August 4, 2003

MEMORANDUM

SUBJECT: Transmittal of Meeting Minutes of the FIFRA Scientific Advisory Panel Meeting Held June 17-20, 2003

TO: James J. Jones, Director
Office of Pesticide Programs

FROM: Paul I. Lewis, Designated Federal Official
FIFRA Scientific Advisory Panel
Office of Science Coordination and Policy

THRU: Larry C. Dorsey, Executive Secretary
FIFRA Scientific Advisory Panel
Office of Science Coordination and Policy

Joseph J. Merenda, Jr., Director
Office of Science Coordination and Policy

Please find attached the meeting minutes of the FIFRA Scientific Advisory Panel open meeting held in Arlington, Virginia from June 17 to 20, 2003. This report addresses a set of scientific issues being considered by the Environmental Protection Agency regarding the potential developmental effects of atrazine on amphibians.

Attachment

cc:

Susan Hazen
Adam Sharp
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Debbie Edwards
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Antonio Bravo
Douglas Parsons
David Deegan
Vanessa Vu (SAB)
OPP Docket

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Stephen M. Roberts, Ph.D.
Steven Heeringa, Ph.D.
Gary E. Isom, Ph.D.
Fumio Matsumura, Ph.D.
Mary Anna Thrall, DVM

FQPA Science Review Board Members
Joel Coats, Ph.D.
Peter Delorme, Ph.D.
Robert J. Denver, Ph.D.
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Sherril L. Green, DVM
Darcy B. Kelley, Ph.D.
Werner Kloas, Ph.D.
Gerald A. LeBlanc, Ph.D.
Carl Richards, Ph.D.
David Skelly, Ph.D.

SAP Report No. 2003-01

REPORT

FIFRA Scientific Advisory Panel Meeting, June 17-20, 2003, held at the Crowne Plaza Hotel Arlington, Virginia

A Set of Scientific Issues Being Considered by the Environmental Protection Agency Regarding:

POTENTIAL DEVELOPMENTAL EFFECTS OF ATRAZINE ON AMPHIBIANS

NOTICE

These meeting minutes have been written as part of the activities of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Scientific Advisory Panel (SAP). These meeting minutes represent the views and recommendations of the FIFRA SAP, not the United States Environmental Protection Agency (Agency). The content of these meeting minutes do not represent information approved or disseminated by the Agency. They have not been reviewed for approval by the Agency and, hence, the contents of these meeting minutes do not necessarily represent the views and policies of the Agency, nor of other agencies in the Executive Branch of the Federal government, nor does mention of trade names or commercial products constitute a recommendation for use.

The FIFRA SAP is a Federal advisory committee operating in accordance with the Federal Advisory Committee Act and was established under the provisions of FIFRA, as amended by the Food Quality Protection Act (FQPA) of 1996. The FIFRA SAP provides advice, information, and recommendations to the Agency Administrator on pesticides and pesticide-related issues regarding the impact of regulatory actions on health and the environment. The Panel serves as the primary scientific peer review mechanism of the EPA, Office of Pesticide Programs (OPP) and is structured to provide balanced expert assessment of pesticide and pesticide-related matters facing the Agency. Food Quality Protection Act Science Review Board members serve the FIFRA SAP on an ad-hoc basis to assist in reviews conducted by the FIFRA SAP. Further information about FIFRA SAP reports and activities can be obtained from its website at http://www.epa.gov/scipoly/sap/ or the OPP Docket at (703) 305-5805. Interested persons are invited to contact Larry Dorsey, SAP Executive Secretary, via e-mail at dorsey.larry@.epa.gov.

SAP Report No. 2003-01

REPORT:
FIFRA Scientific Advisory Panel Meeting, June 17-20, 2003, held at the Crowne Plaza Hotel, Arlington, Virginia

A Set of Scientific Issues Being Considered by the Environmental Protection Agency Regarding:

POTENTIAL DEVELOPMENTAL EFFECTS OF ATRAZINE ON AMPHIBIANS

Mr. Paul Lewis
Designated Federal Official
FIFRA Scientific Advisory Panel
Panel
Date: August 4, 2003

Stephen Roberts, Ph.D.
FIFRA SAP Session Chair
FIFRA Scientific Advisory

Date: August 4, 2003

Federal Insecticide, Fungicide, and Rodenticide Act
Scientific Advisory Panel Meeting
June 17-20, 2003

POTENTIAL DEVELOPMENTAL EFFECTS OF
ATRAZINE ON AMPHIBIANS

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Carl Richards, Ph.D., Director and Professor, Minnesota Sea Grant College Program, University of Minnesota Duluth, Duluth, MN

David Skelly, Ph.D., Associate Professor of Ecology, Yale School of Forestry and Environmental Studies, New Haven, CT

PUBLIC COMMENTERS

Oral statements were made by:

Ronald Kendall, Ph.D., Texas Tech University, Glen Van Der Kraak, Ph.D., University of Guelph, Canada, Ernest Smith, Ph.D., Texas Tech University, John Giesy, Ph.D., Michigan State University, Louis Du Preez, Ph.D., Potchefstroom University, South Africa, K.R. Solomon, Ph.D., University of Guelph, Canada, Timothy Gross, Ph.D., United States Geological Survey and the University of Florida, Robert Sielken, Jr., Ph.D. Sielken and Associates, Inc., James Carr, Ph.D., Texas Tech University, and Ms. Catherine Bens, United States Department of Agriculture, on behalf of Ecorisk, Inc.

John Ashby, Ph.D. and Charles Breckenridge, Ph.D. on behalf of Syngenta

Tyrone Hayes, Ph.D., the University of California, Berkeley, as a private citizen

Nigel Noriega, Ph.D.,USEPA , as a private citizen

Janis McFarland, Ph.D. on behalf of Syngenta

Angelina Duggan, Ph.D. on behalf of CropLife America

Mr. Scott Slaughter on behalf of the Center for Regulatory Effectiveness

Mr. Jere White, Kansas Corn Growers Association and Kansas Grain Sorghum Producers Association, Ms. Stephanie Whalen, Hawaii Agriculture Research Center, Bill Kubecka, DVM, Kubecka Farms, and Mr. Gary Marshall, Missouri Corn Merchandising Council and Missouri Corn Growers Association, on behalf of the Triazine Network

Richard Fawcett, Ph.D., on behalf of the Iowa Corn Growers Association

Mr. Robert Hedberg on behalf of the Weed Science Society of America

Jennifer Sass, Ph.D., on behalf of the Natural Resources Defense Council

Steven Sheffield, Ph.D., George Mason University, as a private citizen

Mr. James Tozzi on behalf of Multinational Business Services

Mr. John Hall on behalf of the Kentucky Corn Growers Association

Mr. Daniel Botts on behalf of the Florida Fruit and Vegetable Association

Written statements were received from:

Center for Regulatory Effectiveness

Ecorisk, Inc.

Natural Resources Defense Council

Rachel Carson Council

Rachel Jordan, as a private citizen

Sielken & Associates Consulting, Inc.

State of New York, Office of the Attorney General

Steven Sheffield, Ph.D., George Mason University, as a private citizen

Syngenta

Tyrone Hayes, University of California, Berkeley, as a private citizen

Triazine Network

Weed Science Society of America

INTRODUCTION

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Scientific Advisory Panel (SAP) has completed its review of the set of scientific issues being considered by the Agency pertaining to the potential developmental effects of atrazine on amphibians. Advance notice of the meeting was published in the *Federal Register* on February 24, 2003 and May 8, 2003. The review was conducted in an open Panel meeting held in Arlington, Virginia, from June 17-20, 2003. The meeting was chaired by Stephen Roberts, Ph.D. Mr. Paul Lewis served as the Designated Federal Official. Steven Bradbury, Ph.D. (Office of Pesticide Programs, EPA) summarized the goals and objectives of the Agency's presentation. Thomas Steeger, Ph.D. (Office of Pesticide Programs, EPA) and Mr. Joseph Tietge (Office of Research and Development, EPA) provided an overview of the atrazine studies and conceptual model for potential studies, respectively. Thomas Steeger, Ph.D. (Office of Pesticide Programs, EPA) completed the Agency's presentation by providing concluding remarks.

In preparing these meeting minutes, the Panel carefully considered all information

provided and presented by the Agency presenters, as well as information presented by public commenters. This document addresses the information provided and presented within the structure of the charge by the Agency.

CHARGE

1. In reviewing the available laboratory and field studies, the Agency used a number of criteria to evaluate individual investigations. Criteria such as experimental design, test protocols, and quality assurance information were used to ascertain the reliability of the generated data in terms of its ability to adequately assess a hypothesis that atrazine elicits developmental effects in amphibians, and if so, the nature and strength of associated dose-response relationships.

a) Does the SAP have any comments and recommendations regarding the EPA's approach and criteria used to evaluate the studies?

b) Given the evaluation criteria employed by the Agency, please comment on EPA's overall characterization of the currently available studies.

c) Please comment on the availability, as of February 28, 2003, of additional, relevant studies in the open literature that were not addressed in the white paper.

d) Since February 28, 2003, is the Panel aware of any studies that would be relevant?

