EFFECTS OF ATRAZINE, CHLORPYRIFOS, AND THEIR INTERACTIONS ON ANTI-PREDATOR BEHAVIOR AND ACTIVITY IN AFRICAN CLAWED FROGS, *XENOPUS LAEVIS*

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The following faculty have examined the final copy of this thesis/dissertation for form and content, and recommend that it be accepted in partial fulfillment of the requirement for the degree of Master of Science with a major in Biology.

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Chris Rogers, Committee Member

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Dorothy Billings, Committee Member

DEDICATION

To my friends, who aided me whenever they could and encouraged me to keep going, and to my family, for helping to lighten my burden.

I'd like the following people to know that this would not have been possible without their help

Natalie
Heather
Elizabeth
Temperance
Ann
Amos
Richard
Cory

Finally, I dedicate this to the memory of Darren Francisco.

"Not all chemicals are bad. Without chemicals such as hydrogen and oxygen, for example, there would be no way to make water, a vital ingredient in beer." --Dave Berry

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ABSTRACT

Two separate experiments were conducted to examine potential mixture effects of chronic, sub-lethal concentrations of atrazine and chlorpyrifos on the alarm response and activity of larva of the African clawed frog, *Xenopus Laevis*. Larvae were exposed to three concentrations of atrazine, (0, 20, and 200 μg/L), three concentrations of chlorpyrifos (0, 1, and 10 μg/L), and all possible combinations of those doses for a period of two weeks prior to behavioral testing.

Activity of larvae was evaluated by measuring both how many seconds the larvae spent active in any way and also by measuring the number of times the larvae crossed the center line of their experimental container. Alarm responses were provoked by introduction of an "alarm substance," to which larvae reacted by exhibiting anti-predator behavior. This alarm response was evaluated by measuring the amount of time spent active before and after alarm substance introduction (depressed activity is the typical alarm response), as well as the amount of time spent opposite the end of the experimental container where the alarm substance was introduced. Behavioral trials were videotaped for purposes of data collection.

Three major null hypotheses were tested: 1) No differences in mean behaviors among atrazine concentrations, 2) No differences in mean behaviors among chlorpyrifos concentrations, and 3) No interaction effects between atrazine and chlorpyrifos on mean behaviors.

In the first experiment, one clutch of *Xenopus Laevis* tadpoles was divided into two replications per treatment totaling 225 individuals. The second experiment tested a total of 270

individuals; Individuals were chosen at random, but in even numbers, from two genetically unrelated clutches. Two replications of each treatment group were also included in this experiment.

Both experiments subjected larval *Xenopus Laevis* to the same period of behavioral analyses. The results affirmed previous research showing atrazine's depressive effect on anuran activity [15]. In experiment 1, 200 μg/L atrazine had a depressive effect on the number of lines crossed before cue addition ($F=4.86$, d.f. = 2,224, $p = 0.0087$). For the variable activity time before cue addition, the tadpoles in the 200 μg/L atrazine treatment showed a significantly decreased amount of time spent active when compared to the 0 and 20 μg/L treatments ($F=3.08$, d.f. = 2,224, $p = 0.0110$).

In experiment 2, for the variable lines crossed after cue addition, the tadpoles in the 200 μg/L atrazine treatment showed a statistically significant decrease in the number of lines crossed when compared to the 0 μg/L negative control for atrazine ($F=3.79$, d.f. = 2,234, $p = 0.0240$). When tadpole weight was compared amongst treatments, it was shown that increasing atrazine concentrations corresponded with decreasing weight ($F=52.04$, d.f. = 2,234, $p <0.0001$).

No significant effects on behavioral traits were observed from the chlorpyrifos treatments. Finally, both experiments showed significant interaction effects that did not conform to a single type of chemical mixture interaction. In experiment 1, there was a significant interaction effect on the number of lines crossed before cue addition ($F=2.70$, d.f. = 4,224, $p = 0.0316$). The analysis of the nine separate treatment groups showed atrazine alone to have a non-significant trend of increasing the number of lines crossed by tadpoles. Chlorpryifos alone also showed a non-significant trend of increasing the number of lines crossed. Tadpoles appeared to

show a synergistic increase in the number of lines crossed at 20 μg/L atrazine mixed with 1 μg/L chlorpyrifos, followed by a significant decrease in the number of lines crossed in the 10 μg/L chlorpyrifos mixture treatments.

In experiment 2, there was a highly significant mixture interaction effect on weight, in which atrazine alone had a depressive effect on weight, with tadpoles in the 20 and 200 μg/L treatments yielding a significantly lower weight than tadpoles in the 0 μg/L concentration negative control. When examining chlorpyrifos alone, tadpoles in the 10 μg/L treatment showed a significantly greater weight than the tadpoles in the 1 μg/L treatment. Ten μg/L chlorpyrifos appeared to have an additive interaction with the atrazine, whereas 1 μg/L chlorpyrifos appeared to have an antagonistic relationship with atrazine, in which the weight of tadpoles exposed to 1 μg/L chlorpyrifos mixed with 20 μg/L atrazine and 200 μg/L atrazine had a larger weight than one would expect were the relationship additive ($F=7.87$, d.f. = 2,234, $p < 0.0001$). The differences in interaction effects highlight the need for further study regarding agrochemical mixture interaction effects on larval anurans.

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Introduction

Over the past two decades, research has shown a general decline in amphibian populations worldwide [1]. This is not only a concern for biodiversity, but for the health of almost every ecosystem in the biosphere. Amphibians live in nearly every biome on the planet and, as a group, they generally possess unique features that make them excellent indicators of environmental health. Amphibians undergo three distinct life stages--egg, larval, and adult--with the adult and larval stages separated by the metamorphic process. Egg and larval life stages are restricted to the aquatic environment which potentially exposes amphibians from the earliest life stages to anthropogenic alterations of aquatic ecosystems. To compound this, larval and adult life stages possess highly permeable skin, making them prone to the effects of harmful components of their environment. All amphibian life stages are highly susceptible to the absorption of toxicants from the water [2]. These characteristics create a situation whereby, if an ecosystem is contaminated or altered, amphibians are usually among the first species to exhibit effects, negative or otherwise. Because of this, amphibians are extremely important 'sentinels' when examining possible toxicological influences in ecosystems [3].

Because of their status as environmental indicators and their noted decrease in abundance in many ecosystems, numerous investigations into the mechanisms of amphibian decline have been performed. These studies reveal a plethora of possible contributing factors to amphibian declines, including habitat loss, introduction of exotic species, diseases and pathogens, UV radiation, and industrial agricultural chemicals. Early records dating to the 1600s indicate that the United States once contained 60 to 75 million hectares of wetlands [4]. However, as of 1994, draining and construction alone had reduced the remaining wetland area to 43.7 million hectares.

Furthermore, the remaining wetland habitat is severely fragmented, greatly reducing the area of contiguous wetland environment. The continuation of draining and construction in wetland habitat has resulted in the continuing loss of 120,000 hectares of wetlands per year [5].

The introduction of exotic species creates serious detrimental effects on amphibian survivorship. Pond owners in the Midwestern states of the U.S. often add game fish such as bluegill (*Lepomis macrochirus)*, small- and large-mouth bass (*Micropterus dolomieu* and *Micropterus salmoides,* respectively*)*, trout (*Salmo sp.; Onchorynchus sp*.; or *Salvelinus sp*.), perch *(Perca sp*.), and pike (*Esox sp*.), all of which prey upon anuran eggs, larvae, and adults [6,7]. Some anurans overwhelm fish predators with high fecundity and synchronized breeding, but this mechanism is often defeated when the fish predators are not native or present in stocked densities [8]. Catfish (*Ictalurus punctatus*) and perch **(***Perca sp***.)** introduced into the waters of California forage by stirring sediments and aquatic plants, locations in which native *Ranid* tadpoles are often found. Furthermore, it was observed that in areas where the catfish and sunfish have been introduced, *Ranid* populations have decreased [9]. In the Sierra Nevada Mountains, introductions of rainbow and golden trout and brook char coincided with population declines of native frog populations [10]. Furthermore, exotic fish species will often carry foreign parasitic and disease-causing organisms [9].

During the late 1970s and early 1980s, North American leopard frog **(***Rana sp***.)** populations showed dramatic declines across Canada, the United States, and Mexico. One proposed explanation for the population decline is a disease called "Red Leg," which results from an infection caused by the gram-negative pathogen *Aermonas hydrophilia*. Red Leg, a syndrome characterized by kidney failure, ulceration, and hemorrhaged blood vessels (the propensity for hemorrhaging to occur on the ventral sections of the hind limbs gives the

syndrome its name), is thought to have been transmitted between populations across the continent [11]. In Oregon, a mold known as *Saprolegnia ferax* has been shown to contribute to high egg mortality in the species *Bufo boreas* since 1989 [11].

Blaustein et al. (1994) have shown that ultraviolet radiation contributes to egg mortality. In anurans, photolyase is an enzyme within eggs which acts to repair UV damage and varies in concentration based on habitat elevation. Eggs, varying in photolyase activity, from several species were subjected to UV-B radiation. Anuran eggs lacking high levels of photolyase activity (varied by species) suffered decreased hatching success when exposed to UV-B radiation, which may be compounded by increases in UV-B due to ozone depletion [12].This can be compounded by the fact that UV radiation may synergize with some chemical contaminants that absorb strongly in some portion of the UV spectrum, making them phototoxic [13].

Sixty-five percent of the entire planet's freshwater resources are relegated to agricultural use, including watering and irrigation [14]. A great many of these agricultural lands are intensely managed, their soils tilled and modified with fertilizers and pesticides. Insecticides and herbicides are heavily applied to agricultural land to control pest growth, petroleum-based products are used in the powering of agricultural equipment, and even antibacterial drugs and hormones are applied with the aim of increasing crop yield. Irrigation water contaminated with agrochemicals is sometimes even shared and recycled between growers, then disposed of in stream, river, or reservoir systems. Because of the fluvial nature of these systems, local contamination becomes a widespread geographical problem, the toxicants polluting both soil and water far downstream of the input site. Furthermore, the agrochemicals not directly disposed of in local aquatic systems often become inputs through run-off from precipitation events.

Although there is much evidence about the acute, lethal effects of anthropogenic contamination on amphibians [2, 3, 15, 16], comparatively little research concerns sub-lethal, chronic impacts on amphibian fitness [17,15, 18, 19]. This may be attributable to the difficulties in measuring how an amphibian is negatively affected once acute reactions such as death are no longer being examined—often such effects are evaluated posthumously, looking at the morphological and physiological impacts of contaminants. However, amphibian behavior can become an excellent frame of reference for research into sub-lethal and chronic contamination [16]. There is research showing that amphibian behavior can be negatively impacted by chronic, sub-lethal exposure to agrochemicals. This is important because behavior has numerous impacts on foraging, survivability, and reproductive success. By measuring the impacts of agrochemicals on amphibian behavior, more can be investigated than purely lethal and physiological effects. Negative impacts on behavior, such as activity levels, could relate directly to foraging behavior. Negative impacts on activity (reduced activity) can indicate reduced foraging activity [20]. Reduced foraging behavior results in reduced size at metamorphosis. Size at metamorphosis is directly correlated to reproductive success [17]. Amphibian behavior is currently subject to intense research and interest. Because of the presence of research concerning normal, healthy amphibian behavior and its impacts on reproductive success, established behavioral experimental approaches can be modified to investigate behavioral effects of sub-optimal conditions [15].

The ultimate goal of a larval amphibian is to survive to metamorphosis. In order to do this, there are a number of key behaviors that must be performed, including kin recognition, foraging, and anti-predator behavior. Of these key behaviors, anti-predator response [21] and foraging behavior are of particular importance to survivorship [22, 17]. As stated earlier, foraging behavior has a major impact on reproductive success. Active predation on tadpole

populations can severely deplete the number of tadpoles surviving to metamorphosis [7, 9, 10, 23]. Because of this a tadpole must compensate behaviorally for the presence of a predator. Toad tadpoles raised in artificial ponds with the odonate predator *Anax junius*, were observed to reduce movement by 41% and avoid predators spatially. Presumably, because of reduced movement and reduced spatial utilization the growth rate of the tadpoles was reduced by 28% and tadpoles were significantly smaller at metamorphosis [24].

Kiesecker [25] investigated western toad, *Bufo boreas*, anti-predator response behavior. Toad tadpoles responding to chemical cues were observed to reduce movement, increase shelter use, and avoid the area where the predator cue was added. Furthermore, in testing what cues the larvae respond to, it was determined that visual cues from insect predators did not result in anti-predator responses. This means that the chemical cue was most likely the only factor eliciting the anti-predator response. In research on four larval anuran species, *Bufo woodhouseii*, *Hyla crucifer*, *Hyla andersonii*, and *Hyla versicolor*, anti-predator responses to chemical cues included decreased movement and increased time spent immobile [23].

