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PESTICIDES AND AMPHIBIAN POPULATION DECLINES IN CALIFORNIA, USA

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Abstract—Several species of anuran amphibians have undergone drastic population declines in the western United States over the last 10 to 15 years. In California, the most severe declines are in the Sierra Mountains east of the Central Valley and downwind of the intensely agricultural San Joaquin Valley. In contrast, coastal and more northern populations across from the less agrarian Sacramento Valley are stable or declining less precipitously. In this article, we provide evidence that pesticides are instrumental in declines of these species. Using *Hyla regilla* as a sentinel species, we found that cholinesterase (ChE) activity in tadpoles was depressed in mountainous areas east of the Central Valley compared with sites along the coast or north of the Valley. Cholinesterase was also lower in areas where ranid population status was poor or moderate compared with areas with good ranid status. Up to 50% of the sampled population in areas with reduced ChE had detectable organophosphorus residues, with concentrations as high as 190 ppb wet weight. In addition, up to 86% of some populations had measurable endosulfan concentrations and 40% had detectable 4,4′-dichlorodiphenyldichloroethylene, 4,4′-DDT, and 2,4′-DDT residues.

Keywords—Chlorpyrifos   Diazinon   Endosulfan   Declining amphibians   *Hyla regilla*

INTRODUCTION

There is a rapidly growing concern about worldwide declines in amphibian populations [1–3]. In California, red-legged frogs (*Rana aurora*), foothill yellow-legged frogs (*Rana boylii*), mountain yellow-legged frogs (*Rana muscosa*), and Yosemite toads (*Bufo canorus*) have undergone drastic population declines over the last 10 to 15 years [4,5]. *Rana aurora* is listed as threatened under the U.S. Endangered Species Act, and both *R. muscosa* and *B. canorus* have been proposed for listing. Pacific treefrogs (*Hyla regilla*) and Western toads (*Bufo boreas*) have undergone less severe declines [4].

The most drastic declines in California are found in the Sierra Nevada Mountains lying east of the San Joaquin Valley [4,5]. Although several hypotheses for these declines have been postulated [6–8], currently no single cause has been positively identified. However, pesticides may play a very important role in population declines of amphibians in this area. Each year, vast quantities of pesticides are applied to the intensely agricultural San Joaquin Valley of California. For example, in 1998, 5.9 million kilograms of active ingredient pesticides, or 60% of the total usage in the state of California, were sprayed there [9]. The prevailing summertime winds transport polluted air masses containing ozone, NO_x gases, particulate matter, and agricultural chemicals into the Sierra Nevada Mountains from the central and coastal valleys [10–13]. Among those chemicals observed in air, rain, and surface waters of the Sierra Nevada Mountains are organophosphorus pesticides such as malathion, chlorpyrifos, and diazinon, which bind with cholinesterase in animals and disrupt neural functioning. According to one study [13], concentrations of diazinon and chlorpyrifos in air are in the nanogram per cubic meter range and are more than 1,000 times greater (66–104 ng/L range) in surface waters at elevations where native anurans are showing the greatest declines. Cholinesterase activity is an excellent bioindicator of exposure to organophosphorus pesticides because they are formulated specifically for cholinesterase (ChE) inhibition and extremely few chemicals have this effect [14]. Cholinesterase has been used successfully in other studies to show exposure of animals to organophosphorus pesticides [15–17].

The Pacific treefrog, *H. regilla,* can be used as a sentinel species for the less abundant *Rana* and *Bufo* spp. because it is widespread throughout the region, its tadpoles are sympatric with the declining amphibian species, and they generally have similar responses to toxicants [7,18]. Tadpole *H. regilla,* however, metamorphose into adults in the same season that they are laid, whereas some *Rana* spp. require more than one summer to metamorphose. Additionally, adult *H. regilla* only visit wetlands to breed, whereas *Rana* spp. spend a majority of their life cycles in or near wetlands. These differences in habits may be related to the continued abundance of *H. regilla* where *Rana* spp. have become scarce if amphibians are primarily exposed to pesticides in their wetland habitats. Adult *Bufo* spend more time in terrestrial environments than ranid adults, but their early life stages are aquatic, and they should experience similar exposures as *Hyla* tadpoles.

