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HATCHING SUCCESS AND PESTICIDE EXPOSURES IN AMPHIBIANS LIVING IN AGRICULTURAL HABITATS OF THE SOUTH OKANAGAN VALLEY, BRITISH COLUMBIA, CANADA (2004–2006)

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Abstract—In 2004 to 2006, in the Okanagan Valley, British Columbia, Canada, we measured pesticides, water chemistry, and hatching success of Great Basin spadefoot *(Spea intermontana)*, Pacific treefrog (*Pseudacris regilla*), Western toad (*Bufo boreas*), and Columbia spotted frog (*Rana luteiventris*). Predator-proof cages containing Gosner Stage 4 eggs were placed in ponds in nonagricultural reference sites in conventionally sprayed and organic orchards. Seventeen pesticides were detected in ponds in sprayed orchards but occurred at low concentrations (ng/L) except for diazinon (1,410 ng/L). Chloride, sulfate, conductivity, nitrate, and phosphorus showed significant differences among sites. Spadefoot mean hatching success ranged from 0 to 92% among sprayed orchards, whereas the range was 48 to 98.6% among organic orchards and 51 to 95.5% among reference sites. Mean hatching success for Pacific treefrog was 22.1 to 76.1% among sprayed orchards, whereas the range was 83.4 to 97.1% among reference sites. Although sample sizes were small and replication was low, we found that trends in hatching success of eggs of Western toad and Columbia spotted frogs were consistent with the other species. Variables that correlated negatively with amphibian hatching success included 12 pesticides and seven water chemistry parameters. However, stepwise regression found that, in 2005, atrazine accounted for 79% of the variation in spadefoot hatching success and, in 2006, atrazine, total nitrate, and chlorpyrifos accounted for 80%. For Pacific treefrog there were no significant correlations with pesticide concentrations; rather, hatching success correlated with water chemistry parameters. The present study also emphasizes the variability in species sensitivity and importance of incorporating water chemistry into the interpretation of water quality for amphibians. Environ. Toxicol. Chem. 2010;29:1593–1603. © 2010 SETAC

Keywords—Amphibian Hatching success Pesticides Water chemistry Agriculture

INTRODUCTION

Pesticides are one of multiple stressors on amphibian populations throughout the world [1]. Pesticide residues are routinely detected in surface waters in agricultural and nonagricultural watersheds [2]; however, quantification of the impacts on reproduction of wild amphibians is still uncommon [3,4].

The global loss of wetlands in agricultural districts is extensive [5] and ponds on farms often provide the last remaining wetland habitats in many agricultural landscapes. Thus, the habitat quality of ponds may be critical to the survival of local amphibian populations. Such is the case in the lowlands of the South Okanagan Valley, British Columbia, Canada, an intensive fruit-growing location, and an area of high amphibian diversity in Canada [6], where less than 40% of the natural wetlands remain [7].

In the South Okanagan Valley, the extirpation of the Northern leopard frog (*Rana pipiens)* and the presence of three nationally listed amphibian species in this intensive agricultural zone has raised concerns that pesticides may hinder the survival and recovery of those populations ([8], http://www.env.gov.bc.ca/wld/documents/recovery/rcvrystrat/great_basin_spadefoot_rcvry_strat_150108.pdf; [9], http://www.env.gov.bc.ca/wld/documents/recovery/rcvrystrat/tiger_salamander_rcvry_strat_150108.pdf). In particular, the South Okanagan Valley contains the majority of the Canadian geographic range of the endangered ([10]; www.sararegistry.gc.ca/sar/index/default_e.cfm) southern mountain population of the tiger salamander (*Ambystoma tigrinum*) and the threatened ([11]; http://www.sararegistry.gc.ca/document/default_e.cfm?documentID=1382) Great Basin spadefoot (*Spea intermontana*). Other species occurring in the South Okanagan Valley include the Western toad (nationally listed as special concern) (*Bufo boreas*) ([12]; http://www.sararegistry.gc.ca/document/default_e.cfm?documentID=226) along with several common species such as the Pacific treefrog (*Pseudacris regilla)*, the Columbia spotted frog (*Rana luteiventris*), and long-toed salamander (*Ambystoma macrodactylum*) [6].

To assess the risk of amphibian populations to multiple stressor effects of agricultural practices in the South Okanagan Valley, during 2004 to 2006 we measured pesticide residues, water chemistry, and hatching success of amphibian eggs in ponds in conventionally sprayed orchards, organic orchards, and reference, nonagricultural ponds. We predicted that amphibian egg hatching success would be significantly lower as pesticide and nutrient exposures increased in ponds.

MATERIALS AND METHODS

Study area

In 2004 to 2006 the study sites in the lowlands of the South Okanagan Valley (350–400 m altitude), British Columbia, Canada (49°3′56.60 and 119°31′21.06) were small ponds in

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three conventionally sprayed fruit orchards, three organic fruit orchards, and three reference sites located 500 m to 1,000 m from conventionally sprayed orchards. Ponds ranged in depth from 1.3 to 3.6 m and approximately 0.01 ha to 3 ha in size. The average size of the orchards was approximately 10 ha. While the sites varied in size they were all located within a 10-km radius of one another. In the area surrounding the amphibian cages (20 m radius), all ponds were free of emergent vegetation. These aspects of the study sites maintained approximately similar sun exposure during egg development. Fruits grown in both conventionally sprayed orchards and organic orchards were predominantly a mix of peaches, apples, and cherries. Apricots, plums, nectarines, and grapes were also occasionally grown on these farms.

In situ hatching success of amphibian eggs

Oviposited eggs were collected when they were less than 6 h old and at Gosner developmental Stage 4 [13]. For each species, all eggs came from the same locations within and among study years. Egg masses were transported at 4°C in small plastic containers and placed in cages in situ in the emergent vegetation zone of the ponds at each study site on the same date among sites within each year. Egg masses for all species were collected from nonagricultural sites. *Spea intermontana* and *P. regilla* were collected from two artificial pond locations approximately 3 km apart (elevation 298 m and 333 m) within the South Okanagan Valley, and approximately 5 to 12 km from the study sites. *Bufo boreas* eggs were collected from a small nonagricultural artificial pond (elevation 475 m) within the South Okanagan Valley and approximately 25 km from the study sites. *Rana luteiventris* eggs were collected from one nonagricultural location (elevation 1104 m, ≈50 km from the study sites), near Anarchist Mountain, along a 3-m stretch of Nine Mile Creek.

The cages excluded invertebrate predators but not bacteria and viruses [14]. Cages were made of inert white Nytex (500 μM; Tekton) that permitted adequate water circulation and light penetration. Cages were attached loosely to small wooden dowels on each side by means of plastic cable ties. This was done to prevent egg dessication by allowing cages to move vertically with changes in the water depth [14]. All cages floated on the surface of the water with eggs in the bottom of the cup suspended at 5 cm from the water surface. Since the eggs were extremely close to the water surface, this ensured that all eggs were exposed to similar oxygen and temperature profiles within the water. Eggs were considered successfully hatched when they produced free-swimming tadpoles at Gosner Stages 20 to 22 [13]. Hatching success (%) was calculated per cage as: (number of free-swimming tadpoles / number of eggs placed in the cage at the outset of the experiment) × 100.

