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THE FUNCTIONAL INTEGRITY OF NORTHERN LEOPARD FROG (*RANA PIPIENS*) AND GREEN FROG (*RANA CLAMITANS*) POPULATIONS IN ORCHARD WETLANDS. II. EFFECTS OF PESTICIDES AND EUTROPHIC CONDITIONS ON EARLY LIFE STAGE DEVELOPMENT

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Abstract—Premetamorphic northern leopard frogs (*Rana pipiens*) and green frogs (*Rana clamitans*) were evaluated at seven wetland sites, four of which were within apple orchards, to determine if environmental conditions associated with orchard management in southern Ontario, Canada, affected frog early development. Synchronous with breeding events (May–July), embryos and tadpoles were exposed, in situ and in the laboratory, to study site pond water for 2 to 3 weeks. Six pesticides commonly applied in apple orchards (Guthion® 50WP, Imidan® 50WP, Thiodan® 50WP, Dithane® DG, Nova® 40W, and Basudin® 500EC) and technical grade diazinon were also evaluated for direct toxicity to green frogs using continuous and discontinuous toxicity tests. Embryos and larvae exhibited poor hatching success and survival at some orchard sites during in situ assays, but no specific water source produced consistently poor developmental success. Reduced tadpole growth occurred at several study sites, showing no clear distinction between reference and orchard sites. Despite the collection of substantial environmental data, only surface water temperature appeared to be correlated with growth rates. Basudin 500EC, technical grade diazinon, and Dithane DG caused mortality, deformities, or growth inhibition during early development at environmentally relevant concentrations (<0.01 mg/L), whereas Imidan 50WP, Guthion 50WP, and Nova 40W produced survival or growth effects at much higher levels (5–10 mg/L). Dithane DG (mancozeb) was acutely toxic during prolonged exposures, and sublethal growth and deformity responses occurred at nominal concentrations greater than 0.01 mg/L. Diazinon was the most toxic to green frogs, with median lethal concentrations of 2.8 to 5 μg/L and median effective concentrations of 6 to 14 μg/L for formulation and technical grades, respectively. The combined results of in situ and laboratory tests of green frog and leopard frog developmental success suggested that embryo–larval development of these two species could be accomplished at all study sites, independent of the association with apple orchards.

Keywords—Amphibian Pesticides Water quality Embryo–larval development Toxicity test

INTRODUCTION

Researchers and regulators have begun to recognize that amphibian responses to pollutant exposure are often not adequately predicted by tests on other aquatic groups such as fish or invertebrates [1–3]. The biphasic life cycles characteristic of amphibians [4–6] add complexity and diversity to their response patterns. The introduction of chemicals or other anthropogenic modifications to an aquatic habitat can produce profoundly different responses in consecutive life stages of a single amphibian species. Aquatic routes of pollutant exposure change between adults, eggs, and larvae with changes in foraging behavior and respiration. Anuran (frog and toad) larvae are omnivorous filter- and macrophagous-feeders on bacteria, plankton, and periphyton [7], and they respire through external gills and their skin [8]. Metamorphosed juveniles and adults are carnivorous, and they respire through lungs and the skin. Larval stages are restricted to the aquatic environment, whereas metamorphosed individuals may spend substantial and species-specific amounts of time away from water. The mechanisms that produce a physiological response to environmental stimuli during early development are also different from those of adults [9]. In most documented comparisons, amphibian eggs and tadpoles appear to be more sensitive to toxicants than are adults [3,10,11].

Bishop [12] and Boyer and Grue [1] have stated that there is insufficient research assessing the effects on amphibian species of inhabiting wetlands that are influenced by a combination of agricultural inputs and eutrophic waters. Few field studies have attempted to evaluate the response patterns of frog tadpoles to chemical contamination in agriculturally influenced freshwater environments by enclosing individuals in cages [13–15]. Most investigators have evaluated the effects of agricultural pesticides on frogs by examining developmental responses produced during laboratory toxicity tests [16–20]. Generally, these studies suggest that premetamorphic amphibians can show a wide range of species-specific sensitivities to various pesticide influxes, and, when exposed to field-relevant mixtures, can experience significantly reduced developmental success.

In Ontario, Canada, fruit production is a large industry with a strong historical foundation. Apple orchards occupy 12,829 ha of land [21], and almost all of that acreage is

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sprayed with insecticides and fungicides. At present, orchards are treated with the greatest quantity and applications of pesticides of all fruit and vegetable crops in Ontario [22]. The waterways maintained in southern Ontario orchards are used by several species of amphibians as breeding habitat in the spring and summer [23,24]. Northern leopard frogs (*Rana pipiens*) breed and lay eggs in late April to early May that develop and metamorphose in 60 to 80 days, whereas green frogs (*Rana clamitans*) breed and lay eggs in mid-May to mid-July, and their tadpoles require two summer seasons to transform [23]. The apple-growing season in southern Ontario usually extends from mid-April to early September. The early life stages of both frog species are thus present in orchard wetlands during the pest management season and are therefore at risk of exposure to pesticide influxes through spray drift, spills, or runoff.

To investigate possible effects on amphibians of living in orchard landscapes, this research tested the hypothesis that the embryo–larval stages of northern leopard frogs and green frogs growing in wetlands in apple orchards were developmentally compromised compared to equivalent-age groups growing in neighboring wetlands in conservation areas. In situ and laboratory pond water assays were completed concurrently with insecticide and fungicide toxicity tests. The survival, growth, and hatching success rates of northern leopard frogs and green frogs were examined over the first 2 to 3 weeks of embryo–larval development in late spring to midsummer in 1993 and 1994. Differences in development in cages set in ponds at study sites were analyzed for significance, and then compared to development in pond water transported to the laboratory where water temperature, an important limiting tadpole growth variable, could be controlled. In addition, four insecticides and two fungicides were selected from those commonly used during fruit production in orchards [25] for toxicity testing on equivalent early life stages. In 1994, in situ and laboratory pond water assays were conducted as continuous 13-d static-renewal exposures. Because it is unlikely that such an extended continuous exposure to a pesticide occurs in orchard ponds [26–28], the effects of a 16-d discontinuous exposure scenario were also evaluated in 1995.

MATERIALS AND METHODS

Description of study sites

A detailed description of study sites is included in the companion paper [29]. Briefly, four wetland study sites were selected within apple orchards in rural areas between Hamilton, Brantford, and Guelph, Ontario, Canada. Two reference wetland sites were selected in neighboring conservation areas in 1993, and an additional reference site was included in 1994. In the following text, orchard study sites were labeled OCD1, OCD2, OCD3, and OCD4, and reference sites were labeled REF1, REF2, and REF3 (see companion paper [29] for map of locations). Three of the four orchard sites were dug-out ponds with associated natural wetland; the fourth (OCD2) was a small canal with minimal water movement. Reference sites were similar in structure to three of the orchard wetland sites in that they were permanent ponds with associated marsh. The two original reference sites (REF1, REF2) were within a few kilometers of three of the orchards. The additional reference site was further removed (~27 km) from all other sites, but assumed to be subject to roughly the same local weather and climate profiles over the period of study.

In situ and laboratory assays of embryo–larval development in pond water from study sites

The survival, growth, and hatching success rates of northern leopard frogs and green frogs were examined in 1993 and 1994 over the first 2 to 3 weeks of embryo–larval development. In situ assays were conducted once for each species in 1993, and both in situ and laboratory pond water assays were conducted once for leopard frogs and twice for green frogs in 1994. Leopard frog tests were conducted July 16 to 31, 1993, and May 20 to June 6, 1994. Green frog tests were conducted July 16 to 31, 1993, and June 8 to 27 and July 7 to 25, 1994.

Fertilized eggs for all assays were produced in the laboratory to ensure control of the genetic source. One or two mature individuals of each sex for each species were used during each fertilization event. Leopard frog adults were obtained from R. Elinson (Hazen Frog Farms, Alburg, VT, USA) in 1993 and from reference populations (REF4) in 1994 [29]. Green frog adults were collected from reference populations (REF1, REF2) in both years. After egg extraction, females were released back into the pond of origin. In 1993, collection of viable fertilized leopard frog eggs was delayed, such that embryo–larval assays were conducted simultaneously with those for green frogs, a full 2 months past the natural breeding window for local populations of leopard frogs. However, the leopard frog adults had been maintained in simulated hibernation since the previous October, and fertilizations produced healthy clutches of eggs.

