Data Evaluation Report on Hermaphroditic, Demasculinized Frogs After Exposure to the Herbicide Atrazine at Low Ecologically Relevant Doses

EPA MRID Number: None

Data Requirement::

EPA DP Barcode None

EPA MRID Not Assigned
EPA Guideline Open Literature

Test material: **Purity:** not reported
Common name Atrazine
Chemical name: IUPAC
CAS name 6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine
CAS No. 1912-24-9
Synonyms
EPA PC Code: 80803

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CITATION: Hayes, T.B., A. Collins, M. Lee, M. Mendoza, N. Noriega, A. A. Stuart, A. Vonk. 2002. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. *Proceedings of the National Academy of Science*, 99 (8): 5476 - 5480.

EXECUTIVE SUMMARY:

The objective of this study was to test the hypothesis that atrazine, at ecologically relevant low doses, may interfere with metamorphosis and sex differentiation via endocrine-disrupting mechanisms. African clawed frogs (*Xenopus laevis*) were exposed to atrazine concentrations ranging from 0.01 to 200 µg/L from 96-hr post-hatch through complete tail resorption (Nieuwkoop-Faber Stage 66). At the end of the exposure period, animal growth (length and weight), time to metamorphosis, gonadal abnormalities and size (cross-sectional diameter) of the larynx dilator muscle were recorded. Exposure to atrazine concentrations ≥ 0.1 µg/L resulted in gonadal abnormalities in 16 - 20% of the animals at all doses tested except for 0.1 ug/L. Abnormalities included multiple gonads or hermaphrodites (multiple testes and ovaries in the same animal). These abnormalities were not observed in the controls. In general, males typically exhibited larger diameter laryngeal muscle diameters than females. In this study, though, atrazine at concentrations as low as 1 µg/L (1 ppb), significantly decrease the proportion of males that were at or above the mean laryngeal size in the control males. According to the authors, these results suggested a threshold effect at ≥ 1 µg/L in which 80% of the exposed males exhibited larynx sizes below the average. The authors hypothesized that the co-occurrence of oocytes and testicular tissue (hermaphroditism) and the decreased male larynx muscle size (demasculinizing) were consistent with increased endogenous estrogen concentrations and that one possible mechanism would be through increased aromatase activity. In support of their hypothesis, the researchers demonstrated that adult male *Xenopus* exposed to atrazine at 25 µg/L showed significantly reduced plasma testosterone.

This study states that the results have been repeatedly verified, but additional data have not yet been provided in the open literature or submitted to EPA. It would be helpful if supporting studies were available to verify the results of this specific study. In addition, more information on the measurement endpoints, effects in the field, percentage of males and females in the study, numbers of frogs exhibiting abnormalities, and sensitivity of *Xenopus laevis* compared to North American frogs would be useful. Also, the lack of a dose- response relative to the phenomenon of hermaphroditism makes it difficult to interpret cause-effect relationships. Although there appears to be a dose-dependent reduction in laryngeal muscle area relative to atrazine concentrations, the reliance on the proportion of animals falling below the average is an indirect measure of the effect. A direct comparison of measured laryngeal muscle area between controls and exposed animals would further clarify the magnitude and extent of any developmental effects. Information on how diminished dilator muscle area or the gonadal deformities might relate to the reproductive success, growth or survival of the affected species in the environment would also provide further insights on the ecological relevancy of effects.

This laboratory study was not conducted for the purposes of reregistration and many of the details typically monitored in a study conducted under Good Laboratory Practice standards were not recorded.

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: Nonguideline study
COMPLIANCE: Not conducted under full Good Laboratory Practices

A. MATERIALS:

1. Test Material Atrazine

Description: Not reported

Lot No./Batch No. : Not reported

Purity: Not reported
Stability of compound under test conditions: Not reported

Storage conditions of test chemicals: Not reported

2. Test organism:

Species: African clawed frog (*Xenopus laevis*)
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Age at test initiation Experiment 1 and 2: 96-hr larvae; Experiment 3: adults (age not reported)
Weight at study initiation: (mean and range) not reported
Length at study initiation: (mean and range) not reported

Source: Eggs and sperm obtained from three adults (3 males and 3 females) from long-term captive colony maintained at the University of California, Berkeley, for Experiment 1 and from adults obtained from Nasco (Fort Atkinson, WI) for Experiment 2. Adults from long-term captive colony maintained at UC Berkeley for Experiment 3.

