Pergamon

Environmental Toxicology and Chemistry, Vol. 14, No. 6, pp. 1011–1018, 1995

Printed in the USA
0730-7268/95 $9.50 + .00

0730-7268(95)00035-6

COMPARATIVE SENSITIVITY OF AMPHIBIAN TADPOLES TO SINGLE AND PULSED EXPOSURES OF THE FOREST-USE INSECTICIDE FENITROTHION

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(*Received* 10 *June* 1994; *Accepted* 2 *December* 1994)

Abstract—The insecticide fenitrothion is sprayed twice, 5 to 10 d apart, to control spruce budworm. To assess the effects of pulsed exposure of fenitrothion to different species of amphibians, we exposed 1- and 8-d-old tadpoles of wood frogs, leopard frogs, green frogs, bullfrogs, and American toads, as well as larvae of the spotted salamander, to 2 to 9 ppm of the insecticide for 24 h. Our results indicate that within each ranid species, day-1 and day-8 tadpoles are equally sensitive, and tadpoles exposed twice, on day 1 and day 8, are no more sensitive to the second exposure than to the first. Bullfrog and green frog tadpoles are more sensitive to exposures than are wood frog and leopard frog tadpoles, exhibiting paralysis at lower concentrations, and they are affected by exposure to the carrier compounds as well as the insecticide. The greater sensitivity of the green frog and bullfrog tadpoles correlates with their smaller size and earlier developmental stage at hatching. American toad tadpoles are more tolerant of pulsed exposure to fenitrothion than are any of the ranids, whereas the spotted salamander larvae are as sensitive as are the bullfrog and green frog tadpoles.

Keywords—Amphibian Pesticide Fenitrothion Tadpoles

INTRODUCTION

The organophosphorus insecticide fenitrothion has been used to control forest defoliators such as pine sawyer beetles, *Monochamus alternatus* [1], and the spruce budworm, *Choristoneura fumiferana* [2]. It has been the insecticide of choice in the intensively managed forests of New Brunswick for over 20 years, where it is usually applied twice to each operational spray block, 5 to 10 d apart [3]. As a result, it has been cited as an example of a pesticide possibly contributing to amphibian decline [4]. Small water bodies are unavoidably contaminated by spraying and, although concentrations in lotic systems are diluted rapidly to levels likely to have little biological impact [5], surface-water concentrations of small shallow ponds may reach a maximum of 2.5 ppm (mg/L) [3].

Fenitrothion has a short half-life of 24 to 48 h, breaking down to less toxic products by hydrolysis and photolysis. It is a nonreversible cholinesterase inhibitor, and animals exposed to it experience depressed brain acetylcholinesterase activity. The rate of recovery is directly related to extent of depression [6]. Populations of aquatic invertebrates in bog ponds are reduced by as much as 50% [7], and simulated exposures indicate that fish and amphibians are likely to be sensitive to pond contaminations [8,9].

Nine anuran amphibians are native to New Brunswick, and they breed in areas potentially subjected to spraying. Initial surveys indicated no reduction of frog populations associated with the spraying [10]. However, transect surveys of adults initiated in 1991 indicated that, while most species were not abundant enough for analysis, mink frog densities were negatively correlated with fenitrothion spraying in previous years [11]. In lab experiments, embryos of three other ranid species (leopard frog, green frog, bullfrog) hatched successfully after 24-h exposures to fenitrothion concentrations as high as 8 ppm, but newly hatched tadpoles were temporarily paralyzed by exposure for 24 h to concentrations as low as 2 to 4 ppm and often died at 8 ppm [9]. Continued use of fenitrothion in control of forest defoliators is likely to have a negative impact on the amphibian community.

Because fenitrothion is usually sprayed twice in succession, 5 to 10 d apart, tadpoles may then be exposed to the pesticide twice prior to metamorphosis. Initial exposure to fenitrothion may render tadpoles more sensitive to a second exposure, for they could still be recovering from depressed cholinesterase activity when exposed for a second time. Sensitivity of tadpoles to fenitrothion exposure may also be correlated with their developmental stage, for cholinesterase inhibition may vary as the rate of neuromuscular differentiation changes during early development. As a result, 1-d-old and 8-d-old tadpoles may differ in sensitivity, confounding the perceived effects of a second exposure.

Different species are also likely to vary in sensitivity. Initial lab experiments on newly hatched ranid tadpoles indicated that leopard frogs may be less sensitive to exposure to fenitrothion than either green frogs or bullfrogs [9]. If a more sensitive species hatches at a smaller size or stage than a less sensitive species, differences in species sensitivity may actu-

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ally be correlated with developmental stage. For example, green frog and bullfrog eggs may hatch at a less developed stage than leopard frog eggs; as they develop further, green frog and bullfrog tadpoles should then decrease in sensitivity as they approach the hatching stage of leopard frogs.