In each year a subsample of 14 to 15 eggs from each clutch of Great Basin spadefoots (spadefoot) were placed in five in situ cages per site (Figs. 1 and 2). Whereas, for Pacific treefrog, whole clutches were placed in each cage; therefore, the number of eggs varied among clutches (Fig. 2). Clutches contained 11 to 31 eggs and were placed in five in situ cages per site in 2006 and in trial 1 of 2005 for a total of 30 clutches in each year (Fig. 2). In trial 2 of 2005, there were only three in situ cages per site; therefore, a total of 18 clutches (Fig. 2). For Western toad, a single string of eggs was collected and subsampled into 15 eggs per cage for a total of five in situ cages per site (Fig. 1). For Columbia spotted frog, two egg masses were separated into subsamples of 14 to 25 eggs and individual subsamples were placed among six in situ cages per site (Fig. 3).

In 2004, spadefoot eggs were collected and placed in the study sites on 27 April 04, and Western toad eggs were placed in cages on 29 April 04. In 2005, eggs were collected and placed into the study sites on 26 April (trial 1) and 30 April (trial 2) (Pacific treefrog) and on 18 May (spadefoot). In 2006 eggs were collected and placed in study sites on 23 April (Pacific treefrog and spadefoot), and 8 May (Columbia spotted frog).

Cages were visited three times per week during the period of development until hatching. To ensure that no cross-contamination of disease or pesticides took place between study sites, prior to use all equipment was disinfected with 10% bleach solution between study sites. Extreme care was taken to reduce water and sediment movement around the cages during examination of eggs to ensure minimum disturbance to egg masses and vegetation, and to avoid sediments being suspended in the water and settling on the eggs.

All procedures were conducted according to the Canadian Council on Animal Care using approved protocols ([15]; http://www.ccac.ca/en/CCAC_Programs/Guidelines_Policies/GUIDES/ENGLISH/toc_v1.htm) from Environment Canada (Delta, BC) and Simon Fraser University (AUP 730B04) Animal Care Committees; research permits were obtained from the British Columbia Ministry of Environment (PE06-21835).

Water chemistry

In 2004 water samples were collected on 29 April and 1 May at each site. In 2005 water samples were collected from each site on 3 May and 25 May. In 2006 water samples were collected from each site on 23 April, 30 April, and 10 May or 16 May.

On each sample date two water samples (1 L) were collected in opaque plastic containers cleaned previously with nonphosphate soap. Each sample was collected within 1 m of in situ cages at each study site. Samples were collected by hand approximately 5 cm below the water surface. Water samples were kept on ice at approximately 4°C until arrival to the laboratory within 24 h postcollection. Water chemistry analysis was done by Pacific Environmental Science Center (PESC), Environment Canada.

Samples for water chemistry were analyzed using standard methods [16] for pH, conductivity, NH3, total nitrogen (TN), phosphorous o-PO4 dissolved, total dissolved phosphorus (TDP), total phosphorous (TP), turbidity, chloride (Cl), fluoride (F), sulfate (SO_4), bromide (Br), nitrate (NO_3), nitrite (NO_2), phosphate (PO_4), and biochemical oxygen demand (BOD).

Pesticides in water

In 2005 a water sample was collected for pesticide analysis from study sites on 20 May 2005 and 3 June 05. Similarly, in 2006, during the in situ egg experiment, one water sample was collected per site on 3 May 06 and on 14 May 06. Samples were collected from all sites on these dates because it coincided with the end of egg development. Due to half-lives of the pesticides being measured, the sensitivity of the analytical methods [17,18], and the lack of spray events during the in situ experiment in 2005 and 2006, pesticide concentrations in the samples collected that day were assumed to be approximately representative of minimum concentrations during the egg development a period of 2 to 3 weeks preceding the water sampling dates.

Water samples were collected in hexane-treated 1-L amber bottles within 1 m of in situ cages at each study site. Samples were collected by hand approximately 5 cm below the water surface. Dichloromethane (100 ml) was placed in each sample bottle to preserve the pesticide concentrations from degradation

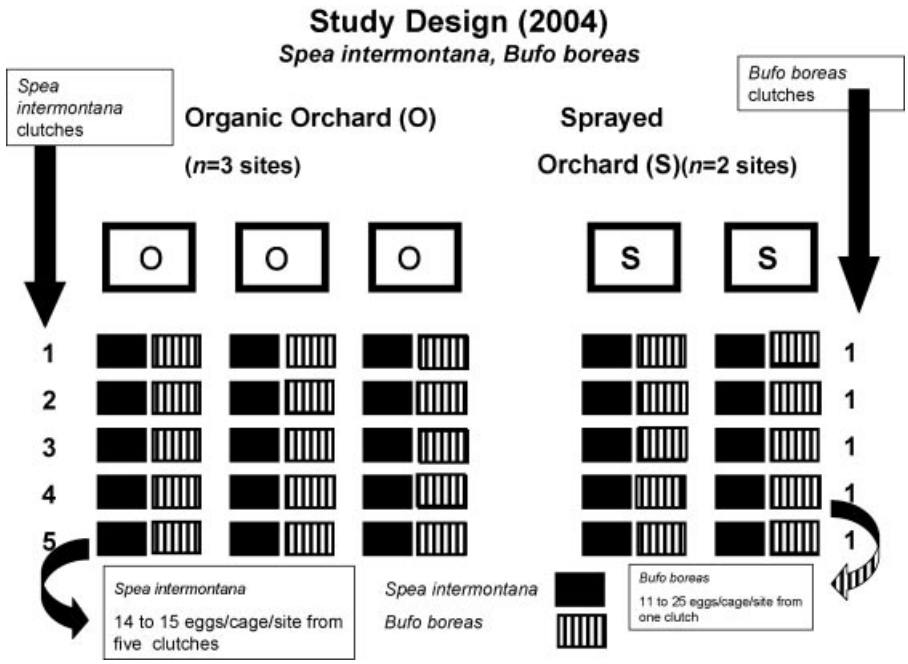

Fig. 1. Study design to measure hatching success of Great Basin spadefoot (*Spea intermontana*) and Western toad (*Bufo boreas*) in the South Okanagan Valley, British Columbia, Canada (2004).

during transport prior to analysis. Samples were labeled with coded identification numbers for blind analysis at the laboratory. Water samples were kept at 4°C until arrival to the laboratory within 12 h postcollection. Pesticide analysis was performed by Axys Analytical.

Pesticides measured in water samples were alachlor, aldrin, alpha- and beta-endosulfan, ametryn, atrazine, azinphos-methyl, buralin, butylate, captan, alpha-, *cis*-, and gamma-chlordane, chlorothalonil, chlorpyrifos, chlorpyrifos-methyl, chlorpyrifos-oxon, cyanazine, cypermethrin, diazinon, azinphosmethyl, linuron, dichlorvos, dieldrin, dimethenamid, disulfoton, disulfoton sulfone, endosulfan-sulfate, endrin, endrin-aldehyde, endrin-ketone, ethalfluralin, ethion, ethalfluralin, fenitrothion, flufenacet, fonofos, alpha-, beta-, delta-, gamma-hexachlorcyclohexane, heptachlor, heptachlor-epoxide, hexachlorobenzene, hexazinone, malathion, methamidophos, methoprene, metolachlor, mirex, naled, *cis*-, and *trans*-nonachlor, octachlorostyrene, oxychlordane, parathion-ethyl, parathion-methyl, pendimethalin, permethrins, phorate, phosmet, pirimiphos-methyl, quintozene, simazine, tebuconazol, tecnazene, terbufos, triallate, trifluralin, 2,4-D, dicamba, 2-methyl-4-chlorophenoxyacetic acid (MCPA), 1-(3-chlorophenyl) piperazine (MCPP), trichlopyr, bromoxynil, and fluazifop.

