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EFFECTS OF LARVAL EXPOSURE TO TRIPHENYLTIN ON THE SURVIVAL, GROWTH, AND BEHAVIOR OF LARVAL AND JUVENILE *AMBYSTOMA BARBOURI* SALAMANDERS

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Abstract—We exposed 10 sibships of the streamside salamander, *Ambystoma barbouri*, to two concentrations of triphenyltin (TPT) (1 and 5 μg/L) and an acetone carrier control for the entirety of the larval period. We measured effects on larval feeding rates, escape behavior, growth rates, and survival to, days to, and size at metamorphosis. Postmetamorphosis, we monitored feeding rates, growth rates, and survival of juvenile *A. barbouri* in order to investigate carryover effects. The 5-μg/L TPT concentration resulted in 93% mortality of the larvae. Exposure to 1 μg/L TPT had no mortality effect and no effect on the escape behavior of larvae. However, larvae exposed to this TPT concentration had significantly lower feeding rates and growth rates and therefore metamorphosed later than the controls but at the same mass. We detected a direct effect of TPT on growth rates beyond the effect through depressed feeding rates. We also found significant evidence for variation among sibships in their sensitivity to TPT toxicity. Once exposure was terminated at metamorphosis, we observed no residual effects of TPT on juveniles. Survival, feeding, and growth rates of juveniles exposed to TPT as larvae were not significantly different from those exposed only to the acetone carrier.

Keywords—Triphenyltin Behavior Survival Growth Amphibian

INTRODUCTION

Today, aquatic systems all around the world face serious threats from anthropogenic contaminants. For aquatic biota, organotins are one of the most harmful organic contaminants [1]. Among organotins, the use of tributyltin (TBT) as a biocide has received considerable attention in the past two decades because of its devastating effects on marine ecosystems. Another important trisubstituted organotin pollutant is the pesticide triphenyltin (TPT), which is primarily used in agriculture in crops such as pecans, potatoes, sugar beets, celery, coffee, and rice [1,2]. Triphenyltin enters the aquatic environment mostly through runoff and leaching from agricultural fields and through some direct application in the case of rice fields. Triphenyltin has also been observed in rainwater after evaporation from an agricultural field [3]. Additionally, TPT is utilized as an ingredient in TBT-based antifoulant marine paints, but only in small quantities (~8%) [4].

While the high toxicity of TBT in marine ecosystems has been well demonstrated, few studies have addressed the ecotoxicological effects of TPT [1]. This is important because comparative studies suggest that TPT can be more toxic than TBT due to increased biosorption and lipid solubility [5,6], reduced elimination [4,7–9], and greater biomagnification [4]. For instance, TPT concentrations in tissues of several fish species in a lake exposed to both TBT and TPT were found to be three times greater than TBT levels, and TPT concentration was also reported to increase with fish age [4]. These findings point to a higher metabolic elimination of TBT compared with TPT, which results in a net accumulation of TPT in exposed organisms. Bioconcentration in fish, such as *Lebistes reticulatus*, exposed to equal concentrations of TPT and TBT were also found to be twice as high for TPT [10]. For benthic organisms and algae exposed to TBT and TPT, greater mortality was reported in the benthic organisms exposed to TBT, but inhibition of growth and photosynthesis were greater for TPT-exposed microalgae [11]. Additionally, recent studies suggest that uptake of organotins might be greater in freshwater than in saltwater because of higher freshwater solubilities [6,10,12]. This is important both because most studies on the toxicity of organotins have been conducted on marine biota and because TPT is the prevalent organotin in freshwater ecosystems. Furthermore, while strict regulations for the use of TBT as an antifoulant have been in place in many regions of the world since the 1980s, environmental standards for the use of TPT as an agricultural fungicide have only been recently implemented [2] and are still needed in many countries [4].

The acute toxicity of TPT on nontarget organisms has been documented in fishes and aquatic invertebrates since the 1970s [13–15]. However, tests at environmentally relevant concentrations were not conducted until recent years [1]. Even with fishes, few studies have looked at the sublethal effects of TPT exposure [16] or at the effects on more than one life stage [7,17,18]. In amphibians, studies on the ecotoxicological impacts of chemical stresses are, in general, less common [19]. Moreover, some evidence suggests that amphibians may be more sensitive to some contaminants than other vertebrates such as fish [20]. Only two studies have looked at the effects of TPT on amphibians [19,21]. Furthermore, no studies have explored the effects of chronic exposure of amphibian larvae to TPT on later life stages after exposure has been terminated (i.e., postmetamorphosis).

In this study, we investigated the behavioral, lethal, and growth effects of long-term exposure to TPT in the streamside salamander, *Ambystoma barbouri*. Larvae were exposed to two

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concentrations of TPT, 1 and 5 μg/L, from hatching until metamorphosis. We examined the sensitivity of *A. barbouri* larvae to TPT by measuring effects on feeding rates, escape behavior, growth rates, duration of larval period, survival to metamorphosis, and size at metamorphosis. After metamorphosis, we continued to monitor for carryover effects of larval TPT exposure for over 16 months by measuring feeding rates, growth rates, and survival on a subset of the terrestrial juvenile *A. barbouri*. Because of our interest in addressing the role of genetic variation in the response of this population to TPT exposure, 10 sibships were randomly selected as test subjects. The two TPT concentrations were selected to examine both the lethal and the nonlethal behavioral and life history effects of this pollutant. The 1-μg/L concentration is lower than concentrations detected in a freshwater habitat following routine TPT application over a nearby agricultural field, 1.5 μg/L [22], and only slightly higher than the highest concentrations of TPT found associated with freshwater marinas, 0.8 μg/L [23]. The 5-μg/L concentration is lower than mean lethal concentrations (LC50) observed for fishes, 30 μg/L and 7.1 μg/L (96 h and 48 h, respectively) [13,15], but higher than concentrations known to cause mortality in *Rana* tadpoles, 1.87 μg/L after chronic larval exposure [19].