The purpose of this study was to examine the possibility that wind-blown pesticides are affecting anuran populations in the Sierra Mountains. To meet this objective, we examined ChE activity and body residues of adult and tadpole *H. regilla* collected from various areas of California.

METHODS

Sampling design and cholinesterase activity

The general sampling design for ChE and residue samples followed a north/south west/east matrix ranging from Lake

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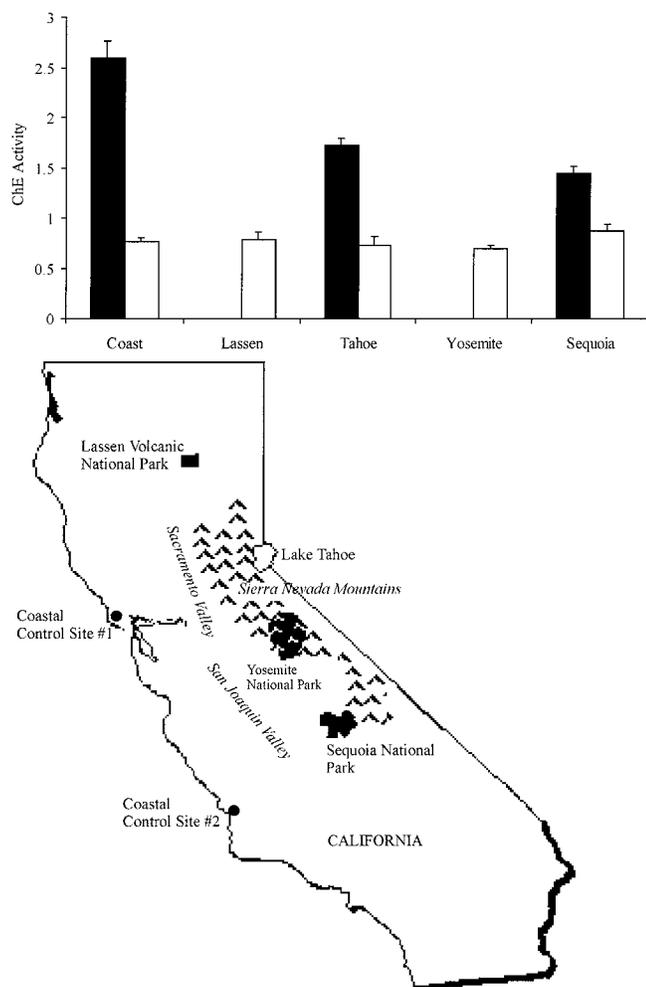Mention of trade or product names does not indicate endorsement by the U.S. federal government.

Fig. 1. Sampling locations in California, USA, and observed cholinesterase activity (mean ± standard error μmol substrate hydrolyzed/min/g tissue) in tadpole (filled bars) and adult (open bars) *Hyla regilla*. No tadpoles were analyzed from the Lassen or Yosemite sites. Coast results reflect combined results from both coastal reference sites.

Tahoe Basin in the north to Sequoia National Park in the south and from coastal sites in the west to approximately 1,500-m elevation in the east (Fig. 1). For ChE analyses, 170 tadpole and 117 adult *H. regilla* were collected from a total of 23 sites in six locations including coastal, foothill, Lake Tahoe Basin, Yosemite National Park, and Sequoia National Park; adults were also collected from Lassen National Park. Tadpoles and adults were collected with dip nets, placed in plastic jars, and frozen on site in liquid nitrogen. They were shipped on dry ice to the Patuxent laboratory, where they were kept at −84°C until analyzed. Tadpoles were partially thawed to remove gut coils, and the remaining whole bodies were homogenized in Tris buffer, with the resulting homogenate centrifuged to remove suspended particles. Adult tongues were dissected and treated similarly as tadpoles. Cholinesterase was measured on the supernatant photometrically [19] with modifications for multiwell-plate readers [20]. Each sample was run in triplicate or until the coefficient of variation for the repeated runs was less than 5%.