Whether or not species differences in sensitivity are at least in part a result of different developmental trajectories, the limited data available suggest that a species more sensitive to one contaminant is likely to be relatively more sensitive to another contaminant. For example, as tadpoles or embryos, leopard frogs and American toads are less sensitive to exposure to both low pH and pyrethroid insecticides than are green frogs. On the other hand, spotted salamanders are considerably more sensitive to both stresses than any of the anurans [12,13]. If relative sensitivity to contaminants remains consistent, then American toads should be relatively tolerant of fenitrothion exposure, while spotted salamanders should be more sensitive. Wood frogs, particularly tolerant of low pH [13], but of unknown sensitivity to pesticides, should then also be more tolerant of exposure to fenitrothion.

Our experiments therefore compare sensitivity of newly hatched tadpoles of four ranids (wood frog, leopard frog, green frog, and bullfrog) as well as newly hatched American toad tadpoles and spotted salamander larvae. We tested the following predictions: (a) newly hatched tadpoles should be more sensitive to a second or pulsed exposure to fenitrothion than they are to a single exposure; (b) 8-d-old tadpoles should differ in sensitivity from 1-d-old tadpoles; (c) green frog and bullfrog tadpoles should be more sensitive to fenitrothion exposure than wood and leopard frog tadpoles, and this greater sensitivity should be correlated with earlier developmental stage at hatching; and (d) a consistent order of sensitivity to contaminant exposure should exist among the amphibians, with wood frogs least sensitive, and American toads, leopard frogs, green frogs, bullfrogs, and spotted salamanders progressively more sensitive.

METHODS

Egg collection and tadpole culture

Egg masses of wood frogs (*Rana sylvatica*), leopard frogs (*R. pipiens*), green frogs (*R. clamitans*), bullfrogs (*R. catesbeiana*), American toads (*Bufo americanus*), and spotted salamanders (*Ambystoma maculatum*) were collected shortly after they were laid from ponds in central Ontario during the spring and summer of 1993. Egg masses were cultured in the lab until the eggs hatched. Tadpoles were maintained at 15°C and fed small amounts of boiled lettuce. Two weeks after hatching, at the end of the exposure experiments, tadpoles were released at the sites where they were collected.

Experimental conditions

Tadpoles of each of the four ranid species were exposed to fenitrothion on day 1 (experiment 1), day 8 (experiment 2), and days 1 and 8 (experiment 3) after hatching. American toad tadpoles and spotted salamander larvae were exposed on days 1 and 8 (experiment 3) after hatching. All tadpoles were 0.5 to 1.0 cm long when exposed. Exposures and recoveries occurred in 1-L beakers half filled with river water, with 10 tadpoles per beaker. Because the six species breed sequentially during the spring and summer, they were tested one or two at a time from late April until early August. With each of the four ranid species, the three experiments were run concurrently. All experiments were run in triplicate.

Exposure to fenitrothion lasted 24 h, and nominal exposure concentrations were 1, 2, 5, and 9 ppm. Before preparing the 1,000-ppm stock solution of fenitrothion from a 480 g/L (96% a.i.), the fenitrothion was dissolved in an equal volume of dawanol, a solvent; an emulsifier, Atlox, was then added in a volume equal to that of the solvent. Appropriate volumes of stock were then added to 500 ml of filtered river water to make up the experimental exposures. For most of the experiments, the three replicates of the nominal concentrations were each subsampled for extractions and residue analyses. Controls consisted of uncontaminated filtered river water, and filtered river water to which both solvent and the emulsifier carriers were added at levels equal to the maximum carrying the fenitrothion. Tadpoles were exposed to the carrier a second time, on day 8, when other tadpoles received their second exposures to the various fenitrothion concentrations. For each of the ranids, a single set of controls served the three concurrent experiments. All exposures and recovery occurred at 15°C in darkness in a controlled environmental chamber.

Egg and tadpole measurements

Egg diameters of 20 eggs from each of 6 to 10 broods were measured for the four ranid species. The eggs were measured at yolk plug stage, Gosner stages 8 to 11 [14]. Hatching stage of at least 20 eggs was determined for 6 to 10 broods of the four ranid species. During each experiment, all tadpoles were examined daily for mortality and prodded for assessment of avoidance response. A normal avoidance response consisted of darting away 5 to 20 body lengths. An abnormal response consisted of moving slowly away for 2 to 3 body lengths, to twitching in place, or not moving at all; we considered these to be increasing degrees of paralysis. At the end of exposures, 2 weeks after hatching, all surviving tadpoles subjected to experimental exposures were measured to determine head width, snout-vent length, and tail length.