2. In its evaluation of existing field studies, the Agency has concluded that these investigations are of limited value. The reasons include: (1) the high variability in environmental conditions and uncertainties in the pre-existing status and condition of field-collected animals, (2) the spatial and temporal aspects of atrazine exposure (*i.e.*, spatial and temporal variability over the course of the studies and the extent to which such aspects of atrazine exposure were empirically measured or otherwise accounted for), and (3) the possible co-occurrence of additional chemical and/or non-chemical stressors.

a) To the extent that the field studies appear to indicate that atrazine may not adversely affect development, please comment on EPA's conclusion that the body of data from field studies does not provide the means to ascertain whether the lack of a relationship between atrazine exposure and developmental effects is due to the absence of a causal relationship or limitation in study methodologies.

b) To the extent that any field studies appear to indicate that atrazine may adversely affect development, please comment on EPA's conclusion that these field studies do not provide sufficient information to resolve the potential role of additional co-occurring stressors.

3. In an evaluation of the existing laboratory-based studies, the Agency concluded that there was sufficient information to establish a hypothesis that atrazine could cause adverse gonadal developmental effects. However, due to different experimental designs and variability in the nature and extent of experimental conditions (*e.g.*, level of excessive mortality, delayed development in untreated organisms, lack of response to positive controls) it was not possible to adequately assess the hypothesis that atrazine causes developmental effects. It was further concluded that the current body of information did not provide the means to characterize the

nature of any associated dose-response relationships.

a) Please comment on EPA's determination that the laboratory studies provide a plausible basis for the means to establish a hypothesis concerning the potential for atrazine to cause developmental effects. Also, please comment on whether the overall body of available data is adequate to demonstrate whether or not atrazine causes developmental effects under the conditions described in these studies.

b) Please comment on EPA's conclusion that given the variability in the available dose-response data across the studies (*e.g.*, an approximately 250-fold difference in reported thresholds for observed developmental effects as well as reports of monotonic and non-monotonic dose-response curves), it is not possible to ascertain the relationship, if any, of atrazine exposure to developmental effects in amphibians.

4. Many of the available studies proposed that aromatase induction results in elevated estrogen levels that lead to feminization (ovotestes/intersex/hermaphroditism) in genetically male amphibians.

a) Please comment on EPA's conclusion that, to date, aromatase induction by atrazine has not been demonstrated in any anuran in controlled laboratory investigations.

b) The variability associated with plasma sex steroid concentrations and aromatase activities is high. Is this variability normal? Please comment on any readily apparent or available methodological improvements (*e.g.*, changes in sampling design, analytical techniques) that could efficiently address this variability in future studies.

c) Please comment on whether there are additional data, other than those summarized in the white paper, that suggest late exposure of amphibians (*i.e.*, juveniles or adults) to estrogens or estrogenic chemicals can induce ovotestes formation.

d) Please comment on whether there are additional data, other than those summarized in the white paper, that suggest alternative mechanisms that could explain the apparent feminization of genetically-male amphibians.

5. With regard to specific endpoints, the Agency does not currently have sufficient information to quantitatively relate gonadal/laryngeal effects to reproductive outcomes. A major underlying uncertainty is the ecological relevance of ovotestes occurrence to the maintenance of anuran populations.

a) Can the Panel provide sources of data on background rates of ovotestes occurrence in amphibian species and any associated considerations for interpreting this information in the context of the reviewed studies?

b) Can the Panel characterize any evidence that suggests that the presence of ovotestes in male anurans results in reproductive impairment via reductions in fertility?

c) The reduction of laryngeal muscle area suggests diminished testosterone in males. If this is found to be a valid observation and if estrogen concentrations do increase as testosterone concentrations decrease, what other endpoints (*e.g.*, secondary sexual characteristics and

reproductive behavior) would likely be affected?

6. While some of the available data indicate there may be an association between atrazine exposure and developmental effects in amphibians, the Agency's evaluation of the existing body of laboratory and field studies has determined that there is not sufficient scientific evidence to indicate that atrazine consistently produces effects across the range of amphibian species examined. However, the current body of knowledge has deficiencies and uncertainties that limit its usefulness in assessing potential developmental atrazine effects and the extent of any associated cause-effect and dose-response relationships. Consequently, the Agency has determined that there are not sufficient data to reject the hypothesis that atrazine can cause adverse developmental effects in amphibians.

Does the SAP concur with these conclusions? If not, what lines-of-evidence would lead to an alternative conclusion?

7. Assuming the Agency determined an ecological risk assessment with a greater degree of certainty concerning developmental effects of atrazine on amphibians were needed, please comment on EPA's conclusion that additional information is required to evaluate potential causal relationships between atrazine exposure and gonadal development. Please also comment on the added utility, if any, of additional information to interpret the shape of dose-response curves for potential developmental endpoints and the extent to which threshold or non-threshold response relationships can be quantified.

8. The Agency has developed a conceptual model from which to develop a set of study protocols for evaluating the potential effects of atrazine on gonadal development in amphibians. The Agency has proposed a research approach using focused, empirical, laboratory studies based on initial investigations with *X. laevis* followed by selective, confirmatory studies with frog species native to North America.

a) Please comment on the proposed sequence of study objectives.

b) Please comment on whether the Agency's first set of proposed studies has accounted for the major sources of uncertainty associated with the potential effects of atrazine on anuran sexual differentiation. In addition to time to metamorphosis, gonadal abnormalities, and sex ratios in the proposed Phase I assays, please comment on any other endpoints that should be considered in this initial phase.

c) Please also comment on the range, spacing and number of atrazine concentrations that should be employed in the proposed testing sequence to resolve uncertainties in the shape and nature of dose-response relationships for any observed developmental effects.

d) Please comment on the Agency's recommendation that *X. laevis* be used as the primary biological model in the proposed studies and whether or not the mechanisms involved in sexual differentiation of the ranid and pipid species are sufficiently similar to predict effects and associated dose-response curves for *Rana* and/or to efficiently design *Rana* studies.

e) In this regard, are there important differences between the species to conclude that any affected developmental processes observed in *X. laevis* would not occur in *Rana*?

f) Alternatively, are there developmental pathways in *Rana*, but not in *X. laevis*, that raise concerns about using *X. laevis* as the primary biological model in any future atrazine studies?

g) Assuming *X. laevis* and *Rana* are sufficiently concordant from a toxicodynamic perspective with regard to potential developmental effects of atrazine, what critical toxicokinetic processes should be considered for extrapolating *X. laevis* dose-response relationships to *Rana* and/or for designing subsequent studies with *Rana*?

DETAILED RESPONSE TO THE CHARGE

The specific issues to be addressed by the Panel are keyed to the Agency's background documents "White Paper on Potential Developmental Effects of Atrazine on Amphibians", dated May 29, 2003, and are presented as follows:

1. In reviewing the available laboratory and field studies, the Agency used a number of criteria to evaluate individual investigations. Criteria such as experimental design, test protocols, and quality assurance information were used to ascertain the reliability of the generated data in terms of its ability to adequately assess a hypothesis that atrazine elicits developmental effects in amphibians, and if so, the nature and strength of associated dose-response relationships.

a) Does the SAP have any comments and recommendations regarding the EPA's approach and criteria used to evaluate the studies?

b) Given the evaluation criteria employed by the Agency, please comment on EPA's overall characterization of the currently available studies.

The Panel considered parts A and B of question 1 together. The Agency's criteria in evaluating the available studies included experimental design, protocols and quality assurance, strength of cause/effect and/or concentration/response, mechanistic plausibility and ecological relevance. These criteria reflect requirements for sound and consistent science in ecological risk assessments.

The Panel concluded that the review was thorough, the approaches and criteria were appropriate and that the conclusions were valid, given the data reviewed. The Panel raised a few minor concerns relating to the Agency's approach. One experiment (Syngenta # 2233-02) categorized as a field study is actually a mesocosm experiment. Mesocosms, while potentially powerful, are not capable of providing inferences regarding the fate of natural populations. With regard to the analysis of laboratory studies, the Panel expressed minor concerns about the reanalysis of the data but agreed that the overall characterization of the studies was reasonable.

