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# In situ effects of pesticides on amphibians in the Sierra Nevada

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**Abstract** For more than 20 years, conservationists have agreed that amphibian populations around the world are declining. Results obtained through laboratory or mesocosm studies and measurement of contaminant concentrations in areas experiencing declines have supported a role of contaminants in these declines. The current study examines the effects of contaminant exposure to amphibians in situ in areas actually experiencing declines. Early larval *Pseudacris regilla* were translocated among Lassen Volcanic, Yosemite and Sequoia National Parks, California, USA and caged in wetlands in 2001 and 2002 until metamorphosis. Twenty contaminants were identified in tadpoles with an average of 1.3–5.9 (maximum = 10) contaminants per animal. Sequoia National Park, which had the greatest variety and concentrations of contaminants in 2001, also had tadpoles that experienced the greatest mortality, slowest developmental rates and lowest cholinesterase activities. Yosemite and Sequoia tadpoles and metamorphs had greater genotoxicity than those in Lassen during 2001, as determined by flow cytometry. In 2001 tadpoles at Yosemite had a significantly higher rate of malformations, characterized as hemimelia (shortened femurs), than those at the other two parks but no significant differences were observed in 2002. Fewer differences in contaminant types and concentrations existed among parks during 2002 compared to 2001. In 2002 Sequoia tadpoles had higher mortality and slower developmental rates but there was no difference among parks in cholinesterase activities. Although concentrations of most contaminants were below known lethal concentrations, simultaneous exposure to multiple chemicals and other stressors may have resulted in lethal and sublethal effects.

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## Introduction

Amphibians are an integral part of their ecosystems, affecting nutrient cycling and serving as high-quality prey for many species (Wake 1991; Whiles et al. 2006). In the last 20–25 years, scientists have documented evidence supporting a global decline in amphibian populations (Houlahan et al. 2000; McCallum 2007). In 2008, the International Union for Conservation of Nature (IUCN) Red List of Threatened Animals cited that 32 % of the 6,200+ species of amphibians are threatened or endangered and that 42 % of the species are declining. Causes for these precipitous declines include one or more of the following: habitat destruction (Houlahan and Findlay 2003); increased UV-B radiation (Castanaga et al. 2009); climate change (Carey and Alexander 2003); disease (Rohr et al. 2008); introduced predators (Knapp 2005); contaminants (Davidson et al. 2002; Sparling et al. 2001) or a combination of two or more of these stressors (Relyea 2003; Sparling 2003). Stuart et al. (2004) specifically identified habitat loss and pollution as the top two causes for the amphibian population declines.

North America has not escaped these dramatic declines (Lannoo 1998). One area of particular concern is the Sierra Nevada of California. Broad scale field sampling and the historical analyses of museum records show a widespread decline of amphibians downwind of the San Joaquin Valley (Fisher and Shaffer 1996). The collapse of a regional frog fauna, 5 of 7 species, in the Yosemite area of the California Sierra Nevada, has been documented (Drost and Fellers 1996).

For example, all five native ranid species in the Sierra Nevada need protection. The Cascade frog (*Rana cascadae*) is declining (Fellers and Drost 1993) and is Near Threatened on the IUCN Red List (www.iucnredlist.org, accessed 1/15/2014), and the northern leopard frog (*Lithobates* [*Rana*] *pipiens*), although globally listed as Least Concern by IUCN, has disappeared from 99 % of its range in California (Rorabaugh 2005). The California red-legged frog (*R. draytonii*) is listed as Threatened by the US Fish and Wildlife Service and Vulnerable by IUCN (Fellers and Kleeman 2007), the foothill yellow-legged frog (*R. boylii*) is in decline and IUCN Near Threatened (Fellers and Drost 1993, Drost and Fellers 1996), the Sierra Nevada yellow-legged frog (*R. sierrae*) has disappeared from over 93 % of historical sites and is IUCN globally Endangered and the southern mountain yellow-legged frog (*R. muscosa*) is federally Endangered (U.S.Fish Wildl. Service 2002) as well as globally listed as Endangered by IUCN.

For more than 40 yrs, organophosphate (OP), legacy organochlorine, and endosulfan pesticides have volatized from the highly agricultural Central Valley and entered the Sierra Nevada ecosystem through wet and dry aerial deposition (Datta et al. 1998; McConnell et al. 1998; LeNoir et al. 1999). Sparling et al. (2001) found quantifiable levels of chlorpyrifos, diazinon, and endosulfan in tissues of adult *Pseudacris regilla* (aka *P. sierra*) and determined that cholinesterase levels were lower in areas that had experienced population declines than in areas that had not. PCBs and toxaphene were found in *P. regilla* tissues from national parks in California (Angermann et al. 2002) and an array of contaminants was found in surface water and *R. muscosa* (now *R. sierrae*) tissues (Fellers et al. 2004). Chlorpyrifos levels exceeded 190 μg/L in Yosemite waters and endosulfan had a maximum detected level of 21.9 μg/L in water (Sparling et al. 2001). Acute and chronic toxicity tests (Sparling and Fellers 2007, 2009) demonstrated that the oxon forms of chlorpyrifos, diazinon and malathion were highly toxic to amphibians and that endosulfan was extremely toxic to *R. boylii*; 100 % of tadpoles exposed to concentrations >0.8 μg/L died during a chronic exposure, a level regularly observed and exceeded in adult *P. regilla* in the Sierra Nevada (Sparling et al. 2001).

All of these studies used indirect methods, including field residue concentrations, biomarker effects, laboratory exposures, and modeling efforts, to evaluate the adverse effects of contaminants on anuran populations in the Sierra Nevada. Direct, in field, evidence on populations is more difficult to obtain. The purpose of this study is to evaluate relationships among environmentally present contaminants and amphibian populations in an in situ experiment occurring in Sierra Nevada meadows over two years. We chose the Pacific treefrog for our test species. This species is IUCN Least Concern and is still widely distributed and found in the three test national parks of Lassen Volcanic, Yosemite and Sequoia. Given that it is abundant and common, and previously studied in the region, we were able to use it for manipulative studies and as an indicator for contaminant exposure. The species in decline in the Sierra Nevada listed under IUCN or USFWS status are too rare for experiments requiring manipulation and translocation. Prevailing wind patterns (Davidson et al. 2002; Davidson 2004) show that Sequoia and Yosemite National Parks receive more wind from the intensely agricultural San Joaquin Valley than does Lassen and, if contaminants are affecting amphibian populations, we hypothesize that effects should be more obvious in these two parks than in Lassen. Thus these comparisons should yield insights on

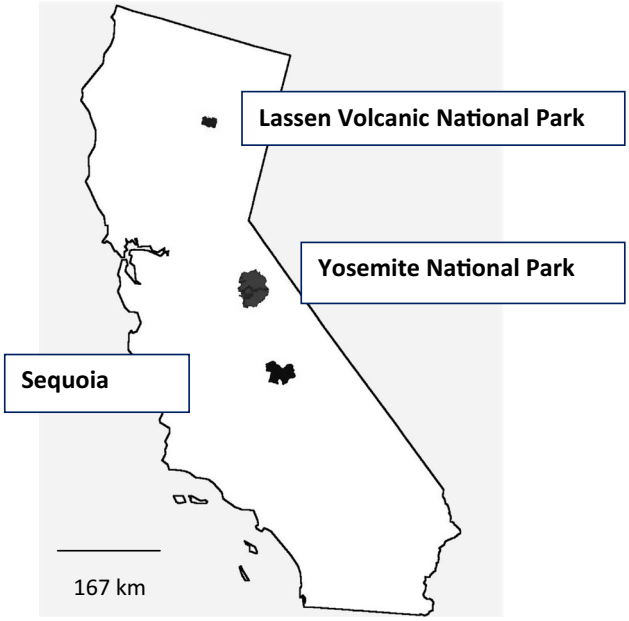


**Fig. 1** Outline map of California showing the locations of the national parks used for an in situ study on *Pseudacris regilla* during 2001 and 2002

potential contaminant impacts. As far as we know, this study is the first to actually examine the effects of contaminants on Sierran amphibians in the field.

## Materials and methods

### Field techniques

Hatchling tadpoles were collected from a single pond in each of Lassen Volcanic and Sequoia National Parks and two ponds in Yosemite between 6 June and 23 June 2001 (Fig. 1). A different set of ponds was used for collection from 31 May to 23 June 2002. All ponds were between 1950 m and 3300 m in elevation. Tadpoles were exchanged among and within parks into three meadows of each park. They were kept cool and transported via automobile to their meadows of deployment and grouped in sets of three (Lassen, Yosemite, and Sequoia). Tadpoles appeared vigorous and healthy upon release into each cage.

In each park three sites with at least three small, contiguous ponds in palustrine wet meadows or larger flooded meadows were selected. One triad consisting of three cages was placed in each pond or area within a larger flooded meadow; each triad consisted of one cage each for tadpoles coming from Yosemite, Lassen Volcanic and Sequoia Parks, three such localities per site. Each site within a park thus had three triads or nine cages and this was repeated at two other sites for each park. This yielded a maximum of 27 cages per site, 9 cages from each of the three parks, and a total of 81 cages among all three parks. This provided sufficient replication in the case of a failure of loss of a small number of cages.

