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

OFFICE OF
CHEMICAL SAFETY AND POLLUTION
PREVENTION

August 19, 2015

MEMORANDUM

SUBJECT: Transmittal of Meeting Minutes of the FIFRA Scientific Advisory Panel Meeting held on "Research to Evaluate the Potential for Juvenile Sensitivity to Pyrethroids"

TO: Jack Housenger, Director
Office of Pesticides Programs

FROM: Fred Jenkins, Jr., Ph.D., Designated Federal Official
FIFRA Scientific Advisory Panel
Office of Science Coordination and Policy

THRU: David Dix, Ph.D. Director
Office of Science Coordination and Policy

Laura Bailey, M.S., Executive Secretary
FIFRA Scientific Advisory Panel
Office of Science Coordination and Policy

Attached, please find the meeting minutes of the FIFRA Scientific Advisory Panel open meeting held in Arlington, VA on May 19-21, 2015. This report addresses a set of scientific issues associated with "Research to Evaluate the Potential for Juvenile Sensitivity to Pyrethroids."

Enclosure

cc:
Jim Jones
Louise Wise
Jack Housenger
William Jordan
Yu-Ting Guilaran
Robert McNally
Donald Brady
Jacqueline Mosby
Jennifer McLain
Dana Vogel
Susan Lewis
Richard Keigwin
Laura Bailey
Anna B. Lowit
Monique Perron
Jaime D'Agostino
William Irwin
Cathy Milbourn
Linda Strauss
OPP Docket

FIFRA Scientific Advisory Panel Members
Stephen Klaine
Kenneth Delclos
Marion Ehrich
David A. Jett
James McManaman

FQPA Science Review Board Members
Russell Carr, Ph.D.
Veronica Berrocal, Ph.D.
Muhammad Hossain, Ph.D.
Allison Jenkins, MPH
Teresa Leavens, Ph.D.
Igor Linkov, Ph.D.
Thomas Murray, Ph.D.
Isaac Pessah, Ph.D.
Bingfang Yan, Ph.D.

FIFRA Scientific Advisory Panel Minutes No. 2015-02

A Set of Scientific Issues Being Considered by the Environmental Protection Agency Regarding Research to Evaluate the Potential for Juvenile Sensitivity to Pyrethroids

---

May 19-21, 2015
FIFRA Scientific Advisory Panel Meeting
Held at the
EPA Conference Center
Arlington, VA

The meeting minutes represent the views and recommendations of the FIFRA SAP and do not necessarily represent the views and policies of the EPA or of other agencies in the Executive Branch of the Federal government. Mention of trade names or commercial products does not constitute an endorsement or recommendation for use. The meeting minutes do not create or confer legal rights or impose any legally binding requirements on the EPA or any party.

TABLE OF CONTENTS

PANEL ROSTER 5

LIST OF COMMON ACRONYMS USED 8

INTRODUCTION 9

PUBLIC COMMENTS 10

OVERALL SUMMARY.....11

EXECUTIVE SUMMARY OF PANEL DISCUSSION AND RECOMMENDATIONS.....13

DETAILED PANEL DELIBERATIONS AND RESPONSE TO CHARGE 26

LITERATURE CITED.....56

NOTICE

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (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 scientific advice, information, and recommendations to the EPA 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. The meeting minutes have been written as part of the activities of the FIFRA SAP.

In preparing the meeting minutes, the FIFRA SAP carefully considered all information provided and presented by EPA and the Council for the Advancement of Pyrethroid Human Risk Assessment, LLC (CAPHRA). The minutes represent the views and recommendations of the FIFRA SAP and do not necessarily represent the views and policies of the EPA, nor of other agencies in the Executive Branch of the Federal government. Mention of trade names or commercial products does not constitute an endorsement or recommendation for use. The meeting minutes do not create or confer legal rights or impose any legally binding requirements on EPA or any party.

The meeting minutes of the May 19-21, 2015 FIFRA SAP meeting held to consider and review scientific issues associated with "Research to Evaluate the Potential for Juvenile Sensitivity to Pyrethroids" were certified by Stephen Klaine, Ph.D., FIFRA SAP Chair, and Fred Jenkins, Ph.D., FIFRA SAP Designated Federal Official, on August 18, 2015. The minutes were reviewed by Laura E. Bailey, M.S., FIFRA SAP Executive Secretary. The minutes are publicly available on the SAP website (http://www.epa.gov/scipoly/sap/) under the heading of "Meetings" and in the public e-docket, Docket No. EPA-HQ-OPP-2015-0130, accessible through the docket portal: http://www.regulations.gov. Further information about FIFRA SAP reports and activities can be obtained from its website at http://www.epa.gov/scipoly/sap/.

SAP Minutes No. 2015-02

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

Research to Evaluate the Potential for Juvenile Sensitivity to Pyrethroids

May 19-21 2015
FIFRA Scientific Advisory Panel Meeting
Held at
One Potomac Yard
Arlington, Virginia

Stephen Klaine, Ph.D.
Chair
FIFRA Scientific Advisory Panel

Date: AUG 1 8 2015

Fred Jenkins, Jr., Ph.D.
Designated Federal Official
FIFRA Scientific Advisory Panel
Staff

Date: AUG 1 8 2015

PANEL ROSTER

FIFRA SAP Chair

Stephen Klaine, Ph.D.
Professor, Department of Biological Sciences
Director, Institute of Environmental Toxicology
Clemson University
Clemson, SC

Designated Federal Official

Fred Jenkins, Jr., Ph.D.
U.S. Environmental Protection Agency
Office of Science Coordination and Policy
FIFRA Scientific Advisory Panel
EPA East Building, MC 7201M
1200 Pennsylvania Avenue, NW
Washington, DC 20460
Phone: (202) 564-3327
jenkins.fred@epa.gov

FIFRA Scientific Advisory Panel Members

Kenneth Delclos, **Ph.D.**
Research Pharmacologist
Division of Biochemical Toxicology
National Center for Toxicological Research
U.S. Food and Drug Administration
Jefferson, AR