Decreased movement can negatively affect not only anti-predator behavior, but also foraging behavior. Anti-predator behavior is directly related to reproductive success in that reduced anti-predator behavior increases risk of mortality before reproductive maturity. Foraging behavior also has a strong impact on reproductive success. This is because a larval amphibian's size at metamorphosis may be positively correlated with its future reproductive success, increased size leading to increased reproductive success, and decreased size leading to decreased reproductive success [26]. Consequently, the time at which a larval amphibian metamorphoses is plastic—it can be varied in one direction or another. Research on this phenomenon has produced the Wilbur-Collins model of metamorphosis, which states that if a larval amphibian can grow in

size, metamorphosis will be delayed and the tadpole will continue to grow. If resources or water quality become limiting, larvae will cease growing and spur development to metamorphosis [26].

Alford and Harris [27] examined the Wilbur-Collins model by subjecting *Bufo woodhouseii* tadpoles to constant, increasing, and decreasing rates of food supply. They showed that an increasing food supply resulted in the delay of metamorphosis and increased growth rate compared to constant food supply. The decreasing food supply rate trials showed a shorter time to metamorphosis and decreased growth rates relative to the constant supply rate.

Crump [22] exposed larval *Hyla pseudopuma* to varying water levels. Tadpoles raised in high water levels and exposed to decreasing water levels showed increases in developmental rates, spurring the metamorphic process earlier than tadpoles raised in constant median water levels. Furthermore, tadpoles raised at constant high water levels were significantly larger at metamorphosis than all others.

Hensley [28] performed a replicate of the Alford and Harris [27] experiment, this time using *Pseudacris crucifer* larvae as test subjects and found that the rate of differentiation again corresponded to growth rates in a manner predicted by the Wilbur-Collins model, even though hind-limb toe differentiation and developmental rate became fixed past a certain developmental stage.

Reques and Tejedo [20] created a number of temporary ponds and varied the duration of time that the ponds remained filled with water in order to test the Wilbur-Collins model. What they found was that *Bufo calamita* larvae metamorphosed more rapidly from ponds with shorter durations, responses that were predicted by the Wilbur-Collins model.

The Wilbur-Collins model provides a prediction of tadpole response to natural stressors in their environments—this is useful because these predictions can be used to evaluate whether

or not artificial stressors, such as agricultural chemical inputs, are causing tadpoles to respond behaviorally in a manner consistent with the Wilbur-Collins model. What has been found is that *Xenopus laevis* tadpoles exposed to atrazine were observed to both delay metamorphosis and also metamorphose at significantly smaller sizes in relation to those not exposed [17]. Atrazine not only resulted in the delaying of metamorphosis and increased exposure time, but also metamorphosis at smaller sizes. This kind of maladaptive reaction is completely inconsistent with the Wilbur-Collins model and may indicate that tadpoles have not had time to develop genetic responses and/or physiological compensations in response to anthropogenic reductions in habitat quality.

The impact of agricultural chemicals on behavior necessary to achieve successful metamorphosis is of particular concern because frogs will often use temporary breeding pools during the spring months. This results in an overlap of the frog breeding season and agricultural irrigation and spray schedules, potentially contaminating freshwater breeding sites with run-off and creating temporary ponds of highly contaminated irrigation water. Larval amphibians may become exposed to potentially harmful chemicals during incubation, development, and metamorphosis. Thus, the potential for negative behavioral effects on larval amphibians is particularly high. Unfortunately, comparatively few studies have assessed toxicological affects of agrochemicals on amphibian behavior. Several studies exposing tadpoles to single toxicants have indicated that exposure to concentrations below the NOEC (20 μg/L) of the herbicide atrazine result in depression of activity (i.e., movement) in *Xenopus laevis* [e.g., 15]. Tadpoles of *Rana sphenocephala* exposed to carbaryl, an acetycholinesterase inhibiting insecticide, exhibited 48.4% reduced activity, spent less time in refuge in the presence of predators, and more time in refuge in the absence of predators compared to control animals [29].

Atrazine has also been shown to reduce activity in *Xenopus laevis* tadpoles, using anti-predator behavior as the axis of measurement. Alarm cue experiments, such as those used by Kiesecker [25], can easily be integrated with toxicological exposure. By exposing tadpoles for a period of time before the behavioral tests, and comparing treatments, behavioral effects from exposure can be measured quantitatively. At concentrations of 200 μg/L, atrazine exhibited a depressive effect on total activity of exposed *Xenopus laevis* tadpoles [15].

To date, there is no research concerning the possible interaction of pesticides and resultant affects on larval amphibian activity. The possibility of interaction effects is important to investigate because tadpoles that develop in irrigation run-off water will be exposed to multiple chemicals for periods of time, and these chemicals may be specifically interacting in ways that could compound potential negative behavioral affects.

In order to address this dearth of information, this study applied a factorial experimental overlay to an established experimental protocol for examining anti-predator behavior. Hews and Blaustein [30], Hews [31], and Kiesecker et al [32] investigated anti-predator behavior using alarm cues. The chemical cues released by damaged conspecific tadpoles were found to stimulate an anti-predator response in experimental animals without the need of any additional stimuli. By removing the presence of a predator from the experiment, the number of possible influences on anti-predator response behavior is reduced. This ability to control the complexity of the experiment lends itself particularly well to the overlay of a factorial toxicological experiment involving multiple contaminants over the basic behavioral experiment.

Atrazine (2-chloro-4-ethylamino-6-isopropylamine-s triazine), a triazine herbicide, has been shown to inhibit photosystem II of photosynthesis and is moderately volatile and soluble in water. In addition, atrazine is resistant to natural degradation in water and maintains at least 85%

of chemical concentrations over a 4-day period [18]. While atrazine is not approved for use in controlling aquatic plant growth, its presence in aquatic systems has been shown to inhibit growth of aquatic plants, which reduces food supply and shelter to anuran larvae [19], in addition to potential impacts on metamorphic and growth rates. Sixty million pounds of atrazine are used annually in the United States alone and during spring run-off concentrations of atrazine greater than 20 μg/L have been found in aquatic systems [33]. Tadpoles exposed to 320 μg/L atrazine increased time to reach metamorphosis and exhibited, among other negative impacts, decreased weight [18]. Exposure to chronic, sub-lethal levels of atrazine resulted in increased surface activity in goldfish. This may be linked to possible acetylcholinesterase-inhibiting action on neuromuscular tissues [34, 35, 36].

Chlorpyrifos (*O*-diethyl *O*-3,5,6 trichloro-2-pyridyl phosphorothioate) is a commercial pesticide that inhibits acetylcholinesterase to cause death. Chlorpyrifos has been shown to be present in streams adjacent to agricultural fields in concentrations up to 3.96 μg/L, with median concentrations of 0.116 and 0.128 μg/L [37]. Although chlorpyrifos displays a very short half-life, from 12 hours to several days in non-flowing, shallow systems [38, 39, 40], its presence can still be associated with reduced hatching success and larval malformations [16]. Exposure to acetylcholinesterase inhibiting pesticides, such as carbaryl (which shares a mode of action with chlorpyrifos) has been shown to decrease activity, leading to longer larval periods and decreased size at metamorphosis [16].

The purpose of this study was to investigate possible changes in activity and anti-predator behavior in larvae of the frog species *Xenopus laevis*, the African Clawed Frog, when exposed to atrazine, chlorpyrifos, and mixtures of both. *Xenopus* larvae were exposed to nine separate levels of treatment, (20 and 200 μg/L atrazine only; 1 and 10 μg/L chlorpyrifos only; 20,1; 20,10;

200,1; 200,10 μg/L atrazine/chlorpyrifos, respectively; and finally a 0,0 μg/L atrazine/chlorpyrifos control). After exposure, individual tadpoles were videotaped both in the presence and absence of a predator alarm substance in order to quantitatively measure activity. Activity was measured both as time spent active and number of crossings of the center line in the experimental container. Although some specific null hypotheses varied between experiments, both experiments included three major null hypotheses: 1) that no differences in mean behaviors among atrazine concentrations would be observed, 2) that no differences in mean behaviors among chlorpyrifos concentrations would be observed, and 3) that no interaction effect between atrazine and chlorpyrifos on mean behaviors would be observed. There are three possible outcomes when examining interactions of two chemicals: first, that there is no interaction; second, that the interaction is synergistic; and third, that the interaction is antagonistic. If there is no interaction occurring between the two chemicals, it would be expected that they would display toxicities equal to the sum of their individual toxicities, or additive interaction. Another possibility is that the atrazine and chlorpyrifos interact in a way that shows a toxic effect greater than the sum of their individual toxicities, termed synergistic interaction. Finally, if the overall toxic effect is less than the individual toxicities of atrazine and chlorpyrifos, it would be indicative of antagonistic interactions. Because of possible acetylcholinesterase inhibition as a result of atrazine exposure, and the fact that AChE inhibition is the primary mode of action of chlorpyrifos, a non-additive, synergistic interaction between atrazine and chlorpyrifos may occur.

Methods & Materials

Two separate experiments that used environmentally realistic concentrations of atrazine and chlorpyrifos were conducted in order to test the effects of chronic, non-lethal chemical exposure on induced anti-predator reactions in *Xenopus leavis.* The first experiment was designed as a 3(levels of atrazine) x 3(levels of chlorpyrifos) x 2(replicate tanks) factorial analysis of variance (ANOVA) and the second as a 3(levels of atrazine) x 3(levels of chlorpyrifos) x 2(replicate tanks) x 2(non-sibling clutches) factorial ANOVA. In both experiments, the three atrazine concentrations used were 0, 20, and 200 μg/L, and the three concentrations of chlorpyrifos used were 0, 1, and 10 μg/L. The combinations of these established nine experimental treatments: 0 μg/L atrazine, 0 μg/L chlorpyrifos, two atrazine only treatments (20/0, 200/0 μg/L atrazine/chlorpyrifos, respectively), two chlorpyrifos only concentrations (1/0, 10/0 μg/L chlorpyrifos/atrazine, respectively), and the four possible mixtures of the concentrations (20/1, 20/10, 200/1, and 200/10 μg/L atrazine/chlorpyrifos, respectively). The first experiment was conducted using randomly assigned larvae from a single breeding pair of *Xenopus laevis*. The second experiment was conducted using two additional, genetically unrelated breeding pairs of *Xenopus laevis* in order to control any sibling effects in the overall data. Because seasonal effects on growth rate can occur, the two experiments were separated by one year.

In Experiment 1 four *Xenopus Laevis* adults, two pairs of males and females, were purchased from Xenopus I, Inc. (Dexter, MI, USA). The two pairs were housed separately in two 38 L aquaria, so as to prevent the mixing of egg clutches from different parents. Each aquarium was filled with 4 L of FETAX water [41]. Using 1000 IU of human chorionic gonadotropin (HCG) subdermally injected into the dorsal lymph sac, the adults were stimulated

to amplex, and removed to holding aquaria following laying and fertilization of eggs. One clutch was used as the experimental animals, and the second clutch was used for preparation of the alarm substance.

Exposure and Maintenance

Beginning at ten days of age, the beginning of the feeding stage, 225 full-sibling *X. laevis* tadpoles were divided evenly, using random choice, and housed in eighteen 38 liter aquaria. Fifty tadpoles were utilized in each of the nine chemical experimental treatments (two replicate aquaria per treatment, 25 tadpoles per aquarium). Each of the eighteen aquaria was filled with six liters of FETAX [41] water and the appropriate experimental concentrations of pesticides were added prior to tadpole addition. 99% pure Atrazine was acquired from ChemService (Westchester, PA USA) and was subsequently dilluted in acetone to make a stock solution of 0.1 g atraizine / 20 ml acetone. 20 μg and 200 μg concentrations were acheived by adding 24 μl and 240 μl, respectively, to the 6 L of FETAX water in the test aquaria. Chlorpyrifos was obtained from DowElano (Indianapolis, IN USA) and was also diluted in acetone in order to create a stock solution of 0.05 g chlorpyrifos / 40 ml acetone. The experimental concentrations of 1 μl l and 10 μl were attained by adding 4.8 μl and 48 μl, respectively, to the 6 L of FETAX water in the test aquaria. Previous research has indicated that acetone at the concentrations in the experimental doses has no affect on *Xenopus laevis* [17], so acetone controls were not considered in the experimental design.

Complete renewal of FETAX water and chemical concentrations was performed on an alternating 3 and 4 day schedule.. Tadpoles were decanted from the aquaria and aquarium walls

were rinsed thoroughly with deionized water and lightly scrubbed with unbleached paper towels, followed by the addition of FETAX water and appropriate chemical concentrations.

Tadpoles were fed ad libitum with a commercial tadpole mash (Xenopus I, Dexter, Michigan USA). The mash was prepared by blending 50 g mash in 500 ml FETAX water. One ml of tadpole mash was added daily to each aquaria. The tadpoles were exposed for a period of at least two weeks prior to behavioral testing. Behavioral testing began at approximately Gosner stage 28-30 and was completed before conclusion of limb development (Gosner stage 39).

