$1.1 \times 10^{-3}$
Littoral zone silt	3238	5770	17	3	$1.1 \times 10^{-3}$
Upwind pesticide use	3238	5773	17	6	$8.2 \times 10^{-3}$

† Test deviance = (deviance of full model less one covariate) – (deviance of full model).

‡ Test df = (df of full model less one covariate) – (df of full model).

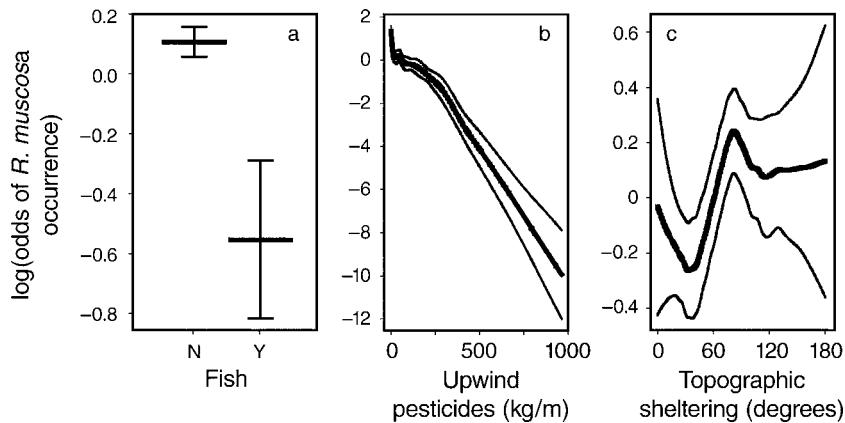


FIG. 2. Response curves for (a) fish presence (Y, yes) or absence (N, no), (b) upwind pesticide use, and (c) topographic sheltering generated from the generalized additive model. For each plot, the y-axis indicates the log odds of *R. muscosa* presence as a function of the predictor variable (x-axis) after the influence of other variables has been accounted for. The thick line indicates the log odds, and the thin lines indicate the approximate 95% CI.

some level, from all water bodies, and at high enough levels in a majority of water bodies to have a stronger negative effect than would fish.

However, evaluating the relative importance of fish vs. pesticides on the occurrence of *R. muscosa* is not straightforward in our study because the strength of the upwind pesticide use variable was sensitive to the extent to which we corrected for spatial autocorrelation. Because the upwind pesticide use variable was spatially correlated with location, if we reduced the deviance accounted for by location (by increasing the “span” of the location variable), the strength of the pesticide variable almost doubled from the values we report here.

None of the models based on pesticide classes or individual pesticides were clearly better than a model based on total pesticides. Roughly half of the classes and individual chemicals produced models with lower AIC values than the total pesticides model, but the difference

in AIC between the models with the lowest values and the total pesticides model was <1%. In addition, the class and individual pesticides models with the lowest AIC values showed no commonality of chemical structure or biological effect.

DISCUSSION

Past observational and experimental research into the causes of decline in *R. muscosa* have strongly implicated introduced fish as a causative factor (Bradford 1989, Bradford et al. 1998, Knapp and Matthews 2000, Vredenburg 2004, Knapp 2005). However, the disappearance of *R. muscosa* from sites without fish (Table 3), and more importantly even from entirely fishless basins (R. A. Knapp, unpublished data) indicates that factors other than fish must have also played an important role in the decline of this species. Our regression results suggest that both fish and pesticides may be contributing

