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

OFFICE OF  
PREVENTION, PESTICIDES AND TOXIC  
SUBSTANCES

June 11, 2008

MEMORANDUM

**SUBJECT:** Transmittal of Meeting Minutes of the FIFRA Scientific Advisory Panel Meeting Held on March 25-26, 2008 to review and consider the Endocrine Disruptor Screening Program (EDSP) Proposed Tier 1 Screening Battery

**TO:** Elizabeth Resek, Acting Director  
Office of Science Coordination and Policy

**FROM:** Jim Downing, Designated Federal Official  
FIFRA Scientific Advisory Panel  
Office of Science Coordination and Policy *Jim Downing*

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

Attached, are the meeting minutes of the FIFRA Scientific Advisory Panel open meeting held in Arlington, Virginia on March 25-26, 2008. This report addresses a set of scientific issues being considered by the Environmental Protection Agency pertaining to the Endocrine Disruptor Screening Program (EDSP) Proposed Tier 1 Screening Battery.

Attachment

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OPP Docket

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**FQPA Science Review Board Members**

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**SAP Minutes No. 2008-03**

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

**The Endocrine Disruptor Screening Program (EDSP)  
Proposed Tier 1 Screening Battery**

**March 25-26, 2008  
FIFRA Scientific Advisory Panel Meeting  
held at the  
Environmental Protection Agency, Conference Center  
Lobby Level, One Potomac Yard (South Bldg.)  
2777 S. Crystal Drive, Arlington, VA 22202**

## 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 Jim Downing, SAP Designated Federal Official, via e-mail at [downing.jim@epa.gov](mailto:downing.jim@epa.gov).

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

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**SAP Minutes No. 2008-03**

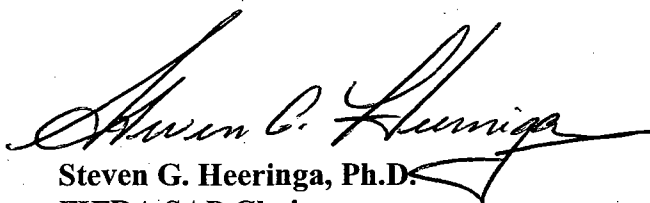
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**March 25-26, 2008**

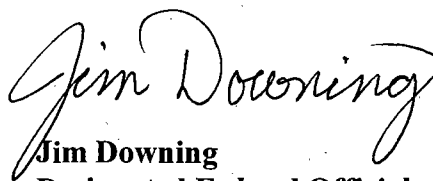
**FIFRA Scientific Advisory Panel Meeting  
held at the**

**Environmental Protection Agency, Conference Center - Lobby  
Level, One Potomac Yard (South Bldg.)  
2777 S. Crystal Drive, Arlington, VA 22202**



**Steven G. Heeringa, Ph.D.  
FIFRA SAP Chair  
FIFRA Scientific Advisory Panel**

**Date: JUN 11 2008**



**Jim Downing  
Designated Federal Official  
FIFRA Scientific Advisory Panel**

**Date: JUN 11 2008**

**Federal Insecticide, Fungicide, and Rodenticide Act  
Scientific Advisory Panel Meeting  
March 25-26, 2008**

**The Endocrine Disruptor Screening Program (EDSP) Proposed Tier 1  
Screening Battery**

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**Designated Federal Official**

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## INTRODUCTION

The FIFRA Scientific Advisory Panel (SAP) has completed its review of the Endocrine Disruptor Screening Program (EDSP) Proposed Tier 1 Screening Battery. Advance notice of the SAP meeting was published in the *Federal Register* on January 24, 2008.

The FIFRA SAP review was conducted in an open panel meeting on March 25-26, 2008 in Arlington, Virginia. Dr. Steven G. Heeringa chaired the meeting. Jim Downing served as the Designated Federal Official. Dr. Linda Phillips, Director of the Exposure Assessment Coordination and Policy Division, OSCP, EPA, provided an overview of the goals and objectives for the meeting. Gary Timm, M.S., of the Exposure Assessment Coordination and Policy Division, OSCP, presented an Introduction and Background of the EDSP. Dr. Les Touart, OSCP, EPA, explained the EDSP Tier I Screening Battery, which was the subject of this meeting.

The EPA is implementing an Endocrine Disruptor Screening Program (EDSP) in response to a 1996 Congressional mandate under the Federal Food, Drug, and Cosmetic Act (FFDCA). Section 408(p) of the FFDCA required the EPA "to develop a screening program, using appropriate validated test systems and other scientifically relevant information, to determine whether certain substances may have an effect in humans that is similar to an effect produced by a naturally occurring estrogen, or other such endocrine effect as [EPA] may designate (FFDCA 21 U.S.C. 346a(p)). In 1998, the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), a panel of experts chartered pursuant to the Federal Advisory Committee Act (FACA) in response to the FFDCA, submitted a final report to advise the EPA on how to develop its Endocrine Disruptor screening and testing program. The screening program was also reviewed and reported on by the EPA's Science Advisory Board and Scientific Advisory Panel (SAB/SAP) as required by the FFDCA. Together, the EDSTAC and SAB/SAP recommended that the EPA address both human and ecological effects and examine effects on the estrogen as well as the androgen and thyroid (EAT) hormonal systems, and that a two-tiered approach be used for screening. The purpose of Tier 1 is to identify substances that have the potential to interact with the EAT hormonal systems using a battery of screening assays. The purpose of Tier 2 testing is to identify and establish a dose-response relationship for any adverse effects that might result from the interactions identified through the Tier 1 screening battery.

The EPA has validated (or anticipates completing validation in 2008) several candidate assays for the Tier 1 battery as follows:

Table 1. Screening assays being considered for the Tier 1 battery.

<b><i>In vitro</i></b>
Rat uterine cytosol & human recombinant estrogen receptor (ER) binding*
Rat prostate cytosol androgen receptor (AR) binding
Human cell line (H295R) steroidogenesis*
Human placental & recombinant aromatase
<b><i>In vivo</i></b>
Uterotropic (rat)
Hershberger (rat)
Pubertal female (rat)

Pubertal male (rat)
Adult male (rat)
Amphibian metamorphosis (frog)
Fish short-term reproduction

\*Consideration of these assays will be contingent on individual assay peer review, which is expected in 2008.

Following an extensive validation process, the EPA has had (or expects to have in 2008) each of these assays peer reviewed independently by a panel of scientific experts. The individual assay peer review process was done to ensure that independent scientific opinions about the candidate assays were obtained and considered. Information regarding individual assay peer review can be found in Section IV.D. of the July 13, 2007 FR notice (<http://www.epa.gov/fedrgstr/EPA-PEST/2007/July/Day-13/p13672.pdf>).

The EPA is now convening an independent scientific peer review of the Tier 1 screening battery and has chosen the Federal Insecticide, Fungicide, Rodenticide Act (FIFRA) SAP process. The FIFRA SAP will be charged with commenting on whether the EPA's proposed battery composition fulfills its purpose (i.e., to identify the potential to interact with the EAT hormone systems). The SAP will specifically be asked to comment on the strengths and limitations of the proposed battery, and to suggest improvements that could be made to the proposed battery considering candidate assays. The SAP's advice will inform the EPA's final decision on the composition of the Endocrine Disruptor Screening Program Tier 1 screening battery.

## PUBLIC COMMENTS

Oral presentations were given by:

**American Chemistry Council –**

**Christopher J. Borgert, Ph.D.**, Applied Pharmacology and Toxicology, Inc.  
**J. Willie Owens, Ph.D.**, Procter & Gamble  
**Sue Marty, Ph.D.**, Dow Chemical Company  
**John C. O'Connor, Ph.D.**, DuPont Haskell Global Centers for Health and Environmental Sciences  
**Richard A. Becker, Ph.D.**, American Chemistry Council  
**Lisa Ortego, Ph.D.**, Crop Life America  
**Reinhard Fischer, Ph.D.**, Bayer CropScience  
**Steven Levine, Ph.D.**, Monsanto  
**Ellen Mihaich, Ph.D.**, Environmental and Regulatory Resources, LLC

**Kristie Stoick, MPH**, Physicians Committee for Responsible Medicine  
**John D. Gordon, Ph.D.**, Xenobiotic Detection Systems  
**Jennifer Sass, Ph.D.**, Natural Resources Defense Council  
**Scott Slaughter**, Center for Regulatory Effectiveness  
**Catherine Willett, Ph.D.**, Science Policy Advisor, Regulatory Testing Division, Research and Investigations, People for the Ethical Treatment of Animals

Written statements were provided by:

**Penelope A. Fenner-Crisp, Ph.D.**, DABT  
**Catherine Willett, Ph.D.**, Science Policy Advisor, Regulatory Testing Division, People for the Ethical Treatment of Animals  
**Troy Seidle**, Science Policy Advisor, Humane Society of the United States  
**Martin Stephens, Ph.D.**, Vice President for Animal Research Issues, Humane Society of the United States  
**Kristie Stoick, MPH**, Research Analyst, Physicians Committee for Responsible Medicine  
**Sue A. Leary**, President, Alternatives Research & Development Foundation  
**Tracie Letterman, Esq.**, Executive Director, American Anti-Vivisection Society  
**Scott Slaughter**, The Center for Regulatory Effectiveness  
**Dee Ann Staats, Ph.D.**, Environmental Science Policy Leader, CropLife America  
**Richard A. Becker, Ph.D.**, DABT, Senior Toxicologist and Senior Director, Regulatory and Technical Affairs Department, American Chemistry Council

## SUMMARY OF PANEL DISCUSSION AND RECOMMENDATIONS

The Scientific Advisory Panel reviewed the documentation provided by the EPA plus the oral and written comments provided by the public. Material describing these EDSP assays, known as Tier 1 assays, along with available peer review and public presentations of each assay was provided to the Panel for evaluation. The Panel was charged with responding to two questions:

1. Please comment on the ability of the proposed Tier 1 screening battery to provide sufficient information to determine whether or not a substance potentially interacts with the estrogen, androgen, and thyroid hormonal systems based on the modes of action covered within the battery.