For Experiment 2, designed as a 4-way ANOVA (3 levels of atrazine x 3 levels of chlorpyrifos x 2 replicate tanks x 2 clutches) six *Xenopus Laevis* adults, three pairs of males and females, were also obtained from Xenopus I, Inc. The three pairs were housed separately in three 38 L aquaria, so as to prevent the mixing of egg clutches from different parents. Each aquaria was filled with 4 L of FETAX water. Using HCG, the injection and stimulation process was repeated as in Experiment 1 and the subjects were removed to holding aquaria following laying and fertilization of eggs. The initial design called for the use of three separate clutches totaling 275 individuals tested; however, one pair failed to produce a viable clutch. This led to the testing of a total of 270 tadpoles from two genetically unrelated clutches, with tadpoles harvested from both clutches for use in making the alarm substance. One hundred thirty-five sibling *X. Laevis* experimental tadpoles were taken from each clutch and divided evenly and housed in eighteen 38 liter aquaria. This resulted in thirty-six 38-liter aquaria, with two replications of each experimental treatment containing twenty-five tadpoles (two aquaria per treatment). Each of the thirty-six aquaria was maintained under exactly the same conditions as delineated in Experiment 1 and the testing completed in an identical manner.

Behavioral Testing

The tadpoles' response to pesticide exposure was evaluated on the basis of anti-predator behavior and activity levels. Anti-predator behaviors and activity were tested according to an already established method used by Hews and Blaustein [30], Hews [31], and Kiesecker et al [32]. This method involves the use of an alarm substance to induce anti-predator behavior in a larval anuran. The alarm substance was prepared by euthanizing a known weight of tadpoles (~5 g). The viscera were removed and the remaining tissues were macerated. Following maceration, the viscera were mixed with 200 ml dechlorinated water. The solution was filtered using a Buchner filter and then centrifuged at 10,000 rpm for ten minutes to separate solids and fluids. Prior to use, alarm substance was separated into measured quantities, stored through freezing with liquid nitrogen, and kept in a -70 °C freezer. Once unfrozen for use, alarm substance was used in testing, with any remaining solution disposed safely. No solution was re-frozen for use on a following day.

Behavioral tests were conducted in an opaque plastic container measuring 28x18 cm and filled with FETAX water to a depth of 4 cm. A single line down the middle delineated the container into two halves (14x18 cm each). The containers were placed behind an opaque cardboard observation blind and tests were run two-at-a-time, each test recorded using a Canon ES 50 8 mm, or a Sony CCD-TRV87 Hi-8 camcorder. Recordings were analyzed in order to minimize any effects an observer might cause through direct observation during the tests. In Experiment 2, observer bias was further mitigated by assigning each tadpole a three- or four-digit random number as an identifier, so that the observers reviewing the film would be unable to determine what treatments the tadpole belonged to until after data were collected.

The experiment was designed to measure activity and predator response in several different ways. The number of times the center line was crossed before and after alarm substance introduction measured both activity and predator response. An active tadpole would presumably cross the center line multiple times. A tadpole exhibiting an anti-predator response might stay in the half of the container opposite the alarm substance introduction, displaying fewer lines crossed after alarm substance introduction than before. These variables were termed PRELINE and POSTLINE, for number of times the center line was crossed before and after alarm substance introduction, respectively.

The amount of time, in seconds, a tadpole spent active before and after alarm substance introduction was another measure of both activity and alarm response. Activity was defined as any directional movement as well as any movement of the body and tail beyond necessary for filtering water across the tadpole's body. As a measure of activity, this variable is self explanatory. Any time spent active was considered activity. As a predator response variable, it was presumed that the tadpole would reduce activity as a predator response. Therefore, it would follow that there would be fewer seconds spent active after alarm substance introduction than before. For data analysis, these variables were designated PREACT and POSTACT, for activity before and after alarm substance introduction, respectively.

A third variable, the cue end in which the alarm substance was introduced (North or South), allowed for the fourth variable to be effectively analyzed. The fourth variable is a measurement of predator response. The amount of time, in seconds, a tadpole spent in a single half of a container, or cue end, before and after alarm substance introduction was measured. This variable allowed examination of whether or not a tadpole would avoid the cue end in which the alarm substance was introduced. It would be assumed that the alarm response would be to favor

the cue end opposite the side of alarm substance introduction. During recording of data, this variable was derived by measuring the amount of time (in seconds) a tadpole spent in the north cue end. This allowed the time spent in the south cue end to be derived mathematically. Combined with the data indicating in which end the alarm substance was introduced, this allowed analysis of whether or not the tadpole favored the cue end opposite alarm substance introduction. For data analysis, this variable was designated PRECUE and POSTCUE, for time spent in a cue end before and after alarm substance introduction, respectively.

In the second experiment, weight of each tadpole, at time of behavioral testing, was also measured. This was an additional measure to see if the chemicals and their interactions were having an effect on tadpole growth.

Finally, in both experiments, the proportion of total number of times the center line was crossed before alarm substance addition (designated PERLINE) and the proportion of total time spent active (in seconds) that occurred before alarm substance addition (designated PERACT) were calculated in order to allow an examination of whether or not there was an alarm response to the addition of the alarm substance. It would be assumed that the tadpole's alarm response would be to exhibit proportionally fewer number of lines crossed and seconds spent active after alarm substance introduction than before.

The behavioral experiment began by releasing of a single tadpole into the container and an acclimation period of ten minutes. From the first release of a tadpole until it was removed at the completion of a trial, cameras stayed recording. Once acclimation was accomplished, the test began and the tadpole was recorded for a pre-alarm-substance period of five minutes. Alarm substance was then introduced by pouring 5 ml from a graduated cylinder gently onto the surface of the water, in either the left or right corner of the side opposite the tadpole's position. Tadpoles

were given a 30 second period to acclimate to alarm substance introduction and tadpole behavior was then recorded during a five minute post-alarm-substance period. Two containers were recorded simultaneously in order to expedite trials. All filming occurred between 8:00 AM and 5:00 PM, during daylight hours. Filming occurred during a period of 9 days for both experiments. Filming began at Gosner stage 28-30 and finished before Gosner stage 39.

The resultant films were observed and variables measured using counters and stop-watches. In order to determine any statistically significant ($p \leq 0.05$) differences in anti-predator behavior or activity, data for each treatment were compared quantitatively using analysis of variance (ANOVA) tests provided in SAS v. 8e software for the Windows OS. Experiment 1 was tested as a 3 x 3 x 2 factorial; however in experiment 2, two separate clutches were tested, making the statistical analyses of the results four-way ANOVAs (3 x 3 x 2 x 2) for the original eight behavioral variables: the number of lines crossed before and after alarm substance addition (designated PRELINE and POSTLINE); the amount of time (sec) spent active before and after alarm substance addition (designated PREACT and POSTACT); the amount of time spent in one half of the experimental container before and after alarm substance addition (designated PRECUE and POSTCUE); the proportion of total lines crossed that occurred before alarm substance addition (designated PERLINE); and the proportion of total time spent active (sec) that occurred before alarm substance addition (designated PERACT). The proportional variables allowed an examination of whether or not there was an alarm response to the addition of the alarm substance. In addition to these eight behavioral variables, Experiment 2 also introduced the variable weight (g), which was measured in order to investigate whether or not there were significant atrazine, chlorpyrifos, or interaction effects on weight.

Results

For the first experiment, the ANOVA for the variable PRELINE indicated a significant atrazine effect, as well as a significant atrazine/chlorpyrifos interaction (Table 1). The atrazine effect was calculated from the mean values of any treatment that had 0, 20, and 200 μg/L atrazine. When Tukey's multiple contrast test was performed on mean atrazine values, the 20 μg/L atrazine treatment was significantly different from the 200 μg/L atrazine treatment, however neither were significantly different from the 0 μg/L atrazine treatment (Figure 1).While chlorpyrifos alone showed no significant affect on the PRELINE variable, when combined in mixture with atrazine a significant interaction was observed (Table 1, Figure 2).

The results illustrated in Figure 2 showed the chlorpyrifos/atrazine mixture interaction had an antagonistic effect on the variable PRELINE when in mixture with 200 μg/L atrazine and a synergistic effect when in mixture with 20 μg/L atrazine. The interaction effect between atrazine and chlorpyrifos is illustrated in Figure 2. As shown, atrazine alone as well as chlorpyrifos alone showed no significant effect on the PRELINE variable. If there were no interactions, the points should have shown additive, parallel lines. The expected lines would show tadpoles in 200 μg/L atrazine/10 μg/L chlorpyrifos having a greater increase in activity compared to those in the atrazine only treatment. Also, tadpoles in the 200 μg/L atrazine/1 μg/L chlorpyrifos would have been expected to show the greatest increase in activity. Instead, adding chlorpyrifos to 20 μg/L atrazine increased activity and adding chlorpyrifos to 200 μg/L atrazine decreased tadpole activity.

Table 1. Results of Factorial ANOVA for experiment 1 for the variable PRELINE. P < 0.05 is considered significant (*); SS values are Type III sum of squares.

Source	DF	SS	F	P
Atra	2, 207	550.67	4.86	0.0087*
Chlor	2, 207	104.43	0.92	0.3998
Rep	1, 207	14.73	0.26	0.6108
Atra*Chlor	4, 207	613.05	2.70	0.0316*
Atra*Rep	2, 207	179.26	1.58	0.2083
Chlor*Rep	2, 207	28.70	0.25	0.7766
Atra*Chlor*Rep	4, 207	263.99	1.16	0.3278

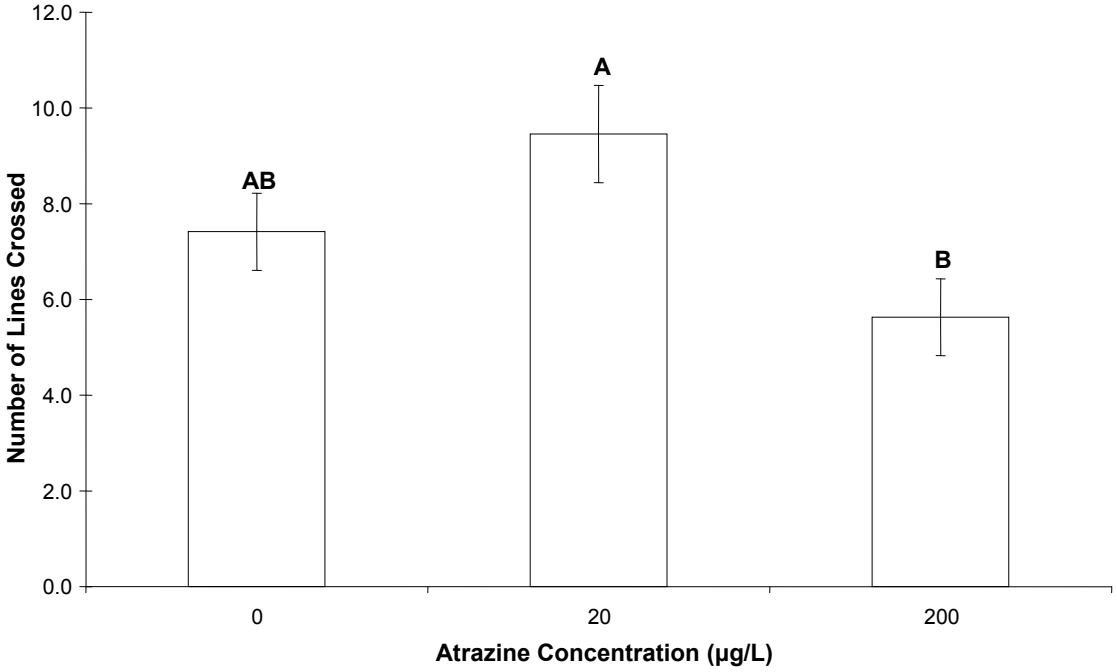

Figure 1. Atrazine effects on the number of lines crossed before cue addition in experiment 1. Data is derived from mean values for any treatment having 0, 20, or 200 µg/L atrazine. Different letters represent significant differences between means (±1 SE) as determined by Tukey's multiple contrast test.