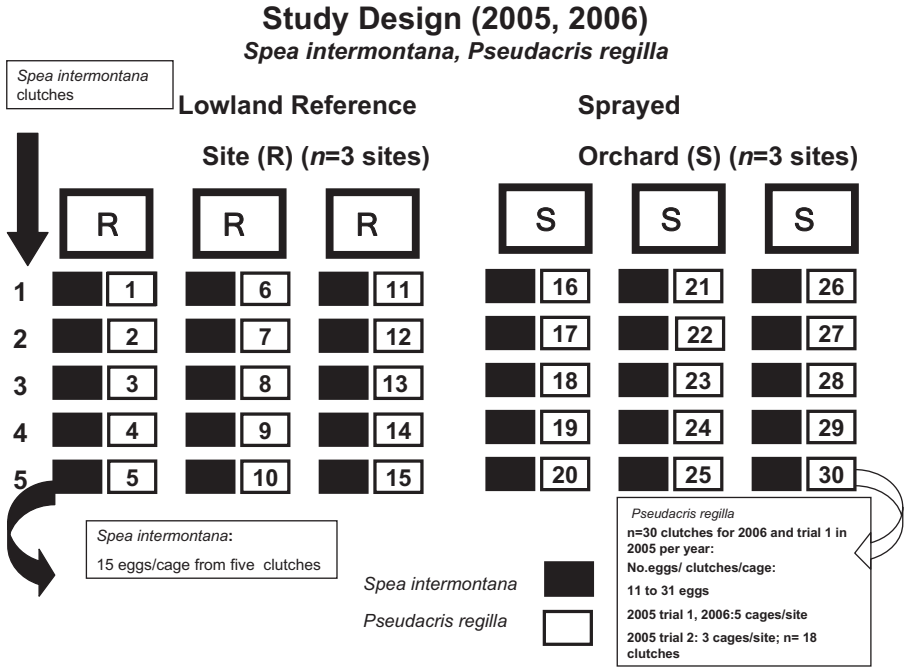

Fig. 2. Study design to measure hatching success of Great Basin spadefoot (*Spea intermontana*) and Western toad (*Bufo boreas*) in the South Okanagan Valley, British Columbia, Canada (2005, 2006).

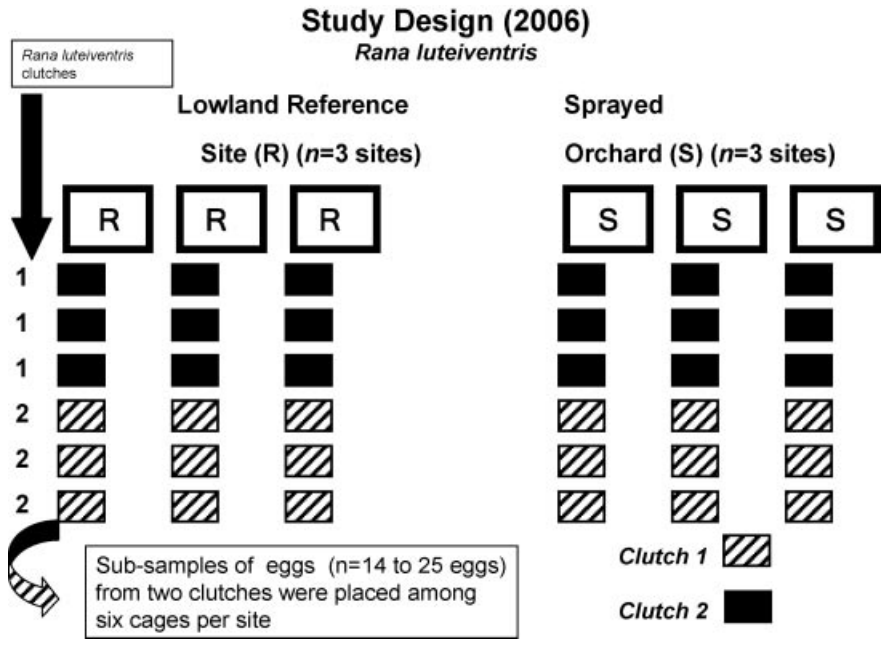

Fig. 3. Study design to measure hatching success of Columbia spotted frog (*Rana luteiventris*) in the South Okanagan Valley, British Columbia, Canada (2006).

For analysis of acid-extractable herbicides 2,4-D, dicamba, MCPA, MCPP, trichlopyr, bromoxynil, and fluazifop the methods were conducted following previous methods [19]. All forms of the analyte were converted to the acid form prior to extraction of the samples. This was done by base hydrolysis (for 2 h) of the samples with 10 M sodium hydroxide at pH 12 to 13. The samples were then extracted with 1×100 ml of dichloromethane (DCM) at pH 12 to remove interferences. The sample was then acidified with 3 M sulfuric acid to a pH value below 2. The resulting sample was then liquid–liquid extracted with 3×100 ml of DCM. The DCM extract was dried over acidified anhydrous sodium sulfate and reduced to just dryness in preparation for the derivatization of the analytes. The extract was then reconstituted with 50 μl of methanol. Derivatization was carried out using diazomethane.

A Florisil column was prepared by filling a glass column (25×1 cm internal diameter [i.d.] with 100 ml reservoir) with hexane. The column was then packed with 8 g of deactivated Florisil. The derivatized sample extract (1 ml in hexane) was loaded to the prepared column. The column was eluted with 50 ml of 15% DCM in hexane and discarded. The column was then eluted with 100 ml of 1:1 DCM: ethyl acetate. The extract was reduced in volume by rotary evaporation and gentle stream of nitrogen, spiked with 10 μl of the labeled recovery standard solution and made up to a final extract volume of 100 μl for instrumental analysis. Aminopropyl-bonded silica solid-phase extraction (SPE) cleanup was then conducted.

The HRGC/HRMS analysis was conducted using an AutoSpec Ultima HRMS equipped with an HP 6890 GC (Agilent Technologies), a CTC autosampler (CTC Analytics), and an Alpha Open VMS data system running on Micromass Opus 6.3X and OpusQuan software. A DB-5 (Agilent) capillary chromatography column ($60 \text{ m} \times 0.25$ mm i.d., and 0.1 μm film thickness) was coupled into the MS source. Using this method, detection limits of 0.05 ng/L for dicamba, MCPA, MCPP, and triclopyr, and 0.5 ng/L for 2,4-D were routinely achieved.

Analysis of all other pesticides in water followed a previous method [18] and used a 1-L aqueous sample containing less than 1% solids, which was spiked with deuterium and 13C-labeled quantification standards in acetone and liquid–liquid extracted with 3×100 ml of DCM. The 300-ml DCM extract was reduced by rotary evaporation to 1 ml prior to column cleanup. Cleanup of the sample extracts was conducted using a microsilica chromatography column. The column was prepared by packing a 10% deactivated 0.75 g silica into a glass wool plugged transfer pipette. The column was wet with 10 ml of hexane. The 1-ml extract was then loaded to the column at a dropwise rate. The column was eluted with 5 ml of 10% methanol in DCM. All eluates were collected, reduced in volume, and spiked with labeled recovery (internal) standards prior to instrumental analysis.

All analyses were conducted using high-resolution gas chromatography / high resolution mass spectrometry (HRGC/HRMS). An AUTOSPEC ULTIMA high-resolution MS equipped with an HP 6890 GC, a CTC autosampler, and an Alpha data system running on Micromass software. A DB-5 capillary chromatography column (30 m, 0.25 mm i.d., and 0.1 μm film thickness) was coupled to the MS source. Immediately prior to running samples the mass spectrometer was tuned to have a static mass resolution of at least 8,000 and operated in the electron impact (EI) ionization mode using multiple ion detection.