The Panel agreed that additional studies are warranted. Studies from several research laboratories provided evidence that atrazine can cause developmental abnormalities in amphibians. Although not considered in the Agency's White Paper, the findings are consistent with studies on effects of atrazine exposure to other vertebrates, both aquatic (e.g. fish) [Moore, 2003] and terrestrial (some rodents). Given the conservation of many basic pathways for endocrine regulation, these studies in other species are relevant to the issues addressed in the Agency's White Paper. However, a range of abnormalities have been reported in amphibians and these are not consistent from study to study (bearing in mind that the literature includes studies

in several anuran species).

c) Please comment on the availability, as of February 28, 2003, of additional, relevant studies in the open literature that were not addressed in the white paper. Since February 28, 2003, is the Panel aware of any studies that would be relevant?

The Agency appropriately identified the extant published literature relevant to atrazine effects on the development of the amphibian gonad. The Panel recommended adding mortality studies to the Agency's assessment because they offer a toxicological perspective regarding the atrazine concentrations used in developmental studies. The Panel also identified selected additional published studies ("open literature") that do not need to be included in the Agency's White Paper, but are relevant to ongoing deliberations and may be useful for the Agency's risk assessment. These studies encompass several topics including:

1) Effects of atrazine on survival and early development of anurans

Allran JW and Karasov WH. 2001. Effects of atrazine on embryos, larvae, and adults of anuran amphibians. Environ Toxicol Chem. Apr;20(4):769-775.

Bevan CL, Porter DM, Prasad A, Howard MJ, and Henderson LP. 2003. Environmental estrogens alter early development in *Xenopus laevis*. Environ Health Perspect. Apr;111(4):488-496.

Morgan MK, Scheuerman PR, Bishop CS, and Pyles RA. 1996. Teratogenic potential of atrazine and 2,4-D using FETAX. J Toxicol Environ Health. Jun 7;48(2):151-168.

2) Hormone effects on survival and early development of anurans

Antila E. 1977. Early steroid metabolism in *Xenopus laevis*, *Rana temporaria* and *Triturus vulgaris* embryos. Differentiation. Aug 11;8(2):71-77.

Fort DJ, McLaughlin DW, Rogers RL, and Buzzard BO. 2003. Evaluation of the developmental toxicities of ethanol, acetaldehyde, and thioacetamide using FETAX. Drug Chem Toxicol. Feb;26(1):23-34.

Nishimura N, Fukazawa Y, Uchiyama H, and Iguchi T. 1977. Effects of estrogenic hormones on early development of *Xenopus laevis*. J Exp Zool. Jul 1;278(4):221-233.

3) Effects of atrazine on vulnerability to infection and immune function

Christin MS, Gendron AD, Brousseau P, Menard L, Marcogliese DJ, Cyr D, Ruby S, and Fournier M. 2003. Effects of agricultural pesticides on the immune system of *Rana pipiens* and on its resistance to parasitic infection. Environ Toxicol Chem. May; 22(5):1127-1133.

Gendron AD, Marcogliese DJ, Barbeau S, Christin MS, Brousseau P, Ruby S, Cyr D, and Fournier M. 2003. Exposure of leopard frogs to a pesticide mixture affects life history characteristics of the lungworm *Rhabdias ranae*. Oecologia. May;135(3):469-476.

4) Normative and experimental data on hormones, sex determination and sexual differentiation

in anurans; hormone sensitive biomarkers

Bögi C, Schwaiger J, Ferling H, Mallow U, Steineck C, Kalbfus W, Negele RD, Lutz I, and Kloas W. 2003. Endocrine effects of environmental pollution on *Xenopus laevis* and *Rana temporaria.* Environ Res. (in press).

Bögi C, Schwaiger J, Ferling H, Mallow U, Steineck C, Kalbfus W, Negele RD, Lutz I, and Kloas W. 2002. Endocrine effects of environmental pollution on amphibians. Proceedings of 2nd Status-Seminar Endocrine Disrupters, 2nd - 4th April 2001, Berlin, Germany, http://www.status-umwelthormone.de, 59-62.

Bögi C, Levy G, Lutz I, and Kloas W. 2002. Functional genomics and sexual differentiation in amphibians. Comp Biochem Physiol B. 133 (4): 559-570.

Catz D, Fischer L, and Kelley D. 1995. Androgen regulation of a laryngeal-specific myosin heavy chain isoform whose expression is sexually differentiated. Dev Biol. 171:448-457.

Kang L, Marin M, and Kelley D. 1995. Androgen biosynthesis and secretion in developing *Xenopus laevis*. Gen Comp Endocrinol. 100: 293 - 307.

Kelley DB. 1982. Female sex behaviors in the South African clawed frog, *Xenopus laevis*: gonadotropin-releasing, gonadotropic, and steroid hormones. Horm Behav. Jun; 16(2):158-174.

Kelley DB and Pfaff DW. 1976. Hormone effects on male sex behavior in adult South African clawed frogs, *Xenopus laevis*. Horm Behav. Jun; 7(2): 159-182.

Kloas W. 2002. Amphibians as model for the study of endocrine disruptors. Int Rev Cytol. 216, 1-57.

Kloas W, Levy G, Bögi C, Opitz R, and Lutz I. 2002. Effects of environmental chemicals on reproductive biology of amphibians. Proceedings of 2nd Status Seminar Endocrine Disrupters, 2nd - 4th April 2001, Berlin, Germany, http://www.status-umwelthormone.de, 55-58.

Kloas W, Bögi C, Levy G, Würtz S, and Lutz I. 2002. Sexual differentiation in amphibians. Proceedings of the 21st Conference of the European Comparative Endocrinologists (Bonn, Germany, 26-30 August, 2002), Monduzzi Editore, 87-90.

Kloas W, Lutz I, and Einspanier R. 1999. Amphibians as model to study endocrine disruptors: II. Estrogenic activity of environmental chemicals *in vitro* and *in vivo*. Drugs and Hormones as Pollutants of the Aquatic Environment – Determination and Ecotoxocological Impacts Sci. Total Environ. 225, 59-68.

Levy G, Bögi C, Lutz I, Opitz R, and Kloas W. 2002. Amphibians as model to study endocrine disruptors: I. *In vivo* effects on reproductive biology. Proceedings of 2nd Status-Seminar Endocrine Disrupters 2nd - 4th April 2001, Berlin, Germany, http://www.status-umwelthormone.de, 99-102.

Levy G, Lutz I, Opitz R, Krüger A, and Kloas W. 2002. Bisphenol A induces feminization in *Xenopus laevis* tadpoles via estrogen-response systems. Proceedings of the 21st Conference of

the European Comparative Endocrinologists (Bonn, Germany, 26-30 August, 2002), Monduzzi Editore, 91-94.

Levy G, Lutz I, Opitz R, Krüger A, and Kloas W. 2003. Bisphenol A induces feminization in *Xenopus laevis* tadpoles. Environ Res. (in press).

Lutz I, Würtz S, Schulz A, Levy G, Bögi C, Opitz R, and Kloas W. 2002. Establishment of estrogen receptor-mRNA as estrogenic biomarker in the amphibian *Rana temporaria.* Proceedings of the 21st Conference of the European Comparative Endocrinologists (Bonn, Germany, 26-30 August, 2002*)*, Monduzzi Editore, 95-98.

Lutz I, and Kloas W. 1999. Amphibians as model to study endocrine disruptors: I. Environmental pollution and estrogen receptor binding. Drugs and Hormones as Pollutants of the Aquatic Environment – Determination and Ecotoxicological Impacts, Sci Total Environ. 225: 49-57.

Marin ML, Tobias ML, and Kelley DB. 1990. Hormone-sensitive stages in the sexual differentiation of laryngeal muscle fiber number in *Xenopus laevis*. Development. 110:703-711.

Miyashita K, Shimizu N, Osanai S, and Miyata S. 2000. Sequence analysis and expression of the P450 aromatase and estrogen receptor genes in the *Xenopus* ovary. J Steroid Biochem Mol Biol. 75: 101-107.

Miyata S, Koike S, and Kubo T. 1999. Hormonal reversal and the genetic control of sex differentiation in *Xenopus*. Zool Sci. 15: 335-340.

Mosconi G, Carnevali O, Franzoni MF, Cottone E, Kloas W, Lutz I, Yamamoto K, Kikuyama S, and Polzonetti-Magni AM. 2002. Environmental estrogens and reproductive biology in amphibians. Gen Comp Endocrinol. 126: 125-129.

Opitz R, Levy G, Bögi C, Lutz I, and Kloas W. 2002. Endocrine disruption in fishes and amphibians. Transworld Research Network, Recent Res Devel Endocrinol. 3: 127-170.

Robertson J and Kelley D. 1996. Thyroid hormone controls the onset of androgen sensitivity in the developing larynx of *Xenopus laevis*. Dev Biol. 176:108-123.

Tobias M, Tomasson J, and Kelley DB. 1998. Attaining and maintaining strong vocal synapses in female *Xenopus laevis*. J Neurobiol. 37: 441-448.