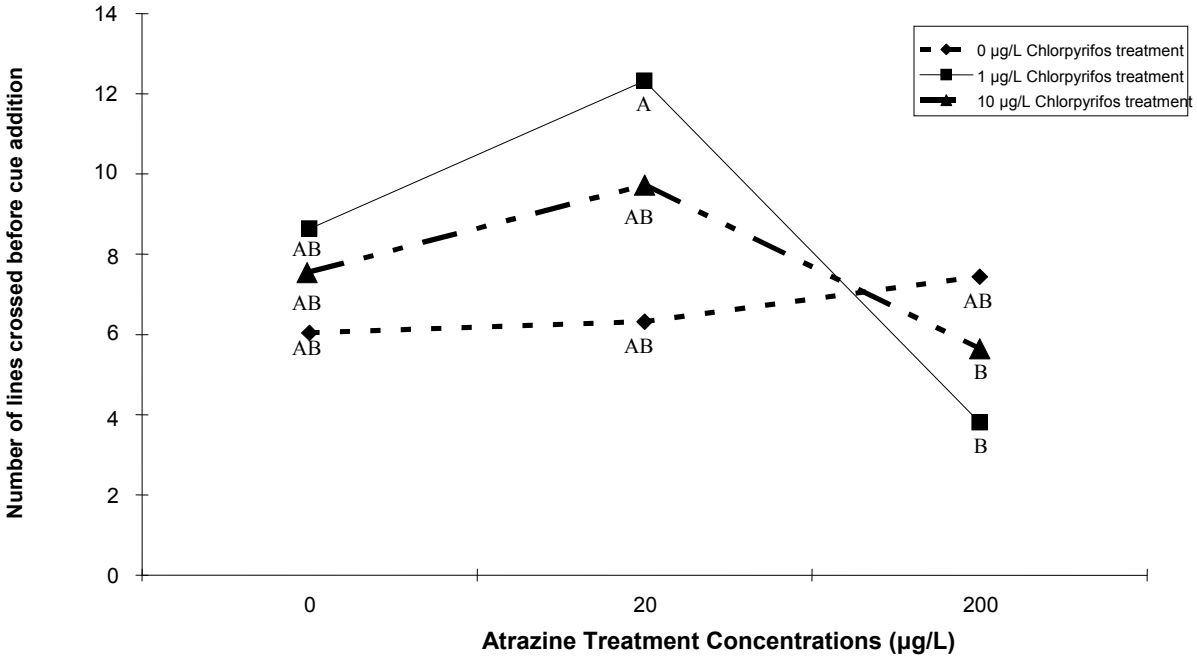

Figure 2. Number of lines crossed (PRELINE) before cue addition in experiment 1: Effects of Atrazine when combined with different concentrations of chlorpyrifos (0 µg/L atrazine concentration indicates the chlorpyrifos only treatments). Key indicates atrazine only treatment with 0 µg/L chlorpyrifos and mixture treatments at 1 and 10 µg/L chlorpyifos when intersecting the 20 and 200 µg/L atrazine concentrations. Letters are a contrast of the 9 mean values (±1 SE) based on Tukey's multiple contrast test.

In the second experiment, the only statistically significant effect for the variable PRELINE was derived from comparing the differences between clutches, in which clutch 1 showed a statistically significant decrease in the number of lines crossed when compared to clutch 2 (Table 2, Figure 3). There were no atrazine, chlorpyrifos, or mixture interaction effects.

Table 2. Results of Factorial ANOVA for experiment 2 for the variable PRELINE . P < 0.05 is considered significant (*); SS values are Type III sum of squares.

Source	DF	SS	F	P
Atra	2, 234	318.68	2.44	0.0894
Chlor	2, 234	51.46	0.39	0.6748
Rep	1, 234	3.25	0.05	0.8237
Clutch	1, 234	337.74	5.17	0.0239*
Atra*Chlor	4, 234	252.05	0.96	0.4275
Atra*Rep	2, 234	153.95	1.18	0.3095
Atra*Clutch	2, 234	244.63	1.87	0.1560
Chlor*Rep	2, 234	380.85	2.92	0.0561
Rep*Clutch	1, 234	4.32	0.07	0.7972
Chlor*Clutch	2, 234	44.69	0.34	0.7106
Atra*Chlor*Rep	4, 234	68.15	0.26	0.9028
Atra*Chlor*Clutch	4, 234	465.26	1.78	0.1334
Atra*Rep*Clutch	2, 234	33.27	0.25	0.7753
Chlor*Rep*Clutch	2, 234	9.22	0.07	0.9318
Atra*Chlor*Rep*Clutch	4, 234	185.14	0.71	0.5867

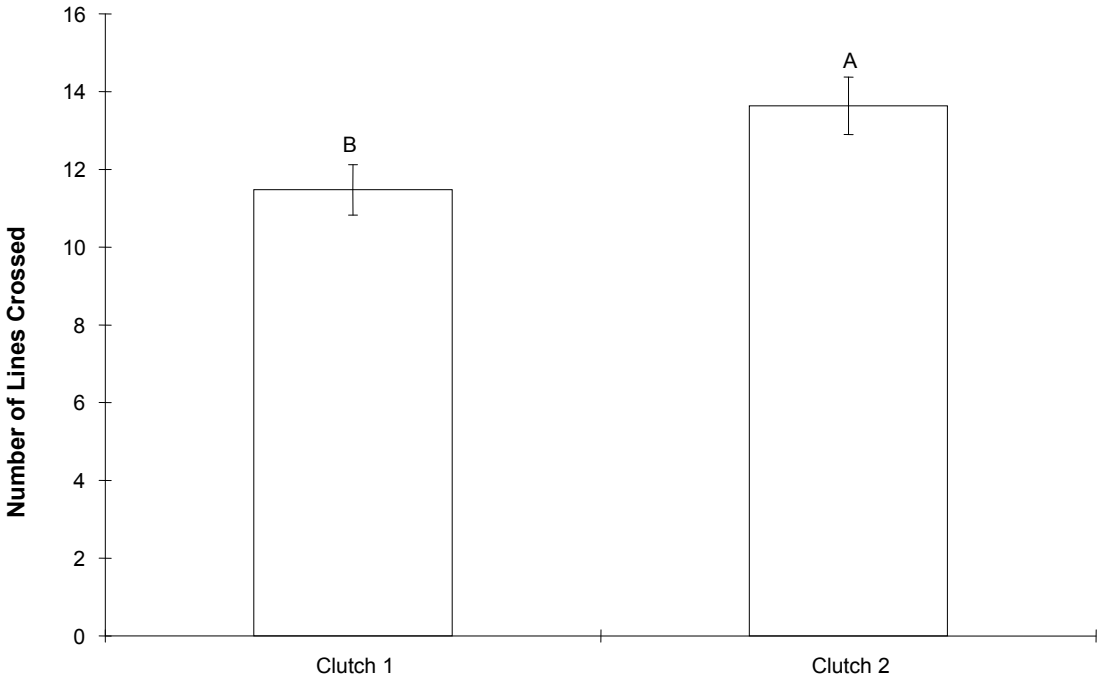

Figure 3. Differences between clutches concerning the number of lines crossed before cue addition in experiment 2. Different letters represent significant differences between means (±1 SE) as determined by Tukey's multiple contrast test.

With regards to time spent active before cue addition, tadpoles in experiment 1 exhibited a significant atrazine effect (Table 3). As illustrated in Figure 4, tadpoles in the 20 μg/L atrazine concentration showed no significant difference compared to those in the 0 μg/L concentration. Tadpoles in 200 μg/L atrazine concentration exhibited a highly significant depressive effect on activity (Table 3, Figure 4).

Table 3. Results of Factorial ANOVA for experiment 1 for the variable PREACT . P < 0.05 is considered significant (*); SS values are Type III sum of squares.

Source	DF	SS	F	P
Atra	2, 207	67451.73	4.61	0.0110*
Chlor	2, 207	5131.69	0.35	0.7047
Rep	1, 207	20820.03	2.84	0.0932
Atra*Chlor	4, 207	42326.45	1.45	0.2202
Atra*Rep	2, 207	41955.73	2.87	0.0592
Chlor*Rep	2, 207	3030.73	0.21	0.8132
Atra*Chlor*Rep	4, 207	25181.27	0.86	0.4889

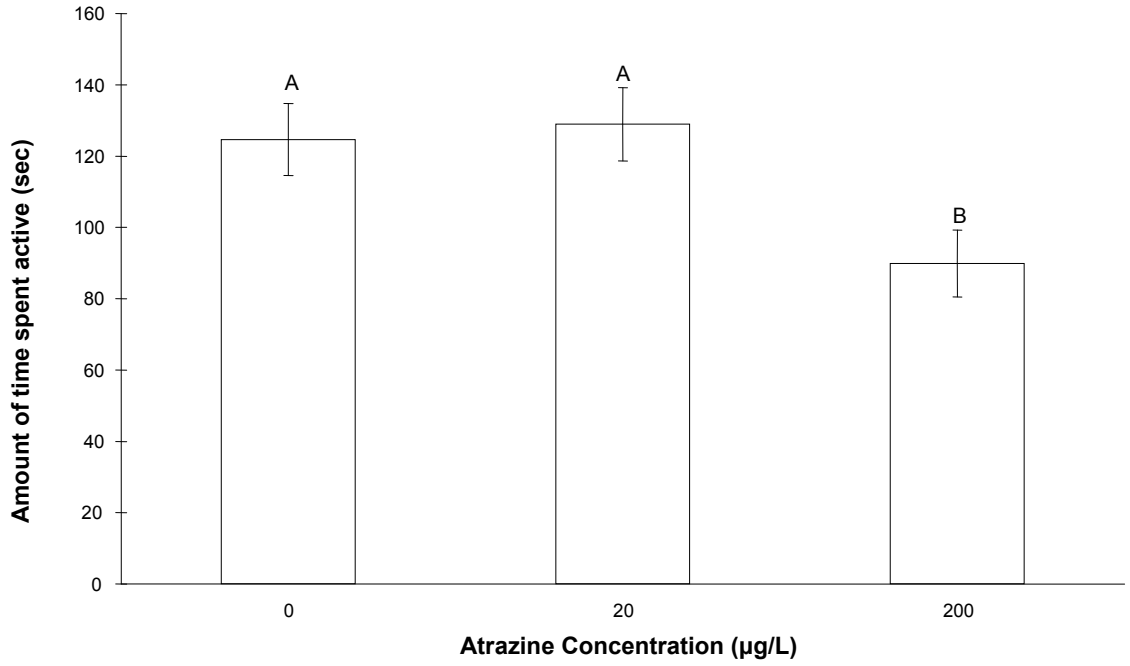

Figure 4. Atrazine effects on the amount of time spent active (sec) before cue addition (PREACT) in experiment 1. Different letters represent significant differences between means (±1 SE) as determined by Tukey's multiple contrast test.

For experiment 2, ANOVA results for the variable PREACT indicated no significant atrazine, chlorpyrifos, or interaction effects. The only significant result was an atrazine *chlorpyrifos*clutch interaction (Table 4).

Table 4. Results of Factorial ANOVA for experiment 2 for the variable PREACT. P < 0.05 is considered significant (*); SS values are Type III sum of squares.

Source	DF	SS	F	P
Atra	2, 234	3188.06	0.35	0.7083
Chlor	2, 234	2831.49	0.31	0.7361
Rep	1, 234	16.06	0.00	0.9530
Clutch	1, 234	103.92	0.02	0.8809
Atra*Chlor	4, 234	7542.00	0.41	0.8024
Atra*Rep	2, 234	6447.05	0.70	0.4984
Atra*Clutch	2, 234	11807.07	1.28	0.2802
Chlor*Rep	2, 234	19760.41	2.14	0.1199
Rep*Clutch	1v	5043.13	1.09	0.2970
Chlor*Clutch	2, 234	1355.56	0.15	0.8635
Atra*Chlor*Rep	4, 234	3218.53	0.17	0.9514
Atra*Chlor*Clutch	4, 234	49282.14	2.67	0.0330*
Atra*Rep*Clutch	2, 234	7989.54	0.87	0.4222
Chlor*Rep*Clutch	2, 234	2278.81	0.25	0.7814
Atra*Chlor*Rep*Clutch	4, 234	16061.45	0.87	0.4826

In experiment 1, the statistical analysis for whether or not the tadpoles favored one side of the experimental enclosure (variable PRECUE) showed that atrazine did indeed yield a significant effect (Table 5); however, the more statistically conservative Tukey's multiple contrast test indicated no separation of means among the treatment levels. With regards to the PRECUE variable in experiment 2, no significant effects were observed (Table 6).

Table 5. Results of Factorial ANOVA for experiment 1 for the variable PRECUE . P < 0.05 is considered significant (*); SS values are Type III sum of squares.

Source	DF	SS	F	P
Atra	2, 207	44802.25	3.08	0.0482*
Chlor	2, 207	21131.70	1.45	0.2365
Rep	1, 207	19235.56	2.64	0.1055
Atra*Chlor	4, 207	32111.87	1.10	0.3561
Atra*Rep	2, 207	7215.16	0.50	0.6098
Chlor*Rep	2, 207	2891.81	0.20	0.8200
Atra*Chlor*Rep	4, 207	10821.74	0.37	0.8286

Table 6. Results of Factorial ANOVA for experiment 2 for the variable PRECUE. P < 0.05 is considered significant (*); SS values are Type III sum of squares.

Source	DF	SS	F	P
Atra	2, 234	1566.45	0.18	0.8382
Chlor	2, 234	9086.99	1.02	0.3605
Rep	1, 234	198.43	0.04	0.8326
Clutch	1, 234	1102.94	0.25	0.6184
Atra*Chlor	4, 234	41313.18	2.33	0.0569
Atra*Rep	2, 234	2945.00	0.33	0.7178
Atra*Clutch	2, 234	6685.67	0.75	0.4717
Chlor*Rep	2, 234	22194.02	2.50	0.0840
Rep*Clutch	1, 234	1120.28	0.25	0.6157
Chlor*Clutch	2, 234	14199.77	1.60	0.2038
Atra*Chlor*Rep	4, 234	7749.45	0.44	0.7819
Atra*Chlor*Clutch	4, 234	25905.07	1.46	0.2150
Atra*Rep*Clutch	2, 234	1764.72	0.20	0.8197
Chlor*Rep*Clutch	2, 234	17731.49	2.00	0.1377
Atra*Chlor*Rep*Clutch	4, 234	3532.43	0.20	0.9386

In experiment 1, the ANOVA for the variable POSTLINE showed a significant atrazine effect for mean values of tadpoles in any treatment that had 0, 20, or 200 μg/L atrazine. A significant interaction effect was also present between atrazine mean values and replications (Table 7). However, when Tukey's multiple contrast test was applied, there was no separation of means for the atrazine or the atrazine/replication treatments.