B. STUDY DESIGN:

Objective: To test the hypothesis that atrazine may interfere with metamorphosis and sex differentiation at ecologically relevant low doses via endocrine-disrupting mechanisms.

1. Experimental Conditions

a) **Range-finding Study**: Not reported
b) **Definitive Study**

Table 1 . Experimental Parameters

Parameter	Details
Acclimation: period: Conditions: (same as test or not) Feeding: Health: (any mortality observed)	Experiments 1 and 2: Embryos hatched in 0.3x modified mammalian Ringer's solution; at 96-hrs post-hatch, larvae transferred to aerated 10% Holtfreter's solution. Fed solution of ground Purina rabbit chow daily. No mortality reported. Experiment 3: Adults acclimated in 10% Holtfreter's solution for 5 days, unaerated; animals fed Purina trout chow daily; water renewed every 72 hours.
Duration of the test	Experiment 1 and 2: Hatching (NF Stage 48) until complete tail resorption (NF Sate 66) Experiment 3: Adults (age not reported) exposed for 46 days.
Test condition static/flow-through Type of dilution system- for flow-through method. Renewal rate for static renewal	 static renewal NA complete exposure solution change every 72 hours
Aeration, if any	Experiments 1 and 2 with larvae employed aeration; Experiment 3 with adults did not employ aeration
Test vessel Material: (glass/stainless steel) Size: Fill volume:	 plastic cages (personal communication, T. Hayes, 2002) 4 L
Source of dilution water quality:	Deionized, distilled water (personal communication, T. Hayes, 2002)

Parameter	Details
Water parameters: Hardness pH Dissolved oxygen Total Organic carbon Particulate Matter Ammonia Nitrite Metals Pesticides Chlorine Temperature Salinity Intervals of water quality measurement	 not reported not reported not reported not reported not reported not reported not reported not reported not reported not reported 22ºC not reported not reported
Number of replicates/groups: negative control: 0.004% ethanol treated ones:	Experiment 1 and 2: 3 replicates
Number of organisms per replicate /group:	Experiment 1 and 2: 30 larvae per replicate
Biomass loading rate	Experiment 1 and 2: 30 larvae/4 L
Test concentrations: nominal:	 Experiment 1: 0.01, 0.1, 1.0, 10.0, and 25 µg/L Experiment 2: 0.1, 0.4, 0.8, 1.0, 25, and 200 µg/L Experiment 3: 25 µg/L
Solvent (type, percentage, if used)	Experiments 1 and 2: 0.004% ethanol in 10% Holtfreter's solution. Experiment 3: 10% Holtfreter's solution Holtfreter's medium:
Lighting	12 hrs light, 12 hrs dark

Parameter	Details
Feeding	Experiments 1 and 2: Purina rabbit chow *ad libitum* (personal communication, T. Hayes, 2002) Experiment 3: Purina trout chow daily
Recovery of chemical Level of Quantitation Level of Detection	Concentrations confirmed by PTRL West, Richmond, Ca and Iowa Hygenic Laboratory, Univ. of Iowa, Iowa City, IO). Not reported. Not reported.
Positive control {if used, indicate the chemical and concentrations}	not reported?
Other parameters, if any	

2. Observations:

Table 2: Observations

Criteria	Details
Parameters measured including the sublethal effects/toxicity symptoms	Experiment 1 and 2: mortality, time to metamorphosis, weight and length at metamorphosis, sex based on gross morphology (all animals) and histology (10 animals/tank), laryngeal size based on largest cross-sectional area of *M. dilator larngis* (transverse histology from 10 males and 10 females from each replicate).
Observation intervals	Not reported
Were raw data included?	No
Other observations, if any	Experiment 3: plasma testosterone in blood collected by decapitation using radioimmuno-assay. Testosterone antisera obtained from Endocrine Sciences (Calabasas, CA).

