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

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

July 14, 2010

MEMORANDUM

SUBJECT: Transmittal of Meeting Minutes of the FIFRA Scientific Advisory Panel Meeting held April 26 - 29, 2010 on the Re-evaluation of the Human Health Effects of Atrazine: Review of Experimental Animal and In Vitro Studies and Drinking Water Monitoring Frequency

TO: Steven Bradbury, Ph.D.
Director
Office of Pesticide Programs

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

Joseph E Bailey 7/14/10

THRU: Laura Bailey, Executive Secretary
FIFRA Scientific Advisory Panel
Office of Science Coordination and Policy

Laura Bailey 7/14/10

Frank Sanders, Director
Office of Science Coordination and Policy

7/14/10

Attached, please find the meeting minutes of the FIFRA Scientific Advisory Panel open meeting held in Washington, DC on April 26 - 29, 2010. This report addresses a set of scientific issues associated with the Re-evaluation of the Human Health Effects of Atrazine: Review of Experimental Animal and In Vitro Studies and Drinking Water Monitoring Frequency.

Enclosure

cc:

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SAP Minutes No. 2010-04

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

Re-evaluation of the Human Health Effects of Atrazine: Review of Experimental Animal and *In Vitro* Studies and Drinking Water Monitoring Frequency

April 26 – 29, 2010
FIFRA Scientific Advisory Panel Meeting
Held at the Hamilton Crowne Plaza Hotel
Washington, DC

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.

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Re-evaluation of the Human Health Effects of Atrazine: Review of Experimental Animal and *In Vitro* Studies and Drinking Water Monitoring Frequency

April 26 – 29, 2010
FIFRA Scientific Advisory Panel Meeting
Held at the Hamilton Crowne Plaza Hotel
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Kenneth Portier, Ph.D.
FIFRA SAP Session Chair
FIFRA Scientific Advisory Panel
Date: July 14, 2010

Joseph E. Bailey
Designated Federal Official
FIFRA Scientific Advisory Panel
Date: July 14, 2010

Federal Insecticide Fungicide and Rodenticide Act
Scientific Advisory Panel Meeting
April 26 – 29, 2010

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INTRODUCTION

The Federal Insecticide, Fungicide and Rodenticide Act Scientific Advisory Panel (FIFRA SAP) has completed its review of the Reevaluation of the Human Health Effects of Atrazine: Review of Experimental Animal and *In Vitro* Studies and Drinking Water Monitoring Frequency. Advance notice of the meeting was published in the *Federal Register* on March 5, 2010. The review was conducted in an open panel meeting held in Washington, DC, on April 26 – 29, 2010. Dr. Kenneth Portier chaired the meeting. Joseph E. Bailey served as the Designated Federal Official.

EPA is undertaking a re-evaluation of the human health effects of atrazine. The re-evaluation plan was presented to the FIFRA Scientific Advisory Panel (SAP) in November 2009 and involves three SAP meetings in 2010. The first was held in February 2010 where the Agency presented its preliminary reviews of several atrazine epidemiology studies on birth outcomes and described a project plan to evaluate atrazine epidemiology data from the Agricultural Health Study (http://aghealth.nci.nih.gov/). The second meeting, held in April 2010, focused on 1) a preliminary review of mammalian *in vivo* as well as *in vitro* studies on toxicological effects and modes of action, and 2) statistical approaches for evaluating monitoring frequency in community water systems (CWS).

For the April SAP meeting, the Agency's scientific review of toxicology studies focused on those studies used to support the reregistration of atrazine and new studies available up to January 30, 2010. Any studies that become available after January 30, 2010 will be integrated into the Agency's weight of the evidence (WOE) analysis scheduled for review by the FIFRA SAP in September 2010. This WOE analysis will integrate *in vivo* and *in vitro* laboratory studies along with epidemiologic data and other human information.

At the April meeting, the Agency solicited comments from the SAP on the toxicological importance and human relevance of a variety of effects including findings on the hypothalamic-pituitary-adrenal (HPA) axis, immune system, prostate, brain, and the enzyme aromatase. In addition, the Agency discussed the scientific issues associated with lifestage susceptibility and linking a chemically-induced activation of the HPA axis to other health outcomes and possible approaches to use key events in a mode of action in risk assessment. Following the April SAP, the Agency will continue to review the scientific literature with particular emphasis on the potential for differential lifestage susceptibility and the temporal aspects of pharmacokinetic and pharmacodynamic events. This temporality is an important scientific issue for the assessment of the health effects of atrazine because the duration of exposure leading to adverse effects is a critical aspect of determining the appropriate frequency of water monitoring in CWS, which will also be discussed in September.

As a condition of reregistration of atrazine, EPA required the registrants to implement a monitoring program in selected CWSs, generally in the corn and sorghum growing area of the US Midwest. In implementing a monitoring program, the frequency of sampling is closely linked to the health effects of concern. If the health effects of concern relate to long-term chronic exposures, a yearly average concentration is sufficient. Less frequent sampling may

provide sufficient data for EPA to be reasonably confident that the results are representative of a yearly average concentration. However, for effects that can occur after shorter exposure durations, more frequent sampling may be needed in order to be confident that the sampling has not missed a high-end or peak exposure that could significantly affect the estimate of short term exposure. Proposed statistical approaches to determine the appropriate monitoring frequency for different durations of interest were discussed at the April meeting. The Agency will be evaluating the temporality of toxic effects as it conducts this reevaluation of atrazine.

Opening remarks at the meeting were provided by Steven Bradbury, Ph.D., Acting Director, Office of Pesticide Programs and Tina Levine, Ph.D., Director, Health Effects Division, Office of Pesticide Programs. Agency presentations were given by Anna Lowit, Ph.D., Elizabeth Mendez, Ph.D. and John Liccione, Ph.D., Health Effects Division; Nelson Thurman, M.S., and Mary Frankenberry, Environmental Fate and Effects Division, all of the Office of Pesticide Programs. Agency presentations were also given by Ralph L. Cooper, Ph.D., Toxicology Assessment Division, and Robert Luebke, Ph.D., Environmental Public Health Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development.

PUBLIC COMMENTS

Oral Statements were presented as follows:

Jere White, Laura Knoth and Richard Fawcett, Ph.D. on behalf of the Triazine Network
Charles Breckenridge, Ph.D., James Simpkins, Ph.D., Robert Handa, Ph.D., Steve Pruett, Ph.D., James Swenberg, Ph.D., Paul Hendley, Ph.D., Tim Pastoor, Ph.D., Robert L. Sielken, Jr., Ph.D. and Paul Mosquin on behalf of Syngenta Crop Protection
Alan Roberson, P.E., on behalf of the American Water Works Association
Jennifer Sass, Ph.D., on behalf of the Natural Resources Defense Council
Scott Slaughter on behalf of the Center for Regulatory Effectiveness
Jay Vroom on behalf of CropLife America

Written Statements were provided by:

Anonymous
Dan Campbell on behalf of Syngenta Crop Protection
Jennifer Sass, Ph.D. on behalf of the Natural Resources Defense Council
M.J. Quinn

List of Acronyms Used

ACTH – Adrenocorticotropic Hormone
AMP – Atrazine Monitoring Plan
ANN – Artificial Neural Networks
AUC – Area Under the Curve
AVP – Arginine Vasopressin
BCR – Bromocriptine
BIC – Bayesian Information Criterion
BMD – Benchmark Dose
cAMP – Cyclic Adenosine Monophosphate
Cmax – Maximum Concentration
CNS - Central Nervous System
CORT – Corticosterone
CREB – cAMP-Regulatory Element Binding Protein
CRF – Corticotropin-releasing Factor
CRH – Corticotropin-releasing Hormone
CWS – Community Water System
CYPs – Cytochrome P450 monoxygenases
DA – Dopamine
DACT – Diamino-s-chlorotriazine
DE – Desethyl Atrazine
DEA – Deethylatrazine
DIA – Deisopropylatrazine
DIP – Desisopropyl atrazine
DMBA - 7, 12-Dimethylbenz(a)anthracene
EtOH – Ethanol
FSH – Follicle Stimulating Hormone
GnRH – Gonadotropin Releasing Hormone
GP – Gaussian Process
GSH - Glutathione
GRE – Glucocorticoid Response Element
HPA – Hypothalamic-Pituitary-Adrenal
HPG – Hypothalamic-Pituitary-Gonadal
IL - Interleukin
IP - Intraperitoneal
IRED - Interim Reregistration Eligibility Decision
LH – Luteinizing Hormone
LOC – Level of Concern
MOA – Mode of Action
NE – Norepinephrine
NK - Natural Killer
P4 – Progesterone
PBPK - Physiologically-based Pharmacokinetic
PDE – Phosphodiesterase
PK – Pharmacokinetics
PoD – Point of Departure
POMC – Proopiomelanocortin
PP1 – Protein Phosphatase 1
PRL – Prolactin

PTU – Propylthiouracil
PVN - Paraventricular Nucleus
RXR – Retinoid X Receptor
SF-1 – Steroidogenic Factor-1
T – Testosterone
TH – Thyroid Hormone
WARP – Watershed Regression on Pesticides
WOE - Weight of the Evidence

SUMMARY OF PANEL DISCUSSION And RECOMMENDATIONS

Charge Issue 1.1 – Atrazine Cancer Classification

In 2003, the human health risk assessment was based on an observed reduction in the release of gonadotropin releasing hormone (GnRH) from the hypothalamus following dosing of female Sprague Dawley rats with atrazine. This lessens the afternoon pituitary luteinizing hormone (LH) surge, leading to a lengthening of the estrus cycle that increases estrogen levels that in turn is associated with an increased incidence of mammary tumors in this rat model. Since this MOA (premature reproductive aging) in the Sprague-Dawley rat is not likely to be operative in humans, EPA concluded that the cancer classification of atrazine was *"not likely to be carcinogenic to humans."* The SAP agreed with the Agency that this remains appropriate since the evidence indicates that the non-genotoxic MOA in experimental animals does not operate in humans. The Panel agreed that more recent information regarding possible atrazine adverse effects on hypothalamic-pituitary function (e.g., sub-lethal reproductive and/or developmental effects) does not affect the previous conclusions concerning the cancer MOA.

While the Panel was not aware of any data implying that excess corticosteroid production (due to atrazine exposure) might result in cancer, one exception to this conclusion could be the possibility of cancer development (particularly skin cancer, lymphoma, cervical cancer, and liver cancer) due to immunosuppression. This was identified as a potential carcinogenic mechanism that should be included when EPA considers carcinogenic endpoints in the integration of epidemiological and laboratory studies as part of a weight of evidence (WOE) approach to identifying hazard. Some panel members advocated that the possible role of atrazine as a tumor promoter (rather than initiator) should be included in the same future considerations.

Charge Issue 1.2 – Mode of Action Involving HPA and HPG Axes

The Agency proposed a preliminary hypothesis that atrazine affects both the HPA and HPG axes, where changes in the former precede those in the latter. The current position of the Agency was examined in detail by the Panel and on the basis of studies using single acute exposures to atrazine, it was considered highly likely that the HPA is the first system to respond. However, the evidence suggests that corticosterone does not mediate the effect of atrazine on this endpoint. The data obtained using repeated or chronic administration of atrazine lack coherence, and the transient increase in profile on the final day following daily dosing over 28 days with a high dosage was indistinguishable from that resulting from a single administration. Evidence on the effects of stress and raised corticosterone levels on the mechanisms regulating the LH surge in sheep and humans further strengthened the view that disruption of normal HPA and HPG function may involve upstream events that separately affect both axes. The Panel believed that the Agency's hypothesis regarding HPG axis disruption by HPA- mediated mechanism(s) was premature, and that significantly more work needs to be done before the hypothesis can be accepted or rejected. Events that perturb the neuroendocrine system operate on different time scales in different species, and this makes it difficult to assess the critical windows of exposure in humans on the basis of data from rodents.

Charge Issue 1.3 – Direct Action on HPG/HPA Axes vs. Non-Specific Stress Response

The Agency presented a preliminary hypothesis that atrazine and its mono-dealkylated metabolites induce changes in the HPA axis due to a direct action on HPA components, and that the observed effects are not a non-specific response to stress. The Panel suggested that the differences between the profiles of responses to physical stress and exposure to atrazine did not provide strong evidence that the observed responses to the latter are due to HPA-specific events. Interpretation of data on the habituation of the corticosterone response was difficult because of the design of the experiments. It was strongly recommended by the Panel that basal levels of corticosterone and of other HPA-linked hormones are obtained prior to treatment, and then at various times during any subsequent testing of the effect of atrazine on HPA function. This would enable distinction between the various alternative hypotheses.

Overall, the Panel agreed that there is insufficient evidence for the EPA to conclude that atrazine works directly on HPA axis tissues in order to stimulate the hormonal responses seen in some of the studies. The Panel considered alternative explanations, including direct action on peripheral tissues, inhibition of phosphodiesterase activity, elevation of thyroid hormone levels and reduction of growth hormone levels. Further, it was noted that the doses of atrazine used to elicit the effects observed were extremely high, and unlikely to occur in a natural setting. The Panel noted that the data from the studies investigating the effects of atrazine on HPA axis function were obtained using different species and sexes, so it is difficult to extrapolate findings from one set of studies to the other. It would therefore be advisable to select a single model system for future work. Despite the weaknesses in the studies, the evidence that atrazine and its metabolites activate the HPA axis is strong, as is the correlation between atrazine-induced corticosterone secretion and reproductive dysfunction, and this warrants concern regarding the effects of this herbicide on human and animal health. The possibility of direct action on the brain or pituitary needs to be further investigated before a conclusion can be made regarding the mode of activating HPA-linked hormone secretion. Other small, non-polar molecules (e.g., simazine and propazine) that have structures and metabolites similar to those of atrazine have been shown to activate HPA function in a similar manner to that of atrazine, but with lesser potency.

Charge Issue 1.4 – Effect on Steroidogenesis vs. Aromatase.

The Agency proposed that there is a general stimulatory effect of atrazine on steroidogenesis rather than a direct effect on aromatase. The Panel advised that aromatase gene expression is under different regulatory controls in different tissues (ovary, adipose tissue, placenta). This tissue-specific regulation stems from the existence of multiple promoter regions that control expression and the differential production of estrogens in response to different and diverse stimulatory factors (including gonadotropin, cyclic adenosine monophosphate (c-AMP), cytokines, glucocorticoids, retinoids). The Panel was not aware of any evidence that atrazine binds directly to the aromatase enzyme to enhance its activity through allosteric modification. There is little *in vivo* evidence of elevated aromatase in rodents exposed to atrazine; rather, elevated levels of estradiol and estrone could be due to raised levels of precursors, decreased

catabolism of steroids or decreased breakdown of cyclic nucleotides by inhibition of phosphodiesterase . The Panel concluded that there is significant evidence that atrazine has the potential to regulate gonadal CYP19 expression, and it is plausible that it stimulates adipose CYP19 expression through its activation of the HPA axis. In order to ascertain whether the effects of atrazine on aromatase gene expression represent a generalized effect on steroidogenesis mediated via SF-1 and cAMP, it is necessary to investigate the potential interaction of atrazine, and metabolites, with other known regulatory sites of aromatase.

Charge Issue 1.5 – Mode of Action and Decrease in Testosterone

The Panel commented on reported decreases in testicular and serum levels of testosterone in adult rats following exposure to atrazine in the peripubertal or gestational periods. In the absence of corresponding effects on levels of luteinizing hormone, it is difficult to interpret those reports. Although it is known that atrazine activates the HPA axis to increase secretion of corticosterone and progesterone by the adrenal gland, and that this suppresses the HPG axis, and also directly inhibits the secretion of testosterone by Leydig cells, there are still uncertainties in the proposed mechanism. Several areas were identified where further information is necessary in order to increase confidence in the hypothesis that the atrazine induced suppression of testosterone levels is mediated through the HPA axis. These include: investigation of the potentially conflicting effects of atrazine on the pituitary secretion of LH; effects of diaminochlorotriazine on the HPG axis, since it barely affects the HPA axis, but still affects the timing of puberty in male rats; the role of activation of the HPA axis in the dam in the possible long term effects that follow prenatal exposure.

Charge Issue 1.6 – Neurotoxicity Endpoints

The Panel considered preliminary conclusions of the Agency that the neurotoxicity endpoints examined are not more sensitive than those evaluated for neuroendocrine function following atrazine exposure (the basis of the current chronic reference dose). The Panel had concerns stemming from inconsistencies in dose-response relationships throughout the studies reported in the Atrazine Issue Paper. As a result the studies were difficult to interpret and it was not possible to be confident in the reliability of conclusions that were drawn. One opinion was that the dose levels used in the acute studies were, in most cases, higher than the current point of departure and that the studies did not justify replacement of the current point of departure with a neurotoxicity endpoint. Although some Panel members agreed with the validity of the Agency's conclusions concerning the technical and reporting flaws in the individual papers, another opinion was expressed about the collective value of the work which might add to the weight of evidence that atrazine may be neurotoxic. In light of this, and the observation of clinical signs of neurological involvement at low doses it was felt that further investigation of the neurotoxicity and neurobehavioral effects at environmentally relevant doses of atrazine across all stages of the life span would be justified. The Panel agreed with the Agency's conclusion that there is no sound evidence for a specific Parkinson's disease effect, though the observed changes in dopaminergic or other neurotransmitter systems may contribute in other ways to the mode of

action of atrazine. More serious concern was expressed by the Panel on the paucity of well controlled dose-response studies designed to examine neurobehavioral and neurodevelopmental endpoints using appropriate dose ranges and the Panel encouraged the Agency to seek such studies conducted with adequate scientific rigor.

Charge Issue 1.7 – Mode of Action for Prostatitis

The Panel evaluated conclusions concerning the mode of action of atrazine in producing toxicological effects in the prostate of rats. An association between exposure of lactating dams in early postpartum and a subsequent increased incidence of lateral male prostate inflammation in offspring has been clearly demonstrated. The postnatal time period is the critical window of susceptibility for increased incidence of prostatitis. However, there was no evidence available to support the hypothesis that this was caused by a deleterious effect on the development of the dopaminergic system in the neonatal brain. Other mechanisms (e.g., atrazine and/or metabolites in milk impacting development of immune system) are plausible, and the current hypothesis could be tested using appropriately designed studies. It is important that the mechanism be better defined in order to identify the possible impacts on human health, and the role of breastfeeding. The Panel agreed with the Agency that further work was necessary to evaluate the hypothesis that atrazine-mediated suppression of prostate cancer in a transgenic rat may be due solely to caloric restriction rather than endocrine-related and that the rat is a reasonable model for human disease.

Charge Issue 1.8 – Effects on Immune System

The Panel advised that, in order to avoid confusion, the general term immunotoxicity or unintended modulation be used to include deleterious effects from both immunosuppression and immunoenhancement. The Panel found that there were many methodological differences between the Rooney et al., 2003 and Rowe et al., 2006 studies that investigated the potential of atrazine to cause immunotoxicity. These included different species, inbred and outbred strains, different lengths of exposure to atrazine, different pharmacokinetic behavior due to differences in routes of dosing, and interspecies differences in metabolism. Other factors (e.g., plastic or metal cages, composition of diet, vivarium environment, and time of year) that could affect the results were not defined in the study reports. Both studies were considered to have provided high quality, technically sound observations that represent real outcomes for the rodent models and experimental systems used. Some of the above unknown factors could also explain discrepancy between the results of repeats of the experiments. Failure to reproduce previously published and legitimate experiments is cause for concern, and diminishes confidence in the conclusions drawn. The Panel identified a need for further experiments that include an examination of the dose response, and a systematic evaluation of timing of exposure and differential gender effects. The Panel cautioned that the negative data in the second set of experiments is not sufficient to negate the published conclusion that exposure to atrazine during development may modulate immune function. Overall, the Panel agreed with the Agency's conclusion that atrazine-induced immunotoxicity is not a more sensitive endpoint than the atrazine-induced effects on

neuroendocrine function. However, it considered the existing developmental immunotoxicity database to be very limited, and identified a need for further studies using an extensive dose range, including lower doses, in both mouse and rat. Such studies should include animal models with genetic predisposition for immune-mediated diseases, and use both adult and developmental exposures.

Charge Issue 1.9a – Determining Appropriate Endpoints and Points of Departure

The Panel was encouraged by the advances in understanding the effects of atrazine on the HPA and HPG axes and recommends that dose-response relationships for each key event within this proposed MOA be characterized for the endpoint selected. It also encouraged the Agency to take into account other effects of low doses of atrazine even if they are not apparently directly associated with the proposed modes of action involving the HPA and HPG axes. It recommended the use of data screening to identify key events, use of benchmark dose analysis to identify sensitive endpoints that lead quantitatively to functional impairment, and use of a weight of evidence approach in selecting points of departure and in assessing a level of their confidence.

The Panel felt that the Agency should consider using measures of internal exposure of target tissues rather than externally administered dose to facilitate a better characterization and comparison of the dose-response relationship from all studies with different species, routes of exposure and study designs. The Agency should obtain data for developing toxicity equivalency factors in order to assess total exposure and dose for both the parent chemical and toxic metabolites. Gaps in the toxicological data identified in earlier sections of the report should be addressed in the selection of endpoints, and associated points of departure for use in human health risk assessment.