Ambystoma barbouri breeds in ephemeral streams in central Kentucky, southern Indiana, and southern Ohio [24]. Females lay their eggs on the undersides of submerged limestone rocks from January through early April. Hatching occurs in April and early May. The larvae live in these streams for approximately 6 to 10 weeks before metamorphosis in late May to mid-July [25]. Little is known about the terrestrial ecology of this salamander, including juvenile growth rates and age at sexual maturity [26]. The streams in which the larvae develop are highly ephemeral, usually drying completely by early to midsummer. Because larvae are typically restricted to the more ephemeral upper portions of streams, they often suffer heavy mortality from stream drying [27]. Fish are generally absent from these ephemeral stretches; however, in some streams, including our study site, larvae suffer heavy mortality in pools with fish [28]. Another important source of mortality is stream flooding during the early spring [25]. Since streams are often embedded in agricultural areas where crops are routinely treated with fertilizers, insecticides, herbicides, and fungicides, we expect stream flooding, along with leaching and agricultural runoff, to be potential sources of environmental toxins to these streams. We do not have data to indicate that *A. barbouri* is, in fact, being exposed to TPT in the wild. However, *A. barbouri* has a rather restricted range, which makes it a species of concern for conservation. Recent findings support the idea that more narrowly distributed, and therefore more narrowly adapted, species are more susceptible to contaminant stress [19]. *Ambystoma barbouri* was also chosen to explore effects of this organotin on a nonanuran amphibian, for which extensive data are available on the ecology and life history of the aquatic larvae.

MATERIALS AND METHODS

Experimental design and treatments

Ambystoma barbouri larvae were exposed to three chemical treatments, a high TPT concentration (5 μg/L), a low TPT concentration (1 μg/L), and an acetone-carrier control. Each treatment was applied to three larvae per sibship in a randomized block design with 10 sibships as blocks, i.e., a total of 9 individuals per sibship and 30 individuals per treatment. *Ambystoma barbouri* eggs were collected from Raven Run Creek in Raven Run Nature Preserve (Fayette County, KY, USA). Ten clutches were brought back to the lab and incubated at increasing temperatures (5–18°C) until hatching. Once all eggs had hatched, nine larvae were randomly selected, measured (snout–vent lengths), and randomly assigned to the three treatments. Individual larvae were raised in 1-L glass mason jars with 600 ml of filtered water, treatment chemicals, and aeration from hatching until metamorphosis. Exposure to the two TPT concentrations and the acetone-carrier control was started within 48 h of the hatching of all clutches and continued through metamorphosis. For the first two weeks of development, larvae were fed ad libitum a combination of *Daphnia* sp. and blackworms, *Lumbriculus variegatus*. They were then switched to an exclusive diet of ad libitum blackworms until metamorphosis. Food was replenished daily if consumed, and after water changes, fresh food was added. For the duration of the exposure period, larvae were inspected daily to add worms, check for mortality, and, later on, check for metamorphs. Larval exposure was conducted at room temperature (21–24°C) and under a 13:11-h light:dark photoperiod. The pH of the filtered water ranged from 7.08 to 8.20.

We used triphenyltin chloride (Aldrich Chemical, Milwaukee, WI, USA; purity $>$ 95%) dissolved in acetone for both TPT treatments. A stock solution was made by dissolving 20 mg of TPT chloride in 20 ml of acetone. Two working solutions were achieved by adding a measured amount of the stock solution to another 20 ml of acetone. Triphenyltin treatments were dosed with 100 μl of the appropriate working solution, while for the control treatment, 100 μl of acetone were directly added to the 600 ml of filtered water in each experimental container. Exposure to acetone at this concentration has been previously shown to have no negative effects on any of the traits measured in this study in either fish or amphibians [19,21,29]. Water changes and chemical renewal were done every 96 h. Actual exposure concentrations were not determined, and only nominal concentrations were recorded. Based on previous studies, actual TPT exposure levels after 96 h were probably only slightly to somewhat lower than nominal values. For example, TPT concentrations were reported to decrease by only 1 to 11% over a 96-h period in static acute tests with TPT hydroxide [15]. Other work showed stable TPT chloride concentrations over a 72-h period with no significant differences between nominal and measured TPT concentrations [19]. Small decreases in concentrations were the result of adsorption of TPT to food items or to direct uptake through the skin of tadpoles [30]. In contrast, another study over a 48-h period showed a 28% decrease in TPT chloride levels due to incorporation of TPT into fish, while adsorption to glassware and loss by evaporation were not significant [17]. Overall, TPT levels appear reasonably stable over the time scale of our study except for uptake by the study organisms.

Larval behavior and growth

Feeding rates were quantified twice over larval development, at days 16 and 24 from the start of exposure, referred to as feeding rates 1 and 2, respectively. In both trials, larvae were given a weighed amount of blackworms over a 24-h period without a starvation period. After 24 h, the remaining worms were recovered and reweighed to determine the amount of worms consumed. For feeding trial 1, each larva was fed 0.200 g ($\pm$0.020) of blackworms, while for feeding trial 2, a larger amount of worms was used, 0.500 g ($\pm$0.020 g). Along

with these feeding trials, we conducted control trials where we assessed the weight loss/gain of the blackworms in the absence of larvae in the same three chemical concentrations used in the experiment (five replicates per treatment). We found no significant difference in mean worm weight loss/gain among the treatments. Worms on average lost 6.2 mg ± 2.2 standard error (SE) (3%) in feeding trial 1 and 40.7 mg ± 4.6 SE (8%) in feeding trial 2. We subtracted these amounts from all feeding rates measured in trials 1 and 2, respectively. This correction resulted in 11 individuals in feeding trial 2 having negative feeding rates. For analysis, these negative values were assumed to be zero. Following each feeding trial, we obtained body mass of each larva. Weights were used to calculate larval growth rates over three time intervals: days 1 to 18, days 18 to 26, and day 26 to metamorphosis. These intervals are referred to as growth periods 1, 2, and 3, respectively.

