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EFFECTS OF LOW CONCENTRATIONS OF FOREST-USE PESTICIDES ON FROG EMBRYOS AND TADPOLES

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Abstract—Management of coniferous forests of eastern Canada may involve spraying with the insecticide fenitrothion and the herbicides triclopyr and hexazinone. Because ranid frogs breed in ponds that are unavoidably contaminated by spraying, we measured the toxicity of these chemicals to embryos and tadpoles of *Rana pipiens* (leopard frog), *Rana clamitans* (green frog), and *Rana catesbeiana* (bullfrog) under lab conditions. Embryos were exposed during late neurula stage and tadpoles within 48 h after hatching to fenitrothion (24 h; 0.5–8.0 ppm), triclopyr (48 h; 0.6–4.8 ppm), and hexazinone (8 d; 100 ppm). We measured hatching success of embryos, and for tadpoles, mortality, ability to swim away when prodded, and total body length one week after exposure. Hexazinone had no effects on embryos or tadpoles, even at the unreasonably high levels to which they were exposed. Hatching success of embryos and subsequent avoidance behavior were unaffected in all species by exposures to triclopyr and fenitrothion. Newly hatched tadpoles of all species were very sensitive to 2.4 and 4.8 ppm triclopyr and to 4.0 and 8.0 ppm fenitrothion, either dying or remaining paralyzed following exposure. Tadpoles initially affected by exposure to lower concentrations of fenitrothion or triclopyr usually recovered within 1 to 3 d. Bullfrog and green frog tadpoles appear to be more sensitive than leopard frog tadpoles, and bullfrog tadpoles were consistently more sensitive than green frog tadpoles.

Keywords—Amphibian Pesticides Fenitrothion Triclopyr Tadpoles

INTRODUCTION

Where forest management involves the use of pesticides, a buffer zone around large water bodies is usually left unsprayed, and sites are contaminated only by unintended spray drift. However, small lakes and ponds, often favored by amphibians as breeding sites, are not protected from contamination by buffer zones, and the eggs and tadpoles of the resident species are likely to be exposed to low concentrations of the sprayed chemicals.

The coniferous forests of eastern Canada are intensively managed. For instance, the organophosphorus insecticide fenitrothion is used in New Brunswick to control the spruce budworm *Choristoneura fumiferana*. The organochlorine herbicide triclopyr has been used to control broadleaf vegetation and permit conifer release. Hexazinone, a second herbicide, is being examined experimentally because of its ability to control hardier weed species such as maple trees. In the future, these herbicides may be used more extensively in New Brunswick coniferous forests. Although amphibians breed in small ponds in the forested regions, their sensitivity to these pesticides is not well known. Experiments on the clawed frog, *Xenopus laevis*, indicate that 24-h exposure at 4 to 10 ppm fenitrothion is sufficient to induce abnormal development and kill embryos and tadpoles, and tadpoles exhibit impaired swimming behavior when exposed to 1 ppm fenitrothion [1]. These results suggest that *X. laevis* may be approximately as sensitive as coldwater fish species to fenitrothion [2]. Studies with synthetic pyrethroids also indicate that amphibians are likely to be approximately as sensitive as fish [3,4].

Fenitrothion has been used in forest pest control in Canada since 1965. Fenitrothion is a nonreversible ChE inhibitor, and animals exposed to it experience depressed brain acetylcholinesterase activity; the rate of recovery is directly related to extent of depression [5,6]. Spraying forests at an application rate of 400 g/ha reduces the reproductive fitness of passerine birds [7,8]. Following operational spray applications in New Brunswick, maximum residue concentrations in exposed water bodies were approximately 0.2 ppm [9], but levels in the surface layers of small ponds sometimes reached 2.5 ppm [10]. Acute toxicity tests with fenitrothion indicate that 96-h LC50s for some fish species are as low as 1 to 3 ppm [11], whereas sublethal effects, including diminished foraging success, may occur at concentrations of <0.1 ppm [6]. Fenitrothion has a short aquatic half-life of 24 to 48 h, breaking down to less toxic products by photolysis and hydrolysis. Aquatic organisms, including amphibian eggs and tadpoles, are therefore likely to be exposed to low concentrations of fenitrothion for relatively brief periods.

