UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON D.C., 20460

OFFICE OF
PREVENTION, PESTICIDES AND TOXIC
SUBSTANCES

January 3, 2008

MEMORANDUM

SUBJECT: Transmittal of Meeting Minutes of the FIFRA Scientific Advisory Panel Meeting Held October 9 - 11, 2007 on the Potential for Atrazine to Affect Amphibian Gonadal Development

TO: Debbie Edwards, Director
Office of Pesticide Programs

FROM: Joseph E. Bailey, Designated Federal Official
FIFRA Scientific Advisory Panel
Office of Science Coordination and Policy

THRU: Steven Knott, Executive Secretary
FIFRA Scientific Advisory Panel
Office of Science Coordination and Policy

Elizabeth Rezek, Acting Director
Office of Science Coordination and Policy

Attached, please find the meeting minutes of the FIFRA Scientific Advisory Panel open meeting held in Arlington, Virginia on October 9 - 11, 2007. This report addresses a set of scientific issues being considered by the Environmental Protection Agency pertaining to the potential for atrazine to affect amphibian gonadal development.

Attachment

cc:

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SAP Minutes No. 2007-08

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

The Potential for Atrazine to Affect Amphibian Gonadal Development

October 9 - 11, 2007
FIFRA Scientific Advisory Panel Meeting
held at the
Environmental Protection Agency Conference Center
Arlington, Virginia

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). The meeting minutes represent the views and recommendations of the FIFRA SAP, not the United States Environmental Protection Agency (Agency). The content of the meeting minutes does not represent information approved or disseminated by the Agency. The meeting minutes 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 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 Environmental Protection Agency, Office of Pesticide Programs (OPP), and is structured to provide balanced expert assessment of pesticide and pesticide-related matters facing the Agency. FQPA 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 Joseph E. Bailey, SAP Designated Federal Official, via e-mail at bailey.joseph@epa.gov.

In preparing these meeting minutes, the Panel carefully considered all information provided and presented by EPA, as well as information presented by public commenters. This document addresses the information provided and presented by EPA within the structure of the charge.

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October 9 - 11, 2007
FIFRA Scientific Advisory Panel Meeting
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Steven G. Heeringa, Ph.D.
FIFRA SAP Chair
FIFRA Scientific Advisory Panel
Date: JAN 3 2008

Joseph E. Bailey
Designated Federal Official
FIFRA Scientific Advisory Panel
Date: JAN 3 2008

Federal Insecticide, Fungicide, and Rodenticide Act
Scientific Advisory Panel Meeting
October 9 - 11, 2007

The Potential for Atrazine to Affect Amphibian Gonadal Development

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INTRODUCTION

The FIFRA Scientific Advisory Panel (SAP) has completed its review of the Potential for Atrazine to Affect Amphibian Gonadal Development. Advance notice of the SAP meeting was published in the *Federal Register* on June 29, 2007. The review was conducted in an open panel meeting October 9 - 11, 2007 in Arlington, Virginia. Dr. Steven G. Heeringa chaired the meeting. Joseph E. Bailey served as the Designated Federal Official. William Jordan, Senior Policy Advisor, Office of Pesticide Programs (OPP), provided opening remarks at the meeting. Arthur-Jean Williams, Acting Director of the Environmental Fate and Effects Division, OPP, provided an overview of the goals and objectives for the meeting. Presentations of technical background materials were provided by Thomas Steeger, Ph.D., and Mary Frankenberry of the Environmental Fate and Effects Division, OPP and by Sigmund Degitz, Ph.D., from the National Health and Environmental Effects Research Laboratory, Office of Research and Development, EPA.

EPA conducted an extensive review of open literature and registrant-submitted studies concerning the potential effects of atrazine on amphibian gonadal development and concluded in 2003 that there was sufficient information to hypothesize that atrazine exposure can result in effects on amphibian gonadal development. EPA also concluded that there was insufficient evidence to refute or confirm that hypothesis because the collective studies failed to show that atrazine produced consistent, reproducible effects across the range of exposure concentrations and amphibian species tested in the studies. EPA summarized the studies and its evaluation of the studies in a White Paper and presented its analysis, conclusions and recommendations to the SAP during the meeting held June 17 – 20, 2003.

The 2003 SAP concurred with EPA's interpretation of the available data and with EPA's recommendations to seek additional data. Additionally, the SAP concurred with the study approach described in the White Paper for addressing uncertainties identified in the available studies

In response to the uncertainties identified in the White Paper and based on the recommendations made by the 2003 SAP, EPA issued a Data Call-in Notice (DCI) on November 12, 2004, to Syngenta Crop Protection, Inc. and other atrazine registrants. The DCI required the registrant to conduct an amphibian study to determine if exposure to atrazine can affect amphibian gonadal development. Secondary objectives of the study were to provide information on the repeatability of previous observations, develop a sound dose-response relationship, and determine the developmental sensitivity of the amphibian species that are being tested.

EPA returned to the SAP October 9 – 11, 2007 with a second White Paper discussing the results of the registrant's amphibian study conducted in 2005 and 2006. In addition, EPA continued to review the open literature studies investigating whether atrazine exposure affects amphibian gonadal development. For the 2007 SAP meeting, EPA included only those studies published in the open scientific literature that tested atrazine alone and examined atrazine's potential effects on amphibian gonadal development. Studies on mixtures of pesticides that include atrazine as well as studies of the potential for atrazine to cause adverse effects other than amphibian gonadal development were not considered for this SAP meeting.

PUBLIC COMMENTERS

Oral statements were presented by:

Keith R. Soloman, Ph.D., and Glen Van Der Kraak, Ph.D., on behalf of Syngenta Crop Protection.
Jennifer Sass, Ph.D., on behalf of Natural Resources Defense Council
Rebeckah F. Adcock on behalf of the American Farm Bureau Federation
Scott Slaughter on behalf of the Center for Regulatory Effectiveness
Richard Fawcett, Ph.D., on behalf of the Triazine Network
Jere White on behalf of the Triazine Network, Kansas Corn Growers Association and Kansas Grain Sorghum Producers Association
Rick Robinson on behalf of the Iowa Farm Bureau

Written statements were provided by:

Timothy Pastoor, Syngenta Crop Protection, Inc.
James A. Carr, Ph.D., Texas Tech University
Richard O. Bennett, Ph.D., Jeffrey N. Holmes and Robert Brodman, Ph.D. on behalf of Partners in Amphibian and Reptile Conservation
Judith S. Schreiber, Ph.D., on behalf of the State of New York, Office of the Attorney General

SUMMARY OF PANEL DISCUSSION AND RECOMMENDATIONS

The FIFRA SAP met October 9 – 11, 2007 to provide advice and recommendations to the U.S. Environmental Protection Agency on the current body of evidence that describes the potential for atrazine to disrupt normal gonadal development in amphibians. The Agency has concluded that atrazine, at environmentally-relevant exposure concentrations, does not affect amphibian gonadal development and that no further testing is required to address the Agency's hypothesis that exposure to atrazine affects amphibian gonadal development. The Agency has sought the advice from the Panel on the soundness of this conclusion.

The Agency reviewed the scientific literature published since the last FIFRA SAP on this topic (2003) to formulate its conclusions on the risks of atrazine to amphibian gonadal development. The peer-reviewed published literature included both laboratory and field evaluations of the impacts of atrazine on amphibian gonadal development. In addition, the Agency issued a Data Call-In Notice (DCI) to the registrant for laboratory evaluations of the effects of atrazine on amphibian gonadal development using an experimental design and protocol that specifically addressed the concern posed by the 2003 SAP. In evaluating these data and in assessing the Agency's use of these data, the Panel modified the Agency's hypothesis statement and confined their deliberations largely to the modified hypothesis stated as follows: the parent compound atrazine causes adverse gonadal development in *Xenopus laevis* within the exposure concentration range of 0.010 to 100 µg/L.