Extraction and residue analysis

Water samples were drawn from exposure beakers 1 h after the onset of the exposures. Extractions of the fenitrothion samples were made according to standard methods [15]. Extract volumes were reduced to 2 ml of solvent. Extractions were analyzed on a gas chromatograph with a flame photometric detector (Hewlett-Packard 5890 GC-MSD) in selected ion mode (SIM), providing detection limits of 13 μg/L of solvent. Reproducibility and linear quality-control checks were performed before the analysis of each pesticide set. Samples were diluted appropriately to ensure that sample chromatographic peaks were within the working range of the calibration standards. Nominal concentrations are used for consistency in our discussion, but residual values are included in Figures 1 to 4.

RESULTS

Residue analyses

Residue analyses were done on extractions of each fenitrothion exposure of the wood frog, leopard frog, and American toad experiments and on half of the green frog, bullfrog, and salamander experiments. In most cases, residual values ranged from 0.75 to 1.05 times those of nominals (Figs. 1–4).

Egg diameter and stage at hatching of ranid species

At the yolk plug stage, the eggs of wood frogs and leopard frogs were similar in diameter (t test, $p = 0.82$), as were the eggs of green frogs and bullfrogs (t test, $p = 0.73$). However, the wood frog and leopard frog eggs were 1.6× greater in diameter, and 4.0× greater in volume than the green frog and bullfrog eggs (Table 1). Both green frogs and bullfrogs hatched at Gosner stages 17 to 18, while wood and leopard frogs hatched at stage 20. This represents a considerable difference in development, particularly in terms of gill development.

Exposures to fenitrothion: Wood and leopard frogs

Exposed to fenitrothion on day 1 after hatching (experiment 1), tadpoles of both species appeared to be unaffected at 1 and 2 ppm, indistinguishable from the unaffected controls. At 5 and 9 ppm, most tadpoles were paralyzed, lacking a normal avoidance response, at the end of the 24-h exposure. They recovered rapidly over the next 24 h. Minimal mortality occurred at both concentrations (Fig. 1).

Exposed on day 8 after hatching (experiment 2), tadpoles again were unaffected by exposure to 1 or 2 ppm. At 5 ppm, their response was similar to their response to day-1 exposure, recovering rapidly from initial paralysis. At 9 ppm, paralysis lasted longer, and mortality was considerable and occurred at least 24 h after the exposure period (Fig. 1).

Exposed on both day 1 and day 8 (experiment 3), tadpoles were again unaffected by exposure to 1 or 2 ppm, indistinguishable from unaffected controls. Exposure to 5 and 9 ppm resulted in responses similar to responses to single exposures on day 1 or day 8, except that mortality of leopard frog tadpoles was minimal after their second exposure on day 8 (Fig. 1).

Therefore, in both species, response to exposure was similar whether it occurred on day 1 or day 8 after hatching, and a second exposure did not result in any greater effects than single exposures held at the same times.

Table 1. Comparison of egg diameter and hatching stage of four ranid species

Species (n)	Egg diameter		Gosner stage (yolk plug)	Hatching Gosner stage
	Mean	(SE)		
Wood frog (10)	2.63	(0.06)	8–11	20
Leopard frog (7)	2.55	(0.05)	9–11	20
Green frog (10)	1.64	(0.04)	8–11	17–18
Bullfrog (6)	1.61	(0.05)	8–10	17–18

(n = number of broods examined).

Exposures to fenitrothion: Green frogs and bullfrogs

Exposed on day 1 (experiment 1), green frog tadpoles were unaffected by exposure to the carrier or to 1 ppm fenitrothion. At 2, 5, and 9 ppm, approximately 50% mortality occurred following exposure, while survivors gradually recovered their normal avoidance response (Fig. 2). At 1 ppm, bullfrog tadpoles were also unaffected, but a carrier effect occurred, with initial paralysis and rapid recovery of survivors, indicating sensitivity to the solvent or emulsifier. The effects of exposure to 2 and 5 ppm were similar to the effects of the carrier control, although the levels of carrier at those exposures would have been considerably less than in the control exposure. At 9 ppm, exposed as well to concentrations of the solvent and emulsifier equal to those of the carrier control, all tadpoles died (Fig. 3).

