

Chapter 12

Ecotoxicological Risk Assessment of Atrazine in Amphibians

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Although the ecological risks of atrazine in surface waters have been extensively reviewed with respect to its potential effects on a number of components of ecological systems, there is a lack of specific data on amphibians, especially for endpoints related to development and reproduction. To assess whether atrazine causes adverse effects in frogs through endocrine-mediated mechanisms, several hypotheses were tested in laboratory and field studies, using guidelines for the identification of causative agents of disease and eco-epidemiology derived from Koch's postulates and the Hill criteria. These criteria were: Temporality; Strength of

Association; Consistency; and Biological Plausibility. Data from the literature and from studies conducted for the purpose of this assessment were used to test the following hypotheses: Atrazine causes adverse effects in amphibians through; 1) estrogen-mediated mechanisms, 2) androgen-mediated mechanisms, 3) thyroid-mediated mechanisms, 4) adverse effects on gonadal development in amphibians, or 5) adverse effects at the population level in exposed amphibians. In a lines-of-evidence approach to address the causal link between atrazine and effects in amphibians, no temporal correlation exists between the occurrence of gonadal effects and the introduction and use of atrazine. The strength of association is not strong. While some concentration-responses have been observed in some studies for some endpoints, these have not been observed under different conditions. The incidence rate of gonadal anomalies and other effects on populations was not related to atrazine exposures. Controlled studies in different laboratories produce inconsistent results and observations are inconsistent among laboratories as well as between laboratory and field studies. The biological plausibility of either of the proposed mechanisms of endocrine disruption is not supported by the laboratory or field observations. The data showed no evidence of effects through thyroid hormone mediated mechanism and little evidence for a mechanism mediated through androgens or estrogens. Overall, there was no compelling evidence to suggest that atrazine causes adverse effects in amphibians that are mediated by endocrine or developmental mechanisms.

Introduction

The potential for ecological risks from atrazine in surface waters has been extensively reviewed with respect to its potential effects on organisms in a number of ecological systems (1,2). Fish full life-cycle studies and mesocosm studies have not suggested effects on reproduction and development at environmentally relevant concentrations. However, there is a lack of specific data on some non-target aquatic and semiaquatic species such as reptiles and amphibians, especially for the newly identified sublethal endpoints related to

development and reproduction. A robust and tested framework within which to conduct risk assessments for endocrine and developmental endpoints is also absent (3). Specifically, standardized tests have not been developed and validated for studies with amphibians or for endpoints other than survival, growth and early development. In addition, there is no guidance available on how to interpret non-linear dose-response relationships or for relating subtle effects at molecular or physiological levels of organization to ecologically-relevant assessment endpoints. With this in mind, we developed several assays for endocrine and developmental disruptors in fish, amphibians, and reptiles, both to refine general assays using relevant methods of exposure and to investigate the possible endocrine and reproductive effects of atrazine. The primary focus of this chapter is on characterizing and assessing endocrine-mediated effects of atrazine on amphibians. Much of this work has been presented and deliberated at a United States Environmental Protection Agency (USEPA) Science Advisory Panel (SAP) meeting in June 2003 (4,5) and this chapter is a summary of key issues related to our findings in the context of other work in the literature.

Laboratory studies using traditional endpoints indicate that atrazine is not highly toxic to frogs and that it does not cause mortality during larval development and metamorphosis at environmentally relevant exposures (2,6-8). Atrazine only causes reproductive effects in birds at large exposure concentrations (9). Acute and chronic exposure studies in mammals do not indicate great sensitivity for the traditional assay endpoints. The exception to this is the increased incidence of mammary tumors in female Sprague Dawley (SD) rats, an effect that was strain-, sex-, and species-specific and judged to not be relevant to humans (10) or other species. Many of the factors controlling amphibian reproduction and sexual development are modulated by the endocrine system (Figure 1).

Amphibians exhibit a variety of reproductive adaptations that include some forms of hermaphroditism in frogs and toads. Hermaphroditism has been reported since the 1860s and the development of this condition varies among species and the magnitude of expression varies among locations (11). Sex reversal has been reported in some species in response to temperature changes, other factors, or spontaneously (12). Other environmental factors, such as disease or parasite loads that may influence this are unknown. Sexual differentiation is a highly labile process in some species of amphibians which illustrates that cross-species extrapolations are not simple nor is it easy to establish cause and effect relationships between various endogenous and exogenous signals that control sexual differentiation.