Cages were made of white Nitex Teflon (Sefar America Inc., Kansas City, MO) and were 60 cm deep × 35 cm dia. Cages were affixed to a metal rod that was driven into the pond bottom (Harris and Bogart 1997) and were in contact with the underlying sediments. Cages were moved into deeper water as ponds dried. A floating platform was placed in each cage for use by metamorphs. Cages were monitored daily. Because tadpoles were confined and unable to freely forage, we provided boiled romaine lettuce ad libitum as food. Approximately 90 % of the lettuce was organic; the other 10 % was carefully washed and rinsed before boiling.

In 2001 a thermometer was included with each cluster of three cages and daily temperatures were read as the cages were checked. In 2002, however we used thermometers that recorded minimum and maximum as well as current temperatures and used the mean of daily minima and maxima for analyses. Tadpoles were partially sampled at 28 days of exposure and the rest were collected as they individually entered metamorphosis, Gosner stage 42–46 (Gosner 1960). Animals were weighed (±0.01 g) with a field balance (Ohaus, Parsippany NJ, USA) and snout vent length (0.1 mm) was measured with calipers. Any observed malformations were classified according to Meteyer et al. (2000). All animal handling procedures were approved by the Patuxent Wildlife Research Center's Animal Care and Use Committee.

### Residue analyses

Water (2001 only) and sediment (2001, 2002) samples for residue analyses were collected soon after snowmelt as part of a larger study that included many of the meadows in this study. These samples were collected only once each year so they portray a 'snapshot' of what was present. Water samples were collected in 8 L stainless steel, chemically cleaned containers and shipped overnight to the USDA analytical laboratory in Beltsville, Maryland USA. Animals for tadpole and metamorph residues were collected from the field at 28 days of exposure and as animals completed metamorphosis, euthanized with tricaine methanesulfonate (MS-222, Sigma, St. Louis, MO) and shipped on dry ice to Patuxent Wildlife Research Center, Laurel Maryland, USA, where they were stored at −80 °C, and then moved to the USDA Agricultural Research Center, Beltsville MD for analysis. Tadpole and metamorph composite samples were pooled for each life stage from the same experimental cage to constitute one residue sample of approximately 10 g. We used the QuEChERS dispersive

solid phase extraction method (Lehotay et al. 2005) for sample extraction and clean up.

Twenty-nine analytes were tested in sediment, 18 in water and 28 in tissues. All chemicals were commonly occurring in other studies within the Sierra Nevada (McConnell et al. 1998; Fellers et al. 2004) and included several currently used insecticides and fungicides, several legacy organochlorine pesticides and five polybrominated diphenyl ethers (BDEs). Samples were analyzed using an Agilent 6890 gas chromatograph coupled to a model 5973 mass spectrometer. Detection limits were determined for each chemical and matrix and expressed as ng/L (water) or ng/g (dry weight for sediment and wet weight for tissues).

### *Flow cytometry*

Various frog tissues were tested for suitability for flow cytometry and optimal results were obtained from frog livers. Previously developed protocols were used to measure DNA content in cells (Sawin et al. 1987; Bickham et al. 1998). All samples for this experiment were run on the same day to reduce variation caused by cytometer drift. Livers were dissected from slightly thawed specimens. Nuclear suspensions were digested with trypsin/detergent solution or physical disruption by douncing. A Coulter Epics Elite flow cytometer (Beckman Coulter, Fullerton, CA) was used in the generation of DNA profiles. The cytometer computer program determined half peak percent coefficient of variation (HPCV), mean, standard deviation, and number of cells for a region determined by the operator. Only samples that measured at least 10,000 nuclei and that satisfied all gating parameters were included in the statistical analyses.

### *Cholinesterase determinations*

We quantified cholinesterase (ChE) activity levels on whole body tadpoles and metamorphs. To provide a representative sample we combined tadpoles into three development groups: Gosner stage 25–35 (young), 36–41 (developed) and 42–46 (metamorphs). For tadpoles we first removed the gut coil because it contains a variable amount of food and mass with little cholinesterase content. For metamorphs we used intact animals because they do not feed during the last stages of metamorphosis and the entire digestive system is living tissue. We used a colorimetric method (Ellman et al. 1961) adjusted for lower volumes of reagents and homogenates in a 96-well spectrophotometer (Biotex Synergy HT microplate reader, Winooski, VT, USA). Each group was statistically analyzed separately.

### *Statistical analyses*

Dependent variables were checked for their adherence to the parametric assumptions of homogeneity of variance and normal distribution. If they deviated substantially from either assumption, data were transformed with log (continuous variables), arcsine (proportions or ratios) or square root (count data) transformations. For statistical comparisons, contaminants that were detected but were below the level of quantification (3 × MDL) were assigned a randomized value within the range of 0 to MDL. Chemicals that were tested but not detected were scored as zero. Before analyses, zeroes were replaced with 0.0001, concentrations were log transformed and regressed against end points (e.g. percent survival). Due to the high number of zeros in residue data, we used the non-parametric Kruskal–Wallis analysis as conducted in SAS to determine if concentrations varied among parks when testing water and sediments. SAS conducts a ranking procedure that uses ANOVA to generate F statistics as the approach. In all analyses we considered the individual cage and not the ponds to be the experimental unit because each cage in a triad contained tadpoles from different parks. Moreover, even though the cages of a triad were in the same meadow, they were analogous to replicate aquaria within a treatment of a laboratory experiment. In regressing tissue residues against survival and similar end points we used the mean value of a contaminant for each cage, not the ponds. In analyses of variance of end points across parks we used an analysis of variance with individual tadpoles as replicates within cages and included both the park of deployment and the park of origin as explanatory variables. When an end point might have been affected by ambient temperature we used analysis of covariance with mean water temperature as the covariate. A posteriori comparisons were conducted with Tukey's tests. Initially, $\alpha$ was set at 0.05 for evaluating statistical significance but when many (>3) analyses were conducted on the same parameters we used an $\alpha$ that was adjusted by Bonferroni's correction for multiple comparisons. All analyses were conducted with SAS 9.2.

## Results

### General water quality

Water quality in the ponds was suitable for amphibians. Dissolved oxygen ranged from 3.4 to 8 ppm, pH from 7 to 7.6, and hardness from 8 to 40 ppm $CaCO_3$. With the exception of one pond that had a measured ammonia concentration of 1.5 ppm, concentrations of nitrates (0.2 ppm), nitrites (0.9 ppm), phosphorus (0.2 ppm) and

**Table 1** Mean, (SEM) and maximum concentrations (ng/L) of contaminants detected in water within montane portions of Lassen, Yosemite and Sequoia national parks, California, 2001

| | MDL (ng/L) | Lassen | Yosemite | Sequoia |
|---|---|---|---|---|
| Number of ponds | | 6 | 6 | 6 |
| α-Chlordane | 4.44 | 0.24 (0.04) | 0.16 (0.01) | 0.13 (0.03) |
| | | 0.44 | 0.19 | 0.24 |
| γ-Chlordane | 3.27 | 0.22 (0.02) | 0.25 (0.04) | 0.16 (0.09) |
| | | 0.29 | 0.35 | 0.47 |
| *Trans*-Nonachlor | 3.28 | 0.16 (0.08) | 0.12 (0.06) | 0.14 (0.10) |
| | | 0.43 | 0.31 | 0.54 |
| Total Chlordanes | | 0.63 (0.09) | 0.53 (0.08) | 0.44 (0.20) |
| | | 0.91 | 0.84 | 1.19 |
| α-endosulfan | 6.74 | 0.56 (0.27) | 0.23 (0.02) | 0.26 (0.06) |
| | | 1.88 | 0.29 | 0.40 |
| β-Endosulfan | 10.1 | 0.51 (0.14) | 0.31 (0.02) | 0.28 (0.09) |
| | | 1.17 | 0.35 | 0.62 |
| Endosulfan Sulfate | 7.86 | 0.94 (0.30) | 0.80 (0.10) | 0.63 (0.08) |
| | | 2.39 | 1.05 | 0.86 |
| Total Endosulfan | | 2.01 (0.70) | 1.33 (0.12) | 1.16 (0.17) |
| | | 5.45 | 1.61 | 1.75 |
| Chlorpyrifos | 6.00 | 1.67 (0.17) | 2.39 (0.36) | 2.00 (0.92) |
| | | 2.39 | 3.54 | 5.64 |
| Malathion | 37.6 | 0.51 (0.51) | 0.09 (0.09) | 1.49 (0.92) |
| | | 3.06 | 0.46 | 3.94 |
| Trifluralin | 2.95 | 0.96 (0.30) | 0.51 (0.09) | 0.45 (0.06) |
| | | 2.42 | 0.70 | 0.63 |
| p,p′-DDT | 84.6 | 0 | 11.4 | 0 |
| Chlorothalonil | 41.8 | 1.20 (1.20) | 0.89 (0.89) | 0 |
| | | 7.22 | 4.45 | |
| α-HCH | 8.71 | 0.22 (0.22) | 0 | 0 |
| | | 1.34 | | |
| γ-HCH | 10.9 | 4.28 (3.15) | 0.33 (0.26) | 0.22 (0.15) |
| | | 19.80 | 1.32 | 0.77 |

Chemicals (MDL, ng/L) analyzed but not detected: *Cis*-nonachlor (2.81), Chlorpyrifos oxon (12.2), p,p′-DDE (27.2), Diazinon (33.3), Oxychlordane (11.7). Concentrations are for chemicals that were detected; for those with values <MDL a randomly chosen value between 0 and MDL was used as a nominal value

ammonia (1.0 ppm) were below the detection limits provided in parentheses.