Charge Issue 1.9b – Toxicokinetic/Dynamic Considerations

The MOA of atrazine for its most sensitive effects involves activation of the HPA axis that through a cascade of events leads to reduced LH and FSH production in the female rat, causing premature reproductive senescence that hastens onset of mammary gland tumors. Although this mechanism of carcinogenesis does not occur in the human female, the atrazine-induced reduction of LH and FSH may occur and produce other adverse effects. The delay in pubertal onset, and other pre- and post-natal development effects observed in animal models, may occur in humans, and the critical atrazine exposure levels for these are not characterized. The threshold dose producing an alteration in the HPA / HPG signal system in rat has been identified, but there appears to be no hard, quantitative link between functional outcomes (reproductive senescence) and duration and intensity of hormonal changes. Further, since this endpoint is not the functional endpoint in the human female, it is not possible to identify from rodent studies the magnitude and duration of endocrine disruption that will produce an adverse effect on health or reproduction in humans. It seems likely that the atrazine exposure producing the functional outcome endpoint is greater than the minimum exposure to produce a measurable change in a hormone signal. However, in order to be safe, it would be prudent to use a point of

departure that was associated with the smallest exposure that produces a measurable change in an endocrine signal.

In order to be able to identify appropriate frequencies of monitoring atrazine concentrations in drinking water, it is necessary to determine whether protection against chronic or acute effects is needed; though the former should protect against both patterns of exposure. In addressing this aspect, a critical factor is the measurement of target organ exposure to be used (a critical maximum concentration for acute exposure and for how long this is exceeded for chronic exposure). One useful measure would be the integrated (or daily average) internal exposure since this would take account of a range of contributing processes. A related approach to monitoring human exposure to atrazine in drinking water would be to use the area under the concentration-time curve for drinking water. This could then be used in a systematic sensitivity analysis to identify key parameters and their relative importance in models of exposure through drinking water. Important variables might be length of pollution events or fluctuations in concentrations, and peak water concentration. However, if the latter does not appear to be critical, there would be no need for frequent monitoring of concentrations of atrazine in drinking water.

The Panel pointed out that the pharmacokinetic behavior of atrazine has not been fully characterized. However, some relevant information has emerged from the available studies that should aid the interpretation of time-dose-response relationships for a number of endpoints. Diaminochlorotriazine is the major metabolite (by an order of magnitude), and binds covalently to available cysteine residues in hemoglobin, serum albumin, and a range of proteins in other tissues (including target tissues). This will have a significant effect on the concentration of free metabolite that is available for elimination or interaction with a target site. Absorption of atrazine administered by gavage seems to be largely limited by dissolution of the bolus, and metabolism by enterocytes and hepatocytes is rapid. The rapid onset of HPA hormonal changes following oral administration of atrazine suggests that only a small internal exposure is required to modify endocrine function.

The concentration of a compound in the gut lumen can markedly influence the pharmacokinetic behavior, and it is difficult to use data on absorption from a high dose in a bolus in the rodent gut to predict uptake from the human gut where atrazine is present at low concentration in drinking water. Further, it is problematic to use the existing rodent models, parameterized using data from oral gavage dosing, to predict pharmacokinetic behavior for other routes of administration (e.g., inhalation, injection, or subdermal implants). It is likely that these alternative dosing methods would lead to higher concentrations of available herbicide in the plasma than is possible by oral dosing. The Panel felt that it should be possible to improve the reliability of extrapolation from rodent models to humans by using the well characterized differences in expression of drug metabolizing enzymes in the intestine between the two species, and dosing regimes that more realistically reflect human exposure. However, on the basis of current knowledge it is difficult to assess the potential exposure of target sites in humans to atrazine and its metabolites, and there is little information on the critical exposure needed to produce an adverse effect. Without this information it is difficult to link toxicity to humans to concentrations in drinking water.

Charge Issue 2.1 – Statistical Approaches to Determine Confidence Bounds

The Agency seeks to set revised levels of concern for concentrations of atrazine in drinking water, using biological information such as that discussed in the first set of Charge Questions. Determination of confidence bounds on estimates of a particular concentration statistic for a specific site can be made by inference from other sites or site-specific determination from monitoring data. The former are appropriate for screening purposes but have unacceptably high uncertainty to be used as a basis for important decisions, and as the time duration of the limit of concern becomes shorter, the need for site-specific monitoring data increases. In order to optimize the utility of sampling effort, consideration should be given to targeting critical periods for sampling (summer, when levels are high, rather than winter). An alternative approach would be to screen sites (using a rapid, inexpensive analytical method) to trigger more frequent quantitative measurements when needed. This would allow adaptive sampling procedures to be developed for implementation within a year.

A parametric approach to setting confidence bounds on a particular quantile of the distribution of atrazine concentrations at a given site of interest is possible, but there are difficulties in meeting the underlying assumptions, and the Panel focused on nonparametric approaches. However, larger sample sizes are needed for the latter, particularly for estimating extreme quantiles. Simulation studies were used to evaluate two different sampling strategies, one based on individual samples, and the other on rolling averages. With the former, unacceptably high sampling frequencies were necessary for estimating the extreme percentiles, and these tended to be overestimated. The precision of the estimates can vary depending on environmental factors such as amounts of rainfall.

The Panel evaluated the reliability of alternative sampling strategies based on statistical sampling from simulated continuous time-series of concentrations. Actual data time series (comprising 30-35 samples for one year) for selected sites were linearly interpolated between samples to yield a daily concentration time series, which was then treated as the "true" chemograph for experiments with sampling strategies. The simulation sampling experiments all underestimated the "true" values of upper percentiles or means, and had a low probability of representing short lived peaks. These methods are flawed since the simulations are benchmarked to a biased representation of actual concentration profiles. Currently, the time frame of interest for the level of concern is not defined, and this will determine the most useful approach to monitoring. It is important to define the importance of peak events, and their duration, the importance of clusters of peaks, their duration, and the intra-cluster peak maxima. Once these have been defined, then appropriate sampling strategies can be defined.

Charge Issue 2.2 – Simulation Methods

Simulations of candidate sampling strategies need to be benchmarked against intensive empirical data for an adequately representative range of sites, or a statistical or deterministic modeling approach that incorporates environmental variables, and uses reliable observations for calibration. However, the Panel emphasized that if inference is to be at the daily level, then sampling needs to be at least daily, for four day rolling averages at least two samples are required

within that period. In order to minimize the work, sampling could be focused on the sites that merit most detailed modeling [identified using a predictive model, such as the Watershed Regressions for Pesticides (WARP)] model, and sampling in periods when atrazine is likely to be present in surface waters.

The small number of chemographs used in the simulations all contain high peaks of atrazine, and are not representative of the majority of community water systems where concentrations never exceeded 3 ppb, and provide a limited range of behaviors against which strategies can be tested. It is difficult to reflect the numerous sources of error in sampling in a simulation, and this is needed to provide a realistic assessment of sampling behavior. One way forward is to use statistical models incorporating environmental explanatory variables since these models have a correlation structure, and geostatistical simulations can be used to simulate chemographs. For such a system, quantities of interest can be estimated using a proposed sampling procedure, and the properties of the distribution of estimates investigated. If short duration exposures are found to be important then representative model sites will need to be sampled frequently (probably daily) and over a number of years.

Charge Issue 2.3 – Placing Confidence Bounds on Rolling Estimates or LOC

Given the large uncertainties in extrapolating toxicological endpoints from model laboratory animals to humans, the Panel would not recommend placing confidence bounds on the LOC since it is unclear how they would be developed. It suggested that the LOC be used as a fixed value and exposure estimates measured against it. In contrast, the reliability of concentration statistics used for exposure characterization should be considered. There are possible tradeoffs between the costs of improving reliability versus accepting probability-based regulatory actions based on low-precision estimates. Confidence limits should be determined and then they can be used to estimate the probability that true concentrations exceed the LOC.

Charge Question 2.4 – Modeling Approaches

The Agency described two methods (linear interpolation and stair-step imputation) for filling in values between actual measurements of atrazine concentrations in water. Neither method will ever allow a predicted value larger than any of the observed values, and will underestimate the maximum value. When the true concentration curve is decreasing, the stair-step method will overestimate values. Linear interpolation will overestimate a decreasing curve when it is concave (the most common case), but not by as much as the stair-step method. For longer-term averages (e.g., 90-day or 26-week), both methods may work reasonably well, since underestimation of peak values may be balanced by the overestimation of post-peak values. However, this is not recommended. Artificial Neural Network methods can be used to predict values larger than those observed in the data, but it is necessary to use sufficient nodes to estimate maxima, and to allow a good fit to the various forms of curve in the profiles, while avoiding over fitting. The Panel believed that the current approach using a neural network with autoregressive errors would be too complicated for a non-expert to implement. Other potential approaches include bootstrapping, kriging, random function models, regression-based models,

and deterministic models. Like the linear interpolation and the stair-step methods, bootstrapping can never predict a value larger than that observed, and will underestimate maxima and short-term averages. The other four methods have definite promise, alone or in combination.

Kriging (a special case of a Gaussian process model) can be used for estimating a maximum, and can smooth noisy data. Since it takes into account correlations between observations over time, it could be particularly useful in the current context. If the functions describing the shapes of the curves from different water sources were known, then random function models could be used in the determination of maximum values, and areas under the curve. Another approach, regression-based models, has been shown to predict longer-term averages well and some ability to predict maxima and extreme quantiles. The Panel felt that it would be valuable to explore the possibility of combining this approach with the statistical methods discussed above. Deterministic models can predict maxima and short-term averages well, though accurate calibration is essential to obtain predictions that closely match observed values. The Panel also suggested considering extreme value theory.

The Panel felt that if the current approach of grab sampling were replaced with the use of autosamplers to produce composite samples over time, then better estimates of rolling averages would be obtained, and the data would be more biologically relevant. It may be possible to use composite daily samples for a rolling average. Another approach worth considering is the use of a weighted rolling average that would reduce the amount of smoothing relative to current methods. Systematic sampling is the most efficient when there are long-term trends; for shorter-term fluctuations, systematic sampling might give more variability than randomly-perturbed fixed-window sampling.

The proposed approach for evaluating interpolation methods and for use in sampling design is sound for the sites and time periods for which there are adequate data. However, the reliability of generalizing results from the relatively small number of sites and site years with relatively continuous data is dependent on how representative the sites and years are. If the duration of a new level of concern drops substantially from 90 days, then further intensive monitoring will be needed, particularly where the level of concern is likely to be exceeded. The appropriate strategy for combining inference, modeling, and monitoring to meet the Agency's needs for risk assessment must be evaluated specifically for new levels of concern, and the required level of reliability. A better strategy for creating new concentration profiles for use in method evaluation would be to use the WARP model, and existing annual maximum prediction data to identify the most vulnerable CWSs, and then combine the regression model with kriging to create a chemograph, followed by geostatistical simulation to determine the variability. The procedure outlined by the Agency for evaluating the effectiveness of different methods for prediction of profiles between measurements is sound, although it does not take into account possible environmental covariates, or make use of knowledge of the shape of the chemograph.

PANEL DELIBERATIONS AND RESPONSE TO CHARGE

As part of the re-evaluation of the health effects of atrazine, three meetings of the FIFRA Scientific Advisory Panel (SAP) are scheduled in 2010. The first of these was held February 2-4, 2010. In preparation for the April meeting, the Agency developed a draft issue paper focused on 1) a review of the recent scientific literature on the mode of action and toxicological profile of atrazine and 2) approaches to determine appropriate drinking water monitoring frequencies. The purpose of the April meeting was to solicit feedback from the SAP on the status and overall scientific direction of the Agency's re-evaluation in these two areas including preliminary conclusions about some aspects of the experimental toxicology data. The Agency will use the Panel's feedback to inform the weight of the evidence (WOE) analysis that will be developed in the coming months and reviewed at the September 2010 SAP. The experimental toxicology portion of the draft April issue paper (Sections 3 & 4, Appendices A & B) represents the state of the science up to January 30, 2010. Since that time, new data have become available and more will become available in the spring and summer of 2010 that will provide further characterization of some key areas, such as describing the causal linkage between key events in the mode of action, the dose-response concordance of these key events, and the relationship between the precursor events with functional outcomes. These new studies will be incorporated into the September SAP.

Question 1.0:

In Vivo & In Vitro Experimental Toxicology in Mammals

In 2003, the human health risk assessment was based on a mode of action in which exposure to atrazine leads to a reduced release of gonadotropin releasing hormone (GnRH) from the hypothalamus thereby lessening the afternoon pituitary luteinizing hormone (LH) surge in female Sprague Dawley rats. As a result, the estrus cycle lengthens. This, in turn, leads to increased estrogen levels and an increased incidence of mammary tumors in female Sprague Dawley rats. EPA determined that atrazine's cancer mode of action (i.e., premature reproductive aging) in the Sprague-Dawley rat is not likely to be operative in humans (a conclusion consistent with the SAP recommendation in 2000). The Agency will be evaluating the cancer classification further as the experimental toxicology data are integrated with the epidemiology literature; new experimental toxicology studies (Section 3.6 of the draft issue paper) do not suggest a change to the Agency's previous conclusion that atrazine is "not likely to be carcinogenic to humans."

Although the cancer mode of action may not be operative in humans, it is not unreasonable to assume that atrazine might cause adverse effects on hypothalamic-pituitary function in humans. Thus, the same endocrine perturbations that induce tumors in rats may play a role in at least some reproductive/developmental effects (not associated with reproductive aging) that may be relevant to humans. Accordingly, the Agency identified disruption of estrous cyclicity and delays in puberty onset (males and females) occurring as a consequence of disruptions to the hypothalamic-pituitary-gonadal (HPG) axis as the critical endpoints of concern (Sections 2.0 & 3.2 of the draft issue paper).

The Agency continues to believe that this disruption of the HPG axis is critical to affecting estrous cyclicity and delayed puberty onset. However, as discussed in Questions 1.2-1.6 (See Figure 3 of the draft issue paper), new information has been published that indicates a more expanded understanding of how atrazine may perturb the pathway leading to reproductive effects. These new data indicate that atrazine disrupts the hypothalamic-pituitary-adrenal (HPA) axis altering the central nervous system's control of the pituitary and adrenal which, in turn, disrupts the HPG axis. Furthermore, a hypothesis for a direct effect of atrazine on the adrenal cortex and the gonads which may impact steroidogenesis directly is also supported by the available data.

Question 1.1

With the caveat that the review of the epidemiological literature is still on-going, please comment on the Agency's preliminary conclusion that new experimental data from in vitro and in vivo laboratory animal studies do not support a change in the conclusions from the 2003 risk assessment that atrazine is unlikely to be a human carcinogen.

Panel Response:

In its 2000 draft Hazard and Dose-Response Assessment and Characterization, and later finalized in the 2003 Interim Reregistration Eligibility Decision (IRED)/2006 Registration Eligibility Decision (RED), the Agency presented its analysis of the human carcinogenic potential of atrazine, based wholly upon the evaluation of results from studies in laboratory animals. Rodent bioassay results revealed no increase in any tumor type in male SD rats or in F344 rats or CD-1 and other mouse strains of either sex. However, an increased incidence and/or early onset of mammary adenomas and carcinomas, mammary fibroadenomas and pituitary adenomas were observed in female SD rats. EPA conducted an in-depth analysis of these tumors, particularly the mammary adenomas/carcinomas, and this included the development of additional data used to describe possible mode(s) of action (MOA(s)) by which these tumors arose, and presentation of conclusions as to the relevance of the hypothesized MOA(s) with regard to the chemical's human carcinogenic potential.

Based primarily on the work of EPA's Office of Research and Development, National Health and Environmental Effects Research Laboratory researchers, a non-genotoxic MOA was postulated for the effects of atrazine on components of the hypothalamic-pituitary-gonad (ovarian) (HPG) axis and the development of the mammary, and to some extent, the pituitary tumors in the SD female rats.

An alternative possible MOA, i.e., genotoxicity/mutagenicity, was not supported, based upon the weight-of-evidence evaluation of a variety of short-term relevant genotoxicity assays. EPA's review of related literature published since 2000 confirms this conclusion. There is no evidence of the ability of atrazine to act as an initiator (i.e., via a mutagenic or genotoxic mode of action). The Panel agrees with the Agency's assessment.

The text quoted below, taken from the 2000 draft document, presents the postulated key events, based upon the data available at that time.

Postulated Key Events:

1. Atrazine exposure affects - either directly or indirectly – the hypothalamus, leading to a decreased secretion of hypothalamic norepinephrine (NE).
2. Hypothalamic NE normally modulates the release of gonadotropin releasing hormone (GnRH) from the hypothalamus. Decreased NE levels result in decreased release of GnRH from the hypothalamus
3. GnRH is the hormone responsible for inducing the pituitary gland to release luteinizing hormone (LH). A decreased GnRH level leads to an attenuated LH release.
4. LH normally provides a signal to the ovaries promoting ovulation. Below some critical level, the decreased serum levels of LH are insufficient to stimulate ovulation.
5. Estrogen from ovarian follicles normally provides a feed back to the hypothalamus to stimulate a pituitary LH surge that promotes ovulation. Following atrazine exposure, there is insufficient GnRH to stimulate ovulation. Under the tonic secretion of LH and follicle stimulating hormone (FSH), the ovarian follicles persist and continue to secrete estradiol. In turn, under the continued stimulation of estradiol, the pituitary lactotrophs become hypertrophied and secrete increasing amounts of prolactin.
6. Estrogen acts on the mammary gland, increasing the risk of mammary tumors, especially carcinomas and adenomas.
7. Prolactin derived from the hyperplastic lactotrophs (prolactin secreting cells) described in step 5 also acts on the mammary gland (in concert with estrogen) to increase the risk of mammary tumors, particularly fibroadenomas.
8. Tumor formation by atrazine does not appear to involve direct mutagenic effects nor does atrazine act as a direct estrogen agonist.

As will be discussed at greater length below, much of the EPA research conducted since 2000 has been targeted toward improving the understanding of the nature of the reproductive/developmental effects of atrazine. Pursuit of the characterization of the mechanisms by which these effects occur has revealed at least one major component (activation of the hypothalamic-pituitary-adrenal (HPA) axis) not identified during the earlier phase of characterizing the mammary tumor MOA. The key events for the effects on the HPA axis have now been postulated.

Postulated Key Events

1. Hypothalamic changes result in an increase in the release of corticotrophin releasing hormone (CRH).
2. Elevated CRH stimulates release of adrenocorticotropic hormone (ACTH) from the pituitary
3. Elevated ACTH stimulates production of corticosterone and progesterone by the adrenals.
4. Some or all of the above HPA changes decrease GnRH release from the hypothalamus.

The convergence of Key Events #2 and 3 in the original mammary tumor MOA analysis and Key Event #4 in the HPA activation pathway is obvious. Discovery that an additional component or pathway may be in play neither negates nor diminishes the conclusions reached

earlier with regard to the characterization of the mammary tumor MOA or its relevance to human health risk assessment. One aspect that remains unclear is whether atrazine directly affects the hypothalamus, setting off the cascade of effects, or only indirectly through the HPA axis or both.

However, one Panel member offered a word of caution: the demonstration of the involvement of the HPA axis in the etiology of the mammary tumor in the SD rat may, in the eyes of some individuals, diminish its biological significance as an indicator of disruption of the HPG axis. The Agency clearly is not following this line of reasoning in its current background document, and the Panel urged the Agency to resist such reasoning. However concern was expressed that papers may now appear in the literature hypothesizing that disruption of the HPG axis reflects only a rat strain-specific effect resulting from a unique or abnormal sensitivity to a general, non-specific stress response, rather than targeted disruption of hormonal pathways.

There is some precedence when interpreting the results of chronic rodent cancer studies to attribute difficult-to-explain tumor incidences to "differential stress" across treatment groups. Rarely are data presented to back up this assertion. Different routes of exposure can result in different rates of tumors at some sites in control animals. There is a general perception that the level of stress in the test animals would be greater in inhalation studies, where they are housed for up to two years in metal chambers with significant airflows and associated elevated noise levels, than in studies where they are held in enclosed polycarbonate cages. Somewhat higher rates of adrenal pheochromocytomas are seen in control male Fischer rats in inhalation than in dosed feed studies. However, no such difference is seen in the rates of mammary tumors in female F344 rats. According to the prevailing hypothesis, this strain would not be expected to differ as it does not senesce into a persistent estrus. There are no National Toxicology Program studies in which SD rats have been used in inhalation studies, but a brief review of SD rat inhalation studies in the open literature revealed that the mammary tumor rates in control SD females are very similar to the rates reported in the controls in the dosed feed studies done on atrazine.

There was general agreement among the Panel members that the findings from the recent (i.e., post-2000/2003) studies on the interrelationships of the HPA and HPG axes and other studies have not provided new data to contradict the conclusion regarding the lack of human relevance of the SD rat mammary tumors. The critical role of inhibition of the LH surge in suppressing estrus cycling in SD rats continues to be supported by the recent literature. The sensitivity of the SD rat to early onset and increased incidence in mammary tumors with increases in endogenous estrogens or from treatment with estrogenic substances is well recognized. Based on the consideration of the existing body of animal studies (pre- and post 2003), the Agency's conclusion is that the data are not sufficient to change the cancer classification of atrazine and that it be considered "Not Likely to Be Carcinogenic to Humans." This narrative/classification is appropriate when there is evidence that the mode of action in experimental animals does not operate in humans.

The Agency has published its work plan, with proposed time lines, for updating its atrazine human health assessment. The present SAP meeting is focused on consideration of the Agency's preliminary evaluation of *in vitro* and *in vivo* laboratory studies (cancer and non-cancer) which have become available since the IRED/RED were completed (as well as

discussion of proposed approaches for determining the appropriate frequency of atrazine monitoring in drinking water and/or its source water). The Agency plans to return to the SAP later this year for a discussion of the epidemiology studies addressing non-cancer endpoints of concern and approaches for integration of the epidemiology and laboratory studies in a weight of evidence hazard characterization. Sometime in 2011, the Agency will be asking the SAP to deliberate on the body of epidemiology studies addressing cancer endpoints and possible approaches for integration of the epidemiology and laboratory studies in a weight of evidence hazard characterization for cancer. In considering the epidemiology data, emphasis should be placed on the temporal relationship, specifically the possible effect of early life exposures. While the Panel is not aware of any data implying that excess corticosteroid production (due to atrazine exposure) might result in cancer, one exception to this conclusion could be the possibility of cancer development due to immunosuppression. In order to investigate this, epidemiology studies should include skin cancer, lymphoma, cervical cancer and liver cancer amongst the endpoints targeted for analysis since they are known to occur in immune-suppressed individuals.