Tobias ML, Marin ML, and Kelley DB. 1991. Temporal constraints on androgen directed laryngeal masculinization in *Xenopus laevis*. Dev Biol. 147: 260-270.

Tobias ML, Marin ML and Kelley DB. 1991. Development of functional sex differences in the larynx of *Xenopus laevis*. Dev Biol. 147: 251-259.

van Wyk JH, Pool EJ, and Leslie AJ. 2003. The effects of anti-androgenic and estrogenic disrupting contaminants on breeding gland (nuptial pad) morphology, plasma testosterone levels,

and plasma vitellogenin levels in male *Xenopus laevis* (African clawed frog). Arch Environ Contam Toxicol. 44:247-256.

Varriale B and Chieffi P. 1997. Oestrogen control of the sexual dimorphism in the Harderian gland of *Xenopus laevis*. J Steroid Biochem Mol Biol. Aug;62(5-6):455-60.

Wetzel DM and Kelley DB. 1983. Androgen and gonadotropin effects on male mate calls in South African clawed frogs, *Xenopus laevis*. Horm Behav. 17: 388-404.

5)Anuran husbandry

Bögi C, Schwaiger J, Ferling H, Mallow U, Steineck C, Kalbfus W, Negele RD, Lutz I, and Kloas W. 2003. Endocrine effects of environmental pollution on *Xenopus laevis* and *Rana temporaria.* Environ Res. (in press).

Major N and Wassersug RJ. 1998. Survey of current techniques in the care and maintenance of the African clawed frog (*Xenopus laevis*). Contemp Top Lab Anim Sci. Sep;37(5):57-60.

Moore A, Scott AP, Lower N, Katsiadaki I, and Greenwood L. 2003. The effects of 4-nonylphenol and atrazine on Atlantic salmon (*Salmo salar* L) smolts. Aquaculture 222: 253-263.

Wright KM. 2001. Diets for captive amphibians. *In* K.M. Wright and R.B. Whittaker (ed.) Amphibian Medicine and Captive Husbandry. Krieger Publishing Company, Malabar, FL. pp. 63-72.

2. In its evaluation of existing field studies, the Agency has concluded that these investigations are of limited value. The reasons include: (1) the high variability in environmental conditions and uncertainties in the pre-existing status and condition of field-collected animals, (2) the spatial and temporal aspects of atrazine exposure (*i.e.*, spatial and temporal variability over the course of the studies and the extent to which such aspects of atrazine exposure were empirically measured or otherwise accounted for), and (3) the possible co-occurrence of additional chemical and/or non-chemical stressors.

a) To the extent that the field studies appear to indicate that atrazine may not adversely affect development, please comment on EPA's conclusion that the body of data from field studies does not provide the means to ascertain whether the lack of a relationship between atrazine exposure and developmental effects is due to the absence of a causal relationship or limitation in study methodologies.

The Panel concluded that the absence of an established causal relationship derived from laboratory studies was not critical in limiting the interpretation of the field studies. Ecological field studies are routinely, and successfully, conducted in the absence of such information. However, the Panel believed strongly that all of the field studies reviewed had serious design or methodological flaws that limit their usefulness in evaluating hypotheses related to the effects of

atrazine exposure on anuran developmental responses. Common, important problems in the field studies considered included inappropriate site selection practices (e.g., designation of control sites with concentrations of atrazine that exceeded some exposure sites) and failure to identify a sampling frame and to choose sampling sites randomly from within it, as well as insufficient statistical power associated with too few sampling sites to evaluate study hypotheses. These problems render interpretation of results problematic, if not impossible.

It also was noted that the field studies focused on measurement of endpoints identified in laboratory studies. None of the field studies measured responses for which field studies are most revealing. Specifically, whereas abundance and age structure were measured occasionally, highly relevant endpoints related to reproduction, recruitment and population viability were entirely absent. It should also be noted that, aside from one mesocosm experiment, all of the field studies were observational. While observational field studies are necessary and potentially yield strong inference, carefully designed field experiments offer opportunities to manipulate the natural environment, thereby controlling for some potentially confounding factors and allowing direct interpretation of responses. Such an opportunity was unexploited in the pool of field studies considered by the Panel.

b) To the extent that any field studies appear to indicate that atrazine may adversely affect development, please comment on EPA's conclusion that these field studies do not provide sufficient information to resolve the potential role of additional co-occurring stressors.

The Panel determined that the field studies provided important information. Most notably, multiple studies conducted by different labs have uncovered gonadal abnormalities in wild populations of anurans. Since the Panel concluded that atrazine exposure is a plausible hypothesis explaining gonadal abnormalities and comparable abnormalities have been described in natural populations of native amphibians, further field studies are warranted and can be conducted in the absence of knowledge concerning a specific mechanism(s) that cause(s) deformities. The Panel concluded that the field studies conducted to date do not, however, provide sufficient information to resolve the potential role of additional co-occurring stressors, for reasons cited in the response to Question 2(a).

3. In an evaluation of the existing laboratory-based studies, the Agency concluded that there was sufficient information to establish a hypothesis that atrazine could cause adverse gonadal developmental effects. However, due to different experimental designs and variability in the nature and extent of experimental conditions (*e.g.*, level of excessive mortality, delayed development in untreated organisms, lack of response to positive controls) it was not possible to adequately assess the hypothesis that atrazine causes developmental effects. It was further concluded that the current body of information did not provide the means to characterize the nature of any associated dose-response relationships.

a) Please comment on EPA's determination that the laboratory studies provide a plausible basis for the means to establish a hypothesis concerning the potential for atrazine to cause developmental effects. Also, please comment on whether the overall body of available data is adequate to demonstrate whether or not atrazine causes developmental effects under the conditions described in these studies.

The Panel concurred with the Agency's determination that the laboratory studies on the

effects of atrazine on anuran gonadal development are sufficient to hypothesize that atrazine interferes with normal development. Available data on the effects of atrazine on gonadal development of frogs are limited; however, existing lines of evidence support the hypothesis. Seven laboratory investigations were designed to evaluate the effects of atrazine exposure on larval frog gonadal development and are described in the Agency's White Paper. Five studies detected abnormalities of gonadal development, including the development of ovotestis (Hayes et al., 2003; Carr et al., 2003) and reductions in primary spermatogonia (males) and oogonia (females) (Tavera-Mendoza et al., 2001a, 2001b). Differences among the types of effects observed in these studies could be due to species differences, differences in exposure, and specific endpoints selected for evaluation. The two studies that reported no effects of atrazine on gonadal development were unpublished reports. One study suffered from very high mortality among organisms in all treatments (Hecker et al., 2003a). Both studies (Hecker et al., 2003a and Hecker et al., 2003b), indicated problems relating to poor water quality. The inability to detect gonadal abnormalities with atrazine exposure in these studies should not detract from the positive results noted in the majority of the studies. Deficiencies in all laboratory studies were noted as related to experimental design, data analyses, or performance standards.

Significant data gaps exist in our understanding of the effects of atrazine on anuran development. These gaps include a lack of understanding of the mechanism by which atrazine might elicit developmental toxicity, the nature of the concentration-response relationship, definition of susceptible windows of exposure, variable terminology used to describe effects, and identification of a threshold concentration. Panel members agreed that sufficient data were available to establish the hypothesis that atrazine interferes with normal gonadal development in anurans but were hesitant to accept the hypothesis with the limited available data. It was agreed that more data are necessary to properly test the hypothesis. These data should be generated under standardized conditions and must be subject to independent verification.

Clarification was requested of the Agency as to whether agreement with the charge statement: "…data is adequate to demonstrate …(that)… atrazine causes developmental effects under the conditions described in these studies" implies acceptance of the hypothesis or implies that sufficient data exist to warrant concern. During the Panel deliberations, the Agency expressed the latter interpretation. All Panel members agreed that sufficient data existed to warrant concern.

b) Please comment on EPA's conclusion that given the variability in the available dose-response data across the studies (*e.g.*, an approximately 250-fold difference in reported thresholds for observed developmental effects as well as reports of monotonic and non-monotonic dose-response curves), it is not possible to ascertain the relationship, if any, of atrazine exposure to developmental effects in amphibians.

A major deficiency that exists among laboratory studies of the effects of atrazine on anuran gonadal development has been the difficulty in defining the concentration-response relationship, and accordingly, a threshold concentration. The magnitude of effects reported in the peer-reviewed scientific literature are summarized in Table 1 below.

