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Abundance, Metamorphosis, Developmental, and Behavioral Abnormalities in *Hyla chrysoscelis* Tadpoles Following Exposure to Three Agrichemicals and Methyl Mercury in Outdoor Mesocosms

C. A. Britson, S. T. Threlkeld

Department of Biology, University of Mississippi, University, MS 38677, USA

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Effects of individual environmental contaminants on amphibian survival, growth, development, and behavior are often examined in the laboratory for only a portion (e.g., 1 - 8 d) of the organism's life cycle (Birge et al. 1983, Bridges 1997, Hudson et al. 1984, and Semlitsch et al. 1995). When experiments are conducted in the field, responses of amphibians to individual contaminants do not include effects on the incidence and rate of metamorphosis (Klaassen and Kadoum 1979, Sparling et al. 1995). In this study we examined the effects of mixtures of three agrichemicals and methyl mercury on the relative abundance, incidence of abnormalities, growth, and metamorphosis of tadpoles in outdoor mesocosms following a natural colonization by breeding gray treefrogs (*Hyla chrysoscelis*). We also examined relationships between these variables and behavioral responses of tadpoles.

MATERIALS AND METHODS

This study was conducted in an enclosed array of 66 500-liter aquatic mesocosms at the University of Mississippi Field Station (UMFS). The study site was bordered on the north and south by constructed ponds and on the west by a spring-fed stream and lowland forest. Access to mesocosms by gray treefrogs was primarily via the north and west. The agrichemicals atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine), chlorpyrifos [*O,O*-diethyl *O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate], and MSMA (monosodium methanearsonate) were present in the mesocosms as part of an experiment examining effects of chemical mixtures in wetland systems. Methyl mercury (methylmercuric chloride) was added in an aqueous acetone solution to half of the mesocosms to bring the concentration of methyl mercury in the upper 1 cm of sediment to 0.4 mg/kg (approximately double the background sediment concentration of 0.2 mg/kg in the southeastern US and the mesocosms). Within four days after application, methyl mercury was not detectable in the water column. Methyl mercury remained in the sediment throughout the experiment as detected by analysis of total mercury present. All mesocosms contained a 15 cm layer of sand and a 5 cm top layer of sediment from a constructed pond at the UMFS. *Juncus effusus* was planted in each mesocosm prior to filling of the mesocosms with spring water from the UMFS.

Using a center point enhanced 2^4 factorial design (Montgomery 1997), agrichemicals were applied to mesocosms on 10 June 1996 at levels of 0, 50 (center point) and 100 % of the expected environmental concentration (EEC). EECs (atrazine, 192 ppb; chlorpyrifos 51 ppb; MSMA, 219 ppb) were derived from a model (US EPA 1995) using normal agricultural application rates and chemical property data. Atrazine, chlorpyrifos, and MSMA were 98% pure from Chem Service, West Chester, PA. Methyl mercury was 97-99% pure from Pfaltz and

Correspondence to: C. A. Britson

Bauer, Waterbury, CT. One third of the mesocosms were sampled for multiple endpoints on days 1, 8, and 64, another thud on days 2, 16, and 70, and another third on days 4, 32, and 94 after initial chemical dosing. Chemical concentrations in water and sediment samples were measured from one-half of sampled mesocosms on all sampling days. Analytical methods are described in Steevens et al. (1997). Percent recovery from spiked samples was always greater than 90%. On day 62 one half of the mesocosms were redosed using a center point enhanced 2^{5-1} fractional factorial design (Montgomery 1997). Within each group of 22 mesocosms (one-third), the center point was replicated 6 times (3 mesocosms not redosed, 3 redosed) and the remaining 16 design points represent all low (0%) and high (100%) EEC combinations (8 mesocosms not redosed, 8 redosed). Mesocosms which received low EEC concentrations of all four chemicals and were not redosed are considered experimental controls. During sampling, notes were taken on occurrence of tadpoles in mesocosms, allowing estimation of a range of colonization dates and chemical exposures. Two additional mesocosms, to which no chemicals were added and from which no endpoints were measured, were also examined for tadpoles.