The herbicide triclopyr is marketed in two quite different formulations. The triethylamine formulation (Garlon 3A®) is not toxic to fish at concentrations below 200 ppm [12], but the butoxyethyl ester (Release® and Garlon 4®), a more effective herbicide, is far more toxic, with acute toxicity tests indicating 96-h LC50s of 1 to 3 ppm [12,13]. Such levels are quite possible; operational applications at a rate of 2.4 kg a.i./ha could result in 4 ppm of the ester in water 15 cm deep

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[13]. The ester is quickly absorbed by exposed fish, which also deesterify it rapidly, with triclopyr acid the primary metabolite and residue [14]. The ester breaks down rapidly in warm, clear, sunlit water, with an estimated half-life of 6 to 24 h [15], and it dissipates quickly in streams. However, its half-life in cool, shaded, turbid water of forest ponds is likely to be considerably longer, as photolysis is a major route of dissipation for the compound [16].

The second herbicide, hexazinone, is considered to be only slightly toxic to animals tested at levels below 200 ppm [17] and appears to have no effect on exposed stream macroinvertebrates or terrestrial microarthropods [18]. Experimental application to a watershed resulted in a maximum stream concentration of 14 ppm, far below any toxic level [19]. Hexazinone degrades slowly, primarily through microbial activity, but its persistence in water or sediment is unlikely to be stressful to the resident animals. We include it in our study, expecting exposed embryos and tadpoles to be unaffected.

Five species of ranid anurans, differing in life histories, breed in New Brunswick ponds; thus, their eggs or tadpoles potentially are affected by spraying. *Rana sylvatica*, the wood frog, and *R. pipiens*, the leopard frog, both breed explosively in spring and metamorphose in early and midsummer, respectively [20]. The remaining three species breed over a more prolonged period during midsummer; tadpoles of both *R. clamitans*, the green frog, and *R. septentrionalis*, the mink frog, overwinter once and metamorphose in the middle of the following summer, whereas tadpoles of *R. catesbeiana*, the bullfrog, overwinter a second time before metamorphosis. All three are likely to be exposed to spraying at more than one stage in their aquatic life histories.

Sensitivity to the pesticides may vary with stage and species. Experimental exposure of leopard frogs and green frogs to low concentrations of pyrethroid insecticides indicated that newly hatched tadpoles are considerably more sensitive than embryo stages [3]. It is also possible that some species are more sensitive than other closely related species; the wood frog is well known to be particularly tolerant of low pH [21], and experimental exposure to pyrethroids indicated that green frog tadpoles are consistently more sensitive than leopard frog tadpoles [3].

We assessed the effects of relatively low concentrations of fenitrothion and triclopyr in lab experiments, looking at embryo hatching success, and for tadpoles, mortality and sublethal effects such as delayed growth, paralysis, and abnormal avoidance behavior. We selected tadpole avoidance response as an indicator of relative good health partly because it is unambiguously monitored, and therefore useful in comparative studies [3,22], but also because its absence could severely reduce antipredator success of affected tadpoles [23]. Retarded development could also reduce tadpole survival. In early spring breeders it is necessary to metamorphose before temporary ponds dry up; delayed early development could result in failure of metamorphosis to occur in time. In all species, retarded development could also result in smaller metamorphs, which are less likely to survive predation [24] or reach sexual maturity. Based on the limited comparative work on amphibian sensitivity to pesticides, we made the following predictions concerning ranid sensitivity to fenitrothion and triclopyr: (a) Frog tadpoles are likely to be as sensitive to experimental exposure as fish; (b) frog species should differ in sensitivity; (c) embryos and newly hatched tadpoles should differ in sensitivity; and (d) sublethal effects should include behavioral and growth changes that potentially reduce later survival. We conducted preliminary tests on wood frog embryos and tadpoles and tested the above predictions on leopard, green, and bullfrog embryos and newly hatched tadpoles.

METHODS

Egg collections and tadpole culture

Egg masses of four ranid species (wood, leopard, green, and bullfrogs) were collected shortly after they were laid from ponds in central Ontario during the spring and summer of 1992. Collected egg masses were cultured in the lab until the embryos reached midneurulation stage (for embryo exposures) or until 1 or 2 d after hatching (for tadpole exposures). Following exposures to the pesticides, surviving tadpoles were cultured for a further two-week recovery period, during which they were fed small amounts of boiled lettuce.

