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

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

MEMORANDUM

DATE: October 19, 2010

SUBJECT: Transmittal of the Meeting Minutes of the FIFRA SAP Meeting Held July 23, 2010 on "Scientific Issues associated with the Comparative Adult and Juvenile Sensitivity Toxicity Protocols for Pyrethroids"

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

FROM: Sharlene Matten, Ph.D.
Designated Federal Official
FIFRA SAP Staff
Office of Science Coordination and Policy

THRU: Laura Bailey
Executive Secretary, FIFRA SAP
Office of Science Coordination and Policy

Frank Sanders
Director
Office of Science Coordination and Policy

Please find attached to this memorandum the meeting minutes of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) open meeting held in Arlington, Virginia on July 20-22, 2010. This report addresses a set of scientific issues associated with "The Comparative Adult and Juvenile Sensitivity Toxicity Protocols for Pyrethroids."

Attachment

cc:

EPA

Stephen Owens (OCSPP)
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Lois Rossi
Anna Lowit, Ph.D.
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SAP Minutes No. 2010-05

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

Comparative Adult and Juvenile Sensitivity Toxicity Protocols for Pyrethroids

July 23, 2010
FIFRA Scientific Advisory Panel Meeting
held at
One Potomac Yard
Arlington, Virginia

NOTICE

These meeting minutes have been written as part of the activities of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Scientific Advisory Panel (SAP). The meeting minutes represent the views and recommendations of the FIFRA SAP, not the United States Environmental Protection Agency (Agency). The content of the meeting minutes does not represent information approved 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 (EPA), 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 Sharlene R. Matten, Ph.D., SAP Designated Federal Official, via e-mail at matten.sharlene@epa.gov.

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

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Comparative Adult and Juvenile Sensitivity Toxicity Protocols for Pyrethroids

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FIFRA Scientific Advisory Panel Meeting
held at
One Potomac Yard
Arlington, Virginia

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

Date: 10/19/2010

Sharlene R. Matten, Ph.D.
Designated Federal Official
FIFRA Scientific Advisory Panel
Staff

Date: 10/19/2010.

Panel Members for the Meeting of the Federal Insecticide, Fungicide and Rodenticide Act Scientific Advisory Panel (FIFRA SAP) to consider and review Comparative Adult and Juvenile Sensitivity Toxicity Protocols for Pyrethroids

July 23, 2010

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INTRODUCTION

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) has completed its review of the Agency's analysis of **Comparative Adult and Juvenile Sensitivity Toxicity Protocols for Pyrethroids.** Advance notice of the SAP meeting was published in the *Federal Register* on **April 30, 2010**. The review was conducted in an open Panel meeting on **July 23, 2010** at One Potomac Yard, Arlington, Virginia. Materials for this meeting are available in the Office of Pesticide Programs (OPP) public docket or via Regulations.gov, Docket No. EPA-HQ-OPP-2010-0378. Steven Heeringa, Ph.D., chaired the meeting. Sharlene Matten, Ph.D., served as the Designated Federal Official. Stephen Bradbury, Ph.D., Director, Office of Pesticide Programs (OPP), and Tina Levine, Ph.D., Director, Health Effects Division, OPP provided opening remarks at the meeting. Presentations of technical background materials were provided by Edward Scollon, Ph.D., OPP, and the Pyrethroid and Pyrethrin Technical Working Group (PPTWG).

Synthetic pyrethroids and naturally occurring pyrethrins[1] have seen increased usage over the past decade as replacements for organophosphate and *N*–methyl carbamate insecticides. At the present time, OPP is actively evaluating the human health risks associated with pyrethroids through its registration review program (http://www.epa.gov/oppsrrd1/reevaluation/pyrethroids-pyrethrins.html). In addition, OPP is assessing pending new uses of pyrethroids requested by pesticide registrants through the Pesticide Registration Improvement Act (PRIA).

The Agency has proposed that pyrethroids share the same mode of action (MOA), namely the ability to interact with voltage-gated sodium channels (VGSCs) ultimately leading to neurotoxicity (USEPA 2009). In June 2009, the SAP met to evaluate the Agency's preliminary conclusions which were provided in the document, "Draft Science Policy Paper: Common Mechanism Grouping (CMG) for Pyrethrins and Synthetic Pyrethroid Pesticides." The panel members agreed with the Agency that pyrethroid insecticides share the VGSC as a common molecular target site. Pyrethroids modify the sodium channel kinetics, resulting in a delayed channel closing and altered nerve cell transmission ultimately leading to fine tremors or choreoathetosis and salivation. Furthermore, the Agency proposed subdividing the pyrethroids into two subgroups, Type I and Type II, largely based on structure (the absence or presence of aα-cyano moiety) and distinct toxicity syndromes. The Panel agreed with the Agency that there was sufficient scientific evidence to subgroup the pyrethroids into Type I and Type II based on structure, the nature and extent of sodium channel modification, ability to interact with calcium and chloride voltage gated channels, and behavioral manifestations at high doses (SAP 2009, USEPA 2010). However, there were a few pyrethroids that did not fit neatly into either subgroup due to intermediate effects and were considered "mixed" pyrethroids.

As part of the pesticide registration process, numerous guidelines studies[2] evaluating the exposure and toxicology of pyrethroids have been conducted and submitted to EPA for review.

[1] This document applies to the naturally occurring as well as synthetic pyrethroids. For ease of discussion, herein, the naturally occurring pyrethrins and the synthetic pyrethroids will be called 'pyrethroids' collectively.
[2] 40 CFR Part 158 Toxicology Data Requirements for Conventional Pesticides. http://www.epa.gov/lawsregs/search/40cfr.html

Among these registration studies, experimental laboratory studies using a variety of durations (acute to chronic), routes (oral, dermal, inhalation), species (rat, rabbit, mouse, dog), and lifestages (juvenile, adult) are available for virtually all of these pesticides registered in the U.S., and are of particular interest for considering the potential for age-dependent sensitivity. In addition, six developmental neurotoxicity (DNT) studies in rats also are available for evaluation.

EPA recently has reviewed these DNT studies (*i.e.*, deltamethrin, bifenthrin, esfenvalerate, beta- and lambda-cyfluthrin, fenpropathrin) and concluded that they did not provide consistent evidence of neurotoxicity or show any increased juvenile sensitivity. Furthermore, they did not contribute significantly to the selection of points of departures (PoD) as part of risk characterization of these chemicals (USEPA 2010). Based on this review, the Agency believed that the results of these six studies could be applied to other members of the class and that no additional DNT studies need to be conducted for the other pyrethroids.[3] This conclusion, however, does not alleviate concern for potentially increased sensitivity to juveniles, particularly from high dose post-natal exposure, as reported in the scientific literature (Shafer *et al.* 2005, USEPA 2010).

Through decades of research, much is known about the exposure, MOA, and toxicological profiles of pyrethroid insecticides (*e.g.*, Soderlund *et al.* 2002, Shafer *et al.* 2005, USEPA 2009). However, even with the robust scientific literature on pyrethroids, gaps in knowledge exist in understanding the potential for postnatal age-dependent sensitivity. In order to assess this potential, the Agency solicited proposals[4] from the pesticide registrants to evaluate differential sensitivity between juveniles and adults. The Agency stated that it would consider protocols that included data from *in vivo*, *in vitro*, and/or *in silico* studies (or combinations thereof).

The Agency received only one proposal, a joint proposal from the PPTWG on May 21, 2010. The PPTWG is a consortium of 24 registrants who hold almost all of the U.S. registrations for pyrethrins and the synthetic pyrethroid insecticides. The Working Group proposal consisted of three areas or "study blocks" to address the data gaps: Block 1 – Further studies of possible age-related sensitivity in the rat, Block 2 – Experimental determination of parameters for PBPK models, and Block 3 – Age-dependent *in vitro* metabolism of pyrethroids in human liver tissue. The one day SAP meeting focused on specific aspects of the PPTWG proposal that were most critical to the PPTWG commencing their experiments. These were the *in vitro* experimental studies to inform the PBPK models, development of the PBPK models, and a review of the use of the acoustic startle reflex as a measure of neurotoxicity potential. The charge questions for this meeting were organized into two broad areas: 1) behavior and 2) PBPK model development.

The Agency expects to follow up this SAP meeting with another SAP meeting in 2011/2012 that will focus on issues not addressed at this meeting, such as, the ontogeny of sodium channels. At that time, the PPTWG will provide the Panel and the public with an update on its research efforts.