2. EPA proposed a Tier 1 screening battery that includes many assays that are complementary in nature in their coverage of the EAT hormonal systems (the strengths of one assay offset the limitations of another), albeit by different taxa, life-stages, endpoints, exposure and use of *in vitro* and *in vivo* methods executed at different levels of biological organization (e.g., cytosolic receptor binding, cell-based assays, whole organism).

a. Please comment on how well the proposed battery minimizes the potential for “false negatives” and “false positives.”

b. Are there any unnecessary redundancies for Mode of Action (MOA) across the battery?

c. Please comment on whether a different combination of validated assays would be more effective in achieving the purpose of the battery than that proposed by EPA.

### Response to Charge Questions:

The Panel discussed assays individually and as a complete set of assays regarding the ability to detect estrogenic, androgenic and thyroid active substances with as few false positives and false negatives as possible. The conclusions drawn upon completion of this review were:

1. Chemicals testing positive in the battery of Tier 1 assays would be identified as potential estrogenic, androgenic and thyroid hormone active substances.
2. The ability to identify endocrine active substances is enhanced in the Tier 1 battery because the tests provide adequate replication and redundancy.
3. It was clear that the inclusion of apical assays of amphibian metamorphosis and fish short-term reproduction were important to detect endocrine active substances that may operate by mechanisms of action yet to be discovered.
4. The 15-day adult male assay proposed during some public comments would not be an appropriate substitute for the male and female pubertal assays because the pubertal assays provide for differences between the sexes and provide the only approach to testing for organizational effects during development.

### Recommendations:

Although the Panel found that the battery of assays presented would serve as an adequate screen for estrogenic, androgenic and thyroid hormone disruptors, a number of recommendations

were made for modifications of the assays and for future research. Among those are the following:

1. Additional assays should be developed and validated for use in the Tier 1 screening battery that screen for ER $\beta$ -specific cell signaling effects.
2. Continuing to monitor ovarian cyclicity of animals in the female pubertal assay after the first cycle may not be a useful endpoint due to high variability that could be caused by other factors.
3. Validation of the human ER $\alpha$  transactivation assay for use in detecting antagonists was considered important and encouraged. Validation of the uterotrophic assay to assess estrogen antagonism, which appears to have been accomplished by OECD, is recommended for the EPA Tier 1 battery.
4. The EPA is encouraged to develop and implement a recombinant AR binding assay as soon as possible to replace or complement the rat ventral prostate cytosol binding assay. Development and validation of an AR transactivation assay was also strongly encouraged.
5. The EPA was encouraged to consider developmental endpoints after perinatal exposure in the Tier 1 battery if possible, but if this is not possible to include this assessment in the Tier 2 battery.
6. The development of standardized laboratory procedures or a centralized laboratory for hormone measurements is recommended.
7. A negative control substance(s) has not been identified for the pubertal assays. This stands as a major limitation to the Tier 1 battery and more compounds should be tested.
8. Several specific suggestions were proposed that could be developed to better address thyroid-related mechanisms of action, such as development of TR transactivation assays, deiodinase assays, and gene expression measurements.
9. Endpoints of thyroid hormone action could be incorporated into the pubertal assays. Possible endpoints that are influenced by thyroid status are plasma cholesterol and heart weight or function. These endpoints would have to be validated.
10. Recognize that new endocrine disruptors are being identified that target the EAT hormonal systems as well as other hormones through novel mechanisms and will require the development of new assay systems currently not considered.

In summary, the proposed set of Tier 1 assays are appropriate to begin screening for disruptors of the EAT axes. However, several assays do not represent the current state of the science, or the proposed screens do not fully address major modes of action and should be updated and extended as soon as possible. The EPA should consider this set of assays to be a work in progress. The Panel expects that the EPA will continue to develop, refine, and review the battery. New endocrine disruptors and new mechanisms of action are likely to be revealed in the future requiring that the current Tier 1 assays be modified and new ones developed and validated.



## PANEL DELIBERATIONS AND RESPONSE TO CHARGE

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

### Charge Question 1

1. Please comment on the ability of the proposed Tier 1 screening battery to provide sufficient information to determine whether or not a substance potentially interacts with the estrogen, androgen, and thyroid hormonal systems based on the modes of action covered within the battery:
  - a. Estrogenicity: acting agonistically by potentiating the estrogen signal

The Panel initiated discussion by listing each of the screens in the proposed Tier 1 battery that would detect chemicals acting via an estrogenic mode of action (MOA). Those assays were identified as: 1) ER binding assay (rat uterus); 2) the human ER $\alpha$  transactivation assay; 3) the rat uterotrophic screen; 4) the pubertal female screen; and 5) the fish short-term reproduction assay (fish screen).

There was general agreement that each assay has individual strengths and weaknesses; however, the combination of screens in the battery was considered robust in addressing this MOA. The ER binding assay, ER $\alpha$  transactivation assay, the uterotrophic assay, and the fish short-term reproduction assay were recognized as giving particularly strong coverage of the MOA. These five assays range from highly specific *in vitro* assays (ER $\alpha$  transactivation assay, ER binding [not peer reviewed]) to *in vivo* assays that examine multiple apical endpoints (pubertal female, uterotrophic, fish short-term reproduction). It was also noted that the amphibian metamorphosis assay has the potential to identify estrogenic compounds since metamorphosis is inhibited by estrogens.

The Panel discussed novel or unrepresented estrogenic modes of action (MOAs) that could contribute to false negatives (Chen et al., 2008). Specifically, the MOA is well covered with regard to effects mediated through the ER $\alpha$  nuclear receptor. It is certainly conceivable that compounds exist that will interact primarily with ER $\beta$ , or membrane receptors, or other yet to be discovered, estrogen signaling mechanisms. Since it would likely be difficult to incorporate new findings related to estrogen signaling (or any hormone system) into the battery of assays in a timely manner, it seems that this is an argument for having apical endpoints within the battery. The need for apical endpoints may detect such effects even in the absence of specific understanding of the MOA.

The Panel discussed the absence of *in vitro* or *in vivo* assays that specifically identify compounds acting through ER $\beta$ . This lack was considered significant because some xenobiotics appear to preferentially bind and act through ER $\beta$ . Further, in cells and tissues that co-express ER $\alpha$  and ER $\beta$ , the activities of each receptor can be antagonistic to one another. Combined effects of ligands at these two receptors might result in no effect in the apical *in vivo* assays. As a result of the absence of an ability to detect such non-selective effects, there is an increased possibility of false negatives. The Panel recommended that additional assays be developed and

validated for use in the Tier 1 screening battery that screen for ER $\beta$ -specific and receptor mediated cell signaling effects.

The inclusion of the fish short-term reproduction assay was viewed by the Panel as an important component of the estrogenicity screens. The measurement of plasma vitellogenin concentrations in particular provides a specific indicator of estrogenic compounds. However, the Panel did hear some concern from public comment regarding the reliability of the fish short-term reproduction assay. This concern addressed the standardization of reproductive success by measurement of fecundity. However, it was noted that this component of the assay was an essential part of apical analysis of hypothalamic/pituitary/gonadal (HPG) activity. A potential drawback of the rat pubertal and uterotrophic assays for estrogenicity is the potential for rat strain differences to affect the sensitivity to the test compounds. For example, specific lineages of Sprague Dawley rats may be relatively insensitive to the estrogenic compound Bisphenol A (BPA) compared to other rat strains for some endpoints. Indeed, BPA was found to be generally toxic but not necessarily endocrine disruptive at the concentrations used in the validations of the prepubertal male and female assays. This concern is somewhat mollified by the fact that BPA scored positively as an estrogen in the *in vitro* estrogen receptor binding and transactivation assays, and in the *in vivo* uterotrophic and fish short-term reproduction assays. Therefore, the inclusion of redundant endpoints is important for cases like BPA.

The female pubertal assay was described as perhaps less important for detection of estrogenic activity than for detecting perturbation of the HPG axis. The point was raised that when one is looking for effects upon female puberty, vaginal opening and onset of first estrus are good endpoints, but ovarian cyclicity of animals may not be a useful endpoint because of high variability caused by other factors. The Panel raised the question as to whether the duration of the female pubertal assay was too long for a Tier 1 battery test.