Experimental conditions

Midneurula embryos and newly hatched tadpoles of leopard frogs, green frogs, and bullfrogs were exposed to the three pesticides. Embryos and tadpoles were exposed and recovered in 1-L beakers half-filled with filtered aerated river water in darkness at 15°C in a controlled environmental chamber. Twenty embryos or 10 newly hatched tadpoles, all <1.0 cm long, were exposed in each beaker. Because the three species breed sequentially during the summer, they were tested one at a time, with the embryo and tadpole experiments of each species overlapping. Experiments began in early May and continued until August.

The first species available to us in the spring was the wood frog, and preliminary range-finding tests with its embryos and tadpoles guided us in selecting exposure levels. Exposure to fenitrothion lasted 24 h, and nominal exposure concentrations were 0.5, 1.0, 2.0, 4.0, and 8.0 ppm. Exposures to the ester formulation of triclopyr lasted 48 h at 0.6, 1.2, 2.4, and 4.8 ppm nominal concentrations. Hexazinone exposures lasted for at least 8 d at 100 ppm.

Fresh 500-ml stock solutions of each chemical were prepared from 96 to 99% technical-grade formulations (240 g a.i. hexazinone per liter, 480 g a.i. triclopyr per liter, and 1,260 g a.i. fenitrothion per liter), resulting in 1,000-ppm solutions of fenitrothion and 480-ppm solutions of triclopyr. Fenitrothion is very weakly soluble in water. Therefore, before preparing the stock solution of fenitrothion, the fenitrothion was dissolved in an equal volume of Dawanol; Atlox, an emulsifier, was then added in a volume equal to that of the solvent. All chemicals were supplied by Natural Resources Canada. The result approximates the formulation used in forest spraying. Appropriate volumes of fenitrothion and triclopyr were added to 500 ml filtered river water to make up the experimental exposures. Each of the three replicates of the nominal concentrations—0.5, 2.0, and 8.0 ppm fenitrothion—were subsampled and combined for extractions and residue analysis. The same procedure was completed for

the 0.6-, 1.2-, and 4.8-ppm nominal concentrations of triclopyr, except the combined subsamples were stored at −15°C pending extraction and residue analysis. Controls consisted of uncontaminated filtered river water, and filtered river water to which both Dawanol and the emulsifier were added at levels equal to the maximum carrying the fenitrothion. Each experiment therefore consisted of 10 treatments and two controls, run in triplicate. Constant brisk aeration of all beakers throughout the exposure periods ensured relatively uniform conditions for the exposed animals.

Measurements

Embryo experiments. Individuals were examined daily and any dead animals removed. Both hatching success and timing of hatching were determined for all embryos following exposures. Any gross morphological abnormalities in the subsequent newly hatched tadpoles were noted. Hatched tadpoles were prodded daily to assess whether they reacted with the normal avoidance response of darting away or whether they reacted abnormally by inactivity, twitching, or weak movement over a short distance. Experiments were terminated 9 d after the embryos hatched.

Tadpole experiments. All tadpoles were examined daily for mortality and prodded for assessment of their avoidance response. Nine days after exposure, snout–vent length of the tadpoles was measured, and in the case of bullfrog tadpoles, stage of tadpole development was determined following Gosner staging [25]. Experiments on tadpoles were terminated 9 d after the conclusion of the exposures.

Residue analysis

Fenitrothion. Fenitrothion was extracted from the water samples [26], and the extractions were analyzed on a GC equipped with a flame photometric detector (sensitivity 13 μg/L). Reproducibility and linearity quality-control instrument checks were performed before the analysis of each pesticide set. The samples were diluted appropriately to ensure that sample chromatographic peaks were within the working range of the calibration standards. Each sample was analyzed in duplicate, with final values reported as averages of the duplicate analyses. Nominal concentrations are used for consistency in our discussion, but residual values are included in Figures 1 to 3.

Triclopyr. Extractions of triclopyr acid were stored at 4°C for final analysis on a GC equipped with an electron-capture detector. As with fenitrothion, each sample was analyzed in duplicate, samples were diluted appropriately, and reproducibility and linearity quality-control checks were performed before analysis. Unfortunately, the lack of an analytical standard meant that triclopyr ester analyses could not be done, and only nominal concentrations are reported here.

