

Endocrine Disruptors in Amphibians: Analysis of the Effect of Two Common Environmental Pollutants

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ABSTRACT

This study examined the effects of common environmental pollutants in amphibians (frogs of the genus *Rana*) and mammals (laboratory mouse, *Mus musculus*). The effects of the pesticide atrazine and the gasoline additive MTBE on growth, development, and sex differentiation were studied in frogs, and the effects on the immune system were examined in mice. Experimental methods included morphology, hematology, histology, and hemolytic plaque assays. Frogs were treated from the time of hatching to metamorphosis. Growth was determined by weight, and development was determined as percent reaching metamorphosis after a given amount of time. Sex differentiation was studied first by morphological examination of the gonads in newly metamorphosed frogs, and was then confirmed histologically. The mice were treated for three weeks, beginning at 16 weeks of age and the effect on their immune system was examined using the hemolytic plaque assay (HPA) to measure the number of activated (antibody-producing) B-lymphocytes. Results show that atrazine has a large but negative impact on growth and development, but no discernable effect on sex differentiation in frogs. MTBE (methyl-tertiary-butyl-ether), a gasoline additive, has little or no effect on growth and development in frogs, but appears to have a pronounced effect on sex differentiation. The effects of contaminants on the immune system in mice are equivocal, but other, unexpected detrimental effects were observed. These results suggest that atrazine and MTBE are both environmental toxins and may act as endocrine disruptors with variable effects on amphibians and mammals. These chemical contaminants may therefore be playing a role in amphibian declines and could also pose hazards for human health.

INTRODUCTION

Amphibians are considered to be good “indicator” organisms with which to study the biological impact of environmental chemical pollution because they depend on water or moisture to reproduce and to breathe through their thin, highly vascularized skin. Recent reports of widespread declines, disease, and deformities in natural populations of amphibians have raised concerns that environmental contaminants may be to blame, especially those that can act as endocrine disruptors. There is ample evidence that gonadal differentiation in amphibian species is highly sensitive to sexual hormones. Estrogens can induce a sex reversal in genetic males, where either an ovotestis is produced or complete and permanent feminization occurs (Lofts 1974). Kloas et al. (1999) reported that bisphenol A and 4-nonylphenol significantly altered the sex ratio in *Xenopus laevis*. Dibutyl phthalate and styrene induced gonadal feminization in *Rana rugosa* (Ohtani et al, 200, 2001). Recently, hermaphroditic gonads induced by Atrazine in *X. laevis* were reported by Hayes et al. (2002). Legitimate concerns have been raised not only about the disappearance and deteriorating health of natural populations of amphibians, but also about the broader implications for human health and well being.

The endocrine system operates through a complex series of events triggered by chemical messengers that choreograph development and function. Chemical messengers

are involved in sexual differentiations. Cell division leads to the construction of tissues and organs that eventually determine future function, such as sperm production and ovulation. Endocrine disruptors work by a variety of mechanism. First, they can impersonate natural hormones by binding to receptors and initiating a new cellular response. Second, an endocrine disrupting chemical may bind and block the receptor, thereby making the regulatory switches unavailable to signal the body's naturally produced hormone messengers. Third, concentrations of the natural hormone can also be affected when man made chemicals promote or interfere with the breakdown of the hormone by the liver enzymes system. Fourth, during development, endocrine disrupting chemicals can alter the number of receptors in developing tissue types, thereby predisposing these tissues to abnormal responses later in life. The changes in expression can lead to functional deficits changes in how an organism's immune, reproductive, and other systems perform (Smolen et al. 1997).

The two chemicals that were tested in this experiment were Atrazine and MTBE. Atrazine (2-chloro-4-ethylamino-6-isopropylamine-1,3,5-triazine): the most commonly used herbicide in the United States and is currently used in more than 80 other countries. MTBE (methyl-tertiary-butyl-ether): a commonly used gasoline additive that increases the efficiency of combustion, thereby reducing particulate emissions.