## Contaminant concentrations in water, sediment and tissues

Of 18 chemicals analyzed in water, 13 were detected in one or more of the three national parks in 2001 (Table 1). No statistically significant differences in concentrations or in the number of different contaminants were detected across parks using Kruskal–Wallis. The chemicals included legacy pesticides such as chlordanes and DDT, endosulfans which were still in use at the time of the study, organophosphorus pesticides and others.

Of 29 chemicals analyzed in sediments, 16 were identified (Table 2). Polybrominated diphenyl ethers were not detected in water but were well distributed in sediments. A few other contaminants were found in either water or sediments but not the other. Again, no significant differences were found in specific contaminants in the sediments across parks with Kruskal–Wallis. In 2002 the number of contaminants in sediment was more evenly distributed among parks with an increase from 4 to 8 in Lassen and a reduction from 13 to 8 in Sequoia.

Tadpoles and metamorphs had a greater array of contaminants than either water or sediment (Table 3). Of 28 chemicals analyzed, 20 were identified in at least trace amounts during the two years of study and 8 were used in analyses. Fipronil, dichlorodiphenyldichloroethane (hereafter p,p′-DDD), dieldrin and mirex were found in tissues but not in any water or sediment sample. In contrast, γ-hexachlorocyclohexane or lindane (hereafter γ-HCH) was found in all water samples but not in any of the tissues.

For differences among parks in 2001 the Bonferroni corrected $\alpha = 0.0062$. Using Kruskal–Wallis analysis, tadpoles deployed at Sequoia had significantly higher concentrations of *cis*-nonachlor $F_{2,54} = 5.77$, $p = 0.0058$), *trans*-nonachlor ($F_{2,54} = 21.88$, $p < 0.0001$), and p,p′-DDE ($F_{2,54} = 8.75$, $p = 0.0006$) than those in the other two parks. BDE 47 ($F_{2,54} = 3.74$, $p = 0.0313$) may have been biologically different among parks but the p value was greater than the Bonferroni correction. Tadpoles at Lassen Volcanic tended to have concentrations that were intermediate between those in the other two parks during 2001. There was a single difference due to park of origin that was significant before Bonferroni correction but not afterwards: ($F_{2,54} = 3.36$, $p = 0.043$)—*trans*-nonachlor was higher in tadpoles and metamorphs from Yosemite than from Sequoia with Lassen Volcanic intermediate. The mean number of total chemicals in tadpole and metamorph tissues at Sequoia ($4.67 \pm 0.5$) was greater than at Lassen Volcanic ($2.99 \pm 0.5$) and Yosemite ($1.7 \pm 0.4$) and greater at Lassen Volcanic than at Yosemite. There were no differences among parks of origin.

In 2002 *P. regilla* deployment parks also showed significant differences in tissue concentrations with Kruskal–Wallis analysis. Sequoia had significantly higher concentrations of α-endosulfan ($F_{2,68} = 9.62$, $p = 0.0002$), and p,p′-DDE ($F_{2,68} = 32.36$, $p < 0.0001$) than at either of the other parks (a posteriori comparisons $p < 0.05$). BDE 47 ($F_{2,68} = 3.60$, $p = 0.033$), and trifluralin ($F_{2,68} = 4.61$,

**Table 2** Mean, (SE) and maximum concentrations (ng/g dw) for sediment contaminants detected in montane portions of Lassen, Yosemite and Sequoia national parks, 2001–2002

| | MDL (ng/g) | 2001 | | | 2002 | | |
|---|---|---|---|---|---|---|---|
| | | Lassen | Yosemite | Sequoia | Lassen | Yosemite | Sequoia |
| Number of meadows | | 5 | 6 | 5 | 7 | 6 | 6 |
| BDE 47 | 3.39 | 0.10 (0.10) | 0.73 (0.73) | 0.54 (0.54) | 0.45 (0.45) | 0.30 (0.30) | 0 |
| | | 0.49 | 4.36 | 2.75 | 3.12 | 1.80 | |
| BDE 99 | 4.07 | 0 | 0.85 (0.85) | 0.80 (0.80) | 0.51 (0.51) | 0 | 0 |
| | | | 5.10 | 4.03 | 3.61 | | |
| BDE 100 | 3.52 | 0 | 0 | 0.15 (0.15) | 0.06 (0.06) | 0 | 0 |
| | | | | 0.74 | 0.43 | | |
| BDE 153 | 8.80 | 0 | 0.41 (0.41) | 0.11 (0.11) | 0.15 (0.15) | 0 | 0.10 (0.10) |
| | | | 2.46 | 0.55 | 1.08 | | 0.59 |
| BDE 154 | 2.34 | 0 | 0.49 (0.49) | 0.17 (0.17) | 0.20 (0.20) | 0 | 0 |
| | | | 2.93 | 0.86 | 1.41 | | |
| Total BDEs | | 0.10 (0.10) | 2.97 (2.97) | 1.49 (1.49) | 1.38 (1.38) | 0.30 (0.30) | 0.10 (0.10) |
| | | 0.49 | 14.85 | 8.93 | 9.65 | 1.80 | 0.59 |
| α-Chlordane | 5.17 | 0 | 0 | 0.28 (0.14) | 0 | 0.31 (0.22) | 0 |
| | | | | 0.77 | | 1.30 | |
| γ-Chlordane | 3.66 | 0.11 (0.11) | 0.21 (0.17) | 0.20 (0.10) | 0.09 (0.04) | 0.33 (0.14) | 0.05 (0.05) |
| | | 0.53 | 1.02 | 0.54 | 0.22 | 0.92 | 0.29 |
| *Cis*-Nonachlor | 6.56 | 0 | 0.11 (0.11) | 0.19 (0.19) | 0 | 0.19 (0.13) | 0 |
| | | | 0.64 | 5.28 | | 0.75 | |
| *Trans*-Nonachlor | 3.54 | 0 | 0 | 0.15 (0.13) | 0 | 0.41 (0.21) | 0 |
| | | | | 0.538 | | 1.17 | |
| Total Chlordanes | | 0.10 (0.10) | 0.39 (0.20) | 0.69 (0.45) | 0.09 (0.04) | 1.25 (0.52) | 0.05 (0.05) |
| | | 0.53 | 1.02 | 2.82 | 0.22 | 3.12 | 0.29 |
| α-Endosulfan | 3.16 | 0 | 0 | 0.29 (0.14) | 0 | 0.31 (0.31) | 0.04 (0.04) |
| | | | | 0.77 | | 1.87 | 0.26 |
| β-Endosulfan | 1.54 | 0 | 0 | 0.06 (0.04) | 0 | 0.47 (0.32) | 0.13 (0.07) |
| | | | | 0.23 | | 1.87 | 0.35 |
| Endosulfan Sulfate | 2.8 | 0.30 (0.18) | 0.49 (0.35) | 1.56 (0.98) | 0.06 (0.03) | 0.37 (0.25) | 0.30 (0.18) |
| | | 1.02 | 2.20 | 5.28 | 0.19 | 1.56 | 1.13 |
| Total Endosulfans | | 0.29 (0.18) | 0.58 (0.42) | 1.60 (0.99) | 0.06 (0.03) | 1.15 (0.72) | 0.47 (0.20) |
| | | 1.02 | 2.20 | 6.16 | 0.19 | 4.13 | 1.26 |
| Chlorpyrifos | 3.25 | 0 | 0.10 (0.10) | 0.31 (0.20) | 0.03 (0.03) | 1.10 (1.07) | 0.02 (0.02) |
| | | | 0.62 | 0.91 | 0.25 | 6.44 | 0.12 |
| Heptepoxide | 1.58 | 0.03 (0.03) | 0 | 0 | 0 | T | 0 |
| | | 0.16 | | | | | |
| α-HCH | 3.12 | 0 | 0 | 0 | 0 | 0 | 1.45 (1.45) |
| | | | | | | | 8.71 |
| p,p′-DDE | 5.66 | 0 | 0 | 0 | 0 | 0.89 (0.89) | 0.49 (0.32) |
| | | | | | | 5.37 | 1.70 |

Chemicals (MDL ng/g) analyzed but not detected: Aldrin (3.67), Chlorthalonil (0.80), p,p′-DDD (6.39), p,p′-DDT (0.87), Diazinon (23.26), Dieldrin (8.89), Fipronil (2.47), γ-HCH (2.47), Heptachlor (5.32), Malathion (17.92), Mirex (6.74), Oxychlordane (3.92), Trifluralin (3.34). Concentrations are for chemicals that were detected; for those with values <MDL a randomly chosen value between 0 and MDL was used as a nominal value

*T* trace, only one site had a detection which was <MDL

$p = 0.0194$) may have differed among parks but not according to the Bonferroni's correction of 0.0062. Lassen Volcanic tadpoles had higher concentrations of trans-nonachlor ($F_{2,68} = 5.84$, $p = 0.0048$) than the other parks. The single difference due to park of origin was in α-endosulfan ($F_{2,68} = 5.06$, $p = 0.0093$) where tadpoles