Two Panel members shared their concern about the implication and human relevance of the results of the studies in transgenic rats on tumor promotion and progression (Fukamachi et al., 2004 and Ueda et al., 2005). While they, by themselves, do not provide the strength of evidence to change the classification of atrazine, they should receive a high level of scrutiny once again when the Agency undertakes an overall WOE assessment for cancer.

One Panel member professed agreement with some but not all of the deficiencies identified by the Agency in its review, stating that a different conclusion could be drawn. In spite of the deficiencies identified in the Agency review of the two studies, the Agency concluded that "... these two sets of data suggest that atrazine does not induce mammary tumors in transgenic rats or ovariectomized rats treated with a mammary tumor inducer thus highlighting the importance of the ovarian hormones in the development of tumors in the mammary gland." The Panel member suggested that the focus for the Agency's conclusion should not be on tumor initiation that these studies were designed to evaluate, but on the tumor promoting potential of atrazine. Tumor promotion is a valid endpoint when considering carcinogenic potential, especially in light of the abundant concomitant tumor initiating factors routinely encountered in the environment by the human population.

Specifically regarding the Fukamachi et al. (2004) study, the Agency determined that the apparent increase in mammary adenocarcinomas lacked a dose-response relationship. While the small sample size and the high tumor incidence in the control group may have clouded the interpretation of the data, the high background rate (approximately 50%) is somewhat understandable given that the rats are designed to be extremely sensitive to 7, 12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumors. Nevertheless, it should be noted that the mammary adenocarcinoma incidence in the females was already at 100% in the lowest dose group (5 mg/kg/day of atrazine), and the number of tumors per rat was doubled (7.10 ± 6.08 versus 3.0 ± 3.69 in the control group). At the next higher dose (50 mg/kg/day) the incidence was also 100%, and the number of tumors per rat increased to 10.33 ± 7.94, the highest of all treated groups. At issue is the drop-off of these two indices at the highest dose (500 mg/kg/day) even though incidence was still higher than the controls. Given that maximum responses were reached at the low and mid dose, other factors could very well come into play at

the high dose. This is not a valid reason to dismiss the results from the low and mid dose groups. The authors noted that tumors induced in their transgenic rats were considered estrogen-independent, because ovariectomy did not affect their development.

The data in the male rats showed a steady increase in tumor incidence over the range of 5 to 50 mg/kg/day [1.89 ± 2.32(controls), 2.56 ± 3.24 (5 mg/kg/day), and 3.00 ± 3.81 (50 mg/kg/day)]. However, there was a slight decline in the number of tumors per rat (2.70 ± 2.06) at 500 mg/kg/day compared with the above value at 50 mg/kg/day.

The study by Ueda et al. (2005) in ovariectomized Sprague-Dawley rats also indicated a tumor promoting potential for atrazine. Of interest is the incidence of DMBA-induced mammary tumors (13/18 (72%), 16/20 (80%), 17/18 (94%) and 15/21 (71%)) in animals with palpable tumors at ovariectomy before atrazine treatment at 0, 5, 50 and 500 mg/kg/day respectively. Although the incidence declined at the high dose, the corresponding tumor volume continued to climb to 114.8 ± 266.8 cm3/rat, compared with 31.8 ± 49.2 cm3/rat (controls), 3.12 ± 44.7 cm3/rat (5 mg/kg/day), and 45.6 ± 109.2 cm3/rat (50 mg/kg/day). Comparable data for rats without palpable tumors are less clear. The tumor incidences were 5.9 (26%), 2/19 (11%), 5/12 (42%) and 11/19 (58%), respectively in the 0, 5, 50, and 500 mg/kg/day groups, with corresponding tumor volumes of 0.9 ± 2.2, 2.4 ± 10.2, 11.7 ± 23.6, and 11.9 ± 20.1 cm3/rat.

While Panel members recognized that these studies were weak because of the use of small numbers of experimental units and insufficient replication, it was thought that they should not be dismissed in future considerations of carcinogenicity. Compounds that act as tumor promoters could make a contribution to the oncogenic process, and have implications for human health.

Question 1.2

Based on an evaluation of the studies examining the mode of action of atrazine on neuroendocrine function (Section 3.2 of the draft issue paper), the Agency has preliminarily concluded that atrazine affects both the HPG axis and the HPA axis. With respect to the temporal concordance, recent studies show that atrazine induces a rapid (within minutes) increase in ACTH and adrenal cortical hormones (corticosterone and progesterone) in both male and female rats (Fraites et al., 2009; Laws et al., 2009; Pruett et al., 2009), while changes in the HPG axis, such as the suppression of the LH surge, may take up to 2-3 days (Cooper et al., 2000). This sequence of HPA alterations followed by HPG changes indicates that the suppression of the surge may be, in part, mediated by activation of the HPA axis (i.e., a corticosterone suppression of the GnRH and LH release). In addition, with respect to dose-response concordance, atrazine-induced increases in ACTH, corticosterone and adrenal progesterone are seen following a single dose of atrazine (50 mg/kg). In contrast, the HPG is not altered following a single dose up to 200 mg/kg. However, when treated for multiple days, the dose necessary to alter the HPA axis is lower than or equal to the one needed to affect the HPG axis.

Please comment on the Agency's preliminary hypothesis for the mode of action involving atrazine's alteration of both the HPA and HPG axes. Does the document adequately and clearly

describe the hypothesis in the context of a "toxicity pathway" (i.e. cellular response pathways that, when sufficiently perturbed, are expected to result in adverse health effects)? Does the document clearly describe the data used to test the proposed hypothesis? To what extent do the available data establish key events in the proposed mode of action hypothesis? What are the strengths and limitations of the data available on this hypothesis?

Please include in your comments a discussion of the Agency's interpretations of the data linking the initial perturbations in HPA axis to changes in the HPG axis.

Please comment on the evidence that the initial perturbations in the HPA axis may lead to impairment in reproductive function and/or developmental consequences. Which event(s) is/are viewed as critical in leading to health consequences? Are there data on other substances that would inform this question?

Panel Response:

The current position of the EPA is that perturbations of the neuroendocrine system leading to reproductive toxicity are the most biologically plausible and sensitive effects attributable to atrazine exposure. Adverse outcomes observed include a delay in onset of puberty in male rats and in female rats, attenuation of the preovulatory LH surge and disruption of estrous cyclicity occurring as a consequence of disruption to the HPG axis leading to early reproductive senescence.

Evaluation of the post-2003 data has further characterized the mechanisms through which atrazine disrupts the normal function of the HPG axis. Most importantly atrazine and some of its metabolites act to:

- attenuate the spontaneous preovulatory LH surge,
- block the gonadal steroid induced LH surge and attenuate concomitant GnRH neuronal activation [measured by a reduction in c-fos expression (a marker of neuronal activity) in GnRH neurons]
- inhibit LH secretion, including a suppression of LH pulse frequency without attenuation of LH pulse amplitude (a clear mark of a central nervous system (CNS) site of action on the hypothalamic GnRH pulse generator activity) (LOEL: 200 mg/kg 4-days: NOEL: 100 mg/kg
- increase the concentration of GnRH in the median eminence (a crude measure of reduced GnRH release).

Evaluation of the post-2003 data has led EPA to identify another disrupted pathway in the MOA of atrazine, namely activation of the HPA axis manifested as increases in levels of ACTH and adrenal cortical steroids (corticosterone and progesterone) in rodent species. It is well established that there is an inverse relationship between the activities of the HPA and the HPG axes, and this has led to the hypothesis that activation of the HPA axis may be causally related to the suppression of the HPG axis, especially in response to stressful stimuli or clinical syndromes such as Cushing's disease. This physiological correlation, coupled with new evidence that atrazine activates the HPA axis has led the EPA to a preliminary hypothesis that the MOA of

atrazine involves changes in both the HPA and HPG axes, such that atrazine and/or its metabolites influence both of these critically important axes sequentially rather than in parallel. This implies that the changes in adrenal hormone secretion drive the deleterious changes in the reproductive axis (i.e., that there is a corticosterone suppression of the GnRH and LH release). The Panel agreed that this preliminary hypothesis remains thus, and it is premature to conclude that matter is settled, due to inadequate supporting data.

Recent studies demonstrate a very credible acute dose-dependent, rapid ACTH and corticosterone response to atrazine within a time domain appropriate for oral gavage drug delivery. Furthermore, there are findings of consistent single-point 15 minute ACTH and corticosterone responses to the atrazine metabolites deethylatrazine (DEA) and deisopropylatrazine (DIA). However, corresponding data on atrazine activation of central elements of the HPA axis, such as corticotropin releasing factor (CRF) expression in the hypothalamic paraventricular nucleus (PVN) or of proopiomelanocortin (POMC) in pituitary corticotrophs, are not yet available. Further, effects of atrazine on cAMP pathways that are involved in stimulation of CRF and other signaling systems are unknown. The initial characterizations are intriguing, but steps to manipulate directly and test, separate elements of the HPA axis (such as with CRF receptor antagonist or controlled glucocorticoid levels) have not been developed. Therefore, currently there is no clear evidence that atrazine stimulates the HPA activation, or that this is required and necessary for suppression of the LH surge.

The strongest data sets are those obtained using single acute atrazine treatments. It is highly likely that the HPA is a "first responder" to atrazine treatment and may, in turn, disturb LH secretion as demonstrated by the Laws 2009 study. The temporal profile and dose response are very credible data. These are further supported by very well-controlled basal (initial) levels ruling out nonspecific (e.g., handling) effects. The rapid ACTH increase and corticosterone response in the Laws 2009 study using atrazine administered by gavage are expressed in an appropriate time window. The first ACTH response was observed at 5 minutes (the first time interval) with the higher doses, as compared with about 2 minutes following restraint or injection. Further, with atrazine treatment, the decline in ACTH response was slower (still elevated at 60 minutes) than in the other treatments and this is consistent with delivery to the stomach by gavage compared with injection or restraint. Nonetheless, whether the HPA response is "necessary and sufficient" remains unresolved. The exact chain of events (or ensemble of effects) is still an open question. For example, increase in CRH has been proposed to act centrally to reduce GnRH secretion and attenuate the LH surge. However, CRH KO mice (Jeong et al., 1999) exposed to restraint stress still display decreased or attenuated LH levels. Therefore, the hypothalamic component of the HPA stress response probably encompasses the CRFs (vasopressin, urocortin) and may also parse between the CRF receptors (probably CRF-R2). The new data described by Syngenta during the public comment period that ovariectomized rats lacking corticosterone because they were adrenalectomized, continue to express an atrazine dose-related inhibition of the estrogen and progesterone (E+P) induced LH surge suggests that corticosterone does NOT mediate this atrazine effect. It is also possible that elevated hypothalamic PVN CRF content and pituitary ACTH secretion resulting from adrenalectomy do not mediate the atrazine effects on the LH surge. Interestingly, adrenalectomy does block the inhibitory effect of atrazine on pulsatile LH secretion as indicated by Syngenta. However, it must be recalled that although a functioning GnRH pulse generator is critical to control of the estrous

cycle in rodents and the menstrual cycle in women, there is no evidence of pulsatile LH secretion during the LH surge in the rats.

In contrast with the data obtained in experiments using single/acute dosing, those obtained using repeated or chronic administration of atrazine lack coherence, and a fundamentally different HPA regulatory mechanism comes into play. The primary source of CRH is from the PVN where mRNA expression increases with stress (and presumably atrazine administration). Moreover, CRF peptide and message is found in other brain nuclei (bed nucleus of the stria terminalis (BNST), central nucleus of the amygdala (decrease) and PV-thalamus, prefrontal cortex) and increases in levels have been demonstrated with the application of stressors and functional neural connections. A chronic stress circuit emerges with repeated restraint evidenced by increases in PVN arginine vasopressin (AVP) mRNA. Additionally, adrenal sensitivity increases and the adrenal gland hypertrophies. There is no evidence for adrenal desensitization: after daily exposure to a very high dose of atrazine (150 mg/kg/d, intraperitoneally), the corticosterone profile (as measured by radioimmunoassay assessment of total corticosterone) following the final dosage on day 28 was indistinguishable from that resulting from a single administration (i.e., there was recovery to baseline levels within 6 hours post treatment (Pruett et al., 2009)). Further, the EPA mentioned unpublished data that showed that chronic atrazine treatment causes only a transient (within days) adrenal hypertrophy with return to normal adrenal weight. Chronic atrazine treatment is also implicated in perturbations in food intake and body weight regulation such that the metabolic state may be interacting with the reproductive axis (through leptin or orexins). The key question of a causal link between initial activation/perturbation of the HPA axis resulting in subsequent suppression of the HPG axis is far from conclusive since evidence for some of the key events is not clear.

There is considerable evidence that acute increases in corticosterone/cortisol do not affect pulsatile GnRH/LH secretion in many species, including rats and primates. The only exception is the sheep where physiological stress levels of cortisol decrease LH pulse amplitude within 1 hour. This is a pituitary phenomenon involving reduced pituitary responsiveness to GnRH (Wagenmaker et al., 2009), and there is very strong evidence (Cooper, 2007) that atrazine does not produce this effect. In contrast to the clear evidence for the absence of effect (apart from in the ewe) of acute rises in corticosterone (or stress) on the GnRH pulse generator frequency, evidence concerning their effects on the GnRH/LH surge is less consistent. For example, Baldwin et al. (1979) showed that a single injection of dexamethasone or cortisol at 2-8 hours after administration of estradiol benzoate did not alter the LH surge in rats, thus suggesting that acute increases in glucocorticoid levels do not suppress the LH surge. However, Roozendaal et al. (1995) showed that restraint stress started at 0, 1 and 2 h before the presumed onset of the pro-estrous LH surge and ending just before the beginning of the dark period (when the surge would have ended) blocked the LH surge in approximately 50% of rats and markedly attenuated the surge in the remaining 50%. However, the latter study does not provide evidence that HPA axis activation (e.g., increased corticosterone) *per se* is the MOA since restraint is a psychological stressor involving a myriad of CNS pathways.

Divergent results have also been observed in the sheep model. In ewes, a common strategy used to synchronize the GnRH/LH surge is to remove a progesterone capsule implanted in the luteal phase, and this initiates the onset of the follicular phase leading to an LH surge,

typically some 48h after the removal. Saifullizam et al. (2010) used this technique and demonstrated that an acute activation of the HPA axis (acute rise in cortisol for 10 h) by insulin-induced hypoglycemia at 30 and 32 h after progesterone withdrawal caused a decrease in estradiol over the following four hours, and delayed the LH surge by 9 h. However, the effect of glucopenia *per se* as the MOA cannot be excluded with this particular experimental strategy. In contrast, Wagenmaker et al. (2010) showed that repeated acute psychological stress (at 12-18, 24-30 and 36-42 h after progesterone withdrawal) (plus other combinations) with concomitant repeated stress-induced increased levels of cortisol, did not block the spontaneous LH surge. In contrast to these divergent effects of acute stress/glucocorticoid effects on the LH surge, maintenance of continuously elevated levels of plasma cortisol as a result of implantation of cortisol capsules produces more consistent results. In the rat, not only were estrous cycles disrupted for periods of 16-52 days, but also the proestrus LH surge was completely blocked analogous to corticosterone treatment (Baldwin et al., 1979). Similarly, in the sheep, a 42h infusion of physiological stress levels of cortisol delayed the LH surge by 10h and reduced its amplitude by approximately 50% (Wagenmaker et al., 2009).

Thus, although there is clear evidence that chronic or sustained corticosterone/cortisol suppresses the GnRH/LH surge in a wide range of species including rodents, sheep and humans (Cushing's Disease), there is a lack of evidence that atrazine causes a sustained increase in corticosterone. Indeed, atrazine induces a transient increase in corticosterone as exemplified by the return to baseline within 6h in the mouse after a dose (150 mg/kg) of atrazine (Pruett et al., 2009) or 1h after a dose (50 mg/kg) in the rat (Syngenta, 2010). Further, daily exposure of mice to atrazine (150 mg/kg/day, ip) over a period of 28 d resulted in a corticosterone profile following the final dosage on day 28 that was indistinguishable from that following a single administration, with recovery to identical baseline corticosterone levels within 6h of treatment (Pruett et al., 2009). These results evidence a lack of sustained increases in corticosterone following chronic atrazine treatment. Late in the meeting, a Panel member expressed a qualification that the corticosterone response might be quite different with two spaced acute administrations of atrazine (e.g., 5 days apart in the rat). The first atrazine exposure could stimulate a facilitatory corticosterone response to the second exposure as observed in paired-stressors. Interestingly, in response to daily dosage with atrazine (6.5, 25 or 100 mg/kg/day), the corticosterone response in the rat (measured at 30 minutes post treatment) was no longer evident on day 7, the first day examined (Syngenta, 2010). The ideal experiment to determine the effect of daily administration of atrazine on basal circulating levels of corticosterone (measured at 24h following the final treatment) in the rat or any species has not been conducted.

The HPA and HPG axes exhibit significant neuroanatomical and functional overlap in the rat, and perturbations of one major central neurotransmitter system (i.e., the noradrenergic system) can affect both, albeit via similar but separate neuronal pathways. Thus, disruption of normal HPA and HPG function may occur by way of an upstream event that separately affects both axes. Further, peripheral changes in response to sympathetic nervous system activation (i.e. epinephrine release from the adrenal medulla) could account for some of the reported changes in HPA/HPG activity. It would be valuable to determine the effects of orally administered atrazine on the following neurotransmitter systems, in order to determine whether they play a role in causing HPA and/or HPG dysfunction caused by the herbicide: (i) the central noradrenergic system at the level of the locus coeruleus, the C1/C5 adrenergic areas of the brainstem, and other

brain areas that utilize norepinephrine to regulate HPA or HPG function; (ii) the oxytocin system; and (iii) the CRF and AVP systems. The EPA's hypothesis regarding HPG axis disruption by HPA-mediated mechanism(s) is based solely on conclusions drawn from correlations in the presented data sets. Significantly more work needs to be done before the hypothesis can be accepted or rejected.

There was general agreement that the LH surge is a key event in the adverse outcome of atrazine treatment. Indeed, without the LH surge there is no estrous cycle in rats or normal menstrual cycle in women. There was considerable discussion on the inadequate and limiting data available and the critical need for more details on toxicological pathways, on CNS site and mechanisms of action of atrazine and on the implications of compromised immunological adverse effects.

The limited data on the pharmacokinetics (PK) of atrazine was of particular concern. The available PK evidence is based on dosing by oral gavage, and it is difficult to use this in the interpretation of toxicological studies based on intraperitoneal (IP) administration. IP administration bypasses first pass intestinal metabolism that has been shown to be important in determining the bioavailability of atrazine and its various metabolites. The nature of the gut contents (and formulation) has been shown in many studies of absorption of therapeutic agents to have a marked effect on the pharmacokinetic behavior of the active ingredient. It is therefore not possible to predict with any certainty the pharmacokinetic behavior of atrazine dissolved at low concentrations in drinking water on the basis of that observed following oral administration in a bolus (methyl cellulose used by Stoker and Cooper, 2007; corn oil by Ross and Filipov, 2006, and Ross et al., 2009) or high doses suspended in water (McMullin et al., 2007).

An additional concern was the paucity of information on the concentrations of freely dissolved atrazine and metabolites in the plasma and other body compartments since it is only this fraction that is available for interaction with receptors. Material that is bound to plasma or tissue components is not available for either interaction with receptors or elimination. Thus, although atrazine is detected in brain tissue for considerable durations, its relevance is completely unknown because its partitioning in the CNS is unknown. There are further problems when considering the effects of parent compound and metabolites since not only do they have different PKs, but their pharmacodynamic activities may also differ. These factors have implications for the interpretation of the relevance of *in vitro* data for *in vivo* systems.

The fundamental question of human relevance of the rat data raised considerable disquiet, as did the repeated question of their relevance in determining water sampling frequency. The rat estrous cycle is 4 days in duration with no discernable follicular phase. The sheep estrous cycle is 16-17 days with a 2-3 day follicular phase and the 28 day menstrual cycle in women has a 14 day follicular phase. Not only are the time windows of ovarian cyclicity considerably different between these species, but the hypothalamic-pituitary mechanisms controlling the LH surge (namely the positive feedback actions of estradiol) are not comparable and, in particular, the lack of necessity for a rise in GnRH release at the time of the LH surge in primates, which is an obligatory component in infra-primate species.

Further, the duration of the neuroendocrine events associated with follicular development vary considerably between species; in humans it takes approximately 14 days for the Graaffian follicle to produce enough estradiol to initiate the LH surge. This contrasts with 2-3 days in the ewe and in all probability considerably less time in rats. Interestingly, if sufficient estradiol is administered to rats it will generate an LH surge every day (pm) until the brain/pituitary is exhausted. If sufficient estradiol is given to a woman during the follicular phase of her menstrual cycle, that phase will be terminated and a new cycle initiated with a spontaneous LH surge occurring 14 days later. Thus, it would not be unreasonable to suggest that events that perturb this neuroendocrine signaling system would also have differing time scales depending on species: longer in women and considerably shorter in rats. A similar scenario is likely to operate concerning puberty where gonadotrophins rise within a matter of days in rats versus years in humans.

There was no general agreement that the very high doses of atrazine used in the rodent studies failed to produce valid physiological data. However, the dosing regimes used in these trials are not fully comparable with the exposure of humans or wild life species through drinking water. It was acknowledged that the EPA had no alternative to working with the available data.