To determine if tongues provided a suitable matrix for ChE analysis, 20 adult male *H. regilla* were placed in 500-ml glass jars half filled with formulated diazinon concentrations ranging from 0 to 9 ppm active ingredient (five frogs at each treatment)

for 5 d. At the end of this period, animals were euthanized via chilling at 20°C and were frozen and stored at −84°C. Later, frogs were decapitated and brain tissue was obtained by gentle dorsal/ventral squeezing of crania, which forced tissue through the foramen magnum. At the same time, tongues were dissected and ChE assays were run for both tissues.

Residue analyses

Forty-eight composite adult and 34 composite tadpole samples were collected in clean glass jars and frozen; they were shipped on dry ice to the U.S. Department of Agriculture–Agriculture Research Station, Beltsville, Maryland, USA. Samples were stored at −40°C until processing. Sample masses were variable, but a maximum of 5 g of tissue was extracted as one sample; larger samples were split into multiple aliquots. Aliquots were extracted in triplicate with 20 ml acetonitrile/dichloromethane. After homogenization with the extraction solvent, samples were centrifuged for 15 min at 2,000 rpm. The supernatant was decanted and pulled under vacuum through a column of anhydrous $MgSO_4$ and a 0.45-μm nylon membrane. The combined extracts were reduced through rotary evaporation. Sample extracts were cleaned through two solid-phase extractors and filtered through a 0.45-μm nylon membrane and a 0.20-μm polytetrafluoroethylene (Teflon®) membrane. To monitor laboratory contamination and extraction efficiency of our target analytes, samples were extracted in batches of 20 with one blank control sample collected from a reference site and one blank spiked with a mixture of our target analytes. Analyses were conducted with a Hewlett Packard model 5890 capillary gas chromatograph (Avondale, PA, USA) coupled to a 5989A mass spectrophotometer using negative chemical ionization in selected-ion-monitoring mode. Detection limits were determined by extracting 10 adult frog samples spiked with a mixture of the target analytes and were, for endosulfans, 0.7 to 1.6 ng/g; for chlorpyrifos, 1.0 ng/g; for diazinon, 2.0 ng/g; for DDT, 4 to 20 ng/g; for hexacyclohexane (HCH), 1.0 ng/g; and for dichlorodiphenyldichloroethylene (DDE), 2.6 ng/g.

Population status

Population status of ranids at the sample sites was determined from over 10 years of study [4,5]. Sites were categorized as good (= areas with declines in less than 25% of historical sites or population levels not less than 50% of historical records), poor (= areas in which ranids were missing from >75% of the historically occupied sites or populations had declined by more than 75%), and moderate (= intermediate areas in which ranids were missing from 25–50% of sites or population declines ranged from 50–75%).

Statistical analysis

Statistical analyses were conducted with SAS [21]. Prior to analysis of variance, ChE activity levels were log transformed to conform to a normal distribution and analyzed with Proc GLM (SAS, Cary, NC, USA) followed by Tukey's honestly significant difference test for a posteriori comparisons. For the study on the appropriateness of tongue as analytical matrix, ChE values did not deviate from a normal distribution and treatment concentrations were regressed against actual scores. Statistical analysis of residues presented a problem in that many samples were below detection limits, which meant that actual concentrations were unknown but below some instrumental level of detection. To discard these samples would

eliminate a large percentage of our data. Thus, when residues were below the detection limits, we substituted one half of the mass-based detection limits to bring all samples into the analyses. In reporting concentrations, however, sites with no values above detection limits are reported as zero. Even after log transformation, residue data were not normally distributed and were therefore analyzed via nonparametric median and Kruskal–Wallis tests. Frequency of occurrence data were analyzed with Proc Catmod (SAS) and chi-square analysis.

