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EFFECTS OF MULTIPLE AGRICULTURAL CHEMICALS ON NORTHERN LEOPARD FROG, *LITHOBATES PIPIENS*, LARVAE

Robert D. Denton: 318 W. 12th Avenue, Department of Evolution, Ecology, and Organismal Biology, The Ohio State University, Columbus, Ohio 43210

Melody J. Bernot: 2000 W. University Avenue, Department of Biology, Ball State University, Muncie, Indiana 47306

ABSTRACT. A primary factor contributing to amphibian declines is the application and accumulation of agricultural chemicals. We examined how a common fungicide, chlorothalonil, affects development in larval northern leopard frogs (*Lithobates pipiens*) in conjunction with atrazine and increased nitrate concentrations in laboratory containers. No synergistic or antagonistic interactions between the treatments were identified. Further, there was no significant difference in weight and Gosner stage between tadpoles exposed to different chlorothalonil concentrations. However, nitrate increased tadpole mortality and decreased growth. According to our results, environmentally relevant concentrations of chlorothalonil may not be directly toxic to leopard frogs but may indirectly influence development.

Keywords: amphibian toxicology, chlorothalonil, mortality, agricultural chemicals

Amphibians' semi-permeable skin, dependence on aquatic habitats, and limited dispersal ability are among the many factors that make these organisms susceptible to environmental contaminants (Blaustein et al. 2003). There is evidence that reduced abundance, lower diversity, and increased instances of malformations occur in intensive agricultural areas (Bonin et al. 1997; Hayes et al. 2002). Therefore, the monitoring of amphibian reactions to agriculturally-derived pollutants is important not only for the retention of biodiversity, but also as an indicator of biotic response to human activities. Chemical contamination is a primary contributor to decline of amphibian populations (Blaustein et al. 2003). Amphibians may be affected by these contaminants either directly, through increased mortality, or indirectly such as through changes in immune response (Blaustein et al. 2003) or sexual morphology (Hayes et al. 2003).

In 2008, Boone showed that interactive effects between agricultural chemicals can be present when individual effects are not. These data necessitate an assessment of synergistic and antagonistic toxicities to understand the complexity of threats to amphibian development. Because there is a complex group of potential stressors for amphibian populations, the control laboratory experiments afford is beneficial for identifying effects of agricultural chemicals on amphibians.

Correspondence to: Robert D. Denton, 318 West 12th Avenue Columbus OH 43210, 765.618.2944, robert.d.denton@gmail.com

The objective of this research was to identify effects of three common agricultural pollutants on amphibian development as well as synergistic and antagonistic interactions of these pollutants. Target compounds were selected to evaluate effects of nutrients (as nitrate), a common herbicide (atrazine), and a common fungicide (chloranthalonil). Although several studies have independently evaluated toxicity of atrazine and nitrate, few data examine interactions of these contaminants on amphibian development. Chlorothalonil is the second most used fungicide in the United States (Gianessi and Anderson 1995), and there is no data available analyzing chlorothalonil's synergistic and antagonistic effects with other agricultural chemicals (Winkler et al. 1996).

METHODS

Methods were similar to those used by Allran and Karasov (2000). 180 northern leopard frog (*Lithobates pipiens*) tadpoles were randomly distributed to laboratory containers (5 tadpoles per replicate) associated with twelve treatment groups (Table 1). Each treatment container was replicated three times for a total of 36 containers.

Table 1.—Chemical treatments and corresponding concentrations used in laboratory container experiments.