Table 7. Results of Factorial ANOVA for experiment 1 for the variable POSTLINE . P < 0.05 is considered significant (*); SS values are Type III sum of squares.

Source	DF	SS	F	P
Atra	2, 207	127.47	3.33	0.0377*
Chlor	2, 207	28.19	0.74	0.4799
Rep	1, 207	69.24	3.62	0.0585
Atra*Chlor	4, 207	59.54	0.78	0.5406
Atra*Rep	2, 207	199.32	5.21	0.0062*
Chlor*Rep	2, 207	27.30	0.71	0.4911
Atra*Chlor*Rep	4, 207	157.82	2.06	0.0870

For experiment 2, the 4-way ANOVA for the variable POSTLINE indicated a statistically significant atrazine effect (Table 8). Tadples in the 200 μg/L atrazine treatment exhibited a significant decrease in the number of lines crossed when compared to tadpoles in the 0 μg/L control for atrazine (Figure 5). A clutch effect was also present, in which tadpoles from clutch 1 showed a significant decrease in the number of lines crossed when compared to tadpoles in clutch 2 (Figure 6). Finally, there was also an interaction between atrazine and clutch treatments (Table 8).

Table 8. Results of Factorial ANOVA for experiment 2 for the variable POSTLINE . P < 0.05 is considered significant (*); SS values are Type III sum of squares.

Source	DF	SS	F	P
Atra	2, 234	376.06	3.79	0.0240*
Chlor	2, 234	39.25	0.40	0.6737
Rep	1, 234	17.43	0.35	0.5539
Clutch	1, 234	334.17	6.74	0.0100*
Atra*Chlor	4, 234	123.09	0.62	0.6484
Atra*Rep	2, 234	4.80	0.05	0.9527
Atra*Clutch	2, 234	338.62	3.41	0.0346*
Chlor*Rep	2, 234	253.83	2.56	0.0796
Rep*Clutch	1, 234	0.14	0.00	0.9576
Chlor*Clutch	2, 234	66.26	0.67	0.5137
Atra*Chlor*Rep	4, 234	83.31	0.42	0.7942
Atra*Chlor*Clutch	4, 234	148.53	0.75	0.5598
Atra*Rep*Clutch	2, 234	40.92	0.41	0.6624
Chlor*Rep*Clutch	2, 234	35.29	0.36	0.7010
Atra*Chlor*Rep*Clutch	4, 234	217.28	1.10	0.3596

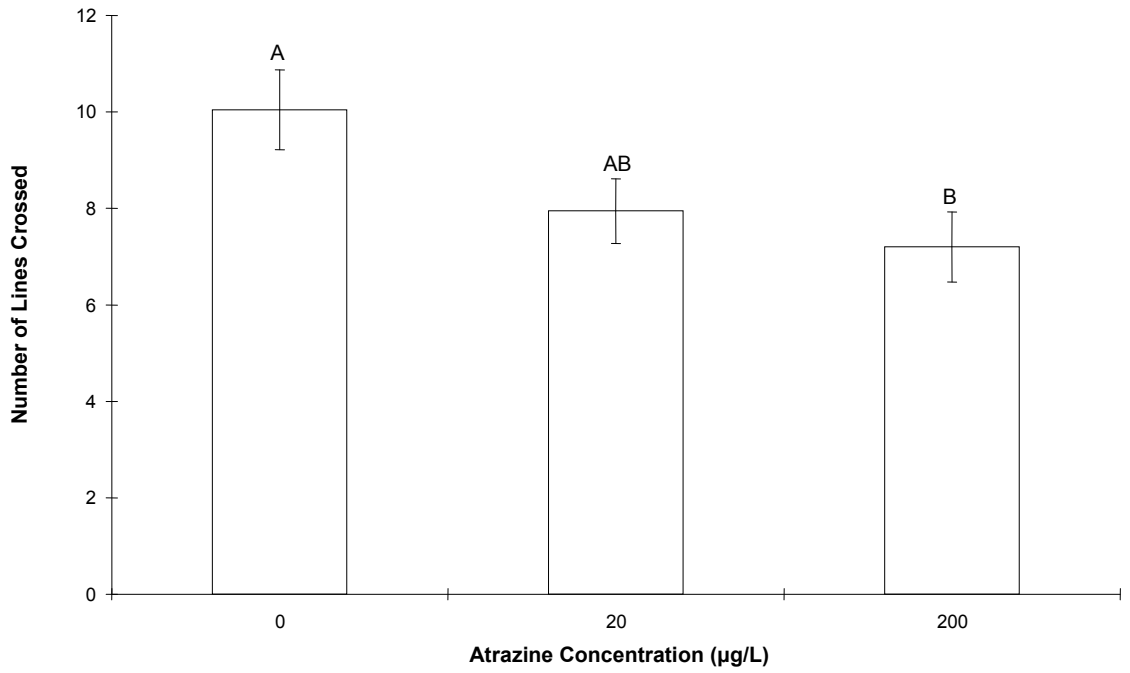

Figure 5. Atrazine effects on the number of lines crossed after cue addition in experiment 2. Different letters represent significant differences between means (±1 SE) as determined by Tukey's multiple contrast test.

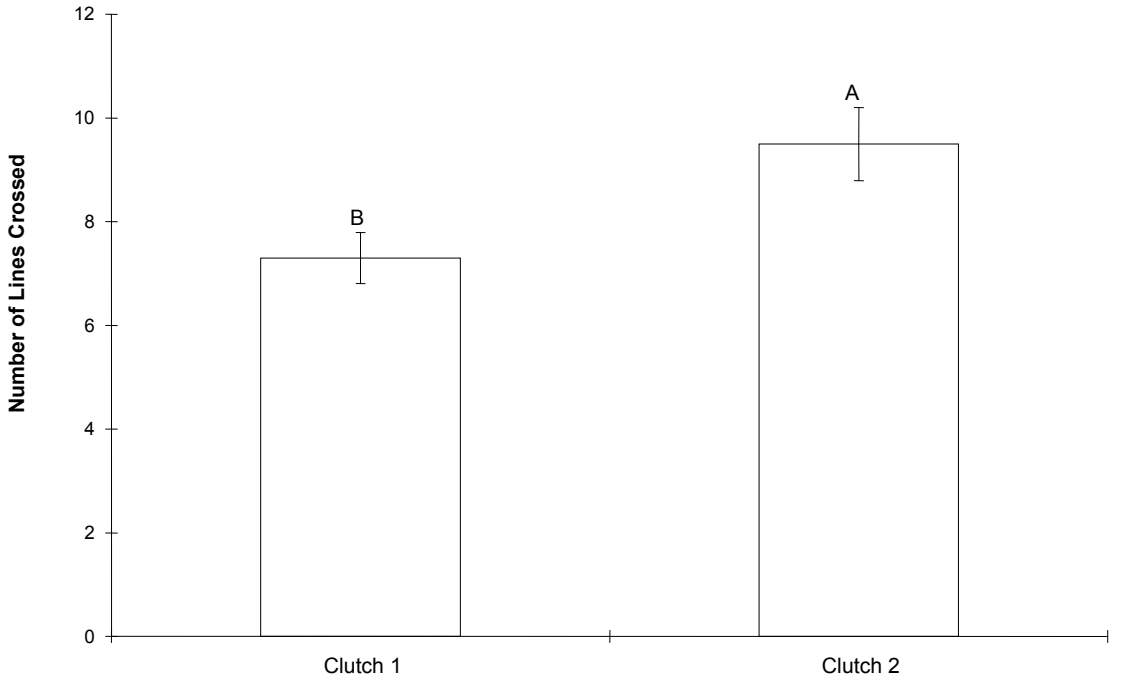

Figure 6. Differences between clutches for the number of lines crossed after cue addition in experiment 2. Different letters represent significant differences between means (±1 SE) as determined by Tukey's multiple contrast test.

In experiment 1, the only significant effect for the variable POSTACT was an interaction between atrazine and the two replications (Table 9). In experiment 2, no significant effects were shown for the POSTACT variable (Table 10).

Table 9. Results of Factorial ANOVA for experiment 1 for the variable POSTACT . P < 0.05 is considered significant (*); SS values are Type III sum of squares.

Source	DF	SS	F	P
Atra	2, 207	21963.28	2.65	0.0727
Chlor	2, 207	17672.22	2.14	0.1207
Rep	1, 207	1406.80	0.34	0.5604
Atra*Chlor	4, 207	13963.08	0.84	0.4988
Atra*Rep	2, 207	25619.53	3.10	0.0473*
Chlor*Rep	2, 207	9687.03	1.17	0.3122
Atra*Chlor*Rep	4, 207	23667.37	1.43	0.2251

Table 10. Results of Factorial ANOVA for experiment 2 for the variable POSTACT. P < 0.05 is considered significant(*); SS values are Type III sum of squares.

Source	DF	SS	F	P
Atra	2, 234	1809.71	0.21	0.8140
Chlor	2, 234	938.98	0.11	0.8987
Rep	1, 234	3447.56	0.79	0.3765
Clutch	1, 234	2421.69	0.55	0.4585
Atra*Chlor	4, 234	22229.04	1.27	0.2843
Atra*Rep	2, 234	5146.70	0.59	0.5574
Atra*Clutch	2, 234	24454.58	2.78	0.0638
Chlor*Rep	2, 234	12221.25	1.39	0.2508
Rep*Clutch	1, 234	404.85	0.09	0.7617
Chlor*Clutch	2, 234	1763.67	0.20	0.8182
Atra*Chlor*Rep	4, 234	17925.97	1.02	0.3974
Atra*Chlor*Clutch	4, 234	18835.84	1.07	0.3709
Atra*Rep*Clutch	2, 234	7373.87	0.84	0.4332
Chlor*Rep*Clutch	2, 234	786.67	0.09	0.9144
Atra*Chlor*Rep*Clutch	4, 234	16691.93	0.95	0.4357

Analysis of the variable POSTCUE (time spent in side opposite cue introduction) in experiment 1 also indicated no significant effects among the experimental treatments, except for

a significant interaction between atrazine and the replications (Table 11). In experiment 2, no significant results were observed for the variable POSTCUE (Table 12).

Table 11. Results of Factorial ANOVA for experiment 1 for the variable POSTCUE. P < 0.05 is considered significant(*); SS values are Type III sum of squares.

Source	DF	SS	F	P
Atra	2, 207	6351.33	0.35	0.7058
Chlor	2, 207	22539.92	1.24	0.2919
Rep	1, 207	740.73	0.08	0.7757
Atra*Chlor	4, 207	32628.52	0.90	0.4670
Atra*Rep	2, 207	153812.38	8.45	0.0003*
Chlor*Rep	2, 207	18417.92	1.01	0.3653
Atra*Chlor*Rep	4, 207	10362.56	0.28	0.8877

Table 12. Results of Factorial ANOVA for experiment 2 for the variable POSTCUE. P < 0.05 is considered significant (*); SS values are Type III sum of squares.

Source	DF	SS	F	P
Atra	2, 234	9286.90	0.66	0.5184
Chlor	2, 234	4969.61	0.35	0.7033
Rep	1, 234	1836.54	0.26	0.6102
Clutch	1, 234	1717.83	0.24	0.6220
Atra*Chlor	4, 234	14442.48	0.51	0.7267
Atra*Rep	2, 234	805.68	0.06	0.9445
Atra*Clutch	2, 234	18846.81	1.34	0.2646
Chlor*Rep	2, 234	7007.75	0.50	0.6089
Rep*Clutch	1, 234	10120.62	1.44	0.2320
Chlor*Clutch	2, 234	3852.56	0.27	0.7611
Atra*Chlor*Rep	4, 234	2868.35	0.10	0.9818
Atra*Chlor*Clutch	4, 234	16561.88	0.59	0.6720
Atra*Rep*Clutch	2, 234	18356.13	1.30	0.2739
Chlor*Rep*Clutch	2, 234	7047.39	0.50	0.6072
Atra*Chlor*Rep*Clutch	4, 234	20114.91	0.71	0.5834

In experiment 1, the proportional variable PERLINE showed only a significant replicate effect (Table 13). In experiment 2, no significant effects were noted for PERLINE (Table 14).

Finally, in experiment 1, no significant effects among main experimental treatments were noted for PERACT, although there was a significant interaction between chlorpyrifos and the replications (Table 15). In experiment 2, no significant effects were noted for PERACT (Table 16).

Table 13. Results of Factorial ANOVA for experiment 1 for the variable PERLINE . P < 0.05 is considered significant (*); SS values are Type III sum of squares.