The Panel concurred that the Agency performed a thorough review of the open literature and appropriately identified those studies that were relevant to the issue at hand. The Panel also agreed that the open literature suffers from various deficiencies due largely to design limitations, poor study conduct and lack of access to raw data for confirmation of results and conclusions. However, the Panel did express concern that the Agency did not utilize any of these studies to evaluate the Agency's hypothesis due to identified deficiencies. The Panel noted that these studies may have provided some added value in evaluating the conclusions drawn from the data provided in response to the DCI (subsequently referred to as the *DCI study*).

The Panel acknowledged that the DCI study was well designed and executed. Some changes to the design, or deficiencies in its execution, were noted but none were considered fatal to the success of this effort or interpretation of the results. The Panel advised the Agency that interpretation of the study results should be limited to atrazine and not transformation products[1] since analyses were not performed for transformation products and the flow-through design would have limited their accumulation in the exposure system. The Panel recommended that the Agency evaluate field monitoring data to establish whether any transformation products of atrazine accumulate in the environment at levels that may warrant direct evaluation of toxicity.

The Panel concluded that the results of the DCI study do not sufficiently address the EPA hypothesis that atrazine, at environmentally-relevant exposure concentrations, adversely affects amphibian gonadal development. In addition to issues addressed above, the Panel noted that parent atrazine exposure concentrations were well characterized during the experiments and were

[1]The term "transformation products" is used throughout this document to denote all chemicals derived from the parent compound through metabolism, abiotic processes and microbial biotransformation.

sufficient for documenting any exposure-effect relationships over the concentration range of 1.0 to 100 µg/l atrazine. They were somewhat less confident of the maintenance of exposures to the lower target levels of atrazine (0.010 and 0.10 µg/l). They generally agreed that primary apical endpoints of gonadal development were appropriate, well characterized, and the results conclusive, but expressed uncertainty in the interpretation and biological relevancy of the secondary endpoints. The Panel recommended verification of histological analyses by independent pathologists.

In evaluating the robustness of the data generated from the DCI study, the Panel considered: strength of concentration-response relationships, strength of cause-effect relationships, mechanistic plausibility, and ecological relevancy of effects. Under these considerations, the Panel viewed the data to be robust and sufficient to address the hypothesis as modified by the Panel and stated above. Some Panel members expressed concern that EPA completely rejected its own hypothesis based solely upon the negative results of the DCI study. This concern related in part to the use of a flow-through exposure system with a species that would not normally encounter such conditions in its natural environment. Additional uncertainty was expressed regarding the apparent differences among strains of *Xenopus* in their propensity to develop testicular ovarian follicles in response to estrogen treatment, as was described to the Panel by a representative of the registrant. The strain used in the DCI studies was apparently an insensitive strain. Panel members were concerned that this apparent insensitivity may have resulted in insensitivity of the apical endpoints to atrazine in general.

The Panel recommended that the DCI study be subjected to additional statistical analyses. The DCI study consisted of an identical exposure experiment performed at two different laboratories. Results from each laboratory were statistically analyzed separately. The Panel noted that combining the data from both experiments would provide greater statistical strength to the analyses, but not likely alter the overall statistical conclusions.

A major issue of discussion by the Panel was the use of *X. laevis* as a model species in the DCI study. The 2003 SAP recommended the use of *X. laevis* because it is amenable to laboratory experimentation. However, the 2003 SAP noted that the biology of this species differs in many respects from that of North American species. The 2003 SAP recommended that studies with *X. laevis* be followed up with comparable studies using a North American species as soon as possible. Such comparative studies have not yet been performed and the Panel expressed uncertainty in extrapolating results from *X. laevis* to North American frog species.

In conclusion, the Panel agreed that the results from the DCI study were sound, but they did not sufficiently address EPA's general hypothesis that atrazine, at environmentally-relevant exposure concentrations, adversely affects amphibian gonadal development. The Panel further agreed that the results from the DCI study were sufficiently robust to test and refute the Panel's modified hypothesis that atrazine, at environmentally-relevant concentrations, adversely affects gonadal development of *X. laevis*. The Panel recommended that the Agency be receptive to additional investigation that would specifically address whether atrazine transformation products pose risk to amphibians in general and whether *X. laevis* is a suitable surrogate for North American frog species with respect to susceptibility to the influence of exogenous chemicals on gonadal development.

PANEL DELIBERATIONS AND RESPONSE TO CHARGE

The specific issues addressed by the Panel are keyed to the background documents, references, and the charge questions provided by EPA.

The Agency has reviewed relevant studies in the scientific literature published since 2003, as well as studies submitted by the registrant in response to a DCI, to evaluate the potential for atrazine alone to elicit effects on amphibian gonadal development and differentiation. The strengths and limitations of the individual studies were assessed, and the extent of concordance for the entire body of information derived from these laboratory and field studies was considered to assess the plausibility that atrazine can cause developmental effects in amphibian gonads, and if so, the nature and strength of associated dose-response relationships. This analysis, while primarily focused on gonadal development and differentiation, included data on other developmental effects, including: time to metamorphosis, growth, sex ratios, and gonadal abnormalities.

The Agency sought comments and recommendations from the SAP on a number of questions surrounding the current body of evidence regarding the potential effects of atrazine on gonadal development in amphibians and the relevancy of these potential effects to an ecological risk assessment of atrazine. The Agency also sought input from the SAP on its conclusion that, based on the results of the most recent studies in response to the DCI, atrazine exposure does not affect amphibian gonadal development and that no further testing is required to address this conclusion.

Charge Question 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 evaluate the reliability of a study's 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) Please provide 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 application of these criteria to the currently available studies.

Panel Response

The Panel's response to Charge Question 1 considers Part a and Part b together.

The 2003 SAP recommended that a tiered approach be used to determine causality between exposure to atrazine and adverse effects in a model amphibian. The tiered approach (controlled laboratory studies followed by field evaluations and mechanistic studies, if warranted) was a logical strategy to establishing causality between atrazine exposure and aberrations in frog gonadal development. Standard aquatic toxicology methods with endpoints associated with apical effects were recommended. The Panel agreed that in most cases the

experimental design implemented these recommendations and the criteria used were sound. EPA noted several issues that limited their use of previous reports and open literature when evaluating hazards of atrazine on gonadal development in amphibians. The Panel largely concurred with EPA's decision-making.

As previous studies used static/static-renewal exposure systems with organisms often housed in containers of questionable and confounding material constructs, the required use of a flow-through system in glass was valid, especially since it appears that the life history of the organism is not impaired under these conditions. However, the Panel noted that the flow-through exposure system may have limited exposure to atrazine degradates that might accumulate in a static-renewal exposure system.

The Panel noted that concurrent performance of the experiment in two laboratories was an excellent approach to facilitate the establishment of causality between atrazine exposure and adverse effects in *X. laevis* and to evaluate the consistency of the results. The Panel also noted that the use of a test organism with well-characterized genetics and life history ensured that exposure occurred during the sensitive periods of gonadal development. One Panel member noted that body burdens of atrazine should have been measured to better evaluate exposure.

Adverse effects of atrazine on amphibian gonadal development were previously reported at 0.10 and 25 µg/L atrazine. Therefore, exposure concentrations bracketing these concentrations were used in the experiments performed in response to the DCI. One laboratory, Wildlife International, Ltd. (WLI), was successful in conducting exposures during the critical window of sexual differentiation bracketing 0.10 µg/L (which was identified as an effective concentration for adverse effects in previous studies). However, the other laboratory, Liebniz-Institute of Freshwater Ecology and Inland Fisheries (IGB), was less successful in reaching this nominal concentration (see response to Charge Question 4). An example of the benefit of inter-laboratory comparison was the analytical chemistry differences observed between the laboratories. A modest effect of gonadal hypoplasia was observed among males exposed to 0.10 µg/L atrazine in the WLI study. This effect was not observed at the same concentration in the IGB laboratory where measured concentrations were consistently below 0.10 µg/L atrazine (~50% of nominal). The possibility exists that the inability of the IGB laboratory to replicate the response observed in the WLI laboratory was due to the lower exposure concentrations during the window of susceptibility. However, other measures (e.g., gonad area or histological abnormalities) in the WLI laboratory did not confirm this effect. Thus, utilizing multiple endpoints to confirm adverse effects was a strength of the study design. Clearly, the quality assurance protocols allowed the observation that measured concentrations during critical windows of development were successful in one laboratory.