Thus, doses of atrazine that attenuate the LH surge in the rat are estimated to be:

LOEL: 3.65 mg/kg/day for 26 weeks to attenuate the LH surge
6.25 mg/kg/day for 4 days to attenuate the LH surge

NOEL: 1.8 mg/kg/day for 26 weeks to attenuate the LH surge
6.25 mg/kg/day for 1 day to attenuate the LH surge

Question 1.3

The Agency has preliminarily concluded that atrazine directly targets cells within the HPA axis (Sections 3.2 & 3.3.2.3 of the draft issue paper) and that the increased activity of the adrenal axis does not reflect nonspecific stress. Evidence for this conclusion is based on the following observations.

a. Several studies report increased adrenocorticotropic hormone (ACTH) and corticosterone levels immediately following a single exposure to atrazine and its two metabolites (deisopropyl-atrazine, DIA; deethyl-atrazine, DEA) but not diamino-s-chlorotriazine (DACT); Laws et al., 2009; Fraites et al., 2009; Pruett et al., 2009).
b. A single intravenous administration of DEA in vivo (Fraites et al., 2009) also causes an immediate increase in ACTH and adrenal hormone release, indicating that a chlorotriazine-induced gastrointestinal distress is not driving the hormonal response.
c. The adrenal corticosterone response to continued oral exposure to atrazine did not habituate in rats (Fraites et al., 2009; Laws et al., 2009) or mice (Pruett et al., 2009).
d. Although, atrazine induced a rapid increase in ACTH, prolactin secretion was not affected in the same animals (Laws et al., 2009). This is unusual in that an increase in both ACTH and prolactin are typically observed in response to a number of physiological and psychological stressors.

Please comment on the extent to which the available evidence supports the preliminary conclusion that atrazine and its intermediate metabolites (DIA and DEA) induce changes in the HPA axis as a result of a direct action on the HPA tissue and such changes are not due to a generalized or non-specific stress response. Are there data on other substances that would support this conclusion?

Panel Response:

Synopsis of available evidence

It was recently reported that the acute administration of atrazine and its metabolites, DIA and DEA, stimulated HPA-linked hormone secretion in male Wistar rats (Laws et al, 2009). This effect was not seen for the most predominant atrazine metabolite, diamino-s-chlorotriazine (DACT). Following a single oral administration of atrazine or DIA, secretion of the pituitary stress hormone ACTH, and the adrenal stress hormones, corticosterone (CORT) and progesterone (P4), all increased. This occurred in a dose-dependent manner, with the peak of stress hormone secretion coming 15-30 minutes after oral administration of the agents. These results were compared with those found following restraint stress, with the major difference being that prolactin secretion was stimulated by restraint, but not following atrazine administration.

In a related set of studies (Fraites et al., 2009), the effects of atrazine, DIA, or DACT on HPA function were examined using female Long-Evans rats as an animal model. HPA- linked hormone secretion was measured on proestrus in these experiments, which utilized either single- or multiple-dosing protocols. Atrazine or DIA given acutely caused a significant ACTH response, as did restraint stress. Following chronic (4X over 4 days) administration of DIA, the ACTH response was significantly attenuated (i.e., showed signs of habituation). This did not occur with the CORT response. The ACTH and CORT responses to repeated restraint stress did not exhibit habituation. The effects of DIA were not due to any GI stress caused by the agents, as vagotomy did not significantly alter the patterns of ACTH, CORT, or P4 release following acute administration of atrazine. ACTH or CORT responses to repeated intravenous DIA were not measured.

A separate set of studies by Pruett et al. (2009) compared both acute and subacute (28-day) HPA and immunotoxicity responses to atrazine and other known neuroendocrine disruptors (ethanol (EtOH), propranil) in female mice. As was found in the studies by Laws et al. (2009) and Fraites et al. (2009), acute atrazine administration significantly activated CORT secretion. In the repeat dose studies, habituation of the CORT response did not occur, whereas this response to EtOH and propanil habituated over the course of the study. Further, both acute and repeated dose atrazine affected many immunological parameters including decreasing white blood cell counts, the lymphocyte/WBC ratio, % of B-lymphocytes and natural killer (NK) cells,in the spleen and the expression of Major Histocompatibility Complex class II protein on B cells in the blood. Single and repeat doses of atrazine also increased the neutrophil/WBC ratio. Finally, acute atrazine decreased cluster of differentiation 4 (CD4) and cluster of differentiation 8 (CD8) T cell counts in the blood, whereas longer term atrazine administration increased these parameters. Overall, both acute and longer term atrazine administration caused different changes in

immunological measures than did restraint stress (for which animals showed adaptation), indicating atrazine alters physiological processes that both activate HPA function and disrupt the immune system. Whether the effects on immune parameters were mainly due to increased CORT secretion cannot be precisely determined by these studies. However, the alterations in immune system parameters by each agent roughly followed the predicted effects of the levels of long-term CORT secretion that they stimulated.

A final set of studies presented at the FIFRA meeting on behalf of Syngenta (Syngenta, 2010) found that, in terms of its actions on the HPA axis, acute atrazine administration stimulated ACTH, CORT and progesterone (P4) secretion within 30 minutes in adult male Sprague-Dawley and female Wistar rats. The effect of atrazine on CORT secretion habituated in male Sprague-Dawley rats, but did not do so in female mice.

Panel discussion regarding supporting evidence

A) Does the current evidence support the hypothesis that atrazine (ATR) and its metabolites desisopropylatrazine and diamino-s-chlortriazine (DIA, DEA) alter HPA function via direct action(s) on HPA tissues?

Overall, the Panel agreed that there currently is insufficient evidence for the EPA to draw the conclusion that atrazine works directly on the HPA axis tissues in order to stimulate the hormonal responses seen in the studies by the Laws, Fraites, Pruett, or Handa groups. Further, it was noted that the doses of atrazine used to elicit the effect were extremely high, and unlikely to occur in an ambient setting.

Main discussion points

1. The data from the studies to date investigating the effects of atrazine on HPA axis function were obtained using different species and sexes, so it is difficult to extrapolate findings from one set of studies to another.

2. In the studies by Fraites et al. (2009), ACTH secretion showed signs of habituation following repeated administration of atrazine. However, in these studies and those done by Pruett et al. (2009) and Syngenta (2010) using female mice, no habituation of CORT secretion following atrazine administration occurred. There are many possible explanations for these results, including a direct action of atrazine and/or its metabolites on hypothalamic-pituitary-adrenal tissues. However, Panel members noted that one major interpretational difficulty for all these studies is that basal CORT levels were not measured prior to atrazine administration. Further, CORT measurements were only taken after atrazine or DIA administration on the final day of the repeat dose studies, and not on alternate days or times. These omissions make the determination of whether repeated administration of atrazine caused an overall increase in basal CORT secretion or a change in the circadian rhythm of CORT secretion impossible. Thus, it was strongly recommended by Panel members that basal CORT values and those of other HPA-linked hormones be obtained at various times during any subsequent testing of the effect of atrazine on HPA function.

3. Given the lack of CORT secretion habituation following repeated dosing by atrazine in females, a plausible hypothesis for the above findings is that atrazine caused a perturbation of a peripheral parameter that in turn stimulates HPA activity. Alternatively, this effect could plausibly be caused by a direct action of atrazine on adrenal function, including an increase in steroidogenesis in the adrenal cortex. Other potential causes discussed by the Panel included a masking effect due to the higher circulating levels of corticosterone-binding-globulin in females compared with those found in males, an alteration in the circadian rhythm of CORT secretion, and an increased sensitivity or number of melanocortin 2 receptors in the adrenal cortex to ACTH. It should be noted that some studies investigating the effects of atrazine on CORT secretion from the adrenal gland indicate a direct inhibitory effect on the synthesis of this hormone in the fish and frog (Bisson and Hontela, 2002; Goulet and Hontela, 2003). However, it is possible that the effects of atrazine on other HPA axis functions may over-ride this hypothetical action.

4. Some of the findings reported by Pruett et al. (2009) regarding the effect of atrazine on the immune system and attributed to CORT could instead be due to increased peripheral catecholamine secretion caused by atrazine and/or its metabolites. For instance, it has been shown that stress-induced catecholamine secretion significantly alters gene transcription in the rat spleen (Gavrilovic et al., 2010), and also alters B and Thymus (T) cell profiles in mice (Edgar et al., 2003). Thus, there may be a role for the sympathetic nervous system in the disruption of immune function by atrazine.

5. Panel members expressed the opinion that the inhibitory effect of atrazine on phosphodiesterase (PDE) activity could explain some of the findings presented. For instance, the apparent lack of CORT secretion habituation to atrazine could plausibly be due to atrazine's hypothesized blunting of PDE activity. PDE inhibition results in increased intracellular cAMP levels. Since cAMP/PKA second messenger signaling pathways are involved in the regulation of a large number of physiological processes, the alteration of the activity of any PDE isoform(s) could cause a response significant enough to stimulate HPA activity.

6. Evidence that atrazine may work via a direct central mechanism to alter normal HPA function includes the finding that subdiaphragmatic vagotomy did not block the HPA response to atrazine administration. Additionally, intravenous administration of DIA activated ACTH and CORT release in a manner similar to that seen following oral administration of the atrazine metabolite. A Panel member noted that the gastric distress data are not definitive, and suggested that the conclusion could be tested by performing a behavioral assay of feeding where rodents ingest inert clay (kaolin) in proportion to gastric distress (pica behavior). EPA commented that those assays had been run recently, and the data indicate that the treated animals did not eat more clay than controls.

7. The lack of prolactin secretion following the administration of atrazine was not viewed as strong evidence that this is an HPA-specific event. This pituitary hormone is often secreted in response to non-specific stressors such as restraint, and its release is generally considered to be directly under control of hypothalamic dopamine (DA), which inhibits its release. Several other instances of a disassociation between prolactin secretion and the administration of a stressor have

been reported (Muir and Pfister, 1986; Rots et al.,1996a and b), and the Panel, in general, felt this finding was not conclusively supportive of the hypothesis in question.

8. It was also suggested that the Agency should consider the effect of atrazine on other hormones that typically are involved in a generalized stress response in order to assess whether effects of atrazine are independent of a stress response. For example, thyroxin levels are typically elevated in a generalized stress response. However, available studies have indicated that thyroxin levels decrease or are not affected by atrazine dosages that affect the HPA axis. This argues against atrazine eliciting a generalized stress response. Growth hormone levels are typically reduced in a generalized stress response. Effects of atrazine on growth hormone levels have not yet been evaluated, but could shed additional light upon the MOA of atrazine. Noteworthy is that growth hormone secretory profiles are sexually dimorphic in rats and this dimorphic secretion profile is responsible for the sexually dimorphic expression of the steroid hydroxylases CYP2C11 and CYP2C12 in rat. Should atrazine affect growth hormone secretion, the expression of sex-specific hepatic enzymes could be altered, resulting in sex-specific effects on steroid metabolism. Such effects could contribute to sex-specific responses to atrazine, such as susceptibility of the HPA axis to habituation by repeated atrazine exposure.

B) Are there data on the effect of similar substances on HPA activity?

Supporting Evidence

Other small, non-polar molecules like atrazine have actions on HPA axis function, albeit in a less robust manner. For instance, simazine has similar metabolites, and has been shown to activate HPA function in a manner similar to atrazine, but with lesser potency (Laws et al., 2009). This agent also causes a disruption of the HPG axis (Laws et al, 2003). The same holds true for propazine (Laws et al., 2003, 2009).

Other Comments

1. When viewed in combination, the studies of Laws et al. (2009) and Fraites et al. (2009) indicate a potential sex difference in HPA responsiveness to chlorotriazines. For instance, the 15 minute peak of plasma ACTH in males was approximately 200 pg/ml following treatment with atrazine (100 mg) whereas in females a dose of atrazine (75 mg) resulted in plasma ACTH levels of over 500 pg/ml. However, as stated above, these studies were done using different strains of rats. Consistency in choosing rat strains in which to study the effects of chlorotriazines on HPA function is suggested.

2. There are several studies that could be performed in order to further delineate whether atrazine and its metabolites activate the HPA axis via direct action at the pituitary or hypothalamus. For instance, the effect of atrazine on blood pressure, peripheral and central cytokine release, and peripheral catecholamine release could be studied. Also, studies investigating the sites of action of atrazine in the brain could be performed, using Fos as a marker of neuronal activity. Finally, investigation of the effects of atrazine on a number of brain signaling systems was suggested. These included the corticotrophin releasing factor and arginine vasopressin systems and the noradrenergic system.

Summary

The evidence that atrazine and its metabolites activate the HPA axis is strong, as is the correlation between atrazine-induced CORT secretion and reproductive dysfunction. This is in itself sufficient to warrant concern regarding the effects of this herbicide on human and animal health. Whether this is due to a direct action of these agents on the brain or pituitary needs to be further investigated before a conclusion can be made regarding their mode of activating HPA-linked hormone secretion.

Question 1.4

Based on a review of in vitro studies evaluating the effects of atrazine on estrogen production, the Agency has preliminarily concluded that atrazine does not have a direct effect on the catalytic activity of aromatase. However, with continued exposure (> 24 hrs) atrazine can cause increased estrone and estradiol production in the H295R and JEG-3 cell lines (Sanderson et al., 2000; Laville, 2006; Higley et al., 2010). These changes in estrogen production have been associated with increased cAMP and CYP19 mRNA (Sanderson et al., 2000, 2001) that are part of a complex mode of action through which atrazine up-regulates the gene expression of aromatase and possibly other enzymes within the steroidogenic pathway (Section 3.3.2.3 & Figure 3, Arrow 7of the draft issue paper). The hypothesis that atrazine alters multiple steroids is supported by increased testosterone concentrations in H295R cells following exposure to atrazine (Higley et al., 2010), and in vitro studies demonstrating that atrazine affects a number of signal transduction pathways and/or transcription factors in a variety of cell lines (Suzawa and Ingraham, 2008; Albanito et al., 2008). Up- regulation of the expression of a group of major steroidogenic genes has been observed in JEG-3 cells (Suzawa and Ingraham, 2008). In vivo, there is little evidence that atrazine alters aromatase per se (Modic, 2004) and the evidence associating atrazine exposures to increases in serum estrogens appears to be related to a general increase in gonadal and adrenal progesterone and androstanedione (Modic, 2004; Laws et al., 2009).

Please comment on the extent that the available data do or do not support the Agency's preliminary conclusion that atrazine has a general stimulatory effect on steroidogenesis, as opposed to a direct effect on aromatase.

Panel Response:

The enzyme, aromatase, is a product of the CYP19 gene and is responsible for the aromatization of androgenic C-19 steroids to estrogens (e.g., 17β-estradiol, estrone). Aromatase is expressed in various tissues in both males and females. Gonadal expression of aromatase is largely responsible for elevated plasma levels of estradiol and related estrogenic hormones in reproductively mature females. Aromatase also is expressed in other tissues including bone, brain, and placenta. In males, immature females, and reproductively senescent females, plasma estrogens are likely the product of aromatase activity in these other tissues. Aromatase gene expression in different tissues is under different regulatory controls. This tissue-specific regulation stems from the existence of multiple promoter regions that control expression and the

differential production of estrogens in response to different stimulatory factors (i.e., hormones) (Simpson et al., 2002).

Ovarian aromatase is under the regulatory control of the gonadotropin follicle stimulating hormone (FSH). FSH, produced by the pituitary gland, stimulates a signal transduction cascade within ovarian granulosa cells resulting in the intracellular elevation of cyclic adenosine monophosphate (cAMP) levels. Elevated cAMP then stimulates the association of the transcription factors Steroidogenic Factor-1 (SF-1) and cAMP-regulatory element binding protein (CREB) to their adjacent response elements resulting in the stimulation of CYP19 expression. Thus, chemicals that enhance the interaction of SF-1 or CREB to their response elements could modulate aromatase expression.

Adipose aromatase appears to be under the joint regulatory control of cytokines (such as tumor necrosis factor (TNFα, and interleukins (IL) IL-6, and IL-11), glucocorticoid hormones, and the transcription factor SP-1. Cytokines stimulate signal transduction pathways within adipocytes that result in the interaction of the signal transducer and activator of transcription 3 (STAT3), and possibly others, with its response element. Glucocorticoid hormones bind and activate the glucocorticoid receptor which binds to the glucocorticoid response element (GRE). The glucocorticoid receptor, activated by glucocorticoid hormone, interacts with this response element to modulate aromatase gene expression. SP-1, which is activated by phosphorylation (by protein phosphatase 1 (PP1), and possibly other phosphatases), in adipocytes binds its response element to activate CYP19 gene transcription. Together, these transcriptional regulators stimulate aromatase activity in adipose tissue. Thus, chemicals that modulate any of these pathways have the potential to modulate aromatase expression.

Finally, placental aromatase appears to be under the regulatory control of multiple factors. Perhaps most relevant to the current issue is its apparent regulation by ligands to the retinoid X receptor (RXRα). RXR ligands include some retinoids, fatty acids, and xenobiotics.

Atrazine has been shown to elevate aromatase activity in cultured cells. The issue at hand is whether this is a specific effect of atrazine on aromatase or a generalized effect on steroidogenesis.

The Panel was aware of no precedent for atrazine binding directly to the aromatase enzyme and enhancing its activity through some allosteric modification. While there is precedent for this type of direct activation for some enzymes, aromatase does not appear to be susceptible to this type of activation by any compounds.

As discussed above, multiple regulatory sites exist through which atrazine could impact expression of the CYP19 gene, thereby altering transcription of the aromatase mRNA and ultimately synthesis of the enzyme. Greatest evidence exists for a role for atrazine in stimulating CYP19 gene transcription via the cAMP/SF-1 pathway. Atrazine has been shown to increase cAMP levels and activate SF-1 via increased phosphorylation. These two mechanisms are likely to contribute to or are responsible for the increase in CYP19 mRNA levels. This SF-1 signaling process is universally involved in regulating adrenal and gonadal steroidogenic pathways. Thus, the effect of atrazine on aromatase activity may be viewed as part of a generalized effect on the

steroidogenic pathways. Indeed, atrazine has been shown to increase expression of at least eight genes involved in steroidogenic pathways that are known to be regulated by SF-1(Suzawa and Ingraham, 2008).

Before the conclusion can be made definitively that atrazine's effect on aromatase gene expression represents a generalized effect on steroidogenesis mediated via SF-1 and cAMP, potential interaction of atrazine via other known regulatory sites of aromatase must be evaluated.

- FSH: Limited studies have examined the effect of atrazine on FSH. Steroidogenesis is promoted by FSH by the stimulation of the development of follicular granulosa and thecal cells and the induction of LH receptors. Future *in vivo* studies should include measurements of FSH to better establish any possible effects of atrazine on this regulatory pathway.
- Cytokines: The Panel is aware of no effect of atrazine on TNFα, IL-6, or IL-11 activity.
- Glucocorticoid hormones: Atrazine does not mimic the effect of glucocorticoid hormones, though atrazine can increase endogenous glucocorticoid levels. This effect would not be part of the generalized effect on steroidogenesis but may result in increased aromatase expression in some tissues.
- SP-1: The Panel is aware of no evidence for an effect of atrazine on SP-1 activity.
- RXR: The Panel is aware of no evidence for an effect of atrazine on RXR signaling.
- Catabolic enzymes: Elevated plasma concentrations of steroid hormones are caused by an imbalance between biosynthesis and catabolism. While the effects of enhanced steroid concentrations after atrazine are likely driven through enhanced biosynthesis, catabolism of hormones may also be impaired by atrazine. As a substrate for CYP3A4, atrazine is also a relatively potent inhibitor of CYP3A4 (IC50 or half maximal inhibitory concentration = 2.8 μM) which may actually impair steroid catabolism and contribute to diminished clearance of steroid hormones such as testosterone and estradiol.

As noted by one Panel member, most of the *in vitro* studies have focused on atrazine without regard to metabolite formation or activity, but the papers that did evaluate the activity of metabolites indicate that the major metabolite, DACT, is not active. The two monodealkylated metabolites, DIA and DEA, are active, although perhaps less so than atrazine itself. The work of Breckenridge et al. (2010) indicates that DACT can block the inhibitory action of atrazine on phosphodiesterase, one of the mechanisms suggested for the increases in aromatase. There is little evidence from the rodent studies reviewed that aromatase is elevated *in vivo*. The evidence that has been cited to support this possibility is the observation that estradiol and estrone levels increase while testosterone decreases. With the exception of the preliminary report of data from an unpublished study in the public comments, the increase in estradiol/estrone levels seems to be a fairly consistent finding. However, in studies where it has been directly evaluated (Modic, 2004; Rivest and Sanderson, 2010) gonadal aromatase is not induced *in vivo*. As pointed out in the Modic discussion, involvement of aromatase in other tissues has not been ruled out. Possible effects on metabolism of estradiol/estrone were suggested as well as a rise in aromatizable androgen substrate produced by the adrenal. The reason aromatase is of interest is the detection of elevated estradiol/estrone levels in many studies; thus most of the *in vitro* studies have focused on aromatase specifically and did not evaluate steroidogenesis more broadly. The study by Higley et al. (2010) does report an increase in testosterone as well as estradiol. The *in*

vivo work of Modic also indicated a transient elevation of testosterone after exposure to atrazine. Other *in vitro* studies (Suzawa and Ingraham, 2008) addressing mechanisms suggest a possible broader effect on steroidogenesis. Again, one issue with many of these *in vitro* studies is the lack of attention to the role of metabolism or a broader range of steroidogenic enzymes. Even when relatively high atrazine doses are administered *in vivo*, metabolites dominate and may well be the active agents.