[3] http://www.epa.gov/oppsrrd1/reevaluation/pryethroids-pyrethrins.html
[4] Letter from T. Levine, February 16, 2010; http://www.epa.gov/oppsrrd1/reevaluation/pyrethroids-pyrethroids.html

PUBLIC COMMENTERS

Oral statements were presented by:

On behalf of the PPTWG:
Thomas Osimitz, Ph.D.; Chair, PPTWG
Larry Sheets, Ph.D., Bayer Crop Science
David Kim, Ph.D., Syngenta
Ronald Hines, Ph.D., Medical College of Wisconsin

Written statements were provided by:

1. J. TerBush, private citizen
2. Thomas Osimitz, Ph.D.; Chair, PPTWG

SUMMARY OF PANEL DISCUSSION AND RECOMMENDATIONS

1.0 Auditory Startle Response or Acoustic Startle Reflex (ASR)

1.1 Auditory Startle Response to Assess Age-Dependent Sensitivity to Pyrethroids

The PPTWG is proposing that the ASR provides a robust and sensitive measure of neurotoxicity and is well suited to assess age-dependent sensitivity to pyrethroids. Please comment on the appropriateness of the ASR technique as a measure of pyrethroid induced toxicity, including suggestions to assure quality of the study design (i.e., appropriate time-to-peak response, variability of peak response, *etc.*) and resulting data.

The Panel concluded that the acoustic startle response is not the best measure for juvenile sensitivity to pyrethroids because the response itself varies with age. Therefore, it would be difficult to discern if any observed change was related to toxicity, variability, or maturity. The Panel stated that the use of an acute dosing paradigm and the ASR as an endpoint to assess peak-time of exposure effects and age-dependent sensitivity to pyrethroids was not sufficient to address the concerns of age-dependent vulnerability. The Panel raised concerns that a peak time for chemical concentration within the brain can differ across ages and thus, makes comparable assessments difficult. The Panel identified problems regarding the use of a neurobehavioral endpoint as an acute response to exposure to determine relative potency of compounds across ages. Making such comparisons across systems at very different levels of maturation does not take into consideration the changing dynamics of the integrated sensory/motor systems in childhood development. These limitations would be present in any neurobehavioral endpoint. Additionally, the Panel was concerned with relying on a response to an acute exposure as a surrogate for an "effect" on the nervous system.

1.2 Age-Dependent Toxicity – Choice of Rat Life Stage to Conduct ASR Studies

The PPTWG has proposed to use conduct ASR studies on 21-day old rats. Please comment on the appropriateness of this age group in regards to *i*) assessing age-dependent toxicity and *ii*) assessing whether the 21-day old rat will adequately inform the Agency regarding toxicity as it relates to children three years of age and younger.

The Panel concluded that the inability to determine a consistent response among different age groups, and most importantly, in a potentially more sensitive age group, makes the utility of the ASR, or any integrated neurobehavioral endpoint, in this context problematic. There was general agreement among the Panel that trying to identify a comparable age for each stage of brain development in the human and the rodent (rat) would be difficult. Measurements of such reflex oriented responses as the ASR require not only an integration of the cellular circuitry of the sensory system, but also the development and maturation of the motor system. While the rodent brain undergoes similar developmental events as those occurring in the human, the process is significantly accelerated and can be difficult to definitively map

(Rice and Barone 2000, Clancey *et al.* 2007). Based on the available published data and the expertise of the Panel members, the Panel agreed that at weaning, the 21-day old rat would not serve as an equivalent model for determining the susceptibility of the human at an age of concern for the Agency, *i.e.*, a toddler between the ages of 1-3 years. Given the differential pattern of development across species, the majority of Panel members suggested that it was imperative to know the developmental ontogeny of any specific functional process prior to using data to assess age-dependent toxicity. For the pyrethroids, this would include information on the ontogeny of sodium channels.

2.0 Physiologically-Based Pharmacokinetic (PBPK) Modeling

2.1 Proposed Modifications to the Tornero-Velez *et al.* PBPK Model

Please comment on the proposed modifications to the Tornero-Velez *et al.* (2010) model as described in sections 4.3 of the PPTWG proposal. Please include in your comments consideration for balancing potentially improved performance resulting from the increased complexity with model parsimony.

The Panel generally supported the proposed efforts to further develop and refine the Tornero-Velez *et al.* family of pyrethroid PBPK models with the aid of new measurements of partition coefficients and protein binding. In general, the Panel indicated that obtaining values for age-specific, chemical-specific parameters in a PBPK model as a function of lipophilicity, steric bulk, electrostatic interactions, polarity, hydrogen bonding, and ionization state (*e.g.*, addressed in Zhang 2005) and tissue descriptors (tissue weight fractions for lipid, protein, and water) (Balaz *et al.* 1999) will aid in reducing uncertainty in these parameters. The Panel recommended that experiments focus on those parameters to which the relevant model responses (*e.g.*, brain concentrations) are most sensitive, and also explore the relative rates of metabolism in tissues. The Panel was split about the prospective usefulness of the proposed Ussing Chamber techniques and human Caco-2 cells. The Panel stated that the six pyrethroids proposed by PPTWG for study were too limited to be representative of all the pyrethroids in this class.

2.2 Microsomal Incubation Studies

Please comment on the strengths and weaknesses of the PPTWG proposal to use hepatocytes in the PBPK effort, including the potential for hepatocytes to decrease uncertainty of model predictions in light of limited data.

The Panel expressed enthusiasm for both the use of hepatocytes and hepatic microsomes in metabolic studies to inform the PBPK model. *In vitro* to *in vivo* extrapolations of data using hepatocytes are considered superior to extrapolations using microsomes. The Panel stated that there was some limitation in the availability of hepatocyte samples particularly within pediatric age groups, but not adult age groups, which might constrain the ability of the measurement program to appropriately represent the full diversity of the human population. However, microsome availability is not as limited as hepatocyte availability.

Considerations that favor the use of hepatocytes included: 1) measurements that help assure that the enzyme activities observed will reasonably reflect *in vivo* conditions and 2) cytosolic, Phase II enzymes, as well as directly oxidative Phase I microsomal enzymes. The Panel commented that the data from *in vitro* hepatocyte experiments should be related to the corresponding whole liver function by considering heterogeneities in the liver (*e.g.*, Andersen *et al.* 1998, Allen *et al.* 2005). Some panelists were concerned with PPTWG's proposal to pool hepatocytes before measuring enzyme activities. These panelists stated that such pooling would obscure any information which may be obtained on human inter-individual variability among the pooled samples.

Panel members noted that there is considerable experience with the use of microsomes for measurements of *CYP* enzyme activities, but the broad applicability of scaling this approach to clearance for other enzyme families (including the carboxylesterases) is far less well established. Enantiomers can also be evaluated easily with both microsomal and hepatocyte systems. With regard to extrapolation from adult to juvenile or other early life stages, another approach would be to model the infant response based on adult data. Simcyp Limited and Simulation Plus, Inc., for example, provide simulation and modeling software that can model the metabolism of "virtual" children based on the input of "adult" parameters. Panel members offered a number of recommendations with regard to future microsomal studies.

3.0 Alternative Study Design(s) For Evaluating Age Differences in Pharmacokinetics

Are there alternative approaches using empirical or data generation techniques potentially requiring less time than the PBPK effort proposed by the PPTWG for evaluating the potential for post-natal sensitivity, particularly with respect to differences in pharmacokinetic profiles, that could be used by the Agency?

The Panel did not recommend any specific approach to evaluate the potential for post-natal sensitivity, particularly with respect to differences in pharmacokinetic profiles. The Panel agreed that development of the PBPK model to facilitate the animal-to-human extrapolation was very important and should be continued. While these efforts are ongoing, the Panel identified several changes/alternatives in the current approach to develop an *in silico/ in vitro/ in vivo* (SVV) PBPK model that could ultimately be used to predict the pharmacokinetics of pyrethroids in mammals, particularly in humans. These included: 1) data-mining efforts to target testing, 2) *in vitro* approaches, *e.g.*, cassette dosing, 3) pharmacokinetic and pharmacodynamic considerations for *in vitro* studies, and 4) non-human primate (monkey) *in vivo* studies. Although these suggestions and alternatives may initially require some additional experiments, they may, in the long term, reduce the time necessary to develop the SVV model of the pharmacokinetics of pyrethroids in humans and enable a more feasible and accurate means of determining the viability of the PBPK model.