With regard to the uterotrophic assay, there had been some discussion (both EPA and public comment) concerning the choice of subcutaneous versus oral dosing. One argument made is to choose the route relevant to human exposure. While this is necessary for risk assessment, the data from the Tier 1 assays are not intended for risk assessment, but rather for detecting a potential for endocrine disrupting activity. In cases where there is little known about metabolic disposition of the test chemical, the approach suggested by EPA, where the subcutaneous route is used for the uterotrophic assay and the oral route is used for the pubertal female assay seems reasonable and could provide useful information, as it did for the two assays discussed above for the detection of BPA activity (negative by oral route in pubertal female, positive in uterotrophic by subcutaneous injection). Additionally, concern was raised that reduction in vitellogenin synthesis could result from acute hepatic injury – toxicity resulting in a false negative.

b. Anti-estrogenicity: acting antagonistically by attenuating the estrogen signal

Discussion was initiated by listing each of the screens in the proposed Tier 1 battery that would detect chemicals acting via an anti-estrogenic MOA. Those assays were identified as: 1) ER binding assay (rat uterus); 2) the human ER $\alpha$  transactivation assay; 3) Pubertal Female (delayed vaginal opening & increased diestrus) 4) the fish short-term reproduction assay (decreased vitellogenin in female).



The Panel felt that the anti-estrogenicity MOA is weakly covered by the screening battery. As currently validated, the only assay that could potentially identify anti-estrogenic compounds is the ER binding assay, although it cannot distinguish agonists vs. antagonists, and is limited to the identification of compounds that can bind to ER $\alpha$ , but not ER $\beta$ . The ER $\alpha$  transactivation assay could be used to identify antagonism, but has not been validated for this purpose. Validation of the transactivation assay for detection of antagonists was considered important by the Panel. They encouraged EPA to validate it as soon as possible. Even with those proposed modifications to the battery, the battery remains limited to screening an ER binding – specific MOA mediated only by ER $\alpha$ . It was also pointed out that there are limitations with the reliance on additional *in vitro* measures based on the inability to identify compounds that are metabolized into a bioactive form.

The pubertal female assay may identify anti-estrogenic compounds, but the validation of this assay to address this MOA was considered inadequate because tamoxifen was tested, which is a selective estrogen receptor modulator (SERM) that is characterized by having mixed (tissue/cell specific) antagonist/agonist activity. In light of the weak coverage for this MOA in the battery, the Panel recommended that the EPA validate the uterotrophic assay, a well characterized assay for estrogens, to identify anti-estrogenic compounds. Adaptation of the uterotrophic assay to anti-estrogens would require the administration of an estrogen (17 $\beta$ -estradiol) to the immature or ovariectomized rat with and without administration of the test chemical. This would require careful selection of an appropriate dose of 17 $\beta$ -estradiol (covered by the female pubertal rat assay). A draft OECD uterotrophic assay protocol has been published (Yamasaki *et al.*, *Arch Toxicol* 81:749-757, 2007) that combines measures of agonism and antagonism in the same protocol. This strategy would restrain animal consumption by using controls for both purposes. While the uterotrophic assay has an acknowledged drawback of low sensitivity, nevertheless it remains a simple-to-perform evaluation of *in vivo* exposure. The Panel recommended strengthening the battery of estrogen-related properties by validating a modified uterotrophic screen to include antagonism.

Anti-estrogenic actions may be identified in the pubertal female assay (delayed vaginal opening) and the fish short-term reproduction assay (decreased plasma vitellogenin in females); however anti-estrogenic actions would result in decreases in activities that appear less reliable and less sensitive than MOAs that induce increases in these end-point measures. Looking for a lack of effect is considered much more difficult than observing an effect or an increased end-point signal. There is a danger of false positives occurring when looking for a chemical that does not produce a signal where one might be expected. For example, hepatotoxins can also reduce vitellogenin levels and hence this end point is weaker due to lack of specificity to endocrine disruption MOAs.

For detecting compounds with anti-estrogenicity, the fish short-term reproduction assay is considered valid since vitellogenin synthesis is quite dependent on estrogens. However, the estrogen induction of vitellogenin synthesis is dependent on adequate prior exposure to thyroid hormone in other vertebrates (Huber *et al.*, 1979, Rabelo and Tata, 1993), so MOAs other than strictly anti-estrogenicity can complicate interpretations of the assay, and must be taken into account.

c. Androgenicity: acting agonistically by potentiating the androgen signal

Discussion was initiated by listing each of the screens in the proposed Tier 1 battery that would detect chemicals acting by a MOA that would potentiate androgen signaling. Those assays were identified as: 1) AR binding (rat prostate cytosol); 2) the Hershberger Assay; 3) pubertal male (pseudoprecocious puberty increased target organ weights) and 4) the fish short-term reproduction assay (masculinization of females reduced egg production)

This MOA is covered by one *in vitro* assay and three *in vivo* assays. Overall, androgenicity is considered well addressed by the Tier 1 battery of screens, and is considered the second most robust with respect to MOA coverage. However, additional and/or modified *in vitro* assays would strengthen the ability to address potential androgenic compounds.

The rat prostate cytosol androgen receptor (AR) binding assay is a well-established *in vitro* assay that will identify compounds interacting with the AR. Limitations to this assay are that it cannot detect agonists vs. antagonists, and there are serious concerns with the ability of individual labs to reliably prepare rat ventral prostate cytosol. The Panel raised concerns for the difficulties in preparing and handling the rat ventral prostate cytosol. Significant inter-laboratory variability in cytosol preparation and quality were reported in the validation process, suggesting that transferability across laboratories will be a problem. The Panel further noted that homogenates must be handled very carefully to get meaningful results. Further, many laboratory issues were reported in the validation process. From the results of the validation process, it is considered most clear that this is not a straightforward or routine assay, nor will it be easily transferable across laboratories. The Panel recommended the EPA develop and implement a recombinant AR binding assay as soon as possible to replace the rat ventral prostate cytosol binding assay. The Panel also strongly encouraged EPA to develop and validate an AR transactivation assay.

The Hershberger assay is considered a strong, reliable, and validated assay that will allow for the identification of compounds that act through an AR-dependent process. The maturity and reliability of the Hershberger Assay for this MOA was quite impressive. For this MOA, end points of the screens were considered to have good predictive abilities; the pubertal male assay using oral administration with metabolic activation was also considered an important component of the Tier 1 battery.

The fish short-term reproduction assay uses the development of male secondary sex characteristics as a robust endpoint to identify androgenic compounds. For most of the same reasons cited earlier as a measure of estrogenicity, the fish short-term reproduction assay serves as an important *in vivo* component of the Tier 1 battery. This assay can detect androgenic compounds providing a strong comparison with the Hershberger and male pubertal assays.

While significant concerns remain about the specificity of the male pubertal assay, an advantage of using an intact animal (rodent and fish) rather than a castrated animal as in the Hershberger assay is that chemicals that potentiate the effects of existing androgens in the system may be revealed. The Panel pointed out that the pubertal male assay relies on preputial

separation, an androgen-dependent process. The Panel raised concerns that this assay (and the fish short-term reproduction assay) could be influenced by non-androgenic MOAs. The pubertal male assay is a necessary assay to detect androgenicity but it may not be better than the adult male assay.

The Panel also expressed concern that only the pubertal period is being considered to address development, and effects during early critical developmental periods (e.g. nervous system effects) are not addressed in the battery. This lack of coverage in known periods specific to development was identified as being a considerable weakness in the battery, especially in light of the highly sensitive nature of developmental effects. Thus, the lack of coverage of developmental functions aside from puberty was considered a significant limitation.

d. Anti-androgenicity: acting antagonistically by attenuating the androgen signal

Discussion was initiated by describing each of the screens in the proposed Tier 1 battery that would detect chemicals acting by a MOA that would act to antagonize androgen signaling. Those assays were identified as: 1) AR binding (rat prostate cytosol); 2) the Hershberger assay; 3) pubertal male (delayed puberty reduced target organ weights) and 4) the fish short-term reproduction assay (attenuated male secondary sex characteristics).

The same four assays that are informative on androgenic activity are useful for detecting anti-androgen MOAs. The general limitations as discussed above were recognized as also relating to the anti-androgen MOA. Many of the same issues identified for anti-estrogenicity also apply. The Panel recommended that the development of an AR transactivation assay and other *in vitro* and cell-based assays for addressing this MOA be made a high priority.

The Panel acknowledged that the Hershberger assay is a particularly strong component of the battery for detecting anti-androgen activity and the AR binding assay does not detect anti-androgen activity. Therefore, a transcriptional activation assay is considered a useful addition to the battery, as was the case for the agonist MOA.