Exposed on day 8 (experiment 2), tadpoles were unaffected by exposure to 1 or 2 ppm fenitrothion. The carrier effect is present in experiments with both species, but represents a second dose of the solvent/emulsifier administered on day 8. At 5 and 9 ppm, green frog tadpoles were less sensitive, with less mortality, than those exposed only on day 1. Bullfrog tadpoles recovered rapidly following initial paralysis after exposure to 5 ppm. Exposed to 9 ppm, most died during exposure or during the first 24 h of the recovery period; the few that did not die appeared to recover fully (Figs. 2 and 3).

Tadpoles that were exposed on both day 1 and day 8 (experiment 3) were again unaffected by exposure to 1 ppm. At 2, 5, and 9 ppm, green frog tadpoles responded to day-8 exposure as they did to day-1 exposure, except with little increase in mortality. At 2 and 5 ppm, bullfrog tadpoles were affected in much the same way as those exposed only to the solvent/emulsifier, and all were killed during day-1 exposure to 9 ppm (Figs. 2 and 3).

Therefore, both species were more sensitive to the experimental exposures than were the wood frog or leopard frogs, although some portion of that greater sensitivity must be a result of exposure to the carrier. No clear difference existed in sensitivity of tadpoles exposed only on day 1 and those exposed only on day 8. Tadpoles exposed on both day 1 and day 8 showed no greater effects than those exposed on only one of those days.

Exposure to fenitrothion: American toads and spotted salamanders

American toad tadpoles and spotted salamander larvae were exposed to fenitrothion on day 1 and day 8 after hatching to compare their responses with those of the four ranids (Fig. 4). No effects were apparent at 1 ppm, and there was no carrier effect. At 2 and 5 ppm, few of the American toad tadpoles exhibited abnormal avoidance behavior, and these recovered rapidly. They were also relatively unaffected by exposure to 9 ppm, particularly to the second exposure, and again all recovered rapidly. In contrast, spotted salamander larvae were affected more like green frog and bullfrog tadpoles, with extensive initial paralysis even at 2 ppm. They were also consistently more sensitive to the second exposure, with almost total mortality at 9 ppm.