Having a positive control was an essential component of the DCI study and estradiol was an effective positive control for feminization given that the tested hypothesis was based upon up-regulation of aromatase (even though increases in estradiol have never been observed) which would lead to an increase in estradiol during critical windows of development. Given the knowledge base with this compound in this species, it was probably the most cost-effective choice.

Questions Concerning the Open Literature Studies

Charge Question 2

The Agency has concluded that the information contained in the open literature published since the 2003 SAP does not provide any additional information that could be used to refute or confirm the hypothesis that exposure to atrazine alone causes adverse developmental effects in amphibian gonads.

(a) Please comment on the comprehensiveness of the Agency's literature reviews relative to the potential effects of atrazine alone on amphibian gonadal development.

(b) Please comment on the Agency's evaluation of the open literature studies and the Agency's conclusion that the data derived from laboratory studies, both individually and collectively, are not sufficient to refute or confirm the hypothesis that atrazine exposure causes developmental effects in amphibian gonads.

(c) The Agency concluded that the field studies are not adequate for assessing the hypothesis at hand. Please comment on the Agency's conclusion. If the SAP concludes that one or more of the field studies do provide the means to assess the hypothesis that atrazine exposure results in effects on amphibian gonadal development, please suggest interpretive and statistical methods that should be employed to evaluate the data.

Panel Response

a) The Panel was not aware of any additional studies that have examined the potential effects of atrazine alone on amphibian gonadal development. However, it should be noted that there is a body of literature that deals with atrazine effects on other aspects of amphibian life histories, development and survival that is not being considered. Gonadal development is only one, and possibly not the most important, potential endpoint.

(b) The Panel concurred with the Agency's concerns and overall assessment regarding deficiencies of open literature laboratory studies. In many cases information is lacking (e.g., tadpole food not tested for atrazine) or techniques are used that EPA does not recommend (e.g., static-renewal). However, the EPA concluded that these concerns provide the basis to disregard all open literature lab studies in their final decision making. Referring to the studies performed in response to the DCI, an EPA presenter stated that "the open literature cannot hope to compete with that standard." The Panel expressed concern that this statement could be interpreted to mean that studies conducted according to approved protocols, and which provide access to raw data (i.e., registrant-submitted studies) will predominate in a situation where weight of evidence is critical and when other scientifically sound studies in the open literature may be available. This seems to be a potentially problematic situation that could be remedied by offering clear guidance to researchers who are not accustomed to conducting studies which might be used in a regulatory context.

A number of Panel members expressed concerns about the lack of further consideration of the open literature laboratory studies. This may be most important in the ways that it influences the interpretation of results from the DCI study. As an example, the DCI study

uncovered evidence that atrazine exposure was associated with reduced metamorphic weight. DCI study investigators described this result as "an anomaly" because it was observed in only one of the two laboratories that performed the studies. However, this finding becomes part of a broader pattern if considered along with the open literature where effects of atrazine exposure on growth and development of tadpoles have been observed by a number of independent research groups.

In light of these prior results, the DCI study provides confirmatory evidence of an effect on larval growth in that portion of the DCI study (IGB laboratory) which was best able to detect such an effect (due to the loss of control replicates at WLI). While the growth and development result is not consistent among studies (and it is not even consistent within the DCI study) it has emerged frequently enough that it should not be dismissed as an anomaly. While the effect on metamorph size was described as small and not biologically significant, a 6% decrease in body weight is large enough to affect postmetamorphic survival in ecological studies of natural populations (Smith, 1987, Altwegg and Reyer, 2003).

The data reported in the open literature are derived from studies that varied in their experimental design, the questions being asked, the endpoints analyzed, and the species under study. There clearly are flaws in the published studies, but this should not, in and of itself, disqualify all of the open literature studies from being considered for application to the evaluation of the central hypothesis. Several laboratory studies (one submitted by the registrant in 2003) found effects of atrazine using a static-renewal paradigm. A major difference between the published studies and the DCI study is static-renewal vs. flow-through. There has been no controlled, side-by-side comparison of these two approaches.

There are many problems with the field studies reported in the open literature. None of the published studies are sufficient to refute the hypothesis that atrazine adversely impacts amphibian gonadal development. However, some of the published studies do provide support for the hypothesis, despite flaws in the design. Whether such flaws are sufficient to remove an entire study from consideration is arguably a subjective determination. Limitations and flaws may render some data suspect, but some Panel members found it remarkable that all of the published data were being discounted.

(c) The EPA has asserted that problems with design render the use of each of the field studies inadequate for evaluation of the hypothesis that atrazine influences amphibian gonadal development. While the Panel agreed with many of the concerns raised, the White Paper failed to acknowledge an important observation raised in the 2003 SAP meeting minutes. Several field observational studies conducted by independent research groups have detected gonadal abnormalities in wild populations of North American amphibians. EPA scientists stated that there was no point in pursuing field studies without a repeatable pattern from lab studies along with knowledge of an underlying mechanism. Some Panel members disagreed as observational field studies provide complementary as opposed to subsidiary data. Some Panel members believed that this line of evidence deserves further attention.

Questions Concerning the DCI Study

Charge Question 3

Please comment on the Agency's evaluation of the final study design. For example, the Agency concluded that the minor changes in the experimental design [*i.e.*, omitting atrazine degradate (DACT, DEA and DIA) analysis and not conducting differential cell counts for ovarian and testicular histology] did not compromise the means to assess the hypothesis that atrazine exposure can affect amphibian gonadal development. If the SAP concludes that the alterations in the study design preclude or significantly compromise the ability to assess the hypothesis, please discuss, to the extent possible, how the specific design modifications could impact the means to assess the hypothesis. Please provide comments on other aspects of the Agency's evaluation as well.

Panel Response

The Panel agreed that it would have been valuable to have conducted atrazine degradate, or transformation product, analyses in the DCI studies for deethylatrazine (DEA), deisopropyl-atrazine (DIA), and diaminochlorotriazine (DACT). However, the omission of these analyses did not compromise the assessment of the hypothesis that exposure to atrazine alone (the parent compound, and within the concentration range studied) can affect gonadal development of *X. laevis*. The limited analysis that was done by Syngenta scientists suggested that the atrazine transformation products were at or below the limit of detection, and subsequent analyses on water samples were not conducted. Atrazine is rapidly metabolized by frogs (it is unclear whether tests have been conducted with tadpoles) and there is little bioaccumulation in fish or frogs. So, it is likely that if atrazine transformation products were generated to any significant degree, they would be rapidly cleared in the flow-through system. Thus, these studies were able to test whether atrazine in its native form affects amphibian gonadal development, without potential confounding effects of atrazine transformation products.

The Panel discussed whether the flow-through paradigm used in the DCI study vs. the static-renewal paradigm used in all of the open literature and prior registrant-sponsored studies could account for the differences in the results among studies. For example, one cannot rule out the hypothesis that transformation products of atrazine are responsible for the effects observed in some static-renewal experiments. These effects might be minimized in the flow-through system since such products would be rapidly cleared. The Panel was not aware of studies in which atrazine transformation products have been tested for effects on amphibians, nor have these transformation products been routinely analyzed in exposure studies.

There is precedent for concern about atrazine transformation products. For example, DEA, DIA and DACT have been shown to delay puberty in rats. Effects were shown on plasma estrone and testosterone, seminal vesicle and epididymal weight and coagulating gland, prostate weight and day of preputial separation (Stoker et al. 2002). There are other precedents for considering the activities of metabolites/degradates, one of the better known being the DDT metabolite DDE and its demonstrated antiandrogenic activity. However, it was pointed out that any *metabolites* that the tadpoles were capable of producing would have been formed in these *in vivo* studies and therefore, would have been tested in the DCI study.

Biotransformation in nature could result in significant accumulation of atrazine transformation products in water, sediments, or soils. Atrazine transformation products have been analyzed in several field studies (Hecker et al., 2004). The Panel recommended that the Agency consider evaluating existing monitoring data, which includes information on environmental concentrations of transformation products, to determine the extent to which they might wish to consider them in the future.