The ability to identify AR antagonists is limited compared with the ability to identify AR agonists. The AR binding assay will identify potential antagonists through their binding to the AR but will not distinguish agonist vs. antagonist. The Hershberger and male pubertal (i.e. delayed preputial separation) assays can potentially identify AR antagonists. In addition, since the fish short-term reproduction assay appears robust for detecting androgen activities through the appearance of secondary sex characteristics, modification of the assay to directly detect anti-androgens should be feasible. One could test whether a chemical blocks the activity of a low dose of an androgen (e.g., trenbolone) as discussed above, and there are published results that trenbolone-treated female fish were shown to be sensitive to androgen antagonists (Ankley et al, 2004). Such a modification to the assay would more directly flag anti-androgenic chemicals as opposed to simply relying on a decrease in fecundity or secondary sex characteristics. The Panel acknowledged that this is an ongoing effort within EPA and that such an assay shows promise for future inclusion in the Tier 1 battery. However, because the Hershberger, rat prepubertal, and fish short-term reproduction assays are *in vivo* assays, a complementary *in vitro* assay will be needed to verify this MOA. Thus, the Panel recommended that EPA develop an AR

transactivation assay as soon as possible. The Panel recommended caution in selecting a specific AR transactivation assay because there are various forms in the literature. The specific assays were described as being very different in regard to their performance, and thus careful consideration of which specific assay to choose was suggested.

Critical developmental windows of sensitivity and long-term effects of exposures during development were again discussed. The Panel raised the question of whether the peri-pubertal period was the most sensitive and appropriate for identifying endocrine disruptive compounds. Hormones have important developmental roles, whereby they 'organize' neural circuitry and physiological functions. Disruption of these actions could lead to long-term, stable alterations in physiology and behavior. The Panel recognized that addressing such effects is beyond the scope of the proposed Tier 1 battery. Nevertheless, the importance of developmental actions of endocrine disruptors should be recognized and addressed in Tier 2.

The Panel discussed standardization of hormonal measures and their relevance to clinical end-points. It was recognized that because measuring hormones is not necessarily a trivial matter there might be a need to develop standardized laboratory procedures for hormone measurements or have all measurements conducted in a single contract laboratory (or laboratories). Currently, there are numerous commercial kits available for measuring hormones in blood plasma or serum, but all kits are not equivalent regarding sensitivity or specificity. Additional factors related to animal husbandry, methods for obtaining samples so as to minimize stress to the animals, and time of day of sample collection are important to standardize so as to be able to compare results across laboratories. The clinical laboratory industry is able to standardize across labs and the Panel suggested that EPA should be able to do the same for contract laboratories.

- e. Steroidogenesis effects: modulating normal steroidogenic processes including aromatase by inducing or inhibiting enzymes in the sex steroid hormone synthesis pathway

The Panel listed each of the screens in the proposed Tier 1 battery that were identified as potentially useful to detect chemicals acting via modulation of steroid hormone synthesis pathways. Those assays include: 1) H295R cell assay (estradiol and testosterone production); 2) aromatase inhibition assay (human recombinant); 3) the rat pubertal male screen; 4) the rat pubertal female screen, and 5) the fish short-term reproduction assay (fish screen).

The H295R cell-line based assay might be a valuable addition to addressing multiple targets in the steroidogenic pathway, but this assay has not yet been peer reviewed. Lack of metabolic activity in this cell line is a limitation. Information presented to the Panel on this assay was limited. The H295 cells seem to be able to detect effects on the enzymes for androgen biosynthesis and on aromatase. However, the cells appear to have no 5 alpha-reductase activity; compounds that affect this enzyme may be detected in the *in vivo* assays by a change in the plasma testosterone:DHT ratio. To validate the use of these transformed cells as a proxy for normal cells, it should be determined whether the steroidogenic enzymes in these cells are regulated similarly to Leydig cells, e.g., transcriptional regulation by Steroidogenic Factor 1, etc. Further, in EPA's technical review document, the result of the *in vitro* steroidogenesis assay is looked on as critical to determining if the effect of a chemical on plasma hormone concentrations is at the level of hypothalamo-pituitary function or on steroidogenesis or steroid metabolism.



However, this will not be a straightforward interpretation since a negative in the steroidogenesis assay along with a positive effect on hormone levels could also be due to the lack of metabolic capability of the H295R cells.

The aromatase assay (human recombinant) seems to be well designed and was considered robust, although it was noted that it only addressed one point in the steroidogenic pathway. The recombinant aromatase assay, while it cannot detect chemicals that influence aromatase expression is valid for the detection of inhibitors and (presumably) allosteric activators. Despite its limitations the aromatase assay is considered to be straightforward, valuable and important, since it might indirectly inform on potential developmental effects mediated by disruption of testosterone to estradiol conversion (especially as it relates to development of the nervous system).

The *in vivo* assays suffer from issues of specificity, as they may not be able to distinguish endocrine MOAs from general reproductive toxicity. However, false positives for endocrine disruption will be detected using these assays, it is recognized that such apical endpoints are necessary to identify compounds that can disrupt hormone-dependent processes. The pubertal and fish short-term reproduction assays could potentially signal alterations in steroidogenesis, but this MOA may be difficult to conclusively identify. This MOA is also covered by the pubertal female, pubertal male and fish short-term reproduction assays. Together, these assays have the ability to identify HPG axis disruption but identifying the specific site of action or MOA may not be straightforward. Concerns of quality control and repeatability among laboratories for each assay were noted.

The appropriateness of the fish short-term reproduction assay with respect to obtaining information on androgen biosynthesis in mammals or other tetrapods was questioned since the active androgen in fish is 11-keto-testosterone. Clarification was provided by EPA when it was pointed out that the pathway to testosterone in fish is essentially the same as in mammalian species with one extra step resulting in the generation of 11-keto-testosterone as the active androgen. Thus, compounds that disrupt 5-alpha reductase in tetrapods would not be detected in the fish short-term reproduction assay. Similarly, compounds that disrupt the generation of 11-keto-testosterone may not affect androgen biosynthesis in mammals. Further screens are necessary to determine if a positive result in this assay is due to alterations in testosterone or 11-keto testosterone synthesis as it appears that alteration in either can result in a disruption of the HPG axis.

f. Hypothalamic/pituitary/gonadal (HPG) axis effects: interference with the hypothalamic-pituitary regulation of gonadal function including the production of hormones and gametes

The Panel listed each of the screens in the proposed Tier 1 battery that would detect chemicals acting via a MOA resulting from disruption of the HPG axis. Those assays include: 1) the pubertal male screen; 2) the pubertal female screen; and 3) the fish short-term reproduction screen.

The MOA of HPG axis effects is covered by the pubertal female, pubertal male and fish short-term reproduction assays and is considered the least well supported in the Tier 1 battery.

With these assays the only MOA that would be bolstered by more than one assay is an MOA that acts via influences on thyroid hormone synthesis (see section g below).

The Panel noted that the EPA has made a good case for the pubertal assays as effective in detecting disturbances in the HPG axis, particularly from the work done at the EPA research labs which have a long history with this assay. There appear to be some transferability problems in the validation studies – those problems are revealed by results from the same assay, but not always in agreement across labs, although the conclusions drawn concerning the compounds' activity were in general agreement. The Panel recommended that EPA consider the immature rat uterotrophic assay for this MOA, as the sensitivity for estrogen agonists appears similar to the ovariectomized model as described during the public presentations and that assay can detect agents that affect the HPG axis.

It was noted repeatedly, and stressed as a major issue, that a negative control substance(s) has not been identified in this group of assays. This fact stands as a major limitation to the Tier 1 battery. Lacking demonstration of expected negative results remains an issue for the validity of these assays.

As noted above, the rat pubertal assays suffer from the absence of an appropriate negative control, since no compound was negative across all apical endpoints in the male and female pubertal assays, including 2-chloronitrobenzene which was chosen for this purpose. In addition, body weight reductions were closely associated with perturbations in the onset of puberty and/or normal cycling. Therefore the specificity of the pubertal assays for detecting alterations in the HPG axis due to purely endocrine-related disruption is currently unclear. Similar concerns regarding non-endocrine based effects on the HPG axis, or directly on gonadal function, are extended to the fish short-term reproduction assay. However, the Panel also recognized that disruption of the HPG axis is clearly separable from the effects on body weight, and this MOA is of concern and should trigger further analysis even without a clear MOA.

Regarding the issue of effects on gametes, the Panel noted that the pubertal assays do not address issues related to gametes. Effects upon gametes will require an additional second tier, multi-generational study.

The Panel also addressed the statistical analysis of the relationship between reduction in body weight due to restricted feeding and HPG reductions in the pubertal rat assays. One EPA presenter displayed a graph showing that weights for some key organs decreased in direct proportion to decreases in body weight. The question was whether a covariate adjustment would be appropriate for further analysis of organ weight differences—that is, distinguishing effects attributable to body weight loss from endocrine disruptor effects. Panel members felt that some sort of covariate analysis might be appropriate, but the actual form of the analysis would not be straightforward. The issue is made more complex by the fact that body weight changes can be viewed as both a response to treatment in its own right in addition to its potential role as a covariate.

If the response to treatment is analyzed in a regression model and change in organ weight is independent of small changes in body weight then a simple linear model of the effect of dose on organ weight might be modeled as follows:

$$\hat{y} = \hat{\beta}_0 + \hat{\beta}_1 \cdot X_1 + \hat{\beta}_2 \cdot Dose$$

where:  $y$  = organ weight for the test subject;

$X_1$  = final body weight for the test subject.