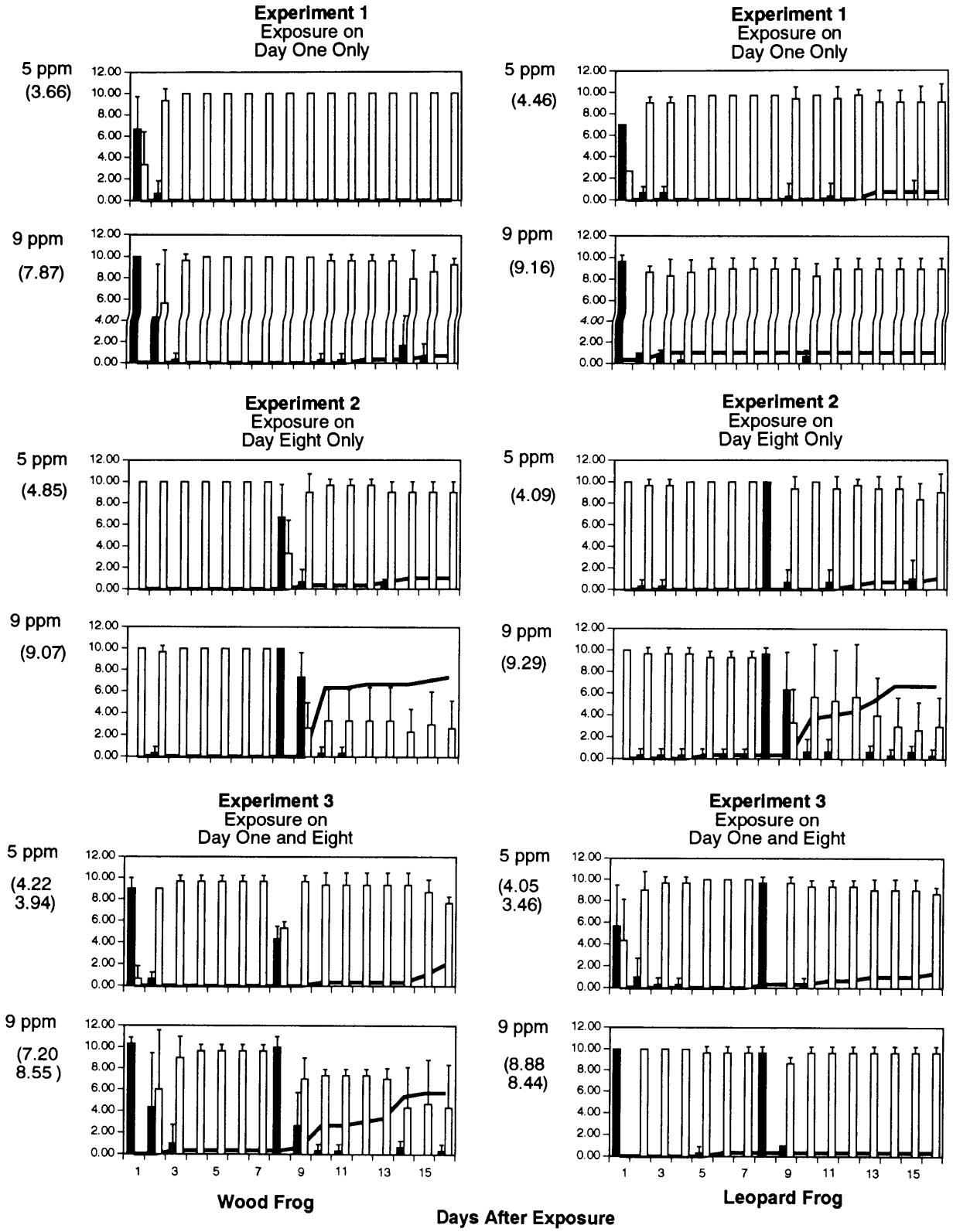

Fig. 1. Effects of exposure of wood frog and leopard frog tadpoles to fenitrothion. Black bars indicate mean number of tadpoles per treatment that were paralyzed, while unshaded bars indicate mean number that responded with a normal avoidance reflex. Error bars indicate standard deviations of triplicate experiment. Solid black lines indicate mean accumulated mortality. Control, carrier (containing solvent and emulsifier), 1-ppm exposure, and 2-ppm exposure experiments are omitted as no paralysis or death occurred in them. Residue concentrations are included in parentheses below nominal concentrations.