RESULTS

Residue analysis

Fenitrothion residues from water samples taken at the onset of exposures were approximately 50% of the nominal values. A small percentage of that loss may be attributable to plating out onto the glass of the beaker containers.

Triclopyr acid residues from water samples taken at the onset of exposures ranged from 0.02 to 0.3 ppm; 24 h later these residue levels rose to 0.07 to 1.2 ppm. This indicates that the triclopyr butoxyethyl ester formulation underwent little hydrolysis to triclopyr acid at 15°C in the darkness of the experimental conditions during the first 24 h of the exposure period. These results suggest that the bioassays were performed with the ester formulation of triclopyr.

Preliminary experiments

Wood frog embryos and tadpoles were exposed to low levels of all three chemicals in preliminary range-finding experiments. Embryos ($n = 100$) exposed to nominal concentrations of either 0.25 or 2.0 ppm fenitrothion or hexazinone for 48 h hatched normally, as did embryos exposed to either 0.15 or 1.2 ppm triclopyr. Tadpoles ($n = 20$ per treatment) were unaffected by a 48-h exposure to 0.15 and 1.2 ppm triclopyr or by 24-h exposure to 2.0 ppm fenitrothion. However, tadpoles exposed to 4.0 ppm fenitrothion were initially unresponsive to prodding, recovering within 24 h.

Embryo experiments

Timing of hatching and hatching success. Midneurula embryos of all three species were exposed to the three chemicals. During exposure, embryos were either at Gosner stage 14 or 15 [25], depending on the egg mass. None of the embryos hatched before completion of the 24- to 48-h exposures. There was <5% hatch failure throughout all treatments, and once hatching began, all members of an egg mass hatched within 36 h. Morphological abnormalities were infrequent (<5% per treatment). Embryos exposed to the three chemicals hatched in synchrony with untreated embryos. No tadpoles died during the 9-d recovery period following hatching.

Sublethal effects. All hatched tadpoles responded to prodding with the normal avoidance response, and their behavior was indistinguishable from tadpoles that had been exposed only to filtered river water. When measured for total body length at the end of the experiments, there were no significant differences among the treatments (ANOVAs, $p < 0.05$). Tadpoles of the three species appear to have been unaffected by their exposure as embryos to fenitrothion, triclopyr, or hexazinone.

Tadpole experiments

Hexazinone exposure. Newly hatched tadpoles of both leopard frogs and green frogs appeared to be unaffected by continuous exposure to 100 ppm hexazinone for 9 d. They exhibited no mortality nor any indication of diminished avoidance response when prodded (Figs. 1 and 2). However, bullfrog tadpoles were initially unresponsive to prodding, gradually recovering over the duration of the exposure (Fig. 3). The occasional mortality of hexazinone-exposed bullfrog tadpoles did not differ from that observed in the control treatments.

Triclopyr exposure. Tadpoles of the three species exposed to 0.6 and 1.2 ppm triclopyr for 48 h suffered minimal mortality. At 0.6 ppm, avoidance behavior was unaffected; however, at 1.2 ppm, more than half of the tadpoles did not respond to prodding during the day following onset of exposure. These tadpoles all recovered within 3 d following