II. RESULTS and DISCUSSION:

Statistical analysis was conducted using SYSTAT® (SPSS, Chicago); sex ratios and mortality were determined using a G test with Wilkin's g-adjustment ; and time to metamorphosis and growth (length and weight at metamorphosis) were analyzed using ANOVA. Animals were scored based on the size of their larynx in relationship to the mean laryngeal size for controls. After scoring, a G test was conducted to determine the

affect of atrazine treatment followed by Kendall's ranked coefficient to determine whether the percentage of below-average animals varied with the dose of atrazine.

Experiments 1 and 2:

At the doses tested, atrazine exposure had no significant ($P > 0.05$) effect on mortality, time to metamorphosis, length, or weight at metamorphosis. Males and females were sexually differentiated at metamorphosis based on gonadal morphology and histology. All atrazine doses tested except 0.01 µg/L produced gonadal abnormalities in 16 - 20% of the animals. Abnormalities included multiple gonads or hermaphrodites (multiple testes and ovaries. These abnormalities were not observed in the controls. The paper contains histological sections through the gonads of an animal exposed to 1 µg/L atrazine that was classified as a hermaphrodite. The sections in the histological slides showed a transition from bilateral testicular tissue to ovarian tissue, moving caudally along the gonads.

Control males had larger larynges than females at metamorphosis, but males exposed to ≥ 1 µg/L had reduced larynges in both Experiments 1 and 2. In the caption of Figure 3, the authors noted that atrazine ≥ 1µg/L reduced laryngeal size in males but did not affect females. Larynges of animals in Experiment 2 were larger than in Experiment 1, suggesting a population difference in absolute size of the larynges. Similar to Experiment 1 though, exposure to atrazine at ≥ 1 µg/L significantly reduced laryngeal size in males. Log-transformed percent of animals above control mean laryngeal size indicated that atrazine exposure ≥ 1 µg/L significantly decreased the proportion of males that were at or above the mean control males (G test, $p < 0.05$). It also suggested a threshold effect at ≥ 1 µg/L in which 80% of the exposed males were below average. The percentage of below-average animals suggested a dose effect with increasing atrazine doses (Kendal rank coefficient; $p < 0.01$).

Experiment 3:

An extrapolatation of Figure 4 of the paper indicated that plasma testosterone levels in control males averaged 4 ng/mL. Plasma testosterone in atrazine-treated males and control females averaged roughly 0.1 and 0.2 ng/mL, respectively, and were significantly lower (ANOVA, $P < 0.05$) than control males.

The authors hypothesized that the 16 - 20% incidence of multiple gonads/hermaphroditism and smaller than average larynxes in males were caused by atrazine- disrupting steroidogenesis. They also concluded that sexually mature males suffered a 10-fold decrease in plasma testosterone. According to the authors, the current findings suggested that atrazine inhibits testosterone and induces estrogen synthesis by an induction of aromatase and consequent transformation of androgens (testosterone) to estrogen. They hypothesized that the loss of masculine features, *i.e.*, decreased laryngeal muscle size, may be due to decreased androgens, and the induction of ovaries may result from increased estrogen synthesis and secretion. According to the authors, the current data raised concerns regarding the effects of atrazine on amphibians: exposure to 0.1 µg/L produced hermaphrodites and exposure to 1 µg/L resulted in a reduction in laryngeal size.

F. REVIEWER'S COMMENTS:

This nonguideline, published study was useful in identifying a potential hazard to amphibians and presented information on measurement endpoints, such as gonadal deformities and laryngeal muscle diameter. The study, however, did not show a clear dose response that demonstrates a causal relationship between atrazine exposure

and developmental effects. While the paper states that the study results have been repeated in 51 trials, the Agency did not receive these replicated data. Results of these repeated trials would help the Agency verify the results found in this study.