To induce ovulation, the adult females of each species were injected with 0.1 μg luteinizing hormone-releasing hormone (L4513, Sigma, St. Louis, MO, USA) or with crude whole frog or toad pituitary extracts. Injections were completed 12 to 24 h before scheduled fertilization events. The males were sacrificed (via double-pithing) to obtain the testes, which were then crushed in aged tap water (i.e., water left at room temperature for at least 24 h) to release the sperm. Eggs were squeezed out of the females, into the concentrated sperm solution. After 15 min, the concentrated solution was flooded with aged tap water, and egg masses were separated into loose clumps to ensure adequate oxygen availability to all eggs. Unfertilized eggs were removed after approximately 2 h, once cleavage was visible. If the fertilization rate was greater than 97%, the eggs were not sorted; the possibility of damaging eggs during sorting was thought to be a greater risk than the random chance of introducing an unfertilized egg to the test. Eggs introduced to any one replicate in a given testing event were either of a single genetic source or an equal mix of two genetic sources.

Field cages for the in situ assays are described in detail in Harris and Bogart [30]. Briefly, an outer cylindrical cage (20-cm depth, 15-cm diameter), composed of rigid 500-μm Nitex nylon (Tetko, New York, NY, USA), housed a shallow plastic basket (5-cm depth, 10-cm diameter) with a nylon mesh bottom. Cages were anchored and maintained with the top slightly above the water line by slipping 0.6-cm-diameter wooden dowels through plastic rings on the cage sides and then tapping the dowels into the mud bottom of each pond. The eggs rested in the inner basket in the oxygen-rich surface water zone until they hatched. Upon hatching, the tadpoles

were gently tipped into the much larger outer cage, and a feeding routine commenced, as outlined below.

In 1994, corresponding laboratory assays were conducted in 250-ml beakers maintained in a temperature-regulated water bath under a 12-h light:12-h dark photoperiod. A fiberglass artificial stream (Frigid Units, Toledo, OH, USA) acted as a water bath; circulation and temperature control were achieved using a Jewel Aquachiller unit (Jewel Industries, Chicago, IL, USA). Parafilm®-covered beakers were held in independent compartments in a perforated polyvinyl chloride (PVC) stand, which allowed the beakers to be one-third to one-half surrounded by circulating water. Water bath temperatures were 19.5 ± 1.5°C for the leopard frog assay, and 19.4 ± 0.6°C and 18.6 ± 0.6°C for the two green frog assays (in order of occurrence). Surface grab samples of pond water from orchard and reference sites were transported on ice to the laboratory for the assay. Pond water solutions were two-thirds renewed every second day; volumes were maintained at 100 ml until hatching, then were increased to 150 ml.

In 1993, cages contained 20 individuals each (~6/L), and two cages were placed at each study site during each test event. In 1994, beakers and cages contained 10 individuals each (~3/L in cage); two or three beaker replicates and four cage replicates were included in each test. The initial number of replicates was equal across treatments in any given test event. Tests were begun once the eggs reached Gosner [31] developmental stage 8/9 (mid–late cleavage, ~9 h postfertilization). Once hatching events were completed, 0.5 g of boiled lettuce was introduced to each cage or beaker, and replenished every second day; rations were increased to 1 g/replicate after approximately 1 week. Anuran larvae hatch with large yolk reserves, and typically do not feed for several days [7]. Feeding was not initiated here until swimming activity was pronounced. Initiation and changes in the feeding routine were simultaneous across treatments. Dead eggs and tadpoles were removed during inspections held every second day for the duration of the test. Tests continued for 2 (1993 both species, 1994 leopard frog) to 3 (1994 green frog) weeks. Upon termination of the test, survival, hatching success, and tadpole growth rates (estimated by snout–tail-tip length) were determined.

Pond water characterization and correlations with in situ tadpole growth

Several water quality variables were monitored throughout the spring and summer in 1994. Surface water maximum and minimum temperatures were recorded daily throughout the spring and summer with max–min thermometers. The pH was recorded each time the ponds were visited (about every second day from mid-April to mid-August) with a portable Fisher pH meter (Fisher Scientific, Toronto, ON, Canada). Dissolved oxygen was also recorded periodically with a field YSI meter. Two sets of water samples were taken from each pond during each caging event for measurement of conductivity, color, turbidity, total dissolved solids, dissolved organic carbon, dissolved inorganic carbon, nitrites, nitrates, ammonia, total Kjeldahl nitrogen, total phosphorus, and pesticide residues. These grab samples were taken as part of a routine analytical program [29], and were not specifically timed with respect to caging events. Sets of samples were collected 4 d before the leopard frog assay and on day 10 of the same event. Further sets of water were collected on days 6 and 19 of the first green frog assay, and on days 5 and 19 of the second green frog assay. Water chemistry was evaluated using Environment Canada [32] methods. Analytical methods for pesticide residues are described in Harris et al. [29].

When significant differences in growth were detected among caging sites, independent Pearson product–moment correlation matrices were constructed with SYSTAT [33] using average snout–tail-tip length values, and the following 12 environmental variables, measured during the caging event in question: color, turbidity, total dissolved solids, conductivity, pH, dissolved organic carbon, dissolved inorganic carbon, total phosphates, total Kjeldahl nitrogen, ammonia, nitrates + nitrites, and total degree days. Total degree days were thought to provide a more accurate reflection than maximum temperature of the cumulative effect of warming trends in surface waters on tadpole development. An estimate of total degree days was calculated for each site during each assay period by averaging maximum and minimum surface water temperature values to obtain a median temperature for every 1 or 2 d of the test. The median was multiplied by the number of days it represented, then these values were summed over the test period. All Pearson correlation probabilities were evaluated with respect to a Bonferroni-adjusted probability (0.05/12 tests = 0.0042).

Pesticide toxicity tests

Green frog eggs and larvae were used for pesticide toxicity tests. Pesticide manufacturers kindly donated samples of Imidan® 50WP (Zeneca Agro, Stoney Creek, ON, Canada), Guthion® 50WP (Bayer/Mobay, Etobicoke, ON, Canada), Thiodan® 50WP (Agrevo Hoescht Noram Canada, Cambridge, ON, Canada), and Dithane® DG and Nova® 40W (Rohm and Haas Canada, West Hill, ON, Canada). Guthion 50WP (50% azinphos-methyl) and Imidan 50WP (50% phosmet) are organophosphorus insecticides, whereas Thiodan 50WP (47% endosulfan) is an organochlorine insecticide. Dithane DG (76–80% mancozeb) is an ethylenebisdithiocarbamate (EBDC) fungicide, whereas Nova 40W (38–42% myclobutanil) is a triazole fungicide. Pesticide solutions were made with pond water from site REF1. Although this pond water was not directly analyzed for initial residues, surface water grab samples were taken throughout the summer for that purpose, as outlined in the previous section. Test conditions were as stated for the laboratory pond water assays. Water bath temperature was 18.6 ± 0.6°C. Toxicity tests were conducted concurrently with in situ and lab pond water assays, July 7 to 19, 1994. Gosner [31] stage 8 embryos through to stage 25 tadpoles were continuously exposed to a treatment for 2 weeks (13 d), with two-thirds renewal of treatment solutions every second day.

In 1995, a second set of toxicity tests was completed that used a discontinuous exposure scenario and four pesticide formulations. A diazinon-based compound, Basudin® 500EC (donated by Ciba-Geigy Canada, Mississauga, ON, Canada), was tested as well as the previously tested compounds, Dithane DG, Guthion 50WP, and Thiodan 50WP. In addition, technical grade diazinon (also donated by Ciba-Geigy Canada) was tested, and responses were compared to those elicited by the Basudin formulation. The discontinuous procedure consisted of a 4-d static renewal exposure initiated at the same developmental stage as in the other assays. After 4 d, treatment solutions were replaced with REF1 pond water and embryos hatched and began feeding in uncontaminated con-

ditions. After 7.5 d in reference water (with renewal every second day), treatment solutions were reintroduced to the same replicate beakers they had initially occupied for a second 4-d exposure. Water bath temperature was 18.1 ± 1.1°C. Frequency and characterization of deformities were calculated at hatching (e.g., on the eighth day of the test). Survival, hatching success, and growth were estimated upon termination of the test event.