RESULTS AND DISCUSSION

Cholinesterase activity

We determined that adult *H. regilla* tongues are a suitable and reliable tissue for ChE analysis. Mean ($\pm$ SD) ChE activity for tongues ranged from 0.934 ($\pm$ 0.254) μmol substrate hydrolyzed/min/g tissue for controls to 0.572 ($\pm$ 0.217) for frogs exposed to 9 ppm diazinon. The regression equation was statistically significant and was

$$\text{ChE activity} = 0.862 - 0.032(\text{dose}), \qquad R^2 = 0.236,$$

$$p = 0.025 \quad (\text{Fig. 2}).$$

Brain ChE activity was higher than tongue and ranged from 5.723 ($\pm$ 1.410) μmol substrate hydrolyzed/min/g tissue for controls to 5.950 ($\pm$ 1.153) for 9 ppm diazinon. However, the regression equation was not significant ($p = 0.969$). The lack of a dose/response relationship for brain ChE can be attributed to difficulty in extracting brain tissue without accompanying cerebrospinal fluid or intracranial tissue and in accurately weighing and diluting the very small amount of brain tissue. The important aspect was that the test showed that tongues provide a sufficiently large, fairly homogenous, highly vascular tissue that could be cleanly extracted and analyzed and that resulting ChE values were clearly interpretable. The relatively low regression coefficient is attributed to the ability of treefrog adults to cling to the inside of jars above the water line, thus increasing variability of exposure.

Because tadpoles at all field sites varied in stage of development from early prelimb bud (Gosner stages 21–26)[22] to limb bud (Gosner 27–35) to jointed hind limb (Gosner 36–39), the analysis of variance model included location, stage of development, and their interaction term. Cholinesterase differed among stages ($p = 0.002$) and locations ($p = 0.0001$) but not in the interaction of stage and location ($p = 0.367$). Tadpoles with jointed hind limbs had higher ChE values (mean $\pm$ SD) (2.38 $\pm$ 0.65 μmol/min/g) than either limb bud (1.69 $\pm$ 0.56) or prelimb (1.69 $\pm$ 0.73 μmol/min/g). Tadpole ChE was depressed at both Lake Tahoe and Sequoia compared with the coastal sites and was less at Sequoia than at Lake Tahoe (Fig. 1). Further, regrouping the sites by status of *Rana* spp. populations showed that ChE was significantly depressed ($p = 0.0001$) at sites with moderate or poor populations compared with those with good populations (Fig. 3).

Amphibian ChE has not been well studied, but in other vertebrates, 50% depression from a reference mean is considered a forensic threshold for proving pesticide-caused inhibition [23]. Increased mortality was observed in *Bufo arenarum* at 56% ChE depression [24]. At Sequoia National Park, 60% of the population had ChE values below the stage-specific 50% threshold, and two wetlands within the park had 82 and 100% of their samples below that level. These values can be compared with 9% of the coastal and 17% of the Lake Tahoe populations below the 50% threshold. Maximum depression
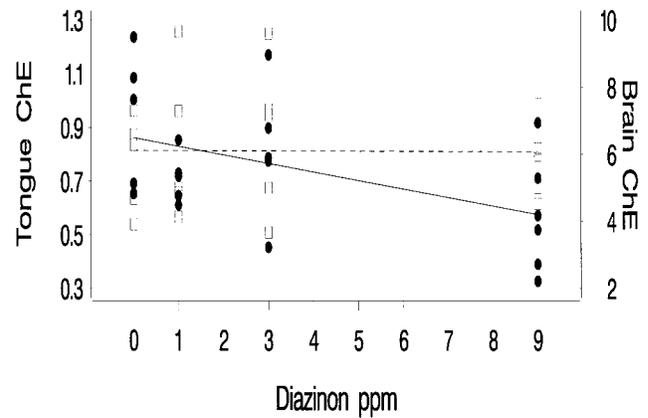

Fig. 2. Cholinesterase activity (μmol substrate hydrolyzed/min/g tissue) in tongues (solid line and dots) and brains (dotted line and squares) of adult *Hyla regilla* exposed to varying concentrations of formulated diazinon for 5 d.

levels are unknown because we do not know at what point in the ChE recovery phase we collected animals.