DETAILED RESPONSES TO CHARGE QUESTIONS
(Note: Information in italics is verbatim of what is written in the Agency transmittal memorandum dated, June 21, 2010.)

1.0 Auditory Startle Response or Acoustic Startle Reflex (ASR)

1.1 Auditory Startle Response to Assess Age-Dependent Sensitivity to Pyrethroids

Measurement of the auditory startle reflex response is a commonly used technique to assess neurobehavioral effects in rats. Auditory startle reflex is a motor reflex characterized by a sequence of reflexive muscle movements elicited by sudden and intense acoustic stimuli measured by a change in motor output. The proposed reflex path is short, consisting of the auditory nerve, posteroventral cochlear nucleus, the nucleus reticularis pontis caudalis, and motor neurons in the spinal cord (Davis et al. 1982). This mechanism is susceptible to a variety of drugs and toxicants making the reflex a useful model of sensorimotor reactivity across animal taxa, including rat and human (Lee et al. 1996). With regard to pyrethroids, auditory startle data in adult rats have demonstrated differing response patterns related to pyrethroid structure (Crofton and Reiter 1984; Tilson et al. 1985; Crofton and Reiter 1988; Hijzen et al. 1988; Hijzen and Slangen 1988); Type I pyrethroids produced an increase in startle amplitude and Type II pyrethroids produced a decrease in startle amplitude. In addition, ASR has been used to demonstrate age-dependent toxicity in rats following high oral doses of pyrethroids (Sheets et al. 1994; Sheets 2000). Therefore, ASR is a potentially sensitive measure to evaluate differences in neurobehavioral effects between adults and pups.

Since ASR is a behavioral measurement, it is important to consider development, dose-response and variability during interpretation of the results. In rats, the onset of ASR response corresponds to the development of the external auditory meatus. In the rat, this usually occurs between 13 and 16 days of age. Sheets et al. (1988) have shown the ability of rats to respond to ASR as early as PND 13; however, the amplitude of response continued to increase through PND 21. Pyrethroids modify the voltage gated sodium channels in the central nervous system and therefore the brain is considered the major target organ for toxicity. Kim et al. (2010) determined the distribution of deltamethrin, a Type II pyrethroid, in brain, fat, liver, plasma, and muscle in PND 10, 21, 40, and 90 rats for up to 510 hours following dosing. Brain concentrations in PND 10 pups were elevated for a longer time relative to the adults. This suggests that pyrethroid kinetics in the brain of pups may not mirror those of adult rats. The Kim et al. (2010) study emphasizes the importance of determining the appropriate time course of effects (i.e., time-to-peak-effect and/or time-to-tissue-recovery) in both adult and non-adult lifestages prior to measuring ASR responses. Additionally, the standard deviation for peak amplitude, the ASR measure proposed by the PPTWG, can vary greatly in guideline DNT studies (20-125%) and literature reviews (Raffaele et al. 2004). However, this variability can be reduced down to 20-30% if the studies are conducted in proven laboratories (Sette et al. 2004).

The PPTWG is proposing that the ASR provides a robust and sensitive measure of neurotoxicity and is well suited to assess age-dependent sensitivity to pyrethroids.

Please comment on the appropriateness of the ASR technique as a measure of pyrethroid induced toxicity, including suggestions to assure quality of the study design (*i.e.*, appropriate time-to-peak response, variability of peak response, *etc.*) and resulting data.

Panel Response

The Panel concluded that the acoustic startle response is not the best measure for juvenile sensitivity to pyrethroids because the response itself varies with age. Therefore, it would be difficult to discern if any observed change was related to toxicity, variability, or maturity. The overall conclusion of the Panel was that the use of an acute dosing paradigm and the ASR as an endpoint to assess peak-time of exposure effects and age-dependent sensitivity to pyrethroids was not sufficient to address the concerns of age-dependent vulnerability. The Panel identified problems regarding the use of a neurobehavioral endpoint as an acute response to exposure to determine relative potency of compounds across ages. Making such comparisons across systems at very different levels of maturation does not take into consideration the changing dynamics of the integrated sensory/motor systems in childhood development. These limitations would be present in any neurobehavioral endpoint. Additionally, the Panel was concerned with relying on a response to an acute exposure as a surrogate for an "effect" on the nervous system.

According to the EPA/OECD DNT testing guidelines, exposure is to occur during a prolonged developmental window under a general assumption that the broader age window would suffice to cover various developmental windows of vulnerability. The Agency noted in its background document that numerous toxicity testing studies, including DNT studies and two-generation reproductive toxicity studies, have not detected a differential sensitivity following *in utero* or pre-weaning exposure to the pesticide class under question. Furthermore, the Agency's recent review (Scollon 2010) suggested that data developed under GLPs following the current DNT guideline and submitted by the registrants demonstrate a high level of variability. This raises the question of the experimental quality of the datasets and their utility for adequately evaluating developmental neurotoxicity. In the review by Shafer *et al.* (2005), independent published studies and unpublished data examining the effect of pyrethroid exposure during various developmental windows suggested an elevated concern for neurological effects. Moreover, the recent work by Kim *et al.* (2010) reported severe clinical signs of toxicity, including death, in 10-day old rats orally dosed with 2 mg deltamethrin/kg, with a decrease in severity observed with increasing age, *i.e.*, PND 21, 40, or 90, at the time of dosing. Further work demonstrated that a higher 4 mg/kg dose of deltamethrin produced higher brain levels at PND 21 as compared to PND 72; yet, a 50% deficit in ASR was at a maximum for both age groups. In the human population, a higher level of pyrethroid exposure is observed in toddlers due primarily to increased hand-to-mouth activity. The Panel commented that the Agency was justified in its concern for early childhood exposure which led to its publically soliciting proposals that would evaluate the potential for differential sensitivity between juveniles and adults.

The Panel discussed the level of documentation supporting the Agency's request for proposals concerning study designs and protocols to evaluate potential differential sensitivity of

juveniles and adults to pyrethroid insecticides and the proposal submitted by the PPTWG in response to the Agency's solicitation. The Panel was in agreement that the written materials provided by the Agency and the PPTWG were sparse in details regarding the PPTWG proposal and the overall goals of the Agency solicitation. This lack of detail created difficulty for the Panel to review the PPTWG proposal and to provide a focused response to the Agency's charge questions. While the Panel concluded that the ASR has many excellent traits for evaluating neurotoxicity in the adult, the Panel was concerned about the high level of variability in the data submitted by the pesticide registrants to fulfill the developmental neurotoxicity testing requirement. In a review provided by the Agency, it was mentioned that this variability decreased with a greater level of experience and expertise in the testing lab (Sette *et al.* 2004). The Panel agreed that testing for ASR requires expertise and optimization of experimental procedures that may be lacking in many of the pesticide registrant studies. A number of points raised by the Panel were related to the details of the testing conditions as a general measure to decrease variability in the ASR data. The Panel provided a number of suggestions to improve the quality of ASR data and to decrease variability, in general. These are summarized in **Appendix 1**. These suggestions relate to the ASR testing within a specific age group and are not presented as an endorsement of the proposed study plans for comparison between young and adult animals.

The Panel found the scientific/biological logic behind the PPTWG proposal to be less than optimal. While a peak time-to-effect approach has been successful for pesticides displaying cholinesterase inhibition as a mode of action, equating ASR as an acute biomarker of effect lacks a scientific basis. The Panel also was concerned with the testing of only a limited number of compounds and with the logic of dose selection. The PPTWG proposes to develop data for only six of the nearly two dozen or so pyrethroids currently registered in the U.S. The rationale for their selection of the four Type I, one Type II and one mixed Type pyrethroids included the observation that these were the ones to which young children were more likely to be exposed, based upon existing indoor use patterns. The Panel indicated that the proposed number and distribution across types of substances to be tested will not be adequate to provide credible answers for the class of pyrethroids as a whole. The Panel recommended that in addition to more Type I and Type II pyrethroids, all compounds of an identified mixed type (*i.e.*, esfenvalerate, in addition to fenpropathrin) should be evaluated in any comparison. The proposal submitted by the PPTWG would evaluate a peak-time to overt toxicity in the young and adult rats. At peak-time, changes in the ASR would be evaluated at lower dose levels. A number of members of the Panel expressed concern that examination of a behavior occurring at a specific time following acute exposure, whether a response observed at higher dose levels or at peak concentration of chemical in the brain remains a measurement of "acute response." In addition, the Panel was concerned that an adverse effect on the nervous system, especially during development, would not be reflected in a change in ASR, or any neurobehavioral endpoint, following a relatively short period of acute chemical exposure. The Panel emphasized that this approach would miss the potential for subclinical toxicity and effects on the nervous system that would become detectable only with complete maturation. The Panel noted that a similar problem would exist for multiple functional endpoints given the developmental differences of the brain and the ontogeny of the various neurobehavioral endpoints used within a DNT study.