To be consistent across formulation types (e.g., wettable powder versus emulsifiable concentrate), test concentrations were expressed as concentrations of active ingredient (a.i.). In 1994, test concentrations were as follows: Guthion 50WP—0.00005, 0.005, 0.05, 0.5, 2.5, and 5.0 mg a.i./L; Imidan 50WP—0.0005, 0.005, 0.05, 0.5, 5.0, and 12.5 mg a.i./L; Thiodan 50WP—0.00047, 0.0047, 0.047, 0.47, 4.7, and 11.75 mg a.i./L; Nova 40W—0.0004, 0.004, 0.04, 0.4, 4.0, and 10.0 mg a.i./L; and Dithane DG—0.00078, 0.0078, 0.078, 0.78, 7.8, and 19.5 mg a.i./L. In 1995, test concentrations for Thiodan 50WP and Dithane DG were the same as those used in 1994, except the highest concentration (11.75 or 19.5 mg a.i./L) was dropped. Similarly, Guthion 50WP concentrations remained the same, except the lowest concentration was changed from 0.00005 mg a.i./L to 0.0005 mg a.i./L. Basudin 500EC concentrations were 0.001, 0.01, 0.1, 1.0, 10.0, and 25.0 μg a.i./L, and technical grade diazinon test concentrations were 0.5, 5.0, and 50.0 μg/L.

To verify the accuracy of the stocks and dilutions, three sets of samples were analyzed in 1995. Methods for residue analysis are discussed in Harris et al. [29]. One sample set was analyzed immediately after being made; the other two sets were stored at 4°C for 4 and 7 d, to quantify amounts of compound degradation over the course of a toxicity test. Pesticide samples exhibited active ingredient concentrations that were lower than those calculated when solutions were initially mixed (see Appendix). Time-zero residues of Imidan 50WP, Thiodan 50WP, and Nova 40W were within 80% of nominal concentrations. These values were within the realm of either manufacturing variability or residue loss during analytical procedures (the latter, notably during analyses of high-dose Guthion 50WP samples). The concentration of mancozeb in all Dithane DG samples was considerably less than expected, suggesting a rapid rate of compound degradation that is consistent with measured degradation rates [34]. Imidan 50WP and Thiodan 50WP appeared to degrade only slightly over time.

Statistics

The field and laboratory pond water tests were evaluated using nested analyses of variance with replicate beakers or cages nested within sites. Initially, all tests were balanced with respect to numbers of subgroups (replicates) per group (site); however, the in situ tests were all unbalanced at test termination. Some cages were vandalized and these cage replicates were not included in the analysis. Initially, orchard and reference sites were grouped into high (orchard) and low (reference) pesticide exposure classes for analyses of variance; however, no significant differences occurred in tadpole growth between exposure classes during any of the in situ assays. Thus, sites were evaluated independently without the exposure grouping.

All statistical analyses were completed using SAS/STAT® 6 [35]. Tadpole length data were checked for normality using probability plots. Data were determined to adequately approximate normality, and no transformations were necessary. The SAS plot procedure was used to evaluate homogeneity of variance; it revealed adequate homogeneity within testing events. An unbalanced nested analysis of variance, using type I sums-of-squares [36] was employed to examine potential differences in tadpole snout–tail-tip length. Variance components for the growth data were calculated using the SAS VARCOMP procedure using type 1 sums-of-squares.

As in the ambient water tests, the replicate beakers in toxicity tests were nested within treatments. The concentrations used in these toxicity tests were orders of magnitude apart, and best represent range-finding tests for these formulations. An estimate of the lethal concentration for 50% of exposed individuals (LC50) was calculated, where possible, for each compound at 96 h and at the termination of the test. The LC50 values were obtained via a trimmed Spearman–Karber method [37]. The deformity data collected in 1995 were subjected to a similar analysis to calculate an effective concentration for 50% of exposed individuals (EC50).

Table 1. Average hatching success and percent survival of northern leopard frog and green frog embryos during in situ assays conducted in 1993 (two cages/site), and in situ and ambient water assays conducted in 1994 (four cages or two or three beakers/site). The two values presented for green frogs in 1994 relate, in order, to the June 8 to 27 and July 5 to 25 test events[a]

			Leopard frogs		Green frogs	
Year	Test location	Test solution	Hatching success (%)	Survival (%)	Hatching success (%)	Survival (%)
1993	Field	OCD1	100	75	95	20
		OCD2	5	0	50	7
		OCD3	100	85	50	45
		OCD4	85	85	100	97
		REF1	100	90	97	87
		REF2	100	95	95	85
1994	Field	OCD1	90	70	95, 97	95, 82
		OCD2	70	62	95, 87	75, 87
		OCD3	85	70	100, 95	90, 92
		OCD4	87	63	90, 97	42, 97
		REF1	90	65	100, 97	97, 95
		REF2	87	60	100, 100	90, 95
		REF3	70	50	NA	NA
	Laboratory	OCD1	90	80	100, 100	87, 100
		OCD2	85	80	97, 100	90, 85
		OCD3	70	50	92, 100	90, 80
		OCD4	80	65	90, 100	87, 80
		REF1	95	50	100, 100	100, 100
		REF2	80	65	97, 100	100, 90
		REF3	85	75	NA	NA

[a] OCD1–4 = orchard study sites; REF1–3 = reference study sites; NA = not available.

RESULTS

In situ and laboratory assays of embryo–larval development in pond water

Survival in reference pond water was greater than 85%, except during the leopard frog test conducted in 1994, when it averaged as low as 50% at REF1 and REF3 (Table 1). Although hatching success of individuals introduced to that test was moderate to high (averaging 82% across treatments), the fertilization rate of the unsorted 1994 egg stock was low, suggesting that the stock was of inferior quality and probably susceptible to the stresses of handling and transport. Aside

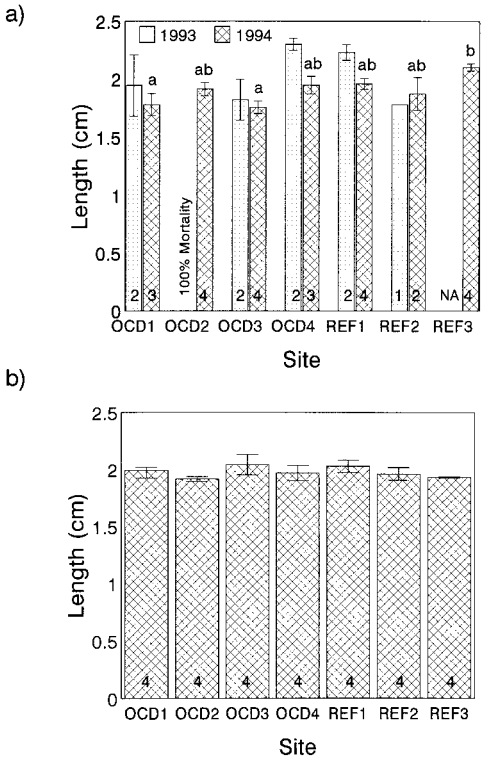

Fig. 1. Leopard frog (*Rana pipiens*) tadpole growth during in situ and laboratory pond water tests. (**a**) Growth produced during two in situ testing events (1993, 16 d; 1994, 18 d). (**b**) Growth produced during the 1994 laboratory pond water test (19 d). Averages represent average growth among cages or beakers within a site. Values at the base of each bar are number of replicates. Sites with at least one similar letter are not significantly different from each other at an α level of 0.05. Error bars represent standard error among replicates. NA = not applicable (site not tested in that year).

