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CONTAMINANT RESIDUES AND DECLINES OF THE CASCADES FROG (*RANA CASCADAE*) IN THE CALIFORNIA CASCADES, USA

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Abstract—Populations of Cascades frogs (*Rana cascadae*) have declined precipitously in the Mount Lassen area, but remain abundant in the other half of their California range in the Klamath Mountains. To evaluate the role of contaminants in Cascade frog declines, we sampled sediment and frog tadpole tissue at 31 sites where Cascades frogs had disappeared and sites where Cascades frogs are still present across the Lassen and Klamath regions. Pacific chorus frogs (*Pseudacris regilla*) were tested and used as surrogates for residue concentrations in Cascades frogs. We analyzed a total of 79 tadpole samples for 73 semivolatile contaminants including pesticides, polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs). The most frequently detected residue was endosulfan sulfate, followed by dacthal, chlorpyrifos, PCB 187, endosulfan II, *trans*-chlordane, and *trans*-nonachlor. Chorus frogs had similar residue concentrations as Cascades frogs for most but not all chemicals, indicating that chorus frogs can serve as a reasonable proxy for chemical concentrations in Cascades frogs. None of the contaminants in tissue or sediment had significantly higher concentrations at sites where Cascades frogs have disappeared than at sites where Cascades frogs are still present. We found no evidence to support the hypothesis that the contaminants analyzed have contributed to the decline of Cascades frogs in northern California, although we were able to analyze only a handful of the over 300 pesticides currently used in the area. Environ. Toxicol. Chem. 2012;31:1895–1902.

Keywords—Amphibian declines Pesticides *Rana cascadae* *Pseudacris regilla*

INTRODUCTION

Amphibian population declines are a key example of the global biodiversity crisis [1]. Although disease has emerged as the leading explanation for amphibian declines [2,3] it is unknown whether disease is acting alone, or whether environmental factors such as climate change or contaminants may have facilitated disease epidemics, possibly by weakening amphibian immune systems. Pesticide exposure has long been hypothesized as a possible cause for amphibian declines; however, there has been surprisingly little field ecotoxicological research examining contaminants and amphibian population declines [4–6].

California is a hotspot of amphibian decline, with many species experiencing sharp range contractions in the last 25 years [7]. Studies in the Sierra Nevada have documented the transport and deposition of pesticides from the Central Valley to remote alpine ecosystems [8–13]. Several studies have found a strong association between declines of numerous California amphibians and agricultural land-use upwind from sites [14,15] or pesticide use upwind from sites [16,17]. Laboratory studies indicate that low doses of pesticides can cause immune suppression in amphibians and in some cases affect disease susceptibility [18–20]. However, to demonstrate that pesticides actually cause declines, one must simultaneously demonstrate the exposure of frogs to contaminants in the field and link those exposures to declines. This is a key piece of missing information on the role of pesticides and amphibian declines and the subject of the present study.

The Cascades frog (*Rana* [=*Lithobates*] *cascadae*) is a montane species that ranges from Washington State to northern California. Like a number of other ranid frogs in California, Cascades frogs have disappeared from large parts of their range [7]. The historic range of the Cascades frog in California consists of two disjunct areas: one around Lassen Volcanic National Park where the frog has almost completely disappeared [21] and another in the Klamath Mountains (including the Marble Mountains and Trinity Alps), where the frog is still widespread (Fig. 1) [22]. The rapid decline of Cascades frogs in the Lassen region has been attributed to several possible causes, including introduced fish [23], disease [21], and pesticide transport from the Central Valley [16]. Although introduced fish clearly have negative impacts on amphibian populations, Fellers et al. [21] argue that introduced fish are unlikely to be responsible for the decline of Cascades frogs in the Lassen region. Fish stocking began in the Lassen region long before declines, and introduced fish are much more prevalent in the Klamath region than the Lassen, yet Cascades frogs remain abundant in the Klamath region. Similarly, Cholodenko found that historic sites for Cascades frogs with and without introduced trout across the Lassen and Klamath regions were almost equally likely to still have Cascades frogs present (L. Cholodenko, 2006, Master's thesis, Sacramento State University, CA, USA). The rapid collapse of Cascades frogs in the Lassen region, with now only a few small remnant populations remaining, fits the pattern of population declines driven by the chytrid fungus *Batrachochytrium dendrobatidis* [24]. However, chytrid fungus is also widespread in the Klamath region [21], raising the question of why similar

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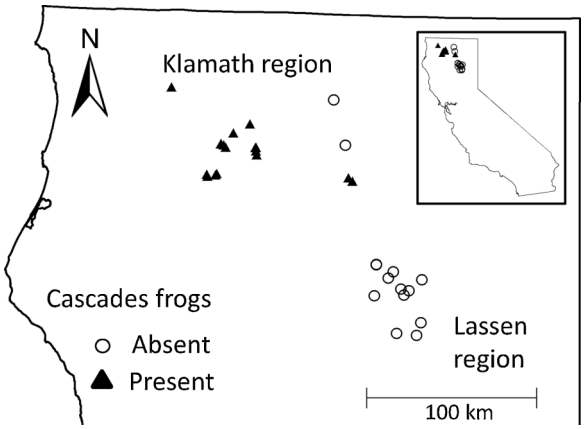

Fig. 1. Residue sampling sites and declines of the Cascades frog. The map indicates our 31 sampling sites in the Cascades Mountains of northern California. Solid triangles indicate sites where Cascades frogs were still present. Open circles indicate sites where Cascades frogs historically occurred but are now absent. Insert map shows sampling sites and the state of California.

declines have not occurred there. In a study across the Klamath and Lassen region, Davidson [16] found that upwind pesticide use was much greater for sites where Cascades frogs had declined than at sites where Cascades frogs are still present. However, the study was based on predominant wind patterns and pesticide application records rather than field measurements of pesticides.