To quantify the effects of TPT on swimming behavior, we tested the escape response of the *Ambystoma* larvae on days 30 to 33 of exposure. Larvae were placed individually in a large arena, acclimated, and gently tapped with a blunt probe behind their hind legs in order to elicit a predator escape response. The arena consisted of a wading pool (34-cm radius) with 3 cm of water (water temperature 22–23°C), at the center of which larvae were acclimated in a mesh cup (2.5-cm radius) for 15 min before each trial. For each larva, three trials were recorded on a Sony Hi8 video camera, from which one trial was later chosen for analysis. Escape responses were analyzed using the Peak Motus motion analysis system (Ver 2.1, Peak Performance Technologies, Englewood, CO, USA). All trials were cropped to 40 frames (0.67 s), with each trial starting one frame before the initiation of movement away from the probe. Salamander movement was obtained by digitizing the center of the head of each individual and tracking its movement over the frames. Distances traveled by the larvae were calibrated by digitizing two points marked in the pool. Maximum acceleration, average acceleration, maximum velocity, and average velocity were automatically obtained for each trial.

Juvenile behavior and growth

Exposure to TPT and to the acetone control was terminated when each individual completed metamorphosis. Time of metamorphosis in these salamanders was defined by the resorption of the external gills. The first metamorph was recorded on day 32 and the last metamorph on day 96 from the onset of exposure. Following metamorphosis, we continued to monitor the effects of larval exposure to TPT on the survival, growth rates, and feeding rates of these organisms for 484 d after the last larva metamorphosed, making the total duration of the experiment 580 d. These terrestrial salamanders are referred to as juveniles, although we cannot be certain they had not reached sexual maturity by the end of the study. Only a subset of the metamorphs was kept for monitoring of post-exposure effects. On day 73 from the onset of exposure and when there were only three larvae in the experiment still to metamorphose, we removed randomly chosen metamorphs from the treatments to reduce our sample size to a maximum of two individuals per sibship (20 per treatment).

Once metamorphosed, each individual was immediately removed from the TPT-treated water, weighed, and placed in a clear plastic container (18.1-cm diameter × 7.6-cm height) with two ceramic tiles (11 cm × 11 cm), a moist cloth, and clean filtered water about 2 cm deep. When individuals were observed out of the water and taking refuge in the moist cloth (usually within 2 d), the water and tiles were removed and salamanders were fed ad libitum a combination of flightless *Drosophila* sp. and one-week-old crickets, *Acheta domestica*. Within 10 d of metamorphosis, feedings were changed to an exclusive diet of three to four one-week-old crickets twice a week. After 60 d (and thereafter), juveniles were fed once weekly 4 to 6 three-week-old crickets. Once most all of the larvae had metamorphosed, juveniles were switched to a moist soil and leaf litter substrate. Leaf litter and water were periodically added to provide cover and moisture. Experimental organisms remained in these containers until the end of the experiment. Juveniles were kept in incubators at 25°C during the day and 22°C at night from metamorphosis to day 176 of the experiment. Temperatures were lowered to 22/20°C day/night from day 176 to day 580. Mortality checks were performed daily for the first 10 d postmetamorphosis and weekly thereafter.

Feeding rates of juvenile *A. barbouri* were quantified 290 d after the onset of larval exposure. Each salamander was given 4 three-week-old crickets (~0.189 g), and every 12 h, the number of crickets left uneaten was checked. The trial was continued for 72 h. We assumed that any missing crickets had been consumed since the last check 12 h prior. Crickets found dead were replaced. Salamanders were given a ranking according to the amount of time it took them to consume all prey. For instance, if an individual consumed all four crickets in the first 12 h, it was given a ranking of one; if it consumed them in 24 h, it was assigned a two; and so forth, up to a ranking of seven for those individuals that had not yet consumed all four crickets after 72 h. Following the feeding trial, we measured body mass of all juveniles. Weights were used to calculate juvenile growth rates over two time intervals, from metamorphosis to day 293 and from day 293 to day 578 of the experiment. These growth rates are referred to as growth periods 4 and 5, respectively.

Statistical analyses

Differences in the survival of *A. barbouri* larvae and juveniles between controls and each TPT treatment were analyzed using chi-square tests. Larval survivorship in the three treatments was compared on the day of metamorphosis, and differences in juvenile survivorship were tested on the last day of the experiment. Days to metamorphosis, body mass at metamorphosis, and both larval feeding rate values were log transformed (natural log) prior to analysis in order to reduce heterogeneity of variances and nonnormality. Because of several zero feeding rates, we added one to the larval feeding rates before taking the natural log in the transformations. Escape response variables were left untransformed for analysis because they were normally distributed and variances were similar among treatments. For larval feeding rates 1 and 2, days to and size at metamorphosis, and escape behavior acceleration and velocity (both maximum and average), treatments were compared with two-way analyses of variance with TPT treatment and sibship as grouping factors using SAS® (SAS Institute, Cary, NC, USA). Since larval weight did not explain a significant proportion of the variance in the four escape response variables, it was not used as a covariate. Because terrestrial feeding rates were not normally distributed, treatment effects were analyzed using the nonparametric Kruskal–Wallis one-way analysis of variance.