Source	DF	SS	F	P
Atra	2, 207	213.23	0.67	0.5114
Chlor	2, 207	328.66	1.04	0.3564
Rep	1, 207	1088.03	6.86	0.0094*
Atra*Chlor	4, 207	1137.05	1.79	0.1314
Atra*Rep	2, 207	719.33	2.27	0.1060
Chlor*Rep	2, 207	707.64	2.23	0.1098
Atra*Chlor*Rep	4, 207	299.24	0.47	0.7562

Table 14. Results of Factorial ANOVA for experiment 2 for the variable PERLINE. P < 0.05 is considered significant (*); SS values are Type III sum of squares.

Source	DF	SS	F	P
Atra	2, 234	234.96	1.16	0.3139
Chlor	2, 234	45.12	0.22	0.7998
Rep	1, 234	142.50	1.41	0.2359
Clutch	1, 234	5.75	0.06	0.8115
Atra*Chlor	4, 234	428.96	1.06	0.3756
Atra*Rep	2, 234	280.46	1.39	0.2511
Atra*Clutch	2, 234	82.47	0.41	0.6650
Chlor*Rep	2, 234	109.36	0.54	0.5823
Rep*Clutch	1, 234	7.46	0.07	0.7859
Chlor*Clutch	2, 234	80.69	0.40	0.6709
Atra*Chlor*Rep	4, 234	121.96	0.30	0.8763
Atra*Chlor*Clutch	4, 234	609.66	1.51	0.1998
Atra*Rep*Clutch	2, 234	321.77	1.59	0.2052
Chlor*Rep*Clutch	2, 234	49.30	0.24	0.7834
Atra*Chlor*Rep*Clutch	4, 234	389.26	0.96	0.4276

Table 15. Results of Factorial ANOVA for experiment 1 for the variable PERACT. P < 0.05 is considered significant(*); SS values are Type III sum of squares.

Source	DF	SS	F	P
Atra	2, 207	873.73	1.50	0.2075
Chlor	2, 207	541.46	0.93	0.3953
Rep	1, 207	813.18	2.80	0.0957
Atra*Chlor	4, 207	1524.78	1.31	0.2663
Atra*Rep	2, 207	1719.18	2.96	0.0540
Chlor*Rep	2, 207	2045.45	3.52	0.0313*
Atra*Chlor*Rep	4, 207	170.44	0.15	0.9643

Table 16. Results of Factorial ANOVA for experiment 2 for the variable PERACT . P < 0.05 is considered significant(*); SS values are Type III sum of squares.

Source	DF	SS	F	P
Atra	2, 234	197.53	1.05	0.3532
Chlor	2, 234	23.77	0.13	0.8818
Rep	1, 234	244.20	2.58	0.1092
Clutch	1, 234	21.09	0.22	0.6371
Atra*Chlor	4, 234	315.92	0.84	0.5035
Atra*Rep	2, 234	126.11	0.67	0.5140
Atra*Clutch	2, 234	19.53	0.10	0.9018
Chlor*Rep	2, 234	52.13	0.28	0.7591
Rep*Clutch	1, 234	63.19	0.67	0.4143
Chlor*Clutch	2, 234	1.28	0.01	0.9933
Atra*Chlor*Rep	4, 234	203.76	0.54	0.7071
Atra*Chlor*Clutch	4, 234	408.62	1.08	0.3664
Atra*Rep*Clutch	2, 234	118.09	0.63	0.5361
Chlor*Rep*Clutch	2, 234	83.44	0.44	0.6435
Atra*Chlor*Rep*Clutch	4, 234	217.79	0.58	0.6801

Specific to experiment 2 was the ANOVA for the variable weight (g). This analysis showed numerous significant effects. There was a highly significant atrazine effect (Table 17), which indicated that increasing atrazine concentrations corresponded with decreasing weight (Figure 7). There was also a highly significant replication effect (Table 17), in which replication 1 showed a significant decrease in weight when compared to replication 2 (Figure 8). There was

a significant clutch effect (Table 17) in which clutch 1 showed a significantly lower weight than clutch 2 (Figure 9). There was a highly significant mixture interaction effect on weight (Table 17), in which atrazine alone had a depressive effect on weight, with tadpoles in the 20 and 200 µg/L concentrations exhibiting a significantly lower weight than tadpoles in the 0 µg/L concentration. Upon examining chlorpyrifos alone, tadpoles in the 10 µg/L concentration showed a significantly greater weight than tadpoles in the 1 µg/L concentration. At a concentration of 10 µg/L chlorpyrifos, there was an apparent additive interaction with the atrazine. In contrast, tadpdole average weight exposed to 1 µg/L chlorpyrifos appeared to exhibit an antagonistic relationship with atrazine; the mean weight of tadpoles exposed to 1 µg/L chlorpyrifos mixed with 20 µg/L atrazine and 200 µg/L atrazine was larger than one would expect were the relationship additive (Figure 10).

Table 17. Results of Factorial ANOVA for the variable tadpole weight (mg) at time of behavioral testing. P < 0.05 is considered significant (*); SS values are Type III sum of squares.

Source	DF	SS	F	P
Atra	2, 234	2.4931	52.04	<0.0001*
Chlor	2, 234	0.1292	2.70	0.0695
Rep	1, 234	0.5280	22.04	<0.0001*
Clutch	1, 234	0.2543	10.62	0.0013*
Atra*Chlor	4, 234	0.7538	7.87	<0.0001*
Atra*Rep	2, 234	0.9331	19.48	<0.0001*
Atra*Clutch	2, 234	0.2569	5.36	0.0053*
Chlor*Rep	2, 234	0.5314	11.09	<0.0001*
Rep*Clutch	1, 234	0.1016	4.24	0.0406*
Chlor*Clutch	2, 234	0.0394	0.82	0.4405
Atra*Chlor*Rep	4, 234	0.1204	1.26	0.2878
Atra*Chlor*Clutch	4, 234	1.0205	10.65	<0.0001*
Atra*Rep*Clutch	2, 234	0.2290	4.78	0.0092*
Chlor*Rep*Clutch	2, 234	0.0485	1.01	0.3651
Atra*Chlor*Rep*Clutch	4, 234	0.3091	3.23	0.0133*

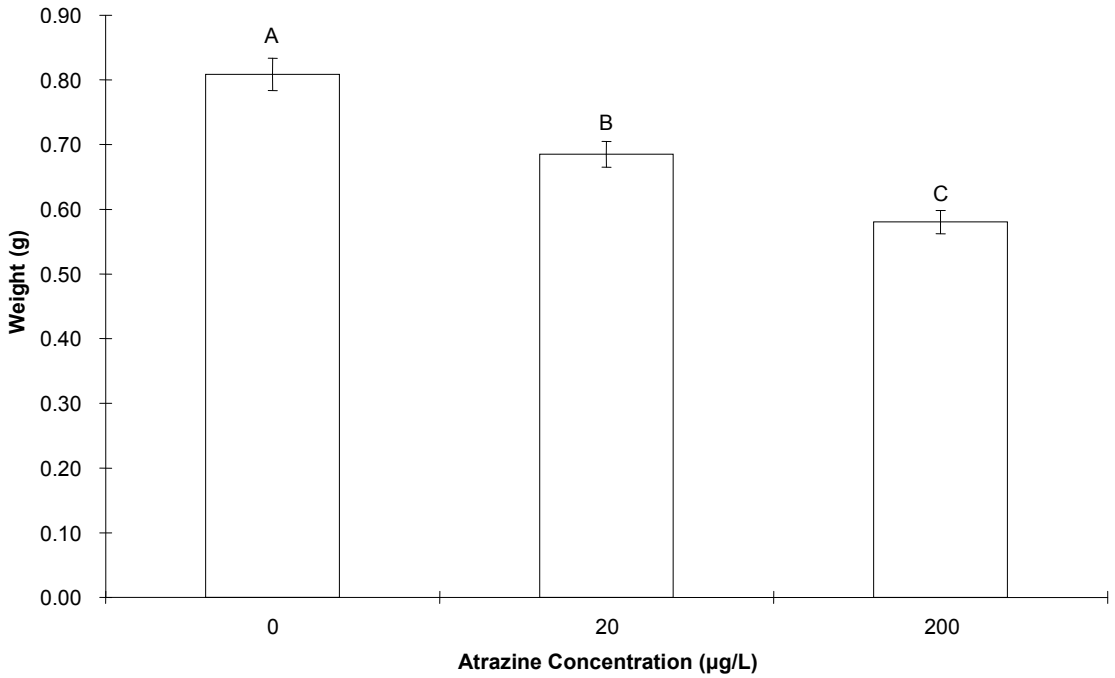

Figure 7. Effects of Atrazine on mean tadpole weight (g) at time of experimentation. Different letters represent significant differences between means (±1 SE) as determined by Tukey's multiple contrast test.

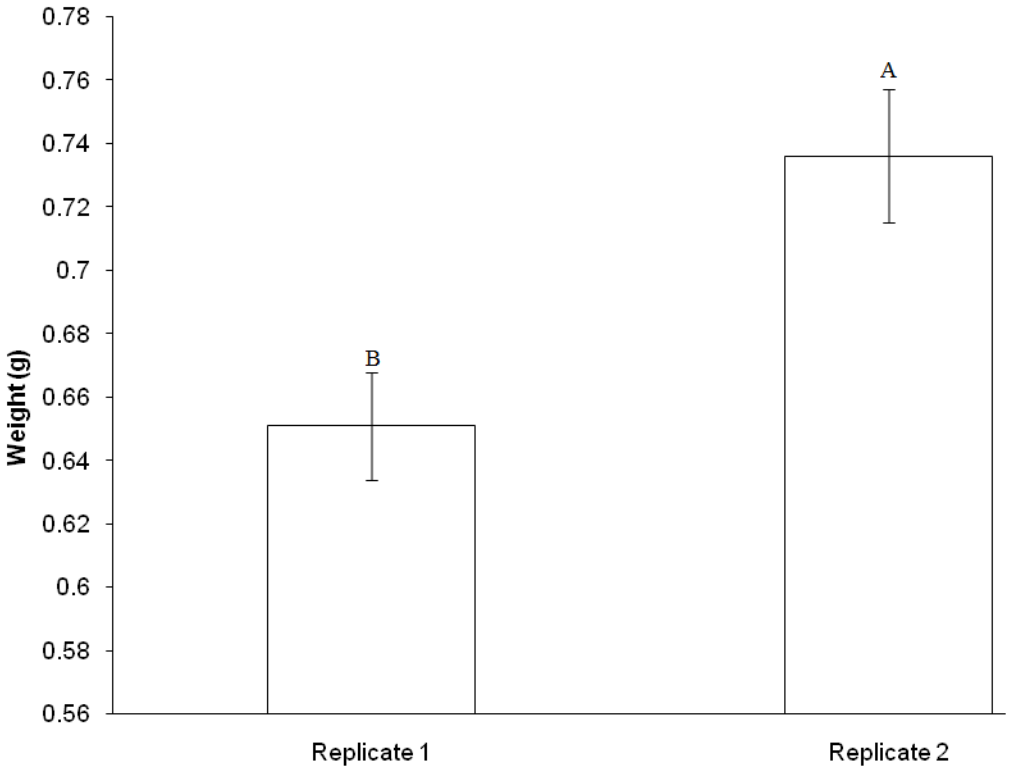

Figure 8. Mean differences between replications in weight (g) at time of experimentation. Different letters represent significant differences between means (±1 SE) as determined by Tukey's multiple contrast test.

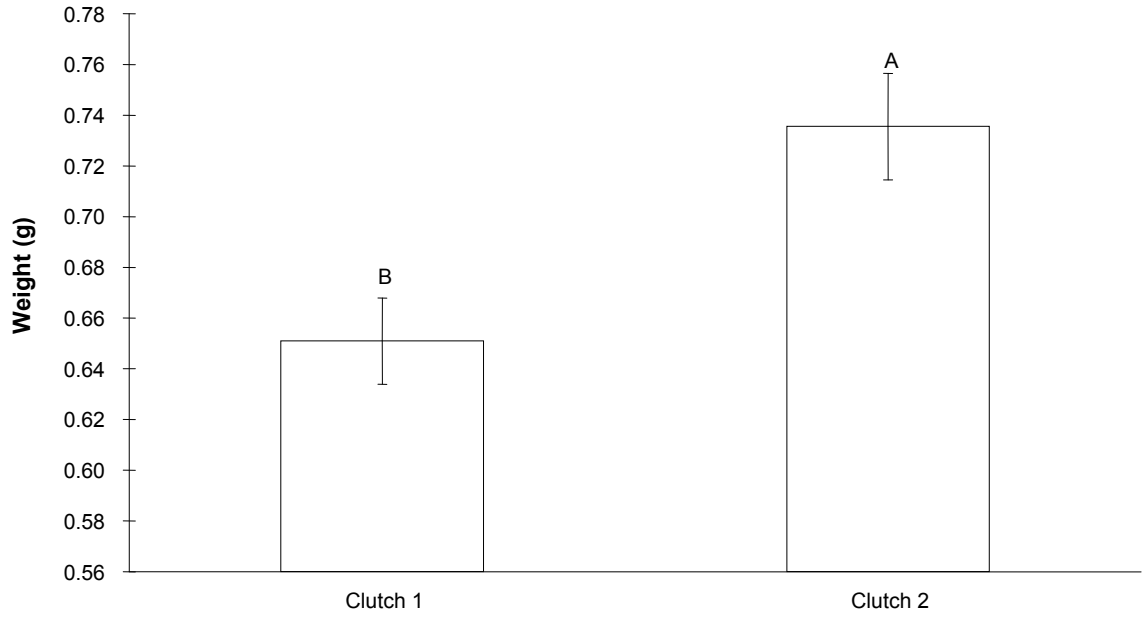

Figure 9. Mean differences between clutches in regards to tadpole weight (g) at time of experimentation. Different letters represent significant differences between means (±1 SE) as determined by Tukey's multiple contrast test.