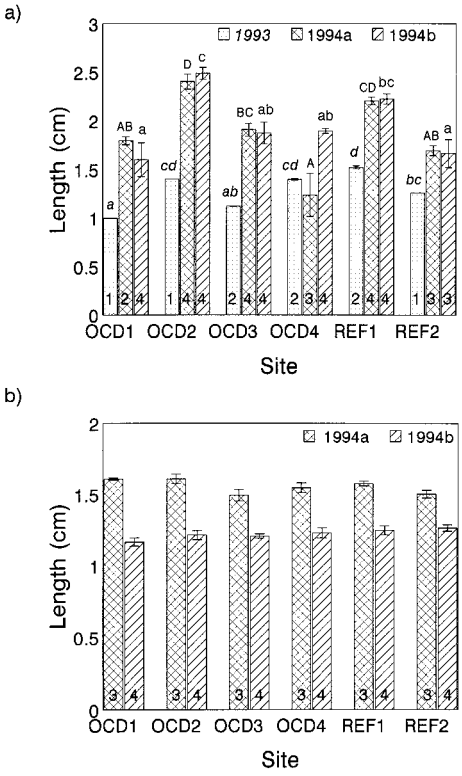

Fig. 2. Green frog (*Rana clamitans*) tadpole growth during in situ and laboratory pond water tests. (**a**) Growth produced during three in situ testing events (1993, 16 d; 1994a, 21 d; 1994b, 21 d). (**b**) Growth produced during the 1994 laboratory pond water test (1994a, 20 d; 1994b, 22 d). Averages represent average growth among cages or beakers within a site. Values at the base of each bar are number of replicates. Sites with at least one similar letter are not significantly different from each other (within a testing event) at an α level of 0.05. Error bars represent standard error among replicates.

from the consistently low survival (63–80%) in the 1994 leopard frog test, survival and hatching success of leopard frogs were low in cages at OCD2 in 1993, but high in all other cases. Survival and hatching success of green frogs were low in cages at OCD2 and OCD3 in 1993. Survival of green frogs was also low in cages at OCD1 in 1993, and in two of four cages at OCD4 between June 8 and 27 in 1994; hatching success remained high (90–95%) in these cages. External deformities were not observed during any of the in situ or laboratory pond water tests.

Tadpole snout–tail-tip length was significantly different among leopard frogs caged in a few orchard and reference site ponds in 1994 (Fig. 1a). Leopard frogs caged in pond water at REF3 were significantly longer than those caged in pond water at OCD1 and OCD3 ($p = 0.01$). Statistically significant differences were not detected when comparing the same sites in 1993. The pond water assay conducted in the laboratory in 1994 did not show growth differences equivalent to those witnessed in situ (Fig. 1b).

The length of green frog tadpoles was significantly different among caging sites upon termination of all three testing events (all p values < 0.0001; Fig. 2a). In 1993, green frogs caged in pond water at REF1 were significantly longer than those caged in pond water at OCD1, OCD3, and REF2. During the first caging event in 1994, green frogs at REF1 were significantly longer than their counterparts at OCD1, OCD4, and REF2. During the second caging event in 1994, only those tadpoles at OCD1 and REF2 were significantly shorter than tadpoles from REF1. Tadpoles caged at OCD2 obtained the greatest lengths in 1994. As occurred during leopard frog assays, no significant growth differences were found in the corresponding laboratory pond water tests (Fig. 2b).

When average length of green frog tadpoles held in situ was directly compared to cohorts held in the same pond water under laboratory conditions, field-caged individuals grew significantly faster ($p < 0.001$) than did laboratory individuals at all study sites except OCD4. In contrast, leopard frog tadpoles developing in cages and in beakers grew at roughly the same rate in 1994. The surface water temperatures in ponds during the 1994 leopard frog assay (median daily temperature range of 13.5–22.5°C) were more closely matched by the laboratory water bath temperature of 19.5 ± 1.5°C than were surface water temperatures in ponds during the subsequent two green frog assays (with water bath temperatures of 19.4 ± 0.6°C and 18.6 ± 0.6°C).

To increase statistical power and to identify the relative importance of microhabitats within a pond on embryo–larval development, the number of replicate cages within a pond was doubled from two to four during 1994 in situ events. The amount of the total variance in in situ tadpole length measurements that could be attributed to variation among replicates was high relative to laboratory results for the 1994 leopard frog assay, but equivalent to laboratory results for both 1994 green frog assays (Table 2). Most of the variance in the three laboratory assays was attributable to within rep-

Table 2. Proportions of variance attributable to study sites, replicates (cages or beakers), and individuals (error) during in situ and laboratory pond water assays

		Proportion of total variance (%)		
Assay type	Year	Study site	Replicate	Error (individual)
Leopard frogs				
Laboratory	1994	0	3.4	96.6
In situ	1993	35.2	48.6***	16.2
In situ	1994	34.6*	35.2***	30.2
Green frogs				
Laboratory	1994a	12.2	15.2***	72.6
Laboratory	1994b	6.5	26.2***	67.3
In situ	1993	60.6***	0	39.4
In situ	1994a	64.1***	15.6***	20.3
In situ	1994b	65.0***	22.1***	12.9

* = $p < 0.05$; *** = $p < 0.001$.

licate, among individuals differences (67.4–96.6%). Variance among replicates was statistically significant in both leopard frog in situ studies, and in all 1994 green frog laboratory and in situ studies (all $p < 0.001$).

Pond water characterization and correlations with in situ tadpole growth

Nutrient and physical analyses of water samples collected in 1994 (Table 3) did not show a clear distinction between water quality at orchard sites versus two of the reference sites (water was not analyzed from REF3). Two of the orchard site ponds were highly nutrient enriched with associated ammonia spikes and dissolved oxygen deficits. The two reference sites and another orchard site showed moderate enrichment, whereas the water quality of the fourth orchard pond could be characterized as noneutrophic.

Analyses of pond water at OCD1 and OCD2 showed large fluctuations in water quality. Surface water temperatures at OCD2 remained several degrees higher than temperatures at other sites throughout the summer in 1994 (Fig. 3a). High ammonia levels (>0.4 mg/L) were also detected in two of eight water samples (Fig. 3c). Color, turbidity, dissolved solids, dissolved organic carbon, nitrogen, and phosphorus values for both orchard sites were relatively elevated, indicating a murkier, more nutrient-enriched aquatic environment. Surface water at OCD1 was also relatively acidic (minimum recorded pH = 6.54), and dissolved oxygen concentrations rarely rose above 5 mg/L (Fig. 3b).

In contrast, pond water at OCD3 and OCD4 was characterized by minimal nutrient enrichment and an absence of ammonia or dissolved oxygen stressors. Water at OCD4 exhibited phosphorus concentrations of approximately 0.01 mg/L (Fig. 3d), which typifies noneutrophic conditions [38]. Total phosphorus concentrations in surface water from OCD3 ranged between 0.01 and 0.03 mg/L, indicating slight enrichment. Water from these two sites also became slightly to highly basic (up to pH 10.4) as the summer progressed.

The reference pond water could be characterized as moderately nutrient-rich. Dissolved carbon, nitrogen, and phosphorus levels generally fell between values for the highly eutrophic and noneutrophic (OCD4) orchard pond waters. Although the elevated ammonia values observed in water from OCD1 and OCD2 were not present, the potential for oxygen stress existed, especially at REF2 where dissolved oxygen levels were consistently less than 2 mg/L (Fig. 3b). The disturbance of anoxic mud at this site may have been responsible for the loss of cage replicates during the leopard frog and green frog in situ assays.