One of the great challenges of contaminant research on amphibian declines is that by the time amphibian declines have been documented, populations are generally extinct or at very low concentrations, often precluding the possibility of examining contaminant concentrations in declining frogs themselves. A strategy to deal with this sampling dilemma is to measure contaminants in a co-occurring but nondeclining frog species as a surrogate for contaminant concentrations in declining species. Several studies have used this strategy [5,6,25] using the widespread Pacific chorus frog (*Pseudacris regilla*) to study declines of ranid frogs; however, to date, no studies have examined whether Pacific chorus frogs are a good surrogate for assessing contaminant concentrations in co-occurring declining amphibians.

After metamorphosis, Pacific chorus frogs and Cascades frogs have fairly different life histories. Pacific chorus frogs reach sexual maturity in less than one year, and adult frogs spend much of their time away from water when they are not breeding [26]. Adult Cascades frogs spend most of their time in and around water, reach sexual maturity in two to four years, and live over five years [27]. However, as tadpoles, the two species are quite similar. They inhabit the same water bodies and even within water bodies are found in the same habitats. We would frequently capture both species in a single sweep of our sampling net. Cascades frogs and Pacific chorus frogs have similar larval periods: two to two-and-a-half months for Pacific Chorus frogs and two months for Cascades frogs [28].

In both the Lassen and the Klamath region, there are healthy populations of Pacific chorus frogs. By studying pesticide residues from Cascades and chorus frogs where they co-occur in the Klamath region, we determined whether pesticide residues were similar in the two species and consequently whether chorus frogs are a good surrogate for measuring pesticide concentrations in Cascades frogs. We then measured pesticide concentrations in chorus frogs in both the Lassen and Klamath regions to determine if pesticide residues in chorus frogs are higher at sites where Cascades frogs have disappeared than at sites where Cascades frogs are still present. We also analyzed sediment samples to determine if pesticide concentrations in sediment are higher at sites where Cascades frogs have disappeared than at sites where Cascades frogs are still present. By examining the pattern of contaminants in frog tissue and sediment, we evaluated whether contaminants may have contributed to declines of Cascades frogs.

MATERIALS AND METHODS

Site selection and sample types

Our first priority for sampling sites was historic locations for Cascades frogs in California. For our purposes, historic locations were sites where Cascades frogs were known to occur before 1990. Historic sites were selected from a database containing museum records, reports from the literature, and other sources [7,14]. To increase total sample size, we also sampled a number of nonhistoric sites where Cascades frogs were currently present. For analysis of population declines of Cascades frogs, we considered a site to have experienced declines if historically Cascades frogs were present and presently the species is absent. Determination of absence was based on unpublished surveys by Davidson, a variety of published sources [22,23], and information in the California Natural Diversity Database [29]. Sites were considered to have not experienced declines if Cascades frogs were still present at the site. We sampled 17 sites where Cascades frogs were still present, and 14 sites where Cascades frogs had disappeared.

We collected a total of 79 tadpole tissue samples at 31 sites (Fig. 1 and Supplemental Data, Table S2) between June 6 and August 23, 2005 consisting of 26 Cascades frog samples, and 53 Pacific chorus frog samples. At most sites, we collected a single tissue sample for Pacific chorus frogs and or Cascades frogs. At six sites we collected eight sets of triplicate site replicates so we could examine within-site variation, for a total of 25 samples (see Supplemental Data, Table S2 for a list of types of samples collected by site). The number of sets of site replicates is greater than the number of sites because at some sites we collected site replicate sets for both Pacific chorus frogs and Cascades frogs. We also collected eight sets of site replicates (a total of 26 samples) that were pooled by site and used as analytic replicates to examine variation in the analytic process itself. We collected 19 paired samples of Pacific chorus frogs and Cascades frogs from 12 sites, allowing comparison of residue concentrations in the two species living at the same site (the number of paired samples is greater than the number of sites because at several sites we collected multiple paired chorus frog and Cascades frog samples). Before processing, the Gosner stage was determined for all tadpoles [30].

Field collection methods

Pesticide residue analysis required 2 g of tadpole tissue. Therefore, a single sample for residue analysis consisted of multiple tadpoles. Because the mass of tadpoles varied at different sites, the number of tadpoles required for a single sample varied. In the field, tadpoles were collected with a dip net and temporarily held in a 125-ml, clean glass jar. Once a sufficient number of tadpoles were collected at a site, tadpoles were put in cryo vials and immediately stored in liquid nitrogen.

We collected sediment samples at sites using a hand-corer (Wildlife Supply Company) to take the top 2.5 cm of sediment from three locations at the site in the same general area as where

tadpoles were collected. The sediment was stored in precleaned 125-ml glass jars with Teflon tops and immediately stored on dry ice. Sediment samples and cryo vials were packed with dry ice and shipped overnight to the Simonich laboratory at Oregon State University, where they were stored at −20°C until residue analysis. All field gear was disinfected between sites using Quat-128 (Waxie Sanitary Supply).

Residue analysis

Our methods were designed to detect over 70 semivolatile organic compounds (SOCs) in tadpole and sediment samples including both current and historic use pesticides, polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs; see Supplemental Data, Table S1 for a complete list along with estimated detection limits in tissue and sediment). Details of the tadpole SOC analysis methods are given in Stanley et al. [31]. Briefly, SOCs were extracted from tadpole samples using matrix-solid phase dispersion. Frozen tadpole samples, containing a pool of tadpoles from a given site, were ground to a sand-like consistency. A 2-g subsample of tissue was further ground with 10 g of octadecylsilyl (C_{18}) and 35 g of sodium sulfate (Na_2SO_4). The tadpole mixture was packed into an empty, 60-ml solid phase extraction (SPE) column, spiked with isotopically labeled surrogate standards and an on-column extraction was carried out, eluting SOCs with 300 ml acetonitrile. The eluted sample was reduced in volume and sample cleanup was carried out using a 20-g silica SPE column. The final sample was reduced in volume and isotopically labeled internal standards were added before instrumental analysis. Moisture content and lipid content were determined using gravimetric analysis on 0.5 g subsamples of the ground tadpole tissue.

