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Public Information and Records Integrity Branch (PIRIB)
Information Resources and Services Division (7502C)
Office of Pesticide Programs
U.S. Environmental Protection Agency
Room 119
Crystal Mail #2
1921 Jefferson Davis Highway
Arlington, VA 22202

AUG 16 2002

Dear Sir/Madam:

Subject: Docket Control No. OPP-34237C –Submission of Additional Comments on Notice Published in the Federal Register May 6, 2002 Atrazine: Availability of Revised Risk Assessments and Related Documents

Enclosed with this letter is a document from the Atrazine Endocrine Ecological Risk Assessment Panel of Ecorisk, Inc, sponsored by Syngenta. This document is a critique of a research paper by Dr. Tyronne Hayes, et al, 2002, "Hermaphroditic, Demasculinized Frogs After Exposure to the Herbicide Atrazine at Low Ecologically-relevant Doses" *(Proceedings of the National Academy of Sciences* 99:5476-5480).

Syngenta wishes to have the enclosed document included in the subject docket, so that the serious scientific flaws in the research that was the basis for the publication are documented.

Syngenta looks forward to future discussions on the chlorotriazine herbicides. Please telephone Janis McFarland at (336) 632-2354 or myself at (336) 632-7207 if there are any questions concerning this submission.

Sincerely,

Thomas J. Parshley

Thomas J. Parshley
Senior Regulatory Product Manager
Regulatory Affairs

CC: Ms. Kimberly Lowe

Attachment

Critique

Of:

Hayes, et al., 2002. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically-relevant doses. *Proc. Nat. Acad. Sci.* 99:5476-5480.

Prepared by:

The Atrazine Endocrine Ecological Risk Assessment Panel

ECORISK, Inc.

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Critique

Of:

Hayes, et al., 2002. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically-relevant doses. *Proc. Nat. Acad. Sci.* 99:5476-5480.

General Comments

The paper fails to present data to support the stated results indicating a response for *Xenopus laevis* exposed to environmentally relevant concentrations of atrazine. This is a major deficiency since the study authors make very bold statements on the association of environmentally relevant concentrations of atrazine and global amphibian declines, but without presenting the actual data for evaluation of scientific validity.

Very little data and insufficient details of statistical analyses are given in the paper. The work reported in the paper was also apparently carried out with little regard for assessment of causality. Hence, the paper grossly overstates the potential for effects on frogs and changes in populations.

Specific Comments

Title:

The title is very misleading in that the authors do not differentiate in the text between either hermaphroditic or mixed sex individuals nor do they discuss how they arrived at description of demasculinized as opposed to feminized frogs. Actual doses were not measured in the tadpoles and the term "dose" is used incorrectly in the title and throughout the paper.

Abstract:

There are no data presented in the abstract to support or refute the hypothesis that atrazine induces aromatase or that the results from studies in *Xenopus* provide an appropriate model for wild amphibians in general.

Introduction:

There are several very comprehensive compilations of reviews on endocrine disruptor contaminants that have been omitted from the introductory references. Additionally, the suggestion that the "causes" of endocrine disruption have been identified is a naive statement and indicates a simplistic approach to ecotoxicology.

The observation of effects on primary and secondary sex differentiation is only discussed in a single sentence and a Figure legend. A more robust analysis of the types of effects observed with their frequencies, etc. must be presented in the publication to determine the scientific validity of the study.

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Materials and Methods:

The methods section lacked sufficient detail to allow the study to be repeated in another laboratory. Specific instances of missing information are listed below.

It is not clear if the adults for Experiment 1, a "long-term captive colony" were an inbred strain of animals and how similar the results obtained with these adults were to the strain of animals used in Experiment 2.

Culture of the embryos for the first four days was not well described.

The feeding rates for the tadpole treatments were not well described.

At 4 days of development, the embryonic sac is still attached to the larvae, yet the method used to determine that the yolk sacs were not affected in netting and transfer of larvae to the exposure tanks is not discussed.

"Nieuwkoop" is spelled incorrectly.

Treatment of tadpoles:

The paper refers to dosing of tadpoles, however, actual doses were not determined. Tadpoles were exposed via the matrix (water). Methods used to verify exposure concentrations were not described nor were any data confirming exposure provided. Additional experimental details regarding the exposures including at least the mean concentrations and variability measurements are necessary to properly interpret the results. The reason for dissolving atrazine in ethanol is not stated. The effect of exposure to ethanol is not stated.