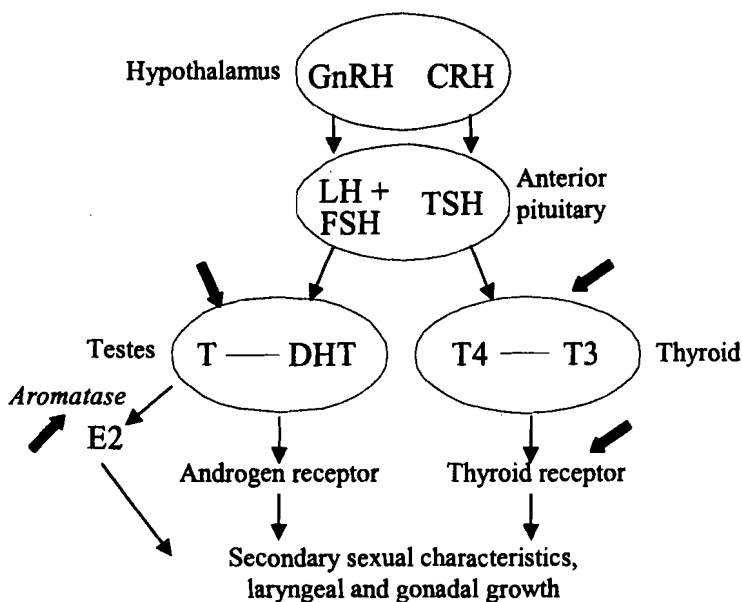


Figure 1. Hypothetical mechanism of induction of secondary sexual characteristics in *Xenopus laevis* showing possible targets for modulation by xenobiotics (heavy arrows). CRH, corticotropin-releasing hormone; DHT, dihydrotestosterone; E2, estradiol; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; T, testosterone; T3, triiodothyronine; T4, thyroxine; TSH, thyroid stimulating hormone.

To assess whether atrazine causes adverse effects in frogs through endocrine-mediated mechanisms, several hypotheses were proposed to address specific mechanisms of action for endocrine modulating substances. In testing these hypotheses, guidelines based on those employed in the identification of causative agents of disease and eco-epidemiology (13,14) were used. These criteria were: Temporality; Strength of Association; Consistency; Biological Plausibility; and Recovery (15).

Assessment of Possible Mechanisms

With respect to potential effects of atrazine on endocrine function in amphibians, the question of possible mechanism can be reduced to several hypotheses which address specific mechanisms of action for endocrine

modulating substances. Also, test endpoints can be defined which include both changes in hormone concentrations or in hormone-mediated processes or structural or functional endpoints that are under the control of steroid hormones such as are illustrated in Figure 2. These are discussed in the following sections:

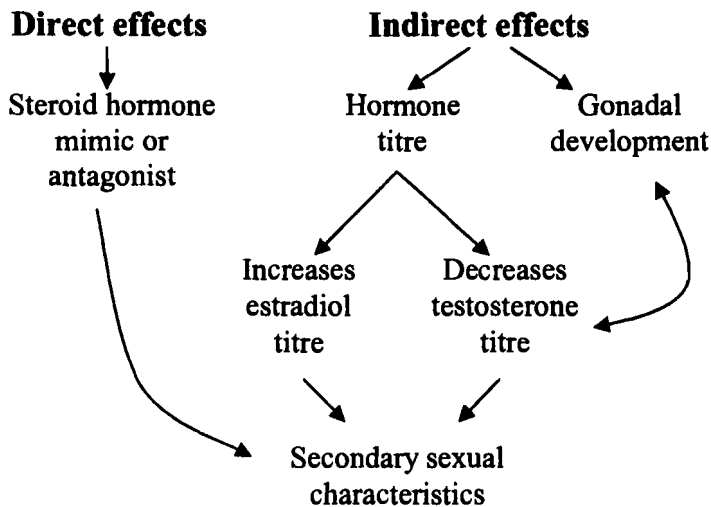


Figure 2. Graphical illustration of the possible pathways through which atrazine may affect the expression of secondary sexual characteristics and gonadal development