Table 1. Effects of Atrazine on General Gonadal Development or Germ Cell Content of Gonads

	Percent Animals with Effects *Atrazine Concentration (mcg/L)*					
	0.01	0.1	1.0	10	20-25	200
Hayes et al. (2002a)	0	~ 18	~ 18	~ 18	~ 18	~ 18
Carr et al. (2003)	–	–	4.5	7	13	–
Hayes et al. (2003)	–	65	–	–	20	–
Tavera-Mendoza et al. (2001a)	–	–	–	–	~ 70	–
Tavera-Mendoza et al. (2001b)	–	–	–	–	~ 30	–

– not tested

From these data, the Panel could draw no conclusions regarding a concentration-response relationship. More data are required to define the concentration-response relationship between atrazine and gonadal development of anuran larvae. However, the Panel believes that the data supports the hypothesis that the effect of atrazine on amphibian general gonadal development occurs with a threshold concentration between 0.01 and 25 ug/L.

It was noted that, in general, maximum effects on gonadal development at atrazine exposure concentrations (when observed) averaged ~20%, irrespective of exposure concentration. One Panel member noted that this may reflect a plateau of the concentration-response curve at this level of effect and that perhaps effects of greater magnitude should not be expected in future studies. Another Panel member disagreed, suggesting that there might be a possibility of monotonic as well as non-monotonic responses.

Precedence was discussed for chemicals having the ability to cause intersex conditions in only a small percentage of the exposed population in laboratory-controlled experiments. Tributyltin is a marine biocide known to cause intersex conditions in some snail species. Incidents of 100% intersex females have been noted in field populations inhabiting tin-contaminated environments (Curtis and Barse, 1990; Gooding et al., 1999; Gooding, 2003; Morcillo and Porte, 1999; and Smith, 1981). During many laboratory experiments (Gooding et al., 2003; Oberdorster et al., 1998; and Smith, 1981), a maximum incidence of intersex of only ~30% has been observed. This discrepancy has been attributed to limitations of laboratory experiments. For example, exposure of maternal organisms during oocyte maturation or of embryos may be required to maximally induce intersex among the resulting organisms. In the absence of such exposure during laboratory experiments, perhaps only the most susceptible individuals develop intersex conditions. The limited susceptibility of anuran larvae to the developmental effects of atrazine, coupled with variations in experimental design, could result in low and variable responses that may mask any suggestion of a concentration-response relationship.

4. Many of the available studies proposed that aromatase induction results in elevated estrogen levels that lead to feminization (ovotestes/intersex/hermaphroditism) in genetically male amphibians.

a) Please comment on EPA's conclusion that, to date, aromatase induction by atrazine has not been demonstrated in any anuran in controlled laboratory investigations.

The Panel agreed with the Agency's conclusion that, to date, aromatase induction by atrazine has not been demonstrated in anurans by controlled laboratory studies. The experimental designs used by several investigators in order to demonstrate effects on aromatase induction using long term exposures are inappropriate to demonstrate any influence of atrazine, if there might be one.

The aromatase hypothesis, as applied to the partial feminizing effects of atrazine on male *Xenopus laevis*, originated with the well-established feminizing effects of estrogen in this species. Exposure of *Xenopus laevis* tadpoles to sufficient estradiol during the critical period resulted in 100% phenotypic females. When backcrossed to genetic males (zz genotype), half of these females produce only male offspring. The results indicated that half of the phenotypic females were genotypic males.

Indirect support for the aromatase hypothesis comes from findings in a human carcinoma cell line (H295R) in which atrazine (and two other triazines) increased aromatase (CYP19) enzyme activity and mRNA levels (Sanderson et al., 2000, 2001). In addition, Hayes et al. (2002a) reported that exposure of adult male *Xenopus laevis* to atrazine decreased plasma testosterone, and these investigators hypothesized that this decrease was due to the induction of aromatase. The Hayes et al. (2002a) study did not directly address the hypothesis that atrazine induces aromatase, and the apparent decrease in plasma testosterone might be explained by other mechanisms (e.g., changes in hormone clearance). Studies submitted to the Agency by Ecorisk (Giesy et al., 2003; Hecker et al., 2003b) were purportedly designed to evaluate the hypothesis that atrazine induces aromatase in *Xenopus laevis*. However, the Panel identified at least two flaws in the design of these studies that limit their usefulness. First, the investigators exposed adult frogs to atrazine for long periods (26, 43 or 47 days). In such long term exposure experiments, compensatory physiological adjustments could have obscured any effects that atrazine may have had on aromatase activity/expression in the short term. Second, the deleterious effects of atrazine on amphibians are hypothesized to occur during gonadal development (during a sensitive premetamorphic tadpole stage). Thus, the appropriate developmental stages were not tested, and developmental changes in sensitivity to atrazine could confound interpretation.

Possible feminizing/demasculinizing effects of atrazine could be produced by modes of action other than induction of aromatase (enhanced estrogen synthesis). For example, atrazine might exert an antiandrogenic effect. Feminization effects caused by estrogen administration to Xenopus tadpoles are dose dependent (Kloas et al., 1999; and unpublished data) and can also be obtained at least in part by antiandrogens such as cyproterone acetate, p,p'-DDE, and vinclozolin (Kloas, 2002; Bogi et al., 2002).

Several potential modes of action should be considered in addition to the aromatase hypothesis. While the available data do not support any one mode of action, the following hypotheses could be proposed:

(1) Estrogenic effects of atrazine:
a) the induction of aromatase resulting in the elevation in plasma and/or intragonadal estradiol levels
b) agonist action mediated by binding to the estrogen receptor (no data available in amphibians)

(2) Antiandrogenic effects:

a) inhibition of 5α-reductase resulting in a decrease in the dihydrotestrosterone (DHT)/ testosterone(T) ratio
b) interference with androgen receptor mediated actions (no data available in amphibians)

(3) Generalized effects on steroid metabolism

(4) Interference via the hypothalamus-pituitary-gonad axis (i.e., Cooper et al., 2000)

Furthermore, atrazine could exert feminizing effects through multiple modes of action. While focus has been placed on a causal endocrine disruptive effect of atrazine, one cannot currently rule out the hypothesis that the feminizing effects of atrazine are due to a generalized (or even specific) interference with genetic pathways responsible for gonadal development. Atrazine could act upstream of the endocrine system (endocrine changes have not been demonstrated in tadpoles in any study) and endocrine (epigenetic) changes caused by atrazine could be a byproduct and not the cause. In addition, atrazine could affect the thyroid system, which is known to influence gonadal development and steroid hormone action in developing amphibians.

b) The variability associated with plasma sex steroid concentrations and aromatase activities is high. Is this variability normal? Please comment on any readily apparent or available methodological improvements (*e.g.*, changes in sampling design, analytical techniques) that could efficiently address this variability in future studies.

Inter-individual variability in plasma sex steroid concentrations and steroid converting enzyme activities in lower vertebrates such as fishes, amphibians and reptiles is often high. This variability may be attributed to sex differences, age, stage of reproductive development, dietary influences, and social dominance, among others. However, the Panel was unable to determine whether the variability reported in the studies submitted to the EPA by ECORISK was due to biological variation or technical limitations. The most common technique used to analyze steroid hormones in animal tissues and plasma is radioimmunoassay (RIA). Although less common, enzyme-linked immunosorbant assay (ELISA) is also used to measure plasma steroids, and this technique was used in the ECORISK studies. It is noteworthy that the estradiol measurements in male *Xenopus laevis* obtained by the ECORISK group by ELISA are one to two orders of magnitude higher than those reported in the open literature in which RIA was used. Also, in the ECORISK studies, there is no mention of attempts to validate the ELISAs for use with *Xenopus laevis* plasma (also, appropriate validations were not conducted in the original reports that described the ELISA techniques which are cited in the ECORISK reports (Hecker et al., 2003b). Recovery analyses (using radioactive hormone) following organic extraction were not conducted in these studies, but are essential to control for variation in extraction efficiency among samples. This alone could account for significant variation in the reported values. No other standard assay validations were conducted (described below) nor were intra- or interassay coefficients of variation reported. These omissions make it difficult to evaluate the validity of the findings reported by ECORISK.

In the studies published by Hayes (2002a), and as indicated during his presentation to the FIFRA SAP, Hayes had been unable to measure plasma estradiol in *Xenopus* using RIA. However, members of the Panel noted that other researchers have successfully measured estradiol in male *Xenopus laevis* (Tobias et al., 1998; Bögi et al., 2003). In addition, in the Hayes et al. (2002a) study, measures of plasma testosterone are based on a sample size of only

four per treatment. The Hayes et al. (2002a) study is the only direct evidence for an endocrine disruptive effect of atrazine in an amphibian, and as such should be replicated and extended from adults to the appropriate developmental stages.

It is essential that the assays used for plasma sex steroid measurements be validated for use in the amphibian species under study. Validations should include, but are not limited to: 1) parallelism between the standard curve and dilutions of plasma/serum; 2) recovery of labeled (i.e., radioactive) hormone added to the plasma/serum sample before extraction; 3) verification that residual plasma sex hormone binding globulins and other plasma proteins do not interfere in the assay; 4) verification of the specificity of the antiserum; and 5) corroboration of results by an independent method (e.g., comparison of ELISA and RIA). As mentioned above, an important quality control is to conduct recovery estimates (using a radiolabeled tracer) for each extracted sample to account for differences in extraction efficiencies.