Colonization of the mesocosms occurred between mid June and early October 1996. Colonizing treefrogs were from a resident population at the UMFS with no known previous exposure to environmental contaminants used in this experiment. On 18 October 1996, 129 days after initial chemical application and 67 days after redosing, all tadpoles were removed. Mass (g) and total length (mm) were recorded for each tadpole as was developmental stage ($25 \cdot 46$: Gosner 1960). Presence or absence of developmental [e.g., missing eyes, lordosis (curvature) of the tail or vertebral column, or limb malformation] or behavioral abnormalities [e.g., swimming (inability to maintain balance) dysfunctions] were also assessed. After initial measurements were taken, tadpoles (≤ 5) from each mesocosm, where present, were chosen randomly, euthanized (via cold shock) and preserved in 10 % buffered formalin. Remaining tadpoles were reared in the laboratory (12L: 12D) until metamorphosis with twice weekly water changes using spring water from the UMFS. Tadpoles were fed a 3: 1 mixture of powdered rabbit chow pellets and flake fish food *ad libitum* twice weekly (Britson and Kissell 1996).

Feeding behavior was tested by placing tadpoles (≤ 10 from each mesocosm) in plastic containers (33.5 x 20 x 11.5 cm) filled with 3 L of spring water. Feeding is characterized by fast, repeated touching of food combined with wavy tail movements (Semlitsch et al. 1995). After an acclimation period of 1 hr, 1 g of powdered food was presented, and the number of tadpoles feeding was recorded every min for 20 min. Mean percent of tadpoles feeding was then calculated. Anti-predator behavior was measured as tadpole sprint speed and distance following a simulated predator attack. Additional tadpoles (≤ 10) from each mesocosm were tested in plastic containers (see above) placed over a 1 cm grid to allow measurement of distance moved. Attacks were simulated by lightly tapping the tail with a blunt probe after a 5 min acclimation period. Each tadpole was tested 5 times with a 1 min rest period between trials. Mean sprint speed (cm/sec) and distance moved (cm) was calculated for each tadpole.

Upon forelimb emergence (stage 42, Gosner 1960), tadpoles were reared individually until metamorphosis was complete (stage 46). Mass, snout-vent length (SVL), and presence or absence of developmental abnormalities were measured for each froglet. All froglets were euthanized and preservedn. Rearing of tadpoles in the laboratory was terminated (16 December 1996) when sufficient time had passed to allow metamorphosis to occur [i.e., more than twice as long as the 35 d developmental period for *H. chrysoscelis* as reported by Ritke et al. (1990)].

Due to uneven colonization across mesocosms (as related to design points rather than geographic position within the experimental facility), an analysis of variance of tadpole

responses against the full treatment structure could not be conducted (i.e., insufficient degrees of freedom). Instead, a multiple, pairwise correlation analysis using the ranks of measured water and sediment concentrations was undertaken to examine relationships between tadpole responses and chemical concentrations. The mean standard normalized (Neter et al. 1990) concentration for all samples taken on days 32, 64, 70, and 94 was calculated and ranked for each treatment combination (water and sediment values ranked separately). This range of dates includes the most likely time period for colonization. However, using concentrations measured over this time scale in the analysis may underestimate the effects of a short-lived, but potentially harmful chemical. Ranked chemical concentrations for mesocosms that contained tadpoles, assuming that values within 0.5 standard deviation units of each other were tied in their rankings, were entered into the correlation analysis. Tadpole responses expressed as percentages were arc sine square root transformed prior to analysis (Neter et al. 1990). A second multiple, pairwise correlation analysis using nominal chemical values was conducted to determine the extent of bias associated with chemicals having longer half lives. Unless otherwise noted (i.e., there is a deviation between results of the two analyses), all correlation coefficients presented were obtained using ranked values. For all statistical analyses the level of significance was set at $\alpha \leq 0.05$.

RESULTS AND DISCUSSION

Treefrogs colonized at least 13 mesocosms representing 10 of the 34 design points (Fig. 1), with 400 total tadpoles recovered on 18 October 1996. From 1 to 127 tadpoles were collected from 12 mesocosms where recovery was possible. It is unlikely that the number of tadpoles collected is an artifact of number of eggs deposited. Average clutch size, deposited at a single site, for *H. chrysoscelis* females is approximately 2000 eggs and ranges from 620 to 4200 (Ritke et al. 1990).