Marion Ehrich, Ph.D., D.A.B.T., A.T.S.
Professor of Pharmacology and Toxicology
Department of Biomedical Science and Pathobiology
Virginia-Maryland Regional College of Veterinary Medicine
Virginia Polytechnic Institute and State University
Blacksburg, VA

David A. Jett, Ph.D.
Director, National Institute of Health CounterACT Program
National Institute of Neurological Disorders and Stroke
National Institutes of Health
Bethesda, MD

James McManaman, Ph.D.
Professor and Chief
Section of Basic Reproductive Sciences
Department of Obstetrics and Gynecology, Physiology
and Biophysics
University of Colorado Denver
Aurora, CO

FQPA Science Review Board Members

Russell Carr, Ph.D.
Associate Professor
College of Veterinary Medicine
Mississippi State University
Mississippi State, MS

Veronica Berrocal, Ph.D.
Assistant Professor of Biostatistics
Department of Biostatistics
School of Public Health
University of Michigan
Ann Arbor, MI

Muhammad Hossain, DVM, MS, Ph.D.
Neurotoxicologist
Department of Environmental and Occupational Medicine
Robert Wood Johnson Medical School
Rutgers University
Piscataway, NJ

Allison Jenkins, MPH
Senior Toxicologist
Toxicology Division of the Texas Commission on Environmental Quality (TCEQ)
Austin, TX

Teresa Leavens, Ph.D.
Independent Pharmacokinetic Consultant
Cary, NC

Igor Linkov, Ph.D.
Team Leader
Risk and Decision Science Focus Area Lead
US Army Engineer Research and Development Center
Concord, MA

Thomas Murray, Ph.D.
Associate Vice Provost for Research and Scholarship
Professor and Chair
Department of Pharmacology
Creighton University School of Medicine
Omaha, NE

Isaac Pessah, Ph.D.
Associate Dean of Research and Graduate Education, Professor
Department of Molecular Biosciences
University of California Davis School of Veterinary Medicine
Department of Veterinary Medicine: Molecular Biosciences
University of California
Davis, CA

Bingfang Yan, Ph.D.
Professor and Department Chair of Biomedical and Pharmaceutical Sciences
Director of the Center for Integrated Drug Development
University of Rhode Island
Kingston, RI

LIST OF COMMONLY USED ACRONYMS AND ABBREVIATIONS

AOP:	Adverse Outcome Pathway
AP:	Apical
ASR:	Acoustic Startle Response
AUC:	Area Under Curve
BBB:	Blood-brain barrier
BL:	Basolateral
CAPHRA:	Council for the Advancement of Pyrethroid Human Risk Assessment
CSAF:	Chemical specific adjustment factor
CE(S):	Carboxylesterase(s)
CPPGL:	Cytosolic protein per g liver
CSPA:	Consumer Specialty Products Association, Inc.
CYP:	Cytochrome P450
DCO:	Detailed Clinical Observations
DLM:	Deltamethrin
fu:	fraction unbound
GC-MS:	Gas chromatography-mass spectrometry
GI:	Gastrointestinal
HTS:	High-throughput screening (HTS)
ISEF:	Inter-system extrapolation factor
LW:	Liver weight
MPPGL:	Microsomal protein per g liver
IVIVE:	*In Vitro* to *In Vivo* Extrapolation
PBPK:	Physiologically-based pharmacokinetic modeling
Papp:	Permeability coefficient
PC:	Partition coefficients
PD:	Pharmacodynamic

INTRODUCTION

On May 19-21 the US EPA Federal Insecticide, Fungicide, and Rodenticide Act Scientific Advisory Panel (FIFRA SAP) met in Crystal City, VA to consider and review scientific issues associated with "Research to Evaluate the Potential for Juvenile Sensitivity to Pyrethroids." The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) authorizes EPA to register pesticides and the Federal Food, Drug, and Cosmetic Act (FFDCA) gives the Agency the authority to establish tolerances for residues on food and/or feed resulting from use of a pesticide. The studies required to allow the Agency to make the appropriate statutory safety findings under both of these acts are stipulated under 40 Code of Federal Regulations (CFR) Part 158. There is flexibility, however, in implementing Part 158. Additional data can be required (§158.75), alternative approaches can be accepted, and studies can be waived (§158.45). The 2007 National Research Council (NRC) report from the National Academy of Sciences (NAS) on Toxicity Testing in the 21st Century describes this new vision for toxicity testing. In response to the NRC report, EPA's Office of Pesticide Programs (OPP) developed a Strategic Direction for New Pesticide Testing and Assessment Approaches (http://www.epa.gov/pesticides/science/testing-assessment.html) which describes OPP's approach to implementing the NAS vision. One of the key components of OPP's Strategic direction is improved approaches to more traditional toxicity tests to minimize the number of animals used while expanding the amount of information obtained. OPP also has a recent document, *Guiding Principles for Data Requirements* (http://www.epa.gov/pesticides/regulating/data-require-guide-principle.pdf) which describes the principles for requiring toxicology data for pesticides, specifically to "only require data that adequately inform regulatory decision making and thereby avoid unnecessary use of time and resources, data generation costs, and animal testing."
OPP is actively working on a reevaluation of the human health effects of the pyrethroids and pyrethrins under the OPP registration review program (http://www.epa.gov/oppsrrd1/registration_review/index.htm), required under FIFRA. Until late 2009, OPP requested developmental neurotoxicity (DNT) studies for pyrethroids. However, the Agency determined that the DNT studies were not providing adequate data to evaluate the potential for post-natal sensitivity to pyrethroids. In July, 2010, the FIFRA Scientific Advisory Panel (SAP) reviewed a proposed research strategy to assess the potential for juvenile sensitivity consistent with the recommendations of the NAS in its report on Toxicity Testing in the 21st Century using a combination of *in vitro studies*, targeted *in vivo studies*, and physiologically-based pharmacokinetic (PBPK) models.