Chemical Treatment	Treatment Concentration	Reference
control	0	
chlorothalonil	0.5μg/L	Caux et al. 1996
chlorothalonil	1.0μg/L	
chlorothalonil	1.8μg/L	
atrazine	18μg/L	Solomon et al. 1996
nitrate	10mg/L	Allran and Karasov 2000
chlorothalonil + atrazine	0.5μg/L + 18μg/L	Caux et al. 1996; Solomon et al. 1996
chlorothalonil + atrazine	1.0μg/L + 18μg/L	
chlorothalonil + atrazine	1.8μg/L + 18μg/L	
chlorothalonil + nitrate	0.5μg/L + 10mg/L	Caux et al. 1996; Allran and Karasov 2000
chlorothalonil + nitrate	1.0μg/L + 10mg/L	
chlorothalonil + nitrate	1.8μg/L + 10mg/L	

Each replicate container consisted of a 1L HDPE container filled with 1000 ml deionized water. Water was changed every three days to ensure that chemical concentrations remained consistent and to remove waste. During water changes, a bulb syringe was used to suction out solid waste at the bottom of the containers. Then, water was decanted out of the tub until it reached a volume of 500ml. At this point, 500ml of fresh water was poured into the containers down the wall in order to minimalize splashing and stress to tadpoles. Target compounds were then added evenly across the water's surface. All containers were placed in an environmental incubator maintained at 23°C for the duration of the experiment. The light cycle consisted of 14 h of fluorescent lighting and 10 h darkness.

Treatment chemical concentrations (Table 1) were chosen to reflect typical ranges previously measured in freshwater ecosystems. Atrazine concentrations seldom exceed 20 μg/L *in situ* (Solomon et al. 1996). Nitrate concentrations are based on concentrations measured by Allran and Karasov (2000). Chlorothalonil concentrations have been measured up to 1.8 μg/L in agricultural areas (Caux et al. 1996). Chemical stressors were prepared using deionized water and stored at room temperature in acid-washed HDPE containers. Chemicals were added to the containers one day before tadpoles emerged from eggs. Tadpoles were added to laboratory containers immediately after they hatched from their eggs. All containers were checked at least every other day for deceased larvae, which were removed immediately. Tadpoles were fed *Xenopus* ground meal *ad libitum*.

Tadpoles were euthanatized by immersion in a tricaine solution at 250mg/L for 10 minutes. The experiment was terminated at day 35 and each tadpole was examined for any noticeable abnormality. Tadpoles were also weighed for wet body mass and assessed for Gosner developmental stage (Gosner 1960) using a dissection scope. All statistical comparisons of means were made with two-way ANOVA tests using IBM's Statistical Package for the Social Sciences (SPSS) v.18. Chlorothalonil concentration and additional chemical treatment (Atrazine, Nitrate) were treated as two separate factors. Separate two-way ANOVA tests were executed for each of the three dependent variables: mortality, Gosner stage, and weight. Least Significant Difference pairwise comparisons between treatments and chlorothalonil concentrations were used to elucidate differences between specific treatments and levels.

RESULTS

Terminated tadpoles ranged in weight from 0.4 to 4.8 grams. Gosner stage ranged from 27 to 38. There were no externally visible deformities. There was, as expected, a positive correlation between stage and weight (exponential regression, $p<0.001$, $F = 168.91$, $df = 1.81$, $R^2 = 0.676$).

Mortality was 53% during the 35 d experiment period. All chemical treatments produced higher mortality than the control groups (Fig. 1). The mortality rates were highest in treatments containing nitrate ($n = 4$) and lowest in chlorothalonil only groups ($n = 8$). All tadpoles in the nitrate-only treatment had died by day 35. All tadpoles in control