Measures of larynx size:

It is unclear whether the methods for determining the size of the larynx is standard method or was arbitrarily chosen. If it is a standard method there should be a citation included. Measurement of all sections of the entire laryngeal muscle would have provided the information to determine the volume of the muscle and the largest section by much less subjective methodology. A more rigorous method for determining the maximum width of the laryngeal adductor muscle should have been used to measure such subtle effects.

Adult Treatments:

Blood collection and plasma preparation methods are poorly described. Sacrifice methodology suggests collection from open vessels but without any indication on procedures used for clotting. Sample collection timing and steps taken to eliminate the possible confounding influence of daily cycles in plasma testosterone content are not described. Also, it is unclear whether blood was collected at the same time of day for each treatment to minimize any impact of a diurnal rhythm in testosterone secretion. It is not clear whether sample collections for Experiment 1

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were timed and whether these were repeated for Experiment 2.

Exposure units should be given as µg/ml, or mg/L or µM/L, rather than "ppm" or ppb.

The analytical methodology and timing regimes used to determine the actual exposure concentration should be described.

It is unclear why atrazine was dissolved in ethanol for this study. Atrazine is soluble in water up to about 30 mg/L, therefore the rationale for use of an ethanol vehicle should be clearly stated. While control tanks were treated with 0.004% ethanol, the ethanol concentration in the treatment groups is not provided for review.

Although differences in water quality can influence the health and response of *X. laevis* under experimental conditions, water quality parameters (dissolved oxygen, conductivity, pH, etc.) are not described or discussed. It is stated that the frogs were exposed in "10% Holtfretter's solution", however since *Xenopus* larval development is sensitive to ionic composition of the medium, much more complete specification of the diluent is required to evaluate any hypothesized effects.

RIA:

The rationale for the repeat of the hormone analyses is not given. It is unclear if the samples analyzed in duplicate or triplicate and what values were used and why.

Intra- and inter-assay variability values are both outside the expected normal range of variability of approximately 10-fold greater. There should be a discussion on possible explanations for these unexpected values. Additionally, it is unclear from the methods how the inter-assay variability was determined.

The largest cross-sectional area through the muscle was initially found to occur one-third (from the rostral or caudal end is not clearly stated) of the way through the larynx using animals from Experiment 1. However, Experiment 2 frogs had dilator muscles that were much larger than those in Experiment 1 frogs. Validation that the largest section of the muscle was also evaluated in animals from Experiment 2 must be described. Lacking that validation, comparison treatments of the extremely different muscle sizes between these two populations should be described in detail to determine if these are valid assessments.

Clearly, subsets of animals were chosen for larynx analysis, as 10 males and 10 females were picked from each replicate and the total replicate size was 30 for each treatment. However, there is no mention of how subsets were selected for laryngeal analysis.

Data Analyses:

Insufficient information was given to evaluate the magnitude of any tank effects and how potential tank effects were incorporated into the statistical analyses. For example, if the tank

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effects were incorrectly treated as fixed rather than random effects, then false positive error rates would increase.

A well-defined study design including procedures for random sampling of individual frogs must be provided in order to evaluate the possibility for selection bias. Information on the association among snout-vent length, tank, and time to metamorphosis for individual frogs is not given. This is an important omission since the size of the laryngeal dilator muscle is known to co-vary with these parameters. Therefore, it is not clear whether the observation of effects on the dilatory muscle could be attributed to atrazine, or rather were due to simply to the size of the frogs. This information is necessary for interpretation of the study results and to evaluate biological plausibility of the study authors' conclusions.

The reviewers have concerns about the appropriateness of the statistical tests reported in the manuscript. Specifically, were the sample sizes sufficiently large to justify the assessment of statistical significance using the asymptotic properties of the G-test? Perhaps more importantly, did the presence of tank effects invalidate the assumption of independence underlying the G-test? There were not sufficient details on the ANOVA tests to determine their appropriateness. Furthermore, non-random sampling and selection bias would invalidate any standard test. There are alternatives to the G-test for evaluating the number of animals with laryngeal size less than the mean size in the controls. If these alternative statistical analyses were conducted and the same results were not found then a discussion on interpretation of the discrepancies is needed to reconcile the results of the various tests.

Data on sample sizes, means, standard errors, etc. is either non-existent or limited. Without such information, it is impossible for the reviewers to evaluate the statistical results provided.