Based on feedback from the SAP and the Agency, the industry research proposal was revised. Since late 2010, the Council for the Advancement of Pyrethroid Human Health Risk Assessment (CAPHRA) has worked with industry and academic scientists to develop assays and models to assess the potential for juvenile post-natal sensitivity to pyrethroids. The on-going research effort is organized around the adverse outcome pathway (AOP) for pyrethroids alterations with voltage-gated sodium channels (VGSC), leading to alterations in membrane excitability and firing potentials and ultimately to *in vivo* clinical syndromes. Specifically, the CAPHRA is evaluating the potency of pyrethroids to human sodium channels and transplantation of adult & juvenile rat synaptic membrane into oocytes. In addition, the CAPHRA is conducting targeted *in vivo* studies on behavioral metrics and developing PBPK models. The research, thus far, has

focused on development of the overall approach using data for deltamethrin and permethrin (Type II and Type I pyrethroids, respectively). The CAPHRA research is at a point where feedback on extending this research to the other pyrethroids would be constructive. The CAPHRA proposal is to use the knowledge gained with deltamethrin and permethrin to develop more targeted datasets using read across and computational approaches (i.e., less data generation) for other pyrethroids. As such, the Agency sought the FIFRA SAP's advice on the current state of the science with the CAPHRA research effort and proposals for next steps which include extension of data on deltamethrin and permethrin to other pyrethroids.

PUBLIC COMMENTERS

There were no oral or written public comments provided for this meeting.

OVERALL SUMMARY

The Panel was charged with advising the Agency on 8 topics areas regarding the development of assays and models to assess the potential for juvenile post-natal sensitivity to pyrethroids insecticides. The following provides a summary of the Panel's advice for each topic area.

- *Topic 1) High-throughput screening studies using human sodium channels expressed in mammalian cells with their regulatory beta subunits (ChanTest Data)*- The Panel concurred that the proposed high-throughput screening approaches would not be useful in depicting the potential for juvenile sensitivity from pyrethroids. They noted that there were several factors that diminished the strength of the data (*e.g.* the large variability in protein expression and many of the electrophysiological parameters).

- *Topic 2) Neurolemma studies: Transplantation of adult & juvenile rat brain synaptic membrane into (Xenopus) \oocytes*- The Panel expressed a lack of confidence in the results of these studies due to various factors (e.g. the small sample sizes of the studies).

- *Topic 3) Targeted in vivo studies in adult and juvenile rat: acoustic startle/detailed clinical observation:* Generally, the Panel thought that the study was well designed. However, they noted that several key pieces of information regarding the studies were omitted. For example rationale for the use of male Sprague Dawley (SD) Rats and the dose selection was lacking. The Panel found that the detailed clinical observation (DCO) method is subjective and not a rigorous unbiased method. Relative to DCO, the automated acoustic startle response (ASR) or tactile startle response (TSR) may be better approaches, however, the Panel found that the use of the ASR for juveniles is problematic in some areas, and that further testing with the TSR is warranted. The Panel encouraged a continued investigation of the comparison of levels of neurotoxicity in young and adult rats with similar levels of exposure.

- *Topic 4) Pharmacokinetic studies*: The Panel commented that the CAPHRA's efforts to develop a human PBPK model appeared to be on the right track for addressing the information needed. However, the Panel detailed a few deficiencies. . One deficiency noted was the lack of data in addressing age-related differences.

- *Topic 5) Physiologically: based pharmacokinetic model in rat*- The Panel had concerns regarding the confidence, accuracy, and uncertainty associated with this model. They also noted that the model appeared to be in a preliminary form. They also recommended further research to better validate the model.

- *Topic 6) Physiologically: based pharmacokinetic model for human*-The Panel noted several concerns regarding the structure of the model. Consequently, the Panel thought that there was insufficient evidence to comment on the reliability, confidence and uncertainty of the age-related brain Cmax predictions of the model.

- *Topic 7) Physiologically: based pharmacokinetic models for humans with other pyrethroids*- The Panel concurred that using the PBPK model to evaluate other

pyrethroids was not yet warranted as the model, according to the Panel judgment was still under development.

- *Topic 8) Integration of lines of evidence:* The Panel expressed doubt that the proposed rat model is a good model to learn about the response of human brains to exposure to deltamethrin. They recommended exploration of alternative experiments to learn about internal dosimetry. Also the Panel recommended the consideration of the Weight of Evidence (WOE) approaches that have been recently developed by the Organization for Economic Co-operation and Development (OECD) for developing Adverse Outcome Pathways (AOPs) (OECD, 2014, Becker et al., 2015).

EXECUTIVE SUMMARY OF PANEL DISCUSSION AND RECOMMENDATIONS

1. ***High-throughput screening studies using human sodium channels expressed in mammalian cells with their regulatory Beta subunits (ChanTest Data):*** *Human voltage-gated sodium channels (Na_{Vs} 1.1, 1.2, 1.3, 1.6) were expressed in human embryonic kidney (HEK) cells and 9 pyrethroids were tested for their effects on channel conductance, as well as the effects of co-expressing the beta 1 and 2 regulatory subunits***.**

a. *Please comment on the ChanTest studies conducted for nine pyrethroids. Please include in your comments a consideration of their robustness (i.e. reproducibility, controls, statistics, background information, Na_V selection, etc). Please comment on the confidence and uncertainties in the ChanTest experiments and related findings.*

The FIFRA SAP Panel unanimously agreed that the ChanTest approach is unlikely to contribute useful information to characterize the potential for juvenile sensitivity of pyrethroids. The approach is fundamentally limited by the heterologous expression system where the Na_V isoforms are over-expressed out of context of their native cell environment, and exposed to acute challenges to most pyrethroids above their solubility range. Experimental factors that weakened the robustness of the data included large variability in protein expression and many of the electrophysiological parameters measured including peak current (I_{peak}), normalized tail current, and persistent (late) sodium current. Presumably the variability in the ChanTest approach extends to all Na_V isoforms and each of the electrophysiological parameters measured. Such variability limits the usefulness of the ChanTest approach to measure small to moderate differences in whole cell current parameters that are necessary before conclusion can be drawn about differential influences on Na_V isoforms and juvenile toxicity. Importantly, many of the most acutely toxic pyrethroids *in vivo* failed to show detectable influences on Na_V channel parameters, bringing into question whether the approach actually reflects an outcome relevant to the pyrethroid AOP.