Sediment samples were prepared for SOC analysis as described in Usenko et al. [32]. Briefly, 12 g of wet sediment was ground with 120 g of Na_2SO_4. The mixture was packed into 66-ml accelerated solvent extraction cells, spiked with isotopically labeled surrogate standards, and pressurized liquid extraction was carried out with dichloromethane. Sample cleanup, gel permeation chromatography, and addition of internal standards were the same as with residue samples. Moisture content was determined using a 2-g subsample of wet sediment and total organic carbon (TOC) was determined by drying 0.2-g subsamples and analyzing for TOC using a CNS-2000 Element Analyzer.

Tissue and sediment sample extracts were analyzed for SOCs using an Agilent 6890 gas chromatograph coupled to an Agilent 5973N mass selective detector. The gas chromatography/mass spectrometry (GC/MS) was equipped with either electron impact ionization (EI) or electron capture negative ionization (ECNI). We used the ionization mode that gave the lowest instrumental detection limit for a given SOC, and selected ion monitoring. One microliter of sample was injected using a pulsed splitless injection and SOCs were separated using a DB5 column (J&W Scientific). A full description of instrumentation details including limits of detection and ions monitored has been published previously [32]. One standard was run for every three to four samples processed on the GC/MS-EI and ECNI to track the condition of the instrument calibration curve and new calibration curves were prepared as necessary.

The sample processing included one laboratory blank run for every batch of samples, consisting of 8 to 12 samples. Final residue values were calculated by subtracting residue concentrations found in the laboratory blanks. If residue concentrations in laboratory blanks were greater than 33% of the sample value for a given compound, the sample value was considered unusable and was not included in statistical analysis. If the blank subtracted residue value was less than the estimated detection limit for a compound, the value was replaced with half the estimated detection limit (½EDL). A chemical was considered to be a "detection" in a sample if the residue value was greater than 33% of the blank value and the blank subtracted residue value was greater than the estimated detection limit for that chemical. For data analysis, we only analyzed and presented results where more than 30% of samples in that analysis are detections. In calculating the percentage of detections in a set of samples, samples where laboratory blanks were greater than 33% of the sample value were excluded. For analysis of analytic variation in the tadpole method, replicate tissue samples collected in the field were ground together in the initial grinding step, and then the three aliquots of ground tissue were treated as individual samples for the remainder of the analysis preparation and instrumental analysis. We calculated residue concentrations in tissue on a wet tissue, dry tissue, and lipid-mass basis. In sediment, we calculated residue concentrations on a wet, dry, and organic carbon mass basis (see Bradford et al. [25] for organic carbon basis methods and Usenko et al. [32] for methods for all other basis).

Statistical analysis

For all statistical analysis, we used the dry mass basis for tissue and the organic carbon mass basis for sediment as the most representative measures of residues. A summary of all the residue concentration measures is in Supplemental Data, Tables S3 and S4. We analyzed variation in tissue residue concentrations in the analytic replicates, site replicates, across sites, and across all samples by calculating coefficients of variation for each set of samples. For analytic or site replicate samples, we calculated coefficients of variation for each replicate set at a site and then calculated the average coefficient of variation across all the sites for each chemical. We corrected the coefficient of variation for small samples sizes following Sokal and Rohlf [33]. All analysis of variation was done exclusively with chorus frog samples. For all statistical analyses—except analysis of variation in analytic replicates—residue concentrations in analytic replicates were averaged, and each set of analytic replicates were treated as a single sample. For all analyses across sites, single site residue values were first calculated by averaging all samples from a site.

We used nonparametric Mann–Whitney tests to evaluate differences in mean residue values between chorus frogs and Cascades frogs and between chorus frogs and sediment collected at sites where Cascades frogs are still present versus sites where Cascades frogs have declined. We used nonparametric Spearman correlation coefficients to determine how well residue concentrations in chorus frogs correlated with residue concentrations in Cascades frogs from sites where the two species were collected together. We also used Spearman correlation coefficients to assess the geographic correlation of residue concentrations between different chemicals. Pearson χ^2 tests were used to compare chemical detection frequencies between chorus frogs and Cascades frogs.

RESULTS

There were no significant differences in tadpole sample collection date, weight, or Gosner stage between sites where Cascades frogs were still present and sites where Cascades frogs were absent that might have biased our analysis of associations

between contaminant residues and frog declines. The mean sampling date for sites with Cascades frogs still present was day 204.8 (with January 1, 2005 set as day 1) and 196.8 for sites where Cascades frogs were absent (Mann–Whitney test, $p = 0.147$). The mean tadpole wet weight was 2.07 g and mean Gosner stage 30.0 at sites where Cascades frogs were still present, and 2.01 g and mean stage 31.1 at sites where Cascades frogs were absent (Mann–Whitney tests, $p = 0.937$ for weight, and $p = 0.549$ for stage).