It would be more appropriate to consider the counts at a specified biologically significant distance below the mean rather than just all counts below the mean. For example, this could be done by evaluating the counts of the number of frogs that had laryngeal size smaller than the mean minus one standard deviation in the vehicle control group, if it was determined that one standard deviation were a biologically significant reduction. The study authors do not discuss the biological significance of different magnitudes of reduction in larynx size either for individual frogs or in populations of frogs. Furthermore, no raw data are given on the magnitudes of any observed decreases or their frequencies. Hence, it is impossible to evaluate the biological significance of the experimental outcomes.

There was no adjustment to the G-test (i.e., the likelihood-ratio test for contingency tables) called "Wilkins's g-adjustment". The authors apparently mean the Williams' scale-factor adjustment (Williams, 1976). The authors apparently mean Kendall's coefficient of rank correlation, τ", or Kendall's rank correlation coefficient and not Kendall's ranked coefficient (e.g. Conover, 1971; Sokal and Rohlf, 1995).

The manuscript states: "Time to metamorphosis and size (length and weight) at metamorphosis were analyzed by using ANOVA with treatment, tank, and sex (sex nested within tank and tank nested within treatment) as independent variables." (This may also be true of some analyses of laryngeal size.) Although this implies that the study authors may have considered the possibility

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of tank effects on the continuous data, the description is insufficient to determine whether tanks are treated as a random or fixed effect. Tanks should be treated as a random effect for contribution to the within-treatment error. If tank is mistakenly treated as a fixed effect in the ANOVA model, then tank-to-tank variability is mistakenly deleted and a false significance is much more likely. The same applies for the standard errors in Figure 3; if the tanks were not treated as random effects, then the standard errors are grossly in error.

Results

Omission of data on mortality and pretreatment hatching among the treatments makes it impossible to determine embryo health in the experiments.

The results state that "up to 20% of animals" (16-20%) had multiple gonads, but the method of determination is not well described. If the results are based on serial sections confirming multiple gonads, the steps taken to ensure that multiple gonads observed were not an artifact of fixation and sectioning must be described. The term "multiple gonads" is not clear. Does this mean that the animals had supernumary gonads? A Table or Figure giving the results by treatment should have been given. It is impossible for the reader to determine if there was a concentration-dependent effect of atrazine on the morphology of the gonads or whether these results were for males or females or both. It is also not clear if as this was the rate for all treatments or just one treatment.

Since no data are provided, it is impossible to determine if there was a concentration-response or if there was a threshold for this effect. Furthermore, no statistics are given for this relationship. It is impossible to know if the response was the same in the two experiments or whether there was a sex-dependent effect. Much more information would be necessary to be able to interpret the significance and severity of the reported observed effects. Answers to the following questions would be necessary to interpret the results: 1) What is the threshold for observing the gonadal abnormalities? 2) Is there a concentration-dependent response? 3) Were these effects observed in all atrazine concentrations or just the greatest? 4) Were the effects repeated between the two experiments? 5) Were these effects observed in treatments other than atrazine? 6) Were the effects of the same range in all atrazine treatments?

Since animals from Experiment 2 were not from the laboratory stock, the comments about 10,000 control observations are not relevant. The study authors should state whether these effects have been previously observed in the Nasco animals and the measures taken to ensure separation of the two stock populations?

Interpretation of the results on the larynx is highly questionable. It is unclear how the measurements of size were standardized. An explanation of the reported size difference of the affected males in Experiment 2 and the control males in Experiment 1 is needed for interpretation of strain-related effects, and for critical consideration of the relevancy of inbred population study data to that of frogs in the wild. The dissimilarity in apparent size of the larynx between the two laboratory strains alone prompts the question of significance of such variability. The authors state that the decrease in testosterone in adult frogs is the likely explanation of the decrease in larynx size (demasculinization) and that this is likely due to a change in the activity of testosterone. The discussion of the mechanism of action of a decrease in testosterone in frogs,

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exposed as adults however is insufficient to verify causality. I
testosterone were observed in adult frogs after the larynx size had be
speculate that this constitutes a disruption of steroidogenesis, but in
support this conjecture. The lower testosterone concentrations in a
just as likely be explained by effects on the affinity and/or numbe
proteins or effects on the hypothalamous and subsequent neuroendoc
of GnRH.