**Table 3** Mean (SEM) and maximum concentrations (ng/g ww) of contaminants in tadpole and metamorph *Pseudacris regilla* collected from an in situ study in national parks of the Sierra Nevada of California, 2001 and 2002

| | MDL (ng/g) | 2001 | | | 2002 | | |
|---|---|---|---|---|---|---|---|
| | | Lassen | Yosemite | Sequoia | Lassen | Yosemite | Sequoia |
| Sample size | | 19 | 15 | 21 | 21 | 24 | 24 |
| BDE 47**,#,1 | 0.15 | 0.03[a] (0.03) | 0[a] | 0.08[b] (0.03) | 0.03[ab] (0.02) | 0.01[a] (0.01) | 0.06[b] (0.02) |
| | | 0.63 | | 0.38 | 0.43 | 0.18 | 0.30 |
| BDE 99 | 0.13 | 0 | 0 | 0.01 (0.01) | 0.00 (0.00) | T | T |
| | | | | 0.23 | 0.03 | | |
| BDE 100 | 0.14 | 0.03 (0.03) | 0 | 0.02 (0.02) | 0.01 (0.01) | 0.01 (0.01) | 0 |
| | | 0.50 | | 0.39 | 0.15 | 0.12 | |
| BDE153 | 0.15 | 0 | 0 | 0.01 (0.01) | T | 0 | 0 |
| | | | | 0.16 | | | |
| BDE154 | 0.12 | 0 | 0 | 0 | 0.00 (0.00) | 0 | 0 |
| | | | | | 0.08 | | |
| Total BDEs*** | | 0.06[a] (0.06) | 0[a] | 0.12[b] (0.04) | 0.05 (0.02) | 0.03 (0.01) | 0.06 (0.02) |
| | | 1.13 | | 0.77 | 0.43 | 0.32 | 0.30 |
| α-Chlordane*** | 0.10 | 0.12[b] (0.03) | 0[a] | 0.18[b] (0.03) | 0 | 0.00 (0.00) | 0.01 (0.01) |
| | | 0.34 | | 0.60 | | 0.04 | 0.12 |
| γ-Chlordane | 0.08 | 0.09 (0.03) | 0.02 (0.02) | 0.07 (0.02) | 0.00 (0.00) | 0 | 0.00 (0.00) |
| | | 0.31 | 0.27 | 0.29 | 0.04 | | 0.12 |
| *Cis*-nonachlor*** | 0.11 | 0.07[a] (0.03) | 0[a] | 0.16[b] (0.04) | T | 0 | 0.01 (0.01) |
| | | 0.29 | | 0.39 | | | 0.24 |
| *Trans*-nonachlor***,## | 0.11 | 0.23[b] (0.03) | 0[a] | 0.34[b] (0.04) | 0.04[b] (0.01) | 0[a] | 0.02[a] (0.01) |
| | | 0.44 | | 0.94 | 0.22 | | 0.17 |
| Total Chlordane ***,# | | 0.51[ab] (0.08) | 0.02[a] (0.02) | 0.76[b] (0.09) | 0.04[b] (0.01) | 0.00[a] (0.00) | 0.05[ab] (0.02) |
| | | 1.12 | 0.27 | 1.36 | 0.22 | 0.04 | 0.36 |
| α-Endosulfan### | 0.09 | 0.05 (0.02) | 0.14 (0.05) | 0.11 (0.04) | 0.01[ab] (0.01) | 0[a] | 0.02[b] (0.01) |
| | | 0.43 | 0.61 | 0.72 | 0.09 | | 0.09 |
| β-Endosulfan | 0.10 | 0.00 (0.00) | 0.01 (0.01) | 0.01 (0.00) | 0.01 (0.01) | 0 | 0.00 (0.00) |
| | | 0.34 | 0.13 | 0.09 | 0.22 | | 0.07 |
| Endosulfan sulfate## | 0.13 | 0.03 (0.03) | 0.11 (0.05) | 0.02 (0.02) | 0.02[a] (0.01) | 0.07[b] (0.02) | 0.01[a] (0.01) |
| | | 0.51 | 0.56 | 0.40 | 0.27 | 0.31 | 0.10 |
| Total endosulfans* | | 0.08[a] (0.04) | 0.26[b] (0.07) | 0.14[ab] (0.04) | 0.04 (0.02) | 0.07 (0.02) | 0.03 (0.01) |
| | | 0.57 | 0.80 | 0.72 | 0.28 | 0.31 | 0.14 |
| Malathion | 0.91 | 0 | 0 | 0 | 0.03 (0.02) | 0 | 0.02 (0.01) |
| | | | | | 0.46 | | 0.27 |
| Fipronil | 0.40 | 0 | 0 | 0 | 0.01 (0.01) | 0 | 0 |
| | | | | | 0.22 | | |
| Trifluralin# | 0.09 | 0.18 (0.02) | 0.27 (0.03) | 0.22 (0.04) | 0.04[a] (0.01) | 0.03[ab] (0.00) | 0.09[b] (0.02) |
| | | 0.30 | 0.60 | 0.66 | 0.22 | 0.08 | 0.36 |
| p,p′-DDD | 0.11 | 0 | 0 | 0 | 0.01 (0.01) | 0 | 0 |
| | | | | | 0.26 | | |
| p,p′-DDE***,### | 0.11 | 0.89[a] (0.33) | 0[a] | 3.19[b] (0.96) | 0.11[a] (0.05) | 1.82[ab] (0.22) | 2.61[b] (0.43) |
| | | 5.12 | | 20.29 | 0.60 | 5.75 | 7.19 |
| Dieldrin | 0.08 | 0 | 0 | 0.03 (0.02) | 0 | 0 | 0 |
| | | | | 0.36 | | | |
| Mirex | 0.14 | 0 | 0 | 0 | 0.02 (0.02) | 0 | 0 |
| | | | | | 0.41 | | |
| Chlorthalonil* | 0.005 | 0[a] | 0.10[b] (0.07) | 0[a] | 0.01 (0.01) | 0 | 0 |

**Table 3** continued

| MDL (ng/g) | 2001 | | | 2002 | | |
|---|---|---|---|---|---|---|
| | Lassen | Yosemite | Sequoia | Lassen | Yosemite | Sequoia |
| | | 0.96 | | 0.25 | | |

Chemicals (MDL ng/g) analyzed but not found: aldrin (0.11) chlorpyrifos (0.12) chlorpyrifos oxon (0.93) diazinon (0.46) p,p′-DDT (0.11) α-HCH (0.10) γ-HCH (0.10) heptachlor (0.14)

[1,] * (2001) or # (2002) denote significant differences across parks for a given year. Three symbols p <0.002 based on the corrected Bonferroni's α for multiple comparisons; two symbols p > 0.002 and <0.01; one symbol: p >0.01 and <0.05. Concentrations with the same superscript are not statistically different from each other. Concentrations are for chemicals that were detected; for those with values <MDL a randomly chosen value between 0 and MDL was used as a nominal value

*T* trace, only one site had a detection which was <MDL

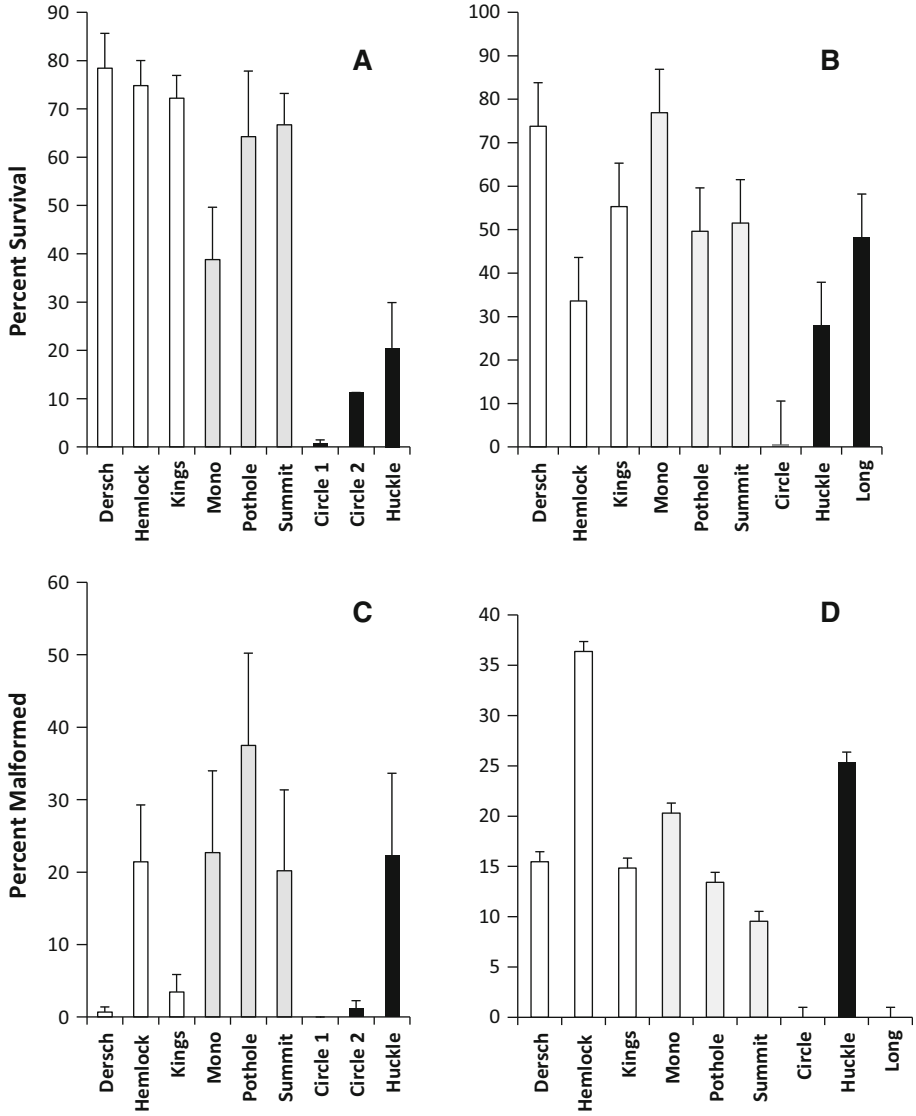


**Fig. 2** Mean (SEM) percent survival and malformations showing hemimelia (shortening of femurs and tibiofibulae) in tadpoles and metamorphs of *Pseudacris regilla* during an in situ study within meadows of three national parks in the Sierra Nevada, USA. **a** percent survival in 2001; **b** percent survival in 2002; **c** percent malformations in 2001; **d** percent malformations in 2002. Color code: *white* Lassen, *gray* Yosemite, *black* Sequoia

coming from Lassen Volcanic National Park had higher concentrations than those from Yosemite or Sequoia (Tukey's test, $p < 0.05$). There was no difference in the mean number of chemicals in tissues across parks (Sequoia = 2.5 ± 0.4, Lassen Volcanic = 1.7 ± 0.4, Yosemite = 1.8 ± 0.2).