When environmental parameters were divided into three time periods coinciding with the three in situ (caging) assays, no significant correlations between average tadpole length and averages of environmental variables were found during the leopard frog caging event (May 20–June 6, 1994; Table 4), or the first green frog caging event (June 8–27, 1994;

Table 3. Water quality at orchard and reference sites during the summer of 1994. Values are averages ($n = 2$) of measures taken during the three in situ embryo–larval assays[a]

Site	Time	Color (Pt-Co[b])	pH	DOC (mg/L)	DIC (mg/L)	Ammonia (mg/L)	Nitrates/ nitrites (mg/L)	TDS (mg/L)	Conductivity (μS/cm[c])	Turbidity (JTU[d])	TKN (mg/L)	TP (mg/L)
OCD1	May 20–June 6	70	7.34	11.7	20.4	0.026	0.03	185	205	2.52	0.804	0.0859
	June 8–27	95	7.09	15.4	21.3	0.057	0.19	148	197	1.88	1.047	0.0902
	July 7–25	170	7.07	17.9	21.7	0.018	0.13	153	195	7.10	1.150	0.0593
OCD2	May 20–June 6	40	7.77	9.6	47.4	0.268	1.06	543	629	18.25	1.135	0.0764
	June 8–27	110	7.87	8.3	52.3	0.233	0.66	461	668	42.35	1.112	0.0389
	July 7–25	100	7.60	24.1	56.7	0.007	0.01	459	692	40.74	0.948	0.0463
OCD3	May 20–June 6	20	8.59	7.4	23.7	0.012	0.03	130	229	2.05	0.493	0.0131
	June 8–27	25	9.77	7.6	13.2	0.003	0.01	107	162	1.92	0.607	0.0248
	July 7–25	45	8.78	10.1	14.9	0.023	0.01	111	159	1.75	0.783	0.0181
OCD4	May 20–June 6	5	7.80	4.8	34.3	0.005	0.02	245	405	0.78	0.212	0.0051
	June 8–27	7	7.89	4.7	29.2	0.004	0.01	255	367	0.49	0.294	0.0099
	July 7–25	10	7.56	5.0	27.6	0.023	0.01	238	352	1.63	0.327	0.0066
REF 1	May 20–June 6	30	7.43	6.3	14.9	0.022	0.02	172	163	2.67	0.387	0.0288
	June 8–27	20	7.40	5.9	15.7	0.001	0.03	110	166	1.73	0.378	0.0215
	July 7–25	55	7.35	6.9	14.4	0.003	0.01	93	150	4.5	0.396	0.0192
REF2	May 20–June 6	25	7.46	6.1	31.6	0.015	0.02	288	331	2.25	0.326	0.0253
	June 8–27	25	7.39	7.5	32.9	0.002	0.01	230	334	3.37	0.431	0.0628
	July 7–25	45	7.25	6.9	31.6	0.003	0.01	187	305	2.56	0.419	0.0472

[a] DOC = dissolved organic carbon; DIC = dissolved inorganic carbon; TDS = total dissolved solids; TKN = total Kjeldahl nitrogen; TP = total phosphates; OCD1–4 = orchard study sites; REF1–2 = reference study sites.
[b] Pt-Co = platinum-cobalt units.
[c] μS/cm = microsiemens per centimeter.
[d] JTU = Jackson turbidity units.

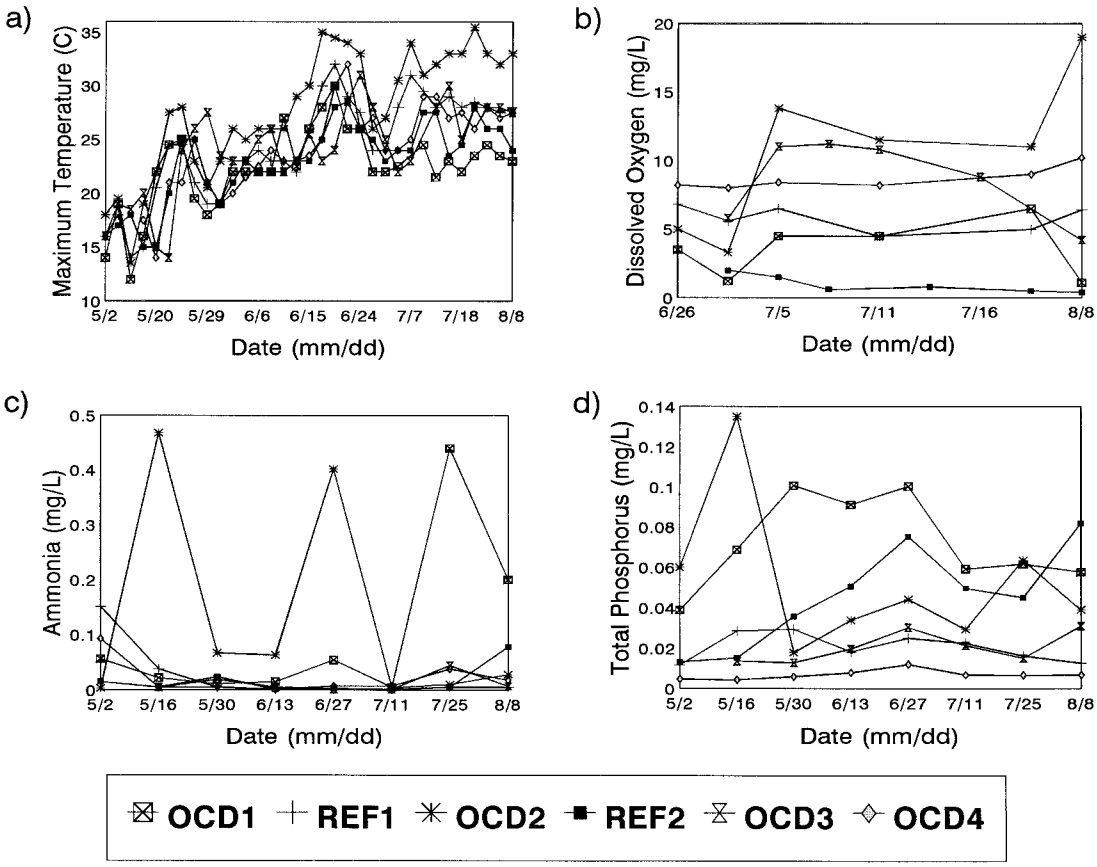

Fig. 3. Physical and chemical properties of pond water at study sites, measured throughout the summer in 1994. (**a**) Maximum daily surface water temperature (°C). (**b**) Dissolved oxygen levels (mg/L) in surface water. (**c**) Ammonia levels (mg/L). (**d**) total phosphates (mg/L).

Table 4). In the second green frog assay (July 5–25, 1994), a significant correlation was found between tadpole length and number of degree days ($r = 0.946$, Table 4).

In 1994, the two sets of water samples taken during each caging event for pesticide residue analysis revealed few detections of orchard-use pesticides, but pervasive low atrazine concentrations in both orchard and reference wetlands. During the leopard frog assay in early spring, one residue of metolachlor (0.29 μg/L) was detected at OCD2, and one detection of atrazine was documented at each orchard site (OCD1—0.07; OCD2—0.37; OCD3—0.08; OCD4—0.07 μg/L), but no orchard-use pesticides were evident in pond water. During the first green frog assay in June, diazinon was found in both samples from OCD1 (0.42–0.78 μg/L), along with one detection of azinphos-methyl (1.0 μg/L). Metolachlor was, again, detected at OCD2 (8.8 μg/L), and atrazine was detected in pond water at every site. Atrazine concentrations were generally low, near analytical detection limits (OCD1—0.051; OCD2—0.063; OCD3—0.063; OCD4—0.055–0.072; REF1—0.053–0.2; REF2—0.039–0.055 μg/L), except for one residue of 15.0 μg/L at OCD2. During the second green frog assay in July, diazinon was detected in pond water at OCD1 (0.22 μg/L) and OCD4 (0.09–0.18 μg/L), and the organochlorine, endosulfan, was found in water collected at OCD1 (0.53 μg/L) and REF1 (0.051 μg/L). One detection of metolachlor was, again, found in water at OCD2 (5.1 μg/L), and atrazine was detected at least once in every pond. As in the June assay, atrazine levels were low (OCD1—0.081; OCD3—0.13; OCD4—0.11; REF1—0.078; REF2—0.055 μg/L), except in water at OCD2 (6.7–10.0 μg/L).

Table 4. Pearson product–moment correlations of environmental variables with tadpole growth. Correlation probabilities were evaluated with respect to a Bonferonni-adjusted probability ($\alpha = 0.05/n = 12$ tests) of 0.0042. Environmental variables tested were color (COL), turbidity (TURB), total dissolved solids (TDS), conductivity (COND), pH, dissolved organic carbon (DOC), dissolved inorganic carbon (DIC), total phosphates (TP), total Kjeldahl nitrogen (TKN), ammonia (NH_3), nitrates/nitrites (NO_3^-/NO_2^-), and total degree days (DEG)[a]

Environmental variable	Leopard frogs (May 20–June 6)	Green frogs (June 8–27)	Green frogs (July 5–25)
COL	−0.420	0.543	−0.186
TURB	0.196	0.687	0.758
TDS	0.363	0.302	0.600
COND	0.324	0.318	0.644
pH	−0.443	0.037	0.103
DOC	−0.587	0.050	−0.213
DIC	0.244	0.281	0.584
TP	−0.246	−0.037	−0.183
TKN	−0.245	0.528	−0.048
NH_3	0.221	0.662	−0.617
NO_3^-/NO_2^-	0.205	0.674	−0.565
DEG	0.206	0.599	0.946*

[a] * Significant correlation; experiment-wise $p < 0.05$.