A robust body of data is required to derive equivalent doses and produce a credible risk assessment under a cumulative risk assessment. The Panel viewed the PPTWG proposal as an ambitious testing program. Alternative approaches were presented by various panel members including examination of additional neurobehavioral endpoints; yet, any such neurobehavioral test requiring the integration of the nervous system would face the same limitations and concerns as mentioned for ASR, especially if used as a response to acute exposure. A number of the Panel members suggested various additional endpoints; however, they were based upon either more extended exposure or time following exposure to demonstrate a delay in the normal developmental pattern or a long-term perturbation. Alternative targets considered included motor activity and perinatal endocrine programming events such as delayed time to vaginal opening, as reported for esfenvalerate (Pine *et al.* 2008). However, these endpoints were measured in the DNT and in the two-generation reproductive toxicity studies submitted to the Agency. According to the Agency, these studies have provided no indication of an increased susceptibility from *in utero* and pre-weaning pyrethroid exposure.

The Panel suggested alternative approaches for consideration by the Agency in determining the potential for age-related susceptibility of the nervous system to pyrethroids. Any evaluation must be conducted when peak concentrations of the relevant chemical occur in the brain. Given the age-related differences in absorption and disposition processes during development, the peak concentration in the brain may occur at different times following a dose in different age groups and species. One suggestion was to consider endpoints other than neurobehavioral, given the limitations of examining an integrated system during development and comparing the response to the mature system. In this case, targeted studies could be performed on dose levels and/or at specific developmental windows to provide appropriate data for the Agency. Such endpoints could include morphological, molecular, or neurochemical developmentally-regulated events occurring in the brain. Also to be considered is the postnatal developmental curve for some of the xenobiotic metabolizing enzymes (Atterberry *et al.* 1997) and the impact this could have on endpoints of neurotoxicity. For example, data for deltamethrin indicated a 50% decrease in ASR amplitude in both PND 21 and PND 72 rats when exposed to the same dose, despite a higher brain concentration of the compound in the weanling rats.

1.2 Age-Dependent Toxicity – Choice of Rat Life Stage to Conduct ASR Studies

Age-dependent toxicity has been observed in rat studies following high doses (i.e., LD_{50} studies resulting in 50% mortality of test subjects) of Type II pyrethroids (Cantalamessa 1993; Sheets et al. 1994; Sheets 2000). However, in sublethal studies using the ASR as a measure of toxicity, ED_{50} (dose at which 50% of the test subjects are affected) values were similar between postnatal day (PND) 21 and adult rats.

These findings suggest that age-dependent toxicity may only be observed at high doses. Based on in vivo (Cantalamessa 1993) and in vitro (Anand et al. 2006) studies, the apparent discrepancy between high- and low-dose age-dependent toxicity is likely attributable to incomplete maturation of the enzymes that detoxify pyrethroids in immature animals, particularly the carboxylesterases and cytochrome P450s. These clearance mechanisms are overwhelmed in younger animals given LD50 doses, leading to increased accumulation of

the pyrethroids in nervous tissue and ultimately increased toxicity.

Carboxylesterases and P450 enzymes are the two major enzyme families responsible for metabolism of pyrethroids. In the rat, it has been shown that carboxylesterase activities are below adult levels at weaning (Moser et al. 1998; Karanth and Pope 2000; Anand et al. 2006; de Zwart et al. 2008; Yang et al. 2009). Information on the ontogeny of carboxylesterase development in the human is more limited. However, increased plasma esterase activity during postnatal maturation has been reported (Ecobichon and Stephens 1973). In contrast, Pope et al. (2005) found carboxylesterase activity in hepatic tissues was similar for humans ranging in age from 2 months to 36 years; however, the sample sizes were small and variability among the age groups was high. Maturation of the P450 enzymes shows a similar trend. 2C19, a CYP450 enzyme which has shown high pyrethroid metabolic activity (Godin et al. 2006), increases rapidly in the human during the first 2 years of life, whereas numerous P450s examined in the rat have minimal expression levels through gestation and do not approach adult levels of expression until PND10 days or later (de Zwart et al. 2008).

Comparisons between lifestages in the rat and human are difficult because of the ontogeny of the brain development and metabolizing enzymes are not an exact match. However, PND 11 rats are considered to be close in development to newborn humans and PND 17 rats are believed to be closer developmentally to human toddlers (Davision and Dobbing 1966; Dobbing and Smart 1974; Benjamins and McKhann 1981). From the aspect of exposure, previous experience with developing cumulative risk assessments for other insecticide groups, ongoing work on HED's Standard Operating Procedures for Residential Pesticide Exposure Assessment, and the Agency's Guidance on Selecting Age Groups for Monitoring and Assessing Childhood Exposures for Environmental Contaminants http://www.epa.gov/raf/publications/guidance-on-selecting-age-groups.htm, the Agency believes that children three years old and younger, particularly those who are mobile (crawling, walking) and who exhibit hand-to-mouth behavior, have the potential for the greatest exposure to pyrethroids. Based on 1) the current understanding that the two major enzyme families responsible for the metabolism of pyrethroids are below adult activity levels at weaning (i.e., PND 21); 2) PND 17 rats are approximately comparable to human toddlers in terms of development; and 3) children younger than 3 years of age are expected to have the greatest exposures to pyrethroids, the Agency is concerned with the PPTWG's proposal to conduct ASR studies in PND 21 rats. Instead, PND 15 to 17 rats, which have been shown to respond to ASR stimuli (Sheets et al. 1988), may better represent the most susceptible human lifestage.

The PPTWG has proposed to use conduct ASR studies on 21-day old rats. Please comment on the appropriateness of this age group in regards to *i*) assessing age-dependent toxicity and *ii*) assessing whether the 21-day old rat will adequately inform the Agency regarding toxicity as it relates to children three years of age and younger.

Panel Response

There was general agreement among the Panel members that trying to identify a comparable age for each stage of brain development in the human and the rodent (rat) would be difficult. What is critical is that the cellular and anatomical development of the brain and its circuitry are interdependent. Measurements of such reflex oriented responses as the ASR require not only an integration of the cellular circuitry of the sensory system, but also the development and maturation of the motor system. While the rodent brain undergoes similar developmental events as those occurring in the human, the process is significantly accelerated and can be difficult to definitively map (Rice and Barone 2000; Clancy *et al.* 2007). Based on the available published data and the expertise of the Panel members, the Panel agreed that at weaning, the 21-day old rat would not serve as an equivalent model for determining the susceptibility of the human at an age of concern for the Agency, a toddler between the ages of 1-3 years. Given the differential pattern of development across species, a majority of Panel members stated that it was imperative to know the developmental ontogeny of any specific functional process prior to using data to assess age-dependent toxicity. For the pyrethroids, this would include information on the ontogeny of sodium channels. Extrapolation from rodent to human on any developmentally-related biological endpoint requires a specific understanding of the developmental features of the endpoint under study.

With specific reference to the ASR, while a response to an auditory stimulus can be detected as early as PND 13 in the rat, the developmental onset of this response varies and matures over the subsequent 25-30 days. This occurs along with the maturation of the internal circuitry as well as maturation of the motor system. In the DNT guidelines (OPPTS 870.6300), the developmental landmarks include body weight, age of vaginal opening and preputial separation. No development landmarks are included in the published guidelines for neurological development such as onset of a startle response. The Panel recommended a re-evaluation of the available literature on pyrethroids to determine whether data exists showing a developmental delay in the startle response, or in other comparable endpoints.

A differential ASR to Type I and Type II pyrethroids has been demonstrated in the adult rat, with an increased response to Type I and a decreased response to Type II pyrethroids. In the developing rat, this differential response has not been reported. Given that the ASR is a reflex response dependent upon a motor response to a sensory stimulus, the ability to detect lower ASR in immature rats may be difficult due to the immaturity of the motor system. Thus, Type II specific effects may not be detected in the immature rat due to the normally low ASR amplitude and would be more difficult to detect with younger and younger ages. Moreover, given the neural circuitry involved in the ASR, changes detected in an immature animal may potentially have a different basis than responses in the adult. The Panel concluded that the inability to determine a consistent response among different age groups, and most importantly, in a more sensitive age group, makes the utility of the ASR, or any integrated neurobehavioral endpoint, in this context problematic.