Under 2-way ANOVA the mean number of different chemicals found in tissues was greater in 2001 than in 2002

($F_{1,115} = 11.91$, $p = 0.0008$). There were also significant differences in the number of different contaminants found in tissues across parks ($F_{2,115} = 7.68$, $p = 0.0007$) and in the interaction of park and year ($F_{2,115} = 6.88$, $p = 0.0015$). Over both years tadpoles deployed in Sequoia had a greater number of chemicals in their tissues than those at Lassen Volcanic National Park (Tukey's, $p < 0.05$).

## Tadpole survival and growth

In 2001, there were significant differences in survival rates among deployment parks and interaction between place of origin and place of deployment for tadpoles (2-way ANOVA, Fig. 2a, b) but there was no overall difference among places of origin. Tadpole survival significantly differed among the three parks with Sequoia (11 ± 4 %) being lowest, Yosemite (52 ± 8 %) intermediate, and Lassen (75 ± 3 %) highest (Tukey's, $p < 0.05$). In 2002, there continued to be a difference among deployment parks (2-way ANOVA, $F_{2,73} = 13.16$, $p < 0.0001$), but not among origin sites or in the interaction between origin and deployment. Sequoia again had the lowest survival (25 ± 5 %), Yosemite had the highest (58 ± 6 %) and Lassen was intermediate (53 ± 4 %); Lassen and Yosemite had significantly higher survival rates than Sequoia (Tukey's, $p < 0.05$). In a full model with both years combined we did not find significant differences between years (2-way ANOVA, $F_{1,128} = 0.39$, $p = 0.5311$), but there were differences among parks ($F_{2,128} = 57.35$, $p < 0.0001$) and in the year by park interaction ($F_{2,128} = 7.08$, $p = 0.0012$). In 2001 tissue concentrations of log *cis*-nonachlor ($r = 0.3723$, $p = 0.0051$) and BDE 47 ($r = 0.4123$, $p = 0.0018$) were significantly and negatively related to survivorship based on Pearson's correlation.

**Fig. 3** Photo of metamorphic *Pseudacris regilla* showing hemimelia or shortening of femurs and tibiafibulae

Some negative relationships also existed between survivorship and p,p′ DDE, *trans*-nonachlor, and α-chlordane but the r values for these were <0.10.

Numerous metamorphs at all parks had malformations characterized by hemimelia (shortened femurs, Fig. 2c, d). This deformity caused severe problems for affected animals. Deformed hind limbs in frogs had a severely limited range of motion and were usually locked at right angles to the body (Fig. 3). The ability of deformed frogs to hop was extremely limited and they often drowned in our cages because they could not crawl or hop onto the platform provided for newly metamorphosing animals. It is probable that we were able to observe this phenomenon because our cages protected these animals from predation and that drowned animals could be recovered. The percent of metamorphs with malformations differed significantly across parks. In 2001, 6.2 % (42/675) of Lassen animals, 25.5 % (70/275) of Yosemite animals, and 7.1 % (24/337) of Sequoia animals showed this hind limb deformity. In 2002, 25.2 % (114/452) of Lassen animals, 14.0 % (71/507) of Yosemite animals, and 7.7 % of Sequoia animals (43/561) showed this hind limb deformity. In a 2-way ANOVA over both years and all three parks we found no significant differences between years in the frequency of malformations ($F_{1,128} = 0.04$, $p = 0.836$), but there were differences among deployment parks ($F_{2,128} = 3.52$, $p = 0.0326$), with Yosemite having higher frequencies of malformations than Sequoia ($p < 0.05$), and Lassen Volcanic frequencies were intermediate between the two other parks. Significance also occurred in the interaction of park and year ($F_{2,128} = 5.48$, $p = 0.0052$). In 2001 Pearson correlations of log transformed tissue concentrations of DDE ($r = 0.3310$, $p = 0.0284$), *trans*-nonachlor ($r = 0.4437$, $p = 0.0007$), α-chlordane ($r = 0.6910$, $p = 0.0003$), *cis*-nonachlor ($r = 0.7233$, $p < 0.0001$) and BDE 47 ($r = 0.6211$, $p = 0.0008$) were significantly and positively related to the percent of amphibians malformed. No chemicals were related to percent malformations in 2002.

By the end of the study each year, most of the surviving tadpoles had metamorphosed. In 2001 the percent of animals that had not yet entered Gosner stage 42 varied among deployment parks ($F_{2,69} = 23.35$, $p < 0.0001$, Fig. 4a) but not origin or their interaction (2-way ANOVA). Most (70 ± 8 %) of those in Sequoia had not started metamorphosis whereas only a few were left in Lassen Volcanic (14 ± 2 %) or Yosemite (17 ± 6 %). In 2002 deployment parks also differed ($F_{2,73} = 10.63$, $p = 0.001$, 2-way ANOVA, Fig. 4c) but no differences occurred among parks of origin or the interaction between park of deployment or origin. Sequoia had a higher frequency of unmetamorphosed tadpoles (52 ± 8 %) than either Lassen Volcanic (5 ± 1 %) or Yosemite (6 ± 3 %, Tukey's $p < 0.05$).

Based on Pearson correlations, percent of tadpoles that were not metamorphosed when the study terminated in 2001 positively correlated with the sediment log concentrations of α-chlordane ($r = 0.075$, $p = 0.035$), BDE 47 ($r = 0.104$, $p = 0.012$), *cis*-nonachlor ($r = 0.135$, $p = 0.0041$) and *trans*-nonachlor ($r = 0.3345$, $p = 0.0096$). In 2002, sediment log BDE 47 concentrations positively correlated with percent unmetamorphosed tadpoles ($r = 0.112$, $p = 0.0055$). Given the number of chemicals used in the Pearson correlations, the Bonferroni's correction was $\alpha = 0.0036$ but we deem that these correlations were sufficiently close to the corrected α level to be considered biologically important.

Average time to metamorphosis reflected the percent of undeveloped animals (Fig. 4b, d). In 2001 tadpoles at Sequoia took significantly longer to metamorphose than those at Lassen Volcanic or Yosemite. In 2002 Sequoia tadpoles still took longer to develop but the differences among parks were less and statistically insignificant.

Two-way ANOVAs showed significant differences in snout vent length (SVL) at 28 days of exposure existed across deployment parks in 2001 ($F_{2,68} = 20.94$, $p < 0.0001$) and origin parks ($F_{2,68} = 7.30$, $p = 0.0013$) and in the interaction of the two terms ($F_{4,68} = 4.31$, $p = 0.0036$). Tadpoles that had been deployed in Yosemite (16.03 ± 0.39 mm) and Lassen Volcanic (15.90 ± 0.24 mm) were 12–14 % larger than those in Sequoia (13.87 ± 0.22 mm, Tukey's a posteriori, $p < 0.05$). Tadpoles that had originated from Lassen Volcanic were about 7 % shorter than those from Yosemite or Sequoia (Tukey's, $p < 0.05$). In 2002, SVL differed across deployment parks ($F_{2,79} = 12.32$, $p < 0.0001$) but not across parks of origin ($p = 0.066$); all three parks differed from one another at $p < 0.05$ (Tukeys test). Tadpoles in