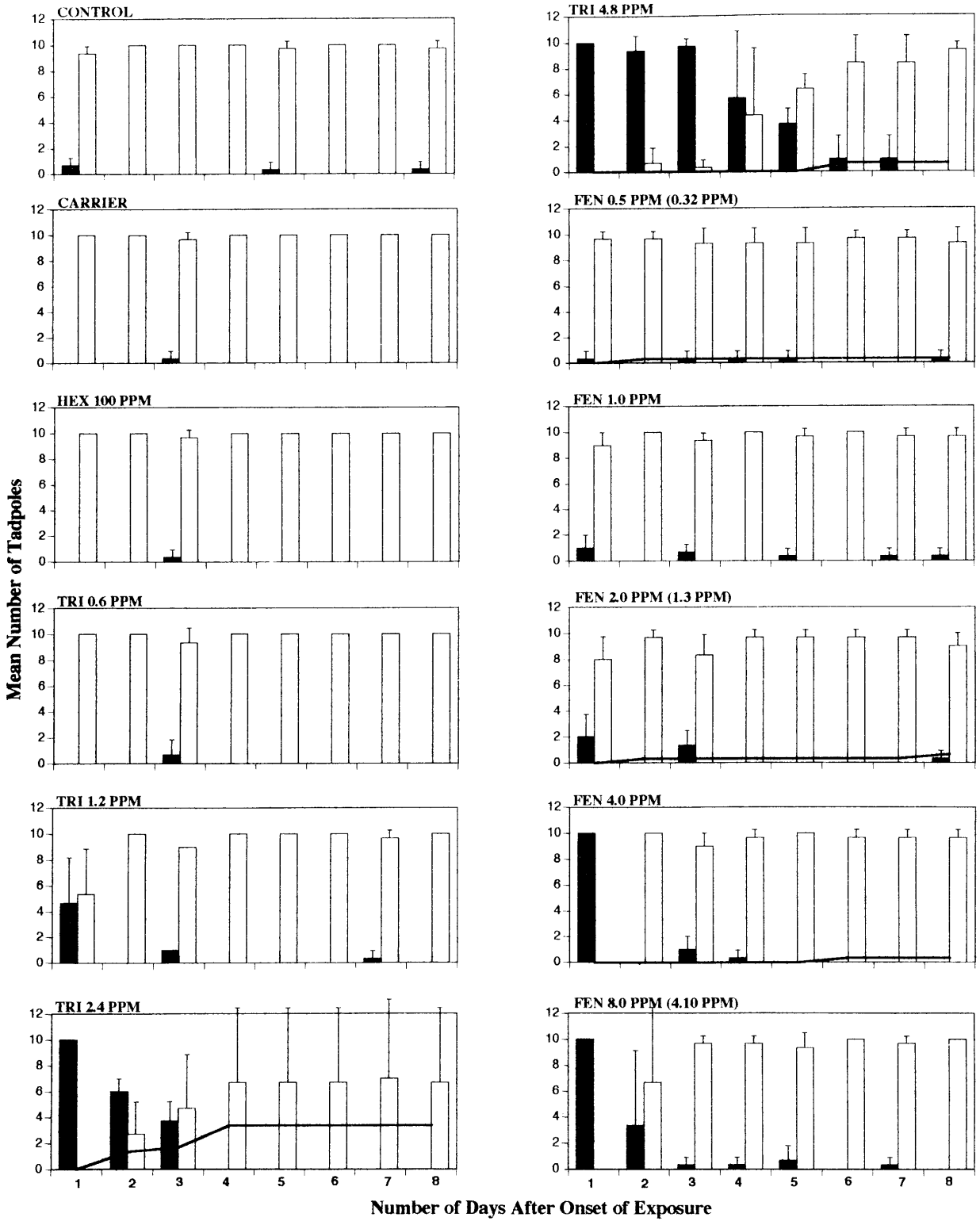

Fig. 1. Response of newly hatched leopard frog tadpoles during and following exposure to triclopyr for 2 d and fenitrothion for 1 d, and during exposure to hexazinone for 8 d. White bars indicate the mean number of tadpoles per treatment able to dart away with a normal avoidance response when prodded. Black bars indicate the mean number of tadpoles per treatment that were unable to respond to prodding by darting away, but twitched in place or were completely unresponsive. Error bars indicate standard deviations of triplicate experiment. The black line indicates mean accumulated mortality. "Carrier" refers to a positive control exposure containing the solvent and emulsifier necessary to solubilize fenitrothion. Nominal concentrations are represented by the numbers in the top left corner of each figure; measured residual concentrations—where available—are in brackets beside the nominal values. Hex = hexazinone, Fen = fenitrothion, Tri = triclopyr.