2.0 Physiologically-Based Pharmacokinetic (PBPK) Modeling

The PPTWG is proposing to use a model developed collaboratively by EPA's Office of Research and Development (ORD) and the University of Georgia as a starting point in their modeling effort. EPA's ORD has published a series of papers that describe the development

and enhancement of pyrethroid PBPK models starting with a deltamethrin model in rats by Mirfazaelian et al. (2006) , improved by Godin et al.(2010), modified for permethrin by Tornero-Velez et al. (in prep.), and finally expanded to include age- and chemical-dependent parameters by Tornero-Velez et al. (2010). In 2007, ORD and OPP jointly presented an issue paper to the SAP (USEPA 2007) which described an approach for using a generic model structure with chemical specific parameters for pyrethroids. The "family modeling" approach was endorsed by the SAP and has been successfully applied in the above PBPK efforts. The Agency believes that it is both reasonable and scientifically sound to use the Tornero-Velez et al. (2010) PBPK model as the starting point for the PPTWG effort to build PBPK models for pyrethroids to assess young children. Furthermore, the PPTWG is proposing to develop PBPK models using in vitro and in vivo rat data, and then using human in vitro data to inform the model to predict human internal dosimetry, similar to the approach which was previously supported by the 2007 SAP.

2.1 Proposed Modifications to the Tornero-Velez *et al.* PBPK Model

The PPTWG proposes to increase the complexity of the Tornero-Velez et al. (2010) PBPK model by modifying some aspects. For example, the PPTWG is proposing to:

a) Predict intestinal permeability through the use of the Ussing Chamber technique with rat cells and human Caco-2 cells, with the potential to increase the number of compartments within the intestinal tract
b) Determine partition coefficients using in vitro techniques
c) Obtain estimates of protein binding

Please comment on the proposed modifications to the Tornero-Velez *et al.* (2010) model as described in sections 4.3 of the PPTWG proposal. Please include in your comments consideration for balancing potentially improved performance resulting from the increased complexity with model parsimony.

Panel Response

The Panel generally supported the proposed efforts to further develop and refine the Tornero-Velez *et al.* family of pyrethroid PBPK models with the aid of new measurements of partition coefficients and protein binding. In general, the Panel indicated that obtaining values for age-specific, chemical-specific parameters in a PBPK model as a function of lipophilicity, steric bulk, electrostatic interactions, polarity, hydrogen bonding, and ionization state (*e.g.*, Zhang 2005) and tissue descriptors (tissue weight fractions for lipid, protein, and water) (Balaz *et al.* 1999) will aid in reducing uncertainty in these parameters. The Panel recommended that experiments focus on those parameters to which the relevant model responses (*e.g.*, brain concentrations) are most sensitive, and also explore the relative rates of metabolism in tissues.

The Panel had a more mixed view of the prospective usefulness of the proposed Ussing Chamber techniques and human Caco-2 cells. Many panel members supported the use of the Caco-2 cell line because it has been widely used for many years by the pharmaceutical industry as a reliable screening tool to assess, in particular, paracellular and transporter-

mediated intestinal permeability. In addition, data obtained from this system generally correlates with absorption characteristics *in vivo*. Apparent permeability estimates from the Caco-2 system can provide information concerning the absorption potential of compounds across the gastrointestinal tract. Estimation of the apparent permeability of the different pyrethroids in this cell culture system will provide an opportunity to rank order these compounds according to their permeability, and hence, absorption potential *in vivo*. Calculation of an "efflux ratio" (the ratio of apparent permeability in the basal-to-apical direction to apparent permeability in the apical-to-basal direction) may provide information regarding the potential contribution of active uptake transporters or efflux transporters (Efflux ratio >2). There was also considerable support for using this system to explore the consequences of stereochemistry for possible differences in behavior of different pyrethroid isomers. With recent improvements, *i.e.*, the 3-day Caco-2 culture, very rapid screening of pyrethroids can occur with this system.

On the other hand, some of the Panel expressed skepticism with these *in vitro* absorption measurements using Caco-2 cells due to concerns about limited solubility of the pyrethroids in water, the need to explore multiple *in vitro* systems (Balimane *et al*. 2006, Cheng *et al.* 2008), and effects of extensive cell culturing on comparative *in vivo* functions. As with all cell lines maintained in culture for an extensive time, the comparative *in vivo* functions could be compromised and should be considered. However, the Panel noted that there are no data to indicate that this has happened in the Caco-2 cells. These panelists were also concerned that the Caco-2 cells may not reveal possible differences related to age because they are of adult origin. Other panel members did not agree with these concerns.

As noted above, many on the Panel were confident in the use of the Caco-2 system and its ability to accurately assess the permeability potential of a series of compounds when permeability is due to passive diffusion mechanisms. The Caco-2 system can identify active processes, but the transporter expression profile is not that similar to the *in vivo* situation. Although age-dependent differences in cell membrane composition may result in differences in the permeability coefficient, these are likely minor. The Caco-2 system can provide some valuable information on permeability (and hence, absorption) without having to resort to *in vivo* absorption determinations (this is very costly in terms of animal resources).

As an alternative to Caco-2 cells, some panel members suggested a preference for more extensive *in vivo* measurements of the detailed time course of pyrethroid transfer first within compartments of the gastrointestinal tract and then into the systemic circulation. Panel members also noted that extensive *in vivo* measurements would certainly be costly from an animal welfare perspective.

The Panel noted that the primary goal of the PBPK model is to assess the concentrations of the parent pyrethroid compounds in the brain because the parent compounds are the toxic moieties. The metabolites, as far as is known, do not contribute appreciably if at all to the neurotoxicity. In particular, the PBPK model discussed here is needed primarily for relating pyrethroid concentrations to differences between juvenile to adult sensitivities to their neurotoxic effects. The prediction of expected and observed biomonitoring results and reverse dosimetry are secondary applications for the model. Some Panel members suggested

that the priorities for measurements of individual compounds should be: 1) metabolism rates, 2) partition coefficients and 3) membrane transport across the intestinal barrier or the blood-brain-barrier. The Panel was in general agreement that partition coefficients should be measured *in vitro*. One panelist suggested that the model not be limited to those results as unchanging values, even for specific age and gender groups. This panelist indicated that partition coefficients should be varied within the model with time to recognize, for example, diurnal and postprandial changes in blood lipid content. In contrast, another panelist stated that by not fixing the values for partition coefficients and blood lipid content, and making these values change according to age and time of day would add a significant amount of complexity to the PBPK model and significantly reduce model parsimony. In addition, this panelist expressed concern about the proposed methods for measuring partition coefficients *in vitro* given the very limited solubility of the pyrethroids in water. Another Panel member noted that partition coefficient measurements should not be a problem even if pyrethroids were insoluble in water. Membrane transport or transport across the intestinal or blood-brain barriers using *in vitro* methods could be problematic because it could be difficult to get them in the cultures. Partition coefficients are often done with water insoluble substances as this is just a 'test tube' test.

Panelists also stated that the proposed number of pyrethroids to be included in the proposed experiments was too limited. An examination of six pyrethroids in detail, as proposed, might not be fully representative of the whole group of pyrethroids registered in the U.S. This limited series of proposed experiments might not even be extensive enough to adequately explore differences between Type I and Type II pyrethroids, as well as the consequences of possible "mixed types" not adequately encompassed within these two categories.

2.2 Microsomal Incubation Studies

Microsomal incubation studies have been used to inform the pyrethroid PBPK models developed by ORD (Mirfazaelian et al. 2006; Scollon et al. 2009; Godin et al. 2010; Tornero-Velez et al. 2010; Tornero-Velez in prep.). The PPTWG has proposed to use intact hepatocytes instead because they may provide a better prediction of metabolism compared to microcellular fractions (Hewitt et al. 2007). Additionally, the PPTWG is proposing to compare the clearance activity of human hepatocytes and microsomal fractions for several age groups. However, the Agency notes that there are a limited number of human hepatocyte samples available to inform the PBPK model. Pooled human microsomes are available representing larger segments of the population relative to hepatocyte availability. The Working Group suggests characterization of the hepatocytes (i.e., CYP450 and esterase composition) and establishing a relationship between hepatocytes and microsomal fraction activities to reduce model uncertainty in light of limited data.

Please comment on the strengths and weaknesses of the PPTWG proposal to use hepatocytes in the PBPK effort, including the potential for hepatocytes to decrease uncertainty of model predictions in light of limited data.