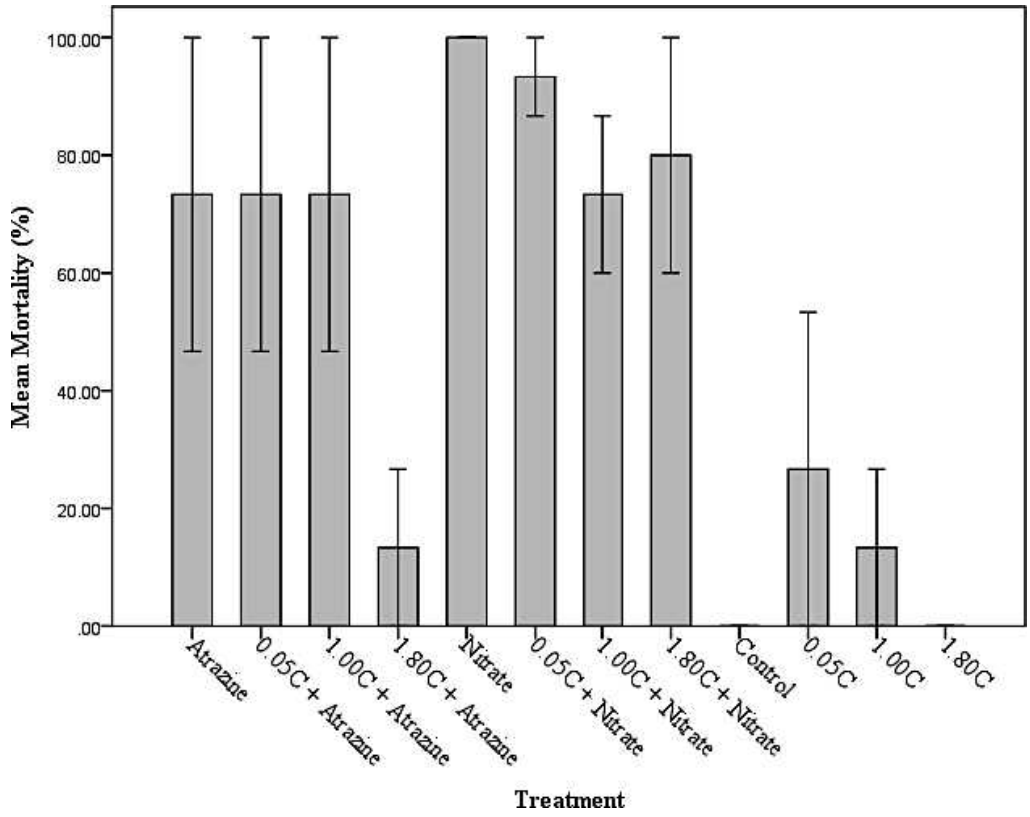

Figure 1.—Average mortality ± one standard error of *R. pipiens* tadpoles for all treatments with one standard error. For concentrations of all chemicals, see Table 1. C=chlorothalonil.

treatments survived the duration of the experiment. Mean mortality was significantly different between treatment groups ($F_{2,11} = 18.87$, $p < 0.001$). Chlorothalonil concentration had no significant effect on mortality ($F_{3,11} = 1.97$, $p = 0.146$). In addition, the interaction between treatment and chlorothalonil concentration had no significant ffect on mortality ($F_{6,11} = 0.92$, $p = 0.50$). Pairwise comparisons indicated that groups treated with Nitrate had higher mortality than groups treated with either Atrazine (Mean Difference = 28.33, SE = 12.62, $p = 0.034$) or chlorothalonil (MD = 76.67, SE = 12.62, $p < 0.001$). Additionally, chlorothalonil treatments had significantly lower mortality than Atrazine groups (MD = −48.33, SE = 12.62, $p = 0.001$).

Because only four containers that included nitrate held surviving tadpoles, nitrate was not included as a factor in both weight and Gosner stage analyses due to unacceptable sample size. The surviving individuals in chlorothalonil groups showed little variation in weight or Gosner stage between the treatments (Fig. 2). Tadpole weight was not significantly affected by treatment ($F_{1,7} = 3.39$, $p = 0.07$), chlorothalonil concentration ($F_{3,7} = 01.03$, $p = 0.383$), or the interaction between treatment and chlorothalonil concentration ($F_{3,7} = 0.51$, $p = 0.679$). Likewise, tadpole Gosner stage was not significantly affected by treatment ($F_{1,7} = 0.97$, $p = 0.33$), chlorothalonil concentration ($F_{3,7} = 0.22$, $p = 0.89$), or the interaction between treatment and chlorothalonil ($F_{3,7} = 1.37$, $p = 0.26$).