Analysis of all the target pesticides was conducted using two instrumental runs. GC operating conditions for organochlorine, organophosphorus, triazine, and pyrethroid pesticides included the following oven temperature program for analyte separation: initial temperature 50°C hold for 0.5 min, ramp at a rate of 20°C/min to 150°C, ramp at a rate of 3°C/min to 230°C, hold for 12 min, ramp at a rate of 10°C/min to 320°C, and hold for 2.8 min. Injection temperature and interface temperatures were set at 220°C and 280°C, respectively.

A second instrument run acquired the organonitrogen pesticides and used an oven temperature program with initial temperature of 75°C hold for 1 min, ramp at a rate of 25°C/min to 150°C, ramp at a rate of 15°C/min to 300°C, and hold for 9 min. Injection temperature and interface temperatures were set at 250 and 280°C, respectively. Detection limit was 1.1 ng/L.

Statistical analysis

For Great Basin spadefoot and Pacific treefrogs, differences in hatching success of eggs among sites and treatments were determined using analysis of variance (ANOVA) and nested ANOVA with the main class effects of site (reference, sprayed, or organic), clutch, and stage of growth. Least squares means were calculated to determine what, if any, differences existed [20]. Due to pseudo-replication in egg samples among sites from single clutches of each of Columbia spotted frog and Western toad, percent hatching success is reported as a general comparison to trends in the other two species but no statistical analyses were conducted for these samples.

Water chemistry results for 2006 were compared among sites using ANOVA. In other years, only two water samples per site were collected for water chemistry and pesticide analysis. Therefore, results for 2004 and 2005 preclude ANOVA comparisons. When water chemistry (2004–2006), pesticide concentrations (2005, 2006 only) and hatching success (2004–2006) were measured in the same year(s), correlations between water quality and hatching success were determined using product-moment analysis. Stepwise regression analyses was then conducted including only variables that were significant in the correlational analyses [20]. All analyses were conducted using Statistical Analysis Software® (SAS 9.1. 3). For stepwise regression, $p = 0.15$ was the significance level for entry into the stepwise regression models. Statistical analyses were considered significant at $p \leq 0.05$ [20].

RESULTS

Hatching success

For all species and years, the lowest percent hatching success was detected at sprayed sites (Figs. 4–6). Hatching success was highest at the nonagricultural reference sites. Among sites within each treatment there were differences in hatching success but no significant clutch effects on hatching success in any year or species.

Mean hatching success for Great Basin spadefoot ranged from 0 to 92% among sprayed orchards, whereas the range was 48 to 98.6% among organic orchards and 51 to 95.5% among reference sites (Figs. 4–6). In 2004 there was no significant difference in hatching success between sprayed and organic orchards ($F = 1.08$; $p = 0.31$) and there were significant differences in hatching success among sites within organic and sprayed treatments. In 2005 and 2006 there were significant differences in hatching success between sprayed and reference sites (2005: $F = 31.41$; $p = 0.001$; 2006: $F = 3.25$; $p = 0.05$). However, in both years there were also significant differences in hatching success among reference and among sprayed sites.

Mean hatching success for Pacific treefrog ranged from 22.1 to 76.1% among sprayed orchards, whereas the range was 83.4 to 97.1% among reference sites (Figs. 5 and 6). In 2005 there were two hatching success trials conducted but there were no significant differences in hatching success between trials ($F = 1.03$; $p = 0.31$); therefore, the results were pooled and compared within sites and between sites and treatments. In 2005 there were significant differences in hatching success between reference sites and sprayed sites ($F = 4.69$; $p = 0.04$). In 2006 there were significant differences in hatching success between reference and sprayed sites ($F = 12.9$; $p = 0.001$). In 2005 and 2006 there were also significant differences in hatching success among reference sites. In 2006 there was a significant difference in hatching success among sprayed sites. However, in 2005

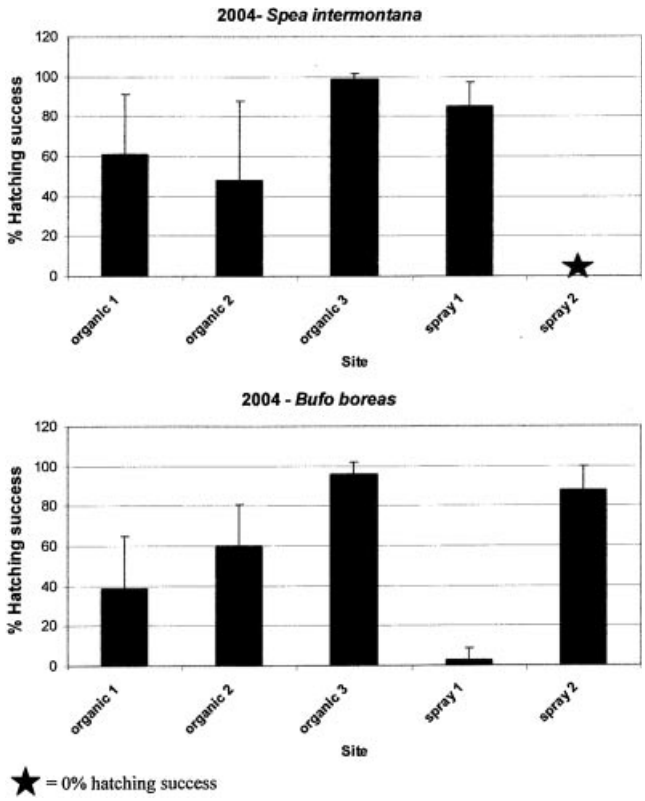

Fig. 4. Hatching success (%) of amphibian eggs from organic and conventionally sprayed (Spray) orchards in the South Okanagan Valley, British Columbia, Canada (2004). No significant differences ($p \leq 0.05$) were seen between treatments (organic vs spray).

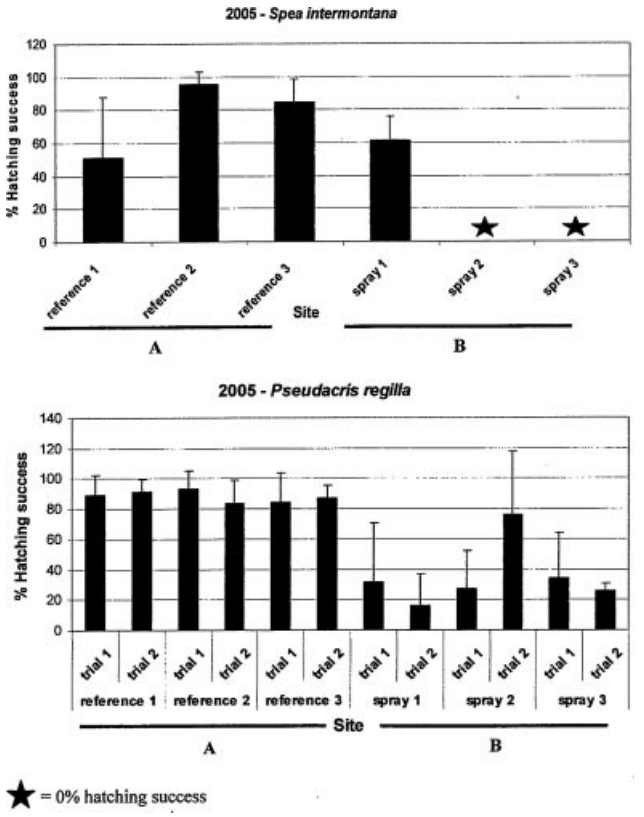

Fig. 5. Hatching success (%) of amphibian eggs from nonagricultural reference sites (Reference) and conventionally sprayed (Spray) orchards in the South Okanagan Valley, British Columbia, Canada (2005). A, B indicate a significant difference ($p \leq 0.05$) between treatments.