The purpose of this experiment was to determine whether Atrazine and MTBE act as endocrine disruptors. I will investigated the effects of widespread environmental contaminants on natural populations of amphibians using an assay for immune function that to our knowledge has not been used with frogs before, but which we have found, from our pilot studies, to be a relatively fast, simple, and effective way to quantify activated B-cell (antibody-producing lymphocyte) production in frogs. This research focuses not only in determining the effects of chemical pollutants on the immune system in general, but also how these effects may play a role in the immunological defense against pathogens. I was also interested in the effects on growth, development, and sexual differentiation with these chemical pollutants.

MATERIALS AND METHODS

Wood frogs (*Rana sylvatica*) larvae were treated with either one of two environmental contaminants, atrazine and MTBE until metamorphosis. Experimental animals were exposed to three different levels of concentrations for both atrazine and MTBE treatments. The tadpoles were collected from the wild one day after hatching. The tadpoles were emersed in the treatment solution until metamorphosis for a total treatment of time of approximately three months. The solutions were changed every three days. There were 6 groups of 15 tadpoles, for a total of 90 tadpoles per each treatment group for a particular contaminant, and a total number of 360 per tested pollutant. A stock solution of atrazine was prepared by dissolving 1 mg in 1 ml 100% ethanol, and then diluted. Controls were treated with ethanol only at equivalent concentration. The experimentals were treated in 1ppb*, 10ppb, and 100ppb (*ecologically realistic) atrazine.

MTBE was dissolved in plain dechlorinated (aged) tap water. Controls were treated in plain aged tap water. The experimentals were treated in 10^{-5} M*, 10^{-4} M, 10^{-3} M (*ecologically realistic) MTBE. Different concentrations were obtained via serial dilutions.

Sexual differentiation in newly metamorphosed frogs was determined via dissection and visual examination of the gonads, and then confirmed via histology. To determine growth and development, the average weight and percent metamorphosed tadpoles was done after a given interval of time.

Adult frogs (*Rana pipiens*) were used for HPA. Each animal was immunized against a standardized antigen (40% sheep red blood cells, SRBCs washed and suspended in saline), injected intraperitoneally on each of five days. On the sixth day, the animals were euthanized with MS222 and their spleens were then surgically removed and used for the isolation of lymphocytes.

Hemolytic Plaque Assay (HPA)

Spleen cells were removed from each spleen using the “balloon” technique followed by mechanical maceration. The spleen cells were flushed out of the spleen into a tissue culture dish with 3 cc of Hank’s buffered saline (HBSS) + 5% heat-inactivated fetal bovine serum (HBSS+) using a sterile syringe and 26 gauge needle inserted into the splenic artery. The spleen cell suspension was then removed to a sterile centrifuge tube, and centrifuged for 5 minutes at 4 degrees C, and the pellet was resuspended in 3 ml HBSS+ and kept on ice. A 50 microliter sample of cells was then suspended in a mixture of 0.5% Trypan blue, which was allowed to stain for 5 minutes, and the live (unstained) cells were counted with a hemocytometer to determine the number of leukocytes (including lymphocytes). The original concentration will then be adjusted to approximately 10^6 cells per ml. A sample of 0.5 ml of the spleen cells were then mixed with 1.5 ml of SRBCs and 0.5 of guinea pig complement, and then left on ice for 5 minutes. Then 100 μ l of the mixture was loaded into both sides of an HPA chamber and allowed to incubate for 3 hours at room temperature. HPA chambers were prepared by placing three narrow strips of double stick tape in the middle and at each end of a sandwich of two cleaned glass microscope slides. Slides are cleaned by dipping in 95% ethanol to which three drops of glacial acetic have been added, and then wiped with a soft, lint-less cloth. Hemolytic plaque were counted using a compound microscope and phase contrast optics, standardized to the initial cell counts to obtain the proportion of antibody producing B-lymphocytes. The results were analyzed using an appropriate statistical test.

RESULTS

In the atrazine experiment on growth and development, the results show that atrazine has a large positive, and statistically significant effect, on growth and development. Growth and development decrease as concentration increases (Fig.1). This indicates that atrazine inhibits growth and development in frogs.