A more defensible approach for high-throughput screening would be to implement primary neuronal networks in cultures isolated from juvenile rodent or human iPSC derived neuronal cells grown in culture. Secondly measure electrical network activity or patterns of synchronous Ca^{2+} oscillations, which are tightly coupled to membrane electrical activity as an integral part of the AOP.

b. CAPHRA thinks that additional data from this line of evidence are unlikely to contribute useful information to characterize the potential for juvenile sensitivity. Thus, the CAPHRA has proposed NOT to continue this line of research. Please comment on this proposal and degree to which the ChanTest data inform the issue of evaluating the potential for juvenile sensitivity.

The Panel generally agreed with CAPHRA that the ChanTest approach using expressed Na_V subunits, with or without ß1and ß2 subunits, does not provide useful information about the potential of differential juvenile toxicity.

c. The ChanTest experiments focus on human sodium channels and generally show weak response. In contrast, in vitro studies in rodents in the literature (e.g. Choi and Soderlund, 2006; Meacham et al., 2008; Tan and Soderlund, 2009[1]) show stronger responses to pyrethroids. Please comment on the extent to which the expressed human sodium channels can be used in combination with these sources to infer relative pharmacodynamic sensitivity between rats and humans.

The Panel unanimously concluded that the ChanTest experiments do not sufficiently discriminate among the electrophysiological properties of expressed Na_V isoforms. Moreover, the ChanTest approach does not have the sensitivity to identify modest to moderate differential influences of pyrethroids of sodium channel gating kinetic parameters. This is a significant concern since the large range of acute neurotoxicological potencies of the pyrethroids examined seem to be largely discordant from their apparent activities towards the Na_V isoforms measured by ChanTest. The data reporting stronger response of rat Na_V to pyrethroids from the cited literature cannot be used in combination with the ChanTest data because the cited data were obtained from a non-mammalian expression system (*i.e., Xenopus* oocytes) which is likely to have differences in protein-protein interactions, have divergent membrane composition, and/or differences in posttranslational modifications, especially protein phosphorylation status. Any and all of these factors could influence interactions between pyrethroids and Na_Vs thereby making comparisons difficult and possibly misleading. The weak effects of pyrethroids in the ChanTest protocol and the lack of neuronal and developmental contexts are unlikely to add knowledge about isoform sensitivity, unless the rat and humans clones are tested under identical experimental conditions with a more robust sample size.

2. ***Transplantation of adult & juvenile rat brain synaptic membrane into Xenopus oocytes:*** *Purified neurolemma membranes from adult and juvenile rats were separately micro-injected into Xenopus oocytes. Patch clamp testing was performed on the oocyte membranes versus various doses of pyrethroids to determine their EC_{50} values for Na_V channel activation. Inhibitors of competing channels were added to isolate the sodium channel conductance (i.e. chloride and calcium channels).*

[1] Choi JS and Soderlund DM. 2006. Structure-activity relationships for the action of 11 pyrethroid insecticides on rat Na v 1.8 sodium channels expressed in Xenopus oocytes. Toxicol Appl Pharmacol. 2006 Mar 15;211(3):233-44. Epub 2005 Jul 26 Meacham, C.A., et al., *Developmentally-regulated sodium channel subunits are differentially sensitive to [alpha]-cyano containing pyrethroids.* Toxicology and Applied Pharmacology, 2008. **231**: p. 273-81. Tan, J. and D.M. Soderlund, *Human and rat Nav1.3 voltage-gated sodium channels differ in inactivation properties and sensitivity to the pyrethroid insecticide tefluthrin.* NeuroToxicology, 2009. **30**(1): p. 81-89.

a. Please comment on the synaptic membrane ("neurolemma") studies conducted for deltamethrin and permethrin. Please include in your comments a consideration of the robustness of data from the synaptic membrane transplantation into oocytes (reproducibility, control compounds, channel modulator reagents, statistics, use of Na blocker tricaine as an anesthetic, etc) and the degree to which these data inform the issue of evaluating the potential for juvenile sensitivity. Please comment on the confidence and uncertainties and associated findings in the oocyte experiments.

The Panel believes that the neurolemma studies were conducted using appropriate techniques and methodology. The Panel applauds the scientists for providing a detailed and accurate documentation of the experiments and of the results found. Nonetheless, despite the appropriateness of the studies performed and the accuracy in documenting the oocyte experiments, the Panel is hesitant in placing a great amount of confidence in the findings of these experiments for three main reasons: the sample sizes used were too small, the estimated uncertainties both in the final results and in the intermediary analyses steps (e.g. the response concentration curves fit) are very large, and the primary data and measurements had to undergo several transformations in order to be compared.

b. In the context of your response to 2a, the CAPHRA has proposed to collect dose-response data in adult and juvenile rat synaptic membrane transplanted into oocytes for five more pyrethroids (including Type I, Type II, and mixed). CAPHRA's proposed path forward: If the additional five pyrethroids show similar patterns to deltamethrin and permethrin (i.e. no lifestage sensitivity observed), no additional pyrethroids will likely be tested in this system. Alternatively, if a different pattern is observed, additional pyrethroids are likely to be tested. Please comment on the CAPHRA's proposed path forward.

The Panel acknowledged that age-related differences in the sensitivity of rat brain sodium channels could be studied using this system. However, the only age-related difference appears to be the lower number of sodium channels in the juveniles as compared to the adults. The system also suffers from other issues that lead to large uncertainties concerning the confidence in the findings. The Panel concluded that the usefulness of using this system for determining age-related differences is minimal.