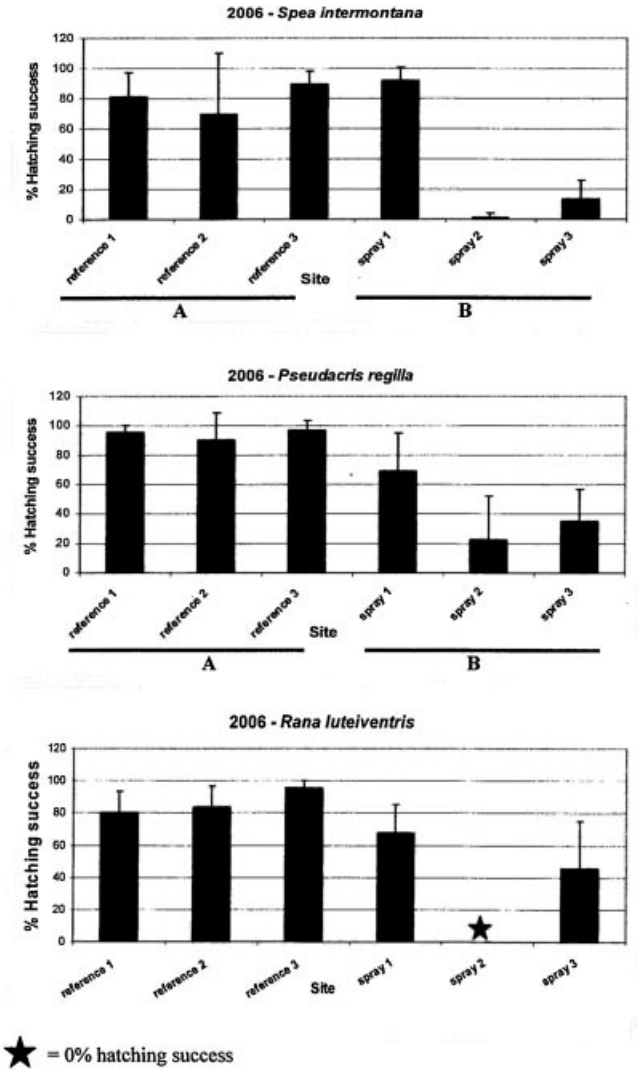

Fig. 6. Hatching success (%) of amphibian eggs from nonagricultural reference sites (Reference) and conventionally sprayed (Spray) orchards in the South Okanagan Valley, British Columbia, Canada (2006). A, B indicate a significant difference ($p \leq 0.05$) between treatments. Where no letters are shown there was no significant difference.

there was no significant difference in hatching success among sprayed sites.

Hatching success of Western toad eggs in 2004 was as low as 0.6% in sprayed orchards and as high as 96% in organic orchards (Fig. 4). For Columbia spotted frog in 2006, mean hatching success ranged from 0 to 67.6% among sprayed and 83.8 to 95.2% among reference sites (Fig. 6).

Water chemistry

For each site and treatment group, water chemistry results were similar among years for many parameters (Table 1) except chloride, sulfate, conductivity, nitrate, and phosphorus, which showed significant differences among reference, organic, and sprayed sites (Table 1). Reference site concentrations were lowest and organic site values were highest for sulfate, conductivity, and chloride in all years (Table 1).

In 2005 mean ammonia concentrations in sprayed ponds were at the lower acceptable limit of the Canadian Water Quality Guidelines (CWQG) ([21], http://www.ec.gc.ca/CEQG-RCQE/English/default.cfm) for the protection of aquatic life. The standard deviation for the mean ammonia level was twice the mean concentration, suggesting that the typical ammonia levels in the sprayed sites were not consistently exceeding the CWQG (Table 1). In all years and all sites except the organic site in 2006, fluoride concentrations exceeded the Canadian Water Quality Guidelines for the protection of aquatic life (Table 1).

Pesticides in water

In 2005 and 2006 no spray events occurred during the experimental period. In 2004, at sprayed site 1, there was a spray on the orchard of endosulfan on 27 April 04 which is the same date the spadefoot eggs were put into the ponds and two days before the Western toad eggs were put into the ponds on 29 April 04. In 2004 there were no other sprays during the experiment.

At all sites, concentrations of many pesticides were below detection limits (Table 2). Diazinon occurred at the highest concentration among all samples. Diazinon was detected at 1,410 ng/L at sprayed orchard 2 and at several hundred ng/L at ponds in sprayed orchards 1 and 3 (Table 2). Concentrations of individual pesticides were detected at up to 76.4 ng/L in reference sites (Table 2). Total pesticide concentrations (sum of all compounds that were detectable at concentrations above 1.1 ng/L) ranged from 60.7 to 198.4 ng/L among three reference sites while concentrations ranged from 118.9 to 1,519.2 ng/L in the sprayed sites (Table 2).

Associations: water chemistry, pesticides, and hatching success

In both study years there were significant correlations among water chemistry, pesticides, and hatching success. In 2005 and 2006, for Great Basin spadefoot hatching success, the significant R values ranged from -0.87 to -0.37 ($p \leq 0.04$–0.001) (Table 3) for the pesticides atrazine, desethyl atrazine, simazine, total permethrins, cypermethrin, azinphosmethyl, chlorpyrifos, diazinon and diazinon-oxon and endosulfan-sulfate, and alpha and beta endosulfan (Table 3). In 2006 concentrations of nitrate, phosphorus, and sulfate were also negatively correlated with hatching success, but not in 2005 (Table 3). For spadefoot hatching success, stepwise regression including all significant variables found that in 2005 atrazine accounted for 79% of the variation (partial R-square $= 0.79$; $F = 130.34$; $p = 0.001$). Stepwise regression for spadefoot hatching success in 2006 found significant variables, including atrazine (partial R-square $= 0.76$; $F = 90.4$; $p < 0.001$), total nitrate (partial R-square $= 0.02$; $F = 3.7$; $p = 0.06$), and chlorpyrifos (partial R-square $= 0.02$; $F = 3.2$; $p = 0.08$).

For Pacific treefrog in 2005 and 2006, significant R-values ranged from -0.38 to -0.69 ($p \leq 0.04$–0.01) for hatching success and water chemistry parameters ammonia, chloride, conductivity, turbidity, and total nitrate concentrations. Chloride concentrations correlated positively with hatching success in 2005. No significant correlations existed between hatching success and pesticide concentrations (Table 3).