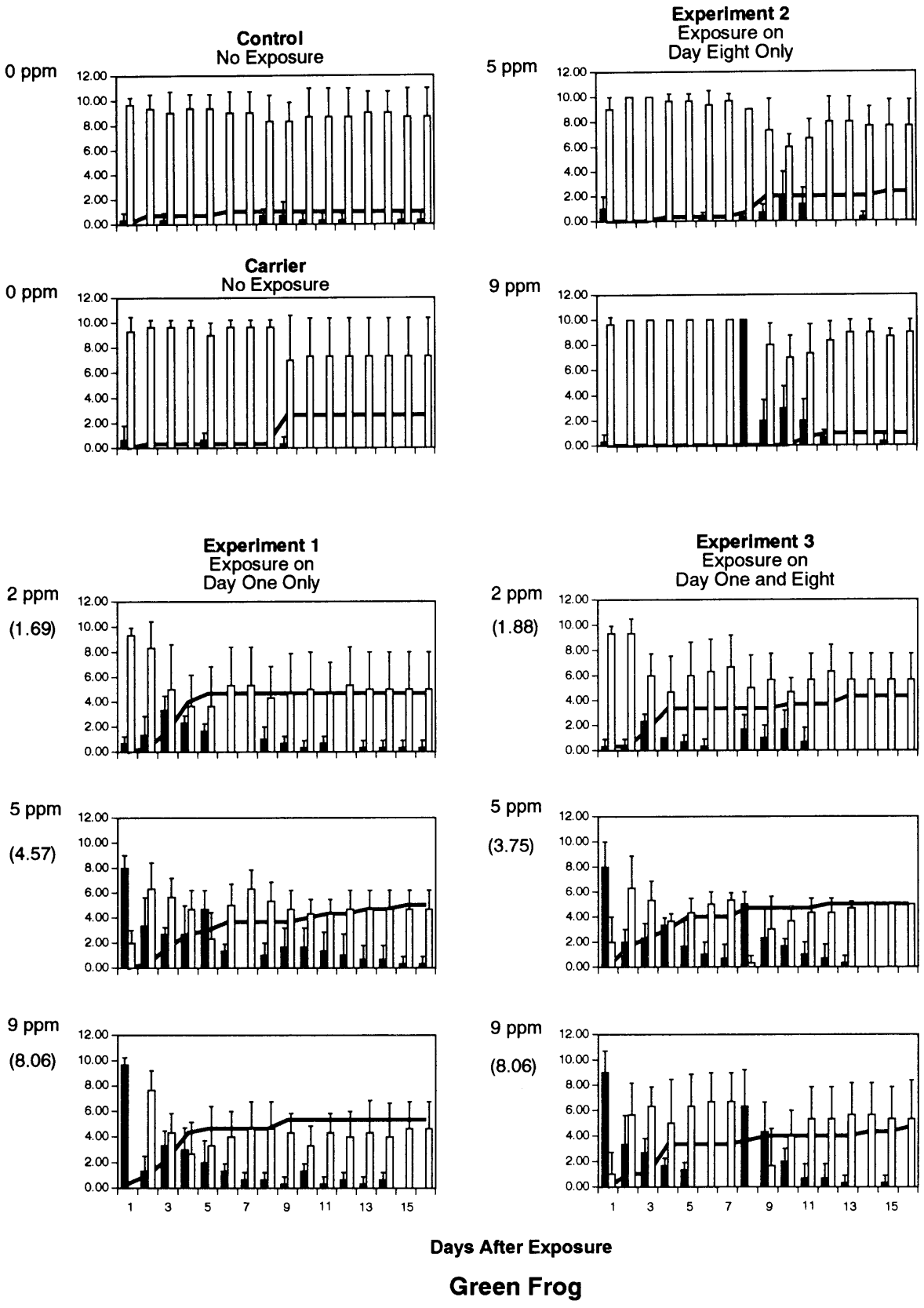

Fig. 2. Effects of exposure of green frog tadpoles to fenitrothion. As in Fig. 1, except that 1-ppm experiments as well as 2-ppm of experiment 2 are omitted. Note that carrier effect is evident after day-8 inoculation.