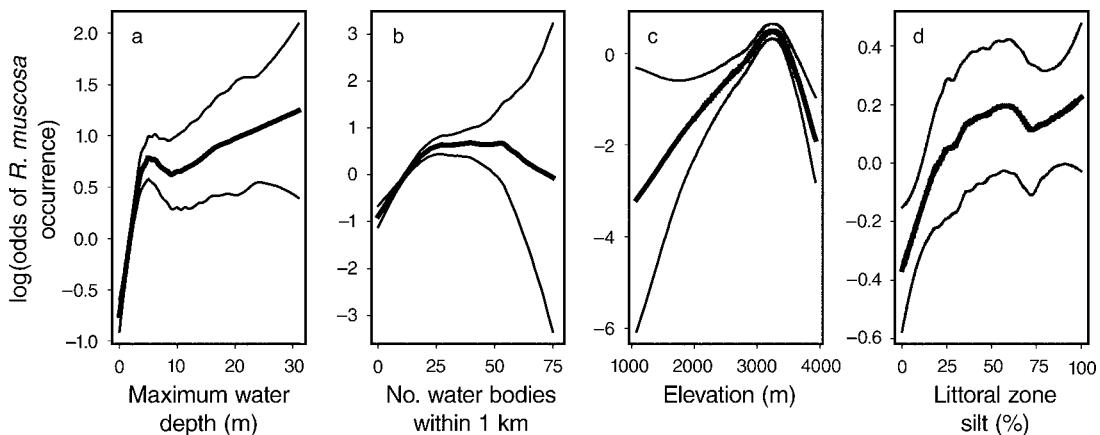


FIG. 3. Response curves for (a) maximum water depth, (b) number of water bodies within 1 km, (c) elevation, and (d) percentage of littoral zone composed of silt, generated from the total pesticides generalized additive model. For each plot, the y-axis indicates the log odds of *R. muscosa* presence as a function of the predictor variable (x-axis) after the influence of other variables has been accounted for. The thick line indicates the log odds, and the thin lines indicate the approximate 95% CI.

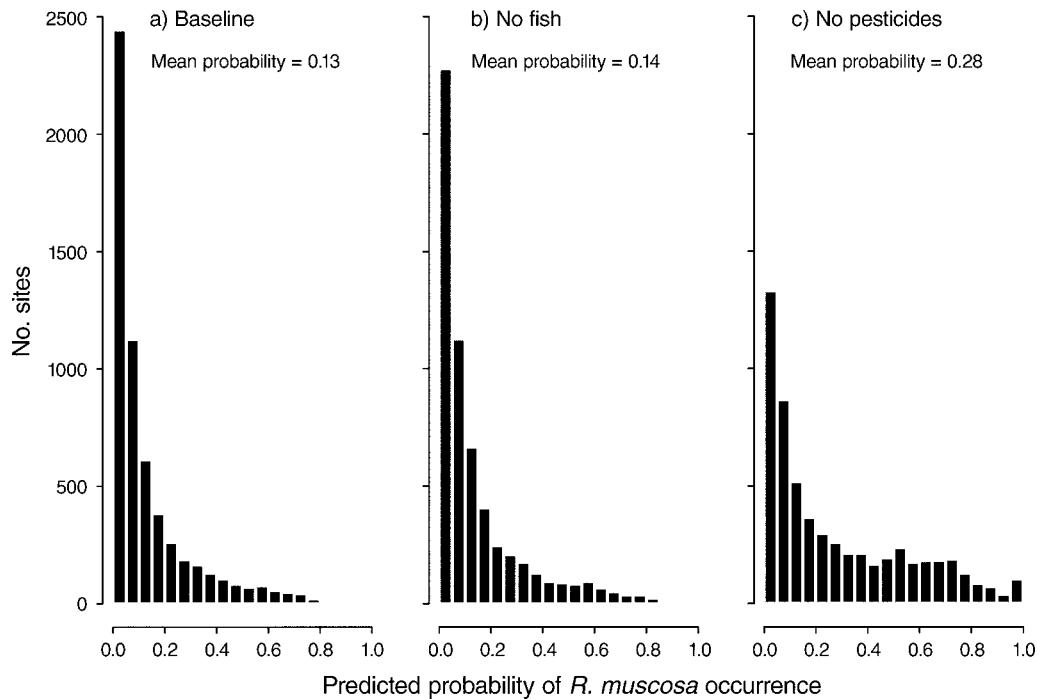


FIG. 4. Distribution of predicted probabilities of *R. muscosa* occurrence produced by the total pesticides GAM, under three different scenarios: (a) baseline with observed variable values for fish and upwind pesticides, (b) “no fish” scenario, with fish absent from all sites and the upwind pesticides variable unchanged from observed, and (c) “no pesticides” scenario, with total upwind pesticide use set to zero for all sites and the fish variable unchanged from observed. In all three scenarios, all other variables are unchanged from observed values.

to the decline of this species. Furthermore, the response curves, odds ratios, and predicted probability simulations suggest that pesticides may be having a much greater overall negative effect on *R. muscosa* presence/absence than are introduced fish.