It was concluded that the data suggest that atrazine caused a
concentrations in adult male frogs at 25 µg/L. To determine if thes
would be necessary to know the number of frogs used in each treatment. Twenty-five µg/L is a much greater concentration than that claimed to cause effects on primary and secondary sex differentiation. The same concentrations must be tested to eliminate the possibility that different mechanisms of action resulted in different endpoints. Also, based on the study authors' hypothesis that atrazine may act by increasing aromatase activity, it is unclear why neither testosterone nor estradiol levels were measured in females treated with atrazine.

The study authors speculate that the reported effects may be causative factors in global amphibian declines, however no data is provided in the manuscript to support such a statement. There is no information provided on how the reported effects could affect populations of frogs. This is particularly speculative since, in an ecological sense, frogs are "r-selected" organisms, and predators and compensatory mortality control populations, not by fecundity. The reviewers would suggest that the discussion should be limited to the actual data reported.

Figure 3:

Since the data portrayed in Figure 3 are the primary information on which conclusions were based, the numerical data and detailed statistical analyses underlying this Figure should be provided.

Figure 3A and 3B show laryngeal area means with claims in the legend of significant effects at various concentrations, however this is confusing since the *Statistical Analysis* Section under *Materials and Methods* do not describe an ANOVA analysis of the laryngeal data. Rather there is only a vague reference to "correlational analyses". If there were ANOVA-based analyses of the laryngeal data, statistical details concerning an ANOVA model for the laryngeal data, including numerical results, details about analyses of differences among treatments, and multiple comparison test results differences between treatments and controls must be provided for confirmation of scientific validity.

In Figure 3D the observation for 0.01 ppb should be disclosed, along with the numerical count data so that concentrations were included in Kendall's rank correlation coefficient and the associated p-value can be determined.

The concentration axis should be expanded to give better resolution of the low exposures in the response region on which the conclusions are based. As presented, it is impossible to determine

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if there was a threshold for effect. Also, because the Y-axis (effect) is not offset, it is not possible to discern what the confidence intervals are in 3A and 3B.

The concentration-response relationship for these effects is very unusual and should be discussed in the manuscript. To go from no effect to roughly a 10% decrease and then stay at that level of effect is unusual. The effect seems to be one of a threshold.

It is noted in the legend to Figure 3 that population differences were apparent with respect to laryngeal size (both males and females). As stated earlier, other differences between the two populations in Experiments 1 and 2 may explain the supposed results.

Data for females should be included in Figures 3A and 3B, to facilitate assessment of statistical significance. If similar effects were observed in males and females, the results are inconsistent with the working hypothesis that atrazine is disrupting steroidogenesis through induction of aromatase, which subsequently causes a decrease in androgens.

Based on the paucity of data presented, the lack of statistical rigor, the lack of conclusive concentration-response relationships, and the differences between experiments, the scientific veracity of the conclusions that there were significant effects on the size of the laryngeal muscle cannot be determined.

Figure 4:

Nanogram per milliliter should be expressed as ng/ml.

Finally, there should have been consideration and discussion of other potential mechanisms to explain the observed effects. Again, the sample size (N values) should be included as well as a discussion on why estradiol measurements were omitted. This is a fatal flaw in establishing a potential explanation of the observed effects.

Discussion:

The authors should provide the calculations upon which they base their statement that the study was replicated 51 times. Without such calculations it cannot be determined whether this number represents the total number of replicates of all treatments, the number of treatments and all of their exposure concentrations or just the highest concentrations used. Additionally if there are indeed 51 replications of this study, results of the other 49 should be included in the manuscript.

The authors indicate that androgens increase laryngeal size however a discussion on the effects DHT on laryngeal size of *X. laevis* should be provided. This is also true for exposure to estradiol. There is a discussion of the effects of estradiol exposure in *X. laevis*, but without presentation of data to support this inference.

The study authors should provide data to support their suggestion that atrazine inhibits testosterone and induces estrogen. First, it is unclear what is meant by "inhibits" or "induces". The reviewers assume that this means the effects are decreases in concentrations of testosterone and increases in concentrations of estradiol. This conclusion is not supported by the data presented. Additionally, concentrations of estradiol in juveniles and adults must be reported if

the suggestion that gonadal effects were due to an increase in estradiol is to be scientifically substantiated.