Regarding the sampling technique for wild-caught *Xenopus laevis*, the ECORISK group trapped frogs, transported them to the laboratory, and then maintained them for 48 hours in the laboratory, purportedly to allow them to recover from capture stress. No attempt was made to evaluate whether animals had indeed recovered from capture stress (e.g. by measuring plasma corticosterone) or if the animals continued to suffer from confinement stress. Stress can have profound effects on plasma sex steroid concentrations, and individuals respond differently to stress. Thus, a major component of the variability in measurements could be due to the method of sampling. Future studies of adults (if, in fact, this life history stage is studied) should strive to collect plasma from animals immediately after capture (in field experiments). Also, the time of day when plasma is collected in both field and laboratory studies should be considered in the design of experiments and reported. There are distinct circadian rhythms in plasma hormones in frogs and thus the choice of time of day or night to sample (it should be noted that *Xenopus laevis* are nocturnal) could impact the results.

In relation to biological variability, every effort should be made to record and correlate physiological parameters that might account for variability in plasma sex steroid concentrations independent of exposure to atrazine or other toxicants. These parameters should include, but are not limited to, body weight (lean vs. fat wet and dry weights), stage of reproductive development (e.g., calculation of gonadosomatic index, plasma vitellogenin concentration in females), and stress (e.g., plasma corticosterone).

Future mechanistic studies should focus on short term exposures in tadpoles/juveniles/adults (e.g. 6 hr, 12 hr, 1 d, 3 d, 7 d, and 14 d). Measures should include estradiol, the androgens T and DHT and activities of aromatase as well as 5α_-reductase. In testing the aromatase hypothesis, biochemical assays could be included that test for direct interference of atrazine with aromatase (or 5α-reductase) enzyme activity. Possible effects of atrazine on aromatase gene expression should be tested using reverse transcriptase-polymerase chain reaction (RT-PCR) techniques such as those described by Miyata et al. (1999) and Miyashita et al. (2000). Particular emphasis should be placed on using these techniques to analyze effects of atrazine on aromatase gene expression in larval amphibians. A complementary approach for testing for estrogenic actions would be to determine effects of atrazine exposure on estrogenic biomarkers now available for *Xenopus* such as plasma vitellogenin (ELISA-kit, BIOSENSE, Norway or TOWA-KAGAKU, Japan) or vitellogenin mRNA (RT-PCR, Kloas et al., 1999).

c) Please comment on whether there are additional data, other than those summarized in the white paper, that suggest late exposure of amphibians (*i.e.*, juveniles or adults) to estrogens or estrogenic chemicals can induce ovotestes formation.

The Panel is unaware of reports that address the possibility of ovotestis formation in *Xenopus* at late exposures. For other amphibians, it is known in bufonids (Ponse, 1945) and some ranid species (Browder, 1975) that there may arise sex reversal at later stages.

d) Please comment on whether there are additional data, other than those summarized in the white paper, that suggest alternative mechanisms that could explain the apparent feminization of genetically-male amphibians.

As mentioned in response to question 4a, a mode of action other than aromatase induction might be responsible for feminizing/demasculinizing effects on gonadal development of genetically male amphibians. One experimental approach to address specifically such effects could be to produce genetic male (ZZ) female phenotypes and use their progeny (all ZZ males) specifically for such experiments. In addition, slight effects on the thyroid system cannot be excluded by the experiments presented up to now.

5. With regard to specific endpoints, the Agency does not currently have sufficient information to quantitatively relate gonadal/laryngeal effects to reproductive outcomes. A major underlying uncertainty is the ecological relevance of ovotestes occurrence to the maintenance of anuran populations.

a) Can the Panel provide sources of data on background rates of ovotestes occurrence in amphibian species and any associated considerations for interpreting this information in the context of the reviewed studies?

The Panel began its response to this question by defining ovotestes as follows: the occurrence of frank testicular tissue and ovarian segments within a recognizable gonad. Witschi (1956) reported 1 case of ovotestes. There is at least one study in which the prevalence of ovotestes in a control population of laboratory frogs has been described (Bögi et al., 2003; Levy et al., 2003). To the Panel's knowledge, the background rates of ovotestes in wild amphibian populations have not been reported. The Panel believed the frequency of occurrence of ovotestes in normal healthy populations of amphibians is probably very low and likely varies among species. This is based on the relatively rare occurrence of ovotestes in Panel members' laboratory amphibians. Without objective analysis and surveys on the background rates of ovotestes in wild populations, it is not possible to assess the impact, if any, that the presence of ovotestes in male frogs may have on anuran populations.

b) Can the Panel characterize any evidence that suggests that the presence of ovotestes in male anurans results in reproductive impairment via reductions in fertility?

To the Panel's knowledge, there are no published reports that indicate that ovotestes in male anurans either do or do not result in reproductive impairment. In amphibians and other species, decreases in sperm production are correlated with a decrease in testicular size. The consequences of gonadal abnormalities for reproductive impairment of individuals or for wild populations of anurans are entirely unknown. Three broad hypotheses exist:

1. Gonadal abnormalities among genetic males may have no effect on local population dynamics. This could occur if abnormal males do not breed or participate in breeding congresses. The breeding system of many anurans leads to a small fraction of males achieving matings with a disproportionate fraction of females.

2. Gonadal abnormalities among genetic males may lead to declines in wild amphibian populations. This could occur if abnormal males participate in breeding congregations and achieve matings with females that lead to incomplete or entire failure of fertilization.

3. Gonadal abnormalities in genetic male frogs may lead to increases in wild amphibian populations. This could occur if abnormal genetic males act as females in the breeding dynamics of natural populations.

Thus, it will be impossible to evaluate the ecological relevancy of reported gonadal abnormalities without measuring endpoints related to reproductive behavior. The Panel therefore recommended that feminized phenotypic males be included in "grow-out" studies for the purpose of using them in breeding experiments to test the hypothesis that ovotestes impair reproductive function.

c) The reduction of laryngeal muscle area suggests diminished testosterone in males. If this is found to be a valid observation and if estrogen concentrations do increase as testosterone concentrations decrease, what other endpoints (*e.g.*, secondary sexual characteristics and reproductive behavior) would likely be affected?

The Panel concluded that the following endpoints could be used. All are endocrine biomarkers. The first six can be evaluated using noninvasive methods and would not require sacrificing the animal:

(1) Snout to vent length, body weight (feminized males should be bigger)
(2) Nuptial pads
(3) Enlargement of the ventral folds of the cloacae
(4) Strength and pattern of the male calling signal
(5) Clasping
(6) Seminal fluid analysis (sperm count, motility evaluation, morphology)
(7) Time course examining synthesis of vitellogenin by the liver in response to estrogen challenge
(8) Oviduct development
(9) Proteins expressed in Harderian glands around the eye (three proteins expressed by females, 1 in males)
(10) Number and size of muscle fibers in larynx, myosin expression in larynx muscle

Continuous studies (e.g., studies that follow atrazine-exposed animals through sexual maturity) would be of great value to field studies. The animal's gonadal development could be assessed using minimally invasive techniques such as ultrasonography, endoscopy, or magnetic resonance imaging.

6. While some of the available data indicate there may be an association between atrazine exposure and developmental effects in amphibians, the Agency's evaluation of the existing

body of laboratory and field studies has determined that there is not sufficient scientific evidence to indicate that atrazine consistently produces effects across the range of amphibian species examined. However, the current body of knowledge has deficiencies and uncertainties that limit its usefulness in assessing potential developmental atrazine effects and the extent of any associated cause-effect and dose-response relationships. Consequently, the Agency has determined that there are not sufficient data to reject the hypothesis that atrazine can cause adverse developmental effects in amphibians. Does the SAP concur with these conclusions? If not, what lines-of-evidence would lead to an alternative conclusion?

The EPA expressed several conclusions in this question. The Panel examined each conclusion separately.

The first conclusion put forth was: "there is not sufficient scientific evidence to indicate that atrazine consistently produces effects across the range of amphibian species examined." The Panel agreed with this conclusion. The Panel previously noted in response to question # 3 that there was sufficient information to establish a hypothesis that atrazine could cause adverse gonadal developmental effects in amphibians. Studies were available that reported results from eight species of frog, including, *Xenopus laevis* (African clawed frog)*, Rana pipiens* (northern leopard frog), and *Rana clamitans* (green frogs) in laboratory or controlled exposure studies (mesocosm studies). Field observational studies reported on effects in *X. laevis, R. pipiens*, *Acris crepitans* (cricket frogs), *Bufo maninus* (cane toad) and *Bufo terrestris* (southern toad). The response of the species, both in terms of the endpoints considered and the magnitude of response, was inconsistent across the species studied and among studies which used the same species. Comparison among the studies was difficult because of the problems identified with respect to the design and conduct of both the laboratory and field studies, which confound their interpretation.