DISCUSSION

Our experiment identified no synergistic or antagonistic interactions between atrazine, nitrate, and chlorothalonil concentration. However, our study demonstrated that these three chemicals do influence amphibian mortality at environmentally-relevant concentrations. Our data indicate nitrate can cause direct mortality

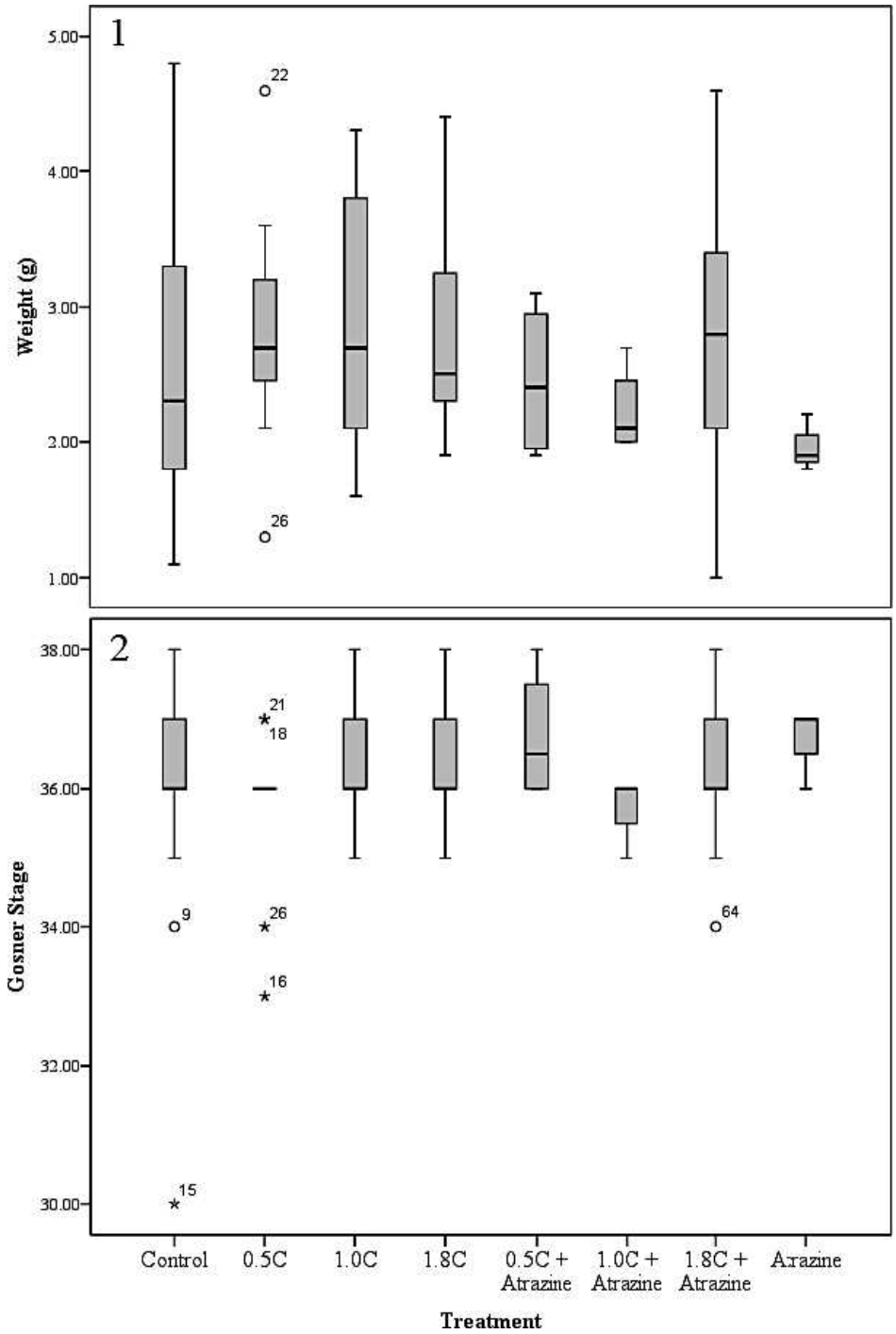

Figure 2.—Mean tadpole weight (1) and Gosner stage (2) of surviving tadpoles across chemical treatment treatments. For chemical treatment concentrations see Table 1. C=chlorothalonil, circles indicate outliers, asterisks indicate extreme values (> 3x the interquartile range), and all numbers represent individual cases.