Larval and juvenile growth rates were calculated as instantaneous growth rates, where growth = [ln (body mass of

each larva at time 2) − ln (body mass of each larva at time 1)]/(number of days between times 1 and 2), and units are ln grams/day [31]. For growth period 1, we did not have weights for the hatchlings (as we only measured snout–vent length then), so we estimated the beginning weight of all larvae to be 0.04 g [32]. Although hatchlings may have varied slightly in weight on day 1, this variation (as estimated by snout–vent lengths also recorded on day 1) did not explain any of the variance in growth rate 1 based on an analysis of covariance using snout–vent length on day 1 as a covariate. Analyses of covariance were used to test for treatment and sibship effects on growth rates in periods 1 and 2 using feeding rates 1 and 2, respectively, as covariates. Differences among treatments in growth periods 3, 4, and 5 were tested using analyses of variance. Type III sums of squares were used in all analyses to account for unequal replication of the treatments. Pairwise comparisons were tested using the least significant difference method.

RESULTS

Larval effects

Exposure to the high TPT concentration of 5 μg/L significantly affected survivorship (chi-square = 87.1, degrees of freedom [*df*] = 1, $p < 0.001$), resulting in 93% mortality of the larvae (Fig. 1a). This mortality was highest between days 16 and 19 of exposure (Fig. 1b). Only two individuals exposed to 5 μg/L TPT for the entire larval period survived to metamorphosis. In contrast, exposure to the low TPT concentration, 1 μg/L, did not cause significant larval mortality. Survival of larvae exposed to low TPT (93.3%) was not significantly different from the 100% survivorship observed in the control treatment.

Treatments had a significant effect on the feeding rates of the larvae (Fig. 2a and Table 1). On day 16 of the experiment, average feeding rates for the low TPT and high TPT larvae were 33 and 71% lower than the control rates, respectively. At day 16, larvae fed significantly less in the low TPT than in the control ($p = 0.0016$), and they also fed significantly less in the high TPT than the low TPT ($p < 0.0001$). At day 24, feeding rates for the low and high TPT concentrations were 42% ($p = 0.01$) and 94% ($p < 0.05$) lower than the control larvae, respectively. We found a highly significant sibship effect on early larval feeding rates but not on late larval rates (Table 1 and Fig. 2b). For example, for feeding rate 1, sibship 1 had rates that were significantly higher than sibships 4, 5, 8, and 9 at the $p < 0.0001$ level. We found no significant interaction of sibship and treatment for either larval feeding rates 1 or 2 (Table 1).

For predator escape response, we found differences among the treatments in the swimming velocities of the larvae, but we found no differences in their acceleration, either maximum or average (Table 2 and Fig. 3). The seven larvae in the high TPT treatment that survived to day 30 of exposure had average swimming velocities that were 46% slower than those exposed to low TPT treatment ($p < 0.05$) and 42% slower than the controls (Fig. 3a). Maximum velocities of the high TPT larvae were almost significantly lower than those of the low TPT larvae (by 40%, $p = 0.0558$). Accelerations did not differ among the three treatments but followed the same pattern as the velocities, with low TPT individuals having slightly higher accelerations than the controls (Fig. 3b). Average and maximum accelerations for the controls and the high TPT larvae tended to be closer in value than average and maximum velocities. Variation among sibships did not explain a significant amount of the variation in any of the four swimming variables.

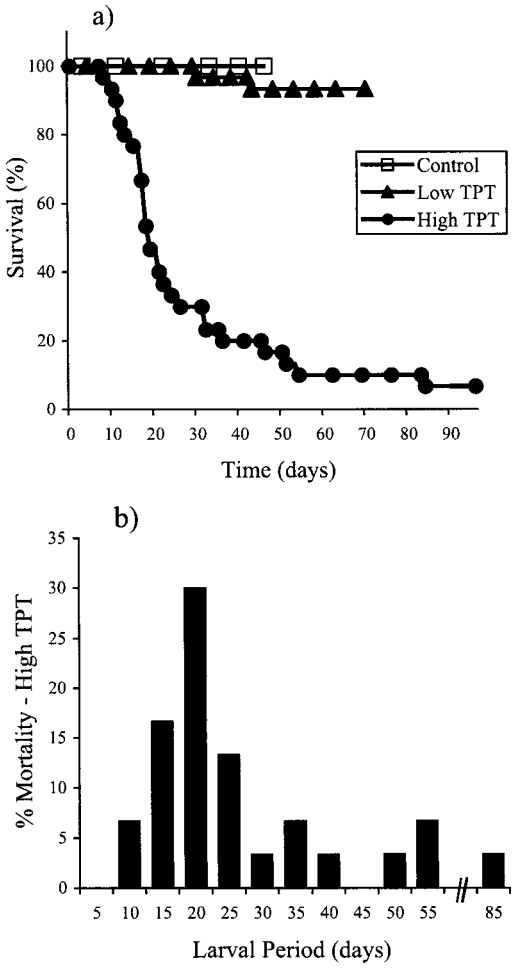

Fig. 1. (**a**) Percent survival of *Ambystoma barbouri* over the entire larval period in the control and the two triphenyltin (TPT) treatments. Data lines end on the day the last metamorph for that treatment was observed; time is days since the onset of exposure. Low TPT = 1 μg/L; high TPT = 5 μg/L. (**b**) Percent mortality in the high TPT treatment over the larval period in 5-d intervals.

Triphenyltin exposure significantly affected larval growth rates both in the early and late larval period (Fig. 4a and Table 3). For growth period 1, growth rates were significantly reduced in both the high TPT treatment (by 93%, $p = 0.0001$) and the low TPT treatment (by 26%, $p = 0.04$). Feeding rate 1 had a highly significant effect on larval growth rates, as did sibships (Table 3); larvae that fed at a higher rate grew faster. During growth period 2, growth rates among the three treatments did not differ (Table 3, $p = 0.11$). Larvae exposed to the low TPT concentration had growth rates that were on average only 13% lower than the control individuals. However, feeding rates during this growth period did have a significant effect on growth rates (Table 3). During this growth period, we also observed the highest mean growth rates of the entire experiment (Fig. 4a). Only 9 individuals of 30 survived to the end of period 2 in the high TPT treatment, yet their mean growth rate was more than six times higher than their mean growth rate in period 1.