DISCUSSION

For Great Basin spadefoot, hatching success in this federally threatened species was significantly lower in conventionally sprayed sites as compared to reference sites in each year of the study; however, there were no significant differences between sprayed and organic orchards. Decreased hatching success of spadefoot eggs correlated with increasing pesticide and nutrient concentrations. For Pacific treefrog, which is not listed as at risk, hatching success was also consistently lowest in sprayed sites but this was not always statistically different from nonsprayed sites

Table 1. Mean (standard deviation) water chemistry parameters measured in nonagricultural reference ponds, ponds in organic and conventionally sprayed fruit orchards in the south Okanagan valley, British Columbia, Canada (2004–2006)

Parameter	Organic sites	Sprayed sites
2004[a]		
	$n = 3$ sites	$n = 3$ sites
BOD [mg/L]	4.3 (3.2)	8.5(25.0)
Cl [mg/L]	72.2(50.4)	40.0(19.5)
F [mg/L]	0.22 (0.27)	0.98(0.43)
SO4 [mg/L]	1631.6(1477.0)	595.5(58.5)
Bromide [mg/L]	0.09(0.08)	0.21 (0.0)
NO3 [mg/L]	1.26(2.2)	0.21 (0.0)
NO2 [mg/L]	0.003 (0)	0.005 (0)
PO4 [mg/L]	0.19 (0.28)	0.025 (0)
pH [pH Units]	7.7(0.12)	8.39(8.2)
Conductivity [μS/cm]	2623.7(1792.6)	1349.5(481.0)
Turbidity [NTU[b]]	2.3(2.6)	3.71(28.8)
NH3 [mg/L]	0.04(0.05)	0.02(0.25)
Nitrogen total [mg/L]	2.2(1.1)	1.62(7.1)
Phos. o-PO4 diss. [mg/L]	0.22(0.39)	0.0(0.1)
Total dissolved phosphorus [mg/L]	0.26(0.43)	0.02(0.07)
Total phosphorus [mg/L]	0.31(0.5)	0.05(0.6)
2005[a]	Reference sites	Sprayed sites
	$n = 3$ sites	$n = 3$ sites
BOD [mg/L]	9.8(5.3)	9.3(6.3)
Cl [mg/L]	19.7(12.1)	35.0(21.2)
F [mg/L]	0.4(0.07)	0.5(0.6)
SO4 [mg/L]	123.3(36.3)	558.8(771.6)
Bromide [mg/L]	0.025(0)	0.11(0.13)
NO3 [mg/L]	0.001(0.0008)	0.04(0.05)
NO2 [mg/L]	0.0025(0)	0.027(0.04)
PO4 [mg/L]	0.025(0)	0.025(0)
pH [pH Units]	8.1(0.5)	8.3(0.1)
Conductivity [μS/cm]	722.5(73.1)	1407.0(1329.7)
Turbidity [NTU[b]]	2.2(2.4)	7.7(8.8)
NH3 [mg/L]	0.04(0.02)	1.3(2.5)
Nitrogen total [mg/L]	1.0(0.21)	2.69(2.8)
Phos. o-PO4 diss. [mg/L]	0.011(0.013)	0.05(0.07)
Total dissolved phosphorus [mg/L]	0.033(0.017)	0.07(0.08)
Total phosphorus [mg/L]	0.06(0.3)	0.12(0.09)
2006[c]	Reference sites	Sprayed sites
	$n = 3$ sites	$n = 3$ sites
BOD [mg/L]	8.1(2.3)	8.1(3.0)
Cl [mg/L]	22.4(8.7)	30.1(14.1)
F [mg/L]	0.25(0.25)	0.3 (0.55)
SO4 [mg/L]	199.7(56.6) A	441.0(588.6) B
Bromide [mg/L]	0.06(0.02)	0.1(0.05)
NO3 [mg/L]	0.008(0.006) A	0.01(0.01) B
NO2 [mg/L]	0.005(0)	0.005(0)
PO4 [mg/L]	0.05(0)	0.05(0)
pH [pH units]	8.1(0.12)	8.4(0.19)
Conductivity [μS/cm]	922.2(132.5) A	1237.6(1114.2) B
Turbidity [NTU[b]]	2.32(1.75)	9.4(9.8)
NH3 [mg/L]	0.025(0.028)	0.02(0.026)
Nitrogen total [mg/L]	1.0(0.65) A	1.6(1.2) B
Phos. o-PO4 diss. [mg/L]	0.007(0.011) A	0.06(0.09) B
Total dissolved phosphorus [mg/L]	0.03(0.02) A	0.086(0.1) B
Total phosphorus [mg/L]	0.06(0.05)	0.1(0.1)

[a] $n = 1$ sample per site;
[b] NTU = Nephelometric turbidity units.
[c] $n = 3$ samples per site. TR = trace concentration $\leq$0.02 mg/L. Minimum detection limits are pH = 0.01 pH; BOD (biological oxygen demand) = 5 mg/L; Cl = 0.1 mg/L; F = 0.01 mg/L; SO4 = 0.5 mg/L; Br = 0.05 mg/L; No3 = 0.002 mg/L NO2 = 0.005 mg/L; PO4 = 0.05 mg/L; NH3 = 0.005 mg/L; total nitrogen = 0.04 mg/L; Phos. O-PO4 diss. = 0.001 mg/L; total dissolved phosphorus =0.002 mg/L. Total phosphorus = 0.002 mg/L; Conductivity = 2 uS/cm; turbidity = 0.05 NTU.

pH = 6.5–9.0 pH; F = 0.12 mg/L; NO3 = 13 mg/L; NO2 = 0.06 mg/L; NH3 = 1.37–2.2 mg/L are Canadian Water Quality Guidelines (CWQG). For the protection of aquatic life. CWQG do not exist for other parameters listed here. Statistical analysis conducted on 2006 only: A, B, C = Different letters indicate significant differences between treatments ($p \leq 0.05$) within years. Where letters are not shown, there were no significant differences between treatments.

Table 2. Pesticide concentrations (ng/L) in water samples from nonagricultural reference ponds and conventionally sprayed fruit orchards in the south Okanagan valley, British Columbia, Canada (2005, 2006)

2005

			Reference Sites						Sprayed orchard					
			1	1	2	2	3	3	1	1	2	2	3	3
Compound	Compound		20-May-05	3-Jun-05	20-May-05	3-Jun-05	20-May-05	3-Jun-05	20-May-05	3-Jun-05	20-May-05	3-Jun-05	20-May-05	3-Jun-05
alpha-Endosulfan	10.1	5.62	2.19	1.5	1.2	0.3	4.57	18	17.2	16	17.6	17.7		
Atrazine	TR	TR	TR	TR	TR	TR	TR	TR	13.4	25.3	14.6	14.6		
Azinphosmethyl	TR	TR	TR	TR	TR	TR	TR	TR	TR	121	46	332		
beta-endosulfan	4.62	7.21	1.46	TR	TR	TR	TR	13.1	3.59	4.93	16.9	5.71		
Chlorpyriphos	TR	TR	TR	TR	TR	TR	TR	TR	TR	TR	2.17	TR		
Cypermethrin	TR	TR	TR	TR	TR	TR	TR	TR	9.71	4.07	TR	TR		
Linuron	78.1	90.7	70.3	117	91.5	75.1	80.9	75.1	55.7	84	90	116		
Desethylatrazine	TR	TR	TR	TR	TR	TR	TR	TR	17.8	19	10	9.43		
Diazinon	68.1	36.5	51.3	56.3	62.5	49.1	198	84.8	710	557	466	216		
Diazinon-oxon	TR	TR	TR	TR	TR	TR	TR	TR	9.54	8.57	2.58	2.56		
Endosulfan-sulfate	16.6	16.9	18.1	16.5	10.5	8.23	7.85	12.2	57.2	55.9	23.6	18.6		
Permethrin	TR	TR	TR	TR	TR	TR	TR	TR	12.6	9.95	TR	TR		
Simazine	2.13	TR	TR	TR	TR	3.48	TR	3.68	12.3	13.1	3.5	3.42		
2,4-D	4.45	8.96	5.65	4.66	7.16	7.27	3.39	3.38	8.16	9.9	4	4.53		
MCPA	3.22	4.3	2.32	2.48	2.27	2.47	1.38	1.49	1.28	1.34	1.71	1.64		
Total sum pesticide exposure[a]	187.3	170.2	151.3	198.4	175.1	146.0	296.1	211.8	928.5	930.1	698.7	742.2		