In conclusion, there is significant evidence that atrazine has the potential to regulate gonadal CYP19 expression through its stimulation of the cAMP/ SF-1 signaling pathway. This effect would be expected to be common to most steroidogenic pathways along the hypothalamic/pituitary/adrenal and gonadal axes. It is also plausible that atrazine stimulates adipose CYP19 expression through its activation of the HPA axis resulting in increased glucocorticoid levels. Many uncertainties remain regarding the potential effects of atrazine on aromatase gene expression and the broader range of steroidogenic enzymes.

Question 1.5

A series of studies evaluating the effect of either peripubertal or gestational exposure of atrazine in the male rat indicate that atrazine decreases testosterone concentrations and may lower androgen dependent tissue weights but that it has little effect on basal LH secretion in the male. This response generally occurs at doses of 50 mg/kg and above. In contrast, atrazine in vitro has been found to consistently increase steroid hormone production. Thus, there is an apparent discrepancy between the in vivo and in vitro data. However, our understanding of the interplay between the adrenal and the gonadal axes may provide an explanation. At the doses used in the in vivo studies, atrazine induces an activation of the adrenal axis (increased ACTH and corticosterone) and the increase in corticosterone does not habituate (i.e., it does not lessen with repeated dosing). There are a number of studies in the literature demonstrating that prolonged corticosterone stimulation will impair testosterone production by a direct action on steroidogenesis in the Leydig cells. Therefore, the in vivo effects on testosterone synthesis are likely the result of an adrenal hormone-mediated down regulation of HPG axis (through corticotropin releasing hormone [CRH] modulation of GnRH pulsatility) or directly on the testis (through a glucocorticoid receptor mediated change in steroidogenesis).

Please comment on the degree to which the proposed mode of action (Figure 3 of the draft issue paper) provides a biologically plausible explanation for the decrease in serum and testicular testosterone identified in the in vivo studies. Please comment on the extent that the available data do or do not support this hypothesis.

Panel Response:

The *in vitro* studies reporting an increase in steroidogenesis were discussed under Charge Question 1.4. The EPA Issue Paper (Section 3.3.2.1) reviewed several studies in which testosterone levels were decreased by exposure to atrazine during the peripubertal or gestational period, and at least one study (Victor-Costa et al., 2010) where exposure to a commercial formulation of atrazine and inerts reduced testosterone in adult rats. There was generally no consistent, corresponding effect on LH, although there was evidence of a trend for a decrease in

LH as the dose of atrazine increased in the study of Stoker et al. (2000). However, it is difficult to detect changes in LH using a single point measurement in intact animals, and thus the lack of reported significant effects on LH levels corresponding to reduced testosterone levels is difficult *to interpret.

While there was no direct assessment of effects on the HPA axis in the studies that report reductions in intratesticular and/or serum testosterone, the proposed MOA involving the HPA axis is plausible. Ample evidence indicates that atrazine stimulates the HPA axis resulting in increased production of adrenal corticosteroids and progesterone. Induction of the HPA axis is well known to suppress the HPG axis, predominantly through the inhibition of hypothalamic GnRH and/or the secretion of gonadotropin inhibiting hormone, resulting in the suppression of LH from the pituitary. Direct suppression of Leydig cell synthesis of testosterone by adrenal corticosteroids has also been demonstrated. Despite these facts, uncertainties in the proposed mechanism remain.

The pituitary secretion of LH is positively influenced by SF-1 signaling and good evidence exists from the *in vitro* studies reviewed under Question 1.4 that atrazine stimulates SF-1 signaling. Thus, the negative impact of atrazine on LH secretion suggests that this suppressive effect outweighs its potential stimulatory effect on SF-1 signaling along this pathway. Alternatively, SF-1 signaling in the pituitary may not be sensitive to the stimulatory effects of atrazine. The relative contribution of these potential conflicting effects of atrazine on the HPG axis need to be scrutinized in order to definitively establish the mechanism of atrazine action on the HPG axis.

A second question that arises concerning the obligatory involvement of the stimulation of the HPA axis in atrazine's reported effects on testosterone synthesis comes from the reported effects of the major atrazine metabolite, DACT, on the time to puberty in males, which is a relatively sensitive endpoint affected by atrazine. DACT has been shown in recent studies to have no effect, or a very weak effect on the HPA axis. Presumably, the effect of atrazine on the timing of puberty in males is related to its actions on the HPG axis. Data on the pharmacokinetics of atrazine indicate that DACT is formed rapidly on exposure to atrazine and is the major metabolite. The data of Ross et al. (2009) indicate that DACT is found at high levels in the brain, and is persistent relative to other atrazine metabolites. As is brought out in the discussions of later questions, there is much that is not known (including levels of DACT accumulated, the proportions of DACT that are free and protein-bound, whether protein binding plays any role in activity) about the fate of DACT in the multi-dose pubertal assay. However, these issues and the overall issue of the DACT effect on the timing of puberty should be addressed in the context of the proposed HPA-mediated inhibition of testosterone synthesis.

Finally, the study of Rosenberg et al. (2008) reported that prenatal exposure to atrazine results in delayed puberty (preputial separation) and a lasting depression of testosterone levels. While there were non-significant reductions in pup body weights that could have contributed to the delayed preputial separation observed in this study, the data do suggest the possibility of a lasting effect on steroidogenesis from prenatal atrazine exposure. There were no data on the HPA axis from this exposure scenario, but one would expect that activation in the dam would be likely. There are data indicating that fetal glucocorticoid exposure can delay puberty (e.g., Smith

and Waddell, 2000). However, Drake et al. (2009) found no effect of glucocorticoid treatment during pregnancy on fetal testosterone synthesis, although it did exacerbate the suppression of testosterone synthesis produced by dibutyl phthalate. If reproducible, the role of the HPA axis in the prenatal effects of atrazine on testosterone synthesis and puberty in the male would need to be evaluated.

In conclusion, the proposal that atrazine-mediated suppression of testosterone levels is mediated through the HPA axis is plausible, but further data are needed to support this hypothesis.

Question 1.6

Based on a review of the neurotoxicity studies (Section 3.4 of the draft issue paper), the Agency has preliminarily concluded that several recent studies provided further support for the concern that dopaminergic neurotransmission may be affected by atrazine, a concern that was raised by studies prior to 2003. However, the Agency has also concluded that several aspects of these studies of the dopaminergic neuronal pathways, in particular the changes noted in stereological evaluations and the observed changes in behavior, should be considered as preliminary findings. This conclusion is based on the notable limitations identified in the data including: (1) lack of clear dose-response relationships, (2) lack of inclusion of suitable positive controls to confirm the competency and reliability of the procedures utilized in examining dopaminergic systems in the brain, (3) limited data to corroborate stereological findings, (4) limited or no supporting histological and behavioral assessments, and (5) no consideration of the potential role of the HPA axis (e.g. alterations in corticosterone). EPA has further determined that two non-dopaminergic neurotoxicity studies--one on brain somatostatinergic systems and the other on neurobehavior in mice -- also have significant limitations (e.g., lack of details on source and purity of atrazine, and age and body weights of the mice; poor quality of amino cupric silver staining photomicrographs; limited presentation of reverse-transcriptase polymerase chain results; lack of data to corroborate conclusion of neuronal degeneration; reference citations did not support statements made in the text; no explanation of biological plausibility of alterations in somatostatinergic receptor subtypes; inappropriate statistical evaluation of data (litter vs. pup); discrepancies in number of pups examined for behavioral endpoints, and lack of objective and validated behavioral tests). Please comment on these preliminary conclusions regarding the neurotoxicity findings.

The Agency has preliminarily concluded that the available studies indicate that the neurotoxicity endpoints examined are not more sensitive than those evaluated for neuroendocrine function following atrazine exposure (e.g., attenuation of LH surge and estrous cycle disruptions in female Sprague-Dawley rats which form the basis for the current chronic RfD). In addition, the Agency has concluded that there is no association between atrazine exposure and development of Parkinson's Disease on the basis of non-specificity of effects on brain dopaminergic systems, lack of histological and behavioral features characteristic of Parkinson's Disease, and results of epidemiological studies. Please comment on this preliminary conclusion.

Panel Response:

The literature review provided by the Agency on pages 41-44, in Appendix A of the EPA Issue Paper and in the presentation given on Monday, April 26, 2010 suggests that dopaminergic neurotransmission may be affected by atrazine as shown by studies conducted prior to 2003 and more recent research. The Panel agreed that while there is evidence that atrazine can alter dopamine transmission, this is not evidence for a specific Parkinson's Disease effect. However, these changes in dopaminergic or other neurotransmitter systems may contribute in other ways to the mode of action of atrazine.

The Agency summarized the strengths, weaknesses and deficiencies in the neurotoxicological studies supplied to the Panel for review, and has made a thorough evaluation of these studies. The deficiencies noted are substantial. The Panel agreed with the Agency's comments that each study presented for evaluation individually suffers from technical errors or problems with reporting. However, because of the cumulative weight of evidence and because of data presented in other sections of the EPA Issue Paper there was disagreement among members of the Panel as to whether the data indicate that the neurotoxicological endpoints present a more sensitive and more credible endpoint than do the neuroendocrine endpoints currently in use. Some Panel members expressed the opinion that, when considered in total, the emerging body of evidence is sufficient to raise concerns that justify further investigation of the neurotoxicity and neurobehavioral effects of atrazine at environmentally relevant doses across all stages of the life span.

The difficulty that the Panel had with the available data stems from the inconsistencies in dose-response relationships and other details of methods reported in the studies reviewed in section 3.4 of the EPA Issue Paper. The inconsistencies rendered the results of the studies difficult to interpret and do not permit confident conclusions regarding the reliability of the effects described. One opinion expressed among some Panel members was that these studies do not suggest that neurotoxicological endpoints are any more significant or more sensitive than those currently in use and thus these neurotoxicological studies do not present data or endpoints that are suitable to displace the currently used point of departure that is related to the neuroendocrine effects of atrazine on the LH surge. The dose levels at which effects were noted in these papers were frequently high, and in most cases higher than the current point of departure, and therefore, do not identify a more sensitive endpoint. The inconsistencies in dose-response relationships throughout these studies are difficult to interpret and do not result in confident conclusions regarding the reliability of the effects described. Similarly, the *in vitro* studies are very difficult to extrapolate to *in vivo* effects. One study that is suggestive of effects at low enough exposure levels in intact animals that might be usable for consideration of a new risk assessment endpoint is the Rodriguez et al. (2005) study. However, very high doses were used in the acute dosage experiments. The dose levels used in the chronic exposure experiments are not clearly defined. Details of methods used to dose the animals by feeding spiked food are not presented. There is no indication of how the atrazine was mixed in the food, of the type of food, or how the amount of food consumed by individual rats was measured to determine the quantity of atrazine administered. The doses are described as 0, 5 or 10 mg/kg, and not in units of mg/kg/day, so it is not clear whether daily dosing was conducted. The data from the study reflect few changes in neurochemistry, and these were not always dose-related. There was

substantial overlap in the stereological results between control and treated animals. Therefore there is little confidence that the observed effects in this study were attributable to exposure to atrazine, and this precludes the use of these data in risk assessment and especially its use to displace the currently well-documented point of departure.

Other Panel members expressed the opinion that when the studies are considered in total, they are sufficient to raise concerns that justify investigation of the neurotoxicity and neurobehavioral effects of atrazine at environmentally relevant doses across all stages of the life span. They questioned whether the consideration of each paper individually is sufficient to cause the Agency to disregard what may be an emerging body of evidence. For example, one Panel member noted additional studies included in the Agency's review for a different section (EPA Issue Paper, Section 3.3.1 - Female Reproductive Development/Toxicology and Appendix A.2) which showed clinical signs at low doses that may indicate neurological involvement. These clinical symptoms include salivation, lacrimation, and perineal staining. When 5 week old female Sprague-Dawley rats were treated with atrazine (30 mg/kg/day) the following results were observed: salivation within 2 weeks of treatment; salivation, lacrimation, and perineal stain within 4 weeks of treatment. The number of days of treatment at the first appearance of these effects was not included in the report (Shibayama et al., 2009). The NOAEL is at 3 mg/kg/day. In addition to the data presented in association with section 3.4, the Panel also discussed data indicating the effects of atrazine on prolactin secretion at doses greater than or equal to 12.5 mg/kg/day (Section 3.3.2.2 and Stoker et al., 1999). Prolactin secretion is regulated by dopamine; thus by inference, data on prolactin secretion can be used as an indicator of the involvement of neurotransmitter function and neurotoxicity. Prolactin was suppressed at doses similar to those at which atrazine was able to suppress LH secretion.

Additionally, a Panel member expressed some concern for the number of published literature studies that the Agency believed were inadequate for use in the endpoint selection process on the basis of inappropriate dosage, lack of a dose response relationship, small sample sizes or technical reasons. The Panel member also suggested that the Agency be sensitive to the restrictions placed on researchers who conduct research with animals (e.g., use of high doses, especially in initial experiments, to increase likelihood of a discernable response; requirements to reduce the numbers of animals used in experiments; over reluctance of institutional review boards to approve, and journals to publish, work that is viewed as replication or simple extension of previous studies; or requirements of editors to simply to shorten the length of papers by reducing the detail of the methods section). To bridge the gap between limitations placed on researchers and the study requirements needed by EPA for regulatory purposes, another Panel member suggested the Agency consider contacting the authors of published papers considered deficient in some manner to request additional information or to help clarify questions the Agency may have. While it is understood that the Agency has requirements for the conduct of studies that generate data used to support regulatory decisions, these Panel members felt that individual studies which might initially be considered deficient in some way could provide some useful information when considered collectively, such as in informing WOE analyses.

The Panel agrees with the Agency's conclusion that the available data do not provide a causal connection to the etiology of Parkinson's Disease. The early studies were focused on a model for Parkinson's Disease; however, the role of dopamine, other catecholamines, and

serotonin in the regulation of the secretion of GnRH, CRH and prolactin, the emerging data provide a plausible link and basis for additional studies related to the hypothesized MOA. However, there is sufficient evidence that atrazine is altering prolactin secretion to suspect that it does affect dopamine secretion at doses in the same range as those that affect LH secretion, and can induce neurological effects within 2 weeks that defines a NOAEL at a lower dose range (Shibayama et al., 2009). At the current time, it is unclear whether these or lower doses are also neurotoxic as defined by the criteria for this section.

The deficiencies reflected in the reports preclude further dose-response analysis, and cause the sensitivity of the neurotoxicity endpoint to be in question. A more serious concern stems from the paucity of well-controlled studies designed to examine neurobehavioral and neurodevelopmental endpoints in appropriate dose response studies. Additional studies that use positive control groups and that are conducted with sufficiently large sample sizes to give ample statistical power could resolve the questions at hand.

Question 1.7

Based on a review of two studies of the potential effects of atrazine on the prostate (Section 3.3.2.2 of the draft issue paper), the Agency has concluded that the results of the Rayner et al. (2007) study of pregnant rats treated during gestation support previous observations (Stoker et al., 1999) that atrazine treatment to the rat dam either perinatally or early postnatally can increase prostate weights (due to an increase in inflammatory infiltrate) in the male offspring. This effect on the offspring was shown to be due to a suppression of prolactin in the atrazine-exposed dams during lactation, and is consistent with the mode of action of atrazine on neuroendocrine function. The Agency also preliminarily concluded that further research is needed to provide more convincing evidence that atrazine-mediated suppression of prostate cancer in the probasin/SV40T antigen transgenic rat (androgen-dependent prostate cancer rodent model) may possibly be due to caloric restriction rather than endocrine-related as postulated by Kandori et al. (2005). Please comment on these preliminary conclusions regarding the recent prostate findings.

Please comment on the extent to which the available data support the proposed mode of action for prostatitis and the appropriateness of the rodent model in the context of human health.

Panel Response:

Stoker et al. (1999) demonstrated that postpartum day 1-4 administration of atrazine (25-50 mg/kg/d) to lactating rat dams dramatically (or completely) inhibited suckling-induced prolactin release in the dams and caused an increased incidence of lateral prostate inflammation in male offspring at 4 months of age. They observed the same effect after treating dams with bromocriptine (a dopamine agonist that inhibits prolactin release) and the effect was reversed by administration of prolactin to the dams, suggesting that the prostatitis is a long-term result of diminished amounts of prolactin (or another substance induced by prolactin in the dam) in the perinatal milk supply. The proposed mechanism to explain these results is that prolactin is important for neonatal brain development and lack of neonatal prolactin exposure leads to a failure of dopaminergic system development and eventual hyperprolactinemia in the adult male.

Hyperprolactinemia is reportedly associated with an increased incidence of prostatitis. This study did not actually demonstrate that hyperprolactinemia occurred in the male offspring, as prolactin levels were measured only at 4 months of age and were no different from those of the controls.

Rayner et al. (2007) examined whether treatment of rat dams with atrazine (100 mg/kg/d) during late gestation has adverse effects on prostate development of the male offspring at 4 months or 7 months of age. A cross-fostering design was used to distinguish between direct effects of prenatal exposure to atrazine via the placenta and indirect effects of lactational exposure to atrazine metabolites and/or altered quantity/quality of milk caused by the dam's prenatal atrazine exposure. This study demonstrated that suckling from an atrazine-exposed dam results in an increased incidence of prostatitis whether or not the male was prenatally exposed to atrazine, and prenatal exposure alone did not cause prostatitis. This finding is consistent with the Stoker et al. (1999) study, but extends the findings to indicate that the postnatal time period, not the prenatal, is the critical window of susceptibility.

Regarding the proposed mode of action for prostatitis, the Stoker et al. (1999) results demonstrate a clear connection between diminished maternal prolactin levels and prostatitis in male offspring. However, the proposal that the prostatitis is due to hyperprolactinemia in the male offspring was not tested in either of the studies. Prolactin levels (and levels of estrone, testosterone, and androstenedione) were measured only at the termination of the experiments at 120 days of age and were no different from those in the controls. It is possible that prolactin levels were elevated in the pubertal period, that this was not recognized, and that a short period of hyperprolactinemia results in prostatitis. This mechanism could be tested by following prolactin levels to determine the timing of an increase, if any, and then using bromocriptine administration to prevent it and then examining the incidence of prostatitis. It is also possible that an alternative mechanism, e.g., an effect of atrazine metabolites in the milk or other differences in milk quality, possibly via effects on the developing immune system, might explain the increase in prostatitis.

The mechanism of action of postnatal exposure (suckling from an atrazine-treated dam) in causing prostatitis needs to be better defined before assessing the possible impact on human health. For example, if the problem were related to diminished prolactin levels in the milk, then we would expect that if there was relevance to human health that the 30% of women who never breast-feed their babies at all (see http://www.cdc.gov/breastfeeding/data/NIS_data/) would have sons who were more likely to have prostatitis. A mechanism related to adverse effects of atrazine metabolites on tuberoinfundibular dopaminergic (TIDA) neuronal development would have different implications for human health, and would be more relevant to the 70% of women who do breastfeed their infants at least some of the time.

The Kandori et al. (2005) findings supporting the idea that atrazine-mediated suppression of prostate cancer in the probasin/SV40T antigen transgenic rat may be due solely to caloric restriction, rather than endocrine-related, is interesting and potentially important; however, these findings are not convincing because of difficulties with the methods and interpretation of some of the data.

1. The atrazine dose administered is uncertain. Dietary administration rather than gavage was used, and the diets had only 60-91% of the intended amount of atrazine. More than one animal was housed in each cage, so individual dietary intake could not be calculated.
2. Testosterone (T) levels were not different when comparing atrazine-treated rats with controls despite previous findings by other researchers that T levels are modified by these same doses of atrazine. This finding might be explained by increased variability in T measurements due to inconsistent atrazine dosing. Furthermore, T was only measured at the end of the study, and this measurement may not reflect the T level prevalent during the majority of the study.
3. The caloric restriction group had highly variable T levels. Previous studies have demonstrated a correlation between caloric restriction and T levels, though there are conflicting reports. It is possible that the caloric restriction effect (to diminish severity of lesions) could be explained by a decrease in T levels, or by other indirect effects on endocrine parameters.
4. There were no differences in any treatment group in the incidence of adenocarcinoma or prostatic intraepithelial neoplasia in any of the prostate lobes. The only finding was a subtle difference in morphometric measurements of the ratio of epithelial area to total prostate area. These measurements were done on only 2-4 total sections from each prostate lobe per animal, so it is unclear whether the results were entirely representative. A more robust difference in outcome would lend more confidence in the conclusions.

Regarding the appropriateness of the rodent model in the context of human health, there is a consistent finding in both rats and humans of an association between prostate inflammation, proliferative inflammatory atrophy lesions, and prostate hypertrophy/prostate intraepithelial neoplasia/prostate cancer. Indeed, anti-inflammatory agents have been tested in humans for their effects on attenuating prostate cancer, albeit with variable efficacy. The link between inflammation and cancer is robust and because of similarities to the rat system suggests that the rat is a reasonable model for human disease.

Question 1.8

Based on data obtained in studies to assess the effects of atrazine on the immune system following developmental and adult exposure, as well as in vitro mechanistic studies, EPA concluded that atrazine has the potential to affect the immune system (Section 3.5 of the draft issue paper). However, the Agency has also concluded that underlying mechanisms of atrazine-mediated immunotoxicity and its relevance to potential adverse health effects in humans are still not thoroughly understood. It is well established that products of the endocrine system modulate immune function. However, adult rodent studies have not determined whether immune system effects are caused by direct effects of atrazine and/or its metabolites or whether they are the result of modulated endocrine hormone production, which in turn affects immune cells. Two published studies indicate that exposure to atrazine during immune system development may result in altered immune function in offspring, although no conclusions were drawn on the potential adversity of the effects, because immunosuppression was observed in one study and immunoenhancement was observed in the other. Please comment on potential explanations for the disparate findings reported by Rooney et al. and Rowe et al. Many immunotoxicologists consider immunomodulation, i.e., suppression or enhancement of immune function, as a

potentially adverse alteration of homeostasis, because both have been associated with disease states. Thus, while the results Rooney et al. and Rowe et al. may appear to be contradictory, together the data indicate unintended immunomodulation at approximately the same dose in two species. Please comment on characterizing the effects of gestational atrazine exposure as immunomodulation, when describing the immunotoxicological outcome of developmental immunotoxicity studies.