Estrogen and Anti-estrogen Mediated Mechanisms

In vitro studies conducted with mammalian estrogen receptor (ER) have indicated that atrazine does not bind to the ER (16). Based on effects on estrogen-mediated determination of sex ratios, atrazine does not mimic the effects of estradiol on sex ratio in frogs, such as *Xenopus laevis* (17,18). Atrazine exposure did not increase plasma estradiol titres in *X. laevis* frogs, exposed in the laboratory (19) or collected from corn-growing areas in South Africa (18), or in *Bufo marinus* collected in sugar cane-growing sites in Florida where atrazine was present in the environment (20). Atrazine has been reported to up-regulate aromatase activity *in vitro* in a human adenocarcinoma (H295R) cell line and fish hepatocytes at relatively high concentrations (21,22). Although atrazine has been postulated to affect hormone titres through induction of

aromatase in frogs (23), it did not significantly increase the activity of gonadal or brain aromatase in laboratory and field studies on exposed frogs (18,19). The lack of response of both estradiol titres and aromatase is consistent with the known role of aromatase in the biosynthesis of estradiol. For the hypothesis that atrazine acts through estrogen or anti-estrogen mediated mechanisms, there was little evidence for a strength of association, for consistency, and biological plausibility. Overall, there was little support that atrazine affects (anti)estrogen-mediated processes in amphibians.

Androgen and Anti-androgen Mediated Mechanisms

Atrazine has been reported to bind to the androgen receptor in mammals *in vitro*, but with very low affinity (24,25). No studies on binding of atrazine to the androgen receptor or androgen receptor-mediated gene activation have been conducted in amphibians, but on the basis of low-affinity binding in mammals, effects via this mechanism would not be expected. Although one study reported that exposure to atrazine decreased plasma testosterone concentrations (23), studies using laboratory and field exposures did not observe this response in *X. laevis* (18,19) or *B. marinus* (20).

Laryngeal development in frogs is a sexually dimorphic process, and the formation of a larynx capable of male calling behavior is androgen-dependent (26). Under normal conditions, male frogs have greater larynx muscle size than do females and this has been used as a measure of possible androgen-mediated effects of atrazine. In the single study reporting that atrazine exposure in the laboratory caused a decrease in larynx dilator muscle size in male *X. laevis* frogs, there was no consistent concentration-response relationship (23). Two other laboratory studies in the same species did not observe this effect (17,19) and no effects on larynx weight were observed in the same species from corn-growing sites in South Africa (27). For the hypothesis that atrazine acts through androgen or anti-androgen mediated mechanisms, there was no conclusive evidence for a strength of association, for consistency, and biological plausibility. Overall, there was little support that atrazine affects (anti)androgen-mediated processes.

Thyroid Mediated Mechanisms

Binding to or interference with the thyroid hormone receptor has not been directly tested in amphibians. No effects on metamorphosis were reported in laboratory studies in *X. laevis* (17,19,23) *Rana clamitans*, or *R. pipiens* (6,28).

Changes in plasma corticosterone and thyroxine concentrations were reported in salamander larvae (*Ambystoma tigrinum*) exposed to atrazine at concentrations of 75 and 250 :g/L (29). The authors did not suggest that this was an atrazine-specific mechanism but rather that it was a compensatory response to stress from an environmental contaminant. In addition, since the development of the laryngeal dilator muscle is dependent on both androgen and thyroid hormone, the fact that effects were not observed in male *X. laevis* in all but one of the studies conducted with atrazine supports the conclusion that atrazine did not disrupt thyroid hormone status in these frogs (17,19,27). Overall, there is no evidence that atrazine affects thyroid-mediated processes in amphibians.

Mechanisms Mediated Through Gonadal Development

Several studies have examined the gonads of developing male and female frog larvae exposed to atrazine, both under laboratory and field conditions. Most of the effects that have been attributed to exposure to atrazine have been reported to have occurred in males and have ranged from morphological anomalies in the gonads to the presence of ovarian tissue in the testes. Two laboratory studies reported gross gonadal anomalies in two species of frogs (*X. laevis* and *R. pipiens*) at concentrations as low as 0.1 :g/L (23,30). However, other laboratory studies have not shown significant increases in these responses in *X. laevis* or *R. clamitans* exposed up to 25 :g/L atrazine (19,28) or have only observed these at greater concentrations (25 :g/L in *X. laevis*) and at lower frequencies (17). Where histology was conducted in these studies, testicular oocytes have been observed, but in small numbers (per testis) and at a relatively low frequency of individuals.