Panel Response

1) Hepatocytes

Panel members expressed considerable enthusiasm for the use of human hepatocytes. Considerations in favor of the use of hepatocytes included: 1) measurements to help assure that the enzyme activities observed will reasonably reflect *in vivo* conditions and 2) cytosolic, Phase II enzymes, as well as directly oxidative Phase I microsomal enzymes. Furthermore, studies have demonstrated that the collection (*i.e.*, time post-mortem), processing, and storage protocols for hepatocytes do not substantially alter metabolic enzyme activities compared to fresh *in vivo* hepatocytes (Guilbouzo *et al.* 1993). In addition, data gained from using hepatocytes will implicitly include transport properties (such as the ATP-binding cassette transporters), which may be very important for the disposition of pyrethroids, but have not yet been directly considered in the written application or verbal presentation of the proposed experiments.

While supporting the use of hepatocytes for enzyme activity measurements, one panelist observed that a weakness in using *in vitro* experimental data on hepatocytes is that the liver is not just a collection of hepatocytes. Additionally, there is spatial heterogeneity in several factors such as oxygen tension, receptor distribution, binding activity, glycolysis, etc. (Lamers *et al.* 1989). Therefore, this panelist observed that it would be desirable to focus efforts on relating data from *in vitro* hepatocyte experiments to corresponding whole liver function by considering heterogeneities in the liver (*e.g.*, Andersen *et al.* 1998, Allen *et al.* 2005). Some panelists were concerned with the PPTWG's proposal to pool hepatocytes before measuring enzyme activities. These panelists stated that such pooling would obscure any information, which may be obtained on human inter-individual variability among the pooled samples. The Panel discussed whether availability of hepatocyte samples within specific age groups would constrain the ability of the measurement program to appropriately represent the full diversity of the human population. Several companies were cited as examples of commercial suppliers of these preparations, *e.g.*, XenoTech, LLC. Puracyp, Inc., KaLy-Cell, Cellz Direct, BD Biosciences[5]. Panelists believed that commercial availability of adult hepatocytes is fairly constant since additional samples are continuously being collected and produced. However, the numbers across the various companies are limited to perhaps a couple of dozen individuals at any one time. The commercial availability of pediatric hepatocytes is far less than for adult hepatocytes, with current availability limited to less than 10 individuals. The Panel commented that it is expected that new samples will only be available from 6-10 individuals per year.

2) Microsomes

The Panel expressed significant support for the proposed microsomal studies, although *in vitro* to *in vivo* extrapolations are considered more superior with hepatocytes than with microsomes. However, microsome availability is not as limited as hepatocyte availability. Panel members noted that there is considerable experience with the use of microsomes for measurements of *CYP* enzyme activities, but the broad applicability of scaling this approach up to clearance for other enzyme families (including the carboxylesterases) is far less well

[5] Disclaimer: The Panel makes no endorsement of any company nor any product. Mention of any company or product is for illustration purposes only.

established. Enantiomers can also be easily evaluated with microsomal systems and hepatocyte systems. “Juvenile” and “toddler” liver microsomes with the full array of specific isoforms can be made commercially as a custom product by companies like BD Biosciences (BD Gentest™ branded products), among others. Thus, V_{max} and Km for specific metabolites (particularly the hydrolytic cleavage products that can have interaction with parent compounds) for each enantiomer can be obtained which will provide a better understanding of potential inhibitory interactions. With regard to extrapolation from adult to juvenile or early life stages, another approach would be to model the infant response based on adult data. Simcyp Limited and Simulation Plus, Inc., for example, provide simulation and modeling software that can model the pharmacokinetics of "virtual" children based on the input of "adult" parameters.

On the other hand, analysis of metabolite production using microsomes can be complex. For example, there are two diastereomers of permethrin (*cis* and *trans*), each has a *R*- and a *S*-enantiomer for a total of four enantiomers with their own set of metabolites that are also likely inhibiting biotransformation of the other enantiomers. Gaughan and Casida (1978) reported more than 30 metabolites of permethrin using TLC analytical methods and only the two diastereomers of permethrin. EPA’s Tornero-Velez *et al.* (2010) PBPK model did not separate out the enantiomers which would likely lead to even more metabolites to measure. Many of these metabolites are conjugates. One panel member suggested that the metabolic stability could be assessed using the substrate depletion approach. Such an approach will generate metabolic rate information without a need for identification and quantitative determination of metabolites in microsomal systems. Alternatively, another panel member stated that if the most toxic enantiomer is not undergoing depletion because its metabolism is being inhibited by a primary metabolite or another enantiomer then the concentration of the enantiomer primarily responsible for the toxicity will be underestimated. This would be the case although the overall concentration of the four enantiomers is measured. The Panel advised that both the advantages and disadvantages of the substrate depletion approach and the metabolite formation approach be considered.

If most Phase II reactions are eliminated from the evaluation through the use of microsomal systems, then the Phase I metabolites may undergo additional secondary reactions with Phase I enzymes. For example, permethrin is hydrolyzed to 3-phenoxybenzoic acid (3-PBA), a reaction mediated by both CYP enzymes and carboxylesterases. With hepatocytes, 3-PBA is likely conjugated via a Phase II reaction (to a sulfate or glucuronide) and would not undergo a second Phase I reaction. Thus, even though concentrations may be below Km values for the enzymes, the formation of multiple metabolites that are also substrates have the potential for inhibitory and possible stimulatory (*CYP3A*) reactions. Therefore, the Panel concluded that it is likely that a *CYP* approach would misclassify the precise paths of metabolism and the relative abundance of metabolites.

The Panel considered whether there was an adequate supply of microsomes to represent the full diversity of the human population. However, the Panel recommended that measurements in microsome samples be retained as individual samples rather than pooled, as an integral part of the measurement program. The Panel noted that microsomes from the collection of

Dr. Ronald Hines[6], Medical College of Wisconsin, are available and that the repository is diverse enough to allow for measurements of sufficient power to represent the human population.

Panel members offered a number of recommendations with regard to future microsomal studies.

1) The Panel noted that while the liver makes the greatest contribution to microsomal oxidation, ignoring other sources introduces uncertainty in metabolic measurements in microsomes. To reduce this uncertainty, some panelists stated that quantification of extrahepatic metabolism would be an important use of microsomal measurements. Therefore, panel members strongly recommended that microsomal oxidation through *CYP* isoforms in the intestine should be incorporated into the model. Very little is known regarding ontological studies of enzyme expression in the small intestine. The Panel recommended that V_{max} and Km comparisons between *CYP* and carboxylesterases be evaluated in enterocytes of the small intestine. For example, one panelist pointed out that gut *CYP3A4* levels are relatively high in human enterocytes and that *CYP1A1* activity is also apparent (Thelen and Dressman 2009).

2) Some panelists expressed concern about the exclusive use of Michaelis-Menten (M-M) enzyme kinetics and scaling in the current pyrethroid modeling family. For example, Kenworthy *et al.* (1999) and others (Houston and Kenworthy 2000; Uchiapichat *et al.* 2004) have observed that M-M kinetics may not always hold either for single compounds or for mixtures of chemicals. The Panel suggested that the Agency carefully consider the metabolic scaling algorithms and enzyme kinetics required and adjust the model accordingly, particularly for analysis of pyrethrin mixtures. Being a composite of enantiomeric compounds, pyrethroids undergo complex Phase I and Phase II biotransformation, and therefore, it is expected that multiple *CYP* isoforms are involved as well as uridinediphosphate-glucuronosyltransferases (UGTs) and sulfotransferases (SULTs). However, at least some Panel members cautioned the Agency that if all possible factors that can influence the activity of an enzyme and how it is modeled, the complexity of the process would preclude the development of a practical and useful model. Added levels of complexity to the model must be weighed carefully as these decrease model parsimony.

3) Some panelists recommended that specific stereoselective interactions be examined further. Incubations would be conducted with individual enantiomers, diastereomers (*e.g.*, *cis* and *trans* permethrin), and the racemate for non-hepatic microsomes in the mixture that is in the commercial pyrethroid product.

4) One panelist suggested consideration of experiments in non-human primates (*i.e.*, monkeys) to elucidate pharmacokinetic parameter values. This would likely better reflect humans than observations made in rodents, although post speciation differences in enzyme action may be limiting.

[6] Ronald Hines, Ph.D., provided public comments during the meeting on behalf of the PPTWG.