Cell counting with ovarian and testicular histological preparations could have provided a means to support the hypoplastic scores at the gross morphological level. However, as pointed out by one Panel member, even with metamorphosed froglets at Nieuwkoop Faber stage 66 it is very difficult to differentiate primordial germ cells and primary spermatogonia. That Panel member suggested that it is impractical and of minimal value to do differential cell counting; the Panel generally agreed with this conclusion. However, concerns were raised regarding the omission of scoring, and the difficulty with interpreting the findings when scores were arbitrarily combined rather than analyzed separately.

Several Panel members also pointed out the importance of grow-out studies for examining morphologic development and how it correlates with reproductive function. On a related point, it was noted that the grow-out study of transgenerational effects of atrazine presented to the Panel by Ecorisk provided useful information for the purpose of assessing exposure effects on F1 gamete quality and F2 viability. However, this study did not fully address the question of reproductive potential because the F1 individuals used for measuring reproductive success were a biased sample selected *because* of their mature condition; and also because the frogs were induced to breed by hormone injections, a procedure which may not fully mimic the outcomes of natural breeding. To address the effects of larval exposures to atrazine on reproductive potential, information on the age and size of the frogs at maturity, in addition to F1 fecundity estimates and egg quality as well as F2 viability, would provide useful and ecologically relevant information.

Charge Question 4

The Agency has described the exposure profiles for studies conducted in response to the DCI and has stated that mean-measured concentrations in the studies were lower than target nominal concentrations. However, the Agency concluded that the frequent analytical measurements provide a sufficiently comprehensive understanding of the exposure profile over the course of the studies. Please comment on the Agency's conclusion that the atrazine exposure concentration profile is reasonably characterized and sufficient for documenting the potential effects of atrazine over a broad range of exposure concentrations. In addition, provide comments on whether the actual concentrations were consistent and sufficiently stable to establish the means to analyze exposure concentration-response relationships.

Panel Response

The frequency of analysis was sufficient to allow a good understanding of the exposure profile, and the exposure profile is well characterized over a broad range of concentrations which encompass concentrations that have been associated with effects on gonadal development in *X. laevis* in the past. In the registrant-sponsored DCI studies, an attempt was made to bracket concentrations that previously demonstrated adverse effects in test species. The lowest of the

putative effect concentrations was 0.10 µg/L. Atrazine also was evaluated at a concentration of 0.010 µg/L in an effort to ensure the inclusion of a no observed effect level. The Panel expressed some minor concerns with respect to the stability of the two lower exposure concentrations (0.010 and 0.10 µg/L) at IGB which were considerably below nominal concentrations, and some concern that the *measured* concentration at the 1.0 µg/L level at IGB was only 74% of nominal during the critical period of gonadal differentiation. The limit of quantification (LOQ) for atrazine was 0.010 µg/L. It is very difficult to carry out an exposure at the LOQ and WLI should be commended in this effort. Since IGB was unable to maintain the exposure concentrations, multiple evaluations of water chemistry were essential in determining accurate exposure concentrations. Given the LOQ, it probably would have been more appropriate to use 0.050 µg/L as the bracketing concentration; the IGB exposure (~50% of nominal) failed to approach the lower values. In particular, the 0.010 µg/L was less than 0.005 µg/L for tank 7 on day 13 and tanks 4, 6 and 8 on day 20. All dates are within the critical window of exposure. All tanks at the 0.10 µg/L nominal concentration during the critical window (day 13 and day 20) were below 0.10 µg/L and were approximately 0.050 µg/L.

However, since adverse apical effects (i.e., morphological/histological) were not observed at 0.10 µg/L in either lab and the WLI laboratory was able to meet the exposure requirements during the critical window of differentiation for both concentrations, the atrazine exposure concentration was adequately characterized when considering the studies together. Again, one Panel member wondered if this is the reason why secondary effects were not observed at the IGB lab for the 0.10 µg/L nominal concentration.

EPA might consider the merit of using average measured concentrations rather than nominal in their reports. Further, the Panel noted that the establishment of an exposure level at the LOQ is not a good practice, and would recommend that in the future EPA consider establishing guidelines for minimum distances between lower doses and LOQ. Overall, however, using multiple concentration analyses and adequate Quality Assurance allowed appropriate exposure over the range recommended by the 2003 SAP.

Charge Question 5

The Agency described atrazine contamination of negative controls in one out of the two studies and concluded that since the experimental design had twice the number of controls relative to other treatments, the data from these atrazine-contaminated controls could be removed from the analyses without invalidating the statistical interpretation of the results.

(a) Please comment on the Agency's decision to omit half of the controls from the WLI study in the statistical analyses and on the conclusion that the study is still scientifically valid. If the SAP has an alternative approach to treating these control data in the statistical analyses, please provide specific recommendations.

Panel Response

The Panel found no problem with the decision to omit half of the controls from the WLI laboratory that were contaminated with atrazine or algae. Based on the results of the power analysis presented to the Panel, loss of these control clusters did not dramatically affect the power of statistical comparisons. Because the original study design had included twice the

number of control clusters as treatment clusters, the two remaining clusters were more than adequate for comparison purposes. For this reason, this deviation from the original study design did not invalidate the WLI laboratory portion of the DCI study.

No alternative statistical methods were offered that could make use of the omitted data. It was understood that the tank was the unit of analysis for the statistical tests but not the unit of treatment, which was the cluster. There was also an appreciation by the Panel on how practical considerations led to the use of four tanks per pump. While the cause of the atrazine contamination or microbial bloom is not known, the clustered design resulted in all four tanks in each affected cluster being lost at once. This loss would have been far greater had more tanks per pump been used.

Charge Question 6

The original White Paper (USEPA 2003) identified measurement endpoints that included the possible enumeration of specific histological structures such as the number of oogonia in ovaries and the number of spermatids in testes. Such a detailed analysis was not conducted in the studies that are in response to the DCI. Rather, a qualitative assessment of the incidence of ovarian and testicular gonadocytes was conducted. The Agency has concluded that the lack of these data does not limit the means to assess the hypothesis that atrazine exposure affects amphibian gonadal development.

(a) Please comment on whether the lack of these histological data limits the utility of the available information to fail to support the hypothesis that atrazine exposure affects amphibian gonadal development.

(b) If the SAP concludes these data are necessary to adequately assess the hypothesis, please provide options to processing and analyzing these data in an efficient and robust manner.

Panel Response

The Panel's response to Charge Question 6 considers Part a and Part b together.

After review of the study conducted in response to the DCI, the 2007 Panel concurred that the omission of 'the number of oogonia in ovaries and the number of spermatids in testes' in *X. laevis* at stage 66 is acceptable because of the gonadal immaturity at this stage and because of the limited number of cell types available to enumerate. The Panel further concurred with the use of a qualitative assessment in lieu of scoring of the histopathological changes.

The Panel recommended that the Agency revisit the statistical analysis in consultation with the pathologist of record. The Panel noted that combining the scores may be acceptable in some cases, but should not be done without the advice of the pathologist of record. Important points to consider are that some pathological changes may be present in most individuals at a score of 1 as they are commonly observed findings; however, at a score of 2, those same changes may be toxicologically significant, and thus it may not be appropriate to combine scores 1 and 2. Further, the Panel reminded the Agency that although much is known about gonadal development in *X. laevis*, little is known about the biological significance of many of the

histological changes observed in the gonads and, thus, caution is advised when analyzing the endpoints and interpreting their significance.

The Panel further noted that, whereas the data collected address the morphological (anatomical) development of atrazine-exposed *X. laevis* gonads, reproductive function is not addressed. To evaluate whether development was complete (or successful) it is necessary to follow through to the adult stage and assess reproductive fitness (i.e., fecundity). The Panel recognized that this was attempted with the grow-out study mentioned by the registrant, but important parameters such as age and size at sexual maturity were not addressed. The Panel recommended that additional grow-out studies be conducted to address these parameters.