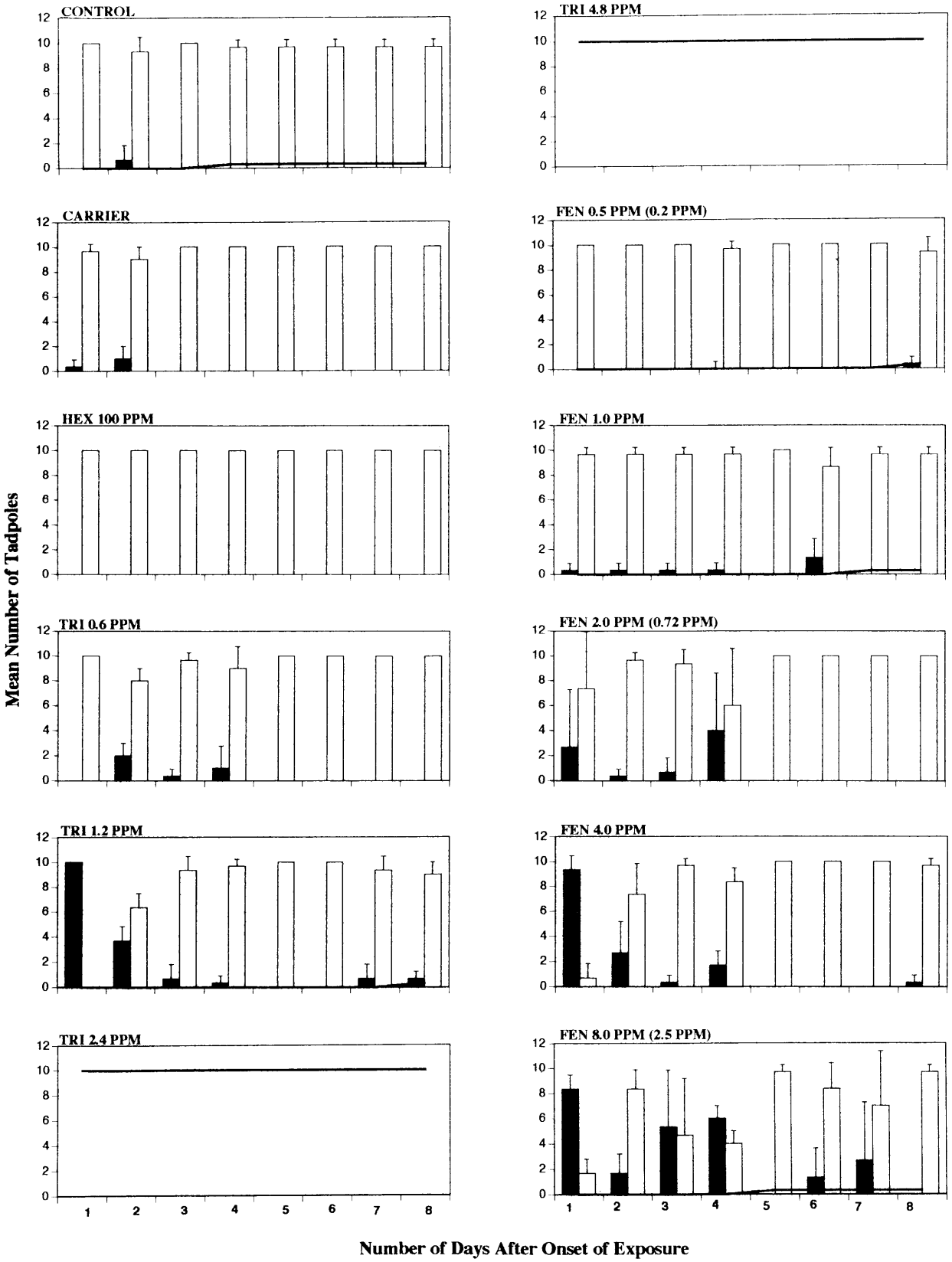

Fig. 2. Response of newly hatched green frog tadpoles following exposure to triclopyr and fenitrothion and during exposure to hexazinone, as in Figure 1. Note that all tadpoles exposed to 2.4 and 4.8 ppm triclopyr died during exposure.