For growth period 2, we also found a highly significant sibship by TPT treatment interaction not seen earlier in the larval period (Table 3). Sibships seemed to differ in their re-

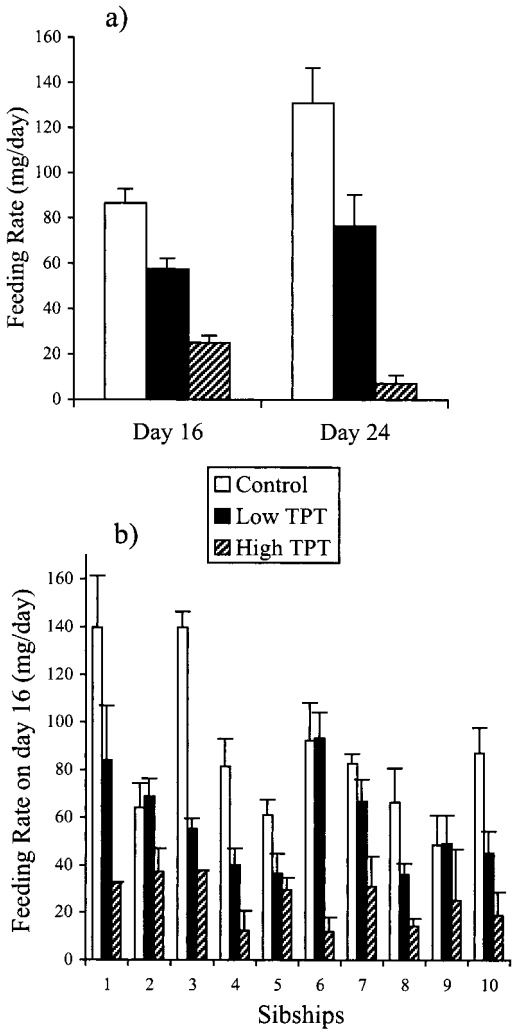

Fig. 2. (**a**) Mean feeding rates, in mg of blackworms consumed per day, at days 16 and 24 of exposure for the larvae in acetone controls and the two triphenyltin (TPT) concentrations. (**b**) Mean feeding rates for the 10 sibships in the study at day 16 of exposure. Low TPT = 1 μg/L; high TPT = 5 μg/L. Bars represent mean values ± 1 standard error.

Table 1. Summary of the analyses of variance for larval feeding rates 1 and 2, days to metamorphosis, and size at metamorphosis. These variables were log transformed prior to analysis. Shown are $F = F$ statistics; df = degrees of freedom; and $p = p$-values for each effect tested; S = sibship; and TPT = triphenyltin

	Feeding rate 1			Feeding rate 2		
Effects	*df*	*F*	*p*	*df*	*F*	*p*
S	9	2.92	0.007	9	1.30	0.27
TPT	2	51.92	<0.0001	2	22.12	0.0001
S × TPT	18	1.29	0.23	16	1.11	0.37
Error	53			42		
	Days to metamorphosis			Size at metamorphosis		
Effects	*df*	*F*	*p*	*df*	*F*	*p*
S	9	3.97	0.001	9	3.42	0.004
TPT	2	37.07	0.0001	2	2.68	0.08
S × TPT	10	1.78	0.10	10	1.25	0.30
Error	38			38		

sponse to increasing concentrations of TPT. Growth rates were rather similar among all sibships for the control treatment, but they differed at the low TPT concentration and were highly variable at the high TPT concentration (Fig. 4b). At the low TPT concentration, half of the sibships showed higher growth rates than their siblings in the control treatment, while the other half showed moderate to sharp decreases in mean growth rates compared with their control siblings. At the high TPT concentration, most sibships responded with sharp decreases in their growth compared with their siblings exposed to the lower TPT concentration, while two of the sibships, numbers 3 and 8, responded with increases in their growth (Fig. 4b). Only one larva represented each of these sibships, and these are the only two individuals to survive to metamorphosis in the high TPT treatment. We have no explanation for why these two individuals had higher growth rates than their siblings in the control and low TPT treatments, especially considering that their growth rates over period 1 were not the highest of their respective sibships. Growth rates over period 3 differed significantly between the control and low TPT larvae ($p = 0.04$). Growth rates for the low TPT larvae were 79% lower than the control growth rates, while growth rates for the two surviving high TPT larvae were only 15% lower than the controls (Fig. 4a). Sibships differed significantly in their mean growth rates over this period. A significant interaction of sibship with treatment for larval growth period 3 indicates that sibships also differed in their sensitivity to TPT with respect to late larval growth (Table 3).

Triphenyltin treatment significantly affected time to metamorphosis (Fig. 5a and Table 1). High TPT larvae metamorphosed significantly later than both the control and low TPT larvae ($p < 0.05$). Larval periods for the two high TPT individuals lasted 62 and 96 d, while the average larval period for the control organisms was only 39 d. Larvae exposed to the low TPT concentration, on average, metamorphosed 5.6 d later than control larvae ($p < 0.0001$). Days to metamorphosis also varied significantly among sibships (Table 1). For size at metamorphosis, there was a trend for an effect of TPT exposure ($p = 0.08$). Body mass of the low exposure metamorphs was 6% higher than that of the control individuals (Fig. 5b). The mean body mass of the high TPT organisms was 76% higher than the controls, but with only two individuals, this difference was not significant. This trend for a larger body mass associated with TPT exposure is congruent with the delayed metamorphosis effect of TPT exposure. We also found sibships to have a significant effect on body mass at metamorphosis ($p = 0.001$).

Juvenile effects

There was no significant postmetamorphic mortality in the three treatments. The two high TPT individuals that survived to metamorphosis were still alive on day 580. For the low TPT treatment, mean survival was not significantly lower than the control survival, 90.4% compared with 90.9%. Two individuals in each treatment died; three of them died within two weeks of metamorphosis while one of the low TPT individuals died more than 350 d after metamorphosis.