Appendix B of the draft issue paper describes experiments conducted by EPA scientists on the potential developmental immunotoxicity of atrazine. The results of the experiments provide conflicting results. Additional studies did not provide a suitable explanation of the differences. The Agency believes both sets of data are of high quality. However, in the context of hazard assessment, such differences are difficult to interpret. Please comment on the information contained in Appendix B and provide suggestions for interpreting such data as part of the atrazine re-evaluation.

The available data do not indicate that atrazine-induced immunotoxicity is a more sensitive endpoint than the atrazine-induced effects on neuroendocrine function, (e.g., attenuation of LH surge and estrous cycle disruptions which form the basis for the current chronic RfD) in female Sprague-Dawley rats. Please comment on the degree to which these preliminary conclusions are supported by the available data.

Panel Response:

Please comment on potential explanations for the disparate findings reported by Rooney et al. and Rowe et al.

Atrazine was obtained from the same supplier for both studies, suggesting that this was not the cause of the different findings. However, there were many differences between the experimental designs of Rooney et al. (2003) and Rowe et al. (2006). The disparate findings of Rooney et al. (2003) and Rowe et al. (2006) could potentially be due to the following:

- Use of different species and/or inbred/outbred animals. Rooney et al. (2003) used outbred Sprague Dawley rats while Rowe et al. (2006) used inbred Th2-skewed Balb/c mice:
  - The use of limited litters from the outbred animal by Rooney et al. (2003) could affect their results. However, Rooney et al. (2003) used numerous litters of Sprague Dawley rats over a 1 ½ to 2 year time period suggesting that the results were not due to a litter effect.
- Assessment of immune function at 8 wks (rats) and 12 wks (mice).
- Different length of atrazine exposure or timing of atrazine exposure:
  - Gestational day 10 (GD 10) for 21 days (Rowe et al., 2006)
  - GD10 to post natal day 23 (PND 23), approximately 34 days (Rooney et al., 2003)
- Oral vs. subcutaneous dosing:
  - Different routes of administration likely result in different kinetics; oral administration is more likely to produce a spike in the plasma concentration;

whereas the continuous minipump administration is likely to result in a smoother plasma profile with a lower maximum concentration.

- Differences in metabolism depending on the species, and on the route of administration (time release pellets vs. a bolus (in 1% methylcellulose) administered by gavage). Different metabolites may predominate with different routes of administration. A large proportion of the parent compound atrazine is metabolized in the gut wall following oral administration, and different primary metabolic routes will be likely to be involved with subcutaneous administration. Thus, actual exposure doses of the different metabolites over time will differ in Rooney et al. (2003) and Rowe et al. (2006) studies. Whether males and females differ in their metabolism of atrazine is not known. Also, administration of atrazine in the diet or in the water would also result in different kinetic and metabolite profiles. This would be particularly important if the metabolites have different immune system actions (enhancement vs. suppression).

• Growth restriction in one model system and not the other:
  - In humans, preliminary findings of an association between atrazine concentrations in drinking water and increased risk of intrauterine growth restriction (Munger et al., 1997) have been reported, as well as a suggestion of an increased risk for small for gestational age birth with atrazine exposure in the third trimester of pregnancy (Villanueva et al., 2005).
  - Rooney et al. (2003) documented a small but significant difference in the weight of 7-day-old male rats after atrazine exposure. It is possible that intrauterine growth restriction is the primary event leading to differences in the immune response.
  - Data to determine if Balb/c mice were smaller at birth are not available (Rowe et al., 2006).
• Unknown critical experimental parameters that have not yet been defined:
  - Caging differences (e.g., animals in polycarbonate cages can be exposed to the plasticizer bisphenol A that is present in that plastic)
  - Composition of diet (e.g., the presence of phyto-estrogens such as those present in soy products)
  - Vivarium environment (e.g., noise levels that can produce stress)
  - Time of year (this is important in seasonally active animals)

Summary: The Rooney et al. (2003) and Rowe et al. (2006) studies, while seemingly producing disparate results, should both be considered to have provided high quality, technically sound observations that represent real outcomes for the rodent models and experimental systems used.

Please comment on characterizing the effects of gestational atrazine exposure as immunomodulation, when describing the immunotoxicological outcome of developmental immunotoxicity studies.

Characterizing the effect of gestational atrazine as immunomodulation is appropriate as long as the term is defined as including both immunosuppression and immunoenhancement. A better overall term may be simply to use the term immunotoxicity or unintended modulation of the immune system. Increases and/or decreases in immune system function can be deleterious.

The overall effects at the organism level of this unintended modulation of the immune system have not yet been fully characterized and may include decreases or increases in an immune response depending on the dose and timing of exposure. The overall adverse health effects of concern are primarily increased risk of infection, neoplasia, allergy and autoimmunity.

Please comment on the information contained in Appendix B and provide suggestions for interpreting such data as part of the atrazine re-evaluation.

Rooney et al. (2003) tested the hypothesis that atrazine may cause developmental immunotoxicity through disruption of prolactin or thyroid hormones. Pregnant Sprague-Dawley (SD) rats were exposed to atrazine (35 mg/kg/d) by oral gavage from (GD) 10 through (PND) 23. This dose of atrazine was chosen to be just above the NOAEL (25 mg/kg/d) for delayed onset of puberty in female offspring. Separate groups were exposed to bromocriptine (BCR) at 0.2 mg/kg/2x/day to induce hypoprolactinemia or to propylthiouracil (PTU) at 2 mg/kg/day to induce hypothyroidism. Neither PTU nor BCR caused immunosuppression in any assay evaluated. Each experiment was repeated once over approximately 18-24 months, and similar results were obtained in both replicates. Atrazine decreased the primary antibody response to sheep red blood cells and the delayed type hypersensitivity response, in adult male offspring only. The authors concluded that developmental exposure caused gender-specific suppression of immune function in adult rats, which appeared to not be mediated through the suppression of prolactin or thyroid hormones.

The above studies were completed prior to the authors moving to a new EPA facility in Research Triangle Park, NC. Additional studies then were initiated in the new laboratory and did not show developmental immunotoxicity. The first of these included two replicate studies to derive a dose response for atrazine developmental immunotoxicity in Sprague Dawley rats using doses of 0, 3.5, 17.5 and 35 mg/kg/day. The rats were from the same supplier, and immune assays were evaluated following the same operating procedures and by the same laboratory personnel. The second experiments used Sprague Dawley, Long Evans, and Wistar rats exposed to atrazine (35 mg/kg/d), under the same study design in order to assess possible strain differences. Again, immune function was not affected in male offspring in these experiments conducted in the new laboratory.

The inability of a laboratory to reproduce previously published and legitimate experiments always raises concerns regarding use of the data to arrive at firm conclusions. However, the investigators have complete confidence in the technical soundness and results of the published 2003 studies and the follow-up studies. Regarding the later negative data, they do not believe it is likely that lots of atrazine used for the studies varied in quality, or that viral, bacterial or parasitic infections may have occurred in rats and affected the data. They believe the differing results are due to an unknown environmental factor that, at this time, will probably remain unknown. Other factors that might have been considered include differences in caging, for instance polycarbonate caging containing immune-modulating bisphenol A, and differences in the rodent housing facility, for instance noise or vibration levels.

Summary: The conclusion that the differing results obtained by Rooney et al. (2003), published versus unpublished studies, are likely to be due to unknown environmental factor(s) between

their old and new laboratories is reasonable. The negative data in the second set of experiments should not negate their published conclusion that exposure to atrazine during development may modulate immune function. This situation does, however, point to the need for further experiments with atrazine in developmental immunotoxicology models, including careful examination of the dose response, as well as systematic evaluation of timing of exposure and differential gender effects.

The available data do not indicate that atrazine-induced immunotoxicity is a more sensitive endpoint than the atrazine-induced effects on neuroendocrine function, (e.g., attenuation of LH surge and estrous cycle disruptions which form the basis for the current chronic RfD) in female Sprague-Dawley rats. Please comment on the degree to which these preliminary conclusions are supported by the available data.

The preliminary conclusion that atrazine-induced immunotoxicity is not a more sensitive endpoint than the atrazine-induced effects on neuroendocrine function, is supported by the currently available data. Attenuation of the LH surge, with a NOAEL of 1.8 mg/kg/d is the appropriate present point of departure. It should be considered that the existing developmental immunotoxicity database is very limited. The Rowe et al. (2006) data, in particular, suggest the need for evaluation of inappropriate enhanced immune responses over a more extensive dose range, including lower doses. Such studies should include animal models with genetic predisposition for immune-mediated diseases, using both adult and developmental exposures.

Considering the immunotoxicity literature published since 2003, six studies using immune cells obtained from adults dealt with *in vitro* exposure to atrazine to define effects on the immune system. An effect on human NK cell function was seen in the 10-30 μM range (Rowe et al., 2007; Whalen et al., 2003), effects on mouse dendritic cells were seen at 1 μM (Pinchuk et al., 2007) and effects on cytokine production by human peripheral blood mononuclear cells seen at 0.3-13 μM (Devos et al., 2003, 2004). The maximum levels of atrazine detected in the spleen and thymus were 2-3 μM following oral doses of 5 and 25 mg/kg atrazine (Ross, 2009). Thus, the *in vitro* concentrations used were potentially relevant to the range of oral doses commonly used in *in vivo* studies.

Two studies dealt with oral gavage administration of atrazine for 14 days. Filipov et al. (2005) used 4 month old male mice (still establishing immunological memory) and noted changes in spleen and thymus cellularity at 125-250 mg/kg/d. A significant decrease in spleen cellularity was still observed 7 weeks after atrazine exposure. In adult mice (Karrow et al., 2005), the primary effect on immunity was noted at doses of 250-500 mg/kg/d for 14 days, with a decreased resistance to tumor challenge.

Intraperitoneal exposure to atrazine (100 mg/kg/d or higher) increased plasma corticosterone in the mouse (Pruett et al., 2003, 2009; Schwab et al., 2005). Whether lower oral doses would also result in significant increases in corticosterone with possible immunomodulatory effects is unclear.

In the immunotoxicity literature published since 2003, for gestational or early postnatal exposure, there are two studies that produced conflicting results (Rowe et al., 2003; Rooney et

al., 2006). These used a very limited dose range (23-35 mg/kg/d for 21- 34 days). A need is indicated for further studies to examine lower atrazine doses in developmental immunotoxicity models, and to clarify the seemingly conflicting results in the mouse and rat.

Question 1.9

After the April 2010 SAP, the Agency will evaluate the weight of evidence (WOE) for atrazine by integrating the experimental toxicology data with the epidemiological studies. As part of this work, the Agency will consider the available data on hormonal changes and functional outcomes that may be used as endpoints for deriving PoDs across different durations of exposure and for different populations (including potentially sensitive ones). Important studies evaluating the dose-response relationships for hormones involved in the HPA axis are still on-going. These studies are expected to provide high quality data that can be used to characterize the entire dose response curve. With the caveat that these data are not available now, given the current understanding of atrazine's mode of action, please comment on what would be appropriate endpoints to consider for use in deriving points of departure in a possible future risk assessment.

Panel Response:

This question addresses the logical next step, following the discussions of upstream events, and seeking to understand the pathways of toxicity and early events within the proposed mode of action (MOA) framework. This step focuses on the manifestation of toxicity downstream in the complex pathway regulatory networks, identifying the endpoints on which the human health risk of atrazine exposure can be assessed.

Endpoints for risk assessment are generally referred to as the functional or structural changes of toxicological significance that can be quantitatively defined with respect to the dose or exposure. They can be life stage- or gender-specific, and either deficits or over-expressions of events. Endpoints that serve as the basis for the point of departure (PoD) generally are identified as the most sensitive, i.e., occurring first along the increasing dose axis. These endpoints are selected for use in evaluating various human exposure scenarios such as duration and frequency of exposure (e.g., acute, short-term, long term, lifetime), the route of exposure, and population characteristics (e.g., pre- and post-natal, and perinatal developmental stages, children, women of child-bearing age, adult males and females, seniors).

It is common to expect that the manifestation of health effects for an endocrine disruptor would be broad and diverse. Other possible MOAs aside, this is true even within the framework of HPA and HPG axis perturbation presented by the Agency. The Panel noted that there are other sensitive endpoints of atrazine toxicity identified in previous assessments by the Agency and other regulatory bodies, and that these may or may not be directly associated with this specific MOA. Examples of effects of atrazine at the low dose range comparable to those endpoints for the PoD used in Agency's 2003 Interim Reregistration Eligibility Decision (IRED) include cardiopathy and atrial fibrillation in dogs, extramedullary hematopoesis in the spleen, and clinical signs of toxicity noted in several toxicity studies.

Many new data sets on newly-identified key events related to possible MOAs for the reproductive and developmental effects observed in animals following atrazine exposure are now available. The new understandings of MOAs that have been presented by the Agency may reveal pertinent endpoints that were previously overlooked. The Panel is encouraged by the advancement of understanding on the effects of atrazine on the HPA/HPG axes and recommends that dose-response relationship for each key event within this proposed MOA be characterized for the endpoint selection. To this end, one Panel member revised the MOA flow diagram presented in Figure 3 of the Agency Issue Paper to provide a more detailed set of key events using the MOA framework and incorporating the endpoint-specific discussions from this April 2010 SAP (See Appendix 1). **It is important to note that the concept of the diagram in Appendix 1 was briefly discussed late during the public meeting, but not reviewed by the entire Panel; thus, it reflects the opinion of one Panel member and not the entire Panel.**

There is a need to revisit all existing toxicity studies on atrazine relevant to characterization of its hazard profile, albeit perhaps only briefly, focusing mainly on sensitive adverse effects. Other new data in domains not considered during the present SAP meeting may also prompt a re-ordering of importance of information driving the hazard assessment. This requires the revisit of prior assessments and decisions on whether to use preexisting or new information in the updated risk assessment (e.g., for selection of PoDs).

Given that the characterization of the MOA(s) for neuroendocrine effects underlying the reproductive and developmental effects is not yet complete, it would be appropriate to screen and critique the data on each key event in the proposed MOA, including the ultimate adverse outcome before settling on a preferred set for final PoD determination. By definition, "key event" is an empirically observable and measurable precursor step that is itself a necessary element of the MOA, or a biologically based marker for such an element.

Benchmark Dose (BMD) analysis should be used to identify the sensitive endpoints. However, because different dose metrics and dose selection rationales have been used in each study, it is often not possible to determine or speculate which endpoints will have lower PoD before the BMD analysis. Thus, datasets for each key event should be subjected to BMD modeling to derive a BMDx or BMDLx (Lower bound of BMDx). Then the most sensitive endpoint can be identified by comparing the BMDs. This will also inform the role that this parameter can play in the generation of duration- and subpopulation-specific values. Endpoints for PoDs can be based on specific key events that relate to functional impairment (e.g., LH surge vs reproductive effects). The PoDs can also be based on systemic toxicity endpoints when the set of data postulated to support a MOA with its key events is not sufficiently coherent to explain and accommodate all possible critical toxicity outcomes to be evaluated by the Agency.

It is important to emphasize that endpoints not in and of themselves considered adverse (e.g., biomarker responses or perturbation levels) are relevant if a relationship to a functional outcome in the animal has been clearly established. In this regard, when the level of a hormone is identified as the endpoint, the focus should be more on homeostatic/physiological considerations as a basis for benchmark analysis, rather than on a traditional 5% or 10% change. Typically, "reversible" or "adaptive" responses would be considered adverse unless they are part of the

"precursory" response with a threshold that will not trigger the next steps of events leading to an adverse effect.

Considerations should be given also to the weight of evidence and certainty of a key event in the MOA scheme (e.g., the "thickness" of the arrows in the MOA framework/continuum in Figure 3 of the Agency Issue Paper, and Appendix 1). This allows input into a statement of the level of confidence associated with a selected endpoint. Overall, it is anticipated that the following information will accompany the endpoint for each step of toxicity perturbation or precursor events:

- Availability of desired data, and the confidence in them
- Strength of the relationship with the functional effect (the extent to which the observed perturbation is close to and reflective of the endpoint (i.e., its relevance)
- Strength of the model fit (usually considered during BMD analysis)

The Agency should also consider using the measure of internal or target tissue dose instead of the administered dose. This would facilitate a better characterization and comparison of the dose-response relationship from all studies with different species, routes of exposure and study designs. Physiologically-based pharmacokinetic (PBPK) models, depending upon their level of maturity and evaluation, might facilitate such analyses. The AUC for the unbound plasma concentration is also a potential metric of internal or target tissue does. The consideration of dose-dependent pharmacokinetics (e.g., glutathione depletion, first pass effect), if feasible, would also allow the consideration of the parent compound, and its metabolites. The Agency should obtain data for developing toxicity equivalency factors in order to assess their total exposure and dose.

Finally, the data gaps from the areas of toxicity discussed previously (e.g., male and female reproductive toxicity, developmental toxicity, neurotoxicity, immunotoxicity) should be addressed in the endpoint selection and the use of the associated PoD in human health risk assessment.

Given that the duration of the toxicological concern is a key factor in evaluating the frequency of drinking water monitoring, please include in your response a consideration of the magnitude and duration of changes in key events in the toxicity pathway that are sufficient perturb normal function and comprise human health. In defining the exposure window of interest, please comment on the toxicokinetic/dynamic considerations with respect to atrazine's effects on the HPA/HPG axis.

The MOA for atrazine's most sensitive effects is hypothesized to involve activation of the HPA axis through increased secretion of adrenocorticotropin releasing hormone (corticotropin-releasing hormone (CRH)) which leads to increased circulating corticosterone, that feeds back negatively to suppress GnRH production by the pituitary. Reduced GnRH production reduces LH and FSH production. In the female rat, these disruptions in the signaling systems of the HPA/HPG axes can contribute to premature reproductive senescence, which includes an elevated estrogen level that hastens the onset of mammary gland tumors. While this estrogen-induced carcinogenesis mechanism does not occur in the human female (estrogen

declines in menopause), the atrazine-induced reduction of LH and FSH may occur in humans and other adverse effects may ensue. In developing animals, atrazine delays onset of puberty in both sexes (Fraites et al., 2009). This effect of atrazine may also occur in humans. Pre- and postnatal development effects may also occur; the atrazine exposure levels that are involved are not yet fully characterized.

Elevated corticosterone in the rat is detected after four oral daily doses of atrazine (12.5 mg/kg), or DIA (10 mg /kg that is equivalent to a total exposure to atrazine of 50 mg/kg). Attenuation of the LH surge is observed after exposure to atrazine (6.25 mg/kg/day) for four days (Table 3, EPA Issue Paper). While a threshold atrazine dose has been identified that produces an alteration in the HPA / HPG signal system in rat (6.25 mg/kg/day for 4 days attenuates the LH surge), there appears to be no hard, quantitative link between functional outcomes and hormonal changes (e.g., premature reproductive senescence, and the duration and intensity of depression of LH surge necessary for senescence to occur). Furthermore, premature reproductive senescence is not necessarily the functional endpoint in the human female, and so it is not possible to identify the magnitude and duration of HPG signal disruption that will produce an adverse effect on reproduction in humans from the data that are available. Responses seem to be graded, so there is likely to be an atrazine dose threshold above which a measurable perturbation in one or more HPA/HPG signals occurs and the magnitude of the perturbation is increased with increased atrazine dose. It seems possible that the atrazine exposure associated with a functional outcome endpoint (e.g., premature reproductive senescence) is greater than the minimum exposure to produce a measurable change in a hormone signal. One Panel member noted that, to be safe, it would make sense to use a point of departure that was associated with the smallest exposure that produces a measurable change in an HPA/HPG signal.

Furthermore, the determination of the frequency of drinking water monitoring depends upon whether it is to deal with acute exposures/effects or chronic exposures/effects. Generally, protection against chronic effects might also translate into protection against acute effects, whereas the reverse is usually not true. The validity of such an assumption would depend upon the PoDs identified for acute vs chronic assessment for atrazine. A critical factor to be considered relates to the appropriate dose measure of target organ exposure, and this has not been established for atrazine. In this regard, it is the maximal concentration (Cmax) that is commonly considered to be the relevant metric for acute effects, and the relevant variables are whether, and for how long, Cmax is exceeded. However, the integrated (or daily average) internal exposure is a useful measure of relating to chronic effects. Such a measure would also account for temporal fluctuations in a number of other determinants, such as exposure/contact frequency and rate, the rate of absorption, and the rate of metabolic transformation and elimination.

In essence, the evaluation of the temporal profile of toxicity vs monitoring frequency requires the consideration of exposure, toxicokinetics and toxicodynamics; not individually or sequentially, but rather in an integrated manner. Based on considerations of parent chemical and metabolite kinetics, the use of area under the curve (AUC) for atrazine in drinking water might provide a relevant measure of exposure and dose, since it incorporates temporal changes in exposure and uptake. Systematic sensitivity analysis could then be applied to identify key parameters and the relative importance of input parameters in models of exposure through

drinking water. Important variables would include the frequency of monitoring or fluctuations in concentrations. However, if the existing data and dose-response analysis do not clearly appear to make a case for the critical role of peak water concentration in the functional outcome following atrazine exposure, then there is no need for more frequent monitoring of concentrations of atrazine in drinking water than is currently mandated.