Charge Question 7

The Agency has described a number of measurement endpoints (*e.g.*, translucent gonads, unpigmented ovaries, pigmented testes) based on histology results that were reported in the studies. The Agency, however, based on its understanding of relevant scientific literature, could not conclude that these measurement endpoints are biologically relevant indicators of effects on growth or reproductive success (*i.e.,* the Agency did not interpret these responses as adverse effects *per se*) nor was the Agency aware of any information that established these responses as precursors to the apical endpoints of primary interest (*i.e.*, time to and size at metamorphosis, sex ratio, and the presence of mixed and/or intersex animals). Please comment on the biological relevancy of these endpoints and the extent to which they may reflect reliable measures of developmental abnormalities.

Panel Response

The Panel was not aware of literature that links translucent gonads, unpigmented ovaries or pigmented testes to effects on growth or reproductive success in amphibians. This is not to say that these phenotypes are not associated with fitness consequences; the Panel was not aware whether such relationships have been tested, nor whether a functional significance of melanocytes on internal organs is known in any species. Pigmentation changes associated with exposure to an irritant are commonly observed in diverse species, but the causes or consequences of these changes are not understood.

The Panel noted that the secondary endpoints that were measured would have utility in supporting observations of effects on endpoints that are more clearly related to reproduction. For example, a subtle, but statistically-significant effect of atrazine on feminization of testis might be supported by commensurate observations of altered testis pigmentation. However, atrazine had no discernible effect on the more apical endpoints (sex ratios, intersex, etc.). In the absence of effects on these primary endpoints, the effects noted on the secondary endpoints are of limited value.

The possibility cannot be excluded that effects on the secondary endpoints may, in and of themselves, indicate an adverse effect or may serve as indicators (biomarkers) of adverse effects. However, the Panel was aware of no precedence for any associations between these endpoints and adverse outcomes on reproductive competence.

Charge Question 8

The Agency's analysis of potential developmental effects in studies responsive to the DCI has focused on histological data as opposed to gross morphological data. The histological data from these studies are based on the analyses of a single certified pathologist. While this approach eliminates the potential variability associated with having multiple pathologists analyze the histological slides, it may introduce an avidity bias.

(a) Please comment on whether a single pathologist is sufficient for interpreting the histology data. If the SAP believes that a single pathologist is not sufficient, please comment on the potential value of convening a pathology review board to evaluate the findings of the DCI study.

(b) Please also comment on the potential value of having a pathology review board evaluate materials (*e.g.*, digital images of gross morphology and microscope slides containing histological sections) from studies published in the open literature. These data could be submitted voluntarily by the authors and could include slides to evaluate similarities or differences in identifying or describing histological features and/or describing and quantifying histological responses.

Panel Response

a. The Panel commended Dr. Wolf, the pathologist who reviewed the DCI study slides, for his efforts and acknowledged the paucity of available amphibian pathologists. The Panel further acknowledged that the Agency's intended use of these results is for regulatory purposes and, thus, must conform to GLP with Quality Assurance. To address Quality Assurance, the Panel suggested that two additional (amphibian) pathologists be enlisted to read and score a pre-determined, randomly selected, 'whole animal' subsample of the slide collection. The subsample of slides would be selected from all treatments and controls and randomly presented to the pathologists. This approach will allow for randomization and stratification of the study to reduce bias (real or perceived) in the scoring. A report on the intra- and inter-agreement/variability between reviewers would strengthen the histological data analysis.

b. The Panel recognized that some benefit may be achieved in establishment of a pathology review board to evaluate digital gross images and microscope slides containing histological sections from studies published in the open literature. However, the conclusions generated from such an established board would need to be interpreted with caution. Disparities in collection techniques, choice of fixative (Bouin's or formalin or ethanol), sectioning protocols, and staining techniques may severely limit quantitative and possibly even qualitative comparisons of the data.

Charge Question 9

After an evaluation of the laboratory-based studies submitted in response to the DCI, the Agency has concluded that these studies do not provide sufficient evidence to support the hypothesis that atrazine causes adverse gonadal developmental effects in amphibians.

(a) In light of the responses to Questions 3 – 8, please comment on whether the results from the study in response to the DCI are sufficiently robust to address the

hypothesis that atrazine exposure causes gonadal abnormalities in *X. laevis*. If the SAP concludes these results are not sufficiently robust, what recommendations can the SAP provide to efficiently and reasonably address remaining uncertainties? For example, if the SAP does not believe the DCI study is sufficiently robust to assess the hypothesis, does the SAP believe either the two experiments or a specific component of the two experiments should be reanalyzed or repeated? Please provide the rationale for recommending any additional analyses and/or experiments.

(b) Please comment and provide recommendations on alternate statistical analyses, if any, to evaluate the data derived from the study. If alternative approaches are suggested, please comment, to the extent possible, on the rationale for these approaches and how they represent improvements in the existing statistical interpretations.

Panel Response

a. The Panel noted that Question 9 was somewhat confusing in that it presents two hypotheses, the first referring to "adverse gonadal developmental effects in amphibians", the second, within Part a, referring to "causing gonadal abnormalities in *X. laevis*". In order to provide a clear response the Panel has restated the hypothesis being considered in this question to better reflect the DCI study, as follows:

Restated Hypothesis: Exposure to the parent compound atrazine causes adverse gonadal development in *X. laevis* within the range of concentrations tested (0.010 to 100 µg/L).

Responses to the more general EPA-stated hypothesis concerning adverse gonadal development in amphibians are addressed in Charge Questions 12 and 13.

The Panel concurred that the results are sufficiently robust to test and refute the hypothesis as it is restated immediately above based on the discussions and considerations identified in responses to Charge Questions 3 through 8. In brief the Panel concluded that:

- The study design was appropriate for testing the parent atrazine and it addressed many of the concerns regarding water quality, loading rates, etc. identified by the 2003 SAP. Panel members agreed that the use of the flow-through exposure system and the lack of analyses for transformation products did not allow for testing of hypotheses related to effects of transformation products on adverse gonadal development.
- Parent atrazine exposure concentration profile was well characterized and was sufficient for documenting the potential effects of atrazine over a broad range of exposure concentrations. Actual concentrations were generally stable. Although the Panel had some concerns about low concentrations at the two lower doses during the IGB portion of the study compared to target exposure concentrations, these concerns are balanced by the robustness of the measured concentration data. One Panel member suggested that atrazine analyses should be provided as measured rather than nominal concentrations.
- The Panel generally agreed that primary apical endpoints were well characterized both technically and statistically for the negative control, positive control and atrazine exposed

groups. However, uncertainty remains with respect to the biological relevance of secondary endpoints.

- With respect to the histological analyses, the Panel recommended a verification of results by independent pathologists.

When evaluating the robustness of the data, the Panel also considered the following elements:

- Strength of a concentration-response relationship: The study provided no evidence for a concentration-response relationship between atrazine and primary endpoints such as sex ratios and intersex testes. Results from one lab in the DCI study provided significant evidence for concentration-response relationships with several secondary endpoints (segmental aplasia, gonadal image area, gonadal segmental hypoplasia and gonadal segmental translucence).

- Strength of cause-effect relationship: The effects observed with atrazine were modest despite robust responses in the positive controls. Furthermore, the noted concentration-response relationships were not reproducible between the two separate laboratories that performed the study using the same study protocol.

- Mechanistic plausibility: There is no currently available proof for the predominant hypothesis regarding the purported action of atrazine on the induction of aromatase. While the aromatase gene is inducible in some cell lines by exposure to high concentrations of atrazine, the Panel was aware of no precedent for the induction of aromatase in frogs exposed to atrazine. Failed attempts to induce aromatase in frogs by atrazine have been reported (Murphy et al., 2006, Coady et al, 2005); however, the studies were not conducted at the thermal optimum for the species tested (closer to 25°C vs. test condition of 37°C).

- Ecological relevancy of effect: Endpoints for which weak evidence exists for an effect of atrazine (see: *strength of concentration-response relationships*) are not recognized as being relevant to reproductive fitness. Conversely, endpoints that are more likely to impact reproductive fitness (sex ratios, intersex conditions) were unaffected in animals exposed in the DCI study to 100 µg/L atrazine.