3. ***Targeted in vivo studies in adult and juvenile rat: acoustic startle/detailed clinical observations:*** *Preliminary experiments that measure acoustic startle and detailed clinical observations have been conducted by the University of Cincinnati. In these experiments, juvenile and adult male Sprague-Dawley rats were treated with deltamethrin and permethrin as model Type II and Type I pyrethroids, respectively. In addition, brain and plasma concentrations were measured in PND 15 and 90 rats exposed to deltamethrin. The preliminary results suggest that juvenile rats are more sensitive to deltamethrin based on changes in detailed clinical signs and, to less extent, acoustic startle. However, there was no greater sensitivity in juvenile rats exposed to permethrin. Preliminary data from whole brain tissue samples with deltamethrin indicated that PND 15 rats had higher deltamethrin concentrations as compared to adult rats given the same dose. The brain concentration and toxicity data from the CAPHRA studies for deltamethrin are consistent to those previously published by the Bruckner lab (Kim et al., 2010[2]) which showed that at a similar dose (2 mg/kg deltamethrin) PND 10 rats had increased severity of clinical signs and higher C_{max} of deltamethrin in the brain as compared to PND 21, PND 40, and PND 90 rats.*

a. *The in vivo behavior studies reported thus far are preliminary evaluations. Please comment on the study design in the preliminary in vivo studies for deltamethrin and permethrin conducted at the University of Cincinnati (Vorhees lab).*

In general, the Panel believed that the study design was carefully planned and permitted rigorous statistical analysis. The Panel commented that more information on the selection of the Postnatal Day 15 (PND15) age in rats was needed, especially since the lack of sensitivity to the Acoustic Startle Response (ASR) observed in PND15 rats was not observed in PND17 and PND21 rats. The rationale behind the use of only male Sprague Dawley (SD) rats in the CAPHRA *in vivo* studies was also not discussed in the study reports and should have been included. The Panel commented that the rationale for the dose selection was also insufficient for several studies, and questioned whether the dynamic range of doses was appropriate.

The Panel also agreed that the description of the pretesting of animals in order to improve performance was sufficient, and a discussion of how this could affect the overall study design and results would have been beneficial. The Panel noted that the effect of litter should have been included in the statistical analysis since the dams were required to care for the pups during transit and the shipping, and this could have induced some level of stress in the dams. Finally, there was no chemical characterization information provided for permethrin.

[2] Kim KB, Anand SS, Kim HJ, White CA, Fisher JW, Tornero-Velez R, Bruckner JV. 2010. Age, dose, and time-dependency of plasma and tissue distribution of deltamethrin in immature rats. Jun;115(2):354-68. doi: 10.1093/toxsci/kfq074. Epub 2010 Mar 8.

b. In 2010, FIFRA SAP commented on some challenges that were anticipated with the use of auditory startle to compare the relative sensitivity of juveniles and adults. In the studies conducted by the CAPHRA, both auditory startle and detailed clinical observations were evaluated. Please comment on the degree to which the auditory startle and detailed clinical observations provide useful data for evaluating the potential for juvenile sensitivity. Please include in your comments discussion of the dynamic range; the direction of the response varying between Type I and IIs; and the type of data obtained (continuous, ranked).

The Panel remarked that one study indicates that the Tactile Startle Response (TSR) test provides a somewhat larger dynamic range; however, more studies may need to be done across ages, compounds and doses, to justify including this paradigm at this late stage. The use of ordinal Detailed Clinical Observations (DCO) data is valuable as secondary observation, but it is subjective and not a rigorous unbiased method for decisions related to hazard assessment across many compounds. Relative to DCO, the automated ASR (or TSR) approach is a better primary method for assessing *in vivo* toxicity. However one panel member noted that the DCO observations, and tremors in particular, might better fit the "adverse" description as opposed to a compound effect. For the PM pre-weaning results, there seems to be no clear relationship between the magnitude of the tremor response and the magnitude of the ASR response. With respect to the ASR data for deltamethrin (DLM), CAPHRA concluded that DLM did not induce statistically significant effects on ASR in PND90 rats (Figure 2-5a) while DLM induced statistically significant effects on ASR in PND15 pups (Figure 2-5b). Shortcomings in the statistical analysis of these data decreased the confidence of the Panel concerning the absence of significant effects on ASR in PND90 rats exposed to DLM.

The ASR has a history as a measure of pyrethroid neurotoxicity and data generated should be comparable to values in the literature. However, the predictability of the ASR model to adverse effects in humans, published or hypothesized, has not been established. The Panel commented that tremors and/or writhing in rats might be more sensitive and relevant to the human condition. Another primary question is whether ASR is linked to any known mechanism of action for pyrethroids, including their influences on sodium channels, or other ion channels. Overall it does appear that use of the ASR for juveniles is problematic because of the overlap with non-specific effects. Going forward, the focus should be on further testing with the TSR, either alone or in combination with the ASR, to see if the test is sensitive enough to separate out the specific effects from the non-specific effects.

c. Please provide comments comparing the temporal pattern and magnitude of the brain and plasma concentration data from the Vorhees & Bruckner labs and utility of such data to aid in the interpretation of the auditory startle and detailed clinical observation data.

For DLM, the time of the rise in plasma and brain levels does not coincide with the peak in ASR effects, but the trends are consistent perhaps with slight delays. It is not expected that these should match perfectly since there are several unknown factors involved in the potential dose- and time-related mechanisms underlying the effect of DLM on ASR.

A plan to continue investigating the comparison in levels of neurotoxicity in young and adult rats with similar levels of exposure (concentrations) within the brain may be appropriate. If the data

support the findings available to date, the Panel would concur that there would be no further *in vivo* work needed to resolve the pharmacodynamics question.