Six current-use pesticides or their breakdown down products, four now banned organochlorine pesticides, five PCBs, and the PAH fluoranthene were found in Cascades and chorus frog tadpole tissue (Table 1). Endosulfan sulfate—a breakdown product of the current-use organochlorine pesticides endosulfan I and endosulfan II—was the most frequently detected chemical in tadpole samples, occurring in 69.2% of samples (Table 1). Six other chemicals were detected in 34 to 53% of samples: dacthal, PCB 187 (hepta), *trans*-nonachlor, endosulfan II, chlorpyrifos, and *trans*-chlordane. Ten other chemicals were detected at much lower frequencies, occurring in 2 to 20% of useable samples (Supplemental Data, Table S3). Average residue concentrations for all the chemicals with at least a 30% detection frequency ranged from 0.08 to 0.5 ng/g dry weight tadpole tissue (Table 1 and Supplemental Data, Table S3).

In general, more chemicals were detected in sediment than in tissue, and they were detected at much higher frequency (Table 1). Although only two chemicals (endosulfan sulfate and dacthal) were detected at greater than 50% frequency in tissue samples, 16 chemicals were detected at greater than 50% frequency in sediment. Chemicals measured in sediment included the same six current-use pesticides or their breakdown down products measured in tissue, plus two additional current-use pesticides found only in sediment (hexachlorobenzene and trifluralin), four now banned organochlorine pesticides (*trans*-nonachlor, cis-nonachlor, *trans*-chlordane, and dieldrin), the same five PCBs as in tissue, and 17 PAHs. Mean pesticide and PCB residue concentrations in sediment ranged from 0.08 to 15 ng/g organic carbon weight, and mean PAH concentrations ranged from 68 to 12,000 ng/g organic carbon weight (Table 1 and Supplemental Data, Table S4).

Coefficients of variation (CV) for tissue residue concentrations in the analytic replicates averaged 25% and ranged from 13 to 39% depending on the chemical (Table 2). Variation in site-replicate tissue concentrations averaged 62%, ranging from 30 to 103%. Coefficients of variation across sites averaged 117%, ranging from 67 to 216%. The average ratio of within site CVs to across-site CVs was 0.62.

In comparing pesticide residues in Pacific chorus frog tadpoles and Cascades frog tadpoles collected together from the same sites, we found no significant difference in the mean number of chemicals detected (mean 3.82 for Cascades frogs, 3.06 for chorus frogs, Mann–Whitney test, $p = 0.26$). In chorus frog and Cascades frog samples collected together, there were six chemicals detected frequently enough to allow comparison of detection frequency and mean residue concentrations between the two species (chlorpyrifos, dacthal, endosulfan II, endosulfan sulfate, *trans*-nonachlor, and PCB 187). For these six chemicals, none of the differences in detection frequency for individual chemicals by species was statistically significant based on Pearson χ^2 tests. For these same six chemicals, mean residue concentrations in Cascades frogs and chorus frogs collected at the same sites were not significantly different (Table 3). The ratio of mean residues concentrations in chorus frogs to Cascades frogs ranged from 0.55 to 1.76. Cascades frogs had higher concentrations for dacthal and endosulfan II, whereas chorus frogs had higher concentrations for the other four chemicals; but, in general, their concentrations were similar. In the paired samples, only five chemicals (chlorpyrifos, dacthal, endosulfan II, endosulfan

Table 1. Chemical detection frequencies and mean residue concentrations in Pacific chorus frog and Cascades frog tadpole tissue, and in sediment[a]

	Tissue detection frequency	Sediment detection frequency	Tissue mean weight, ng/g	Sediment mean weight, ng/g
Current use pesticides				
Chlorpyrifos	48.9%	62.9%	0.484	0.754
Dacthal	53.1%	66.7%	0.261	0.534
Endosulfan II	35.4%	41.7%	0.171	0.234
Endosulfan sulfate	69.2%	97.2%	0.535	3.005
Hexachlorobenzene	0.0%	87.5%		15.233
Trifluralin	0.0%	60.0%		0.181
Banned organochlorines				
trans-Chlordane	35.3%	88.9%	0.100	0.521
cis-Nonachlor	12.7%	83.3%	0.057	0.373
trans-Nonachlor	33.8%	91.7%	0.082	0.620
PCBs				
PCB 118 (penta)	5.6%	33.3%	0.184	0.896
PCB 138 (hexa)	1.6%	66.7%	0.744	1.130
PCB 153 (hexa)	16.7%	77.8%	0.344	1.138
PCB 183 (hepta)	13.0%	75.0%	0.054	0.319
PCB 187 (hepta)	38.0%	86.1%	0.114	0.708
PAHs				
Benzo[*b*]fluoranthene-L	0.0%	58.3%		256.3
Benzo[*e*]pyrene-L	0.0%	41.7%		102.1
Chrysene + triphenylene	0.0%	30.6%		184.1
Fluoranthene	19.7%	79.4%	2.133	970.6
Fluorene	0.0%	30.0%		513.3
Phenanthrene	0.0%	100.0%		12,041.8
Pyrene-LA	0.0%	36.1%		858.6

[a] Tissue residue concentrations are ng/g dry weight, sediment residue concentrations are ng/g organic-carbon weight. Only chemicals with at least a 30% detection frequency are included in the table. Samples sizes and concentrations based on wet and lipid weight for tissue and wet and dry weight for sediment are in Supplemental Data, Tables S3 and S4.

PCB = polychlorinated biphenyls; PAH = polycyclic aromatic hydrocarbons.