We found no significant differences in the feeding rate of terrestrial *A. barbouri* due to larval TPT exposure (Kruskal–Wallis = 0.20, $df = 9$, $p = 0.90$). On average, both control and low TPT individuals consumed four crickets in 1.9 d, while the two high TPT *Ambystoma* survivors took 1.5 d to eat the same number of crickets. We found a significant sibship effect

Table 2. Summary of the analyses of variance for maximum swimming velocity, mean velocity, maximum acceleration, and mean acceleration of the *Ambystoma barbouri* larvae. Shown are $F = F$ statistics; df = degrees of freedom, and $p = p$-values for each effect tested; S = sibship; and TPT = triphenyltin

	Maximum velocity			Mean velocity		Maximum acceleration		Mean acceleration	
Effects	*df*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
S	9	0.41	0.92	1.09	0.39	1.50	0.18	1.18	0.33
TPT	2	3.06	0.06	5.37	0.009	2.02	0.15	2.18	0.13
S × TPT	15	0.27	0.99	0.65	0.81	1.16	0.34	0.67	0.80
Error	39								

(Kruskal–Wallis = 18.3, $df = 9$, $p = 0.03$) on juvenile feeding rate, as was detected on early larval feeding rate. Sibships 3, 4, and 7 had juvenile feeding rates more that 2.5 times that of other sibships. However, we found no relationship between juvenile and larval feeding rates. Only one of the sibships, number 3, also had one of the highest feeding rates measured as larvae.

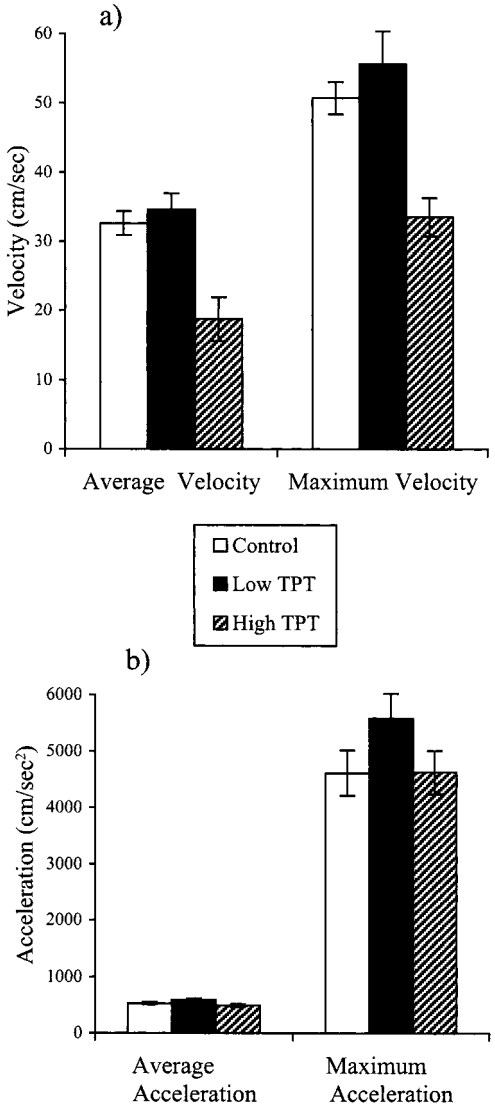

Fig. 3. (**a**) Mean and maximum swimming velocities for larvae exposed to the acetone control and to low and high triphenyltin (TPT) concentrations. (**b**) Mean and maximum swimming accelerations for larvae exposed to the three treatments. Low TPT = 1 μg/L; high TPT = 5 μg/L. Bars represent mean values ± 1 standard error.

As expected, growth rates for the terrestrial salamanders across the three treatments were 95% lower than pooled larval growth rates. We found, however, no significant differences among treatments in terrestrial growth rate periods 4 and 5. Sibships differed significantly in growth rate during the first terrestrial period, but not later in the second. We also found no significant sibship by treatment interactions for either growth period.

DISCUSSION

Overall, our results indicate that long-term TPT exposure had important lethal, life history, and behavioral effects on *A. barbouri* larvae. Exposure to 5 μg/L TPT depressed feeding behavior, escape behavior, and growth rates before resulting in almost 100% mortality of the larvae. Only two salamanders survived to metamorphosis, and their size at metamorphosis as well as their duration of the larval period was almost double the control treatment. At 1 μg/L TPT, larval exposure reduced both feeding behavior and growth but caused no mortality. These individuals also delayed metamorphosis but did not increase their size at metamorphosis significantly.

Our results also suggest that not all genotypes of *A. barbouri* were equally affected by TPT toxicity. We found differential sensitivity of sibships to TPT in larval growth rates. In the midlarval period, growth rates of 5 of the 10 sibships used in the study showed decreases when exposed to the 1-μg/L TPT level, while the other five sibships showed increases in growth. As the concentration increased to 5 μg/L, most of the surviving siblings in this treatment had reduced growth, but still two sibships, which had lower growth rates in the low TPT treatment than the control, now showed important increases in growth. This could mean that, while some genotypes might be relatively unaffected by this toxin, other families might be severely impacted by exposure to TPT. If this is the case, TPT contamination can be expected to reduce overall genetic diversity of exposed populations as has been observed with other environmental toxins [33].