in *L. pipiens*. Other studies using comparable nitrate concentrations identified variable responses among species (reviewed in Rouse et al. 1999). Allran and Karasov (2000, 2001) also demonstrated that nitrate slows development of *L. pipiens* larvae.

There was no significant difference in mortality, weight, or Gosner stage between tadpoles with different chlorothalonil concentrations. Our data show no evidence that low environmental concentrations of chlorothalonil in freshwater influence *L. pipiens* development. Previous field documentation of amphibian declines in response to chlorothalonil focused on cranberry bogs, where the chemical can bind to humic material and increase chlorothalonil exposure (Winkler et al. 1996). At the time of this experiment, the chlorothalonil concentration of 1.0μg/L was estimated to be the lowest observable effect concentration for several fish species. Larger chlorothalonil concentrations have been identified in runoff (~ 272μg/L, Shuman et al. 2000), and expected environmental concentrations have been calculated (164 μg/L, McMahon et al. 2011). McMahon et al. (2011) found significant mortality in *L. sphenocephalus* (Southern leopard frog) and *Hyla cinerea* (green treefrog) at concentrations as low as 0.0164 μg/L in laboratory experiments with single doses of chlorothalonil. However, McMahon et al. showed that *L. sphenocephalus* had significantly higher mortality at low and high concentrations compared to intermediate concentrations (~1.64 μg/L). The concentrations used in our experiment could have fallen in this intermediate zone of tolerance. Similar to chlorothalonil, atrazine treatments did not influence Gosner stage and tadpole weight when compared to nitrate and chlorothalonil treatments. This result is consistent with previous studies (Allran and Karasov 2000, 2001). Studies that have shown developmental deformities in *L. pipiens* from atrazine exposure have assessed higher concentrations (Hayes et al. 2003) or have been descriptive field assessments (Bonin et al. 1997), potentially influencing observed effects. However, atrazine is known to have indirect and sublethal effects that were not measured in the experiment (reviewed in Rohr and McCoy 2010).

One possible explanation for why there were no synergistic or antagonistic effects of chemical treatment could be the dissimilar modes of action of the three contaminants. Boone and Bridges-Britton (2006) suggested that non-additive results may be due to chemicals that show different modes of action. Given that other studies have found greater additive effect on *L. pipiens* with atrazine and alachlor (Howe et al. 1988), it was surprising that atrazine when combined with chlorothalonil had little effect on the weight and Gosner stage when compared to chlorothalonil-only treatments. From an ecological perspective, these chemicals also affect amphibians indirectly. Greater mortality in a natural wetland setting could be facilitated by direct chemical effects on both individual larvae and their food sources (Caux et al. 1996). Additionally, agrochemicals can increase amphibian susceptibility to parasites (Rohr et al. 2008). Future testing is required using chlorothalonil, but our data supports theories that chlorothalonil tolerance is highly species specific (McMahon et al. 2011), and that amphibian mortality is most likely in environments where chlorothalonil is allowed to bind to humic material, resulting in very large bioconcentrations (Winkler et al. 1996). The lack of synergistic and antagonistic effects of atrazine and nitrate is consistent with previous work (Allran and Karasov 2001).

Atrazine, nitrate, and recently chlorothalonil have both been hypothesized to be factors in amphibian reductions, but studies, including our data, indicate these chemicals do not cause negative consequences by themselves. However, there are many factors that influence amphibian larval development and many environmental situations that can lead to higher concentrations than those used in this experiment. For management purposes it is vital that contaminant studies continue to be done both in the field and in the laboratory to discover any and all negative consequences these chemicals may have on freshwater integrity.

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