$\hat{\beta}_1$  = estimated relative change in organ weight for a unit change in BW

$\hat{\beta}_2$  = estimated relative change in organ weight for a unit change in Dose

$\hat{\beta}_0$  = intercept included to account for potential non-linear effects close to zero Dose & BW

Of course, this simple linear model can be extended to parameterizing dose through indicator variables representing the distinct dose levels or possibly through more complex and possibly nonlinear regression models.

However, if, independent of dose effects, organ weights change with small (e.g. 10%) changes in body weight, the previous model will confound the effects of body weight change and the effect of change in organ weight due to the chemical dose. Data external to the experiment and based on fasting studies can be used to estimate the relationship of organ weight change to change in body weight (see Figure 1 below). The relationships estimated from these data could be used to "calibrate" the above regression model. The calibration takes the form of a separate model (as depicted in the following linear model) that predicts organ weight change for a test animal that is attributable to body weight change due solely to fasting weight change.

$$\hat{z} = \hat{\gamma}_0 + \hat{\gamma}_1 \cdot (X_1) + \hat{\gamma}_2 (X_{1,f} - X_{1,s})$$

where:  $\hat{z}$  = predicted organ weight due to  
final body weight;

$X_{1,s}$  = starting body weight for fasting study test subject;

$X_{1,f}$  = final study body weight for fasting study test subject.

It is important to note that the estimated parameters for this model of predicted organ weight are based on data that are obtained outside (external to) the specific assay run.

The external adjustment for predicted organ weight due to body weight and not to dose can be incorporated into the analysis of the assay using the following general regression:

$$\hat{y} = \hat{\beta}_o + \hat{\delta}_1 \cdot \hat{z} + \hat{\beta}_2 \cdot Dose$$

where:  $y$  = organ weight for the test subject;

$\hat{z}$  = the predicted organ weight for the test

subject based only on starting body weight and  
change (start to finish) in the body weight.

The difficulty in drawing inference from this model is that it combines models fitted to data from two distinct studies—a model of organ weight as a function of body weight and body weight change from the fasting studies with a separate model that relates organ weight to assay study dose and the “calibrated” dose-independent value of body weight. Again, the models presented here are meant only to illustrate the relationship of dose effect to predicted organ weights in the simplest of linear forms. A hierarchical Bayesian approach that combines information on the model uncertainty from both data sources might be considered. See Raghunathan et al. (2007) for an illustration of the application of Markov Chain Monte Carlo approach to integrating data from two sources in the model estimation. There may be other more standard approaches to incorporating an external calibrating regression model into the final analysis of the dose/effect experiment. The Panel recommended appropriate EPA/ORD statisticians be consulted for the details of this analysis.

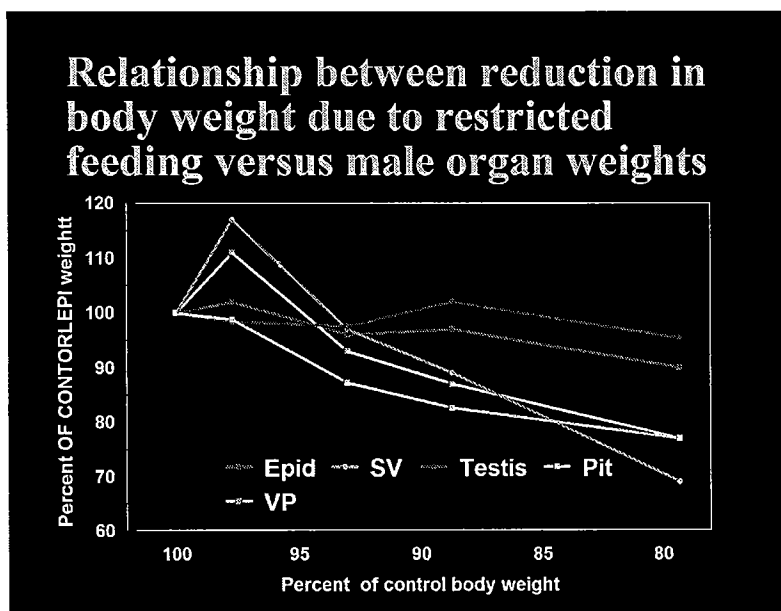


Figure 1 Example of organ weight changes as a function of body weight. REF: Endocrine Disruptor Screening Program: Tier 1 Screening Battery (T1S): The ability of the T1 Battery to detect lower potency, environmentally relevant EDCs, Earl Gray, ORD, NHEERL. (Slide 11, EPA Supplemental Presentation ORD NHEERL SAP 3-26-08. PPT in the EPA Docket for the SAP meeting)

g. Hypothalamic/pituitary/thyroid effects: modulation of the processes associated with direct thyroid hormone receptor interaction as well as those processes involved indirectly (e.g., synthesis, secretion, elimination of thyroid hormones) in thyroid function



The Panel continued by listing each of the screens in the proposed Tier 1 battery that could detect chemicals acting via a MOA resulting from disruption of the hypothalamic-pituitary-thyroid (HPT) axis. Those assays include: 1) the pubertal male assay; 2) the pubertal female assay; and 3) the amphibian metamorphosis assay (AMA).

The ability to detect a MOA involving the thyroid axis is considered to be among the weakest in the Tier 1 battery. In the male and female pubertal assays serum thyroid hormones, thyroid stimulating hormone (TSH), and thyroid histology will be measured. In the amphibian metamorphosis assay, measures of thyroid histology and tadpole development are the primary endpoints. These assays have the potential to identify thyroid disruptors but may not allow for identification of the specific MOA. Many compounds may lower plasma T4 through changes in hormone degradation and clearance without acting directly on components of the HPT axis (e.g., hypothalamus, pituitary TSH secretion, thyroid hormone biosynthesis). Thus, although it will be difficult to distinguish a specific thyroid disruptor from a more general toxicant given the limited assays available to address this MOA, the observation of toxicant-induced reduction in serum thyroid hormone should be considered adverse.

The Panel expressed concern regarding the portability/transferability of these assays to external labs. This is considered a major limitation in light of the strong (sole) reliance on AMA to address the disruption of thyroid hormone action in peripheral tissues leaving this component (MOA) weakly addressed.

The pubertal assays will measure serum TSH and thyroid histology. These assays have the potential to identify thyroid hormone disruptors but may not allow for differential identification of the MOA. Many compounds lower plasma T4 unrelated to a direct impact on the thyroid axis (i.e., they do not influence TSH). Thus it will be difficult to distinguish a specific thyroid disruptor from a more general toxicant given the limited assays available to address this MOA.

The amphibian metamorphosis assay is relevant because the thyroid systems of tetrapod vertebrates are highly conserved, both in terms of morphology and physiology. The amphibian thyroid system is regulated, and responds in a similar manner to perturbation as that of other tetrapods, including mammals (Fort et al., 2007). However, it should be noted that the thyroid system of a developing animal may be regulated differently from that of the juvenile or adult. As such, the regulatory relationships among the different organs and the actions of hormones on tissues (morphological and gene expression) vary among stages of the life cycle. Thus, the morphological or molecular signatures of perturbation of the axis in a tadpole can differ markedly from those of the adult animal. As one example, histological findings from the OECD Phase II Study showed that treatment with T4 caused alterations in thyroid gland morphology that were in some cases opposite to what one would expect based on similar experiments with adult tetrapods.

The amphibian metamorphosis assay is currently the only assay that addresses the potential for compounds to interact with the thyroid hormone receptors (TRs) as an analogue. Metamorphosis is a thyroid hormone dependent process, and compounds that accelerate metamorphosis may be presumed to function as thyromimetics, although there could be other

MOAs such as the up-regulation of the corticosteroids which can synergize with endogenous thyroid hormone to accelerate metamorphosis. This assay has limited power in identifying compounds that inhibit thyroid function. The apical endpoints are not very sensitive to such compounds. The thyroid histopathology is a sensitive marker for compounds that influence thyroid hormone synthesis, and possibly feedback on TSH (although the feedback relationship in tadpoles is still under investigation and may not mirror those in adults) (Fort et al., 2007).

The battery aimed at analysis of the HPT axis, which includes effects on thyroid receptor activity, is far less developed than the battery for the estrogen or androgen systems. Alterations in the thyroid endocrine system are only addressed by the male and female prepubertal assays, and the AMA, with no *in vitro* assays. The common endpoint in all three assays is thyroid gland histology, which screens for direct toxicity at the gland itself, or secondary effects on T4 and TSH levels. Due to technical reasons, measurement of TSH and T4 is not included in the AMA but is measured in the rat pubertal assays. The Panel was concerned about the potential for confounding effects in the rat pubertal assays for detecting alterations in the HPT axis, particularly the influence of body weight on T4 levels, as described for the HPG axis. However, it should be emphasized that the ability of a chemical to cause a reduction in serum T4 by any mechanism would deprive the fetus of essential hormone. Thus, any observed reduction in serum T4 must be emphasized in a weight-of-evidence interpretation of the Tier 1 assays.

Replacement of the pubertal male or female assays with an alternative assay is not considered a reasonable alternative because it is possible to miss gender specific effects from certain chemicals.