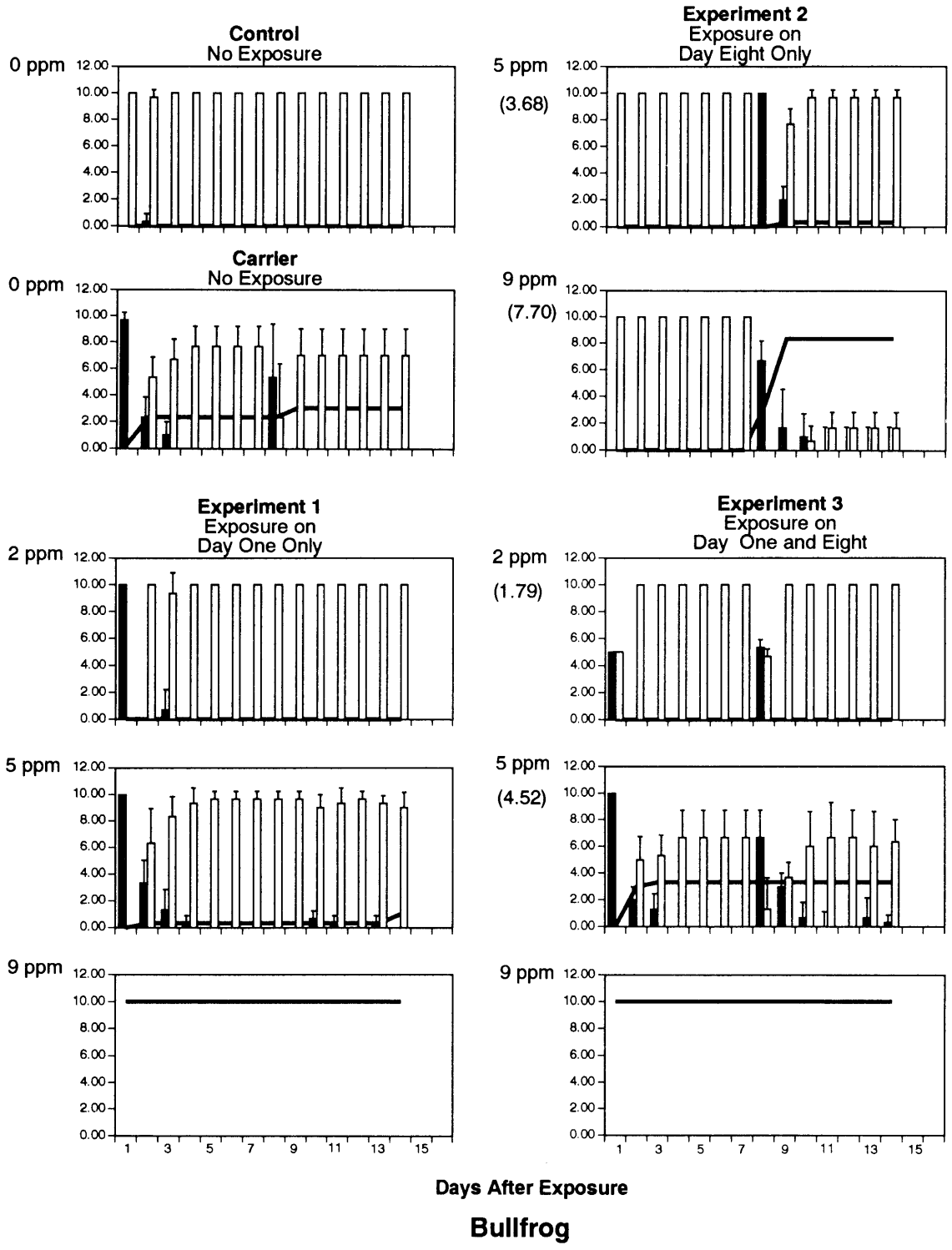

Fig. 3. Effects of exposure of bullfrog tadpoles to fenitrothion. As in Fig. 1, except that 1-ppm experiments as well as 2-ppm of experiment 2 are omitted. Note the considerable carrier effect on day 1 and day 8.

DISCUSSION

Our results indicate that differences in sensitivity of tadpoles to fenitrothion exist between the two developmental stages of the four ranid species that we tested. In wood frog and leopard frog experiments, almost all of the observed mortality occurred after day-8 exposures to 9 ppm fenitrothion, whereas in green frog and bullfrog experiments, mor-