Pesticide toxicity tests

Green frog hatching success was 100% in all 1994 pesticide treatments with the exception of the two highest Dithane DG concentrations (Table 5). In 7.8 mg a.i./L and 19.5 mg a.i./L of Dithane DG, hatching success was 0%. Exam-

Table 5. Hatching success (%), and trimmed Spearman–Karber median lethal concentration (LC50) and median effective concentration (EC50) values (in mg/L) for green frog embryos and tadpoles exposed to pesticides in solution. Hatching successes <100 are followed by the concentrations at which they occurred (in brackets). The 1994 test involved a 13-d continuous exposure. The 1995 test involved a 16-d discontinuous exposure. Values are expressed as averages with associated standard errors (SEs; tests are based on two replicates/treatment/year)[a]

			LC50 (mg/L ± SE)				
	Hatching success (% ± SE)		1994		1995		EC50 (mg/L ± SE)
Chemical	1994	1995	96-h	13-d	96-h	16-d	1995
Basudin® 500EC	NT	100 40 ± 28.28 (25 μg/L)	NT	NT	>0.025	0.0028 ± 0.0003	0.0059 ± 0.0024
Diazinon	NT	100	NT	NT	>0.05	0.005 ± 0.001	0.014 ± 0.001
Dithane® DG	100 0 (7.8–19.5 mg/L)	100 0 (0.78–7.8 mg/L)	2.21 ± 0.25	0.023	0.96 ± 0.25	0.20 ± 0.04	0.04 ± 0.02
Guthion® 50WP	100	99 ± 0.29	>5.0	2.61 ± 0.55	>5.0	>5.0	>5.0
Thiodan® 50WP	100	99 ± 0.29	>11.75	0.015	>4.7	0.015	2.43

[a] NT = not tested.

ination of dead embryos showed that they had died almost immediately upon introduction to the test solutions; lysing eggs had stopped developing at Gosner [31] stage 8, the development stage chosen for test initiation. In 1995, most of the discontinuous tests resulted in 99 to 100% hatching success (Table 5); exposure to Dithane DG at 0.78 mg a.i./L and Basudin 500EC at 25.0 μg a.i./L resulted in 0% hatching.

To determine the consistency of LC50 values derived with different egg stocks, the 96-h LC50s calculated for Dithane DG during three separate testing events were compared. An analysis of variance revealed no significant difference ($p = 0.17$) between LC50s derived with eggs treated in 1994 (LC50 = 2.21 ± 0.3 mg a.i./L, Table 5) and 1995 (LC50s = 0.93 ± 0.9 mg a.i./L and 0.98 mg a.i./L).

Approximate LC50 values were calculated at 96 h for Dithane DG, and at test termination for Dithane DG, Basudin 500EC, diazinon, Guthion 50WP, and Thiodan 50WP (Table 5). The other compounds, Imidan 50WP and Nova 40W, were not acutely or chronically lethal at or below 10.0 to 12.5 mg a.i./L. Thiodan, Basudin, and diazinon were the most toxic compounds, producing 16-d LC50s of 0.015 mg a.i./L, 0.0028 mg a.i./L, and 0.005 mg/L, respectively in the 1995 discontinuous assay. The LC50s calculated at test termination for the 13-d continuous tests with Dithane DG and Guthion 50WP were predictably lower than comparable LC50s calculated for the 16-d discontinuous tests. However, Thiodan 50WP produced the same LC50 value (0.015 mg a.i./L) during both procedures. Most individuals in concentrations at or above 0.047 mg a.i./L Thiodan did not begin to die until shortly after feeding commenced. Commencement of feeding corresponded to chemical exposure during the continuous test, but not during the discontinuous test.

The deformity frequencies observed at hatching during 1995 discontinuous tests were used to calculate EC50s. Deformities were produced in larvae exposed to Basudin 500EC, diazinon, Dithane DG, and Thiodan 50WP (Table 4). Deformities produced by diazinon and Basudin appeared at 0.5–50 μg/L and 1–25 μg a.i./L, respectively, and were characterized as abdominal and head edemas and blistering, ventral and lateral flexure of the tail, stunting of the tail, and underdevelopment of the gills. Deformities produced by Dithane appeared at 0.078 mg a.i./L, and were characterized as dorsal and lateral flexure of the tail, with a small percentage of abdominal edemas and resulting gill displacement. Thiodan caused skeletal deformities, expressed as dorsal and lateral flexure, at concentrations of 4.7 mg a.i./L. Also evident at this concentration was a general low level of tadpole activity; tadpoles did not swim unless repeatedly prodded, and swimming movements were uncoordinated and undirected.

Nova 40W and Imidan 50WP caused significant reductions in tadpole length at relatively high exposure concentrations of 10.0 to 12.5 mg a.i./L ($p = 0.024$, $p = 0.0003$, respectively; Fig. 4). Tadpoles exposed to much less Dithane DG or Guthion 50WP exhibited dose-dependent reductions in length with increasing chemical concentration (Figs. 5 and 6). Growth inhibition was significant during discontinuous exposure tests at a Dithane concentration of 0.078 mg a.i./L ($p = 0.0004$), and during both continuous and discontinuous tests at a Guthion concentration of 2.5 mg a.i./L (continuous $p = 0.005$; discontinuous $p = 0.0003$). Thiodan 50WP exposure at 0.0047 mg a.i./L did not significantly alter growth rates in either the continuous ($p = 0.1095$) or discontinuous ($p =$

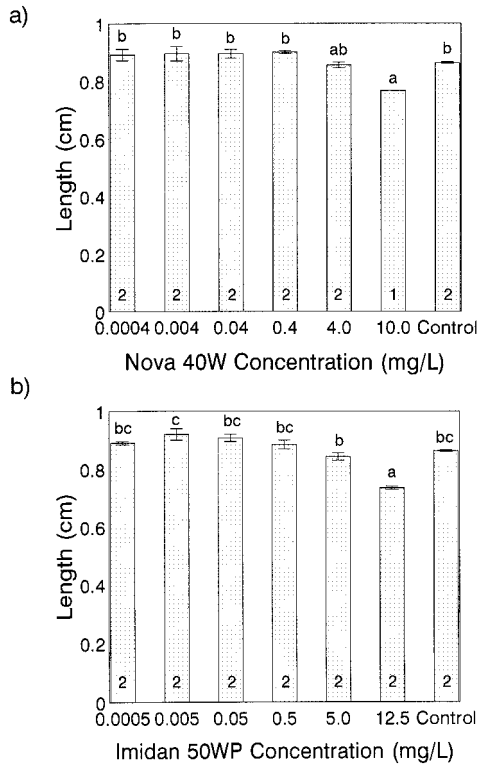

Fig. 4. Effects of a 13-d continuous exposure to a pesticide formulation on growth rate of green frog tadpoles. Average tadpole length in (**a**) Nova® 40W, and (**b**) Imidan® 50 WP concentrations of 0.0004 mg/L to 12.5 mg/L compared to average length achieved in reference/control pond water solutions for each test event. Values at the base of each bar are number of replicates. Treatments with the same letter at the top of the bar are not significantly different from each other at an α level of 0.05. Error bars represent standard error among replicates.