Our finding that topographic sheltering is significantly related to frog presence represents a second, independent line of evidence that windborne contaminants are negatively effecting *R. muscosa* populations. Although we focused on pesticides, because upwind pesticide use is strongly correlated with upwind agricultural land use and distance to the Central Valley, the pattern of declines associated with upwind pesticide use could be driven by any wind-blown agricultural substance that negatively affects frogs (e.g., fertilizers [Marco 1999]). However, we suggest that pesticides are the most likely substance to be affecting frogs due to their potential

toxicity even at low concentrations (<1 ppm; e.g., Gopal et al 1981, Hayes et al. 2002) and documented long-range transport. While the statistically significant effect of the topographic sheltering variable does not specifically implicate pesticides over other types of contaminants, this result is consistent with the significant relationship between the total upwind pesticides variable and frog presence/absence, and provides an additional piece of evidence suggesting that airborne pesticides are negatively impacting *R. muscosa* populations.

One of the most intriguing results from our analyses is the suggestion from the shape of the upwind pesticide response curve that small differences in the amount of upwind pesticide use, even at the very lowest levels, may have a large effect on the probability of frog occurrence. Davidson (2004) found similar results in an analysis of the association between upwind pesticide use and

TABLE 3. Presence/absence of *R. muscosa* and fish for all water bodies and for water bodies deeper than 3 m.

Fish	All water bodies			Water bodies >3 m deep		
	<i>R. muscosa</i>		Total	<i>R. muscosa</i>		Total
No	Yes	No		Yes		
No	4986 (87%)	742 (13%)	5728	953 (77%)	285 (23%)	1238
Yes	946 (86%)	157 (14%)	1103	553 (85%)	94 (15%)	647
Total	5932 (87%)	899 (13%)	6831	1506 (80%)	379 (20%)	1885

population declines of five California amphibians. Much more extensive field sampling for pesticides is needed at remote locations such as our Sierra Nevada sites to determine if locations with the lowest levels of upwind pesticide use do indeed contain very low pesticide concentrations. If low levels of pesticides do have important negative effects on amphibians (e.g., Hayes et al. 2002), it is likely that their effect is not through lethal exposures, but rather is caused by sublethal exposures and synergisms with other factors.

Laboratory studies have shown that pesticide exposure may interact synergistically with predation stress (i.e., presence of newts) to make pesticides much more lethal (e.g., Relyea and Mills 2001, Relyea 2003). We looked for an interaction between fish and upwind pesticide use in our data set by comparing the strength of the negative effect of fish on probability of occurrence at sites with low upwind pesticide levels versus sites with high upwind pesticide levels. This analysis suggested that the negative effect of fish was the same at both high and low upwind pesticide sites. However, the potential for a fish  $\times$  pesticide interaction (and our ability to detect such an interaction) was limited in our study by the relative paucity of sites at which fish and frogs co-occur. In addition, we can not rule out an interaction between predators and pesticides without data on other predators in addition to fish (e.g., invertebrates).