The Panel noted that it may be important to examine the effects of pyrethroids in young and old rats at a matched internal dose (e.g. brain/plasma concentration), but the difficulties in obtaining a high enough dose in adults and the temporal variability in these doses may be prohibitive. This difficulty must be weighed against the added value of these data to the overall question of whether young rats/humans are more affected by a given external dose, regardless of intrinsic differences. The Agency will need to consider what information will be critical for the final hazard assessment and regulatory activity.

d. Please comment on the use of a 5 ml/kg dosing volume in the CAPHRA studies and how any impact on pyrethroid kinetics affects correlations with behavioral effects.

The Panel concluded that the dose volume does not seem to appreciably influence the DLM experiments in terms of the correlation to the timing of ASR effects. The Agency should consider the question: Is a slower release and delayed or extended period of the ASR desirable? For example, is it better to give a smaller volume and test at 2 hours, or larger volume and test at 4 or 6 hours? The answer may lie in the optimal study design for comparable results across compounds, as well as how best to design the model in order to simulate exposure in humans.

The Panel questioned the justification of using 5 mL/kg in the CAPHRA studies given that previous studies in the literature used 1 mL/kg. CAPHRA stated that the methods were developed to allow for the testing of additional pyrethroids with very low toxicity that would require very high dosages. Considering the Agency goal to modify toxicity testing not only to reduce animal use but also to test more human relevant doses, it may be worth considering if it would really be necessary to push the doses of low potency compounds to such high levels.

Panel members agreed that there is a concern that doses administered to a pup with a full stomach of milk may be problematic. It was stated that 15-day old rat pups were not removed from the dam prior to dosing which did not allow gastric emptying. It is possible that the milk in the stomach altered the absorption and pharmacokinetics of the pyrethroid.

4. ***Pharmacokinetic studies:*** *A number of pharmacokinetic studies using deltamethrin were performed by the CAPHRA in order to further refine and validate the developing rat PBPK model and construct a developing human PBPK model. For refinement of the rat PBPK model, tissue: plasma partition coefficients (in vivo measurements in PND 21 and adult rats), age dependent plasma protein binding (in vivo measurements in PND 10, 15, 21, and 90 rats), and cytochrome P450 (CYP) and carboxylesterase (CES) metabolism (in vitro measurements using rat liver and plasma preparations from PND 15, 21, and 90 rats) were evaluated. Further in vivo pharmacokinetic studies in rats were conducted with single IV (PND 90), single oral (PND 90), and multiple oral (PND 15, 21, and 90) doses to generate plasma and tissue data to validate the developing rat PBPK model. Additional experiments with deltamethrin were conducted for constructing the developing human PBPK model. Parameters evaluated in vitro included age dependent plasma protein binding derived in plasma from human donors aged from birth to adults, transport across the blood-brain*

barrier using a human brain microvascular endothelial cell line, estimates of gastrointestinal absorption in caco-2 cells, and ontogeny data for CES, CYP1A2, and CYP2C8 determined in human liver tissue from donors (age 1-18 years old). Further in vitro experiments are currently underway by the CAPHRA to determine human adult and juvenile liver metabolism of deltamethrin by CYP and CES enzymes using human liver preparations or recombinant human enzymes.

a. Please comment on the in vitro experiments to support the PBPK model development in the rat conducted thus far for deltamethrin.

Based on the needs of information for the development and validation of the PBPK model for deltamethrin, the *in vitro* work conducted so far by CAPHRA appears to be relevant towards the stated goal of constructing a developing human PBPK model. CAPHRA has made considerable progress with proposed kinetic studies for deltamethrin, and, overall, the Panel thought that the experiments completed or underway were excellent in quality and appropriate for addressing the age-dependence of liver and plasma metabolism and protein binding. The age-dependence of these kinetic factors is particularly important to be captured in the model, and the experiments have quantitated important issues that are critical for the In Vitro- In Vivo Extrapolation (IVIVE) approach. The Panel recommended that the CAPHRA program should complete the propose metabolism work, in particular utilizing enzyme preparations from various age groups. The Panel identified a few deficiencies in the work including the lack of data or experiments addressing age-related differences in permeability and active transport at the blood-brain barrier, lack of in vitro studies that addressed possible age-related changes in the oral absorption of deltamethrin, lack of studies to address potential sex-specific differences in kinetics, and lack of studies to address extrahepatic metabolism.

b. Please comment on the in vivo experiments to support the PBPK model development in the rat conducted thus far.

The CAPHRA program systematically carried out and collected high quality data for numerous toxicokinetic and tissue distribution studies that had several strengths including: (a) use of various age-groups to determine age-related changes in toxicokinetics; (b) administration of deltamethrin by intravenous and oral dosing to establish bioavailability; and (c) dosing over a range of deltamethrin concentrations doses to provide important information on the detoxification capacity (saturation). However, the Panel expressed some concerns about the study design and the data analysis, including the lack of repeated exposure studies, lack of information regarding sex selection for the study, lack of adequate discussion for results from studies, and lack of adequate quantitation of bioavailability. Two issues that generated significant discussion among Panel members were the potential influence of vehicle on absorption and subsequent impacts on lymphatic versus portal system distribution. This is an important issue because the assumption of bypassing the liver in the approach described may have influenced modeling results, which predicted a higher brain Cmax in the adults than in children.

c. Please comment on the in vitro experiments in the human tissue conducted thus far. Please include in your comments a discussion of the ongoing in vitro experiments with recombinant

enzymes for use in PBPK models and associated confidence and uncertainty with the use of such data.

The Panel noted the approach that CAPHRA was using for the IVIVE with recombinant enzymes seemed to be thorough and a logical method strategy to predict metabolism in the liver across age groups. The Panel noted several areas of confidence for the approach including the following: ontogeny data on human liver carboxylesterases; ontogeny data on CYP2C8 and CYP1A2 in the liver; and, determination of enzyme kinetics with recombinant CYPs and CESs. However, the Panel had concerns about some aspects of the study that would generate uncertainty in the use of the data. These included lack of estimation in hepatic clearance in primary human hepatocytes from various age populations (cryopreserved hepatocytes), and, use of different laboratories to determine ontogeny expression, which adds another source of variability to the data.