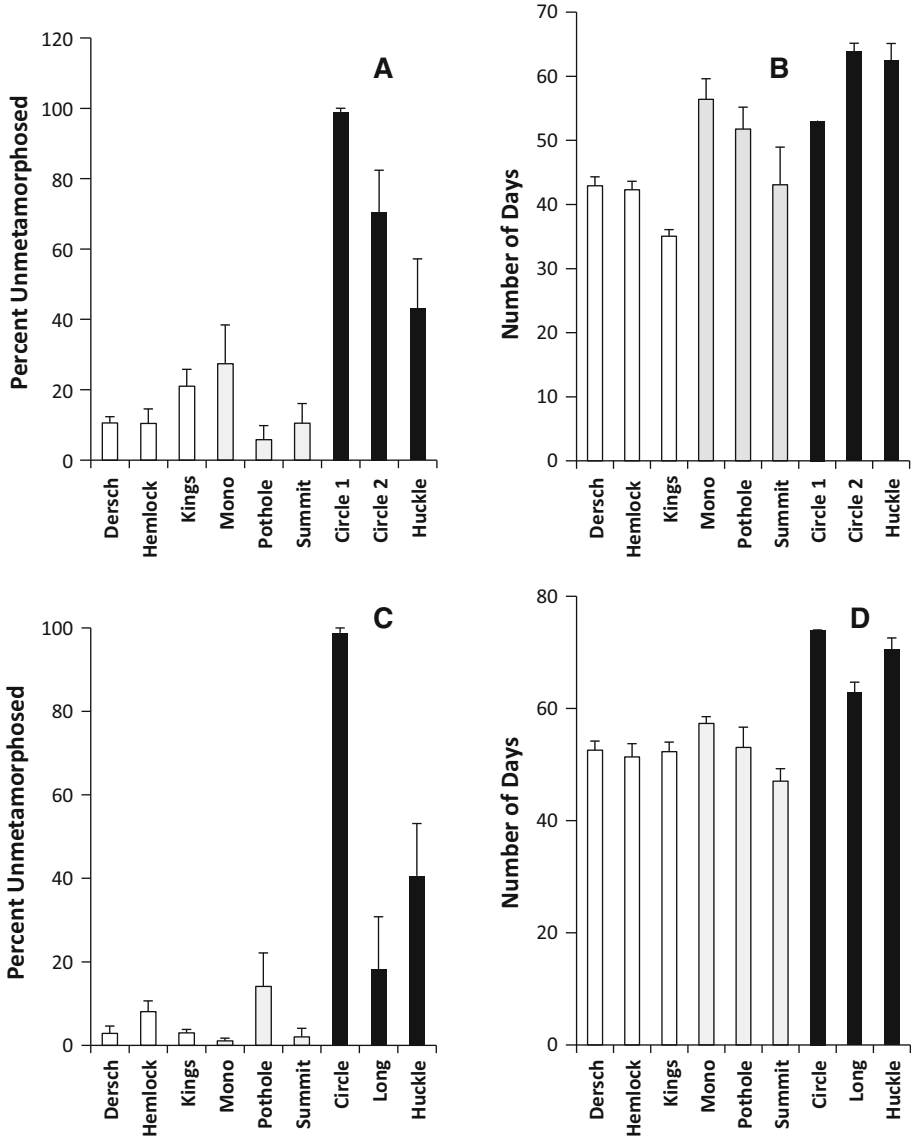


**Fig. 4** Mean (SEM) percent late unmetamorphosed and time to metamorphosis in larval *Pseudacris regilla* during an in situ study within meadows of three national parks in the Sierra Nevada, USA. **a** percent unmetamorphosed 2001; **b** days needed for development in 2001; **c** percent unmetamorphosed 2002; **d** days needed for development 2002. Color code: *white* Lassen, *gray* Yosemite, *black* Sequoia

Lassen Volcanic (14.26 ± 0.31 mm) and Yosemite (14.95 ± 0.30 mm) were about 17 % larger than those in Sequoia (12.54 ± 0.20 mm).

Among metamorphs, SVL differed among deployment ponds ($F_{2,53} = 4.36$, $p = 0.0176$, Fig. 5a) and ponds of origin ($F = 4.99$ $p = 0.010$) but not in their interaction during 2001. In 2002 there were no differences based on Bonferrroni-corrrected $\alpha = 0.01$. However, at higher p values that might be considered biologically relevant, tadpole SVL differered across deployment ($F_{2,53} = 4.36$, $p = 0.0176$, Fig. 5b) and origin ($F_{2,53} = 4.99$, $p = 0.0103$) parks. Metamorphs deployed in Sequoia (14.85 ± 0.35 mm) were smaller than those at Lassen Volcanic (15.79 ± 0.31 mm) or Yosemite (16.74 ± 0.26 mm). Among parks of origin, those coming from Yosemite (17.09 ± 0.34) were still larger than those from Lassen Volcanic (15.31 ± 0.30 mm) or Sequoia (15.30 ± 0.25 mm). In 2002 SVL differed among deployment ponds ($F_{2,58} = 21.52$, $p < 0.0001$) but not across ponds of origin or their interaction ($p > 0.27$). At the end of the study tadpoles in Sequoia (14.96 ± 0.35 mm) were 7 % shorter than those at Yosemite (16.07 ± 0.26 mm) or Lassen Volcanic (16.21 ± 0.21 mm). Metamorphs at Lassen and Yosemite were larger than those at Sequoia by about 10–12 %.

Data on mass were not collected in the field during 2001. In 2002, the log of the mass differed among deployment parks for 28-day tadpoles ($F_{2,79} = 3.49$, $p = 0.036$) but not among parks of origin; there was a significant interaction term ($F_{4,79} = 5.98$, $p = 0.0003$). Metamorphs at Yosemite were about 15 % heavier than those at Sequoia ($p < 0.05$, Fig. 5c) while those from Lassen varied among meadows and did not differ from either of the other parks.

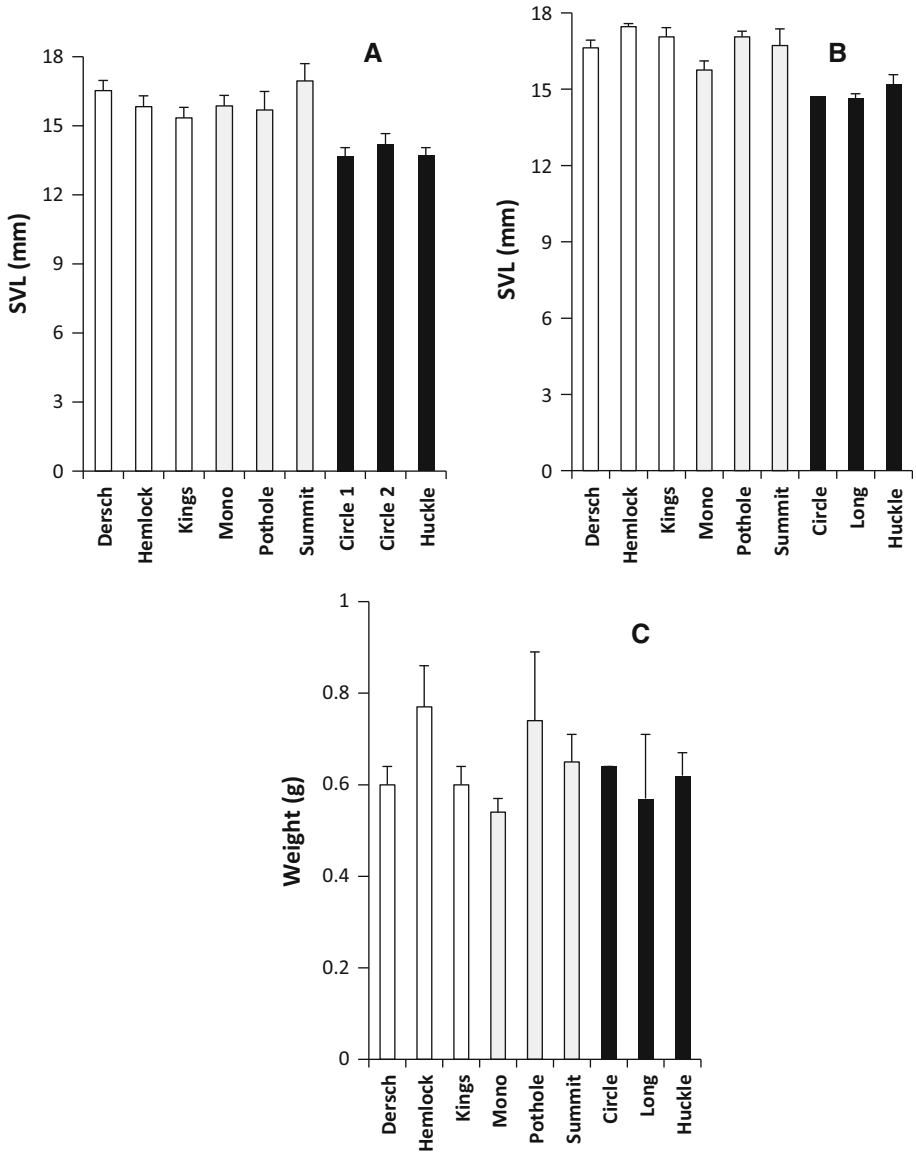


**Fig. 5** Mean (SEM) snout vent length (SVL) and body mass of *Pseudacris regilla* metamorphs collected from an in situ study in meadows of three national parks in the Sierra Nevada, USA. **a** SVL 2001; **b** SVL 2002; **c** body mass 2002. Color code: *white* Lassen, *gray* Yosemite, *black* Sequoia

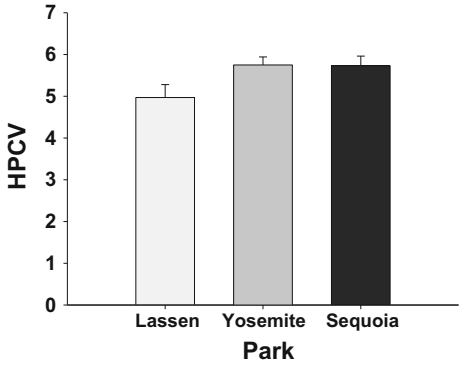


**Fig. 6** Mean (SD) half peak coefficients of variation (HPCV) determined through flow cytometry on livers of *Pseudacris regilla* collected from an in situ study conducted in three national parks of the Sierra Nevada, USA, 2001. Color code: *light gray* Lassen, *gray* Yosemite, *black* Sequoia

## Flow cytometry

Half peak coefficients of variation (HPCV) differed significantly among parks ($p = 0.04$, Fisher's PLSD test, Fig. 6). Tadpoles at Lassen Volcanic had lower HPCV values and presumably less chromosomal damage than those from either Yosemite or Sequoia, suggesting that there was more chromosomal damage at Yosemite and Sequoia than at Lassen Volcanic.