The major concern of the Panel regarding the assays for disruption of the HPT axis, however, was the complete dependence on the amphibian metamorphosis assay to detect peripheral target tissue effects of altered thyroid hormone signaling. In this regard, the AMA is not unnecessarily redundant but in fact essential. In addition, the AMA is the only assay in the battery that uses an amphibian, and examines effects of chemicals during an early developmental time frame. However, the Panel was concerned that weakly acting chemicals that affect thyroid hormone receptor or deiodinase activity in peripheral tissues would score positive in only the AMA, and it was not clear from EPA's presentation whether such a finding would be enough to move above the weight of evidence bar for Tier 2 testing. Thus, the Panel strongly recommended development of additional *in vitro* assays for thyroid hormone receptor binding (both  $\alpha$  and  $\beta$  subtypes), thyroid hormone receptor transactivation (mammalian  $\alpha 1$ ,  $\beta 1$ , and  $\beta 2$  isoforms), and monodeiodinase (Types I, II, and III) activity assays to support and extend the AMA in future testing regimens. This point is discussed further in subsequent sections.

The Panel also recognized the significant degree of effort required to develop and validate the AMA for inclusion in the Tier 1 battery, as is true for all of the assays presented. However, there are limitations in the assay that bear mentioning. The sophisticated design of the flow through systems recommended by EPA may limit the transferability of the assay from lab to lab and the peer review of the assay also noted this concern. Furthermore, the sensitivity of the assay is currently unclear.

Some specific suggestions were proposed that could be developed to better address thyroid-related MOAs and include:

1. *In vitro* TR transactivation assays.
2. *In vivo* TR reporter assays (transgenic tadpoles expressing reporter genes that are activated by TRs)
3. Gene expression changes in amphibian tadpoles. Many TR target genes have been identified in tadpoles, and their expression is sensitive to changes in thyroid hormones.
4. *In vitro* transthyretin binding (TTR) assay. A well-studied MOA for thyroid disruptors in mammals is competition for binding to TTR. *In vitro* assays using recombinant TTRs have been developed and are relatively straightforward to perform. With regard to this MOA, it should be noted that the specificity of mammalian and non-mammalian TTRs are different. Mammalian TTRs are T4 binding proteins, while non-mammalian TTRs are T3 binding proteins. Thus, compounds acting through this MOA could potentially influence mammals and non-mammalian species differently (i.e., a compound that disrupts TTR binding in a mammal may not in an amphibian, and vice versa).
5. Thyroid hormone metabolism (deiodination, glucuronidation, sulfation).
6. Development of simple endpoints in the pubertal assays for thyroid hormone action. These might easily include specific gene targets in a variety of tissues, but also specific elements of cardiovascular function or lipogenesis.

### **Charge Question 2**

EPA proposed a Tier 1 screening battery that includes many assays that are complementary in nature in their coverage of the EAT hormonal systems (the strengths of one assay offset the limitations of another), albeit by different taxa, life-stages, endpoints, exposure and use of *in vitro* and *in vivo* methods executed at different levels of biological organization (e.g., cytosolic receptor binding, cell-based assays, whole organism).

- a) Please comment on how well the proposed battery minimizes the potential for “false negatives” and “false positives.”
- b) Are there any unnecessary redundancies for MOAs across the battery?
- c) Please comment on whether a different combination of validated assays would be more effective in achieving the purpose of the battery than that proposed by EPA.

The second set of questions was addressed by evaluating each proposed assay in terms of the physiological process it is expected to assess. These evaluations are separated into the three hormone classes, i.e., estrogen, androgen and thyroid, as targeted by EPA. The frequency of matches among multiple assays and processes are intended to provide insight regarding assay redundancy that should provide safeguards against false positives or false negatives. Individual assays in the battery that relate to the same physiological process offer complementary information to the process. As a framework for the discussion, a flow diagram was prepared to illustrate the processes being examined (Figure 2, below).

## Components of Estrogen/Androgen Activity

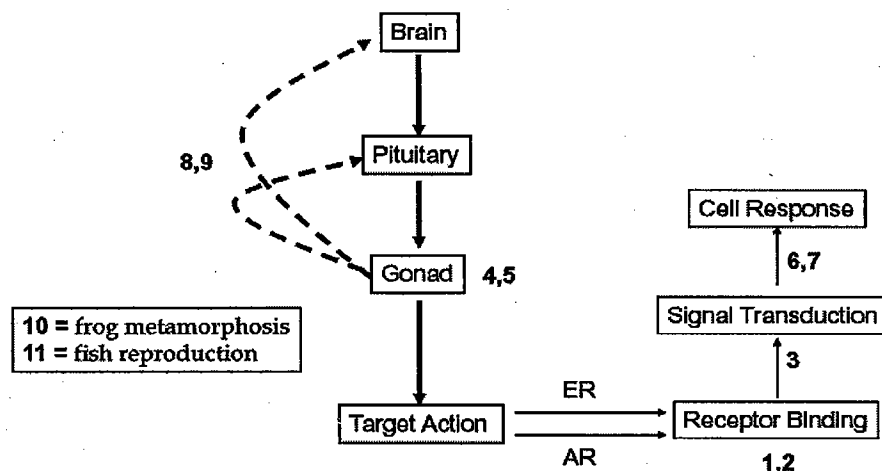


Figure 2

### Estrogenic Responses

Nine modalities were associated with EAT activities as indicated in Figures 2, 3 and 4. These include 1) Receptor/Target Binding, 2) Cell Signaling, 3) Cell Response, 4) Enzyme Activity, 5) Organ Response, 6) Male System Integrity, 7) Female System Integrity, 8) Development (thyroid), and 9) Comparative System Integrity. Each modality or basis of causation represents individual EAT molecular targets, mechanisms of action, or physiological processes. The overall integrity of the complete system is considered separately in the frog and fish short-term reproduction assays (Figure 2). Each proposed Tier 1 assay is first considered in reference to each of these nine modalities and assigned either a positive or negative score. In several instances, categories received a letter score (A) suggesting the modality was assumed in the overall function of the assay. Once all categories were assigned, modality redundancy values were determined by summing scores across all assays. Complementation of modalities was assigned by summing scores downward across modalities for each assay. Through this process definitive evaluation of redundancy for the Tier 1 battery can be determined. The grid used to determine the associations for the nine modalities in regard to estrogen responses is shown as Figure 3.

## I. Estrogen Assays

The Key at the Left Refers To the Numbers in Figure 2 (above) Which Indicates the Physiologic Process for Which Each Assay Was Assessed

Estrogen grid	Assay Type	1	2	3	4	5	6	7	8	9	10	11	Redundancy
Bases of Causation													
1.	Receptor/Target Binding	+	-	+	-	-	-	A	-	A	-	-	3
2.	Cell Signalling	-	-	+	-	-	-	A	-	A	-	-	2
3.	Cell Response	-	-	-	-	-	-	+	-	+	-	-	1
4.	Enzyme Activity	-	-	-	+	+	-	-	-	-	-	-	1
5.	Organ Response	-	-	-	-	-	-	+	-	+	+	+	3
6.	Male System Integrity	-	-	-	-	-	-	-	-	-	+	+	1
7.	Female System Integrity	-	-	-	-	-	-	+	-	+	+	+	3
8.	Development (thyroid)	-	-	-	-	-	-	-	-	-	+	+	1
9.	Comp System Integrity	-	-	-	-	-	-	-	-	+	+	+	2
	COMPLEMENTS	0	N A	1	0	0	N A	4	N A	5	4	4	
KEY													
1=ER Receptor Binding													
2=AR Receptor Binding													
3=ER Signal Transduction													
4=Aromatase													
5=H295R Cell line													
6=Hershberger													
7= Uterotropic													
8=Male Puberty													
9=Female Puberty													
10= amphibian metamorphosis assay													
11= Fish Screen													

Figure 3

From the estrogenic action grid it is apparent that each modality retains at least one degree of redundancy within the battery. Analysis of complementation for modalities indicates that *in vivo* assays are well represented with a breadth of complementary endpoints. *In vitro* assays on the other hand have few or no complements such as indicated for receptor binding or transactivation assay.

In addition to identifying specific redundancies, this evaluation provides a mechanism to address charge Question 2(a) regarding false positives and false negatives. The assays suggest that a range of both specificity and sensitivity are incorporated into the battery. As such, this design will likely provide a balanced approach to identifying false positives and false negatives. Redundancy within the battery is essential to limit both false positives and false negatives. The question, however, is: what degree of redundancy is necessary to ensure confidence in the battery of assays? Given an initial evaluation of the preliminary data there may be a tendency towards positives. This may be due to the fact that EPA has not provided a thorough evaluation with a sufficient number of compounds, or the screen is too sensitive, or perhaps the battery



lacks specificity. If the latter, it is assumed the battery will need to be refined to eliminate potential false positives likely due to toxicities other than EAT.

A good example of this may arise with the fish short-term reproduction screen. As demonstrated, both vitellogenin (VTG) and secondary sexual characteristics (SSC) endpoints for this assay are well accepted, both within EPA and the OECD. Both peer review and public comments questioned the interpretation of the fish fecundity endpoint. As described, the Panel was concerned over the variability of results and possibility of a false positive scoring based upon alterations of fecundity by mechanisms other than those involving EAT activities. EPA should be alert to possible non-endocrine mediated refinements of the fish short-term reproduction assay to ensure the reductions in fecundity are truly representative of EAT mechanisms and not generalized toxicity. It should be recognized that the role of the fecundity assay is paramount for evaluations of the HPG axis. The number of assays targeting this assessment is weak.