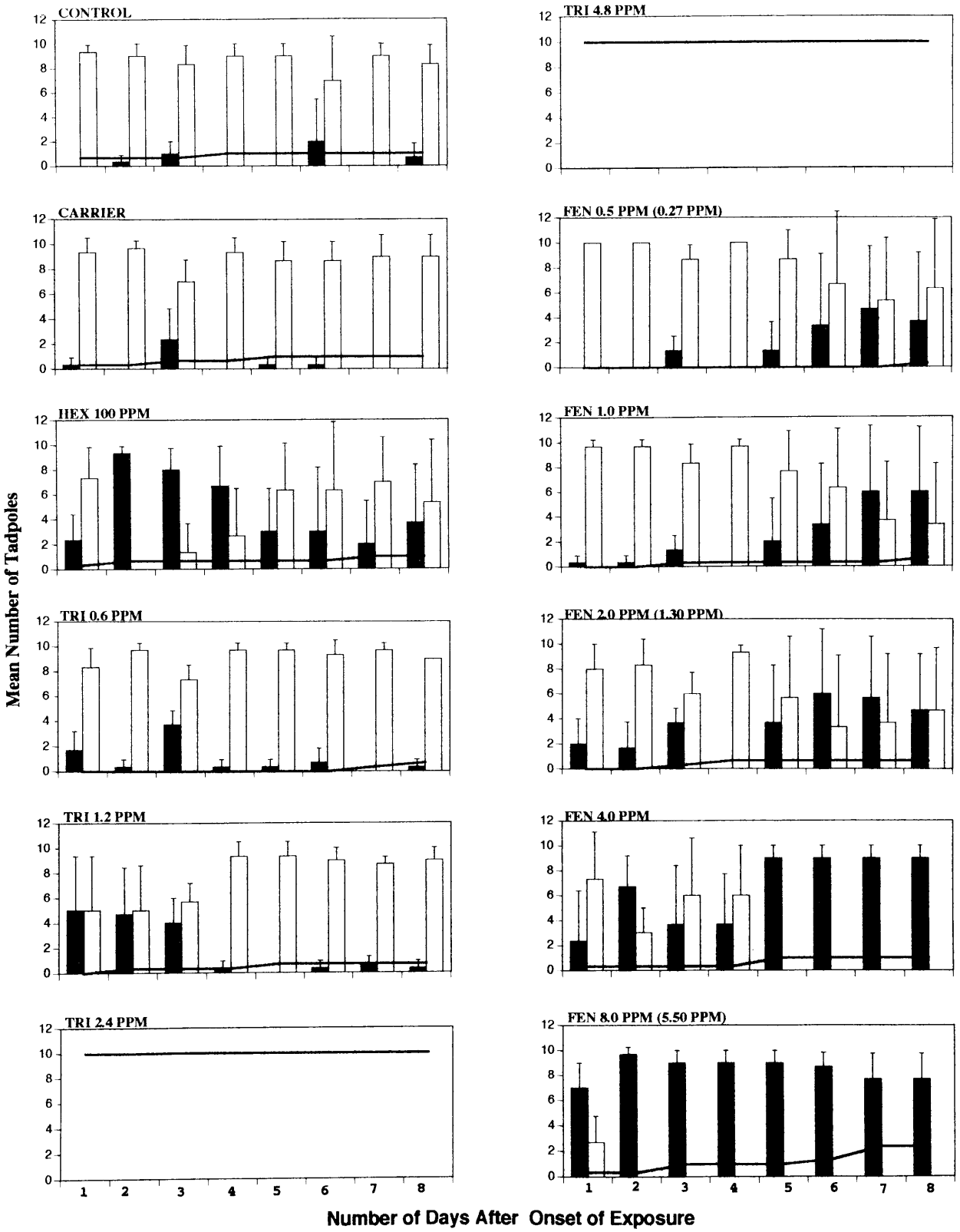

Fig. 3. Response of newly hatched bullfrog tadpoles following exposure to triclopyr and fenitrothion and during exposure to hexazinone as in Figure 1. Note that all tadpoles exposed to triclopyr 2.4 and 4.8 ppm died during exposure, and that all became paralyzed following exposure to 4.0 and 8.0 ppm fenitrothion.

onset of exposure (Figs. 1, 2, and 3). At 2.4 and 4.8 ppm triclopyr, all green frog and bullfrog tadpoles died. Although relatively few leopard frog tadpoles died following exposure to 2.4 and 4.8 ppm triclopyr, all were initially unresponsive to prodding. Most of those exposed to 2.4 ppm recovered within 3 d, whereas those exposed to 4.8 ppm took several days longer.