Comments on the toxicokinetic/dynamic considerations with respect to atrazine's effects on the HPA/HPG axis

It should be pointed out that the pharmacokinetics of atrazine have not been fully characterized. Early studies (Timchalk et al., 1990; McMullin 2003) used 14C-atrazine with only radioactivity measured over time. As the radioactivity would distribute among atrazine and its metabolites, the plasma radioactivity vs. time profile would reflect the weighted average concentration of atrazine and its metabolites and it would be difficult to obtain useful pharmacokinetic information about any single chemical species. McMullin et al. (2003) also quantified the plasma concentrations of atrazine and its three chloro-metabolites individually after a 90 mg/kg oral gavage dose to rat. The DACT metabolite was the dominant species; its concentration exceeded that of atrazine by 200 fold or more during the 0.5 – 72 h sampling period. This suggests that the radioactivity half-life of 11 h in rat mostly reflects the half-life of DACT. When the concentration of atrazine and its metabolites have been determined individually in plasma after an oral dose (McMullin 2003 and 2007; Ross 2009), the concentration-time profile of atrazine has been erratic, and assay sensitivity has limited investigator ability to obtain kinetically useful data. AUC values have not been proportional to dose, and have generally not even shown a consistent trend toward more- or less-than dose proportional. Attempts at development of a comprehensive PBPK model for atrazine and its three chloro metabolites (McMullin, 2007) have had mixed success. The model for DACT and for DACT after administration of either of the mono-N-alkyl metabolites, as well as for the mono-N-dealkylated metabolites, showed reasonably good agreement with their experimentally determined plasma concentrations. However, after atrazine administration, the model predicted concentration-time profiles for atrazine and its three metabolites did not agree with the experimentally determined values. The values for some of the model parameters were distinctly non-physiological; e.g., the elimination rate constant for atrazine was reported as 188 h-1, which implies a systemic clearance of about 75 L/h in rat, and this is far in excess of cardiac output.

The half-life of the plasma concentration of total radioactivity is about 11 h in rat after a 30 mg/kg oral dose (Timchalk et al., 1990). Using a ¾-power interspecies scaling relationship for clearance and assuming that the volume of distribution is proportional to body weight, the half-life in humans would be expected to be about four times that in rat (Lin, 1998), or about two days. From this expectation, it follows that a single acute dose of atrazine in humans could alter HPA/HPG signals for several days. If DACT does not perturb the neuroendocrine system, however (Fraites et al., 2009), signal disruption could be less long lasting. However, there are no reliable estimates of the half-lives of atrazine and its active metabolites in humans and it is not possible to state with certainty the duration of signal disruption after acute oral atrazine exposure. It would appear to be conservative to suggest that the persistence of neuroendocrine disruption after an acute atrazine dose could be at least one day and that once-daily dosing would result in continuous HPA/HPG disruption until one day after the last dose of atrazine.

The pharmacokinetic studies of Dooley et al. (2006, 2007) in rats raise some interesting points that need to be heeded when interpreting the toxicological significance of pharmacokinetic (PK) profiles, and hence the exposure corresponding to a particular dose. They showed that DACT, which is quantitatively the main oxidative detoxication product of atrazine, binds covalently to hemoglobin and to serum albumin. The latter is a major component of blood. Since it is only the freely dissolved pool of compound that is available for detoxication, elimination, and interacting with components of the metabolic pool to produce a toxic response, then studies that use total radiolabel rather than detection by mass spectrometry will overestimate the exposure of the active site to atrazine, and hence underestimate the toxicity. Further, the binding of DACT to available cysteine residues is not limited to hemoglobin and serum albumin, but occurs with other tissue proteins. The study of Dooley et al. (2008) shows covalent binding of DACT to a range of proteins in the pituitary. However, there is no indication of the proportion of the protein that has bound DACT, or whether this is physiologically or toxicologically significant. It is likely that binding of DACT to proteins will occur in most tissues.

Complex interactions between a number of variables (dose availability in gut, gut motility, membrane permeation involving diverse carriers in same or opposite directions, detoxication by enterocytes affect rate of appearance of compound in hepatic portal vein (Pang, 2003)). Many of the studies use high doses, but the PK profiles in both mice and rats dosed by oral gavage indicate that atrazine is absorbed slowly and eliminated rapidly. Furthermore, there is a biphasic penetration curve in both rats (McMullin et al., 2003, 2007) and mice (Ross et al., 2009). This is probably a result of an initial faster absorption of dissolved atrazine, followed by a slower phase where the rate limiting step is dissolution of atrazine from the bolus. Where dissolution is slow compared with gut permeability, then the former process becomes rate limiting for absorption (Usansky and Sinko, 2005). Recent work (Parrott et al., 2009) using a dog model showed marked differences between absorption rates of drugs in fed and fasted dogs, and emphasized the importance of the effects of gut contents on the pharmacokinetic behavior of drugs.

The absorption of atrazine after an oral gavage dose of 150 mg/kg suspended in 1% carboxymethylcellulose appeared to be erratic, slow and subject to presystemic metabolism (McMullin et al., 2007). The slow absorption was attributed to the low water solubility of atrazine; and although this seems a likely explanation for the slow absorption, empirical evidence in its support was lacking. Other possible explanations are that an intestinal export transporter such as P-glycoprotein blocks atrazine's passage across the intestinal epithelium and that the passive permeability of the intestinal epithelium to atrazine is very low. In consideration of the absorption of atrazine from drinking water, the low-water-solubility effect would not be operative since all the atrazine would be in solution and the amount of atrazine in the gut at any particular time would be very small and not above its water solubility. On the other hand, intestinal exporter transport and intestinal epithelial or hepatic presystemic metabolism, if they occur, would be expected to proceed at maximal rates as the concentration of atrazine would be low and saturation of these processes would be minimal. Also, a low passive intestinal permeability would be independent of the concentration of atrazine in the gut.

The onset of HPA hormonal effects in the rat after dosing with atrazine is very rapid; ACTH, CORT, and P4 circulating levels were elevated 2-5 fold 15 minutes after an oral dose of

75 mg/kg atrazine or an equimolar dose of its metabolite DIA (Fraites et al., 2009). DIA is at least equipotent with atrazine, if not more potent. The PK analysis of McMullin et al. (2007) suggested that only a small fraction of a 150 mg/kg oral dose of atrazine was rapidly absorbed. This indicates that the 15 minute-effects observed by Fraites were produced by a relatively small fraction of the 75 mg/kg oral dose that had arrived at the systemic circulation by 15 minutes post dosing. The PK analysis of McMullin et al. (2007) also suggested a substantial (64%) presystemic conversion of atrazine to mono-dealkylated metabolites.

An array of enterocyte enzymes operates during absorption from the gut, and subsequently when arterial blood returns compound escaping first pass metabolism to the gut. *In vitro* metabolism of atrazine using rat and mouse hepatic microsomal preparations produced the N-dealkylated products (desethyl atrazine and desisopropyl atrazine) but not DACT: Ross and Filipov (2006) concluded that the large quantities of DACT produced *in vivo* were due to extra-hepatic metabolism, possibly by enterocytes. The pattern of metabolites may vary with dose since different groups of enzymes will become important. At high doses of atrazine, it would be expected that there would be significant metabolism by enterocyte cytochrome P450 dependent oxygenases (CYPs) with a high Km.

Given the relatively well-characterized differences in expression between rat and human drug metabolism enzymes in the intestine (Cao et al., 2006), refinements to rat and human PBPKs particularly through biotransformation parameters should allow estimates of plasma concentrations (either Cmax or AUC) at specific times. The biotransformation of atrazine has been well-characterized in each species and catalytic efficiency parameters (Km/Vmax) are available and the indications are that CYP1A1 and CYP1A2 are the primary CYPs responsible for atrazine demethylation (Lang et al., 1997). In addition to CYP1A1, higher Km CYPs which are also present in intestinal epithelium carry out the biotransformation of atrazine (i.e. CYP3A4) and this will have kinetic implications when high concentrations of atrazine are present (e.g. with high dose oral exposures).

At lower doses, detoxification by enzymes in the liver and with lower Km would become more important. In most of the studies, the rapid appearance of DACT is consistent with primary detoxification in the gut wall. In the study of Ross et al. (2009), the concentrations of atrazine found in the plasma and in some tissues were an order of magnitude lower than those of DACT. If DACT is toxicologically active, even if it is less so than atrazine, then it may still be important. Ross and Filipov (2006) also found that at low *in vivo* doses of atrazine, glutathione (GSH) conjugation was a relatively minor route of detoxification, but that this became more important at higher doses (125 mg/kg and 250 mg/kg). This latter effect raises the possibility that at very high doses there may be depletion of the GSH pool that could impact on normal metabolism of intrinsic compounds involved in regulation of a range of physiological processes. While it is possible to use a model based on rat data to predict absorption of therapeutic drugs from the human gut (Zakeri-Milani et al., 2007, Zhao et al., 2003), this is not the same as predicting bioavailability. The latter is the fraction of the absorbed material that escapes metabolism by the gut wall and liver. Marked differences have been observed between humans and rats in the bioavailability of orally administered compounds. This has been attributed by Cao et al. (2006) to differences in enterocyte and hepatocyte metabolism. These workers observed large interspecific differences in levels of expression of oxidative enzymes, but found

significant correlations between the diffusion and carrier-mediated intestinal absorption of drugs. These findings further emphasize the difficulty in relating the behavior of atrazine in rodent studies where high doses of the compound are administered by oral gavage with that in humans where intake is primarily by drinking water in which the concentrations are low (typically in the low µg/L range). Further it is difficult to extrapolate from the models based on oral dosing to one based on the inhalation route, or slow release from subdermal implants. It is likely that these two routes would result in higher concentrations of free (available) atrazine in the plasma than is possible with oral administration since the compound would not be exposed to first pass metabolism (gut and liver) before being available to target tissues. This should result in a greater availability for same dosage.

In order to find equivalent human and rat exposures that correlate with the NOAEL in rat, the AUC of free concentrations of atrazine and DACT in plasma (and possibly target tissues) following acute and chronic exposures need to be evaluated. More realistic exposure scenarios are needed, but it would be difficult to follow low doses that are rapidly absorbed and metabolized (as might be case if dosed in water) since the analytical limit of detection (LOD) becomes a problem. It would be particularly helpful to have a measure of the effects of chronic exposure to atrazine in drinking water on the levels of free atrazine and free and bound DACT in the plasma. However, if the concentration in water were 3 µg/L, and water consumption 2 L/day, then internal exposure would be very low compared with that observed in high-dose rodent experiments. If all of the atrazine were instantaneously and homogeneously distributed, without metabolism, throughout a 70 kg human, then the tissue concentration would be of the order of 0.1 µg/kg bw. This would pose an analytical challenge. Even if the concentration in drinking water were 100 µg/L, the maximum average tissue concentration (around 3 µg/kg) would still be challenging to quantify.

On the basis of current knowledge it is difficult to assess the potential exposure of target sites in humans to atrazine and its metabolites. There is little information on the critical exposure needed to produce an adverse effect. For disruption of a developmental process, the critical lesion might result from a short (possibly as low as an hour) exposure to a low concentration of atrazine. For other toxicological end points, a longer exposure to a higher concentration (greater area under the plasma concentration- time curve) could be needed to produce adverse effects. Currently there is little information about critical concentrations (related to Cmax) or critical exposures (AUC) associated with the various adverse effect endpoints. When information becomes available on the nature of the critical lesions, then it will become possible to design experiments to assess the critical doses needed to produce them. However, it will require more realistic dosing regimes, and careful selection of PK models to predict the resulting exposure of target sites in humans.

Question 2.0: Approaches to Evaluating Water Sampling Strategies & Frequency of Monitoring

In conjunction with the toxicological review presented in the issue paper, the Agency has also discussed methods for re-evaluating the sampling frequency that is necessary for determining, with confidence, concentrations of the pesticide in water that sources drinking water. These have included different methods for estimating pesticide concentrations between known sampling

events and examining the performance of different sampling strategies for averaging periods of different durations. The Agency seeks feedback from the Panel with regard to how the uncertainty and variability in both the monitoring data and in the toxicity data (i.e., point of departure) can be integrated to characterize and to interpret the potential significance of atrazine concentrations in drinking water.

General Observations of the Panel:

The Agency is anticipating the likely need to revise the FIFRA-motivated atrazine monitoring strategy because of (1) revisions to the human-health concentration objective, or Level of Concern (LOC), and (2) consideration of the lessons learned from extensive monitoring already conducted during the past 10-20 years. This FIFRA monitoring is in addition to Safe Drinking Water Act compliance monitoring, which is not currently being evaluated as part of this SAP, but could later be affected by the SAP analysis or lessons learned from subsequent monitoring. Against this background, the Panel was asked to provide advice to the Agency on if/how a revised monitoring strategy should be designed, once the exposure objectives have been defined. This has to be achieved within the following constraints and characteristics specified by EPA:

- The sampling target is untreated source water for surface-water supplied public, community water systems (CWS). Note that this may not always be the original flowing stream source, but sometimes a pretreatment storage reservoir.
- The chemicals of interest include atrazine, its chlorinated degradates and simazine.
- The objective will be to characterize source-water concentration characteristics for individual systems in relation to specific concentration benchmarks (one or more LOCs), which will be determined from the toxicological analyses.
- The LOCs—which will have been determined from exposure levels and durations of potential concern—may be anywhere in the range of 1 day to 90 day rolling average concentrations, on a time-weighted basis.
- The Agency desires an approach that will enable reliable detection of potential exposure conditions that exceed LOCs, and desires to do this with quantitative estimates of reliability.
- Overall, the approach needs to address (1) how to identify the specific CWS that should be monitored, (2) how to monitor concentrations at these sites for a particular LOC in order to achieve a specified reliability, and (3) the data analysis and decision process that will be applied to the monitoring data, and which may then modify monitoring requirements as information is gained over time.

Question 2.1

Given the nature of the temporal patterns of pesticide occurrence in surface waters described in Section 5.2, including serial correlations from day to day, periodicity in elevated concentrations within seasons and from year to year, below quantitation data, and uncertainty in the shape of the pesticide distributions in surface waters--what statistical approaches should the Agency consider in determining confidence bounds on exposure estimates from monitoring data? Please comment on how the approach may vary depending on the duration of concern.

Panel Response:

Determination of confidence bounds on estimates of a particular concentration statistic for a specific site can be made in two basic ways.

1. **Inference from other sites**: First approximations are possible using the types of results reported by Crawford (2004), in which aggregated results for a selected set of sites are used to estimate precision and bias characteristics of specific sampling strategies, which can then be used to infer expected confidence for additional sites; or by using an empirical statistical model, such as Watershed Regression on Pesticides (WARP) model, to predict concentration statistics and confidence bounds for unmonitored sites.

2. **Site-specific determination from monitoring data:** For monitored sites, concentration statistics and confidence bounds can be directly estimated from the sample data.

Estimates by inference from other sites are appropriate and useful for study design and screening-level analysis, but usually have unacceptably high uncertainty for important decisions, such as deciding whether or not to limit atrazine use in a watershed. Generally, the usefulness of inference declines as the time duration of the LOC gets shorter and the need for site-specific monitoring data increases. The focus of the following discussion is on estimation of confidence bounds from monitoring data.

In considering confidence bounds on exposure estimates from monitoring data, a key consideration is what is being estimated. Possibilities include a specified quantile of the atrazine values within a year, a specified quantile of the rolling average over a specified time frame (e.g., 5, 7, 14, or 90 days) or the mean of the rolling average over the specified time frame. Sampling strategies consistent with these goals will be considered. These comments draw heavily on Appendix A of the ILSI (1999) report and will be contrasted with some of the materials supplied by EPA and Syngenta.

Some consideration should be given to targeting critical periods for sampling and sampling strategy. The sampling period and strategy could be targeted to individual CWSs. Perhaps a low level of sampling during the winter months when atrazine levels are historically low could be coupled with a more intensive sampling program during the spring and summer months when levels could be high. More frequent sampling could be considered for those smaller sites that have more variability during the growing season and less frequent sampling could be used for sites with less variability, or ones that are located on larger reservoirs that tend to lessen peak concentrations, but have higher long term averages. For a given CWS system, historical information could be used to establish such a sampling strategy, which could potentially be adjusted from year to year. Alternatively, less accurate, but rapidly processed, antibody-based sampling methods could be used for screening purposes. If screening detected a potential issue, then more intensive sampling could be conducted. This would allow adaptive sampling procedures to be developed for implementation within a year.

If setting confidence bounds on the pth quantile of the distribution of atrazine level at a given site is of interest (Note: The maximum is the 1.0 quantile or the 100^{th} percentile of the distribution.), one approach is to assess the distribution of the atrazine levels over the course of a

year and use properties of that distribution to set confidence intervals on the quantile(s) of interest. However, the challenge with this approach is that the distribution of levels cannot be determined with certainty, and the extreme quantiles are heavily dependent on the choice of distribution. The presence of temporal correlation further complicates this problem. For this reason, here the focus is on nonparametric approaches.

Without making a distributional assumption, sample size becomes an even more important consideration when estimating extreme quantiles. Both EPA and Syngenta have adopted this approach. Specifically, to estimate the quantile p using sample size n, the sample size must be greater than $p(n + 1)$. In other words, to estimate the 95th percentile, at least 20 observations are needed, and at least 100 observations are required to estimate the 99th percentile.

Let X_p be the pth quantile of a distribution of values with underlying cumulative distribution function, F; that is, $F(X_p) = p$. For a sample of size n, let $x_{1n}, x_{2n}, \ldots, x_{nn}$ be the sample order statistics, *i.e.*, $x_{1n} < x_{2n} < \ldots < x_{nn}$. To estimate the pth quantile, use x_{jn}, where $j = p(n+1)$. A 100(1-α)% confidence interval takes the form $(X_{(RLCL)}, X_{(RUCL)})$ where $RLCL = p(n+1) - z_{\alpha/2}(np(1 - p))^{0.5}$ and $RUCL = p(n+1) + z_{\alpha/2}(np(1 - p))^{0.5}$.

Suppose that the goal is to ensure with probability $(1 - \alpha)$ that the maximum value in the sample will exceed the pth quantile of the population. For large p, $1 - \alpha \approx 1 - p^n$. Solving for n provides the minimum sample size required to obtain the estimate with the desired precision is:

$$n = \log(\alpha)/\log(p)$$

Table A1 from the ISLI (1999) report providing minimum sample sizes is reproduced below. Note that this is equivalent to the procedure described in the Syngenta report, "Investigation of Statistical Confidence in Upper Quantiles and "Peak" Concentrations of Currently Available Atrazine Drinking Water Monitoring Data."

Table A1. Smallest sample size required for the maximum observation to exceed 100p% of the sampled population with (1-α)% confidence

	(1−α)%						
p	50%	75%	90%	95%	98%	99%	99.9%
0.800	4	7	11	14	18	21	31
0.850	5	9	15	19	25	29	43
0.900	7	14	22	29	38	44	66
0.950	14	28	45	59	77	90	135
0.960	17	34	57	74	96	113	170
0.970	23	46	76	99	129	152	227
0.980	35	69	114	149	194	228	342
0.990	69	138	230	299	390	459	688
0.995	139	277	460	598	781	919	1379
0.999	693	1386	2302	2995	3911	4603	6905

Two simulation studies were conducted to evaluate sampling strategies (ISLI, 1999). For all herbicides considered (including atrazine), monthly or twice-monthly sampling was found to be insufficient for accurate estimation of percentiles at or above the 90th percentile. Four-time-a-week or weekly sampling was adequate for estimating the 0.50, 0.90, and 0.95 quantiles, but not

the 0.99 quantile. Standard deviations of the 99th percentile estimates were large when using ten-times-a-week sampling, indicating a more frequent sampling frequency may be needed. Further, in wet years when the concentrations tend to be higher, the precision of the estimates of extreme quantiles is lower than in dry years. Also, the extreme quantiles tended to be overestimated. Perhaps the over-estimation of the quantiles is a consequence of the correlation in the sample values, resulting in an effective sample size smaller than the total number of observations. Relative standard errors were also proposed by Syngenta. Although this is a legitimate approach, it lacks an associated confidence level. Consequently, this would not be as satisfactory a procedure.

The above was based on individual observations. Alternatively, if a rolling average is used to assess the levels of atrazine, because observations close in time are likely to be correlated, the mean over the period of interest (e.g., 90-day rolling average) has a standard error of:

$$s = \sqrt{\frac{\sigma^2}{n} + \frac{\sum_i \sum_j \sigma_{ij}}{n^2}}$$

where n is the number of observations within the 90-day period. Further, because rolling averages are being used, the correlation between consecutive rolling averages is likely to be high. The nonparametric methods above could be used. However, if correlation among sample values results in an effective sample size smaller than the number of sample values (see above), then the rolling averages might lead to substantial bias, resulting in over-estimates of the upper quantiles. Another possibility is the use of extreme value theory. This approach is discussed under Question 2.4.

Question 2.2

The first two simulation methods presented in Section 5.5 are applicable to the specific data sets they describe, although some generalities regarding shape patterns appear to exist. Given this information, please comment on the strengths and weaknesses of the approaches and on the practical merits of pursuing them or some other numerical approach with a larger set of higher concentration systems. Please comment on how the methods for determining confidence bounds might apply given these considerations.

Panel Response:

The primary objective of the two methods described in Section 5.5 is to evaluate the reliability of alternative sampling strategies by statistically sampling simulated continuous time-series of concentrations which were derived from less than continuous monitoring data. These methods follow the general concept used in Crawford (2004), in which actual data time series for selected sites were linearly interpolated between samples to yield a daily concentration time series which was then treated as the "true" chemograph for experiments with sampling strategies. But, the important difference between the Section 5.5 methods and Crawford (2004) is that the Section 5.5 actual data time series are only 30-35 samples for a year, versus very intensive sampling for much of the year at the sites studied by Crawford.