5) Some panelists thought that the unusually low ratio of concentrations observed for the brain versus the blood for some pyrethroids should be further evaluated. To unravel this apparent anomaly in partitioning, the Panel recommended further investigation of the possibility of active efflux of pyrethroids from the brain back to blood, as well as specific *in vitro* measurements of equilibrium partitioning in the brain versus analogs to the plasma.

3.0 Alternative Study Design(s) For Evaluating Age Differences in Pharmacokinetics

The Agency gives special consideration to the potential pre- and postnatal lifestages regarding potential exposure to pesticides. Pre-natal exposure to pyrethroids has been evaluated extensively in over 80 developmental toxicity, reproductive toxicity, and DNT test guideline studies and no sensitivity from in utero exposure has been observed. As previously described, there are gaps in knowledge surrounding the potential for post-natal sensitivity and, as described in Question 1.2, the Agency considers children less than 3 years of age to be the most susceptible population. The PPTWG has proposed a robust PBPK model development effort to describe pyrethroid dosimetry across several lifestages; however, these models will not be ready for use by the Agency until approximately 2013.

Are there alternative approaches using empirical or data generation techniques potentially requiring less time than the PBPK effort proposed by the PPTWG for evaluating the potential for post-natal sensitivity, particularly with respect to differences in pharmacokinetic profiles, that could be used by the Agency?

Panel Response

The Panel did not recommend a specific approach to evaluating the potential for post-natal sensitivity, particularly with respect to differences in pharmacokinetic profiles. The Panel agreed that development of the PBPK model to facilitate the animal-to-human extrapolation was very important and should be continued. While these efforts are ongoing, the Panel identified several changes/alternatives in the current approach to develop an *in silico/ in vitro/ in vivo* (SVV) PBPK model that could ultimately be used to predict the pharmacokinetics of pyrethroids in mammals, particularly in humans. These included: 1) data-mining efforts to focus future testing programs, 2) *in vitro* approaches, *e.g.*, cassette dosing, 3) pharmacokinetic and pharmacodynamic considerations for *in vitro* studies, and 4) non-human primate (monkey) *in vivo* studies. Although these suggestions and alternatives may initially require some additional experiments, they may, in the long term, reduce the time necessary to develop the SVV model of the pharmacokinetics of pyrethroids in humans and enable a more feasible and accurate means of determining the viability of the PBPK model.

Data-mining and modeling

The Panel indicated that a more targeted testing approach could be developed to guide the choice of endpoints incorporated into the design of future toxicity studies used to quantitatively characterize age-related sensitivities. One way to focus *in vitro* studies would

be a critical and thorough review of the existing pyrethroid database pertinent to the age-specific acute toxicity question at hand (*e.g.,* pharmacokinetics, pharmacodynamics, acute toxicity, pre- and post-natal developmental toxicity). This new data-mining effort would update the pre-existing comprehensive review paper by Shafer *et al.* (2005) to include new studies conducted post-publication and include studies that were submitted to the Agency for pesticide registration. This effort should include comments on the strengths and limitations of each study. From this analysis, one would compile a database on the dose-response relationships of all relevant endpoints for each pyrethroid and perhaps identify age-specific sensitivity data as well as any data gaps. These data would not be limited to acute neurotoxicity as proposed by the PPTWG. For example, another possible endpoint might be long-term effects on reproductive functioning caused by developmental alterations in endocrine signaling reported for pyrethroids (see Shafer *et al.* 2005).

Modeling the dose-response relationships (PBPK/PD) and time course pattern for all relevant toxicity endpoints for which data are available would also be a valuable exercise. These data may include the time course from time-to-peak-effect evaluation which is associated with specific neurobehavioral endpoints, *e.g.*, motor activity. Useful data for modeling application can also come from studies showing differential severity of toxicological response solely due to different dosing volume. The expectation is that these exercises can provide insights into model integrity as well as inform the choice of dose-metrics pertinent to each specific endpoint that can potentially be used to establish the point of departure for risk assessment. A parallel effort to ensure reliable modeling of target age groups with significant exposure, in this case, young children, is also essential for a successful application of PBPK model for risk assessment.

Possible in vitro approaches

The Panel had the following caveat regarding additional *in vitro* studies: All future testing should be designed to determine whether there are any quantitative differences in sensitivity between adults and juveniles. The Agency reminded the Panel that, currently, the mandatory default 10X safety factor is being retained in the assessment process for all pyrethroids, because the question of whether there are age-related differences in sensitivity to members of this class remains unanswered. The Panel indicated that what is important would be to identify those "critical studies" for which the Agency has characterized a NOAEL and/or LOAEL in the adult then use these studies to focus on determining whether the juvenile would be expected to be more, less, or similarly susceptible at the same dose levels.
The Panel considered *in vitro* approaches that could generate data for input parameters to the rat and human models, especially those with significant impact to model outcome as indicated by sensitivity analysis, and pertinent to the potential candidates of dose metrics for interspecies extrapolation. The Panel did not prioritize their suggestions.

1) One suggestion was to measure the blood and brain levels of pyrethroids over the first four hours after either intravenous (i.v.) or subcutaneous (s.c.) (use s.c. if it is more feasible/reliable at PND 10) administration of a prototypical Type II pyrethroid (*e.g.*, deltamethrin) in PND 10, PND 21 and PND 90 rats. High pyrethroid blood levels in neonate rats within the first couple of hours after oral dosing have previously been

proposed to be due primarily to the combined result of rapid absorption, low first pass metabolism, rapid distribution into the brain, and limited subsequent metabolism. At least one effect and one no-effect dose should be tested in all age groups, although a wider range and number of doses (*e.g.*, two or three effect level doses) might be needed. Whether the 10-fold higher levels seen within the first two hours after oral dosing with Type II pyrethroids are due totally to absorption and first pass metabolism is not clear. If it is, then gut absorption and renal excretion may be even more important than metabolism for initial Type II pyrethroid concentrations in the blood and brain. Consequently, more detail needs to be provided for modeling the absorption and excretion of Type II pyrethroids in neonates than now proposed. If significant age differences are found in blood and brain concentrations after either i.v. or s.c. administration then more consideration should be given to the vast differences in body and organ fat levels, gut absorption, and possible renal excretion in neonates versus adults that affect Type II pyrethroid concentrations. If the i.v. or s.c. studies were designed more elaborately and toxic effects were closely monitored during the first four hours, then it would be easier to discern if there were significant differences in PND 10 animals versus adults with respect to the Type II pyrethroid pharmacodynamic aspects of neurotoxicity and lethality than can be currently determined from available data from oral administration.

2) Another suggestion was to use a Cassette-Dosing approach to expedite the data gathering for the PBPK model. Cassette-Dosing is a high-throughput screening tool used in the drug discovery process for the rapid (although imprecise) assessment of the pharmacokinetics of a series of compounds. This approach involves the simultaneous administration of multiple compounds to a single animal.

Pharmacokinetic Considerations

1) *Mortality is a poor measure of age-dependent sensitivity.* One panel member noted that the age-related difference in sensitivity with mortality as an endpoint reflects a saturation of detoxication ability which is lower in juvenile animals than in the adults. Therefore, running an experiment at a level that causes mortality does not reflect the difference in sensitivity between juveniles and adults that would be operational at realistic exposure levels.

2) *Beyond Michaelis-Menten (M-M) kinetics.* As previously mentioned in the Panel's response to Charge Question 2.2, the current data rely solely on M-M kinetics, and M-M kinetics may not always hold true either for single compounds or for mixtures of chemicals (specifically pyrethroids). One panelist suggested that the Agency reanalyze the available datasets using sigmoidal and/or inhibition approaches to modeling *CYP* and other enzymes. However, another panelist cautioned that trying to consider non-M-M kinetics, inhibition kinetics or activation kinetics would add levels of complexity to the modeling process when the model is not intended to give exact estimates.

3) *Pharmacodynamic versus pharmacokinetic considerations.* One panelist pointed out that pharmacodynamics, with respect to the toxic mode of action, should also be considered in

addition to pharmacokinetics. Pyrethroid insecticides share the voltage-gated sodium channel (VGSC) as a common molecular target site. Pyrethroids modify the sodium channel kinetics, resulting in a delayed channel closing and altered nerve cell transmission ultimately leading to fine tremors or choreoathetosis and salivation. VGSCs exhibit a complex regional and temporal ontogeny, and embryonically expressed forms of these channels are replaced by expression of adult forms during neurodevelopment (Shafer *et al.* 2005). The complex ontogeny of VGSCs could result in altered sensitivity of the developing central nervous system to perturbations by pyrethroids (Shafer *et al.* 2005). Although toxicokinetic factors are thought to account for differences in susceptibility to pyrethroids between young and adult animals (Sheets *et al.* 1994), toxicodynamic factors have not been systematically studied (Shafer *et al.* 2005). A recent study (Mackenzie *et al.* 2009) links changes in VGSCs to auditory deficits which provide a possible rationale for the use of the ASR in determining toxicity, despite its short-comings.