Cholinesterase in adults also differed among locations ($p = 0.025$) but the only difference was that frogs collected from Yosemite National Park had lower levels than those from the foothills. No observed ChE value was less than 50% of the coastal reference mean. Mean tadpole ChE activity values were higher than those of adults because of differences in tissues analyzed. Tadpole whole bodies included brains, which have the highest concentration of ChE of all tissues, plus the spinal cord, which also should have high concentrations of the enzyme. Adult tongues lacked these ChE-rich tissues. Because of this difference in tissue, statistical comparisons of adult and tadpole ChE activity would be meaningless.

Residue analyses

Of the pesticides analyzed, chlorpyrifos and diazinon are potent ChE inhibitors. Both chemicals are nonpersistent, with half-lives of only a few days in tissues [25–27] and a few weeks in water [28,29]. Thus, they are difficult to detect and their detection in tissues indicates very recent exposure. Their median lethal concentrations (LC50s) are in the parts per billion range, making them very highly toxic to aquatic organisms [28]. Specifically, for diazinon, LC50s ranged from 2.8 to 5.0 μg/L in *Rana clamitans* tadpoles [30]. The LC50s for chlorpyrifos were 1 μg/L in *Bufo americanus,* 177 (24 h) to 10 (6 d) μg/L in *Rana tigrina,* and 3,000 μg/L in *Rana pipiens* [31].
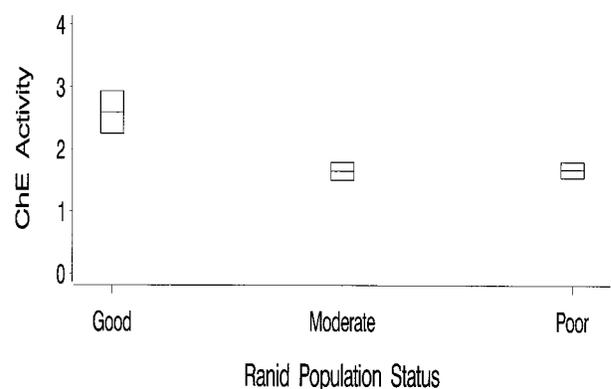

Fig. 3. Mean $\pm$ standard error of cholinesterase activity (μmol/min/g tissue) in *Hyla regilla* tadpoles by status of *Rana* spp. populations.
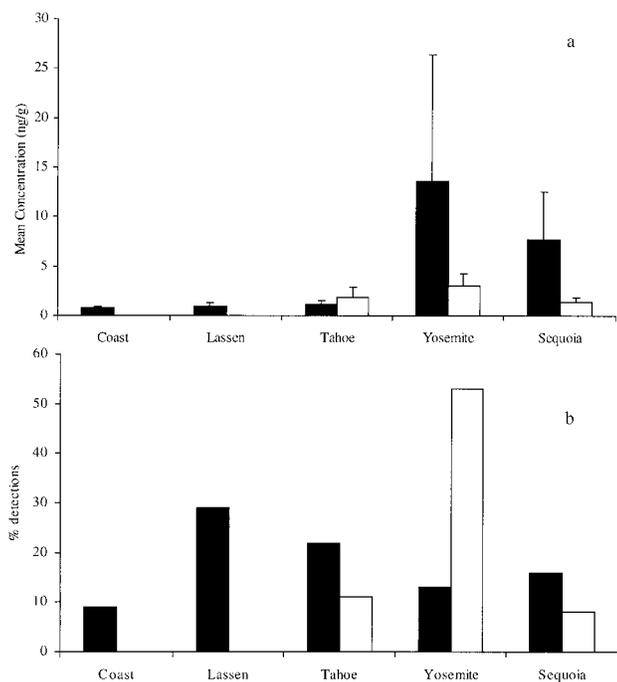
Fig. 4. Organophosphate pesticide residue results. (**a**) Mean concentration (ng/g) $\pm$ standard error for chlorpyrifos (filled bars) and diazinon (open bars) concentrations in whole *Hyla regilla* tadpole and adult tissue samples from the four major sampling locations and the coastal reference site. (**b**) Percentage of the samples analyzed in which chlorpyrifos and diazinon were detected.