As evaluated, the Section 5.5 time series are too small to yield realistic estimates of the "true" values for upper percentiles or the mean, all of which will be biased low and have a low probability of representing short-lived peaks. All simulations presented to this SAP have begun with some observed time series that have been expanded to daily data through some means of interpolation. Consequently, the peaks are attenuated, the series is overly smooth, and the sampling procedures are truly better than they appear. Evaluations of inferences from different sampling strategies based on these estimates of "truth" are benchmarked to a poor and biased representation of the actual concentration conditions. This makes both methods described in Section 5.5 flawed for reliable analysis of sampling strategies that are substantially affected by relatively short-term high concentration conditions, such as the high percentiles emphasized. In particular, both EPA and Syngenta have provided the SAP with several simulations. Each simulation is being used to address a different question. This is probably a consequence of the final duration of concern not being fully determined yet, and the simulations being developed in anticipation of what might be decided as to the timeframe of interest. Depending upon what is finally decided from the biological perspective, the various approaches might be useful. However, before a definitive simulation is possible, the following questions must be answered: (1) How important are the peaks in atrazine concentration? (2) How long do the peaks last? (3) How important are clusters? and (4) If clusters are important, what maximum values and time scale need to be considered?

Creation of "true" time series for analysis of sampling requirements to address a specific concentration objective, once defined, needs to be based on either (1) intensive empirical data (at least for critical periods, and relative to the time-scale of the concentration objective), such as used in Crawford (2004), for an adequately representative range of sites, or (2) a modeling approach that reliably simulates reality based on (a) the relatively small number of actual sample analyses available (essentially for "calibration") and (b) appropriate predictor variables, such as the timing of pesticide use, precipitation, and streamflow conditions using either a statistical or deterministic model. Note that both the Agency and Syngenta have already been pursuing the first approach using available data. However, the Panel emphasized that if one wants to draw inference at the daily level based solely on the data collected, then sampling must occur at least daily. If one wants 4-day rolling averages, then sampling must occur at least twice within the 4-day period (and that may not be enough). Simulations, models, or any other approach that suggests otherwise are making some strong assumptions about what happens at the finer time scale. If a good understanding of the system exists, then one may be able to model the results, in which case sampling could be confirmatory. But, this requires knowledge of the system and sufficient supporting data to construct such a model for each site, something that is not present for atrazine, at least not yet. It does seem reasonable to concentrate sampling effort during the time of exposure. However, as has been noted several times, the sampling scheme developed for atrazine may not be applicable to other contaminants.

A few data series are repeatedly used as the foundation for all simulations and many of these chemographs have extremely high variability in concentrations of atrazine and the Panel is uncertain about how representative these chemographs are of most of the CWSs. It was noted by both EPA and Syngenta that approximately 95 % of all of the data collected as part of the Atrazine Monitoring Program had concentrations below 3 ppb, but these observations are from

much sparser sampling. Although the chemographs used are adequate, they represent quite a limited range of CWSs. For this reason, serious concerns could be raised about all of the simulations conducted by either EPA or Syngenta. Simulating data to explore the behavior of sampling schemes is challenging because there are numerous sources of error, all of which should be reflected in the simulation to provide a realistic assessment of sampling behavior.

In the absence of additional "near continuous" data sets for estimating "true" concentration time series, a modeling approach similar to that described by Sullivan et al. (2009) and Vecchia et al. (2008) would be a potential approach for simulating "true" concentration time series from relatively small data sets. But the present formulation of these approaches is designed for multi-year trend assessment and is not optimized for representing actual extremes in a particular year. This type of statistical model, however, uses seasonal patterns and daily stream flow or precipitation conditions to fit the model to monitoring data. The simulated time series reflect the influence of these factors on concentrations, including simulation of extreme conditions not directly measured. A potential approach for simulation that includes more variability is to use WARP or some other regression-based method, as mentioned above, to develop a reasonable chemograph for a particular system based on explanatory variables. Such a model would have a correlation structure, such as the exponential covariance structure. Then geostatistical simulation can be used to simulate curves that are potential realizations from that system. For each curve, quantities of interest can be estimated using a proposed sampling procedure, and the properties of the distribution of estimates investigated.

Development of such statistical models, as well as an overall need for representing a broader range of hydrologic systems than currently possible, argue for careful and selective development of additional "intensively sampled" sites, including multiple years, if short duration exposures (such as a day or week) are deemed important. If these types of short-term exposures become the target, then temporally intensive, multi-year data from a relatively few additional sites will be needed more than moderate intensity data from many sites.

A closely related need, not directly brought up in the charge sub-questions, is to efficiently identify and target the relatively small proportion of sites that merit the most detailed sampling and model analysis. This can be done with a version of the WARP model as implied in the EPA Issue Paper. The WARP model is a multiple regression model that estimates atrazine concentration statistics from data on watershed characteristics, such as pesticide use, soil properties, and so forth. Essentially, this approach enables use of information on geographically distributed explanatory variables to estimate the probability that specific concentration statistics, such as the annual maximum of a specific duration average, will be exceeded. For any particular LOC objective, a WARP model can be used to estimate the probability that any particular site will exceed the LOC and this can be used to systematically allocate sampling priorities.

The second part of this Charge Question requested the Panel's comments concerning methods for determining confidence bounds and how they might apply given the data limitations discussed above. This was covered in the response of the Panel to Charge Question 2.1.

Question 2.3

As described in Section 5.4.2, the Agency is considering the use of a confidence interval or prediction interval approach to characterize the uncertainty of exposure estimates derived from monitoring data of varying sampling frequencies. Please comment on the strengths and weaknesses of either placing confidence bounds on the rolling average estimates and comparing the upper limit from monitoring against the level of concern (LOC) or, conversely, placing confidence bounds on the LOC.

Panel Response:

It is the Panel's recommendation that confidence bounds not be placed on the LOC. How confidence bounds would be developed was not immediately obvious to the Panel. Given the safety factors included in the development of the LOC and the inherent uncertainty in transferring toxicological endpoints from animals to humans, placing confidence bounds on an LOC was not considered appropriate. The LOC should be used as a fixed value and exposure estimates measured against it.

In contrast, the reliability of the concentration statistic used for exposure characterization should be factored into the assessment of exceedance. Exposure concentration can be estimated in a number of different ways with quite different reliabilities. There are distinct tradeoffs that must be considered regarding costs of improving reliability versus accepting probability-based regulatory actions based on low-precision estimates. Confidence limits should be determined and then used to estimate the probability that true concentrations exceed the LOC.

Question 2.4

Please comment on the relative merits of the various modeling approaches the Agency described in Section 5.4.1 and 5.6 for interpolating pesticide concentrations between sampling points and, in particular, on the strengths and weaknesses of these methods as the frequency of samples decreases. Considering the health endpoint(s) being considered for atrazine, particularly data for the HPA axis, and the exposure time frame needed to induce the health effect(s) which is shorter than that used in the 2003 risk assessment, please comment on the advantages and disadvantages of each model for evaluating the likely occurrence and exposure via drinking water of short, moderate, and long duration concentrations. Please comment on the Agency's proposed approach for evaluating these methods, as described in Section 5.7.1. To what extent should the Agency consider other factors, such as the shape of the chemograph (Section 5.5.3), weather patterns, stream flow, and/or pesticide use patterns in evaluating the modeling approaches?

Panel Response:

Section 5.4.1 describes two basic methods for filling in values between actual measurements: linear interpolation and stair-step imputation. Neither of these methods will allow a predicted value to ever be larger than any of the observed values, and thus will clearly underestimate the maximum value if the maximum does not occur on a sampling day. Where the

shape of the true concentration curve is decreasing, the stair-step method will overestimate true concentration values. Linear interpolation will also overestimate concentration for a decreasing curve when it is concave, but not by as much as the stair-step method. From the examples in Figure 7 of EPA's Issue Paper, it would seem that most curves will generally be concave for more of the year than convex, as there is an initial peak following application of the pesticide, followed by a proportional decrease from the peak. The shape of the peak or multiple peaks will change from site to site based on physical characteristics of the system such as drainage basin size, percent of row crop, and whether the intake is located on a reservoir or in a stream.

For a longer-term average, such as a 90-day or 26-week average, both the linear interpolation and stair-step methods may work reasonably well. In this case, the underestimation of peak values may be balanced by the overestimation of post-peak values. However, this is not a particularly strong endorsement of either method.

In order to estimate a maximum value when it is out-of-sample, it is necessary to use a method that can predict a value larger than those observed in the data. An artificial neural network (ANN) is an example of such a method and Section 5.6 and Appendix C give a good description of how ANNs can be used for this problem. One technical note on ANNs: Appendix C describes the importance of not using too many nodes, so that over-fitting does not occur. This is absolutely correct. However, there is also a concern when using too few nodes, particularly when trying to estimate the maximum, although it is less of an issue for a longer-term average. As described, each node can be used for modeling an inflection in the concentration curve. And often the nodes combine non-linearly to allow modeling of additional inflections with fewer nodes. However, the nodes do have a particular shape (that of a logistic function), and therefore, some shapes of concentration peaks may require an extra node or two to model accurately. For example, compare the four-node model in Figure 26 of EPA's Issue Paper to the three-node model in Figure 27. Both fit pretty well; however, the three-node model is smoother than the four-node model, and hence, it tends to underestimate the maximum values. A steeper curve is necessary for a better fit to the sharp peaks, and thus the four-node model produces better estimates of the maximum as well as any short-term averages in the neighborhood of the maximum. One simple model selection metric that has been used is the Bayesian Information Criterion (BIC) (Schwarz, 1978), which is described in the context of ANNs in Lee (2004). The idea is to choose the model that maximizes a penalized log-likelihood, where there is a penalty for additional nodes. Thus extra nodes are used in the model only if they can be justified by a sufficient improvement in the fit. The BIC is based on an approximation to the Bayes factor, but does not require use of priors or Bayesian statistics. It has been shown to be asymptotically unbiased for model selection for a variety of models. Akaike's Information Criterion (AIC) (Akaike, 1974) is another metric used in the literature.

The current approach involving use of a neural network with autoregressive errors seems too complicated for a non-expert to implement. Using another node or two might eliminate the need for autoregressive errors.

Section 5.4.1 also mentions five other potential approaches: bootstrapping, kriging, random function models, regression-based models, and deterministic models. Bootstrapping shares the same problem with linear interpolation and the stair-step method in that it will never

predict a value larger than that observed, and thus it will not be an effective method for estimating a maximum or a short-term average. The other four methods have definite promise, alone or in combination.

Kriging, a special case of a Gaussian process (GP) model, interpolates the observed data with a smooth curve, which allows the curve to move above the observed values around the area of the highest values, if needed. Thus the approach can be used for estimating a maximum. A GP, like a neural network, can also be used to smooth noisy data, rather than strictly interpolating. Gaussian processes explicitly take into account the correlations between observations over time, and thus are an approach that could be further investigated in this context. They are also simpler to implement than a neural network model. The report does note that they are best used for concentration curves with high correlation, and that for pesticides with little correlation over time (such as chlorpyrifos), they may not be a good choice of model. Some initial exploration on CWS data by one Panel member found that the GP fit usually did not noticeably exceed the observed maximum, possibly because the peak is so sharp, and thus the correlation structure is a bit different around the maximum compared to the rest of the space. Non-stationary GPs that account for changing correlation do exist, but are likely to be too complex for practical use in this context. As pointed out by a Panel member, estimation of correlation structure should probably be done more globally because empirical variograms are highly variable.

Random function models can be used for data that are treated as functions. Thus knowledge of the shape of the curve could be useful in comparing curves from different water sources and could aid in the determination of a maximum value. Approaches such as those in Ramsay and Silverman (2002, 2005) may be useful. Vecchia et al. (2008) provide an example of a basic approach of this type in the context of pesticide concentrations.

Regression-based models have been explored in the context of this problem, particularly Larson et al. (2004) and Stone and Gilliom (2009), and have shown good ability to predict longer-term averages, and some ability to predict maxima and extreme quantiles. On their own, their accuracy in predicting maxima may not be sufficient. EPA might find it useful to explore the approaches of combining these models with the other statistical models above. ANNs, GPs, and functional models can all be modified to include covariates, and this information may additionally improve the accuracy of the estimates.

Deterministic models are built from knowledge of the physical and chemical laws of the process of contamination of water by a pesticide, and if built well, can be very helpful for predicting maxima and short-term averages. An important issue for deterministic models is accurate calibration, which involves the setting of the inputs and possible tuning parameters so that predictions closely match observed values. Such calibration can sometimes be done globally, but it might need to be done for each watershed individually. Calibration can be assisted through the use of GP models (Kennedy and O'Hagan, 2001; Taddy et al., 2009).

One additional modeling approach is extreme value theory. A very recent paper by Padoan et al., (2010) provides methods for estimation of extreme values from a correlated process, and it also includes references for related literature. A project group web page looking

at spatial extremes can be found at: http://www.cces.ethz.ch/projects/hazri/EXTREMES/reports.

Most of the current sampling is grab sampling, but, biologically it makes more sense to use autosamplers to provide samples composited over time which would give better estimates of rolling averages. It may be possible to composite daily samples for a rolling average. A weighted rolling average could also be considered. The current methods weight all points equally, but point values could be time-averaged with points closer to the current reference point getting more weight. This would produce less smooth curves than the methods previously discussed.

Systematic sampling is the most efficient approach when there are long-term trends. For shorter-term movements, systematic sampling might produce more variability in estimates than randomly-perturbed fixed-window sampling.

A Panel member queried whether the EPA should also evaluate the data for clusters of spaced, repetitive atrazine exposures since repeated exposures could prime individuals to subsequent HPA/HPG responses.

2.4b Please comment on the Agency's proposed approach for evaluating these methods, as described in Section 5.7.1. To what extent should the Agency consider other factors, such as the shape of the chemograph (Section 5.5.3), weather patterns, stream flow, and/or pesticide use patterns in evaluating the modeling approaches?

The proposed approach for evaluating interpolation methods, as well as sampling design questions, is sound for the sites and time periods for which there are adequate data. However, the reliability of generalizing results from the relatively small number of sites and years with relatively continuous data is dependent on the representativeness of the sites and years. If the duration level of a new LOC drops substantially from 90 days, there will be a need for new sites with intensive monitoring.

In particular, for the public water systems for which short duration LOCs are likely to be exceeded, and thus, where reliability requirements are high, there probably will be no substitute for intensive monitoring. In this situation, time-intensive monitoring using inexpensive tests for atrazine, such as immunoassay field kits, may be a cost-effectively approach to identify samples that merit complete chemical analyses.

The appropriate strategy for combining inference, modeling, and monitoring to meet the Agency's needs for risk assessment must ultimately be evaluated for specific objectives. Once we know the specific LOCs of interest and the reliability requirements, the strategy can be developed.

If the exposure window of interest is narrow, it quickly becomes evident that there are not enough examples of closely monitored sites for effective evaluation. For example, there is a need for better geographic distribution. Using the CWS data that is observed only weekly and interpolating the other days is not sufficient for understanding the true shape of the chemographs, nor for understanding the true variability involved; it oversimplifies the problem and will lead to overly optimistic results. As discussed in the response to Question 2.2, a better strategy for

creating new "truths" to use for methodology evaluation would be to first use WARP and existing AMP data to identify the most vulnerable CWSs, and combine the regression model with kriging to create a "truth" and then use geostatistical simulation to get a better idea of variability. Simulating with additional variability is critical for creating a more realistic set of "true" comparisons.

Section 5.7.1 provides a procedure for evaluating the effectiveness of different methods for prediction between observations. The approach is sound, although it does not take into account possible covariates, such as weather patterns, stream flow, and/or pesticide use patterns, nor does it make any use of what is known about the shape of the chemograph (Section 5.5.3). By not including covariates, the regression-based models are necessarily excluded from consideration, as would be any combination of regression-based models with other models. It would definitely be helpful to consider covariates such as these and the other key variables identified in Stone and Gilliom (2009). Similarly, random function models and deterministic models are dependent on information about the shape of the curve, so that information would be needed if those approaches are to be considered.

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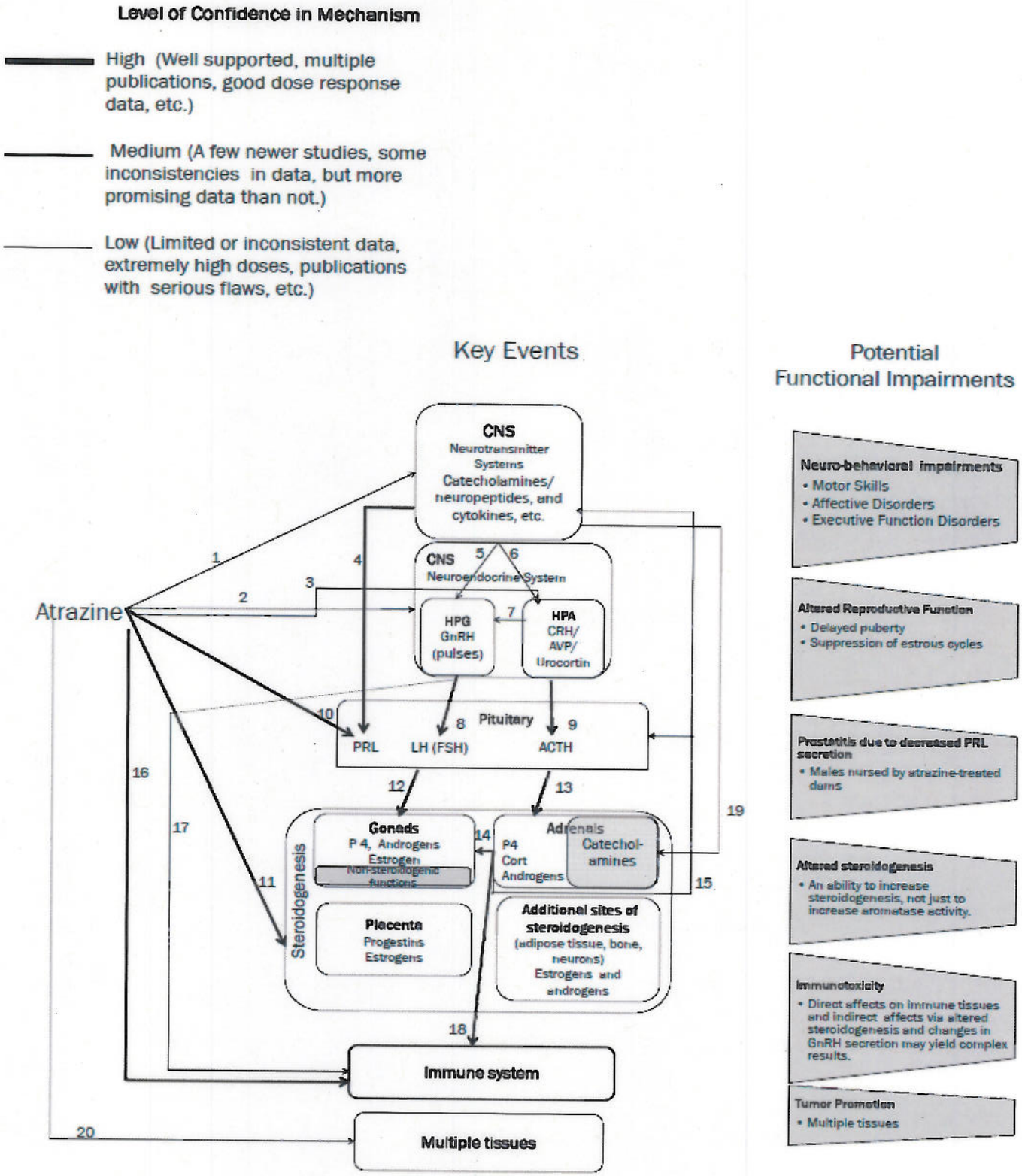
Appendix 1 – Revision of Figure 3 in EPA Issue Paper as Proposed by One Panel Member and Not Discussed by the Full Panel.



Key to the pathways. The numbers below correspond to the numbers labeling the key events in Figure 1 of Appendix 1. Each pathway corresponds to a process discussed in the Minutes of the FIFRA SAP held April 26-29, 2010.

1. Direct action on CNS (Actions upstream or not necessarily directly associated with the HPG and/or HPA axes)
2. Direct action on the HPG axis
3. Direct action on the HPA axis
4. Altered dopamine secretion results in changes in PRL secretion (increasing DA suppresses PRL)
5. Altered HPG function due to alteration in upstream CNS functions.
6. Altered HPA function due to alteration in upstream CNS functions.
7. Suppressed GnRH secretion due to increased CRF inputs.
8. Suppressed LH secretion due to decreased GnRH secretion. (FSH included in parenthesis as a reminder that GnRH also regulates FSH secretion and that both are required for proper ovarian function.)
9. Increased ACTH secretion due to increased CRH/CRF secretion
10. Direct affects on pituitary PRL secretion
11. Direct affects on steroidogenesis
12. Altered gonadal function due to changes in LH and FSH secretion
13. Increased ACTH secretion results in increased adrenal steroid synthesis.
14. Increased adrenal steroid secretion, especially corticosteroids, inhibits gonadal function via direct action on the gonads.
15. Increased adrenal steroid secretion, especially corticosteroids, inhibits gonadal function via feedback to the hypothalamus and pituitary.
16. Immunotoxic effects resulting from direct actions of atrazine on the immune system.
17. Immunotoxic effects resulting from reduced exposure of the immune system to GnRH.
18. Increased adrenal steroid secretion exerts immunotoxic effects.
19. Sympathetic nervous system activity increases catecholamine secretion by the adrenal medulla.
20. Direct affects of atrazine as a tumor promoter.