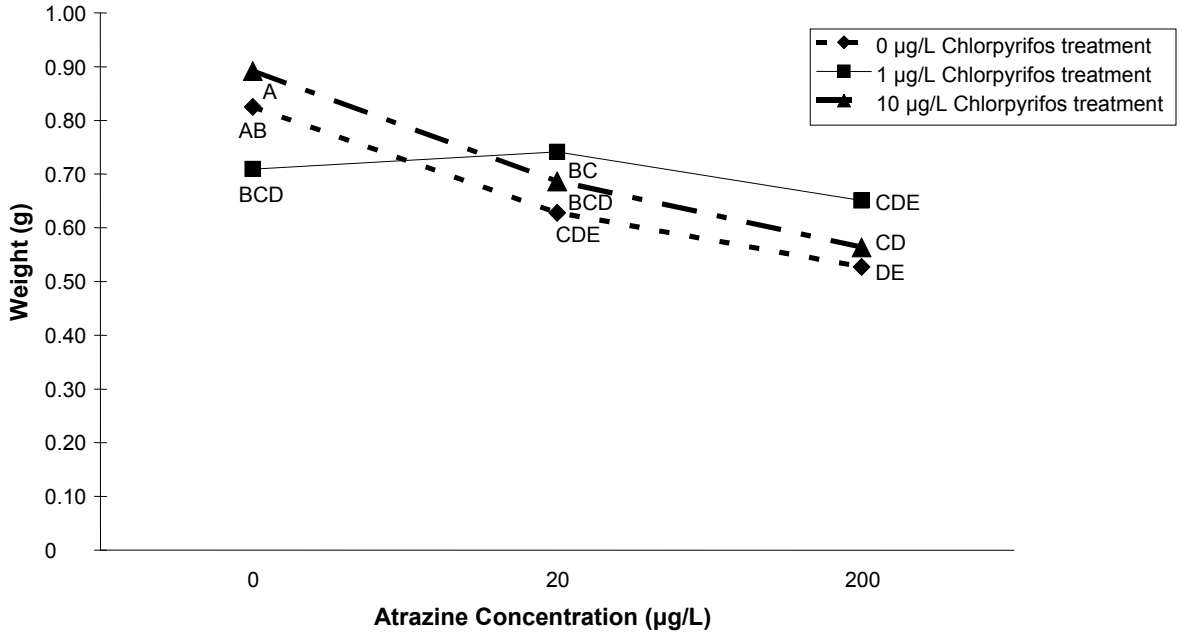

Figure 10. Weight (g) at time of experimental testing. Effects of atrazine when combined with different concentrations of chlorpyrifos (0 µg/L atrazine concentration indicates the chlorpyrifos only treatments). Key indicates atrazine only treatment with 0 µg/L chlorpyrifos and mixture treatments at 1 and 10 µg/L chlorpyifos when intersecting the 20 and 200 µg/L atrazine concentrations. Letters are a contrast of the 9 mean values (±1 SE) based on Tukey's multiple contrast test.

Discussion

The general goal of this research was to determine whether or not there were single chemical or mixture interaction effects of atrazine and chlorpyrifos on the activity levels and anti-predator response behavior of *Xenopus Laevis* larvae. Three specific null hypotheses (as well as more minor hypotheses) were tested in this study: 1) that no differences in mean behaviors among atrazine concentrations would be observed; 2) that no differences in mean behaviors among chlorpyrifos concentrations would be observed; and 3) that no interaction effect between atrazine and chlorpyrifos on mean behaviors would be observed. The original hypothesis regarding possible chemical interaction effects held that there would be synergistic interactions between chlorpyrifos and atrazine, based on previous studies showing that atrazine has been shown to have possible acetylcholinesterase-inhibiting effects [34, 35,36]. The herbicide atrazine, in separate studies, has been shown to depress larval activity levels even at concentrations below the NOEC of 20 ul/L [15]. Atrazine has also been shown to have a depressive effect on larval size and weight at metamorphosis [18]. There is even some evidence to suggest that atrazine may have acetylcholinesterase-inhibiting effects [34, 35, 36]. Acetylcholinesterase inhibitors, the same mode of action through which the organophosphate insecticide chlorpyrifos works, have been shown to decrease activity and size at metamorphosis [16].

The environment in which amphibians spawn often contains multiple agrochemicals derived from landuse runoff. Investigation of chemical mixture interactions provides a more environmentally realistic examination of the kinds of anthropogenic challenges facing larval amphibians. Further elucidating these challenges may help to explain the continuing declines in

worldwide amphibian populations [1]. Of primary importance for amphibians living adjacent to agricultural fields would be potential effects of chemical mixtures containing herbicides, insecticides, and fertilizers. The possible interactions that might occur between these different chemicals could be additive, synergistic, or antagonistic. In a recent study by Schuler et al. [42], atrazine, non-toxic when present as a single contaminant in tests with *Chironomus tentans* larve, potentiated (a form of synergism) the toxicity of chlorpyrifos when animals were exposed to the two chemicals in combination. Other studies using midge have indicated a similar response [43,44]. Based on this evidence that atrazine and chlorpryrifos could share similar acetylcholinesterase-inhibiting effects, it was hypothesized that atrazine and chlorpyrifos would show synergistic interactions in this study. The results of this study will first be examined with respect to the single chemical and mixture effects on the activity and predator cue alarm response of *Xenopus laevis*. Following this discussion, the implications of replication and clutch effects and their interactions with the main chemical treatments will be addressed.

Atrazine Effects on Behavior

Of the eight behavioral responses measured for tadpoles in experiment 1, statistically significant effects attributable to atrazine were found in two cases; the variables preline and preact (Tables 1 & 3, respectively). The atrazine effect on the variable PRELINE, number of times the center line was crossed before alarm substance addition, was depressive. Tadpoles in 200 μg/L atrazine exhibited a significant decrease as compared to those in the 0 μg/L treatment (Table 1). Atrazine also showed a depressive effect on the variable PREACT, the amount of time (s) spent active before alarm substance introduction. Again, those tadpoles in 200 μg/L atrazine showed a significant decrease as compared to those in the 0 μg/L and 20 μg/L treatments. These

activity results have been corroborated in previous work conducted by this laboratory in which Sirimalle [15] found that a concentration of 200 μg/L atrazine resulted in a decrease in lines crossed prior to cue addition and, in a separate experiment, the same concentration also had an overall depressive effect on time spent active when in the presence of conspecifics [45]. Unfortunately, these results were not entirely consistent with atrazine effects observed in the second experiment. It is of importance to note that the number of lines crossed before the cue was near significance (Table 2, p = 0.0894). The only two significant atrazine effects observed were for the variables POSTLINE and weight (Tables 8 & 17, respectively). Tadpoles exposed to atrazine exhibited a depressive effect on the number of times the center line was crossed after alarm substance addition (Figure 5). The most striking response to atrazine was evident in the only non-behavioral variable, weight at time of behavioral tests. Atrazine exhibited a distinctly depressive effect on weight; tadpoles in the 20 μg/L treatment were significantly smaller than those in the 0 μg/L treatment, and tadpoles in the 200 μg/L treatment were significantly smaller than those in the 0 μg/L and the 20 μg/L treatments (Table 17, Figure 7). Reduced body size, as indicated by weight, has been reported in *X. laevis* exposed to atrazine in other studies in this laboratory. While the results between experiment 1 and 2 did differ, both are consistent in that atrazine was associated with decreased activity in either the pre or post-cue period of filming. The dissonance between significant atrazine effects in expermient 1 and 2 may be explained, in part, by variability in behavioral responses between genetically different clutches.

These responses confirmed the results of previous research into the behavioral effects of sub-lethal, chronic atrazine exposure on activity levels [26, 15, 45]. The results also confirmed weight effects of atrazine in previous research [17]. Previous research also shows decreased mass in the presence of atrazine in leopard frogs (*Rana sphenocephala*), American toads (*Bufo*

americanus), streamside salamanders (*Ambystoma barbouri*), and wood frogs (*Rana sylvatica*) [46,47,48]. Depressive effects on tadpole activity could lead to a disruption in the Wilbur-Collins model. A tadpole that is less active would presumably forage less, which would help explain why larvae exposed to atrazine show both a delay in metamorphosis and a decrease in size at metamorphosis [17,46,47,48]. This detrimental effect would be compounded by the fact that many amphibian larvae are herbivorous and would have their food source reduced by atrazine's herbicidal effects [46,47,48].

Did atrazine cause differences in response to the alarm due, suggesting a disruption of anti-predator behavior? A behavioral response to an alarm cue in tadpoles may manifest in a number of visible effects. It was anticipated that the alarm cue would depress activity so as to decrease predator detection, as measured in two ways—a decrease in seconds spent active and number of times the center line was crossed in the presence of the alarm cue compared to these behaviors before alarm cue. Because tadpoles in the distinct atrazine treatments exhibited significant differences in activity when not in the presence of the predator, similarity in a predator response, such as a decrease in an individual's activity, may be masked by these initial differences in activity among individuals. For example, tadpoles exposed to 200 μg/L exhibited significantly lower number of lines crossed and number of seconds active before the alarm cue compared to controls (in experiment 1); after cue addition however, no differences in number of lines crossed was observed among chemical treatments. To account for differences among individual activity within a treatment and make comparisons after cue addition more compatible, the variables PERLINE and PERACT were measured in order to portray accurately the two major activity measurements as they related specifically to the alarm response. PERLINE represents the proportion of the number of times the center line was crossed by an individual

before alarm substance addition out of the total number of times the center line was crossed before and after alarm substance addition by that same individual. PERACT represents the proportion of the amount of time (s) spent active before the alarm substance was introduced out of the total amount of time (s) spent active before and after alarm substance introduction by the same individual. Consider that two animals may be quite different in activity levels before a predator cue, but both initiate a similar and proportional decrease in activity with the cue. That would suggest that their response to a predator, namely to slow down and/or freeze, remained similar. Thus, the proportional variables were a more accurate portrayal of how the alarm response was influenced by the experimental chemical concentrations.

For both Experiments 1 and 2, the two proportional variables PERLINE (Tables 13 & 14) and PERACT (Tables 15 & 16) exhibited no statistically significant effects due to either atrazine or chlorpyrifos, nor were there any interaction effects. This meant that the alarm response itself was not significantly affected by the chronic, sub-lethal doses of atrazine and chlorpyrifos. In other words, regardless of amount of initial activity, animals decrease the proportional rate of activity similarly. This finding is consistent with previous studies conducted by this laboratory. An examination of the effects of chronic, sublethal atrazine exposure on antipredator response in larval *Xenopus laevis* showed no significant change in alarm cue response at three atrazine concentrations identical to those used in this study [15]. With regard to major impact of these chemicals on behavior, these results suggest that atrazine does indeed decrease activity in general, possibly having a deleterious effect on growth rates and time to metamorphosis. However, atrazine did not cause animals to respond to a predator cue by inappropriate behavior (eg., increasing activity levels, thereby increasing visibility).

Chlorpyrifos Effects on Behavior

Chlorpyrifos alone showed no significant effects on any of the variables measured in either experiment. Previous studies that have examined interaction effects between chlorpyrifos and atrazine have been conducted within known lethality ranges for the insecticide (eg., Schuler et al. [42], used EC1 – EC50 concentrations of chlorpyrifos for midges). In this study, however, pesticide concentrations were chosen that would be more typical of environmentally realistic conditions and much below lethality. Based on a wide geographical range of agricultural streams in the USA, a maximum detection of 0.40 ug/L has been reported [49]. Models based on extrapolated field data and biotransformation studies predict chronic 60-day chlorpyrifos concentrations of 0.4 μg/L in small lakes and reservoirs that drain agricultural land [50]. Similarly, half-life estimates of several days to less than 12 hours have been reported for pools and outdoor microcosms [38,39,40]. Previous work with chlorpyrifos has indicated chloryrifos shows peaks followed by rapid loss even in the intervals between water changes used in this study [18], more typical of a pulsed exposure. This would suggest that concentrations of chlorpyrifos used in this study may not have been sufficiently great enough or of persistance to cause sub-lethal effects. Consequently, the prediction for interaction effects, specifically potentiation, between chlorpyrifos and atrazine may only be observed using concentrations that do cause some degree of lethality. Originally, chlorpyrifos was expected to show similar effects to carbaryl, such as decreased weight [16]. Perhaps, even though chlorpyrifos and carbaryl share the same acetylcholinesterase-inhibiting mode of action, the effects of one cannot be extrapolated to the effects of the other. The absence of significant effects on *Xenopus laevis* behavior at identical concentrations is consistent with previous findings in this laboratory [45].