A series of laboratory studies on the effects of atrazine on gonadal and kidney development in male and female *X. laevis* tadpoles were reported in a thesis and two published papers (31-33). In males, reductions in testicular size were reported in one experiment. Unfortunately, the data in the published papers and in the thesis on which they are based are inconsistent and some experiments showed responses while others (only published in the thesis) did not. These data cannot be interpreted.

A field and a microcosm study on *X. laevis*, reported no relationship between exposure to atrazine and other agricultural chemicals used in corn production and gonadal anomalies (27) and the incidence and frequency of testicular oocytes (34). Studies on *X. laevis* exposed to time-weighted mean concentrations of atrazine of 0, 1, 12 and 30 μ g/L in semi-field microcosms from shortly after hatch until after metamorphosis showed a low frequency of

metamorphs (stage 66) with abnormalities but no concentration response to atrazine. Frequency of incidence of gross gonadal anomalies (visible without histology) ranged from 8% of males in the controls to 7% of males exposed to 30 $\mu\text{g/L}$ atrazine. Rigorous histological examination of the testes in the microcosm-exposed metamorphs revealed the presence of one or more testicular oocytes per frog at frequencies ranging from 57% in the unexposed control metamorphs to 40% in those exposed to 30 $\mu\text{g/L}$. The mean number of oocytes per testis ranged from 9.5 in the controls to 12 in metamorphs exposed to atrazine at 30 $\mu\text{g/L}$. The total number of oocytes per testis ranged from 1 to 60 but no concentration response was observed for either frequency of occurrence or number of oocytes. Although the number of frogs with testicular oocytes was similar to those in metamorphs after 10 months of additional exposure, the mean number of testicular oocytes per testis was smaller (>3 per testis) in control and exposed frogs examined 10 months after metamorphosis.

In a field study of *R. pipiens* carried out in the US Midwest, the greatest frequencies of gonadal anomalies occurred in sites with the lowest concentrations of atrazine (30). The authors suggested that this could be due to an inverted "U" concentration response, resistance developed in some populations, or that it was caused by other agents. In fact, the measured atrazine concentrations likely had no relation to exposures during development as the samples for residue analysis were taken at the same time as the metamorphosed frogs. Thus, exposures during development were not known. The most plausible conclusion is that there was no relationship with atrazine. The reported frequency of anomalies was different between *R. pipiens* and *X. laevis*, but there was no indication of a concentration-response nor any effects on the frequency of other cell types in testes of field collected *X. laevis* from corn-growing sites in South Africa (27). The historical incidence of intersex in the cricket frog (*Acris crepitans*) did not show a statistically significant relationship to concurrent atrazine exposure concentrations (35) or a temporal relationship with the introduction of atrazine (36).

In a preliminary study, adult male *B. marinus* from sugar cane-growing areas where atrazine was used showed female-specific skin coloration patterns and the presence of oocytes in the testes, possibly as a result of development of Bidder's organ. Bidder's organ is a rudimentary ovary which is known to be responsive to a wide variety of endocrine factors and, in some genera of amphibians, such as *Bufo*, is retained in association with the testes or ovaries in adults. Exposure measurements were incomplete and give the large number of pesticides used in sugar production, causality cannot be assessed from this study.

There was no evidence for temporality, little evidence for strength of association, some evidence of consistency, and little evidence of biological plausibility, particularly as gross gonadal anomalies and testicular oocytes have been reported from unexposed controls and from historical observations. Overall, there is little evidence that atrazine affects gonadal development in amphibians, however, there is uncertainty with respect to background incidence of some of the responses observed in the gonads of male frogs. Also, it is unclear how atrazine can cause effects on gonads above a threshold of 0.1 :g/L without any apparent concentration-response relationship, such as has been suggested.

Mechanisms Mediated Through Effects at the Level of the Population

Although few studies have addressed this question relative to causality, there is no evidence of effects at the population level that have been linked to atrazine exposures. Apparently robust populations of *R. pipiens* were observed across a range of atrazine exposures in the US Midwest (30). Preliminary observations in *B. marinus* from South Florida showed an abundance of frogs in sugarcane-growing areas where atrazine was present (20). No differences in sex and age classes in *X. laevis* from reference and corn-growing sites in S. Africa were observed (37). There was no evidence for temporality, for strength of association, for consistency, and for biological plausibility. Overall, there is no evidence that atrazine affects frogs at the population level. Based on the frequency and magnitude of gonadal anomalies in frogs that have been reported to have occurred in laboratory studies and similar observations in apparently robust field populations, responses in these endpoints would not be expected to translate into changes in the assessment endpoint of populations. Because most frogs are 'r-selected', they produce many eggs, few of which survive to become reproductive adults. Survival of California red-legged frog (*Rana aurora draytonii*) tadpoles to metamorphosis under field conditions has been reported as low as 5% (38). Thus, even if the subtle effects described to have occurred in the gonads of the frog species studied were attributable to atrazine, they may have little relevance at the population level. Without consistent, robust, and mechanistically understood responses, it is not possible to determine if they might be adverse responses in other amphibian species or not.