5. ***Physiologically-based pharmacokinetic model in rat:*** *Using data described in Question 4, the CAPHRA has developed a PBPK model for deltamethrin using age-specific metabolism parameters in rats to simulate plasma and brain internal exposures in young and adult rats. This PBPK model relies on in vitro to in vivo extrapolation (IVIVE) to use age-specific metabolic data collected in vitro to estimate hepatic metabolic clearance in vivo. The deltamethrin brain or plasma concentrations estimated by the model in rats are then compared to measured concentrations from in vivo rat studies to verify the model. Non-chemical specific physiological parameters for rats were obtained from the published literature, including body weight, cardiac output, hematocrit levels, tissue volumes, and tissue blood flows. As discussed in Question 4, recently generated data by the CAPHRA and published data were used for compound-dependent parameters, including partition coefficients, metabolic rate constants, absorption rates, protein binding, compartments and tissue permeability*.

a. *Please comment on the robustness of the rat PBPK model for simulating internal exposures in the developing rat. In your response, please include evaluation of the structure and parameters used to build the model, as well as its ability to accommodate different oral absorption scenarios (i.e. different vehicles used for in vivo studies) and discussion of confidence, accuracy and uncertainties associated with the deltamethrin developing rat model. Please also comment on the sensitivity analyses of parameters CAPHRA has completed thus far.*

While the Panel recognized the fluidity of the modeling process and commended the researchers and modelers on the iterative nature of the experimental work with model development, the Panel were reticent to comment on the confidence, accuracy and uncertainty of a model that did not appear to be in the final form. The PBPK model structure was based upon the model by Tornero-Velez et al. (2010) and was expanded to account for vehicle-specific absorption of deltamethrin, plasma metabolism age-specific plasma protein binding, and the metabolic contribution of CES to pyrethroid hydrolysis. The Panel felt the incorporation of this additional information increased the confidence and accuracy of the model, however, the Panel expressed concern regarding the adequacy of the model structure and parameter values due to lack of consistency of the model predictions with observed data and experimental data to support certain

model structures *(e.g.* lymphatic versus portal uptake of deltamethrin). The lack of a quantitative assessment of the goodness of fit adequacy of fit for the absorption parameters decreased the confidence in the model predictions. One parameter, which appeared to be influential and for which no age-dependent changes were incorporated was the permeability-surface-area cross product in the brain. This appeared to be a parameter that was highly uncertain and the Panel recommended that potential age-related changes in the permeability of the blood-brain barrier should be investigated and incorporated in the model. The Panel was unable to provide comments on the sensitivity analysis because information was not provided in the overview documents or presentations for the rat model. The Panel did recommend that a global sensitivity analysis, in addition to a local analysis, be performed for the modeling work.

b. In the context of your response to 5a, please comment on the extent to which additional data are/are not needed to refine the developing rat model.

The Panel recommended several studies that are needed to better validate the predictions of the current model as well as provide further refinement with respect to predicted brain concentration of deltamethrin in older versus younger rats. These include repeated dosing of pyrethroids to look at the potential accumulation in the brain, studies to determine lymphatic versus portal absorption, quantitation of bioavailability, and, evaluation of age-dependent differences in the blood-brain barrier. Based on the discussions of the Panel during the meeting, the recommended studies will provide important data to have confidence in the use of the PBPK model for risk assessment for children.

6. ***Physiologically-based pharmacokinetic model for human:*** *Similar to the rat PBPK model, the human PBPK model integrates non-chemical specific physiological parameters for humans from the literature. Compound-dependent parameters, such as partition coefficients and oral absorption parameters, were adapted from the rat PBPK model. Recently generated data by the CAPHRA were used for the remaining compound-dependent parameters, such as metabolic rate constants, protein binding and tissue permeability. With respect to metabolic constants, CYP and CES enzymes involved in metabolism of a given pyrethroid will be identified and the in vitro metabolic constants for those enzymes will be determined for integration into the PBPK model. Intrinsic clearance for each active enzyme will be scaled to in vivo using scaling factor data collected by the CAPHRA and the SIMCYP database. The ontogeny of enzyme expression (also from the CAPHRA data and the SIMCYP database) will be incorporated into the process of obtaining distributions of age-specific intrinsic clearance for each enzyme. Preliminary simulations have been conducted for deltamethrin to demonstrate the process used with the PBPK model.*

a. Within the context of understanding potential juvenile sensitivity, characterize the robustness of the PBPK model for extrapolating age-specific internal tissue exposures for humans. In your response, please comment on the structure and parameters used to build the model and include discussion of confidence, reliability, and uncertainties associated with the deltamethrin human model. Please include in your comments a discussion of the data from the McCarver/Hines Laboratory (submitted as part of the CAPHRA package) for providing ontogeny of CES enzymes.

The human PBPK model was developed with the intent of predicting human tissue exposure to deltamethrin. Parameters of the model included body weight, tissue volume, tissue body weight fraction, tissue permeability, partition coefficients, etc.

While appreciating CAPHRA's effort to develop a human PBPK model, the Panel had several concerns, mostly regarding the model structure. Relative to the model parameters, the Panel highlighted the fact that there were discrepancies between the model parameters reported in the presentation and the overview documents making it appear as if the model was still under development.

For the model structure, the major concerns of the Panel were relative to the parameterization of the human brain in the PBPK model, especially in light of its influence on the C_{max} predictions, and, the pathway of absorption that is structured to completely bypass the liver and portal system by switching to the lymphatic system. The Panel believed that the differences between rats and humans pose a major challenge especially with respect to the metabolism of deltamethrin. Further, the Panel was concerned about the capacity of the human PBPK model to reproduce the large variability observed among humans and was perplexed about the usage of data from 1-year olds and the use of the Monte Carlo approach since neither was justified or documented..