2006

			Reference Sites						Sprayed orchard					
			1	1	2	2	3	3	1	1	2	2	3	3
Compound	Date of sampling:		3-May-06	14-May-06	3-May-06	14-May-06	3-May-06	14-May-06	3-May-06	14-May-06	3-May-06	14-May-06	3-May-06	14-May-06
alpha-Endosulfan	9.77	17.2	5.7	9.05	11.2	14.5	1.18	12.7	4.67	2.1	10.2	9.37		
Atrazine	TR	TR	TR	TR	TR	TR	TR	TR	14.5	16.3	13.3	13.4		
Azinphosmethyl	TR	12.9	TR	6.69	TR	6.82	103	9.94	5.08	50.3	20.8	13.8		
beta-endosulfan	5.26	6.89	TR	4.24	3.6	5.59	TR	5.23	TR	TR	3.6	2.88		
Chlorpyriphos	TR	TR	TR	TR	1.03	TR	TR	TR	1.82	1.34	2.36	1.7		
Desethylatrazine	TR	0.277	TR	TR	TR	0.731	TR	TR	29.3	32.1	12	14.9		
Diazinon	28.2	60.6	47.6	TR	76.4	TR	83.4	1410	46	175	32	43.3		
Diazinon-oxon	TR	TR	TR	37.5	TR	49.7	TR	TR	1.3	TR	TR	TR		
Endosulfan-sulfate	6.26	14.8	8.61	TR	6.32	TR	7.54	24.8	18.4	19.3	11.6	10.8		
Hexazinone	TR	TR	TR	5.29	TR	7.03	TR	TR	TR	TR	TR	TR		
Methamidophos	TR	7.99	TR	TR	TR	TR	26.3	5.03	TR	4.04	TR	8.92		
Pendimethalin	TR	TR	TR	TR	TR	TR	TR	TR	TR	TR	3.1	TR		
Simazine	TR	2.1	TR	TR	TR	TR	1.07	1.34	14	11.8	4.05	2.95		
2,4-D	6.57	7.86	7.95	8.7	13.7	17.3	18.3	41.8	7.52	14.3	4.11	7.96		
Dicamba	TR	TR	TR	TR	TR	TR	TR	1.37	TR	TR	TR	TR		
MCPA	2.58	1.84	2.32	1.71	5	3.53	4.28	6.98	1.78	2.23	1.8	2.01		
MCPP	2.06	0.596	2	0.46	1.19	1.32	1.64	TR	1.14	1.07	TR	1.21		
Total sum pesticide exposure[a]	60.7	133.1	74.2	73.6	118.4	106.5	246.7	1519.2	145.5	329.9	118.9	133.2		

TR = trace concentrations at or below 1.1 ng/L in sample

[a] Concentrations of all detectable pesticides were summed. 2-methyl-4-chlorophenoxyacetic acid (MCPA); 1-(3-Chlorophenyl) piperazine (MCPP).

Table 3. Product-moment R-values for hatching success versus pesticide concentrations in the south Okanagan valley, British Columbia, Canada (2004–2006) ($p \leq 0.04$- 0.001)

	Great basin spadefoot			Pacific tree frog		
	(Spea intermontana)			*(Pseudacris regilla)*		
Compound	2004	2005	2006	2005	2005	2006
				Trial 1	Trial 2	
Ammonia				−0.69	−0.50	
Chloride				0.54		−0.42
Conductivity					−0.60	−0.38
Turbidity					−0.49	
Total Nitrate		0.32	−0.53		−0.56	
ortho-phosphate		0.38	−0.64			
Total dissolved phosphorus		0.55	−0.58			
Total phosphorus			−0.51			
Sulfate						
pH	−0.56					
alpha-endosulfan	NA	−0.87				
beta-endosulfan	NA	−0.37				
Endosulfan-sulfate	NA	−0.55	−0.75			
Atrazine	NA	−0.88	−0.83			
Desethylatrazine	NA	−0.82	−0.78			
Simazine	NA	−0.59	−0.72			
Permethrin	NA	−0.49				
Cypermethrin	NA	−0.47	−0.81			
Azinphosmethyl	NA	−0.66				
Chlorpyrifos	NA	−0.54	−0.79			
Diazinon	NA	−0.66				
diazinon-oxon	NA	−0.67				

NA, pesticide analysis not conducted in 2004.

due to high variation in hatching success among replicates within some study sites. Similarly, hatching success for Western toad and Columbia spotted frog were lowest in ponds in the sprayed fruit orchards, although we were unable to quantify the statistical significance of those trends. Also, moderately low rates of hatching success in the organic fruit orchards, in some cases, were as low as those occurring in sprayed orchards, but we did not measure pesticides at these sites. Regression analysis supported the prediction that hatching success of Great Basin spadefoot eggs would decrease with increasing pesticide exposure, in particular, associations with atrazine, endosulfan, and chlorpyrifos concentrations were significant. Yet there were also water chemistry factors in ponds that were associated with low hatching success in spadefoots, although the association with these was not as strong as with pesticide exposure. For Pacific treefrog, the only significant associations with hatching success were with water chemistry parameters as predictors of egg fate. Furthermore, at one of the sprayed sites (no. 1) hatching success of all species, except Western toad, was as high as in the reference or organic sites and there were some significant differences in hatching success among sites within treatments. This indicated that the variation in hatching was not just species-specific but was also due to heterogeneity in environmental conditions among ponds that may or may not have acted in association with pesticide exposure.

Interspecies variation in sensitivity of amphibians to pesticide exposures is well known [4,22]. In the present study, Great Basin spadefoots appeared more sensitive than Pacific treefrogs to environmental conditions, including exposures to pesticides. Spadefoots also showed more statistically consistent differences between sprayed and reference sites. However, the occurrence of low to no hatching success in sprayed sites in spadefoots, tree frogs, Western toad, and Columbia spotted frog in most years of the study was similar. While pesticide concentrations were highly variable among sites and between exposure dates within sites in each year and between years, and eggs for each species were not exposed at exactly the same time, it is notable that 100% egg mortality occurred only within the sprayed sites.

Amphibian eggs in all ponds in the South Okanagan Valley are exposed to a mixture of pesticides similar to conditions in other areas of North America such as the Central Valley of California and vegetable and fruit production areas in southern Ontario, Canada. In 1991 to 1993, in an intensive vegetable production area of Ontario, up to 101 ng/L diazinon, 6,470 ng/L atrazine, and 210 ng/L azinphosmethyl were detected in surface waters of the Holland River [23]. The concentrations in Ontario were comparable to the South Okanagan Valley for diazinon levels detected in reference sites and azinphosmethyl levels in sprayed sites but far exceeded concentrations of atrazine found in any of the Okanagan study sites. The most comparable data to ponds in orchards in the South Okanagan Valley are ponds sampled in apple orchards in southern Ontario, Canada, in 1993 to 1994 [24,25]. In Ontario apple orchards, azinphosmethyl concentrations were 60 to 1,000 ng/L, diazinon occurred at 30 to 780 ng/L, and endosulfan was 51 to 530 ng/L [24,25]. However, herbicide concentrations in the South Okanagan Valley were again much lower than the atrazine concentrations of 70 to 15,000 ng/L found in orchard ponds in southern Ontario [24,25]. Concentrations of 1 μg/L diazinon in orchards from Canada are still an order of magnitude lower than peak concentrations of 0.1 to 1 mg/L diazinon in runoff from orchards in the Central Valley of California [26]. The findings of a relatively high number of pesticides in the South Okanagan ponds are certainly not unprecedented and further confirms that amphibian eggs are exposed to a mixture of chemicals in most agricultural systems.