The devastating mortality observed at 5 μg/L is comparable with mortality rates observed at very similar TPT concentrations in early life stages in fish [17,34]. However, contrary to previous work on tadpoles [19], we did not observe most of the mortality occurring close to the time of metamorphosis. Nor did we observe the highest mortality rate in the earliest and typically more susceptible larvae. Instead, we observed a peak in mortality rate halfway through the larval period, during days 16 through 19 of exposure. The lack of a peak of mortality associated with metamorphosis could be due to the fact that the morphological and physiological changes associated with metamorphosis in salamanders are not as dramatic as they are in anurans [35]. Alternatively, the high mortality at 5 μg/L in middevelopment may have filtered out susceptible individuals

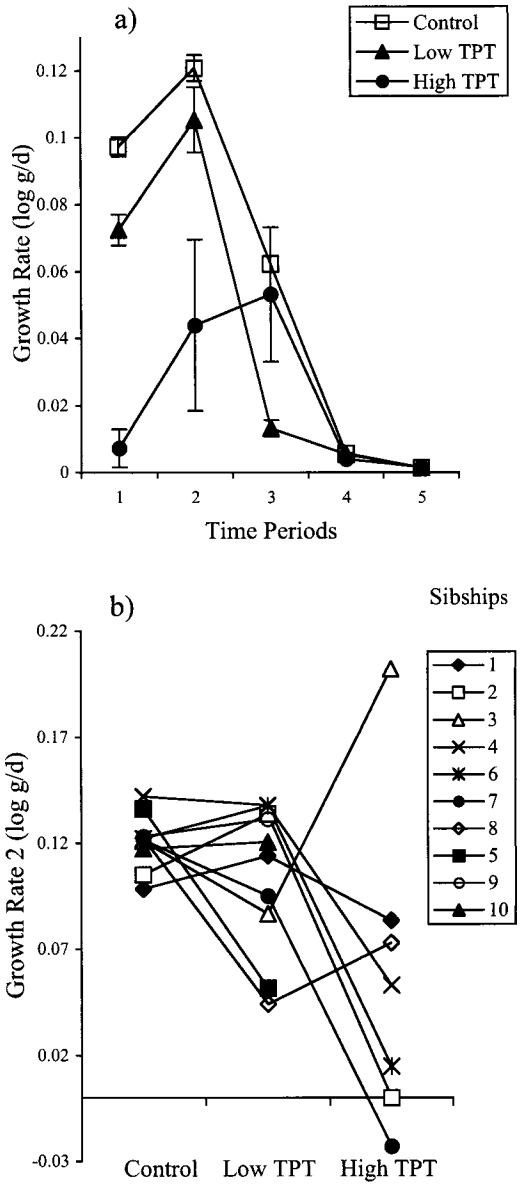

Fig. 4. (**a**) Growth rates for larval and juvenile *Ambystoma barbouri* individuals in the control and two triphenyltin (TPT) treatments. Time periods are (1) days 1 to 18 of larval exposure; (2) days 18 to 26; (3) day 26 to metamorphosis; (4) metamorphosis to day 293; and (5) days 293 to 578. Symbols represent means ± 1 standard error (SE). (**b**) Interaction of TPT treatment with sibship effect on growth rate over period 2 (days 18–26 of larval exposure). Each line represents 1 of the 10 sibships. Growth rate 2 in the high TPT treatment is not shown for sibships 5, 9, and 10 since there were no survivors in the treatment. Low TPT = 1 μg/L; high TPT = 5 μg/L.

so that only the very few least sensitive salamanders remained at the onset of metamorphosis. The absence of a mortality effect at the concentration that best approaches levels detected in nature (1 μg/L) also contrasts with the significant mortality observed after long-term exposure to 0.81 and 1.87 μg/L TPT in *Rana* tadpoles [19].

For amphibians inhabiting seasonally ephemeral habitats, a delay in time to metamorphosis could have serious negative impacts on their overall fitness. For *A. barbouri*, a major source of mortality is stream drying [25,27]. At low levels of TPT, 1 μg/L, exposed larvae completed metamorphosis on average 6 d later than control organisms. This could mean that if ephemeral *A. barbouri* habitats are exposed to relatively low concentrations of TPT, delayed metamorphosis can result in increases in larval mortality that will, in turn, cause decreases in recruitment into the breeding adult population. Moreover, for a species that has a restricted range and is dependent on only a few large populations for long-term persistence, decreases in adult recruitment will impact overall population size and structure and could result in local extinctions. In the event of exposure to higher TPT concentrations, few individuals would survive; however, survivors would be much larger than usual. And, while larger sizes at metamorphosis are generally thought to be positively correlated with juvenile fitness [36], because of the ephemerality of the habitat and the greater delay in time to metamorphosis, those individuals would also suffer significant additional mortality. Recent work with other anthropogenic contaminants shows that delays in time to metamorphosis coupled with increases in sizes at metamorphosis are the result of interactions with thyroid hormones or prolactin [37]. Further studies are needed to address how TPT might be interacting with hormones associated with the onset of metamorphosis in amphibians.

The behavior of the larvae was strongly impacted by TPT exposure. Larval exposures at both concentrations significantly reduced feeding rates so as to have an effect on life history traits. Analyses of covariance detected both early and midlarval feeding rates explaining a significant amount of the variation in larval growth rates. At the same time, we still found a direct effect of TPT on growth rates, at least in early development, which suggests a stress effect. This means that TPT may have also had other physiological or metabolic effects, which reduced larval growth above and beyond depressed feeding rates. However, the mechanism underlying this direct effect is unknown and should be explored in future research.

The swimming performance of the larvae was also negatively affected by TPT exposure, although only at the higher concentration. Triphenyltin caused larvae to have lower swimming velocities, but it did not reduce either maximum or average accelerations. Slower swimming velocities could have important consequences for *A. barbouri* since they coexist with predatory fish. Evidence suggests that faster amphibian larvae generally have higher survival rates [38]. However, acceleration would seem to be key to escaping a fish and quickly bursting into refuge. At the same time, low swimming velocities could potentially affect the ability of the larvae to catch prey or could affect their swimming endurance, which might be key in allowing larvae to avoid stream portions where fish are present.