Without inclusion of any direct measures of endocrine disruption nor any rationale for extrapolation from frogs to humans, the authors statement that the study results indicate endocrine disruption has significant implications for environmental and public health cannot be scientifically demonstrated. The authors conclude that the concentrations of atrazine that were effective at causing the observed effects on the gonads were environmentally relevant, yet the accuracy of this statement cannot be evaluated since data on the concentration-response relationship has not been provided. Furthermore, this lack of data precluded the ability of readers to assess the degree of severity of the response. The authors should provide the concentration-response relationships and associated statistics for scientific verification of the probability and severity of the reported occurrences prior to any conjecture that environmentally relevant concentrations of atrazine are likely to produce these effects, and even more dubious that atrazine is a likely contributor to global declines in amphibian populations.

The authors' conclusion that the concentration of atrazine that will cause laryngeal muscle size responses in amphibians is 600-fold less than that to significantly up-regulate aromatase in vitro and 30,000,000 times less than that required to cause reproductive effects in rats is made based on scientifically inappropriate comparisons and therefore is not based in fact. A basis upon which to make these comparisons is not provided in the study, however, calculations to authenticate these suppositions indicate that the authors have made evaluations based on "parts per million" (ppm), without regard to the actual units used in the comparison studies. Amphibian studies conducted via a water-born exposure, express exposure concentrations as mg/L of atrazine. Like apples to oranges, these results are then compared to concentrations in rodent studies in which the exposure is through diet and exposure units are expressed as mg atrazine/kg of diet or as mg atrazine/kg body weight/day.

The finding that effects can occur at concentrations 10,000 times less than has been previously reported as no effect levels for atrazine is not supported with concentration-response information for the larynx or gonads. Again, there is no concentration-response data provided to support the statement that the concentrations of atrazine that elicited responses in the current study were 30,000 times less than those reported by Allran and Karasov (2001).

Relevance of recommended atrazine application rates to the study results to is incorrectly represented in the manuscript. The application rates cited are the atrazine concentrations after mixing in the spray tank, not the concentration occurring in the environment after application. Pathways of exposure must be appropriately characterized in order to compare concentrations to those in tank mixes, groundwater, or precipitation. Furthermore, constant exposures under laboratory conditions are unlikely to represent the shorter duration exposures experiences under field conditions.

The authors of the paper inappropriately extrapolate results on morphological change findings to population-level effects. The concentration-response and severity of effects must be considered, especially for "r-selected" species of anurans, where large numbers of eggs are laid and many of the juveniles die in a density-dependent or compensatory manner. It is completely speculative to

extrapolate from very subtle histological effects to population-level effects. It is highly unlikely that such effects, especially the effects reported for the larynx, would result in any population-level effects. The effects, reported in this study, should represent indicators of potential effect concentrations that need to be investigated to determine if they translate into population-level effects.

The authors suggest that there is a high probability that amphibians could be exposed to 0.1 or 1.0 ug atrazine/L and that this is linked to the potential for these species to decline or go extinct. There is no evidence given to support this conclusion.

Summary:

In general, this is a very poorly constructed manuscript. The reviewers are surprised by the errors and incompleteness of the manuscript that should have been noted by a critical review of the manuscript prior to publication. This indicates that the manuscript was either not reviewed or reviewed by individuals inexperienced in conducting toxicological studies. Conclusions are reached based on very limited data. Furthermore, actual data to support the conclusions are not included in the paper. Finally, unsupported wide-sweeping conclusions are made relative to frog populations. These statements are clearly speculation that goes far beyond the data presented in this paper. In particular, there is no attempt to relate the reported phenomena to ecologically relevant endpoints. It is impossible from the information presented to determine if any of the reported effects have any potential population-level effects. It is impossible to know from the results provided if the responses would adversely affect mating, fertility, fecundity or overall productivity. Since native North American species of frogs are "r-selected" and there is a great deal of compensatory mortality, it is unlikely that the observed effects attributed to atrazine would be likely to be causing adverse effects in wild populations of frogs. Since neither quantitative measures of the severity or incidence of gonadal effects, nor concentration-response relationships for the reported effects are provided the data quality and scientific reliability of this research cannot be determined.

References

Allran JW, Karasov WH. 2001. Effects of atrazine on embryos, larvae, and adults of anuran amphibians. *Environmental Toxicology and Chemistry* 20:769-775.

Conover, W. J. 1971. Practical Nonparametric Statistics. John Wiley & Sons: New York.

Sokal Robert R. and Rohlf, F. James 1995. Biometry: The principles and practice of statistics in biological research. W. H. Freeman and Company: San Francisco.

Williams, D. A. 1976. Improved likelihood ratio tests for complete contingency tables. Biometrika 63:33-37.