## Cholinesterase activity

Mean cholinesterase (ChE) activity was significantly different among the three developmental groups ($F_{2,165} = 97.86$, $p < 0.0001$). There were no significant differences in mean ChE activity across parks for either tadpole group (Fig. 7a, c) in 2001; however, a significant difference in ChE activity had developed in metamorphs across deployment parks ($F_{2,54} = 34.46$, $p < 0.0001$, Fig. 7e), although not among parks of origin or the interaction between deployment and origin. Metamorphs at Lassen ($1.55 \pm 0.03$ µmol/g min) had significantly higher ChE activities than those at Yosemite ($1.15 \pm 0.04$), which were significantly higher than at Sequoia ($0.76 \pm 0.02$, Tukey's, $p < 0.05$ for all comparisons). In 2002, there were

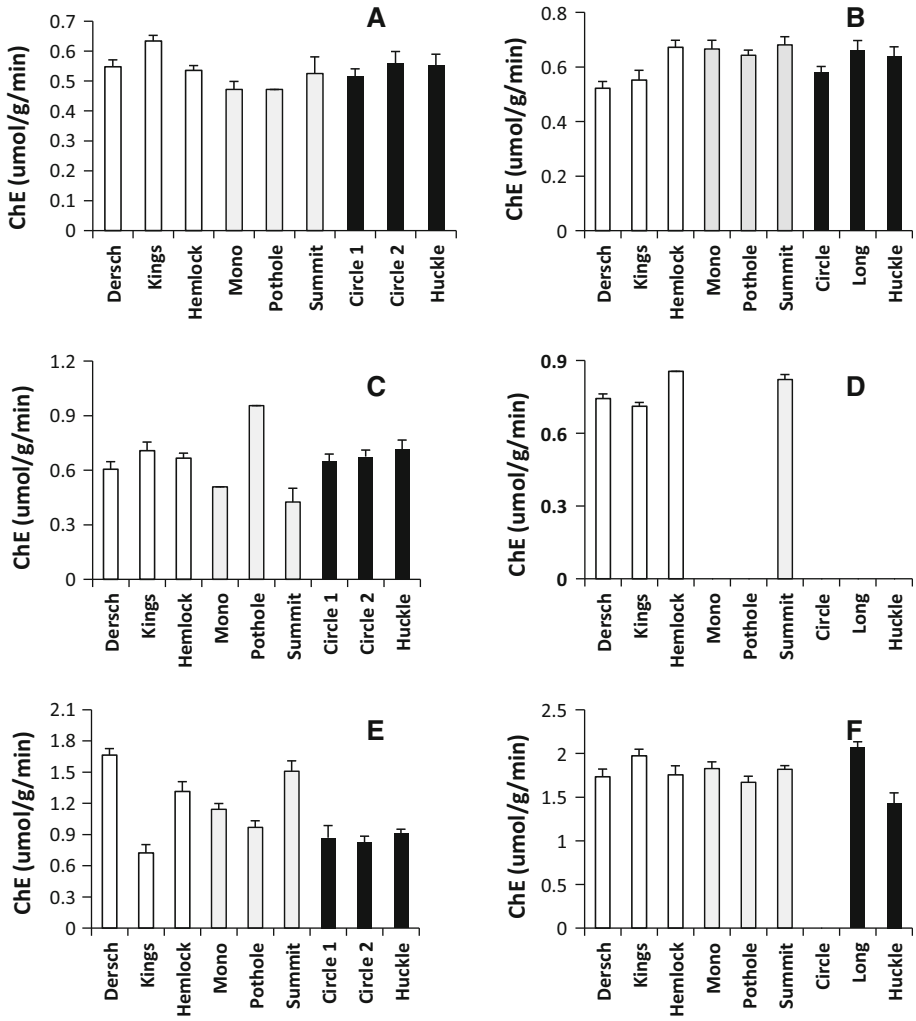


**Fig. 7** Mean (SEM) cholinesterase activity levels in *Pseudacris regilla* whole bodies collected during an in situ study from meadows of three national parks in the Sierra Nevada, USA. **a** 2001 early tadpoles; **b** 2002 early tadpoles; **c** 2001 late tadpoles; **d** 2002 late tadpoles; **e** 2001 metamorphs; **f** 2002 metamorphs. Blanks occur because of no or insufficient animals of a given state for a particular meadow. Color code: *white* Lassen, *gray* Yosemite, *black* Sequoia

still significant differences among developmental stages ($F_{2,128} = 8.91$, $p < 0.0001$) but no significant differences were found among parks of origin or deployment for any of the stages (Fig. 7b, d, f).

ChE activity levels may be influenced by ambient water temperature (Johnson et al. 2005) so we conducted an ANCOVA on ChE across parks with mean water temperature used as a covariate. In 2002, the year with the better representation of temperature, water temperature was significantly and positively related to ChE activity in metamorphs ($F_{1,46} = 6.77$, $p = 0.0125$) but, with the covariate, statistical significance now occurred among parks ($F_{2,46} = 4.44$, $p = 0.0172$).

## Discussion

### Chemical residues

The concentrations of individual contaminants in water (ng/L) or sediment (ng/g dry weight) were below values found to be acutely toxic in the laboratory. Chronic, hatching to metamorphosis, lethality has been determined only with a few of these contaminants but toxic concentrations were also higher than those recorded in this study (Sparling and Fellers 2009). Mean values reported were small due to the fact the anything less than 3 × MDL was considered to be below quantification and assigned a random value between 0 and 1 MDL and in many samples contaminants were not detected and therefore assigned a zero. However, sublethal effects can occur at substantially lower concentrations than those resulting in direct mortality. Also, recorded water and sediment contaminant concentrations reflect a moment in time and may not include the highest concentrations occurring during the breeding seasons of 2001 and 2002.

Other studies (LeNoir et al. 1999, Angermann et al. 2002, Fellers et al. 2004) have found similar contaminants in Sierra Nevada meadows in the same parks as this study. Sequoia, in particular, has had relatively high concentrations of many different contaminants compared to other western parks (Hageman et al. 2006). The geographical locations of the study parks are the key to understanding contaminant concentrations. Prevailing northwesterly wind patterns course through the highly agricultural San Joaquin Valley of California, carrying volatized contaminants, which are deposited with wet or dry precipitation into Sequoia (Davidson et al. 2002). Yosemite also receives westerly winds from the upwind San Joaquin whereas Lassen is situated at the north end of the Central Valley with prevailing northern winds and is much less exposed to agricultural contaminants.

BDE's, malathion, trifluralin, and endosulfan were in use during the time of this study but endosulfan has since been banned for the most part. BDEs are used as fire retardants and are mixed with building materials, plastics, electronics and textiles (Coyle and Karasov 2010). They have become nearly ubiquitous as contaminants. BDEs can decrease survival and retard growth and development in amphibians (Coyle and Karasov 2010) and may be endocrine disruptors (Carlsson et al. 2007). Malathion is a highly toxic, commonly used organophosphorus insecticide. Its acute 96 h LC50 in amphibians is around 2 mg/L and its oxon form is approximately 10 times as toxic as the parent form (Sparling and Fellers 2007). Between 240,000 and 280,000 kg of active ingredient (a.i.) of this pesticide were applied in California during 2001 and 2002 (http://www.cdpr.ca.gov, accessed 1/15/2014). Trifluralin is a pre-emergent herbicide with an estimated 96 h LC50 of a few mg/L in amphibians (Sayim 2010, Weir et al. 2012). Between 421,000 and 496,000 kg a.i. of this herbicide were used in 2001 and 2002. Endosulfan is an extremely toxic chlorinated pesticide with a chronic LC50 around 0.5 μg/L in *Rana boylii* (Sparling and Fellers 2009). About 69,000 kg a.i. of this pesticide were applied in each of the 2 years. Production and use of endosulfan was phased out within the United States starting in 2010. Of these chemicals in use at the time of the study, endosulfan, trifluralin and BDEs have moderate to very long half-lives and can bioaccumulate.

Noticeably absent from the results of our tissue residue analysis was the most highly used organophosphorus pesticide in California, chlorpyrifos. From 644,000 to 762,000 kg a.i. of this pesticide were applied in each year of the study. Chlorpyrifos and its oxon are also highly toxic to amphibians (Sparling and Fellers 2007, 2009). They were found in the water and sediments of ponds used in this study and have been found in several other studies in the Sierra Nevada (McConnell et al. 1998; LeNoir et al. 1999; Albert et al. 2007). Chlorpyrifos has a half-life of several days to a few weeks and does not bioaccumulate. These factors may have accounted for the lack of its detection in amphibian tissues. The other contaminants were mostly legacy pesticides whose use in the United States ceased from several to many years ago, but are so persistent that they can still be found in the environment. They can have multiple effects on amphibians including reduced growth, delayed development, genotoxicity, and endocrine disruption (Sparling 2003). There is very little information relating tissue concentrations of contaminants in amphibians to toxic effects.