Despite the robustness of the DCI study for addressing the restated hypothesis, several concerns were noted by Panel members. Some Panel members were concerned by EPA's total rejection of its original hypothesis that atrazine causes adverse gonadal development in amphibians based on negative data from the DCI study alone. In part, their concern was based on the uncertainty caused by the relevance of the exposure system to the natural life history of *X. laevis*. They pointed out that the flow-through paradigm is likely not as close to what a *X. laevis* tadpole would experience in the wild where they are commonly found in standing water (including sewer ponds). A static-renewal paradigm may more closely simulate those environmental conditions.

Based on information presented by Syngenta, some Panel members were also concerned about the specific strain of *X. laevis* used in the studies and the apparent differences in the

presence of testicular ovarian follicles (TOF) among different strains of *X. laevis*. It was not clear whether the differences in presence of TOFs are the result of differential sensitivity of the strains or different environmental conditions where the strains are located. The *X. laevis* used in the DCI study were derived from the strain with no reported TOFs. While the DCI study included positive controls, the possibility of differential sensitivity to estrogenic compounds introduces added uncertainty to the interpretation of the results. A comparison of responses to a positive control (i.e., estradiol) by different strains could help reduce the uncertainty.

As noted earlier, the Panel concluded that the current study did not address potential effects caused by exposure to atrazine transformation products (DACT, DEA and DIA). The Panel recommended that the Agency use existing monitoring data which includes information on environmental concentrations of transformation products to determine the extent to which they might want to consider transformation products in future evaluations. In addition, a literature search should be conducted to determine if information exists on the potential for transformation products to interact with the endocrine system (e.g., receptor binding assays, etc.).

b. The Panel agreed that the statistical procedures used to analyze the data of the DCI study are, on the whole, appropriate. In some instances, alternative methods of analysis are possible.

The Panel recommended that, if possible, an analysis of the combined data from the WLI and IGB laboratories be conducted. The usual procedure to analyze data from a study (experiment) that is repeated two or more times involves two stages. In the first stage each study is analyzed and interpreted separately. The analysis of the combined data from the two studies in the second stage results in more powerful tests than analyses of the studies separately. Thus, for example, a treatment effect may be established in the combined analysis but not in the analysis of either study by itself. More importantly, only analysis of the combined data allows research questions like the following to be tested and interpreted.

- Do differences between controls and levels of atrazine differ in the two studies (labs)?
- Do potential atrazine dose-response relationships differ in the two studies?
- Does the unexplained variability (experimental error) in the two studies differ? If so, why?

When two or more studies are repeated, analysis of the combined data extracts maximum information.

The Panel recommended the nature and properties of the experimental units used in the DCI study be reviewed. Experimental unit denotes the group of material to which a treatment is applied in a single trial of an experiment (Cochran and Cox, 1957.) They noted three reasons for such a review. First, the choice of unit, along with the method of randomization of treatments to units, determines how the experimental error for the experiment is to be computed. Failure to properly identify the experimental unit results in a biased estimator of experimental error and incorrect tests. Second, measurements reflect properties of the units as well as the effect of the treatments, and therefore, may be important in interpreting the effects of treatments. Also,

lessons learned from performance of the units in the DCI study may be used to improve experimental designs in future studies using similar protocols.

The Panel recommended the use of confidence intervals to interpret tests of hypotheses. A test of the hypothesis of no difference is seldom realistic. Because a confidence interval provides an estimate of the size of an effect, it is generally more useful in experimental work than a test of significance.

The high quality of data collected and the previous mention during this SAP meeting of the idea of developing a standard protocol, led the Panel to also suggest and recommend that more sophisticated statistical methods be considered. Specifically, the data analysis presented for the DCI study reveals no information on any associations that may or may not be present between the measured variables. Also, there should be more consideration given towards the male/female ratio in the tank. From the data reported in the DCI study, it is observed that the differences between male and female means are significant in several of the endpoints. However, the overall tank means are not weighted or standardized for this differential (i.e. reported data could be influenced by having a skewed male/female ratio). These two methods can be approached by transposing the data set into a multivariate data set where each larva and its corresponding measurements are the units of analysis. This is not such a stretch considering that the individual tanks have been accepted to be the unit of analysis. By using each larva as an observation, one can incorporate all measurements to that sample from which it was measured, as well as incorporating the observed sex of each sample at metamorphosis. This would enable a multivariate approach such as a MANOVA (multivariate analysis of variance) or even a canonical analysis, and hence, a better understanding of possible associations and correlations between the measurements and observations within and across treatment effects, and would also enable the easy inclusion of the male/female as an appropriate measured variable.

This said, it is unlikely that this more complex analysis will find additional significant results. What it has the potential to do is demonstrate where observed significant effects might be the result of common underlying processes.

Concluding Questions

Charge Question 10

Is the SAP aware of any other laboratory-based or field-based studies not included in this White Paper that contradict the Agency's conclusions that 1) the designs associated with current studies available in the open literature are not appropriate for evaluating the hypothesis that atrazine affects amphibian gonadal development and 2) the available data in the open literature combined with the results of DCI study indicate that atrazine does not cause adverse effects on gonadal development in *X. laevis* when investigated under conditions consistent with those recommended by the SAP in its previous report (SAP 2003). If so, please identify the studies and briefly outline how the results from these studies would contradict the conclusion that atrazine at concentrations up to 100 µg/L does not cause adverse effects on amphibian gonadal development.

Panel Response

There are two subquestions in this charge. The first, restated, is: *are there studies published in the open literature that challenge the design of the study submitted in response to the DCI?* Such studies might, for example, show that *X. laevis* does not perform well under flow-through conditions; or that, initiating exposures at stage 46 limits the sensitivity of the tadpoles to agents that influence sex differentiation. The Panel was not aware of any studies that would call into question the design of the DCI study.

The second question, restated, is: *are there studies published in the open literature that were performed according to design recommendations provided by the 2003 SAP that challenge the conclusion that atrazine does not cause adverse effects on gonadal development of X. laevis at exposure concentrations as high as 100 µg/L?* The Panel was not aware of any such studies.

Charge Question 11

The Agency is not aware of data that establish a mechanistic basis for how atrazine could affect amphibian gonadal development. Please identify and comment on any studies that demonstrate the mechanistic steps by which amphibian gonadal development could be affected by atrazine, and thereby contradict the Agency's overall conclusions based on the studies evaluated for this SAP review. If the SAP is aware of any relevant study(ies), please comment on the data from this study(ies) and how the data indicate and quantify a mechanistic pathway from atrazine's molecular site of action to histological and apical endpoints associated with adverse effects on amphibian gonadal development. Please also comment on any dose-response relationships associated with the steps in the reported toxicity pathway.

Panel Response

The hypothesis most often cited in the open literature for purported estrogenic effects of atrazine on amphibian gonadal development is that the compound increases aromatase gene expression and/or activity during the critical period of gonadal development. Such an effect could result in a shift in gonadal sex steroid biosynthesis in genetic males from testosterone to estradiol. Published evidence for this has been indirect, and includes studies such as those comparing the reported effects of atrazine to the effects of androgen receptor antagonists and estrogen receptor agonists, or reported reductions in plasma testosterone which appear to be the most consistently reported effect of atrazine in the literature. The Panel concurred with the EPA analysis that the best evidence in support of the aromatase hypothesis remains the published studies in cultured human cell lines. Direct evidence for induction of aromatase by atrazine *in vivo* in amphibians is lacking. The Panel is not aware of any studies that have examined the expression of the aromatase gene, or activity of the enzyme in the presumptive gonads of tadpoles during the key developmental stages at which atrazine is proposed to act. Furthermore, aromatase assays that have been conducted on tissues from adult amphibians that have been exposed to atrazine were done at 37° C while the thermal optimum for *X. laevis* is approximately 25° C (see response to Charge Question 9). These studies, which reported no effects of atrazine on aromatase enzyme activity in adult amphibian tissues, are suspect owing to the thermal conditions used in the experiments.