Conclusions

Using the guidelines suggested for assessing endocrine disruptors by the International Program on Chemical Safety (15) in a lines-of-evidence approach

to address the causal link between atrazine and effects in amphibians, the following general conclusions are possible:

With respect to temporality, no correlation exists between occurrence of gonadal effects and the introduction and use of atrazine. Intersex, other gonadal anomalies, and testicular oocytes were observed before the introduction and use of atrazine as herbicide. There is little evidence to support the strength of association. While some concentration-responses have been observed in some studies for some endpoints, these have not been found under slightly different conditions. The incidence rate of gonadal anomalies and other effects on populations is not related to atrazine exposures and the possible influence of a number of plausible confounders has not been specifically addressed. The observations reported are not consistent. Controlled studies in different laboratories produce very different results and the outcomes for many of the endpoints and observations are inconsistent between laboratory and field studies. Similarly, the biological plausibility of the proposed mechanisms is not supported by the laboratory or field observations. There is no evidence of effects through an (anti)thyroid hormone mediated mechanism and little evidence for a mechanism mediated through (anti)androgens or (anti)estrogens. The criterion of recovery cannot be specifically addressed since no consistent and robust responses have been identified from which to measure recovery.

Application of Koch's postulates (13) as modified for assessment of substances instead of infections leads to similar conclusions. That is, 1) That exposure to atrazine is always associated with effects in amphibians is not satisfied as there are no consistent or robust effects observed in the field. 2) That the substance must be isolated from the environment of the affected organism is not satisfied because of the background incidence of many of these effects and their occurrence prior to the introduction and use of atrazine. 3) That the substance causes the responses experimentally is not fully satisfied by the inconsistent laboratory and field observations in controlled exposure situations.

Understanding of mechanisms of action of endocrine disruptors reduces uncertainties and may offer useful biomarkers for further study. These biomarkers can be useful in risk assessment and for confirming responses under field conditions. Thusfar, laboratory responses are inconsistent and, where some of these potential biomarkers have been measured in the field, no trends have been apparent. The lines of evidence thus does not support a causal link between atrazine exposures and possible endocrine-mediated effects in amphibians. The lack of consistent biomarkers makes it impossible to conduct a risk assessment as no hazard has been identified.

A number of uncertainties have been identified. These have been discussed in more detail and expanded upon by the SAP (5). These relate to laboratory assays and method studies on the influence of husbandry and source of animals on responses to atrazine as well as positive and negative controls and the effects of seasonal and circadian cycling on hormonal and other responses in frogs. Several uncertainties exist with respect to knowledge of basic biology and stages of development of amphibians. Efforts should be made to standardize tests so that gonadal anomalies such as gross morphology and the presence of testicular oocytes are assessed at similar stages of gonadal development. None of the laboratory studies performed to date have controlled for stage of gonadal development at sampling. Samples were collected at the same point in larval development (at complete metamorphosis), but different species and different populations of frogs vary in the timing of gonadal development relative to somatic development. Some of the anomalies observed may be "normal" and their type and incidence may change with stage of gonadal development and the time of observation.

A significant uncertainty exists in our basic understanding of reproductive and endocrine responses in frogs under natural conditions that are free of anthropogenic contaminants as well as the relevance of any effects caused by known endocrine-active substances on reproductive success of frogs. Uncertainty also exists with regard to temporal variability of exposure of amphibians to atrazine under field conditions and, although the bioconcentration of atrazine is known to be small, uptake and depuration kinetics as well as tissue and organ distribution of atrazine in larval and adult frogs has not been studied.

If and when consistent and robust responses to atrazine are identified in frogs, the mechanisms by which these effects are mediated should be elucidated. This will better allow the application of biomarkers to assess the significance of responses in the field situations and increase the precision and accuracy of extrapolation of effects to other amphibians and assessment of their relevance at the level of the population.

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