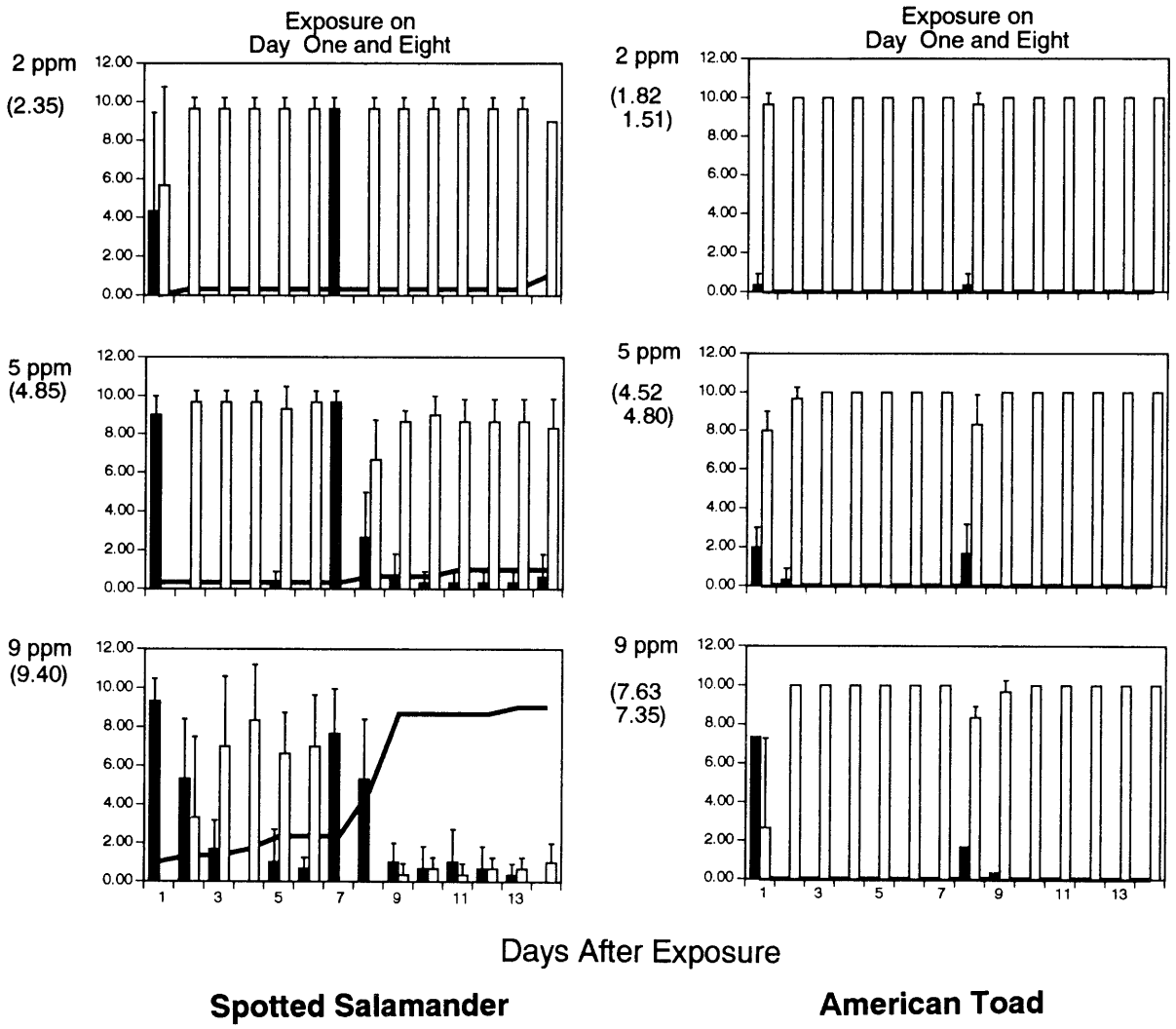

Fig. 4. Effects of exposure of spotted salamander larvae and American toad tadpoles to fenitrothion. As in Fig. 1, except that control, carrier, and 1-ppm experiments are omitted.

tality was greater following day-1 exposures. However, tadpoles exposed on both day 1 and day 8 after hatching were no more sensitive to the second exposure than were those exposed to fenitrothion for the first time on day 8, indicating that exposures that far apart, on those particular stages, and at those concentrations will not result in an increase in direct mortality or more prolonged paralysis. On the other hand, if a tadpole is paralyzed by the first exposure, subject to potential predation or delayed growth until it recovers, it will also be paralyzed by the second exposure and face the same risks again. There may also be more sensitive stages in tadpole development—for example, around the time of metamorphosis, where pulsed exposures may have greater impact.

Certainly green frog and bullfrog tadpoles are more sensitive to fenitrothion exposure than are wood and leopard frog tadpoles, and are also sensitive to exposure to the higher concentrations of the emulsifier/solvent used to carry the insecticide. All four species were affected by exposure to 5 ppm, with green frogs recovering more slowly. At 9 ppm, bullfrog tadpoles died, although their sensitivity to the solvent/emulsifier control probably added to their sensitivity to fenitrothion. However, both green frogs and bullfrogs were affected by exposure to 2 ppm, levels at which any carrier effect ought to have been minimal, for they would have received only one-quarter of the solvent/emulsifier that the tadpoles in the carrier control received. Considering that fenitrothion levels in New Brunswick pond surface water have not been observed to exceed 2.5 ppm, tadpoles may not be as seriously affected by the carrier as they are by the pesticide.

The smaller size of the eggs of both green frogs and bullfrogs, along with their earlier stage of development at hatching, does correlate with their greater sensitivity to experimental exposures. Both features make sense in life-history terms, for both species produce very many eggs in the middle of the summer; tadpoles of neither species grow quickly enough to metamorphose until the middle of the following summer

(green frogs) or the one after that (bullfrogs), so there should be less selective pressure on them to be as large as possible and grow quickly to metamorphosis, compared with wood and leopard frog tadpoles. However, the cost may be greater sensitivity to contaminants, correlated with smaller size and earlier stage of development at hatching. Although the eggs we measured varied very little between broods within each species, egg size in some species is known to vary with size and age of the female [16]; exposing smaller eggs of leopard or wood frogs would also allow us to assess the relationship between sensitivity and developmental stage.

The prediction that a species is consistent in its relative sensitivity or tolerance to contaminants is only partly supported: American toad tadpoles were more tolerant of exposure than we predicted, and the spotted salamander larvae were less sensitive than the pyrethroid experiments [12] suggested they would be. Each species is likely to vary in sensitivity depending upon the pesticide, its concentration, and the stage exposed [17], and exposures of other developmental stages to other concentrations could modify our results. Nonetheless, a comparison of the six species included in our experiments indicates that the American toad, wood frog, and leopard frog are all more tolerant of exposure to fenitrothion than the bullfrog, green frog, or spotted salamander.

Our experiments confirm that newly hatched amphibians are sensitive to exposure to, but not killed by, fenitrothion concentrations approaching those that may rarely occur under the carefully managed conditions of New Brunswick forests. The compounds that carry the insecticide also appear to affect some species, but not others. Previous experiments have satisfied us that embryos are unlikely to be affected by exposures [9]. However, experimental exposures of later developmental stages of tadpoles, particularly premetamorphic stages when the tadpoles are not feeding and are likely to be under developmental stress, will be necessary to understand more fully the impact of contaminants such as fenitrothion on amphibian survival.

Acknowledgement – This research was supported by funds from the Canadian Wildlife Service and the Wildlife Toxicology Fund. We are grateful to Chris Metcalfe for his advice on water chemistry and to Tracy Metcalfe, who undertook the extraction.

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