Triphenyltin exposure to the 1-μg/L concentration did not cause larvae to swim slower when an escape response was elicited. On the contrary, swimming velocities and accelerations tended to be, on average, 12% higher in the low TPT than the control treatment. Such a response might be evidence of a hormetic effect of TPT whereby physiological processes are enhanced to temporarily overcompensate for inhibitory effects. If this trend holds true, higher swimming velocities when exposed to this toxin could translate to higher activity levels for the larvae, which would increase their predation risk. Similar enhanced swimming effects have been observed in fish, where exposure to 2 μg/L TBT resulted in rainbow trout hatchlings swimming at greater speeds, for greater distances, and for a longer time due to neurotoxicological effects in the midbrain [39]. Anecdotal observations on the behavior of larvae at the 5 μg/L TPT also tell us that larvae often appeared

Table 3. Summary of the analyses of covariance for larval growth periods 1 and 2 and the analyses of variance for the larval growth period 3 and juvenile growth periods 4 and 5 in *Ambystoma barbouri*. Durations of growth periods are shown in parentheses (in days). Shown are F = F statistics; df = degrees of freedom; and p = p-values for each effect tested; S = sibship; TPT = triphenyltin; and FR = feeding rate

	Larval growth									Juvenile growth					
	Period 1 (1–18 d)			Period 2 (18–26 d)			Period 3 (26 d–metamorphosis)			Period 4 (metamorphosis–293 d)			Period 5 (293–578 d)		
Effects	*df*	*F*	*p*	*df*	*F*	*p*	*df*	*F*	*p*	*df*	*F*	*p*	*df*	*F*	*p*
S	9	4.60	<0.001	9	1.75	0.11	9	3.11	0.007	9	2.66	0.03	9	0.89	0.55
TPT	2	36.08	<0.001	2	2.32	0.11	2	4.36	0.02	2	1.81	0.19	2	0.31	0.73
S × TPT	18	1.42	0.17	15	2.87	0.004	10	2.63	0.02	10	1.21	0.34	10	0.98	0.49
FR	1	12.28	0.001	1	5.80	0.02									
Error	49			41			38			20			19		

disoriented and showed signs of ataxia, as seen in fish exposed to similar levels of TPT [16,17].

An important finding of our study was the failure to detect chronic effects on the terrestrial juveniles after exposure was terminated at metamorphosis. Larval TPT exposure did not affect the survival, growth, or feeding behavior of juveniles for an average of 17 months postmetamorphosis. This is an unexpected result since evidence suggests that toxic effects early in life may result in permanent modifications in older life stages [40]. However, we do not have data on how exposure of larval *A. barbouri* might affect the reproductive biology and fecundity as well as the overall health, survival, and longevity of reproductive adults. This is important in light of evidence that suggests that organotins can have severe impacts on the breeding activity of marine gastropods due to endocrine disruption [41]. Ultimately, we would like to know if exposure of amphibians to this organotin in the early aquatic life stage has an impact on the reproductive success of adults and on the fecundity of their offspring. In addition, it is likely that, under field conditions, *A. barbouri* might not only be exposed to TPT in the early aquatic stage but also in the terrestrial life stage. The potential effects of exposure to agricultural toxins in multiple life stages for organisms with complex life cycles need to be addressed in future studies. Furthermore, under natural conditions, exposure to TPT toxicity might often occur in combination with other natural stressors such as habitat ephemerality and predation risk and possibly with other anthropogenic pollutants. Studies that incorporate multiple stressors will be necessary to understand how they interact to determine the overall effect felt by natural populations of amphibians. It is now long recognized that standard toxicity tests are unable to predict the long-term effects of toxins like TPT in a natural system. Future work combining full life-cycle studies with multiple stressors studies is needed to better understand the effects of environmental contaminants on biota.

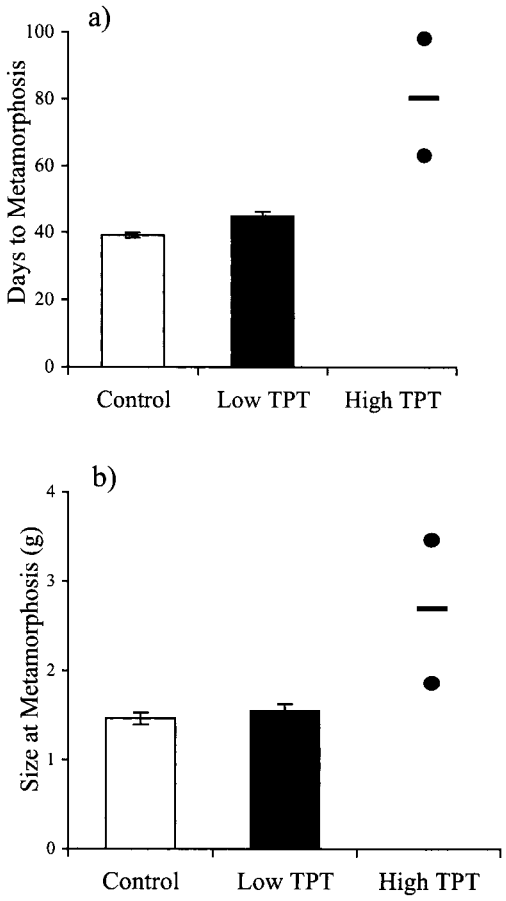

Fig. 5. (**a**) Mean number of days to metamorphosis of larvae in control, low triphenyltin (TPT), and high TPT treatments. In the high TPT, the two data points represent the only two individuals that survived to metamorphosis, and the line between them indicates the mean of the two. (**b**) Mean body mass at metamorphosis of larvae in control and low TPT and high TPT treatments. In the high TPT, the two data points represent the same two individuals that survived to metamorphosis, and the line between them indicates the mean for the treatment. Low TPT = 1 μg/L; high TPT = 5 μg/L. Bars represent mean values ± 1 standard error.

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