Because of the variability and nature of the measurement endpoints, the statistical analyses in this study relied heavily on nonparametric methods. Access to the raw data supporting this study would help the Agency to verify the study's conclusions regarding mortality, time to metamorphosis, growth, gonadal development, laryngeal diameter (size) or plasma testosterone levels. Although the range of gonadal abnormalities was provided, the prevalence of gonadal effects is difficult to interpret. EPA needs more specific data on the range of gonadal abnormalities, the incidence for each dose tested, the relative percentages of males and females, the numbers of animals involved in the 16-20% abnormality incidence, and the impact of gonadal abnormalities on reproductive success in the environment. Without this information, it is difficult to interpret the error bars in Figure 4 of the paper and to establish the ecological relevancy of the data. EPA also needs clarifying information concerning the sample sizes involved in the analyses, the number of doses samples and the method of blood collection (i.e., decapitation could contaminate blood samples).

In its evaluation of this study, the Agency had questions concerning the use of ethanol as a co-solvent. Since atrazine concentrations used in these studies were less than the 30 mg/L water solubility limit, a co-solvent should not be necessary. Previous studies conducted by the authors (personal communication: Tyrone Hayes 2002) used dihydrotestosterone and 17-β estradiol as positive controls and required ethanol as a co-solvent. The current study, though, did not utilize these steroids and a co-solvent should not have been necessary. The Agency also has questions concerning the conditions of the study. The study states that 30 organisms with an estimated maximal weight of 1.5 g were held in 4 liters of test solution. These conditions would result in a loading rate of 11.3 g/L, which is 27 times the recommended loading rate for static renewal toxicity tests.

While the paper discusses that the study results have been repeated in 51 trials, none of the data are presented; therefore, it is not possible to gauge the researchers' success at replicating the current results.

Although only the range of gonadal abnormalities (16 - 20%) is provided, no information is presented on what the incidence was for each dose tested. The data suggest that there was a threshold effect level at 0.1 µg/L but that the effect did not increase with increasing dose.

While the authors suggest that up-regulation of aromatase activity is a plausible rationale for the observed effects and that reduced testosterone levels in atrazine treated adult male *Xenopus* further support this hypothesis, no direct measure of aromatase activity. Further testing with additional dose concentrations is a needed to establish a causal relationship between atrazine exposure and gonadal effects in amphibians. is provided nor can a dose response be established. Additionally, if aromatase activity were increased and there was subsequent decline in testosterone, estrogen levels would likely increase; however, there are no data available on the estrogen concentrations. Since a single concentration (25 µg/L) of atrazine was used to test the hypothesis concerning aromatase, it isn't possible to establish a dose response.

The ecological relevancy of this data is not clear. No information is available on whether similar gonadal abnormalities impact reproductive success in the environment.

The lab study utilizes relatively sub-standard conditions in that 30 organisms with an estimated maximal weight of 1.5 g were held in 4 liters of test solution. This results in a loading rate of 11.3 g/L, which is 27 times the recommended loading rate for static renewal toxicity tests.

As reported, the prevalence of gonadal effects is difficult to interpret. The results section apparently combines the results of both larval studies and fails to provide dose response information and any indication of variance. Since the presentation of dose response data is a fundamental standard for toxicological studies, this omission is curious, and suggests that the report is incomplete for some reason. Also, what is the relative contribution of the two major abnormality types to the overall prevalence of gonadal abnormalities? There are no data presented on the relative percentages of males and females in the study, nor any other data indicating numbers on which the apparent 16-20% abnormality incidence was calculated.

Results from the adult atrazine exposure do not indicate the sample sizes involved in the analysis. In fact, it does not indicate the basic information on how many males were exposed or held as controls. Since this information is not available, one cannot make sense of the error bars (what are they representing?) in Figure 4, nor whether or not this is a credible analysis. In the RIA section of the Methods & Materials, the report states that plasma was sampled at 3 doses. However, the Adult Treatments section indicates that only one dose was utilized. The method of blood collection, decapitation, seems rather crude and likely to contaminate blood with other bodily fluids, which could confound the analysis. A more acceptable approach, for example, would be a cardiac puncture.

H. REFERENCES:

Nieuwkoop, P. D. and J. Faber. 1994. Normal table of *Xenopus laevis* (Daudin). North-Holland Publishing Company, Amsterdam.