Also, Spence [18], using chlorpyrifos concentrations of 1.25 and 5 μg/L found no effects on metamorphic timing or size characteristics of *X. laevis*.

Chemical Interaction Effects

In Experiment 1, the only significant interaction effect between the two chemicals was displayed by the variable PRELINE (Table 1). As shown in Figure 2, if there had been additive responses or no interaction, the slopes of the three lines would have been parallel. Instead, when examining the 10 μg/L chlorpyrifos slope at 20 μg/L atrazine, there is an evident increase in the number of times the center line was crossed when compared to an expected parallel slope. This suggests an antagonistic interaction in which the depressive effect of atrazine on activity is reduced by mixture interaction with 10 μg/L chlorpyrifos. This is reinforced by examining the 1 μg/L slope where it intersects at 20 μg/L atrazine. There is a much more pronounced increase in the number of times the center line is crossed, indicating that 1 μg/L chlorpyrifos interacts to a greater antagonistic degree with 20 μg/L atrazine than the 10 μg/L chlorpyrifos interaction with the same atrazine concentration.

When the 200 μg/L atrazine concentration is examined, there appears to be a synergistic interaction with the chlorpyrifos rather than an antagonistic interaction. Again, both the 10 μg/L and 1 μg/L chlorpyrifos lines show a non-parallel slope when compared to the 0 μg/L chlorpyrifos line. The synergistic effect is greater when the 1 μg/L chlorpyrifos interacts with 200 μg/L atrazine as opposed to when the 10 μg/L chlorpyrifos treatment interacts with the same atrazine concentration.

Given the limited mixture results, the importance of this should not be overstated. Since only one behavioral measure in only one of the two experiments exhibited a significant chemical

mixture response, there is a possibility that this could simply be an experiment-wide Type I error. These results also suggest that, with response to this particular behavioral measure, there is no generalized trend in how atrazine and chlorpyrifos interact, with antagonistic interactions appearing to occur at 20 μg/L atrazine and synergistic interactions appearing to occur at 200 μg/L atrazine. Given these limitations in the consideration of this result, there is an important implication to note. The lack of a simple individual chlorpyrifos effect would suggest that such interactions could occur even when chlorpyrifos is not present at levels in aquatic habitats sufficient to behaviorally affect larval amphibians directly. This means that even if chlorpyrifos is present at what could be considered safe levels, its potential interactions with other agrochemicals, such as atrazine, may still make it damaging to amphibian reproductive success.

In Experiment 2, the only variable showing chemical interaction effects was the non-behavioral variable, weight (Table 17). Analysis of the observations shows that, when comparing the 0 μg/L chlorpyrifos line to the 10 μg/L chlorpyrifos line, there is a strictly additive interaction, or no interaction (Figure 10); however, the 1 μg/L chlorpyrifos line shows a distinct antagonistic interaction with atrazine at both 20 μg/L atrazine and 200 μg/L atrazine. Atrazine alone had a depressive effect on the weight of the tadpoles, but when interacting with 1 μg/L chlorpyrifos, there was an increase in tadpole weight, as compared to both the 0 μg/L chlorpyrifos line (atrazine only) and the 10 μg/L chlorpyrifos line. While the reason for an antagonistic interaction remains unknown, it could be hypothesized that the acetylcholinesterase inhibition effect of the chlorpyrifos may have an increasing effect on tadpole activity and, therefore, foraging rates. However, the lack of chlorpyrifos effects alone suggests that some other unknown interaction could be causing the increase in weight, especially considering that the 10 μg/L chlorpyrifos treatment showed lower weights at 20 μg/L atrazine and 200 μg/L

atrazine than the 1 μg/L chlorpyrifos at those same atrazine concentrations (Figure 10). Spence [18] reported no chlopyrifos-atrazine interaction effects on time to metamorphosis or size measures at metamorphosis for *X. laevis*; similarly chlorpyrifos exposure in the absence of atrazine did not contribute to differences in body size (concentrations for both chemicals were similar to those used in this study).

There were avery limited number of chemical mixture interaction effects in this study; also few trends in those interactions suggest an easily understood relationship in how those interactions occur or how consistent such interactions might be among populations. While experiment 1 indicated a switch from antagonistic interactions to synergistic interactions with increasing atrazine concentration, experiment 2 indicated either additive or antagonistic interactions. The presence of the interaction effects alone suggests that classical examinations of toxicity through NOEC and LC 50 experiments present a woefully incomplete picture of what is occurring in the environment, with regards to chemical interaction effects on behavior.

Other Interaction Effects and Their Significance

The results of this study indicated several instances of significant differences among replications and among clutches, as well as significant interactions between replication, clutch, atrazine, and chlorpyrifos treatments. For example, in Experiment 1 the variables POSTLINE, POSTACT, POSTCUE, and PERLINE exhibited significant replication or replication interaction effects. (Tables 7, 9, 11, & 13 respectively). In Experiment 2, significant clutch or clutch interaction effects were observed for the PRELINE, PREACT, POSTLINE and weight variables (Tables 2, 4, 8, & 17 respectively). The weight variable also exhibited replication and replication interaction effects (Table 17).

The advantage of using a Factorial ANOVA is that main treatment effects are examined independently for statistical purposes. For instance, an F test associated with the atrazine treatment examines the differences in response to the atrazine while factoring out differences that might be attributed to other main effects, such as chlorpyrifos or clutch. Including in the design used in both experiments in this study was the factor 'replicate'. Replicate was included not because there would be any biological significance or meaning to a difference among animals in the same replicate number, but because animals could not all be raised either individually or combined in the same aquarium due to density effects. It was therefore necessary to spread desired sample sizes among tanks (replicates). Any differences attributed to replicates would simply reflect experimental error, in the sense that the unique combination of 25 animals in one tank might be slightly different than a combination of 25 animals in another tank, even when animals were assigned at random and otherwise encountered the same experimental conditions for the other factors in their treatment group. Thus, by including replicate in the experimental design, differences among replicates are removed from consideration of other main, and biologically important, factors. Therefore, no attempt to explain significant differences that involve replicate are made. On the other hand, clutches were included in experiment 2 specifically to determine if responses might differ among distinct groups of full-siblings, suggesting genetic variation in response which is of biological significance. The significant differences in experiment 2 both between clutches and the significant interactions that include clutch highlight the role of genetic diversity within and between clutches in measuring responses to environmental contaminants. It is not unexpected that behavioral responses within a population of individuals will exhibit extensive phenotypic variation due to both genetic differences among individuals and phenotypic flexibility; this variation will contribute to

statistical experimental error. While some portion of this significant 'within' and 'among' clutch variation may simply represent inherent variation, it also emphasizes the problem with determining toxic effects among individuals in any experiment. Using laboratory cultures of animals that have been maintained for many generations (or groups of full siblings only) may well eliminate variation in response, making the probability of observing a main effect more likely. At the same time, ignoring natural variation in response to a stressor may provide a biased indication of the stressor's impact on a population. That significant clutch and clutch interactions were observed in this experiment (both between the outcome of experiment 1 and 2 and within the clutches of experiment 2) is an important biological indication of the potential range of responses that might be observed in natural populations.

Previous research concerning the order Anura has indicated significant differences in tolerance to carbaryl, not only between species, but within populations and among full sibling clutches of the Sotuhern Leopard Frog (*Rana sphenocephala)* as well [51]. Variations in virus susceptibility in the presence of contaminants occurs between different amphibian species [52]. Amphibian behavioral effects from atrazine exposure show conflicting results among different species. Both streamside salamander (*Ambystoma barbouri*) and wood frog (*Rana sylvatica*) larvae show elevated activity in response to atrazine [47,48], while African clawed frogs (*Xenopus laevis*) have demonstrated decreased activity in response to atrazine exposure [15].

Recent studies concerning chemical interactions and natural stressors continue to provide conflicting results. The results of these studies continue to demonstrate the sheer amount of variability that can occur when looking at interaction effects. Recent attention has focused on effects within more realistic mesocosm structures. Chemical effects on larval survival vary by species and can interact with population demographic features, such as sex ratio or age structure.

The presence of carbaryl in a mesocosm virtually eliminates spotted salamanders (*Ambystoma punctatum*) and shows significant reduction in the mass of surviving larvae, significantly reduces American toad (*Bufo americanus*) survival, and has no significant effect on southern leopard frog (*Rana Sphenocephala*) survival [46]. Atrazine has been shown to significantly reduce hatching ratios and larval survival in streamside salamanders (*Ambystoma barbouri*) [47]. Atrazine reduces survival of woodfrog larvae (*Rana sylvatica*) by reducing food availability, which heightens competition between individual larvae and between larvae and snail populations [48]. Boone and James [46] found no significant interactions between carbaryl and atrazine, but show significant, non-additivie interaction effects between atrazine, density, and hydroperiod with regards to spotted salamanders, leopard frogs, and American toads. Rohr et al. [47] showed additive interactions between chronic atrazine exposure, food levels, and hydroperiod on streamside salamander survival, behavior, and physiology at metamorphosis. Rohr and Crumrine [48] found additive interactions between acute treatments of atrazine and community structure in mesocosms populated with woodfrog tadpoles, snails, and dragonfly larvae.

Interaction studies can also provide insight into virus susceptibility. In tiger salamanders (*Ambystoma tigrinum*), high concentrations of atrazine alone show increased ranavirus infection rates among atrazine treatments and high nitrate concentrations significantly lower infection rates in the presence of atrazine. However, both contaminants show significant decreases in leukocyte levels, suggesting immunosuppressive effects [52]. All of these more complex studies attempt to increase the number of variables involved in chemical interactions, but each shows the present lack of large scale study needed to understand these interactions effectively.

Summary

Significant differences were determined between mean behaviors among atrazine concentrations for the variables PRELINE and PREACT in Experiment 1 (Appendix, Tables 1 & 3, Figures 1, 2, & 4) and the variables POSTLINE and weight in Experiment 2 (Appendix, Tables 8 & 17, Figures 5, 6, 10, 11, 12, & 13). In general, a increase in exposure to atrazine tended to decrease various aspects of activity, although not the generalized response to a predator alarm cue. There were no differences in mean behaviors among chlorpyrifos concentrations in Experiments 1 and 2. Finally, there were limited interaction effects between atrazine and chlorpyrifos on mean behaviors. In Experiment 1, significant interaction effects were observed in the variable PRELINE (Figure 3). In Experiment 2, the variable weight showed significant interaction effects (Figure 10). Overall, the lack of consistency in interaction effects prevents the formation of any hypotheses regarding the general effects of the interaction between chlorpyrifos and atrazine. However, the presence of these effects is a clear mandate for additional research regarding the possible interactions among agrochemicals

This paper shows that interaction effects can and do occur between agrochemicals and that these effects are both generally under-researched and difficult to interpret. Furthermore, individual chemicals show significant interaction effects with environmental factors on amphibians. There are a vast multitude of anthropogenic chemical interaction effects that may be affecting amphibian behavior and survival. When state or governmental authorities rely on current research that fails to incorporate interaction effects when determining safe runoff and spray levels for agrochemicals, they are using data that is relevant to the lab only. The sheer number of different variables that can be affected by mixture interactions highlights the shortcomings of current environmental policies. With amphibian populations declining

worldwide, it is of the utmost importance that decisions regarding conservation and environmental policy and standards be made based on research that accurately reflects ecological conditions faced by fauna.

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APPENDIX

Appendix: Summary of All Significant Effects for Experiments 1 & 2.

Statistically significant effects are marked with a star (*), Non-significant effects are marked with NS.

Source	**PRELINE**		**PREACT**		**PRECUE**		**POSTLINE**		**POSTACT**		**POSTCUE**		**PERLINE**		**PERACT**		**Weight**
	Exp 1	*Exp 2*	*Exp 1*	*Exp 2*	*Exp 1*	*Exp 2*	*Exp 1*	*Exp 2*	*Exp 1*	*Exp 2*	*Exp 1*	*Exp 2*	*Exp 1*	*Exp 2*	*Exp 1*	*Exp 2*	*Exp 2*
Atra	*	NS	*	NS	*	NS	*	*	NS	NS	NS	NS	NS	NS	NS	NS	*
Chlor	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Rep	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	*	NS	NS	NS	*
Clutch		*		NS		NS		*		NS		NS		NS		NS	*
Atra*Chlor	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	*
Atra*Rep	NS	NS	NS	NS	NS	NS	*	NS	*	NS	*	NS	NS	NS	NS	NS	*
Atra*Clutch		NS		NS		NS		*		NS		NS		NS		NS	*
Chlro*Rep	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	*	NS	*
Chlor*Clutch		NS		NS		NS		NS		NS		NS		NS		NS	*
Atra*Chlor*Rep	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Atra*Chlor*Clutch		NS		*		NS		NS		NS		NS		NS		NS	*
Chlor*Rep*Clutch		NS		NS		NS		NS		NS		NS		NS		NS	NS
Atra*Chlor*Rep*Clutch		NS		NS		NS		NS		NS		NS		NS		NS	*