Some Panel members indicated that there are recent papers regarding the aromatase hypothesis not discussed in the open literature review, again using cell culture based assays, that should be briefly mentioned. First, aromatase activity (but apparently not protein levels) can be induced by atrazine two to three fold in cultured primary human granulosa cells; therefore, this effect is not limited to transformed cancer cell lines (Holloway et al., in press). Second, a pair of papers presented data that demonstrate that atrazine can activate aromatase gene expression in an SF-1 dependent manner. SF-1 is an "orphan" nuclear receptor known to be an important physiological regulator of aromatase gene expression in vertebrates. The authors also suggest that atrazine acts as a direct agonist ligand for SF-1, but the exact nature of the interaction is unclear (Fan et al., 2007a and 2007b).

In general, the atrazine concentrations used to induce aromatase activity in cell lines are higher than those reported to cause gonadal abnormalities in the open literature, although significant induction can be observed in both sets of studies cited above with as little as 10^{-7} M atrazine. The dose response curves in both studies are monotonic rather than U shaped, as expected for a simple mass action driven interaction.

Some Panel members also asserted that it is possible that atrazine may act by alternate mechanisms other than induction of aromatase gene expression and/or activity which may be responsible for effects on *X. laevis* gonadal development under certain conditions. For example, SF-1 has gene targets other than aromatase and is expressed in the hypothalamus as well as the adrenals and gonads. However, the importance of a role for atrazine-activated SF-1 activating genes other than aromatase in regulating gonadal morphology is speculative; therefore, at this time, there are no new data on potential mechanisms of atrazine effects not mentioned in the 2003 White Paper other than the aforementioned cell culture experiments. These new results should be considered by the EPA, but in and of themselves are insufficient to explain atrazine's potentially detrimental effects on *X. laevis* gonadal development, if they exist at any dose.

Charge Question 12

In its 2003 White Paper the Agency proposed a research approach using focused, empirical laboratory studies based on initial investigations with *X. laevis,* potentially followed by selective, confirmatory laboratory studies with frog species native to North America. However, the 2003 SAP did not identify any important differences between amphibian species to conclude that any affected developmental and/or mechanistic processes observed in *X. laevis* would not be applicable to indigenous ranid species. Please comment on the Agency's recommendation that data derived from *X. laevis* in the studies evaluated for this review are sufficient to conclude that additional testing with indigenous species is not warranted.

Panel Response

In response to a comment by one EPA presenter noting the challenges of having responsibility to protect thousands of species based on environmental toxicity data from a small number of surrogate species, a number of Panel members stated that they understood the challenges involved in performing risk assessments and the need for using surrogate species. However, the Panelists also believed that the questions being asked of them needed to be answered from a scientific perspective.

From this scientific perspective, the Panel disagreed with the Agency's recommendation that data from the DCI study are sufficient to conclude that testing with native North American species is not warranted. The Agency's decision is based on the presumption that *X. laevis* is a suitable surrogate for native North American species. However, there are reasons to question such a conclusion. Unlike North American species, *X. laevis* is a fully aquatic amphibian in both larval and adult stages. Aspects of its biology are suggestive of paedomorphosis; that is, the retention of larval characters in the adult form. Again, this is unlike North American anurans. These and other points were also raised during the 2003 SAP meeting. The Agency's question to this SAP suggests an interest in a more specific response.

As one example of a comparative study between *X. laevis* and a native species, *Rana pipiens*, Glennemeier and Denver (2002) reported on the development of responsiveness to stressors by the hypothalamo-pituitary-interrenal (HPI) axis. During development, tadpoles of different stages were subjected to one of two stressors (shaking or injection of adrenocorticotropic hormone [ACTH]) or a control treatment. The investigators then measured whole body corticosterone concentrations as an index of HPI activity. The patterns of whole body corticosterone content during development differed strongly between species. Corticosterone content in *R. pipiens* was low during premetamorphosis and prometamorphosis, increasing greatly during metamorphic climax. By contrast, corticosterone content was at its maximum in *X. laevis* during premetamorphosis, declined in prometamorphosis and increased again during metamorphic climax. Further, while both species responded to experimental stressors, the pattern of response differed. As an example, elevation of corticosterone content in response to ACTH injection was maximal in *R. pipiens* in premetamorphic stages and decreased in later stages. In *X. laevis,* elevation of corticosterone in response to ACTH did not differ statistically among stages. These results suggest that sensitivity to stressors may have a different temporal pattern relative to other developmental processes, such as gonadal development or other critical developmental pathways.

In the following quotation from the discussion in the paper, the authors compare the responses of their focal study species to other North American species that have been studied: "While changes in whole body corticosterone content in *R. pipiens* follow those observed in the blood of other species, *X. laevis* exhibits a somewhat different pattern. Our findings with *X. laevis* largely confirm those of Kloas and colleagues (1997) who reported whole-body corticosterone content to be highest at early limb bud stages but decreased to lower values during prometamorphosis." Panel members have heard evidence that the tendency to form testicular ovarian follicles may differ among populations of *X. laevis*. Based on our knowledge of variation among species in response to environmental stressors, it is reasonable to predict that interspecies differences in response to stressors will exist when important endpoints are measured. Concerns about ecological relevancy to North American species and ecosystems prompted the 2003 SAP to suggest that studies of native species be carried out as early as possible. Those concerns remain.

The 2003 SAP concluded that a *Rana* species should be used as a further test species. The choice of *X. laevis* as a primary biological model is well justified since developmental and sexual differentiation of *X. laevis* have been extensively studied and the published literature is extensive. This is not the case for any North American species. However, the primary ecological concern for the Agency remains for the 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 for *X. laevis* 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.

Several Panel members in 2003 stated that there is little or no evidence to demonstrate that there are significant differences between *Rana* or *Xenopus* in basic 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. EPA noted that surrogate species are used for all toxicity tests; however, one Panel member noted that, except for *X. laevis*, typical current surrogate species are native to North America. The Panel further noted that the widespread occurrence of atrazine in water might be considered when determining whether or not information related to native species is needed.

Gonadal differentiation in two native frog species exposed to estrogenic and antiestrogenic compounds has been examined (Mackenzie et al., 2003). These investigators assessed whether the native ranids would respond to exogenous compounds that might influence gonadal development before pesticide exposures were attempted with these species. The study assessed the response of two native North American amphibian species, the northern leopard frog (*Rana pipiens*), and the wood frog (*Rana sylvatica*), exposed as tadpoles to estradiol, ethinylestradiol, nonylphenol, an aromatase inhibitor, flavone, and an antiestrogen. After assessing various endpoints histologically it was concluded that *R. pipiens* appears to be much more susceptible to sex reversal and development of intersex gonads following these exposures than was *R. sylvatica*. While the wood frogs showed alterations in their gonads, these were less dramatic than in the northern leopard frogs. One Panel member wondered if studies on an additional species would satisfy a future panel. In response, other Panel members noted that, as a strategic decision from the Agency's perspective, future studies based on a North American species will be less susceptible to criticisms related to ecological relevance. Another Panel member expressed appreciation for the relevance of data from a North American species, but also expressed concern that unless the culture and bioassay conditions for a North American amphibian are sufficiently well established, such that regulatory quality data could be acquired, then the requirement at this time for additional tests on a North American amphibian might be premature.

In an effort to provide additional information on interspecies variation among amphibian species, a list of relevant studies can be provided (Bridges, 1997, 1999a, 1999b, 2000, Rouse, et al., 1999). This list is not intended to represent an exhaustive list of papers on this topic but is provided as an introduction to the field. For instance, there is a wide variation in tolerance levels among amphibians, even between closely related species (Bridges et al. 2002). Therefore, conclusions drawn from studies on only a few species cannot reveal the full effects of potentially harmful chemicals to amphibians in general (McDiarmid and Mitchell, 2000). Several published articles have reported differences in the responses of several amphibian species to exposure to acid rain and heavy metals in the form of coal ash (Rowe et al., 1996; Rowe et al., 1998; Rowe et al., 2001; Hopkins et al., 1997; Harte, 1989; Beebee et al., 1990; and Clark and LaZerte, 1987). In addition, the table in Appendix 1 illustrates the range of 96 hour LC_{50} values for two amphibians exposed to carbaryl, copper, PCP or permethrin, and how those values compared to other commonly tested aquatic vertebrates (modified from Bridges et al. 2002).