It bears mention that *in vivo* screens will be much more costly and more difficult to repeat with confidence than *in vitro* screens. False positive findings could result in a substantial burden of expense and interpretation. The regulatory process also faces mandates to “reduce, replace and refine” protocols that consume test animals, yet *in vivo* screens could be fraught with errors that lead to unfortunate repetition. The Panel recommended that substances not be administered near the maximum tolerated dose (MTD), that appropriate negative controls be identified and utilized in the screens, and that confident results from *in vitro* screens be given primacy when conducting “weight of evidence” assessment of a battery of results.

An additional concern related to the handling of weak versus strong agonists and antagonists for each assay. As demonstrated, there is an inherent differential degree of sensitivities for each of the assays to weak agonists/antagonists. It was unclear to the Panel how these differences would be interpreted in relation to the “weight of evidence” approach for evaluating the assays.

Inclusion of the H295 cell assay may provide redundancy for the aromatase assay and significantly add to the breadth of the assays by inclusion of gene expression for aromatase and other CYPs/reductases in the pathways. However this will require additional resources and costs. This may be offset by potential benefits to evaluate additional mechanisms of aromatase gene transcription – a now well documented MOA resulting in altered steroid levels *in vitro* and *in vivo*.

## II. Androgen Assays

A similar grid showing the testosterone relationships was developed (see Figure 4 below). In the Tier 1 battery for androgens (e.g. testosterone), assays were divided into a set of responses. These response modalities included: 1) Receptor/Target Binding; 2) Cell Signaling; 3) Cell Response; 4) Enzyme Activity; 5) Organ Response; 6) Male System Integrity; 7) Female System Integrity; 8) Development (thyroid); and 9) Comparative System Integrity.

The Key at the Left Refers To the Numbers in Figure 2 (above) Which Indicates the Physiologic Process for Which Each Assay Was Assessed

TESTOSTERONE	Assay Type	1	2	3	4	5	6	7	8	9	10	11	Redundancy
Basis of Causation													
1.	Receptor/Target Binding	-	+	-	-	-	A	-	A	A	-	-	3
2.	Cell Signaling	-	-	-	-	-	A	-	A	A	-	-	2
3.	Cell Response	-	-	-	-	-	A	-	A	A	-	-	2
4.	Enzyme Activity	-	-	-	+	+	A	-	A	A	-	-	4
5.	Organ Response	-	-	-	-	-	+	-	+	+	+	+	4
6.	Male System Integrity	-	-	-	-	-	+	-	+	-	+	+	3
7.	Female System Integrity	-	-	-	-	-	-	-	-	+	+	+	2
8.	Development (thyroid)	-	-	-	-	-	-	-	-	-	+	+	1
9.	Comp System Integrity	-	-	-	-	-	-	-	-	-	+	+	1
	COMPLEMENTS	N A	0	N A	0	0	5	N A	5	5	4	4	
KEY													
1=ER Receptor Binding													
2=AR Receptor Binding													
3=ER Signal Transduction													
4=Aromatase													
5=H295R Cell line													
6=Hershberger													
7= Uterotropic													
8=Male Puberty													
9=Female Puberty													
10= amphibian metamorphosis assay													
11= Fish Screen													

Figure 4

For a test chemical with androgenic properties an androgen would register a positive response in the androgen receptor binding assay thus satisfying the general property of receptor/target binding. Androgens could also influence enzyme activity in the aromatase and H295R cell line assays. The aforementioned assays complete the complement of *in vitro* assays in the Tier 1 battery. Whereas the *in vitro* assays convey specificity and sensitivity, they represent standalone assays and do not provide complementary or redundant assessments of androgenic activities.

*In vivo* assays, such as the Hershberger assay, assess androgenic activities with assumed MOAs that mediate receptor/target binding, cell signaling, cell response and enzyme activity. As a measure of these responses, the apical end points in the Hershberger assay examine organ response and male system integrity. Androgens would not be expected to provoke a response in the *in vivo* uterotrophic assay.

Androgens will affect organ responses and male system integrity in the male pubertal assay, mediating these effects through receptor/target binding, cell responses, cell signaling and/or enzyme activities.

Although the amphibian metamorphosis assay targets effects on the thyroid hormone system, exposing both male and female frogs to an androgenic compound may influence their sex specific development in this assay through effects on organ responses, male and female system integrity, developmental changes and effects on comparative system integrity. Likewise, androgens may affect similar response measures in the female pubertal assay. EPA should be alert to these possible results when analyzing the data from the assays.

The comparative fish short-term reproduction assay would also be sensitive to the effects of an androgen. In this assay system, androgen would affect organ responses, male and female system integrity with the apical endpoints being manifest on development and comparative reproductive system integrity.

In summary, the *in vivo* assays included in the Tier 1 battery provide for both redundancy and complementarity for androgenic responses. Due to the complex, multiorgan, multi-parameter nature of the *in vivo* assays, the complementarity and redundancy will be essential toward weight of evidence decisions that determine whether test chemicals are recommended for Tier 2. False positives can be eliminated by weight of evidence decisions pertaining to positive results across a minimum of two or more *in vivo* assays. Based upon the redundancy and complementarity of assays, false negatives would be extremely rare and would be largely eliminated as demonstrated in the test grid. As indicated in the list of response measures, the mechanisms of action covered by the assays often may not be distinct, particularly in the *in vivo* assays, and therefore include a broad range of endpoints with redundancies that are necessary to discriminate positive and negative results.

### III. Thyroid Hormone Assays

There are differences in the regulation of thyroid function and thyroid hormone action, compared to that of sex steroids, which must be considered in answering this question (Zoeller et al., 2007). The HPT axis itself is quite similar to the HPG axis in its overall regulation; specifically, the hypothalamus controls the pituitary secretion of thyrotropin (TSH), which in turn controls the synthesis and secretion of thyroid hormones (T4 and T3, TH). However, TH circulates through the blood bound to specific binding proteins, is taken up into cells and tissues via specific transport proteins, and must be metabolized by deiodinases before interacting with the TH receptor (TR). Each of these points of regulation of thyroid hormone action may be a site of toxicant action. There are two TRs, TR $\beta$  and TR $\alpha$ ; importantly, TR $\beta$  mediates the negative feedback effect on the hypothalamus and pituitary, whereas the TR $\alpha$  is expressed in a developmental and tissue-specific manner to mediate specific effects of TH on various developmental and physiological processes (Zoeller et al., 2007).

An important mechanism by which xenobiotics can affect the thyroid system is by activating liver enzymes to increase T4 clearance from serum and thereby causing a reduction in circulating levels of T4. The interpretation of this event as benign (i.e., "non specific") or adverse is a central issue of importance to the ability of the Tier 1 battery to minimize false negatives. To illustrate this, consider the work of Klaassen and Hood. (Klaassen and Hood, 2001). These investigators studied the effects of four microsomal enzyme inducers on the HPT



axis – Phenobarbital (PB), pregnenolone-16a-carbonitrile (PCN), 3-methylcholanthrene (3MC) and Aroclor 1254 (PCB). Each of these chemicals produced a highly significant dose-dependent decrease in serum total and free T4. However, only PB and PCN produced an increase in serum TSH with commensurate changes in thyroid gland histology and cell proliferation. In contrast, neither 3MC nor PCB exposure caused an increase in serum TSH or altered thyroid gland histology. These data demonstrate that a variety of microsomal enzyme inducers are capable of causing a reduction in serum T4, but not all of these are capable of causing a “compensatory” increase in serum TSH. This failure to increase TSH by PCBs is important because we know that PCBs can interfere with TH signaling in a variety of specific ways (Gauger et al., 2007; Langer et al., 2007; Meeker et al., 2007; Otake et al., 2007; Turyk et al., 2007; Bansal and Zoeller, 2008; Radikova et al., 2008; Yang et al., 2008). Thus, if EPA interprets a decrease in T4 in the absence of a concomitant increase in serum TSH (and attendant changes in thyroid histopathology) as a “non-specific effect”, false negatives will not be minimized.

There are three assays in Tier 1 that capture measures of thyroid disruption. These include the two pubertal assays and the amphibian metamorphosis assay (Figure 5). The interpretation of results in the pubertal assays will determine the degree to which the Tier 1 battery minimizes false negatives and false positives. However, it is currently not possible to estimate what these levels of false results might be, as illustrated below. For false negatives, consider the profile of effects shown by linuron where, T4 is reduced, but TSH is not increased and there is no change in thyroid histopathology, as described in the EPA’s integrated summary for the 15-day adult male assay. In this case, Linuron was considered by EPA as a “negative”. To determine whether this is a true negative, one would need a measure of thyroid hormone action in these animals. A particularly important case in point would be the situation of a pregnant animal (rodent or human) in which maternal serum T4 levels are reduced during the first trimester for humans or 17.5 days for rats. During this period, the fetus requires thyroid hormone, but cannot synthesize thyroid hormone itself. (Zoeller and Rovet, 2004) Therefore, the only source of TH is the mother. Thus, if measures are incorporated into a developmental study (Tier 2) such that this profile of effects of linuron could be evaluated, then it would be empirically determined whether this profile in the Tier 1 battery is a false negative or a true negative. This same logic holds for false positives.