On the other hand, one panelist stated that pharmacokinetics instead of pharmacodynamics should be the focus of attention because of concerns with the utility of the ASR (see Panel Response to charge question 1.1) as well as the utility of other tests of neurotoxicity for comparing very young rats to adult rats. This panelist suggested that protecting children be examined by pharmacokinetics instead of pharmacodynamics because the data from the former are straightforward and interpretable based on metabolism studies using the current state of the science; whereas, this might not be true for neurotoxic endpoints.

Non-human primate studies

One panelist proposed that pyrethroid studies could be conducted using monkeys as a means of model validation. Such studies could potentially yield valuable data that may be more interpretable for producing a better SVV model than the one being developed (*i.e.*, Tornero-Velez *et al.* family of PBPK models). The appropriate time span for testing the pharmacokinetics of pyrethroids to represent the newborn to 3-year old human is much longer in the monkey (being at least 4 months) compared to only a few days in the rat (*e.g.*, PDN 10-17). In addition, there is the possibility of examining the effect of multiple exposures of pyrethroids on the same animal over some period of time. The relative rate of development of brain, gut, kidney and liver in monkey are more in synchrony with human than that of rat. Also, the body fat ratio during monkey development would be more like human. This panelist stressed the importance of having a suitable species to validate the final SVV model.

Other comments concerning the PPTWG proposal

One panel member suggested that the Agency evaluate the PPTWG proposal in the context of answering questions similar to those used by the U.S. Army Research Laboratory's Human Research and Engineering Directorate (HRED) during the development and review of the detailed study protocols (see **Appendix 2).**

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Appendix 1: Expanded References and Suggestions for Conducting ASR Testing

References for Development of ASR:

1) Auditory acuity in the rat improves progressively between 16 and 20 days. During this time both sensitivity and range of frequencies to which the animal can respond increase with postnatal age (Crowley and Hepp-Raymond 1966).

2) The startle response can be elicited by a tactile stimulus and the sensitivity matures earlier than auditory sensitivity (Gottlieb 1971). However, modification of the startle response is progressively elaborated over a period of days during the 2nd and 3rd weeks of life. This also occurred with a tactile stimulus (Parisi and Ison 1977). Both serotonin and norepinephrine have been implicated in the modulation of the startle response in the adult rat. Both the noradrenergic and serotonergic systems are undergoing intense maturation during the 2-3rd weeks of life in the rat with regards to cellular differentiation and synthetic enzyme activity.

3) Kungel *et al*. (1996) reported that exposure to cysteamine to reduce somatostatin levels in the developing rat brain between PND 10 and PND 17 animals resulted in ASRs that were similar between exposed and control animals at PND 13 while by PND 18, the ASR was lower in the cysteamine-dosed rats as compared to controls reflective of a delay in the maturation of the ASR between PND 13 and PND 18.

Points to Consider in Conducting ASR to Decrease Variance in Data Sets:
[Note: These points are not arranged in any order of priority.]

1) The EPA DNT guideline requires a minimum sample size (n) of 10 male and female rats at each dose level. One would assume, based upon the variability across a large majority of the data sets for ASR, that a sample size of 10 is not adequate to detect a 20-30% difference. Based upon the available data, a power analysis would be required to determine the necessary sample size. This may be different across various test methods, commercial apparatus, and SOPs, but needs to be sufficient to detect a specific level of difference. An inadequate sample size and high level of variability found in individual test labs will not provide data sufficient for determining chemical-related effects.

2) In the current EPA DNT guideline, auditory startle response for habituation is performed on PND 60. The mean response amplitude is calculated for 10 sequential trials – referred to as a block for a total of 5 blocks. There are no specific requirements for the parameters of the startle stimulus, *i.e.*, dB level, Hz range, pure tone versus white noise, background noise levels, inter-trial intervals and fixed or random delivery. Each of these components has been demonstrated in the literature to modify the ASR and can alter the sensitivity of the test.

3) Interpretation of an ASR is confounded if the hearing ability of the animal is altered. While both the EPA and WHO IPCS neurotoxicity risk assessment guidance documents discuss these features, the DNT guideline does not address this issue with regard to either data collection or within the SOP to confirm a relatively uniform hearing capability at the stimulus level. While hearing threshold evaluation requires auditory brainstem response recordings, a crude evaluation of the response of animals across a range of stimulus intensities can confirm no gross differences in hearing.

4) Weight and size of the test animal can influence the measured ASR. This requires that each AS chamber be calibrated and sensitivities adjusted for testing of animals to ensure the responses are kept within the scale of the system. This can be required in adult animals between ages of 5 – 10 weeks and is definitely required for the testing of younger animals. In addition, within the SOP it should be required that the sound levels are measured in dBc using a sound level meter and calibrated and equalized across all chambers. An acclimation period within the chamber is required. For adult rats, this is often 5 minutes. This can vary in the younger rodent.

5) In control rats, there is usually a high correlation between the peak amplitude and the average amplitude; however, this may vary with exposure and thus, both types of data should be analyzed. Standardize control animal response and any specific age with regards to response amplitude, lag-time to response, etc. Use uniform testing at an age that is considered "adult," an age that must be post puberty.

6) Habituation to an auditory startle stimulus can vary as a function of age or exposure. Using the commercially available ASR systems, the generation of a habituation curve normally requires a test session of approximately 100 stimulus deliveries. However, other earlier data using a direct delivery of the stimulus upon visual confirmation of an absence of movement of the rat indicated that habituation occurs much earlier. Thus, examination of the individual startle responses made by the animal can provide a greater level of sensitivity. If a pre-pulse startle inhibition (PPI) protocol is employed, habituation occurring in the later portions of the test session represents a floor-effect and the ability to detect PPI can be diminished. Thus, PPI calculated during the test period of a non-habituated startle response will provide data less compromised. Prepulse paradigms can be used to record gap detection.

7) In combination with new commercial apparatus to measure ASR, data generated for evaluating the basic neuroscience of the ASR and the association to neurological diseases such as, schizophrenia, provide additional methods to assess components of the ASR. These new ways of evaluating the ASR waveform should be evaluated for inclusion into neurotoxicity testing to provide more sensitive and possibly less variable and more relevant response data.

8) Using the commercial apparatus for ASR, a measurement can be obtained during a time of no-stimulus presentation and if sampled over the course of the test session can provide an indication of activity levels.

9) The following elements needed for any neurobehavioral test need to be standardized: housing, husbandry, handling (*i.e.* bedding changes), litter sizes, strain and time of day when the test is performed. In immature rodents, additional efforts are required to address the temperature and humidity regulation of the environment and to minimize stress. Ultrasonic vocalizations between animals must be considered in the logistics of handling animals during the pre-weaning periods.

Appendix 2: Protocol Review Criteria Provided by One Panelist Based on Those used by the U.S. Army Research Laboratory's Human Research and Engineering Directorate (HRED)

Review Criteria	Yes	No
Is the experimental design clearly stated?		
Is the methodology described in sufficient detail to evaluate the statistical analyses possible with this experimental design?		
Are the experimental conditions (treatments) to be compared well defined?		
Are the proposed statistical analyses appropriate to the design, the sample size, and the type of data that will be collected? (*e.g.*, interval versus ordinal variables, fixed versus random effects models, assumptions for the use of the proposed analyses met, etc.)		
Is the proposed sample size appropriate to the experimental design? (e.g., Could the sample size be smaller and still provide statistical power? Is the sample too small for the planned analyses?)		
Is the process of assigning experimental units (subjects) to experimental conditions (treatments) appropriate and clearly detailed or defined?		
Does the design make the best use of available statistical techniques? (e.g., Was blocking utilized correctly or a Latin Square design proposed correctly based on the needs and assumptions of the research?)		
Does the design and methodology create any sources of bias or threats to the validity of the proposed analyses and interpretation of results that need to be addressed?		
Based on a review of the entire proposal, are the research design and analyses proposed in this protocol sufficient to answer the primary research questions?		
Will any results based on these data and analyses be generalizable?		