It was not possible to analyze all of the various pesticides and other contaminants to which amphibians in the Sierra Nevada might be exposed. In 2001 68,693 metric tons of a.i. pesticides were applied in California with

30,666 metric tons or 44 % of the total applied in the five counties of the San Joaquin Valley (www.cpdr.ca.gov, accessed 1/15/2014). In one county alone 456 different chemicals were applied in 2001. Many of the chemicals were applied in low amounts but 67 were applied in quantities between 545 and 2,272 kg; 21 between 2,273 and 4,545 kg; 34 between 4,545 and 45,455 kg; and 8 in excess of 45,455 kg a.i. Although not all of these highly used chemicals would be carried into the foothills or mountains by the prevailing NW winds into Sequoia and Yosemite (Davidson 2004), the amount available for transport will increase with higher rates of application; these numbers give an idea of the magnitude of the potential problem. In 2002 78,221 metric tons of a.i. were applied statewide with 47 % of that occurring in the San Joaquin counties. In comparison, 741 metric tons of a.i. pesticide were applied in 2001 and 581 metric tons in 2002 in the five counties surrounding Lassen Volcanic National Park, about 2 % of the amount applied in the San Joaquin Valley. Due to known deposition of aerosols of many pesticides, the risk of exposure in Sequoia and Yosemite is quite high compared to Lassen (McConnell et al. 1998; LeNoir et al. 1999).

Whereas the concentrations of individual contaminants were low in the environment and in tissues, the numbers and kinds of contaminants an animal is exposed to are important because chemicals can interact and produce antagonistic, additive or synergistic effects. Other additive or synergistic effects of chemicals in amphibians have been reported (Boone and James 2003; Rohr and Crumrine 2005; Orton et al. 2006; Albert et al. 2007). In addition, contaminants may synergistically interact with other stressors such as disease (Kerby and Storfer 2009), predation or interspecific competition (Albert et al. 2007; Boone et al. 2007).

## Tadpole survival, development and growth

Differences in survival among locations of origin in 2001 may have been due to multiple factors. Tadpoles may have experienced greater stress as the travel distance between site of origin and site of deployment increased. However, we did not see an origin effect in 2002 even though the travel distance was the same as in 2001. Alternatively, contaminants can be transferred from mothers to their eggs and subsequently to their larvae (Hopkins et al. 2006). We did not analyze residues in tadpoles prior to transferring them, but there is a small possibility that those coming from Sequoia had an initial contaminant burden. It is also possible that *P. regilla* in Sequoia have adapted to conditions there and hence had higher survival than transplants. However, in both 2001 and 2002, survival rates of tadpoles collected from Sequoia and deployed into either Lassen or Yosemite were more than twice as great as those translocated back into Sequoia.

In general, malformations in amphibians have been associated with several environmental factors, especially trematode parasites. However, parasitically induced malformations are typically asymmetrical (Meteyer et al. 2000, Goodman and Johnson 2011). Uniform, bilateral malformations as found in this study are more likely due to exposure to contaminants than to parasites (Ankley et al. 1998). However, the only association between the hemimelia seen in this study and any chemical was with *trans*-nonachlor and it was in the opposite direction of what would be anticipated if the chemical caused the malformations; the observations we collected on the relationship between malformations and potential contaminant exposure are equivoval. As far as we have been able to determine, this particular malformation has not been previously identified in *P. regilla.*

Body size and mass have been related to survival. In tadpoles smaller animals are subject to longer periods and greater numbers of predators, especially gape-limited predators, than are large animals (Relyea 2007). Smaller sizes at the completion of metamorphosis have also been associated with reduced survival of juvenile frogs (Wilbur and Collins 1973). Slow development can be harmful to amphibians if pools dry up before they complete metamorphosis (Petranka 2007). In the Sierra Nevada, almost all precipitation occurs during the winter as snow. After the snowpack melts, pools evaporate and many disappear by mid-summer. If development is delayed, many tadpoles may perish. Of particular concern in the Sierra Nevada are *Rana sierrae* and *R. muscosa* tadpoles because they require up to 3 years to metamorphose and developmental delays could be compounded over that time. Endosulfan and BDEs have caused delayed development under laboratory conditions (Balch et al. 2006; Sparling and Fellers 2009).

## Genotoxicity and cholinesterase depression

The role of contaminants other than polycyclic aromatic hydrocarbons (PAHs) in producing genotoxicity in amphibians has not been well studied but has been shown in agricultural fields exposed to various agrichemicals (Lowcock et al. 2011) and in the laboratory with chlorpyrifos (Yin et al. 2009). Elevated HPCV values from flow cytometry suggest increased genotoxicity in the present study.

Cholinesterase depression has been used often as a biomarker of exposure to organophosphorus and carbamate pesticides. Sparling et al. (2001) showed that depressed ChE activity was associated with malathion, diazinon and chlorpyrifos concentrations in *P. regilla* collected from the Sierra Nevada. All three of these contaminants irreversibly

bind with ChE and animals need to produce more enzyme to survive, which requires time. In the current study, ChE depression was not associated with any specific contaminant but ChE-depressing compounds have short half-lives in the environment and do not bioaccumulate. Because organophosphate pesticides permanently bind to cholinesterase, it is possible that their effects extend past detectable concentrations; thus the pesticides may have been at low concentrations at the time of sample collection but present at higher concentrations earlier.

## Conclusions

This study suggests some linkages between contaminant exposure and negative physiological effects under natural conditions in *P. regilla,* though there are several inconsistencies in the results as well. Specifically:

- Thirteen contaminants were found in water and 16 in sediments of the study ponds. These and other contaminants for a total of 20 were also found in the tissues of amphibians reared in the ponds.
- Several physiological factors including reduced survivorship, increased genotoxicity, reduced rates of growth and development, reduced cholinesterase activity levels and hind limb malformations were identified; however the latter was not associated with any elevated levels of contaminants.
- In absolute magnitude, Sequoia, considered the most impacted park, had the highest concentrations of DDE in both metamorphs and tadploes, the lowest ChE levels in metamorphs in 2001, the highest indication of chromosomal damage in 2001, the smallest SVL in both metamorphs and tadpoles in both 2001 and 2002, the lowest survivorship in both years and the longest time to metamorphosis in both years. Due to high variability not all differences were always significant, but the pattern was quite consistent.
- However Lassen, considered the cleanest park, had equivocal results, but was generally less affected in all biomarkers measured but frequently not different from or occasionally more impacted than Yosemite.
- Effects were inconsistent from year to year and were more pronounced in 2001 when most of the environmental and tissue contaminant 'loads' were higher than in 2002. We cannot determine if the incidence or severity of these physiological factors were specifically related to the overall presence of contaminants although meadows and parks with higher contaminant loads expressed stronger effects than those with lower contaminant load. A longer time series would be necessary to disentangle annual variation from a consistent pattern of exposure and biological effects.
- Most of the physiological effects observed are consistent with those found in laboratory studies on specific contaminants; this merits further investigation.

There are some issues in assuming a cause-effect relationship between contaminants and effects too strongly in this study. Of greatest concern is that the concentrations of contaminants in tissues, sediments and water were well below laboratory concentrations known to be toxic. As mentioned in the methods section, the residues reflect a snapshot in time of the varying concentrations that actually occurred in the field and several of the contaminants have short half-lives. Also, there is little information relating body residues in anurans to effects. Moreover, tadpoles at all sites were exposed to several contaminants simultaneously and interactive effects of these chemicals are not well known. The hypothesis concerning greater effects in Sequoia and Yosemite National Parks than in Lassen Volcanic was consistently supported in only 2001 and not 2002; thus annual variations in contaminant concentrations and toxicity need to be studied in greater detail. For the single samples of water residues the three parks were comparable in their concentrations and types of contaminants. The hypothesis that Sequoia and Yosemite had a greater variety of contaminants than Lassen was supported in sediment residues in 2001 but the difference did not hold for 2002. A longer but considerably more expensive study that collects and analyzes sediment and water samples repeatedly within and over a few years could provide a more reliable relationship between contaminant concentrations and population effects.

In 2001 the greatest variety and often highest concentrations of residues in amphibian tissues occurred in Sequoia and the differences were commensurate with the frequency and severity of observed effects. In 2002 residues were more equally distributed while concentrations and effects were reduced compared to 2001. This suggests that amphibians could experience considerable annual variation in contaminant exposures. Winter rainfall and wind patterns are the likely drivers behind this variation as the prevailing winds in the Central Valley consistently blow from the agricultural regions with heavy pesticide use in the direction of both Yosemite and Sequoia, whereas the prevailing winds at Lassen come from the north. Finally, population effects can be determined only over several years. Even stable populations of frogs and toads often experience very high tadpole mortality (Calef 1973) so losses experienced in this study may not affect long term population status.

This study provides evidence suggesting that there is a potential relationship between the presence of contaminants in the Sierra Nevada and the health of *P. regilla*. While *P. regilla* is not a species of concern at this time,

laboratory tests show that the species' sensitivity to pesticides is similar or even lower than that of some ranid species that have been tested (Sparling and Fellers 2009). Therefore, compared to this species, we might anticipate equal or stronger lethal and sublethal effects are occurring in the species that are declining.

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