Recent experimental studies indicated that the removal of nonnative trout from several Sierra Nevada lakes resulted in rapid increases in *R. muscosa* densities (Vredenburg 2004; R. A. Knapp, unpublished data). The results of our study that suggest important roles for both fish and pesticides in driving the decline of *R. muscosa* may at first make the observed rapid increases in frog densities following fish removal alone seem paradoxical. However, the fish removal studies were conducted in lake basins located in areas of low upwind pesticide use. As a result, in these basins, our GAM predicts a large positive effect of fish removal on the probability of *R. muscosa* presence (average probability of occurrence prior to and following fish removal is 35% and 46%, respectively). In contrast to the rapid recovery of frogs following fish removal, four reintroductions of *R. muscosa* into other sites in the Sierra Nevada failed (Fellers et al. 2004). While comparing natural recovery following fish removal to translocation into already fishless lakes is not perfect, the reintroductions were all conducted into sites with very high upwind pesticide use. As a result, our GAM predicts a very low probability of *R. muscosa* presence at these sites (average probability of occurrence = 0.0004). This suggestion that upwind pesticides may have influenced the outcome of these studies is consistent with the results of recent research (Fellers et al. 2004) indicating that *R. muscosa* at the reintroduction sites had higher pesticide levels in body tissue than did those immediately adjacent to one of the fish removal sites. It remains to be determined whether other factors, such as disease, also contributed to the

success or failure of fish eradication and frog reintroduction efforts.

An earlier analysis of upwind pesticide use and amphibian population declines for five California anurans (Davidson 2004) found that declines were more strongly associated with cholinesterase-inhibiting pesticides (mostly organophosphates and carbamates) than with total pesticide use or any other class or group of pesticides. The reason for the lack of a similar association in the current study is unknown, but may be due to several important differences between the two studies. These include (1) different time periods of pesticide use (1974–1991 in the Davidson (2004) study vs. 1991–2000 in the current study), (2) inclusion of five amphibian species vs. solely *R. muscosa*, (3) different geographic scope (all of California vs. the central and southern Sierra Nevada only), (4) different size upwind triangle, (5) inclusion vs. exclusion of winter pesticide use, and (5) different independent variables included in statistical models. We conclude that the fact that cholinesterase inhibitors were not more strongly associated with the distribution of *R. muscosa* than other pesticide classes weakens the generality of the earlier finding of a strong association between declines and upwind use of cholinesterase inhibiting pesticides (Davidson 2004).

Although the current study focused only on a single amphibian species (*R. muscosa*), our results suggest an urgent need for further observational and experimental studies of the role of pesticides in amphibian declines in general. Experimental studies are needed to examine possible mechanisms whereby low-level pesticide exposures could lead to large-scale population declines. We believe the most plausible hypothesis is that pesticide exposure causes immune suppression that in turn makes amphibians more susceptible to disease. Several studies on amphibians have indeed found that pesticide exposure can weaken immune response and increase disease susceptibility (Taylor et al. 1999, Kiesecker 2002, Gilbertson et al. 2003). However, to date no experiments have been conducted using pesticide levels as low as those likely found in remote Sierra Nevada sites (e.g., LeNoir et al. 1999, Fellers et al. 2004, Hageman et al. 2006). In addition, very little is known about the geographic and temporal distribution of pesticides in Sierra Nevada water bodies. Measurements of chemical concentrations have been made for only a handful of different pesticides, and only at a small number of sites. Measurements of actual pesticide levels are needed across a much larger spatial scale to assess the level and type of exposures actually experienced by animals. However, the use of hundreds of different pesticides and the extreme temporal variability in pesticide applications means that large scale geographic analyses of pesticide effects will for the foreseeable future be dependent on proxy measures of exposure (such as upwind pesticide use) rather than direct field measurements.

In amphibian decline research, there is a growing awareness of the need to examine multiple factors and interactions (Kiesecker et al. 2001, Linder et al. 2003). However, for practical reasons most field studies continue to focus on single factors. Our finding that both fish and pesticides are associated with declines strongly supports the need for additional multi-factor studies. Studies of a single causal factor that find that the factor is associated with a phenomenon do not indicate that other factors are not also contributing to the phenomenon. Our results show that multi-factor studies can also move beyond the question of which factors are contributing to declines, and investigate the relative importance of different factors in causing declines. This information is critical for the development of effective conservation efforts and environmental policies. In the case of policies to address declines of *R. muscosa*, our findings on the relative effects of fish and pesticides suggest that pesticides deserve greater attention and concern than they have received to date.

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#### APPENDIX

Details on model validation and pesticide analyses (*Ecological Archives* A017-022-A1).