Although diazinon was measured at the highest concentrations in water samples from sprayed ponds, several other

pesticides were detectable at low concentrations and were also significantly correlated with reduced hatching success in the South Okanagan. Pesticides such as endosulfan, chlorpyrifos, and atrazine and desethylatrazine and synthetic pyrethroids correlated with decreased hatching success. In the laboratory, only the most sensitive amphibian species and life stages experience acute toxicity at the part per billion concentrations similar to those that were found in samples from Okanagan Valley ponds. Acutely toxic concentrations of endosulfan (LC96 h) to larval amphibians range from 1,800 ng/L for *Rana tigrina* [27] to 509 μg/L for *Rana hexadactyla* [28]. Diazinon was acutely toxic (LC48 h) at 14 mg/L in *Bufo japonicus* [29]. At 1.3 μg/L for 96 h, endosulfan induced 17% tadpole mortality in Australian treefrog (*Litoria freycineti*) [30], yet following 96-h dosing with 0.03 to 0.4 mg/L endosulfan, *Rana sylvatica* embryos hatched successfully [31]. For chlorpyrifos, 1 μg/L in American toad (*Bufo americanus*) to 3 mg/L in northern leopard frog (*Rana pipiens*) is the 96-h LC50 range for toxicity in larval amphibians [32]. Azinphosmethyl acute toxicity ranges from 109 μg/L to 4.14 mg/L in larvae among the tested native amphibians of North America [33]. At 1.0 μg/L, α-cypermethrin reduced hatching success by 18% in *Rana arvalis* eggs exposed for 48 h, whereas 0.1 μg/L had no effect on hatching success but had significant effects on deformity rates and survivorship to metamorphosis [34]. The LC50 concentrations of atrazine that are acutely toxic to amphibians range from 0.4 to 127 mg/L in amphibians exposed from fertilization to 4 d posthatching [22] but statistically lower survivorship has been reported at concentrations of 3 μg/L for 30-d exposures in larval amphibians of four species native to North America [35].

While concentrations causing acutely toxic responses in amphibian larvae are an order of magnitude above concentrations of individual pesticides detected in South Okanagan ponds, total pesticide exposure for eggs was as high as 1.5 μg/L in sprayed sites and may have acted together as a mixture. Among five amphibian species native to North America, combined pesticide exposures caused lower survival and growth of larvae than any of diazinon, carbaryl, malathion, and glyphosate alone [36]. While those effects were never worse than diazinon or malathion alone at 2 mg/L [36], the combined impact of the pesticides is similar to that predicted by the total concentrations of pesticides [36].

In a study examining interactions of atrazine and chlorpyrifos in four aquatic vertebrates, organisms were exposed to binary mixtures of these chemicals in bioassays [37]. Atrazine alone did not affect organisms at concentrations up to 5,000 μg/L; however, the presence of atrazine at 1,000 μg/L did result in a significant increase in the acute toxicity of chlorpyrifos in *Xenopus laevis* tadpoles. Mixed results were found with *Pimephales promelas*, fathead minnow, with some bioassys showing greater than additive toxicity while others showed an additive response. No effect of atrazine on chlorpyrifos toxicity was observed for bluegill (*Lepomis macrochirus)* or *Rana clamitans* tadpoles [37].

In the South Okanagan Valley, pesticides were also measurable in water from reference sites that were at least 500 m from a sprayed orchard, indicating that most ponds in the lowland valley of the South Okanagan are exposed to pesticides. The same situation was reported in southern Ontario, where reference sites were located 500 m to more than 1 km from agricultural areas and atrazine was detected in reference ponds at concentrations as high as 200 ng/L [24,25]. Concentrations of diazinon (3.1–3.4 ng/L), and endosulfan-sulfate (2.2–2.9 ng/L) measured in surface waters from the Tablelands of Sequoia National Park, California, which is exposed directly to prevailing winds from agricultural regions in California [38], are relatively lower than concentrations of diazinon of 76 ng/L and of endosulfan-sulfate at 18 ng/L in Okanagan lowland reference ponds in the present study.

When comparisons are made among hatching success rates in reference areas in agricultural watersheds, and reference sites in nonagricultural watersheds, hatching success is substantially lower in the reference sites in agricultural areas. While the sprayed sites showed significantly lower hatching success in the South Okanagan, the mean hatching success rates in reference sites were often about 80% (20% egg mortality). This is similar to egg mortality in agricultural landscapes such as the Holland River, Ontario, and orchard areas of southern Ontario, where rates of egg mortality were 15 to 18% in anurans and toads in reference areas only 500 m from sprayed sites [23–25]. Whereas in reference areas in eastern Ontario in sites located several kilometers from agriculture, the mean egg mortality rate was only 3% in northern leopard frog (*Rana pipiens*) when the same in situ caging system was used as in the Okanagan [39].

Water chemistry and nutrient concentrations in ponds in the Okanagan were also similar to conditions reported for agricultural ponds in North America [25]. The only exceptions were sulfate concentrations that were highly elevated in ponds in organic orchards and some sprayed sites in the Okanagan. Some water chemistry factors showed trends with hatching success, suggesting that concentrations not currently considered toxic to aquatic life may influence amphibian development possibly due to additive or synergistic effects with pesticides and/or other environmental factors. For example, in 2005 mean ammonia concentrations in sprayed ponds were at the lower acceptable limit of the Canadian Water Quality Guidelines (CWQG) for the protection of aquatic life [21] and were also correlated with reduced hatching success of Pacific treefrog eggs. However, it may not have been the mean levels but the occasional elevated ammonia concentration that was toxic to the amphibians. In contrast, fluoride also exceeded the CWQGs in most sites and years in the South Okanagan Valley; however, it did not correlate with egg mortality in any year or site. This is not surprising given that CWQGs are not set based on toxicity to amphibians; rather, they are determined based on toxicity to fish and invertebrates [21]. McKibbin et al. [40] also found that, for Oregon spotted frog (*Rana pretiosa*), increasing concentrations of chloride correlated with hatching success, consistent with our 2005 results showing a positive association between chloride and Pacific treefrog hatching success.

Increasing nitrates, sulfate, and phosphorus also correlated with decreasing hatching success in the South Okanagan Valley. The toxicity of ammonia and associated nitrates, sulfates, and highly acidic pH to amphibians is known [41]; however, concentrations of nitrates and sulfates in the South Okanagan Valley sites were lower than concentrations that have been reported as toxic to amphibians in dose-response studies [4]. The use of sulfate as a fungicide in orchards, particularly organic orchards ([42]; http://attra.ncat.org/attra-pub/PDF/apple.pdf) probably contributed to very high concentrations detected in ponds in orchard sites.

All ponds remaining in the lowland elevation areas of South Okanagan Valley are within an agricultural production zone. In the case of Great Basin spadefoot, the South Okanagan Valley is the core of the range of this species in Canada [11] but it is also the northern periphery of the geographic range of this species. With many species at risk showing population trends in which their geographic range has collapsed to the periphery rather than the core of their range [43] the global

relevance of the environmental quality of northern wetlands within and outside agricultural sites becomes even more urgent.

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