Table 2. Variation in chemical tissue residue concentrations in Pacific chorus frog tadpoles[a]

	Analytic replicates	Site replicates	Across all sites	All samples	Ratio site replicates to across site CV	Ratio analytic replicates to across site CV
trans-Chlordane	0.29			4.78		
Chlorpyrifos	0.39	0.43	0.87	1.01	0.50	0.45
Dacthal	0.26		0.93	1.00		0.28
Endosulfan II		1.03	0.90	1.34	1.15	
Endosulfan sulfate	0.13	0.71	1.01	1.08	0.71	0.12
PCB 187 (hepta)	0.20	0.30	2.16	2.01	0.14	

[a] Coefficients of variation (CV) for analytic and site replicates and across sites and across all samples. For analytic and site replicates the values are average CV values averaged across all sites. Blanks in the table indicate that the CV was not calculated because there were less than 30% detections in the set of samples. PCB = polychlorinated biphenyls.

sulfate, and *trans*-nonachlor) had adequate detections to calculate correlations between chorus frog and cascades frog samples. Fewer samples were available for calculating correlations than for calculating means because, if either one of a paired sample was not usable, both had to be left out in calculating correlations. For endosulfan II, endosulfan sulfate, and dacthal, the correlations were significant and the correlation coefficients ranged from 0.68 to 0.85, whereas for *trans*-nonachlor and chlorpyrifos, the correlations between chorus frog and Cascades frog tissue residue concentrations were not significant (Table 4).

We found no pattern of higher chemical residue concentrations at sites where Cascades frogs had disappeared than at sites where Cascades frogs were still present (Table 5 and Supplemental Data, Fig. S1). Of the five chemicals (chlorpyrifos, dacthal, endosulfan II, endosulfan sulfate, and PCB 187), we could analyze in chorus frog tadpoles tissue at the two sets of sites, only dacthal had significantly different concentrations. Dacthal concentrations were significantly higher in chorus frog tadpoles collected at sites where Cascades frogs were still present (mean 0.45 ng/g dry wt) than at sites where Cascades frogs had disappeared (mean 0.2 ng/g dry wt, Mann–Whitney test, $p = 0.003$). In sediment, residue concentrations for pesticides were generally higher at sites where Cascades frogs had disappeared than at sites where Cascades frogs were still present (Table 5); however, not one of the differences was significant. Polycyclic aromatic hydrocarbons and PCBs in sediment showed the opposite pattern, with all higher concentrations at sites where Cascades frogs were still present than at sites where Cascades frogs had disappeared, again with none of the differences significant.

In general, chemical residue concentrations were not highly correlated between different chemicals across sites. There were 21 possible pairwise correlations for site average residue concentrations in chorus frogs for the seven chemicals with adequate detection frequencies, of which only one was significant (endosulfan sulfate and dacthal, Spearman correlation = 0.475, $p = 0.016$). There were more correlations between chemicals in sediment than in tissue, with 44 of 153 possible pairwise correlations significant. Seventeen pairwise chemical correlations had significant correlation coefficients of 0.6 or greater, nine between different PCBs.

DISCUSSION

Field study of the role of pesticides in amphibian population declines has been challenged by the difficulty of measuring pesticide residues in declining species. We found that the nondeclining Pacific chorus frog is a reasonable surrogate for evaluating chemical residues in declining Cascades frogs, at least for some chemicals. There were no significant differences in mean chemical residues between the two species. For endosulfan II, endosulfan sulfate, and dacthal, the correlation in residue concentrations between the two species were significant and reasonably high, especially given the high variation within analytic and site replicates. However, for *trans*-nonachlor and chlorpyrifos, the correlations were not significant. This suggests that the ability of one species to serve as a surrogate for residue concentrations in another species needs to be tested rather than assumed and will depend on the specific chemical in question and the question to be answered. For example, for the chemical *trans*-nonachlor, we found chorus frogs were a better surrogate for mean residue concentrations than they were for assessing geographic patterns because of the low geographic correlation of *trans*-nonachlor concentrations in chorus frogs and Cascades

Table 3. Mean chemical residue concentrations (ng/g dry wt) in Pacific Chorus frogs and Cascades frog tadpoles collected together from the same sites[a]

	Mean residue level ng/g dry wt		
Chemical	Chorus frog	Cascades frog	p value[b]
Chlorpyrifos	0.42	0.43	0.829
Dacthal	0.42	0.34	0.264
Endosulfan II	0.22	0.13	0.122
Endosulfan sulfate	0.47	0.62	0.488
trans-Nonachlor	0.05	0.13	0.377
PCB 187 (hepta)	0.04	0.08	0.202

[a] A total of 19 samples of each species were collected from 12 different sites.
[b] Mann–Whitney nonparametric test for difference of mean values between Pacific Chorus frogs and Cascades frogs.

Table 4. Spearman nonparametric correlations between chemical residue concentrations in Pacific Chorus frogs and Cascades frogs collected together from the same sites[a]

Chemical	No.[b]	Correlation[c]	p value[c]
Chlorpyrifos	16	0.595	0.1195
Dacthal	28	0.851	<0.001
Endosulfan II	38	0.684	0.001
Endosulfan sulfate	36	0.783	<0.001
trans-Nonachlor	28	0.393	0.164

[a] A total of 19 samples of each of the two species were collected together from 12 sites. All reported values are based on groups of samples with greater than 30% detections.
[b] Number of samples—both detections and half the estimated detection limit (½EDL) substituted values. Both samples in a paired collection had to be usable for the sample to be included in the analysis.
[c] Spearman correlation coefficients.