Fig. 5. Chlorinated pesticide residue results in *Hyla regilla* adults and tadpoles from California. (**a**) Mean concentration (ng/g) $\pm$ standard error for DDTs (4,4'-DDT + 2,4'-DDT + 4,4'-dichlorodiphenyldichloroethylene) (hatched bars), endosulfans (α-endosulfan + β-endosulfan + endosulfan sulfate) (filled bars), and HCHs (γ-hexachlorocyclohexane + γ-hexachlorocyclohexane) (open bars). (**b**) Percentage of the samples analyzed where these chemicals were detected.

Tadpoles, which were not sampled at Yosemite, had marked increases in chlorpyrifos concentrations at Sequoia and diazinon at Lake Tahoe compared with other sites (Fig. 4a). Among adults, populations in Yosemite and Sequoia National Parks had elevated concentrations of both chlorpyrifos and diazinon compared with other sites, and maximum concentrations exceeded 190 ppb at Yosemite. However, because of the frequency of samples below analytical detection limits at all study sites, no significant differences were found in residues of either age or across sites, even after substituting 1/2 detection limits for values below detection limits.

When both tadpoles and adults are included, more than 50% of the *H. regilla* at Yosemite National Park had measurable levels of chlorpyrifos or diazinon, compared with only 9% at the coast (Fig. 4b). Moreover, there was a significant difference in the frequency of detection of diazinon among locations ($p = 0.004$), with high occurrences at Yosemite and in the foothills. At coastal sites, only one adult had detectable levels of chlorpyrifos and no adult or tadpole had detectable levels of diazinon.

Other non–ChE-inhibiting pesticides were found in *H. regilla* tissues, including endosulfans (α- and β-endosulfan and endosulfan sulfate collectively), DDTs (4,4'-DDE, 4,4'-DDT, and 2,4'-DDT; DDx collectively), and α- and γ-hexachlorocyclohexane (HCH). The DDT and derivatives and HCH are persistent pesticides, and their presence may be due to either recent or historical deposition; use of DDx in the United States and Canada has been prohibited for more than 25 years. Endosulfan has a persistence and toxicity similar to that of diazinon and chlorpyrifos, with 96-h LC50 values ranging from 1.8 μg/L for *R. tigrina* [32] to 123 μg/L for *Bufo melanostictus* [33]. Extensive paralysis and increased mortality was observed in *R. sy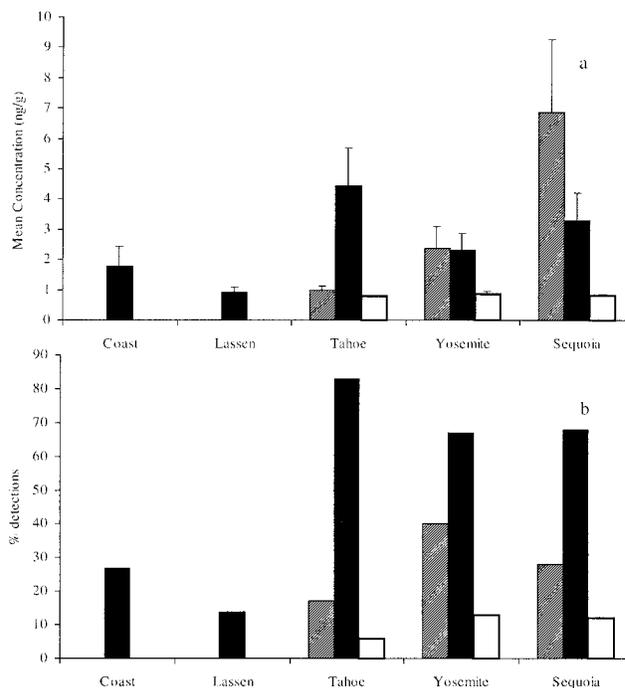lvatica, R. clamitans,* and *B. americanus* at the lowest exposure concentration of 53 μg/L [34]. Thus, the detection of endosulfan in frogs denotes recent exposure and suggests risk.