Fenitrothion exposure. Leopard frog and green frog tadpoles were unaffected by exposure to 0.5 and 1.0 ppm fenitrothion. Although a few were initially unresponsive to prodding following exposure to 2.0 ppm, all recovered within a few days. At 4.0 and 8.0 ppm, tadpoles appeared to be paralyzed following exposure. All recovered within 3 d, except for the green frog tadpoles exposed to 8.0 ppm, which recovered more slowly (Figs. 1 and 2). In contrast, bullfrog tadpoles were consistently more sensitive to the exposures. The extent of their unresponsiveness to prodding following exposure to 0.5, 1.0, and 2.0 ppm fenitrothion increased with level of exposure. Moreover, this unresponsiveness increased rather than diminished as time passed. Following exposure to 4.0 and 8.0 ppm, all surviving bullfrog tadpoles appeared paralyzed and had not recovered at the end of the experiment (Fig. 3). Following exposure to 8.0 ppm, bullfrog tadpoles were smaller and had developed more slowly (Gosner stage 19) than tadpoles recovering from exposure to any of the other treatments (Gosner stages 22–24).

DISCUSSION

Ranid tadpoles are likely to be paralyzed or killed by residues of fenitrothion or the ester formulation of triclopyr that could occur in small ponds as a result of forest management spraying programs. Paralysis is likely to render tadpoles more vulnerable to predation, and when it is associated with slower growth it could also reduce later reproductive fitness. Residues, at approximately 50% of nominal values, were lower than we expected. Although a small percentage adhered to beaker glass and air stones, we are uncertain at what stage the remaining loss occurred. As we have no reason to doubt the nominal values, we have chosen to consider them to be accurate, for they demand a more conservative interpretation of the data.

Experiments with fish eggs indicate that the egg chorion is not an effective barrier to fenitrothion [27]. However, the jelly layer surrounding frog eggs may provide them with some protection from fenitrothion and triclopyr, for even with our highest exposure levels of 8 ppm and 4.8 ppm, respectively, there was no apparent effect on embryo hatching success. We have also seen that although fish eggs are harmed by exposure to pyrethroids, amphibian embryos hatch without apparent abnormalities [3], indicating protection from direct exposure to these insecticides. It remains possible, however, that higher exposure levels may affect earlier embryo stages, for example, at gastrulation, as in the case of *X. laevis* [1].

Unlike embryos, tadpoles are approximately as sensitive to these chemicals as fish. Both the herbicide triclopyr, in its ester formulation, and the insecticide fenitrothion are likely to have a severe impact on young tadpoles of ranid frogs exposed for 24 h to 2.4 or 4.0 ppm, respectively. Those not killed by the exposure suffer paralysis from which they may not recover. Exposure to lower concentrations of either 1.0 or 2.0 ppm fenitrothion, or 0.6 or 1.2 ppm triclopyr, is likely to paralyze the more sensitive tadpoles, and such exposure levels may occur in ponds in a managed forest system. In contrast, hexazinone use is not likely to have any direct effect on the resident amphibian tadpoles at the concentrations to which they might be exposed.

The timing of contamination is also critical, for a spraying event that coincides with frog egg development is likely to have few direct effects on later tadpole abundance. However, with ranids breeding sequentially throughout the summer, and breeding periods of later breeding species such as green frogs, mink frogs, and bullfrogs extending over many weeks, there is no portion of the spring and summer when pond contamination will not affect at least one of the species. The later breeding species also have prolonged tadpole stages extending through at least one winter, and tadpoles may be exposed to each of the forest-use chemicals at different stages in their aquatic life histories. Reexposure could either increase sensitivity or confer some resistance, and further investigation on the effects of pulsed exposures is necessary. However, we conclude that continued use of both fenitrothion and triclopyr ester in forest management must assume a negative impact on amphibian communities in the small forest ponds contaminated as a result.

Although the three ranid species we tested were relatively consistent in their responses to the three chemicals, there appear to be small but distinct differences between the species. Bullfrog tadpoles are the most sensitive of the three species, and green frog tadpoles appear to be more sensitive to fenitrothion and triclopyr than leopard frog tadpoles. These species differences may imply differences in the sensitivity of newly hatched stages that are worth further investigation. The embryos of different species appear to hatch at slightly different developmental stages. Because we tested newly hatched tadpoles of each species, irrespective of exact developmental stage, we may not have compared identical stages. As a result, it is possible that the observed differences are actually differences in the sensitivity of early tadpole stages.

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