Table 5. Mean chemical residue concentrations in Pacific chorus frog tadpole tissue and in sediment at sites where Cascades frogs are still present compared to sites where Cascades frogs are now absent[a]

	Cascades frogs present	Cascades frogs absent	*p* value
Tissue ng/g dry weight			
Chlorpyrifos	0.53	0.49	0.481
Dacthal	0.45	0.20	0.003
Endosulfan II	0.19	0.14	0.430
Endosulfan sulfate	0.52	0.58	0.325
PCB 187 (hepta)	0.05	0.11	0.810
Sediment ng/g carbon weight			
Chlorpyrifos	0.56	0.63	0.191
Dacthal	0.40	0.65	0.117
Endosulfan II	0.23	0.27	0.811
Endosulfan sulfate	2.82	3.63	0.444
Hexachlorobenzene	34.99	1.67	0.200
trans-Chlordane	0.49	0.67	0.679
cis-Nonachlor	0.39	0.41	1.000
trans-Nonachlor	0.56	0.81	0.711
PCB 118 (penta)	1.03	0.58	0.419
PCB 138 (hexa)	1.63	0.67	0.811
PCB 153 (hexa)	1.71	0.64	0.744
PCB 183 (hepta)	0.49	0.18	0.879
PCB 187 (hepta)	1.06	0.41	1.000
Benzo[*b*]fluoranthene-L	370.82	39.37	0.845
Benzo[*e*]pyrene-L	139.06	24.23	0.647
Fluorene	1,376.45	31.37	0.817
Phenanthrene	18,789.30	101.55	0.200
Pyrene-LA	1,302.50	15.75	0.777

[a] *p* value is for a Mann–Whitney nonparametric test for a difference of mean values between residue concentrations at sites where Cascades Frogs are still present versus sites where Cascades frogs are now absent. Tissue samples are from chorus frog tadpoles. All calculations include half the estimated detection limit (½EDL) substituted values where these constitute less than 30% of total samples.

frogs. It is unclear why the two species had similar concentrations for some chemicals and not others. It could be due to differences in diet or micro-habitat choice. Additionally, Cascades frog tadpoles may spend more time in the benthos than Chorus frog tadpoles (C. Davidson, San Francisco State University, San Francisco, California, USA, personal observation) and therefore may be more exposed to contaminants in sediment.

Most of what we know about pesticide residues in frogs in the wild in California is based on the analysis of Pacific chorus frogs [5,6,25,34,35] because they are abundant and widespread. Additional studies like ours comparing residue concentrations in Pacific chorus frogs to those in declining amphibian species would help confirm the utility of Pacific chorus frogs as a surrogate and therefore improve our ability to extrapolate from studies on Pacific chorus frogs to other amphibians.

It is difficult to evaluate the biological effects of the contaminant concentrations that we measured in frog tissue and sediment. Laboratory exposure studies are typically based on exposure to known chemical concentrations in water rather than resulting tissue concentrations, and no toxicology studies have been done on Cascades frogs. We can get a very rough estimate of possible water exposure concentrations for our frogs by extrapolating from a study by Fellers et al. [4] that measured endosulfan I and *trans*-nonachlor concentrations in tissue of adult and subadult mountain yellow-legged frogs (*Rana muscosa*) and in water where the frogs were collected. For endosulfan I, they measured both tissue and water from three sites with an average concentration of 0.534 ng/g wet weight in tissue and 0.000483 μg/L in water. Concentrations in tissue were, on average, 1,206 times higher than in water, indicating bioaccumulation was taking place. For *trans*-nonachlor, Fellers et al. [4] measured both tissue and water at a single site and found concentrations of 0.68 ng/g wet weight in tissue and 0.000031 μg/L in water, so tissue concentration was 21,000 times higher than in water. If the ratios between water and tissue concentrations found in metamorphic *R. muscosa* frogs by Fellers et al. [4] were applied to the Cascades and Pacifica chorus frog tadpoles in the present study, then our finding of mean endosulfan I and *trans*-nonachlor concentrations of 0.01 and 0.036 ng/g wet weight tissue, respectively, suggest that frogs in the present study may have been exposed to water concentrations in the range of 0.0000083 μg/L endosulfan I and 0.0000016 μg/L *trans*-nonachlor—well below 1 part per trillion. This extrapolation, however, does not account for possible species differences, differences in exposures through air, food or sediment, and that the relationship between water and tissue concentrations in metamorphic frogs may be different than in tadpoles.

The U.S. Environmental Protection Agency's ECOTOX database [36] has amphibian toxicology studies for endosulfan, chlorpyrifos, and chlordane, but no information regarding the four other chemicals we found most frequently in frog tissue: endosulfan sulfate, dacthal, *trans*-nonachlor, and PCB 187. For endosulfan, median lethal concentrations (LC50) for amphibians ranged from 1.65 to 9,000 μg/L, from 66.2 to 5,471 μg/L for chlorpyrifos, and 50 to 4,000 μg/L for chlordane. Sparling et al. [37] found LC50 concentrations for Pacific chorus frog tadpoles of 15.6 μg/L endosulfan, 66.5 μg/L chlorpyrifos, 0.55 μg/L endosulfan, and 365 μg/L chlorpyrifos for foothill yellow-legged frog tadpoles (*Rana boylii*). The extrapolated endosulfan and *trans*-nonachlor exposure concentrations for our frogs are more than four orders of magnitude below short-term water-exposure concentrations known to be acutely lethal to amphibians. No information was available for Cascades frogs or Pacifica Chorus frogs on what concentrations of the chemicals we measured—acting alone or in combination—may cause sublethal effects, such as the weakening of immune systems.

We found no evidence to support the hypothesis that the pesticides and other contaminants we analyzed in the present study have contributed to the sharp decline of the Cascades frogs in the Lassen region. The pattern of chemical residue concentrations in frog tissue and sediment indicated that chemical residue concentrations are similar at sites where Cascades frogs have declined and at sites where Cascades frogs are still present. Only for dacthal were mean residue concentrations significantly different between decline sites and still present sites. Dacthal residues were significantly higher at sites where Cascades frogs were still present, opposite the pattern that would support pesticides as a contributing factor to declines.