The number of malformations or behavioral dysfunctions detected from tadpoles at each design point ranged from 0 to 38 with six tadpoles having more than one malformation or dysfunction. No more than 3 malformations or dysfunctions were observed in any tadpole. Lordosis and missing eyes were the most frequently observed malformations and were found in 35 and 19 tadpoles, respectively. No more than one eye was missing per tadpole and there was no pattern of eye absence (i.e., always the right or left eye).

There was a positive relationship between the number of abnormalities and surviving tadpoles ($r = 0.65$, $P = 0.04$), although none between proportion of tadpoles with abnormalities and tadpole abundance ($r = -0.27$, $P = 0.45$). Schoener (1979) and Willis et al. (1982) showed that injuries in lizards and snakes respectively do not necessarily reflect severity of the injury-causing agent when other sources of mortality or loss are expected. In the case of abnormally developing tadpoles, we might expect the proportion of abnormalities to be proportional to number killed, although uncertainty regarding loss to predators in mesocosms, metamorphosis out of mesocosms, and number of initial colonists makes such an analysis impossible.

The total number of tadpoles (Table 1) collected per mesocosm was negatively correlated with the ranked concentration of atrazine in water ($r = -0.59$, $p = 0.04$). As concentration increased, including redosing, the number of tadpoles collected decreased. In the correlation analysis using nominal values, the relationship between atrazine and tadpole abundance was not significant ($r = -0.43$, $p = 0.15$). In addition, nominal values of methyl mercury were negatively correlated with tadpole abundance ($r = -0.72$, $p \leq 0.01$; Fig. 2). Variances in tadpole density can have

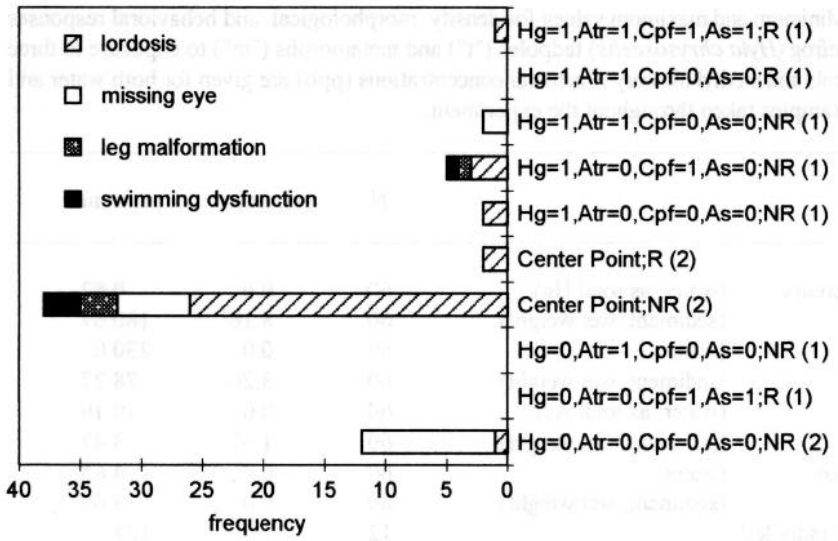

Figure 1. Frequency of developmental or behavioral abnormalities in gray treefrog (*Hyla chrysoscelis*) tadpoles collected on day 129 of the experiment. Design points for mesocosms (n) from which tadpoles were collected are listed on the right. Nominal concentrations are representedby “0” (low, 0%), center point (50%), and "1" (high, 100%). Hg = methyl mercury, Atr = atrazine, Cpf = chlorpyrifos, and As = MSMA. "R" and "NR" represent redosed and not redosed mesocosms.