To the Panel's knowledge, there are few, if any, papers that document response differences to environmental stressors (in the wild) between *Rana* spp and *Xenopus*, but given existing information in the open literature as cited above, it is reasonable to expect that there may be, and that conclusions drawn from studies only on laboratory *Xenopus* cannot fully reveal all of the effects of potentially harmful chemicals on amphibian species in general. Given these discussions, the Panel, from a scientific perspective, disagreed with EPA's recommendation that data from the DCI study are sufficient to conclude that testing with a native North American species is not warranted.

Charge Question 13

Based on the available data provided by the DCI studies, the Agency has concluded that atrazine does not adversely affect amphibian gonadal development. The Agency has further concluded that no additional studies are required to address the hypothesis that atrazine adversely affects amphibian gonadal development. Please comment on the Agency's recommendation that the current body of data is sufficient to refute the hypothesis that atrazine by itself can adversely affect amphibian gonadal development and that no additional data are required to address this hypothesis.

Panel Response

The general consensus of the Panel was that the current data alone are not sufficient to refute EPA's hypothesis that atrazine by itself can adversely affect amphibian gonadal development. As noted in response to Charge Question 9, the Panel concluded that the DCI study is sufficient to test and refute the hypothesis that atrazine by itself can adversely affect *X. laevis* gonadal development under the conditions tested. However, the data do not adequately address the broader hypothesis that atrazine affects gonadal development of all amphibians. The Panel concluded that the assumption that *X. laevis* is a suitable surrogate species for all amphibians is not well-supported at this time. To determine whether *X. laevis* is an appropriate model species requires comparison to a native North American species.

During discussion of this charge question, the Panel identified a number of concerns/uncertainties regarding the assumption that the DCI study is sufficient to encompass potential effects in all amphibians. The concerns/uncertainties identified by the Panel, in keeping with their function, are from a scientific perspective and, therefore, may not be consistent with those from a regulatory perspective. Some of the concerns/uncertainties identified were related to the general topic of the relation between surrogate species and the species they represent. Other uncertainties were specific to the chemical in question, atrazine. The points below outline the uncertainties that the Panel discussed and considered.

***Xenopus laevis* as a surrogate species** - The Panel acknowledged the use of surrogate species as an efficient, logical and accepted risk assessment approach. It further acknowledged that toxicity data related to amphibians is not currently a specified data requirement for pesticide risk assessments, and recognized the challenges faced by the Agency with respect to obtaining quality toxicity data for amphibians, especially in light of currently available protocols, knowledge and expertise related to native amphibians. However, from a scientific perspective, the Panel noted that unlike other toxicity tests or surrogate test organisms, an existing body of

knowledge and research related to effects in other amphibian species for a range of other chemicals does not exist to support this type of assumption at this time.

- Uncertainty remains as to where in the species sensitivity distribution *X. laevis* lies with respect to other species (in particular native North American species), in the types of gonadal abnormalities of concern in this case, and across a range of chemicals/stressors.
- There are differences in species ecology which could affect outcomes.
- *X. laevis* is not a species native to North America.

Additional information which was considered came from other species where responses to exposure to atrazine have been shown to differ. For example, experiments with rats and mice have demonstrated a well-studied difference in carcinogenic response to atrazine. Traditional 2-year cancer bioassays showed an increase in mammary tumors in Sprague-Dawley rats receiving atrazine in their diet. This response differed in magnitude and tumor type from the response of Fischer rats and from the complete absence of a carcinogenic response in mice. Further studies have suggested that this is the result of a strain specific differential phenotypic response to atrazine-induced interference with gonadotropin releasing factors from the hypothalamus.

These uncertainties with respect to the assumption of representativeness of the test species are not to say *X. laevis* is not a good surrogate species, but that the necessary supporting data are not available at this time. Some Panel members recognized that generating the necessary data to allow a thorough science-based comparison will take time and should be considered a longer-term goal. At a minimum, consistent with the recommendations of the 2003 SAP, and the response to Charge Question 12, the Agency should consider testing for effects of atrazine using North American native species to reduce the uncertainty.

Atrazine - With respect to atrazine, a variety of concerns/uncertainties were identified. As noted by one Panel member, all well-executed studies open more questions than they answer. Some of the questions which resulted from the DCI study include the following:

- Were the subtle effects noted in the IGB laboratory truly indicative of an effect of atrazine or were they chance, random responses?
- Do atrazine metabolites/transformation products have any adverse effects that were not noted in the flow-through study due to dilution/removal of these products? As previously discussed in the response to Charge Questions 3 and 9, the current study design did not allow for thorough testing of effects from transformation products which are found in environmental samples, nor was an analysis of the potential for exposure to transformation products of larval amphibians undertaken.
- Are there environmental variables (e.g., nitrates, other water quality parameters) that modify the toxicity of atrazine resulting in differences between labs?
- The Panel recognized that there are both direct effects (effects on the organism itself, such as acute toxicity) and indirect effects (effects on the organism that are caused by an impact to some element of its habitat, such as food or prey base) from atrazine to consider. The indirect effects involve both the effects of transformation products on gonadal development as well as the interaction of atrazine (or its transformation products) with other chemicals typical to the agricultural environment that many frog species

inhabit. Some Panel members noted that assessment of the effects of atrazine on amphibian gonadal development could include both direct and indirect effects.

At a more general level, and in a broader context, it was noted that the 2003 SAP considered two primary lines of evidence in making its recommendations. The first involved laboratory-based evidence that atrazine exposure was related to abnormal gonadal development and other responses, providing some plausible, albeit confounded, results to support the general hypothesis the DCI study tested. The second line of evidence was based on the detection of gonadal abnormalities in wild populations of amphibians. Since 2003, very little new evidence has emerged to evaluate the role of atrazine in producing these abnormalities which are heterogeneous in space and, in some cases, apparently related to gradients of exposure to atrazine and other pesticides. Given the possibility of interspecies differences in response to atrazine exposure, the lack of study on native North American species means that the role of atrazine in producing abnormal development in field populations of native North American species remains unknown. Even if the Agency concludes that laboratory studies provide no basis for further exploration of the atrazine hypothesis, these observations of natural populations remain unexplained.

As noted earlier in the response to this question, toxicity testing of amphibians is relatively unexplored compared to testing with other major taxonomic groups. It is evident that there will be a continuing need to assess the potential effects of pesticides and other chemicals on amphibians given the growing awareness and concern of the scientific community and the public. At several points during the Panel's discussions, the need for basic research on amphibian ecology was noted, as was the need to examine background levels of effects in native species.

Ultimately, decisions must be made as to whether the strength of answers to the major questions asked in comparison to the perceived relevance of the unanswered questions justify decision making with no call for additional data. From the scientific perspective, the Panel agreed that the relevance of the uncertainties justifies the generation of additional data.

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APPENDIX 1

96 Hour LC_{50} Values for Amphibians and Aquatic Vertebrates Exposed to 4-nonylphenol, Carbaryl, Copper, PCP or Permethrin

Chemical	**Southern Leopard frog tadpoles** (*Rana sphenocephala)*	**Boreal toad tadpoles** (*Bufo boreas)*	**Bluegill Sunfish** *(Lepomis macrochirus)*	**Fathead minnow** *(Pimephales promelas)*	**Rainbow trout** *(Oncorhynchus mykiss)*
4-nonylphenol (mg/L)	0.34 (0.31-0.37)	0.12 (0.09-0.15)	N/A	0.27	0.19
carbaryl (mg/L)	8.4 (7.4-9.6)	12.31 (10.3-14.7)	6.2	5.21	1.88
copper (mg/L)	0.23 (0.21-0.25)	0.12 (0.07-0.18)	7.3	0.47	0.88
PCP (mg/L)	0.14 (0.12-0.17)	0.37 (0.25-0.42)	0.192	0.25	0.016
Permethrin (µg/L)	18.2	>10	6.2	9.38	3.31