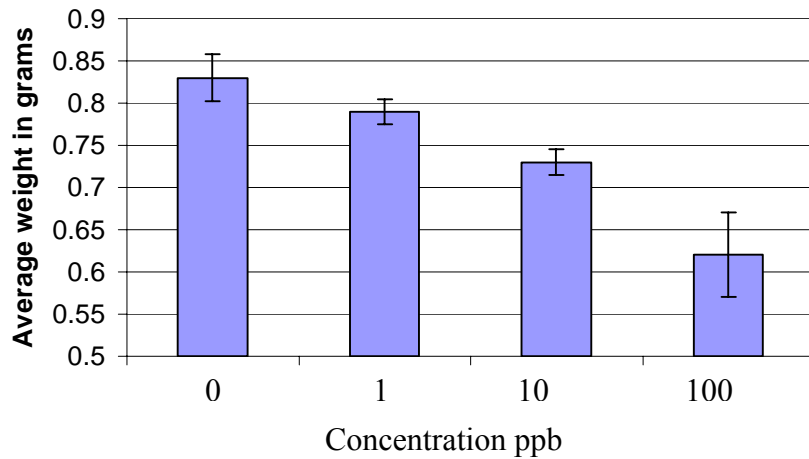


Figure 1. Histogram showing that atrazine causes a decrease in growth and development (ANOVA; significant $p < 0.001$).

Regarding sexual differentiation, atrazine had a small effect on sexual differentiation. There is little or no effect at the two lowest concentrations, but frogs exposed to the highest concentration had a skewed sex ratio in favor of females (Fig.2).

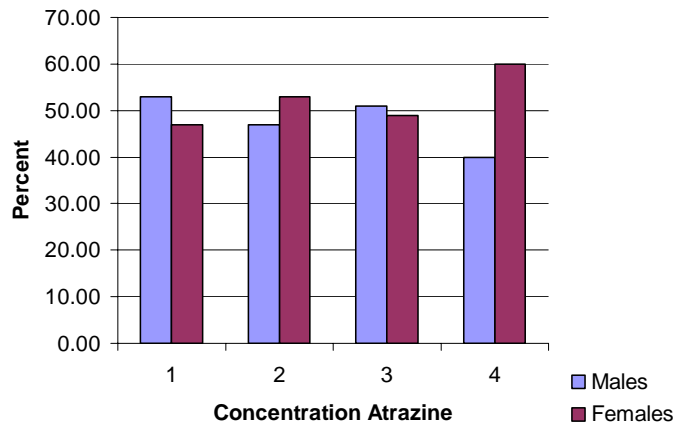


Figure 2. Histogram shows that atrazine has a small but significant effect on sexual differentiation; 1 = 0ppb, 2 = 1ppb, 3 = 10 ppb, 4 = 100 ppb; (ANOVA; $p < 0.05$).

My study in the affects of MTBE on growth and development showed a small but insignificant positive effect on the average growth size in frogs (Fig. 3).

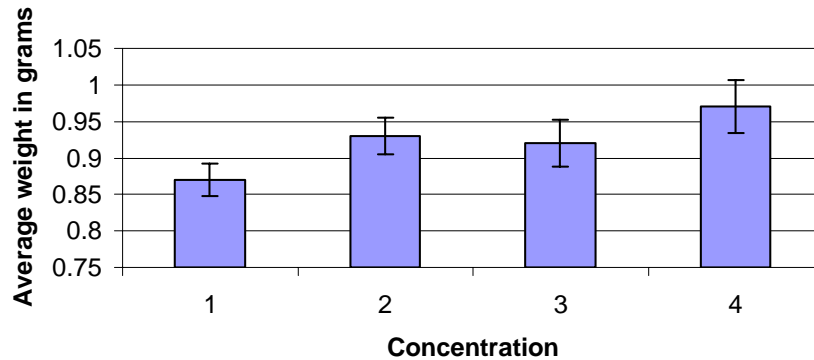


Figure 3. Histogram showing that MTBE has a slight positive on growth and development (not statistically significant); 1 = 0 M, 2 = 10^{-5} M, 3 = 10^{-4} M, 4 = 10^{-3} M.