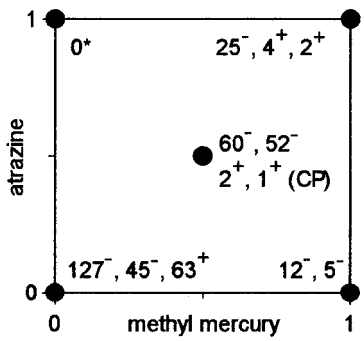

Figure 2. Distribution of gray treefrog (*Hyla chlysoscelis*) tadpole abundance across the nominal concentrations [representedby “0” (low, 0%), center point (CP, 50%), and "1" (high, 100%)] of methyl mercury and atrazine. "+" and "-" represent redosed and not redosed mesocosms respectively. An "*" indicates that tadpoles were observed prior to day 129, but none were observed at the time of collection.

Table 1. Minimum and maximum values for density, morphological, and behavioral responses of gray treefrog (*Hyla chrysoscelis*) tadpoles ("t") and metamorphs ("m") to exposure to three agrichemicals and methyl mercury. Chemical concentrations (ppb) are given for both water and sediment samples taken throughout the experiment.

		N	minimum	maximum
methyl mercury	(water, as total Hg)		0.0	0.82
	(sediment, wet weight)		8.16	180.67
atrazine	(water)		0.0	230.0
	(sediment, wet weight)		3.26	78.27
MSMA	(water, as total As)		0.0	10.19
	(sediment, wet weight)		1.67	3.47
chlorpyrifos	(water)		0.0	4.65
	(sediment, wet weight)		0.0	23.69
number of tadpoles[t]			1	127
tadpole mass/length[t] (g/mm)			0.01	0.03
developmental stage[t]			25	46
% lordosis[t]			0	100
% missing eyes[t]			0	8.0
% leg malformation[t]			0	8.3
% swimming dysfimction[t]			0	8.3
% feeding[t]			30.0	74.0
swimming speed[t] (cm/sec)			4.91	12.41
days to metamorphosis[t]			15	45
metamorph mass/SVL[m] (g/mm)			0.01	0.04
% lordosis[m]			0	100
% missing eyes[m]			0	57.1
% leg malformation[m]			0	100
% swimming dysfunction[m]			0	11.11

marked effects on tadpole size and rates of metamorphosis and are critical to interpretation of experiments examining amphibian development and metamorphosis. As demonstrated in field and laboratory experiments, body size decreases and days to metamorphosis increases with an increase in density (Gromko et al. 1973; Wilbur and Collins 1973). Both responses can lead to a decrease in recruitment of juveniles into a population as well as future breeding success (Smith 1987). Our results show negative relationships between days to metamorphosis and the ranked concentration of atrazine in water ($r = -0.79$, $p < 0.01$), tadpole size, developmental stage upon collection, and froglet size at metamorphosis. Only significant correlation coefficients for relationships among tadpole response variables, exclusive of relationships among tadpole responses and chemical concentrations which are discussed in the text, are given in Table 2. There was a positive relationship between the ranked sediment concentration of methyl mercury and days to metamorphosis ($r = 0.67$, $p = 0.02$), and a negative relationship between the ranked sediment concentration of chlorpyrifos and developmental stage ($r = -0.60$, $p = 0.03$). These relationships are consistent with models for amphibian metamorphosis (Wilbur and Collins 1973) when effects of density are considered. It would be premature to conclude that effects

Table 2. Significant Pearson correlation coefficients for the relationships among multiple responses of gray treefrog (*Hyla chrysoscelis*) tadpoles ("t") and metamorphs ("m") exposed to three agrichemicals and methyl mercury (exclusive of relationships among tadpole responses and chemical concentration). Percentages were arc sine square root transformed prior to correlation analysis.

response variable	response variable	N	r	p
number of tadpoles^t	tadpole mass/length^t (g/mm)	11	-0.69	0.01
	developmental stage^t	12	-0.78	< 0.01
	% missing eyes^t	12	0.67	0.01
	days to metamorphosis^t	11	0.73	0.01
	% missing eyes^m	11	0.87	< 0.01
tadpole mass/length^t (g/mm)	developmental stage^t	11	0.87	< 0.01
	% lordosis^t	11	0.73	0.01
	% missing eyes^t	11	-0.59	0.05
	days to metamorphosis^t	11	-0.77	< 0.01
	metamorph mass/SVL^m (g/mm)	9	0.68	0.04
developmental stage^t	% missing eyes^t	12	-0.59	0.04
	days to metamorphosis^t	11	-0.88	< 0.01
	metamorph mass/SVL^m (g/mm)	9	0.65	0.05
	% missing eyes^m	11	-0.60	0.05
% lordosis^t	% feeding^t	7	-0.75	0.05
	days to metamorphosis^t	11	-0.78	< 0.01
	metamorph mass/SVL^m (g/mm)	9	0.84	< 0.01
% missing eyes^t	days to metamorphosis^t	11	0.67	0.02
	% missing eyes^m	11	0.68	0.01
% leg malformation^t	% swimming dysfunction^t	12	1.00	< 0.01
	% feeding^t	7	-0.89	< 0.01
% swimming dysfunction^t	% feeding^t	7	-0.89	< 0.01
days to metamorphosis^t	metamorph mass/SVL^m (g/mm)	9	-0.72	0.02