None of the chemicals showed the general pattern of greater concentrations in the Lassen region than the Klamath area, as predicted based on predominant wind patterns and location of pesticide applications [16]. With the exception of dacthal, chemical concentrations appeared to be randomly scattered across the entire Cascades area, with no clear geographic patterns (see Supplemental Data, Fig. S1 for a map of tissue residue patterns). Local site conditions such as water depth, water temperature, and sunlight exposure may be driving factors affecting residue concentrations found in sediment and tadpoles. The high analytic and within site variation relative to across site variation may have made it difficult to observe across site geographic patterns of residues. This is especially true for endosulfan II and endosulfan sulfate, where coefficients

of variation for within site replicates were 115 and 71% of across site CVs, respectively.

Our laboratory methods were designed to look for 73 different chemicals. The present study—along with a similar study in the Sierra Nevada [6]—are the broadest examination of contaminant residues in amphibians to date. No other study that we know of has looked at more than a handful of different chemicals. Of the 73 chemicals that we looked for, we detected 34 (16 in tissue, 33 in sediment). We were able to examine the geographic pattern of 18 chemicals (5 in tissue and 18 in sediment) in relation to the pattern of declines of Cascades frogs. In 2005, a total of 371 different pesticides were reported used in the Sacramento Valley, California, USA [38]. The current-use pesticides that we could potentially measure with our methods accounted for less than 10% of the total weight of pesticide applications in the valley in 2005 [38]. Thus, the patterns of residue contamination for these remaining pesticides, as well for many nonpesticide contaminants, remain relatively unknown. Because of their persistence in the environment, we were much more likely to detect compounds such as organochlorine pesticides and PCBs than current-use organophosphate and carbamate pesticides with relatively short half-lives in the environment. We detected few current-use pesticides even though we know they are used in great quantities in the nearby Sacramento Valley [38] and some undergo long-range transport. For example, our methods were setup to detect diazinon, which is heavily used in the Sacramento Valley, yet we failed to detect it in our samples. The lack of correlation in geographic patterns between chemicals suggests that although applications are highly geographically correlated, each chemical may have its own transport, deposition, and breakdown patterns. Therefore, the lack of association with declines of Cascades frogs for any of the 18 chemicals we were able to analyze should not be taken as representative of contaminants in general.

Because of the great expense and difficulty of field residue studies, and the ability to detect only a limited number of the many chemicals that frogs encounter, further field residue studies may not be the most productive way to study whether pesticides are contributing to amphibian population declines. It may be more expeditious to first identify contaminants that plausibly could be contributing to declines based on use and transport properties and then pursue laboratory or mesocosm studies on mechanisms whereby they may be involved in declines. Then only once a promising mechanism is established, one could search for evidence of it in the field. For example, if laboratory studies show a pesticide suppresses some specific aspect of the amphibian immune system resulting in increased mortality due to disease, then field studies could look for evidence of suppression of that aspect of the immune system in wild frogs in relation to pesticide concentrations.

The hypothesis that pesticides are responsible for amphibian population declines presents a challenge for researchers because of the large number of different pesticides in use, the difficulty in determining animal exposures in the wild, and the many possible biological effects of pesticides. The pesticide hypothesis for amphibian declines is like a stool with a hundred legs. Studies such as this one can knock a few legs out from the stool, but cannot prove that pesticides are not playing a role in declines. The challenge for researchers is how to balance the risk of falsely rejecting the entire pesticides hypothesis based on limited evidence, and the risk of using limited research and conservation resources on a hypothesis that ultimately may not be true but cannot be disproved.

SUPPLEMENTAL DATA

Tables S1–S4.
Fig. S1. (442 KB PDF).

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REFERENCES

1. Wake D, Vredenburg V. 2008. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proc Natl Acad Sci U S A* 105:11466–11473.
2. Rachowicz LJ, Knapp RA, Morgan JAT, Stice MJ, Vredenburg VT, Parker JM, Briggs CJ. 2006. Emerging infectious disease as a proximate cause of amphibian mass mortality. *Ecology* 87:1671–1683.
3. Skerratt LF, Berger L, Speare R, Cashins S, McDonald KR, Phillott AD, Hines HB, Kenyon N. 2007. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth* 4:125–134.
4. Fellers GM, McConnell LL, Pratt D, Datta S. 2004. Pesticides in mountain yellow-legged frogs (*Rana muscosa*) from the Sierra Nevada mountains of California, USA. *Environ Toxicol Chem* 23: 2170–2177.
5. Sparling DW, Fellers GM, McConnell LL. 2001. Pesticides and amphibian population declines in California, USA. *Environ Toxicol Chem* 20:1591–1595.
6. Bradford D, Knapp RA, Sparling DW, Nash MS, Stanley K, Tallent-Halsell N, McConnell L, Simonich S. 2011. Pesticide distributions and population declines of California, USA alpine frogs, *Rana muscosa* and *Rana sierrae*. *Environ Toxicol Chem* 30:682–691.
7. Jennings MR, Hayes MP. 1994. *Amphibian and Reptile Species of Special Concern in California*. California Department of Fish and Game, Inland Fisheries Division, Rancho Cordova, CA, USA.
8. Aston LS, Seiber JN. 1997. Fate of summertime airborne organophosphate pesticide residues in the Sierra Nevada mountains. *J Environ Qual* 26:1483–1492.
9. LeNoir JS, McConnell LL, Fellers GM, Cahill TM, Seiber JN. 1999. Summertime transport of current-use pesticides from California's Central Valley to the Sierra Nevada mountain range, USA. *Environ Toxicol Chem* 18:2715–2722.
10. McConnell LL, LeNoir JS, Datta S, Seiber JN. 1998. Wet deposition of current-use pesticides in the Sierra Nevada mountain range, California, USA. *Environ Toxicol Chem* 17:1908–1916.
11. Zabik JM, Seiber JN. 1993. Atmospheric transport of organophosphate pesticides from California's Central Valley to the Sierra Nevada Mountains. *J Environ Qual* 22:80–90.
12. Hageman KJ, Simonich SL, Campbell DH, Wilson GR, Landers DH. 2006. Atmospheric deposition of current-use and historic-use pesticides in snow at national parks in the western United States. *Environ Sci Technol* 40:3174–3180.
13. Hageman KJ, Hafner WD, Campbell DH, Jaffe DA, Landers DH, Simonich SLM. 2010. Variability in pesticide deposition and source contributions to snowpack in western US national parks. *Environ Sci Technol* 44:4452–4458.
14. Davidson C, Shaffer HB, Jennings M. 2002. Spatial tests of the pesticide drift, habitat destruction, UV-B and climate change hypotheses for California amphibian declines. *Conserv Biol* 16:1588–1601.
15. Davidson C, Shaffer HB, Jennings MR. 2001. Declines of the California red-legged frog: Climate, UV-B, habitat and pesticides hypotheses. *Ecol App* 11:464–479.
16. Davidson C. 2004. Declining downwind: Amphibian population declines in California and historic pesticide use. *Ecol App* 14:1892–1902.