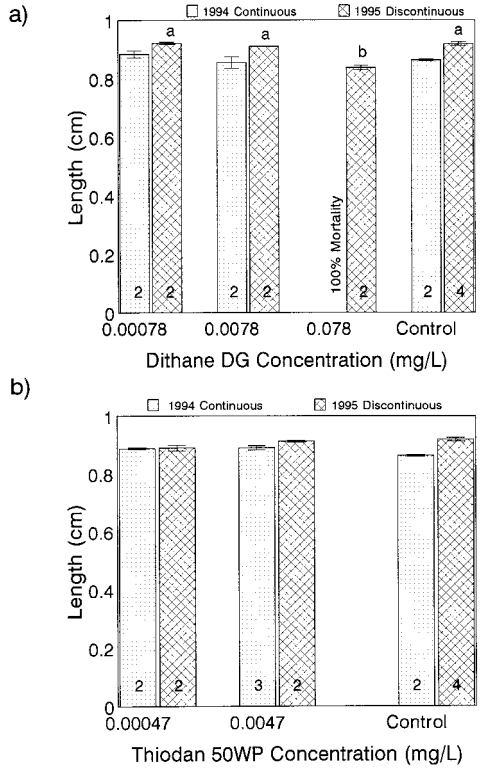

Fig. 5. Effects on growth rate of green frog tadpoles of a 13-d continuous exposure or a 16-d discontinuous exposure to a pesticide solution. Average tadpole length in (**a**) Dithane® DG, and (**b**) Thiodan® 50WP at concentrations ranging from 0.00047 mg/L to 0.078 mg/L compared to average length achieved in reference/control pond water solutions for each test event. Values at the base of each bar are number of replicates. Treatments with the same letter at the top of the bar are not significantly different from each other at an α level of 0.05. Error bars represent standard error among replicates.

0.0826) assays (Fig. 5). Basudin 500EC did not significantly alter growth rates at or below an exposure concentration of 1.0 μg/L during a discontinuous test ($p = 0.053$; Fig. 6). The active ingredient in Basudin, diazinon (50%, w/v), did cause significant reductions in tadpole length at an exposure concentration of 5.0 μg/L ($p = 0.01$; Fig. 6).

DISCUSSION

Poor hatching success and survival, and significant differences in growth rates observed during some in situ assays suggest that variability in pond water characteristics among study sites affected both embryonic and larval development of leopard and green frogs. During those tests in which in situ hatching rates of 95 to 100% were measured in reference pond water (indicating that the stocks were developing normally within the constraints of the cage design), unsuccessful embryonic development, as shown by hatching and survival rates, occurred sporadically at OCD1, OCD2, and OCD4. Tadpole in situ growth was not consistently more rapid in cages at reference sites relative to cages at orchard sites. Upon evaluation of correlative significance, no one measured environmental parameter fully explained the different growth rates, although water temperature was clearly a critical variable. Pesticides were detected in pond water, but could not be directly linked to differences in embryo–larval development among sites, and were not found at concentrations that affected green frog development during laboratory toxicity tests. Replicates within sites contributed a statistically significant amount of variability to the design; it is thought that this was due to the random occurrence of either highly dominant or genetically inferior individuals within particular replicates.

Several water quality conditions that had the potential to induce stress responses in tadpoles existed at specific sites (both orchard and reference) during test events. Diamond et al. [39] reported a no-observed-effect concentration (NOEC, derived using 21-d growth data) of 0.27 mg/L ammonia for leopard frog embryos. That NOEC was exceeded in 12% of water samples collected from the pond at OCD1, and in 25% of water samples collected from the pond at OCD2 in 1994. None of those water samples were collected during caging events, but because water quality was not continuously monitored it is still possible that ammonia toxicity was involved in low caging hatching success, survival, or growth rates. Also, because elevated ammonia was evident in pond water, it may act as an environmental stressor on wild tadpole populations inhabiting ponds at OCD1 and OCD2. The same may be said of the low dissolved oxygen levels in pond water at some study sites. Amphibian larvae begin the transition from gill to lung respiration before metamorphosis [7] and air breathing begins with the appearance of occasional air breaths. Under hypoxic conditions, the frequency of air breathing increases, increasing the risk of predation on larvae [8]. Apparent increased rates of air breathing in wild green frog tadpoles were seen on three occasions at OCD1.

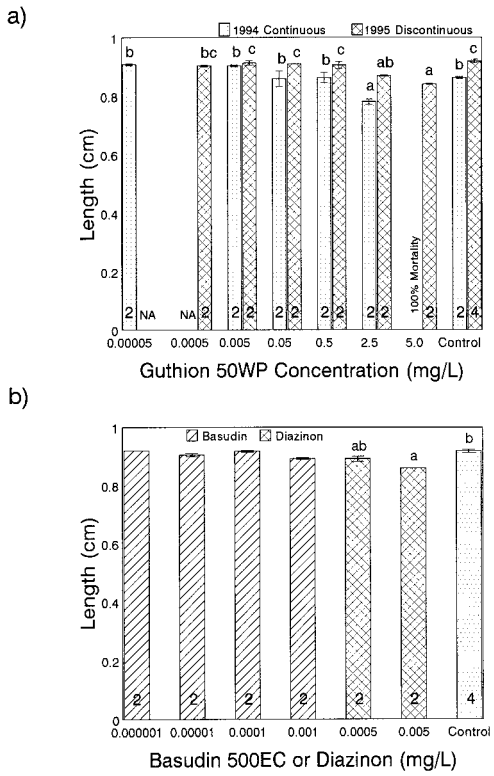

Fig. 6. Effects of a 13-d continuous exposure or a 16-d discontinuous exposure to an organophosphorus insecticide solution on the growth rate of green frog tadpoles. Average tadpole length in (**a**) Guthion® 50WP and (**b**) Basudin® 500EC and diazinon at concentrations ranging from 0.001 μg/L to 5.0 mg/L compared to average length achieved in reference/control pond water solutions for each test event. Values at the base of each bar are number of replicates. Treatments with the same letter at the top of the bar are not significantly different from each other at an α level of 0.05. Error bars represent standard error among replicates.

Differences in average caged tadpole length were not duplicated in the lab for either test species, and tadpole growth was elevated in the field compared to the lab. The reduced proportion of total variance attributable to site in the laboratory relative to in situ green frog tests confirmed that the laboratory assay eliminated much of the field environmental variability. The most obvious environmental difference was the controlled laboratory temperature, which was greater than 10°C lower than some pond water temperatures during the green frog assays. Duellman and Trueb [4] stated that, after food availability is considered, temperature is the major external factor controlling the duration of larval development in amphibians. Warkentin [40] reported that green frog tadpoles at Gosner [31] stages 24 to 37 consumed significantly more food when maintained at laboratory temperatures of 23 to 26°C than when maintained at 20°C. Growth rates of caged green frog tadpoles at OCD2 were very high, probably due to elevated surface water temperatures, which rose well over 30°C during midday from the middle of June to the end of August. The increased rate of development produced by high water temperatures might decrease the time to metamorphosis enough that it enables some species to complete development despite the presence of other stressors such as sporadic high ammonia and pesticide influxes.

At extremely elevated water temperatures, however, larval development may be compromised [4], especially in the presence of other stressors. Johnson [41] showed that thermal tolerance of Pacific treefrogs (*Hyla regilla*) was significantly compromised after exposure to organophosphorus pesticides similar in chemical composition to those applied in study orchards (i.e., azinphos-methyl and diazinon). Also, Materna et al. [17] found that survival of leopard frog (*Rana* sp.) tadpoles exposed to the pyrethroid insecticide, esfenvalerate, was significantly reduced at elevated water temperatures (e.g., at 22°C compared to at 18°C). High temperatures at OCD2 could, then, also be partly responsible for the sporadic high mortality rates witnessed at that site.

Food availability, the primary environmental determinant of larval development rates identified by Duellman and Trueb [4], was partly controlled during in situ assays, but still could have been a variable contributing to differing tadpole growth rates. All caged tadpoles had access to equivalent amounts of boiled lettuce, and were never left without access to that food source; however, it was possible that individuals at some sites were benefiting from additional natural food sources. Kupferberg et al. [42] found that Pacific treefrog tadpoles could potentially grow faster on some natural diets of filamentous green algae and epiphytes, but they did not test whether tadpoles would, if given the choice, selectively feed on those food types. Jenssen [43] reported that wild green frog tadpoles fed continuously and indiscriminately on algae and microcrustaceans. It seems unlikely that leopard and green frog tadpoles were preferentially feeding on natural phytoplankton and zooplankton, but they might have indiscriminately ingested enough natural food of a higher nutritional value to affect growth rates in a site-specific manner.