of atrazine and methyl mercury on days to metamorphosis, negative and positive respectively, would cancel each other out if the chemicals occurred simultaneously in a natural wetland. Ecological (i.e., release from competition) and physiological (i.e., thyroid hormones) responses associated with metamorphosis need to be evaluated as well in multiple chemical experiments using known initial tadpole densities.

Percent of leg malformations and swimming dysfunctions in tadpoles was positively correlated with the ranked sediment concentration of chlorpyrifos (for both correlations, r = 0.62, p = 0.02). There was also a positive relationship between the frequency of lordosis (upon collection) and the ranked sediment concentration of MSMA (r = 0.57, p = 0.05). For tadpoles surviving to metamorphosis, the positive relationship between the frequency of lordosis and the ranked sediment concentration of MSMA was also significant (r = 0.70, p = 0.01). Tadpole feeding behavior was affected by the presence of these abnormalities. During feeding, as proportion of developmental abnormalities in a tadpole group increased, percent of tadpoles engaged in

feeding decreased. A reduction in food intake may result in a decrease in growth or survival if the minimum tadpole size needed for metamorphosis to occur is not reached before pond drying (Bridges 1997; Semlitsch et al. 1995). Ouellet et al. (1997) showed a positive, though not significant, relationship between the proportion of hind-limb malformations in free-living anurans from control (0.7%) and pesticide-exposed habitats (12%). Ouellet et al. (1997) state that malformations appeared to interfere with swimming and saltatory movements in juveniles and may be evidence of a survival (to adulthood) handicap.

Tadpole swimming speed can be a measure of the individual's ability to escape predation (Bridges 1997). Swimming speed was negatively correlated with the ranked sediment concentration of methyl mercury ($r = -0.68$, $p = 0.04$), but positively related to the ranked concentration of atrazine in water ($r = 0.89$, $p < 0.01$). Individual variation between tadpoles may have affected these contrasting results. Speeds for all but one tadpole ranged from 4.9 to 8.1 cm/sec. The remaining tadpole swam at a rate of 12.4 cm/sec. Removal of this single speed value from the data set eliminated the significance of these correlations.

There was a negative correlation between the percent of missing eyes and the ranked concentration of atrazine in water ($r = -0.58$, $p = 0.04$). This malformation was significantly correlated with several ecological variables. For example, as percent of missing eyes increases, tadpole body size and developmental stage (upon collection) decreases and days to metamorphosis increases. These results suggest that tadpoles with one eye are less likely to find food resources needed for growth and will remain in the larval stage for a longer period of time.

In this experiment we observed significant correlations between the incidence of developmental malformations in anurans and presence of chemical contaminants. Given the preliminary nature of our data, it would be premature to speculate that these chemicals caused deformities or that deformities, when present, are indicative of significant, long-term changes in the population. We can conservatively conclude that environmental conditions (e.g., water quality) during likely periods of egg deposition and larval development are known and our results may lead researchers in more specific directions when examining the phenomena of amphibian malformations. Environmental stressors other than agrichemicals have also been implicated as possible stimuli for the development of abnormalities in amphibians. Blaustein et al. (1997) demonstrated that ambient UV-B radiation will cause malformations in *Ambystoma macrodactylum* embyros. Blaustein et al. (1997) suggest that these deformities may affect behavior and long-term survival of amphibian embryos but could not conclusively demonstrate this relationship because their experiment ended at hatching. Although UV-B was not measured in our experiment, it could have affected our results only through interaction effects with other stressors in the experimental system since it was not manipulated independently.