17. Davidson C, Knapp RA. 2007. Multiple stressors and amphibian declines: Dual impacts of pesticides and fish on yellow-legged frogs. *Ecol App* 17:587–597.
18. Davidson C, Benard M, Shaffer HB, Parker J, O'Leary C, Conlon JM, Rollins-Smith LA. 2007. Effects of chytrid infection and carbaryl exposure on survival, growth and antimicrobial peptide defenses in foothill yellow-legged frogs (*Rana boylii*). *Environ Sci Technol* 41: 1771–1776.
19. Gilbertson M, Haffner GD, Drouillard KG, Albert A, Dixon B. 2003. Immunosuppression in the northern leopard frog (*Rana pipiens*) induced by pesticide exposure. *Environ Toxicol Chem* 22:101–110.
20. Taylor SK, Williams ES, Mills KW. 1999. Effects of malathion on disease susceptibility in Woodhouse's toads. *J Wildl Dis* 35:536–541.
21. Fellers G, Pope K, Stead J, Koo M, Welsh H Jr. 2008. Turning population trend monitoring into active conservation: Can we save the Cascades Frog (*Rana cascadae*). *Herpetol Conserv Biol* 3:28–39.
22. Welsh HH, Pope KL, Boiano D. 2006. Sub-alpine amphibian distributions related to species palatability to non-native salmonids in the Klamath mountains of northern California. *Diversity Distrib* 12:298–309.
23. Fellers GM, Drost CA. 1993. Disappearance of the Cascades frog *Rana cascadae* at the southern end of its range, California, USA. *Biol Conserv* 65:177–181.
24. Briggs CJ, Vredenburg VT, Knapp RA, Rachowicz LJ. 2005. Investigating the population-level effects of chytridiomycosis: an emerging infectious disease of amphibians. *Ecology* 86:3149–3159.
25. Bradford DF, Stanley K, McConnell LL, Tallent-Halsell NG, Nash MS, Simonich SM. 2010. Spatial patterns of atmospherically deposited organic contaminants at high-elevation in the southern Sierra Nevada mountains, California, USA. *Environ Toxicol Chem* 29:1056–1066.
26. Rorabaugh JC, Lannoo MJ. 2005. Species account: *Pseudacris regilla*. In Lannoo MJ, ed, *Amphibian Declines: Status and Conservation of United States Amphibians*. University of California Press, Berkeley, CA, USA, pp 478–484.
27. Briggs JL, Storm RM. 1970. Growth and population structure of the Cascade frog, *Rana cascadae* Slater. *Herpetologica* 26:283–300.
28. Nussbaum RA, Brodie ED Jr, Storm RM. 1983. *Amphibians and Reptiles of the Pacific Northwest*. University of Idaho Press, Moscow, ID, USA.
29. California Department of Fish and Game. 2005. California Natural Diversity Database. California Department of Fish and Game, Natural Heritage Division, Sacramento, CA, USA.
30. Gosner KL. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16:183–190.
31. Stanley K, Simonich SM, Bradford D, Davidson C, Tallent-Halsell N. 2009. Comparison of pressurized liquid extraction and matrix solid phase dispersion for the measurement of semi-volatile organic compound accumulation in tadpoles. *Environ Toxicol Chem* 28:2038–2043.
32. Usenko S, Landers DH, Appleby PG, Simonich SL. 2005. Current and historical deposition of PBDEs, pesticides, PCBs, and PAHs to Rocky Mountain National Park. *Environ Sci Technol* 41:7235–7241.
33. Sokal RR, Rohlf FJ. 1995. *Biometry*, 3rd ed. W.H. Freeman and Company, New York, NY, USA.
34. Angermann JE, Fellers GM, Matsumura F. 2002. Polychlorinated biphenyls and toxaphene in Pacific tree frog tadpoles (*Hyla regilla*) from the California Sierra Nevada, USA. *Environ Toxicol Chem* 21:2209–2215.
35. Datta S, Hansen L, McConnell L, Baker J, LeNoir J, Seiber JN. 1998. Pesticides and PCB contaminants in fish and tadpoles from the Kaweah River Basin, California. *Bull Environ Contam Toxicol* 60: 829–36.
36. U.S. Environmental Protection Agency. 2012. ECOTOX database. Duluth, MN, USA.
37. Sparling DW, Fellers GM. 2009. Toxicity of two insecticides to California, USA, anurans and its relevance to declining amphibian populations. *Environ Toxicol Chem* 28:1696–703.
38. Department of Pesticide Regulation. 2006. Pesticide Use Report: 2005. California Department of Pesticide Regulation, Sacramento, CA, USA.