The one set of caged green frogs that suffered high mortality in two of four cages at OCD4 in 1994 also exhibited lower average length measures of surviving individuals. These individuals were the only ones that did not exhibit significantly faster growth in the field compared to the laboratory. Young-of-the-year green frogs captured there in 1994 were also significantly smaller than sampled individuals from all other sites [29]. These size reductions and those of the one set of caged tadpoles may or may not have been caused by the same environmental phenomenon, but it was apparent that unfavorable conditions existed within the pond for unknown time intervals in 1994, and slowed growth relative to reference individuals.

In most cases, growth rates achieved during in situ assays did not reflect relative size of young-of-the-year captured at study sites [29]. The most obvious contradiction was seen at OCD2, where caged green frog tadpoles exhibited relatively rapid growth rates, but wild young-of-the-year were smaller than those from any other study site except OCD4. Even though this particular dichotomy may reflect the existence of multiple stressors at OCD2 (as discussed in previous paragraphs), the overall inability of in situ and laboratory pond water growth assays to predict size of young-of-the-year places the relevance of short-term growth rates to time-to-transformation and size-at-transformation of wild tadpoles in question. Size-at-transformation and rates of development are driven by a set of complex environmental interactions, involving inter- and intraspecific competition for food, predation rates [6], and water quality. The unpredictable nature of early development in wild stocks of leopard and green frogs in study site ponds suggests that either short-term in situ and laboratory tests do not encompass a

sufficient number of the major environmental developmental determinants (e.g., predation, microhabitat use, food quality), or that wild stocks were more tolerant of reduced water quality than were healthy egg stocks produced in the laboratory. Support for the latter phenomenon may be taken from Hecnar [44] who found a threefold difference in LC50 values between two populations of American toads (*Bufo americanus*) exposed to ammonium nitrate fertilizer. He suggested the different sensitivities might have been due to differences in exposure histories of the populations related to the intensity of agricultural activity in the two regions.

The relatively short time windows encompassed by the in situ assays, coupled with the lack of correlations between environmental variables and witnessed developmental differences meant that it was invalid to dismiss the potential for detrimental effects on wild tadpoles of detected pesticide residues in pond water. Toxicity tests using Gosner [31] developmental stages 8 to 25 revealed that four of the chosen six pesticide formulations produced significant effects on survival, deformity rates, or growth rates, but none of these effects were produced at concentrations detected in orchard pond water during this study.

The organophosphorus insecticide, Imidan 50WP, and the triazole fungicide, Nova 40W, pose no threat to the successful early development of green frogs. The organophosphorus formulation, Guthion 50WP, and the organochlorine, Thiodan 50WP, did not produce effects in green frogs at concentrations that might be encountered in pond water via runoff or drift events [29,45], but growth inhibition and acute lethality did occur at levels probable during a chemical spill or sprayer backflush event (C. Bishop, personal communication); ponds are routinely used to fill sprayers. Basudin 500EC, technical grade diazinon (the active ingredient of Basudin), and the EBDC formulation, Dithane DG, were toxic to green frog early life stages, and pose risks to embryos and larvae in the field.

Diazinon is commonly used in both agricultural and urban environments [28,46]. It was detected in 38 to 50% of samples collected from storm water detention ponds in Guelph, Ontario, at average concentrations of 0.04 to 1.04 μg/L [46], and Frank et al. [47] detected a maximum diazinon residue of 10.0 μg/L in farm well water in the Holland Marsh agricultural region of Ontario. Water samples collected at orchard site ponds revealed a maximum diazinon residue of 0.78 μg/L. The LC50 values of 2.8 to 5 μg/L for green frogs exposed discontinuously to diazinon in formulation and as technical grade, respectively, fall within the range of values detected in well water samples, but not within the range detected at orchard sites. More frequent exposure to diazinon residues in the range of concentrations detected by Struger et al. [46] is possible for frogs inhabiting wetlands adjacent to vegetable crops [28,45], and could seriously affect the survival and development of green frog early life stages.

The EBDC fungicide, Dithane DG, affected tadpole survival and growth at concentrations at or below the detection limit for its active ingredient, mancozeb. The detection limit for mancozeb, which constitutes 80% of the formulation, is 0.05 mg/L. Dithane caused significant growth inhibition between 0.0078 and 0.078 mg a.i./L, and the EC50 for the creation of deformities (mostly skeletal) in tadpoles at hatching was 0.038 mg a.i./L. Dithane was not detected in pond water samples collected in 1994, but toxicity test results imply that zero detections do not guarantee a lack of deleterious effects to the early life stages of green frogs.

The death of green frog embryos upon exposure to Dithane at concentrations above 0.78 mg/L was an immediate response to chemical introduction; however, stability tests suggested that only about 70% of expected mancozeb was present immediately after mixing in pond water. Ethylenebisdithiocarbamates do decompose rapidly in water [34], and the major degradation product of mancozeb, ethylene thiourea, is acutely toxic and teratogenic to many aquatic organisms [48]. Therefore, green frog embryo–larval mortality could have been due to the production of ethylene thiourea. Regardless of which compound produced the effects witnessed in tadpoles, the effects were severe, and the spraying and improper storage of Dithane DG in orchards near ponds may pose a risk to the early life stages of green frogs.

The toxicity tests with pesticide formulations detected only direct effects on embyro–larval development, and not the potential cumulative effects of multiple pesticide exposures in concert with other environmental stressors such as elevated water temperature, high ammonia concentrations, and low dissolved oxygen levels. Mixtures of pesticides are often sprayed in the orchards as well [25], and green frog responses to pesticide mixtures were not evaluated. Also, pond water collected from REF1 (the source of water for treatment solutions) contained trace amounts of atrazine. Although that confounds the evaluation of direct effects of pesticide formulations on amphibian embryos and larvae, pond water analyses from all study sites show that it is the most realistic exposure scenario for wild eggs and tadpoles.

Despite the limitations of the in situ and laboratory tests performed in this study, they were useful in providing a means of comparing study sites for short-term effects on early life stages. Incidences of low survival rates in embryos and tadpoles caged in orchard ponds suggested that acute lethality could occur. The lack of substantial mortality in reference pond cages (with the notable exception of the poor stock of leopard frog eggs in 1994) suggested that the causative agent(s) were more likely to be encountered in orchard ponds or canals. The paucity of correlations between pond water properties and tadpole growth, and the lack of significant differences in tadpole growth in laboratory pond water assays suggested that surface water temperature in combination with other unidentified environmental variables were responsible for significant differences in 2-week growth rates exhibited in tadpoles at different sites. The changes in survival, deformity, and growth rates of tadpoles exposed to pesticide formulations suggested that recorded pesticide influxes in pond water at orchard sites could contribute to a suite of environmental stressors acting on populations to reduce developmental success.

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APPENDIX

Characterization of pesticide formulations used in toxicity tests. Stability of selected pesticide treatment concentrations over time (10.0 mg/L = stock; 0.1 or 1.0 mg/L = dilution)

Pesticide (active ingredient)	Formulation concn. (mg/L)	Nominal concn. (mg/L)	Storage time (d)	Nominal concn.	
				Measured (mg/L)	Expected (%)
Guthion® 50WP (azinphos-methyl)	10.0	5.0	0	3.4	68
			4	4.1	82
			7	3.4	68
	0.1	0.05	0	0.049	98
			4	0.042	84
			7	0.047	94
Imidan® 50WP (phosmet)	10.0	5.0	0	4.0	80
			4	3.0	60
			7	2.3	46
	0.1	0.05	0	0.052	104
			4	0.030	60
			7	0.032	64
Thiodan® 50WP (endosulfan)	10.0	4.7	0	3.816	81
			4	1.56	33
			7	1.55	33
	0.1	0.047	0	0.052	110
			4	0.051	109
			7	0.054	115
Dithane® DG (mancozeb)	10.0	7.6–8.0	0	5.4	68–71
			4	4.0	50–53
			7	5.0	63–66
	1.0	0.76–0.8	0	0.4	50–53
			4	0.3	38–39
			7	0.3	38–39
Nova® 40W (myclobutanil)	10.0	3.8–4.2	0	3.5	83–92
			4	3.5	83–92
			7	3.8	90–100
	0.1	0.038–0.042	0	0.031	74–82
			4	0.039	93–103
			7	0.033	78–87