The concentration of total endosulfans ($p = 0.02$) and total DDx ($p = 0.008$) from adults and tadpoles collectively differed significantly among locations (Fig. 5a). In each case, mean concentrations tended to be low (often zero) in coastal sites and at Lassen National Park and higher at the other locations. The maximal concentrations of HCH (1.57 ppb), endosulfans (21.9 ppb), and DDx (38.7 ppb) occurred either at Sequoia or Yosemite National Parks.

Among all the pesticides tested, DDx and endosulfans had the highest frequency of occurrence across locations (Fig. 5b). For adults, the frequency of DDx detection differed significantly among locations ($p < 0.04$). Only one tadpole had measurable levels of DDx, and it was found in Sequoia. The higher frequency of detections for adults may be related to their older age and opportunity to accumulate DDx. All sites had adults with measurable concentrations of endosulfans, but the frequency of occurrence was higher at Sequoia and Yosemite National Parks and Lake Tahoe Basin than at Lassen or coastal sites ($p < 0.05$). For tadpoles, Lake Tahoe Basin, Sequoia, and the foothills region had higher frequencies of detections for endosulfans, but the differences were not statistically different. For adults and tadpoles collectively, the difference among locations was significant ($p = 0.013$). The HCH was only found in adults and, although 33% of those sampled from Sequoia had detectable concentrations, compared with none of those at the coast or Lassen National Park, the difference was not statistically significant.

Overall, the contaminant profiles showed a clear trend of widespread DDx, lower frequencies and concentrations of less persistent pesticides in coastal sites and Lassen National Park,

and a mixed but increasing occurrence of endosulfan, HCH, and diazinon from west to east across the Central Valley into the mountains. These profiles are consistent with ChE depression and the demography of declining amphibians.

CONCLUSIONS

Collectively, the evidence that wind-blown pesticides from the Central Valley have a role in the decline of amphibians in the Sierra Nevada is building. Other studies [10–13] have shown that 50 to 100% of the precipitation and water samples collected from Sequoia National Park and Lake Tahoe Basin had measurable concentrations of chlorpyrifos, malathion, diazinon, and other pesticides. The concentrations of these pesticides were often within an order of magnitude of the LC50 for aquatic organisms. Our study demonstrates that the concentrations and frequency of detections for pesticides in amphibian tissue follow north–south and west–east patterns consistent with intensified agriculture upwind of the areas with the most serious amphibian declines. Some of these pesticides, in turn, are reducing ChE activity in tadpoles. Depressed ChE has been associated with reduced activity, uncoordinated swimming, increased vulnerability to predators, depressed growth rates, and greater mortality in tadpoles [24,34–37]. The evidence that tadpole *H. regilla* demonstrate greater depression than adults is consistent with their relative dependence on aquatic environments and the detection of pesticides in these waters and their sources. Because the declining *Rana* spp. are more reliant on these environments both in terms of longer existence as tadpoles and a closer association as adults, ranids may be more at risk to aqueous pesticides than *H. regilla*. Additional research on sublethal effects, survivorship, and comparative sensitivity to key pesticides needs to be conducted.

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