The experiment examing the effects of MTBE on sexual differentiation showed that MTBE had a major effect on sexual differentiation (Fig. 4). At the two intermediate concentrations there is a big effect on sexual differentiation which disappears at the highest and lowest concentrations. This could indicate that there is a dose effect whereby sex differentiation is most strongly affected at intermediate concentrations of MTBE.

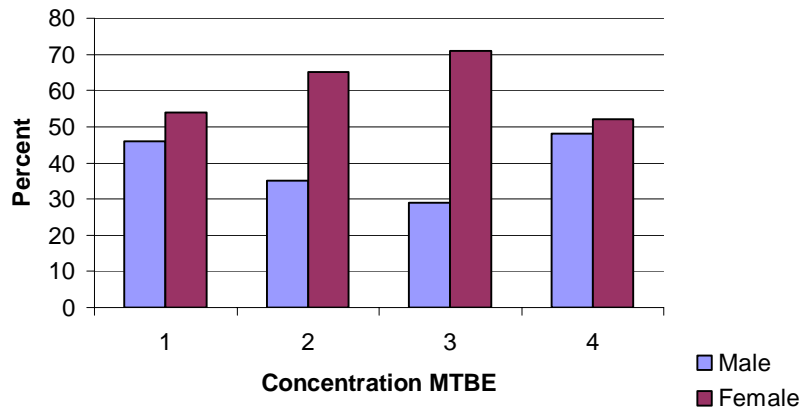


Figure 4. Histogram showing the effects of MTBE on sexual differentiation in frogs (x-axis labeled as in Fig 3).

Mice were also treated with both atrazine and MTBE. No effect on the immune system was evident (data not shown), but the mice showed loss of fur and whiskers around the snout while under the treatment of Atrazine at the lowest concentration (1ppb; Fig. 5). This result indicates that atrazine probably interferes with growth factors necessary for the maintenance of whisker follicles on the snout.



Figure 5. Atrazine caused mice to lose their snout whiskers, indicating that atrazine is probably interfering with growth factors involved in whiskers follicle maintenance.

DISCUSSION

Endocrine disruptors have been documented in a wide variety of vertebrates, in both laboratory and field conditions. Numerous pesticides, industrial chemicals, and commercial products that have been released into the environment are indeed endocrine disruptors. The future-well-being of humans and other organisms will depend on achieving a thorough understanding of this impact in order to achieve a balance between technological development and environmental health. This project has generated information concerning two widely used chemicals that have become important environmental pollutants, atrazine and MTBE, whose health effects are currently controversial. I have examined their effects on amphibians, with a focus on, growth, development, sex differentiation, and the immune system.

This study has contributed to our understanding of recent reports of amphibian declines and deformities. Damage of the immune system by environmental chemical pollutants would leave frogs vulnerable to all sorts of pathogens and diseases. Furthermore, since amphibian immune systems are structurally and functionally similar to that of other vertebrates, including mammals, our results will elucidate potential environmental risks to human health.

Our study has indicated that both atrazine and MTBE may act as endocrine disruptors in amphibians, with effects on growth, development and sex differentiation. However, it remains an open question whether these pollutants are involved in deformities in frogs as well, most of which are now known to be produced by parasites (Sessions et al. 1999). One possible unanticipated outcome of the proposed study is that the parasites show greater sensitivity to the chemical pollutants than the frogs, in which case the parasite becomes the indicator organism. This result would also be a valuable contribution to our understanding of the relationship between chemical pollution, immune function, and disease in amphibians and mammals.

Further research can be done by examining levels of testosterone to determine if these chemicals are interfering with the sexual differentiation of male frogs. It will also

be important to examine the immune function in more detail to examine if there are any other problems that occur when exposed to these chemicals.

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