Thyroid	Assay Type	1	2	3	4	5	6	7	8	9	10	11
Bases of Causation												
Hypothalamus		-	-	-	-	-	-	-	+	+	+	-
Pituitary		-	-	-	-	-	-	-	+	+	+	-
TH synthesis/release		-	-	-	-	-	-	-	+	+	+	-
TH levels		-	-	-	-	-	-	-	+	+	+	-
Serum binding proteins		-	-	-	-	-	-	-			?	-
Tissue Responses		-	-	-	-	-	-	-	+	+	+	-
Liver metabolism		-	-	-	-	-	-	-				-
Complements		-	-	-	-	-	-	-	4	4	4	-

**KEY**

- 1=ER Receptor Binding
- 2=AR Receptor Binding
- 3=ER Signal Transduction
- 4=Aromatase
- 5=H295R Cell line
- 6=Hershberger
- 7= Uterotropic
- 8=Male Puberty
- 9=Female Puberty
- 10=amphibian metamorphosis assay
- 11= Fish Screen

Figure 5

Considering this, the AMA is essential to capturing endpoints of thyroid hormone action in Tier 1, but it must be recognized that there are differences in amphibian and mammalian physiology that could produce a situation in which the AMA does not complement the two pubertal assays.

The Panel's recommendations are:

1. The 15 day adult male assay does not serve to replace the amphibian metamorphosis assay (AMA) and the pubertal assays. Differences in metabolism between males and females are important to capture. The AMA represents the only endpoints of TH action in the Tier 1 battery.
2. A significant effect of test substance on serum T4 must be considered a positive finding, whether or not serum TSH is affected, and whether or not the AMA is positive.

#### IV. General Recommendations/Goals for Future Research.

While a different combination or a revised set of Tier 1 assays would likely result in significant improvements in detecting EDCs, the proposed set of Tier 1 assays are an appropriate starting point to detect EDCs based on the current state of the science. The EPA should consider this set of assays to be a work in progress. The Panel expects that the EPA will continue to develop, refine, and review the battery. For example, the Panel was concerned about the apparent paucity of *in vitro* assays and the abundance of *in vivo* assays. Would a different combination of validated assays be more effective in achieving the purpose of the battery than that proposed by EPA? In its response to this question, the Panel took the opportunity to suggest how the EPA might modify existing assays, complete the validation of others and develop and validate new assays. To briefly recap, there were suggestions for improved pubertal assays, the inclusion of developmental/organizational assays, more specific thyroid assays, the addition of more negative control data, and a broader array of signal transduction assays as well as new assays for emerging endocrine disruptive substances.

A. The Panel discussed the potential for false positives, especially the use of near MTDs. At the MTD, many of the animal's normal defenses are saturated and therefore the overall health of the animal is compromised. Under these conditions, it is difficult to determine whether any positive effects seen would be truly endocrine disruption or merely overall general toxicity, and whether the data have been confounded by the overall impact on multiple physiological systems.

In light of these concerns about the integrity of the biochemical and physiological conditions of the animals, caution is urged in the interpretation of endocrine effects observed only at the MTD. EPA indicated that production of false positives depends on responses at lower doses and that doses at 0.25 or 0.5 MTD are generally used. For chemicals with unknown toxicity, a preliminary range-finding test may be useful.

B. The potential for false negatives was also discussed, and centered around two areas of concern. First, a single rat strain (Sprague-Dawley) is recommended for the Tier 1 battery validation and the future Tier 1 and Tier 2 screens. The EPA defends the choice of rat strain based on their previous experience in assay validation, and the lack of time before the screens are mandated to begin. However, there is substantial evidence for the effect of genetic background on sensitivity to hormones and EDCs alike (reviewed in Spearow and Barkley, 2001, for a recent example see Thigpen et al 2007), and therefore choice of animal strains remains a highly valid concern. For that matter, only one fish species and one amphibian (and a non-native species as well) were chosen to include in the battery. The Panel recommended that the EPA, along with other Federal funding agencies such as the NIH or NSF, support research on determining the genetic basis for strain differences in hormone (and EDC) sensitivity. This investment would be of great value to the EPA's goal of minimizing false negatives in EDC screens as well as to the basic science community interested in hormone and endocrine disruptor modes of action.

Second, working at or near the MTD level of dosing may also mask the effects of chronic, low dose effects of EDCs, or miss them altogether. Concern was raised that a chemical that scores as a positive in acute toxicity may be disregarded as having an EAT mode of action that would only be revealed at far lower doses. Low dose effects of one EDC, bisphenol A, which is a reference chemical for Tier 1 validation, is reviewed in vom Saal and Hughes (2005).

C. Many of the compounds proposed for the initial battery already have an extensive data base. Some of the existing data would have addressed endpoints involving the action of the endocrine system. Therefore, it would be important to evaluate the data available, and to use existing data that are of high quality in the ultimate overall weight of evidence analysis of the compound. Use of existing high quality and relevant data might prevent the unnecessary repetition of tests, and would reduce the number of animals required for this program.

D. Tests for disruption of organizational effects of hormones, not just expression of hormone action in the peripubertal or adult animal, were also recommended by several Panel members. The *in utero* to lactational assay was suggested as an example. EPA indicated that it was evaluated but it was deemed too complicated, too long, and too expensive to include in a Tier 1 battery. EPA indicated that rodent *in utero* to lactational tests are in process of validation for Tier 2 screens.

E. The Panel re-emphasized the importance of development of transactivation assays for AR, ER $\beta$  and TR that will add redundancy via an *in vitro* component but also provide a means for adding a degree of specificity and potential MOA for a given chemical. Specific suggestions were provided in previous sections.

F. Improve standardization of all assays because there appears to be an inherent lack of standardization based upon the absence of inter-laboratory comparisons for some assays.

G. Interpretations of the amphibian and fish short-term reproduction assays need to consider specific metabolic differences between mammalian and non-mammalian species. For example, fish, frogs and higher vertebrates have some significant differences in steroid synthesis and metabolism. These differences are also receptor specific and underscore the problems of using the same criteria for adjudicating the potential risks of substances for higher and lower vertebrate systems.

H. Inclusion of the H295 cells may provide redundancy with the aromatase yet significantly add to the breadth of the assays by inclusion of gene expression for aromatase and other CYPs/reductases in the pathway.

I. Validation of gene expression assays using quantitative PCR would significantly impact several of the *in vivo* assays. For example, the thyroid hormone receptor  $\beta$  and TH/ZIP genes are strongly and specifically thyroid hormone regulated in *Xenopus laevis* tadpoles (Yaoita, et al 1990, Brown et al, 1996, Furlow and Brown, 1999, Opitz et al, 2006). Such an assay would be more quantifiable and less subjective than visual inspection and assignment of developmental stages in the amphibian metamorphosis assay. Furthermore, identification of specific, diagnostic molecular markers of thyroid hormone action in rodent peripheral tissues is lacking and should be pursued. The EPA reminded the Panel that cholesterol determination is included in the rat pubertal assays and was suggested as a possible marker of thyroid hormone actions on the liver. Altered serum cholesterol levels may not be specific enough to serve as a diagnostic marker for disruption of peripheral thyroid hormone action, however. Heart myosin heavy chain isoform expression (i.e. switching from MHC-alpha to MHC-beta expression indicates reduced thyroid hormone activity in the heart) or altered Spot 14 or Deiodinase Type I expression in the liver are potential targets for assay development in rodents (for a recent example see Shi et al, 2008).

In addition, liver enzyme gene expression assays would also aid in the determination of MOAs for disruption of estrogen, androgen, and thyroid hormone systems. Recent progress has been reported in development of xenobiotic response signatures in the liver by microarray analysis (Natsoulis et al, 2008, Zidek et al, 2007). Validation of panels of PCR primers for specific detection of steroid and thyroid hormone metabolizing enzymes may be an important avenue to consider. Such an assay would be more specific than detection of a general xenobiotic response that would be almost certainly generated by chemical exposure at or near the MTD, or measuring circulating hepatic enzymes as a marker of general hepatotoxicity.

J. Finally, the EPA needs to be aware that new EDCs will continue to be identified and will require the development of new EAT (and possibly glucocorticoid and PPAR) assays. Recent evidence points to the potential role of mitogen-activated protein kinases (MAPKs) pathways in the nuclear receptor mediated signal augmentation for certain EDCs (Jansen et al. 2004). The prolonged half-life of nuclear receptors, the recruitment of novel coactivators as well as the involvement of a secondary binding domain in nuclear receptors may also contribute to the signal potentiation phenomenon of trichlorocarbon (TCC) (Syms et al., 1985; Gregory et al., 2001;

Heinlein and Chang, 2002; Chang and McDonnell, 2005; Wang et al., 2006). Clearly, comprehensive investigations are required in order to identify the potential mechanisms for TCC. Chen et al. (2008) has identified TCC as a possible environmental hazard that could act through such mechanisms. These new findings will require that the current Tier 1 battery be modified as new assays are developed and validated.



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