The second conclusion put forth was: "the current body of knowledge has deficiencies and uncertainties that limits its usefulness in assessing potential developmental atrazine effects…". The Panel agreed with the conclusions in the Agency's White Paper that there were deficiencies and uncertainties with respect to the methods, conduct, and results of the studies submitted, as were identified in responses to questions 2 and 3. Among the major factors identified were difficulties with the husbandry in laboratory studies, presence of atrazine in control exposures and reference sites and a lack of consideration of and/or information on the presence or potential impact of other stressors in observational field studies. Given these deficiencies and limitations, the Panel concluded that the current data would not be suitable for ecological risk assessment. Further, it was recognized by the Panel that in order to conduct a scientifically sound ecological risk assessment, the Agency needs to have results from studies where other factors can be ruled out as a cause in either the presence or the absence of effects.

The third conclusion put forth was that the uncertainties and deficiencies limited "the extent of (the identification of) any associated cause-effect and concentration-response relationships." The Panel concluded that although they agreed that a causal relationship can be hypothesized between atrazine and effects on gonadal development, the uncertainties and deficiencies in existing studies precluded acceptance of the hypothesis (see response to question 3). Further, the exact nature of the response in gonadal development in amphibians (shape of the concentration/response function, presence of a threshold) cannot be characterized at this point for the species tested. The Panel also addressed this issue in its response to questions 3(b) and 7. Finally, the Panel noted that knowledge of the concentration/response function is a necessary element to

conduct an ecological risk assessment.

The final conclusion put forth was: "the Agency has determined that there are not sufficient data to reject the hypothesis that atrazine can cause adverse developmental effects in amphibians." The Panel agreed that the available data suggest that atrazine can affect gonadal development in amphibians. However, the available data do not allow a proper characterization of the nature and magnitude of the response at either the organism or population level, nor do they offer sufficient support for the identification of a plausible mechanism.

The Panel further agreed that information gained from the available studies contributes to our knowledge and will be useful in the design and conduct of future studies. It was further concluded by the Panel that the adverse effects, in this case the presence of abnormalities in gonadal development described, need to be connected to the assessment endpoints of reproductive competence (i.e., fertilization success and subsequent potential effects at the population level).

7. Assuming the Agency determined an ecological risk assessment with a greater degree of certainty concerning developmental effects of atrazine on amphibians were needed, please comment on EPA's conclusion that additional information is required to evaluate potential causal relationships between atrazine exposure and gonadal development. Please also comment on the added utility, if any, of additional information to interpret the shape of dose-response curves for potential developmental endpoints and the extent to which threshold or non-threshold response relationships can be quantified.

The Panel agreed with the conclusion that additional information is required to evaluate potential causal relationships between atrazine exposure and gonadal development. Several points were made in regard to this conclusion. There is a need to confirm the causal relationship that is suggested by the existing data, and some similarity of data, or patterns or trends, from different labs needs to be presented to show repeatability of the effects. One of the tenets of the scientific method is the repeatability of experiments. Further, as previously noted, it is necessary to characterize the nature of the dose-response (or more correctly, concentration-response) function. Finally, there is a need to identify a plausible mechanism, supported by data. The characterization of a mechanism can, in part, aid in the extrapolation of results from surrogate test species to species of concern in the environment.

With respect to concentration-response curves, the Panel emphasized that these are extremely important to the question of any detrimental effects of a toxicant to an organism and necessary for risk assessment. Regardless of whether behavior demonstrates either a monotonic or atypical concentration-response relationships for a given endpoint, it should be possible to ascertain the shapes of the curves, given enough concentrations, replications, and controlled conditions. Repeatability in other laboratories should be feasible, if the same species, stage, water, concentrations and timing are utilized.

It was further put forth that studies on the quantitative structure-activity relationships (QSAR) can often provide information about a mechanism of action or provide a rationale for the data that are generated from comparative testing. Experiments using a series of closely related compounds (cyanazine, propazine, simazine, terbuthylazine, etc.) could elucidate patterns that would help explain the interaction between the molecules and the putative receptor, addressing the causal relationship. This approach seems to be lacking so far. It could be

valuable in the *in vivo* tests for gonadal development as well as for induction of aromatase or expression of mRNA for aromatase.

There are few studies among those reviewed in the Agency's White Paper that acknowledge the existence of metabolites that may be biologically significant. Three chlorinated metabolites are potentially bioactive in the same mode as atrazine -- deethyl atrazine, deisopropyl atrazine, and didealkyl atrazine, which is also called diamino chlorotriazine. There also is one major dechlorinated metabolite (hydroxy atrazine) that should be evaluated as well, based on its presence as a primary degradate of atrazine. These have been addressed in the context of water quality and mammalian toxicology, but scarcely in the amphibian development studies. They should be evaluated because they are commonly occurring transformation products, and they also could be interesting if included in the QSAR studies suggested above.

8. The Agency has developed a conceptual model from which to develop a set of study protocols for evaluating the potential effects of atrazine on gonadal development in amphibians. The Agency has proposed a research approach using focused, empirical, laboratory studies based on initial investigations with *X. laevis* followed by selective, confirmatory studies with frog species native to North America.

a) Please comment on the proposed sequence of study objectives.

The Panel was in agreement that a logical progression of studies as proposed by the Agency would elucidate effects and document mechanisms associated with any gonadal abnormalities due to atrazine exposure. The Panel had a number of suggestions as to how this model could be improved and which of the studies provided the most immediate and useful tests of the atrazine hypothesis. There was consensus that laboratory experiments (Phase 1 of the proposed approach) should proceed immediately. These studies would confirm whether gonadal deformities occur with exposure to atrazine and bracket concentrations at which effects are observed. One Panel member suggested that Phase 1 testing include characterization of a concentration-response relationship using some cellular/molecular marker of atrazine's effect. Such an endpoint would likely be sensitive and detectable in a timely fashion.

Panel members concurred that the studies identified under Phase 5 should be initiated as early as possible within the framework of the study plan. These studies would require grow-out of individuals with gonadal deformities to appropriate life stages to examine the effects of the reported gonadal deformities on fecundity and fertility. The Panel concluded that these studies were essential since true ecological effects are dependent on the hypothesis that reproduction of populations is impaired by individual reproductive impairment.

The Panel believed that, subject to study design constraints (i.e., both logistical and the necessity for a sound statistical design), the ideal study would address the effect of atrazine on gonadal development/morphology and have sufficient individuals to allow for the continuation of the study for assessment of the effects on fertility/reproduction. If such a study were not feasible, grow-out studies under Phase 5 should begin as soon as possible as noted above.

There was consensus among the Panel that the Agency should proceed with confirmatory studies with a North American *Rana* species due to potential differences in response with those species compared to *Xenopus*.

Some Panel members believed that the importance of field studies should be more prominent in the Agency's approach. Field studies often alter, sometimes drastically, conclusions derived from laboratory studies. The same study organisms under multiple, interacting stressors in a field situation often exhibit a dramatically elevated sensitivity to a particular compound in comparison to exposure to it in isolation in a lab situation. These Panel members contended that field studies are vital for addressing population-level effects of atrazine and generating insights about ecologically relevant endpoints and therefore they should not be consigned to a second priority to Phase 1 lab studies.

Experimental, field-based studies could be quite incisive. More specifically, a highly revealing experimental system in the field would be a small, temporary wetland with established, seasonal breeding populations of amphibians. Multiple wetlands can be surrounded with drift fences such that all individuals entering (pre-breeding adults) and leaving (post-breeding adults and metamorphs) are captured, marked, measured, and released. Many such wetlands could be included in a given study with subsets left as controls and others experimentally treated with various levels of atrazine. A wealth of demographic information could be gleaned from such an experimental study (studies by KA Berven [1990] provide a useful example of this approach and the information that can be gained from it). Moreover, high densities of such wetlands with little or no previous exposure to atrazine are readily available, thereby limiting many of the complications associated with contaminated controls that have arisen in many of the studies conducted on the topic to date in agricultural areas. Additional benefits would accrue if laboratory studies were able to identify a suite of external morphological characteristics that were associated with feminization/demasculinization and were indices of internal gonadal abnormalities noted during laboratory studies. Use of such indices would potentially obviate the need to sacrifice animals in the field for time-consuming histopathological analysis and thereby increase efficacy of field surveys.

b) Please comment on whether the Agency's first set of proposed studies has accounted for the major sources of uncertainty associated with the potential effects of atrazine on anuran sexual differentiation. In addition to time to metamorphosis, gonadal abnormalities, and sex ratios in the proposed Phase I assays, please comment on any other endpoints that should be considered in this initial phase.