The results from this study suggest a link between exposure to chemical contaminants and larval development, survival, growth, and metamorphosis in *H. chrysoscelis* tadpoles. The causative nature of this link could not be determined given the uncertainty in the exact number of eggs deposited in each mesocosm as well as the specific date of deposition. Unless experiments are conducted to examine responses across the complete anuran life cycle (i.e., egg-to-egg), and with multiple interacting stressors present, little insight will be gained into how individuals and populations are affected by long-term, outdoor exposure.

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REFERENCES

Birge WJ, Black JA, Westerman AG, Ramey BA (1983) Fish and amphibian embryos -- a model system for evaluating teratogenicity. Fund Appl Toxicol 3:237-242

Blaustein AR, Kieseeker JM, Chivers DP, Anthony RG (1997) Ambient UV-B radiation causes deformities in amphibian embryos. Proc Natl Acad Sci 94: 13735-13737

Bridges CM (1997) Tadpole swimming performance and activity affected by acute exposure to sublethal levels of carbaryl. Environ Toxicol Chem 16: 1935-1939

Britson CA, Kissell Jr RE (1996) Effects of food type on developmental characteristics of an ephemeral pond-breeding anuran, *Pseudacris triseriata feriarum.* Herpetologica 52:374-382

Gosner KL (1960) A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16: 183-190

Gromko MH, Mason FS, Smith-Gill SJ (1973) Analysis of the crowding effect in *Rana pipiens* tadpoles. J Exp Zool 186:63-72

Hudson RH, Tucker RK, Haegle MA (1984) Handbook of toxicity of pesticides to wildlife. US Fish Wild1 Serv Resour Publ 153

Klaassen HE, Kadoum AM (1979) Distribution and retention of atrazine an carbofuran in farm ecosystems. Arch Environ Contam Toxicol 8:345-353

Montgomery DC (1997) Design and analysis of experiments. John Wiley & Sons, New York

Neter J, Wasserman W, Kutner MH (1990) Applied linear statistical models: regression, analysis of variance, and experimental designs. Richard D. Irwin, Inc, Boston, MA

Ouellet M, Bonin J, Rodrigue J, DesGranges J, Lair S (1997) Hindlimb deformities (ectromelia, ectrodactyly) in free-living anurans from agricultural habitats. J Wildl Dis 33:95-104

Ritke ME, Babb JG, Ritke MK (1990) Life history of the gray treefrog (*Hyla chrysoscelis*) *i n* western Tennessee. J Herpetol 24:135-141

Schoener TW (1979) Inferring the properties of predation and other injury-producing agents from injury frequencies. Ecology 60: 1110-1115

Semlitsch RD, Foglia M, Mueller A, Steiner I, Fioramonti, Fent K (1995) Short-term exposure to triphenyltin affects the swimming and feeding behavior of tadpoles. Environ Toxicol Chem 14:1419-1423

Smith DC (1987) Adult recruitment in chorus frogs: effects of size and date at metamorphosis. Ecology 68:344-350

Spading DW, Lowe TP, Day D, Dolan K (1995) Responses of amphibian populations to water and soil factors in experimentally-treated aquatic macrocosms. Arch Environ Contam Toxicol 29:455-461

Steevens J, Allgood J, Benson WH, Threlkeld ST (1997) Chemical mixtures in a wetland mesocosm: fate of chlorpyrifos, atrazine, monosodium methanearsonate, and methyl mercury. Abs Soc Environ Toxicol Chem 18th Annual Meeting:263

US EPA (1995) The generic expected environmental concentration program (GENEEC) Vers 21; tier one screening model for aquatic pesticide exposure. Environmental Fate and Effects Division, Office of Pesticide Programs, Washington

Wilber HM, Collins JP (1973) Ecological aspects of amphibian metamorphosis. Science 182:1305-1314

Willis L, Threlkeld ST, Carpenter CC (1982) Tail loss patterns in *Thamnophis* (Reptilia: Colubridae) and the probable fate of individuals. Copeia 1982:98-101