There was Panel consensus that the Agency's first set of proposed studies have accounted for the major sources of uncertainty associated with the potential effects of atrazine on anuran sexual differentiation.

The Panel had a number of suggestions on potential endpoints that could be measured in Phase 1. Many of these endpoints have been presented in response to question 5c. The Panel was in consensus that a clear set of definitions concerning the terminology for classifying gonadal deformities should be developed by the Agency. This is essential for quantifying results of past and future studies.

Regarding the major sources of uncertainty associated with the potential effects of atrazine on anuran sexual differentiation, the Panel agreed with the Agency that the lack of standardization of husbandry protocols for laboratory *Xenopus laevis* and *Rana pipiens* likely played a significant role. The Panel is aware that the Agency has expertise in these areas. The Panel concurred that ASTM guidelines for water quality should be followed. For example, pH, conductivity, ammonia (total, ionized and unionized forms), nitrate, nitrite, dissolved oxygen,

chlorine or chloramine levels, copper and iron levels should be standardized among experiments. Alterations in any of these parameters may alter experimental results, particularly those involving growth rates and development. With FETAX, Holtfreter's solutions with adequate calcium are adequate for raising tadpoles.

Animals should be loaded in flow-through tanks at a density according to ASTM guidelines. Flow through tanks are preferable, but data collected from static renewal tanks would be acceptable provided animals are loaded according to ASTM guidelines and water quality is assessed on a daily basis (and maintained within the ASTM guidelines).

Xenopus laevis are carnivores. Therefore, diet should contain at least 14% protein. Diets formulated for herbivores or for omnivorous fish or turtles are not suitable. Diets formulated especially for *Xenopus* are commercially available for both tadpoles and adults. The quantity of feed (g/animal) should be based on the manufacturer's recommendation and adjusted as the animal grows.

The following reference is recommended as a guide for housing and husbandry of anurans: Amphibian Medicine and Captive Husbandry. (Eds.): KM Wright and BR Whitaker, Krieger Publishing Co., Malabar, FL**.**

One Panel member felt that a larger number of male/female pairs should be used to develop tadpole treatment groups. The current use of three pairs is minimal and may contribute to variation in results among experiments reported to date.

One Panel member believed that a stock colony of *Xenopus* animals should be developed that researchers could draw upon. This would minimize potential variation among populations studied in different laboratories. This stock colony should include phenotypic females with a ZZ genotype so that sex ratios can be accurately determined.

c) Please also comment on the range, spacing and number of atrazine concentrations that should be employed in the proposed testing sequence to resolve uncertainties in the shape and nature of dose-response relationships for any observed developmental effects.

The Panel recognized that it must answer this question in the context of realistic constraints on the cost and effort that can be devoted to any single study to determine if aqueous concentration of atrazine bears a relationship to gonadal irregularities in amphibians. The scope of any new experimental study will be determined by the number of concentrations and controls tested, the number of intra-laboratory replications (e.g. tanks) for each concentration level, and the number of test animals per experimental replication. Range and spacing of the experimental concentration levels are related to the number of feasible experimental points and may also be governed by the hypothesized functional form of any underlying relationship of concentration levels to response.

The Panel considered the components of the experimental design for a new study in the following order: 1) selection of controls; 2) range of observations for experimental concentrations; 3) number of independent replicates per treatment; 4) number of test animals per experimental replicate; and 5) number and spacing of experimental concentrations.

1) Selection of controls. The experiment should include untreated control replicates and a positive control treatment in which test animals are exposed to a concentration of 17β-estradiol. The Panel supported the Agency's statement that a positive androgen control group is not needed.

2) Range of observations: The experimental concentrations used in the experiment should include an untreated control and span the range of ecologically relevant atrazine concentrations to include at least one concentration exceeding the upper percentile bounds that have been measured in natural, aquatic environments.

3) Number of replications per treatment: From the *Xenopus* Hayes et al., (2002a) and Carr et al. (2003) studies and *Rana* study (Hayes et al., 2002b), estimates of the empirical intra-replication (intra-tank) correlation can be calculated. The estimated intra-class correlation should be used to determine the number of replicates per treatment arm and the allocation of total sample size to replications and test animals per replication. Since there will be uncertainty in the estimation of intra-class correlation for a future study, the Panel advised the Agency to err in selecting values that are near the high end of the range of values observed in the previous studies. Underestimation of the intra-replicate correlation in planning the sample size allocation can seriously attenuate the true power of the tests of the hypothesis for the chosen endpoints. In contrast, overestimation of the intra-class correlation will produce some cost inefficiency in the design, but will not generally endanger the power to detect significant true effects in the experimental study.

4) Number of test animals subjects per experimental replicate: Having established a working value for the intra-replicate correlation for the class of outcomes of interest and the desired levels of statistical power for the specific hypothesis tests, the optimal number of animal subjects per replicate can be determined using standard sample size allocation formulas. The allocation is obviously constrained by bio-loading and water quality considerations that are discussed in the Agency's White Paper.

5) Number and spacing of experimental concentrations: Determination of the optimal number and spacing of treatments is governed by the hypothesized shape of any underlying concentration-response relationship. The Panel had determined that data from existing studies support testing the hypothesis that there is a relationship between atrazine concentrations and gonadal abnormalities in amphibians. Hayes, in his presentation to the Panel, provided data and arguments that the relationship is not monotonic --potentially an "inverted" response. However, Hayes' conclusion has not been replicated in other studies. The Panel believes there is insufficient data for an understanding of the shape of the concentration-response curve. Data are needed to evaluate, at the low end of the curve, whether a non monotonic relationship exists. A robust design approach would be to follow the pattern of previous studies and use multiple concentration points to accommodate the possibility that any effect is monotonic, or alternatively that there is a simple non-monotonic relationship between atrazine concentration and rates of gonadal abnormalities in the amphibian test subjects. There is an advantage to retaining concentration points that have been used in the prior research by Hayes, Carr, Hecker and others: 0,0.01,0.1, 1, 10 and 25 ug/l. The Panel also recommended adding an upper concentration level that exceeds the 25 ug/l value at which Hayes' and Carr's studies have detected increases in the number of abnormalities. Such a spacing of concentration treatments would be sufficient to test the hypothesis of an effect of atrazine concentration on gonadal abnormalities and to secondarily test whether any real effect is monotonic or non-monotonic across atrazine concentration levels.

d) Please comment on the Agency's recommendation that *X. laevis* be used as the primary biological model in the proposed studies and whether or not the mechanisms involved in sexual differentiation of the ranid and pipid species are sufficiently similar to predict effects and associated dose-response curves for *Rana* and/or to efficiently design *Rana* studies.

The Panel concluded that Rana should be used as a collaborating species. The choice of *X. laevis* as a primary biological model is well justified since developmental and sexual differentiation of *Xenopus laevis* have been extensively studied, the published literature is extensive, and this is not the case for any North American species. However, the primary ecological concern for the Agency remains effects, if any, on North American anurans. *Rana* is the best studied North American genus and is the best choice for study. Sexual differentiation in ranids has not been as extensively studied as in *Xenopus* but, given strong conservation of basic developmental mechanisms and no known dissimilarities, there is no reason at this time to believe that mechanisms will differ substantively.

e) In this regard, are there important differences between the species to conclude that any affected developmental processes observed in *X. laevis* would not occur in *Rana*?

Several Panel members stated that there is little or no evidence to demonstrate that there are significant differences between *Rana* or *Xenopus* developmental pathways that would preclude the Agency from using *Xenopus* as a model in future studies. However, some Panel members noted that there are significant differences between the two groups of species in timing of life cycle events such that concerns about differences in developmental pathways cannot be eliminated.

f) Alternatively, are there developmental pathways in *Rana*, but not in *X. laevis*, that raise concerns about using *X. laevis* as the primary biological model in any future atrazine studies?

No differences have been identified to date that would raise concerns about differences in developmental pathways.

g) Assuming *X. laevis* and *Rana* are sufficiently concordant from a toxicodynamic perspective with regard to potential developmental effects of atrazine, what critical toxicokinetic processes should be considered for extrapolating *X. laevis* dose-response relationships to *Rana* and/or for designing subsequent studies with *Rana*?

To the Panel's knowledge, there are no pharmacokinetic studies on *Xenopus laevis* or *Rana pipiens* that would allow direct comparisons between uptake, metabolism, or depuration. However, given that *Xenopus* is a fully aquatic species and *Rana* is semi-terrestrial, there are bound to be differences. The significance of such differences, if any, is uncertain. Measuring whole body burden and/or residues in specific tissues will yield information on this topic.

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