The Panel suggested revisions to the PBPK model focused on the definition of clearance, the definition and usage of age grouping when modeling the liver function, the usage of western blot analyses,, and the determination of internal concentrations within an organ. Due to the various concerns relative to the model structure and status of the model parameter calibration, the Panel did not believe it had sufficient evidence to comment on the reliability, confidence, and uncertainty of the age-related brain C_{max} predictions.

b. Please comment on the proposed use of SIMCYP for providing enzyme ontogeny patterns and deriving population distributions for metabolic parameters in humans. Please include in your comments whether or not other tools with similar capacity to SIMCYP are available.

The Panel recognized that there is general acceptance of the SIMCYP Simulator by the modeling community as a tool to provide enzyme ontogeny patterns and derive population distributions for metabolic parameters in humans. On the other hand, the Panel pointed out potential challenges for the pediatric modeling of pyrethroids due to high-levels of protein binding, extensive metabolism by multiple enzyme systems, and potential involvement of active transport. In addition, there are knowledge gaps regarding the metabolism of pyrethroids in humans. These knowledge gaps include but are not limited to: (1) the involvement of both hydrolysis and oxidation in metabolism (whether and to which extent these two elimination pathways differentially contribute to the overall clearance of a pyrethroid is not clear); (2) the presence of both hydrolysis and oxidation enzymes in the liver and extrahepatic tissues (there is no information on organ-specific clearance of a pyrethroid, as well as age-dependency in this regard); and, (3) the potential for differential induction of cytochrome P450s over carboxylesterases in humans by pyrethroids, which are activators of pregnane X receptor (how the differential induction might lead to changes of the contribution of respective elimination pathways is not clear). The Panel also recommended that an alternative modeling platform should be used as a comparison with the SIMCYP Simulator.

7. ***Physiologically-based pharmacokinetic models for humans with other pyrethroids:*** *Thus far, the CAPHRA has focused its human PBPK efforts on deltamethrin and, to a lesser extent, permethrin. Soon laboratory efforts will turn to other pyrethroids. CAPHRA's proposal is to conduct fewer studies and in lieu of such data, use read across and computational approaches.*

a. *Please comment on the appropriateness of the current human PBPK model to be used for other Type I, Type II, or mixed type pyrethroids. Please include in your response evaluation of the path forward provided by the CAPHRA regarding in vitro and in vivo studies for other Type I, Type II, or mixed type pyrethroids.*

While the Panel provided comments on the appropriateness of the use the PBPK model to read across for different pyrethroids, it also pointed out that these comments and recommendations were provided on a concept that had not yet been thoroughly vetted. The model appeared to still be under development and the experimental work has not yet been completed. In theory, if the issues regarding rat and human models were addressed to decrease uncertainty in the models' structures and increase accuracy of the models' predictions, the Panel thought a read across with chemical-specific parameters would be appropriate. The Panel did not anticipate that the pharmacokinetic model structure would need to be different for the different pyrethroids; however, the parameter values would differ. As pointed out by one panel member, one challenge of modeling these compounds is the variability in metabolism, and that the metabolic clearance for individual pyrethroid compounds must be determined to allow models to be developed with accurate predictions. The Panel made several recommendations regarding the proposed path forward that was presented by CAPHRA with focus on partition coefficients, permeability-surface-area cross products, *in vitro* kinetics since isomeric forms will likely differ, and evaluation of PBPK models for all pyrethroids for which data is generated. The Panel also commented that the Adverse Outcome Pathway approach is a combination of pharmacokinetics and pharmacodynamics and both have to be adequately understood to read across (i.e. extrapolate) to other compounds. This charge question focused only on extrapolation of the pharmacokinetics across the family of pyrethroids.

8. ***Integration of lines of evidence:***

a. *The tissue dosimetry data from the rat suggest higher brain levels in juveniles compared to adults. In contrast, the preliminary PBPK modelling for human predicts slightly lower brain concentrations in young children compared to adults. Please comment on these differences, including comments on the key inputs that lead to this difference, human variability associated with the key parameters, and the confidence and uncertainties associated with the difference between the rat and human internal dosimetry.*

The Panel raised several concerns regarding the human PBPK model in previous charge questions. Of particular concern was the model structure and parameters (e.g. brain permeability surface area cross product and blood flow to the liver) that highlighted the large uncertainties surrounding the findings obtained thus far. Therefore, the Panel was not confident in the

conclusions that CAPHRA has reached with respect to the differences between rat and human internal dosimetry.

The Panel strongly questioned the rat as a good model to learn about the response of the human brains to exposure to deltamethrin and suggested CAPHRA look into alternative experiments to better quantify internal dosimetry. In particular, the Panel suggested CAPHRA explore the possibility of using either guinea pigs or ES1 plasma carboxylesterase knockout mice as potentially more appropriate animal models for extrapolation to human.

Further, to pursue a weight of evidence approach and combine findings from multiple disparate experiments, the Panel recommended considering the quantitative Weight Of Evidence (WOE) approaches that are being formalized by the Organization for Economic Co-operation and Development (OECD) for developing Adverse Outcome Pathways (AOPs) (OECD, 2014, Becker et al., 2015).

b. The CAPHRA has proposed to continue in vivo behavioral testing for deltamethrin and permethrin in definitive dose-response evaluations. Pyrethroids have been studied for decades and thus there is a large body of evidence for these pesticides. Given your response to 8a along with 1) the extensive body of scientific literature on pyrethroid toxicity syndromes and high dose studies in juvenile rats (e.g., Sheets et al, 1994[3]); 2) neurolemma studies supported by the CAPHRA (Question 2) along with additional in vitro studies (e.g. Meacham et al., 2008); and 3) recent in vivo studies from the Vorhees & Bruckner labs, please comment on the additional scientific value that would be provided in conducting further in vivo rat experiments to assess potential for PD sensitivity of human infants and children.

From a general standpoint, the Panel thinks that CAPHRA's plan to direct the research effort mostly on the pharmacokinetics aspect and the effort to increase the level of understanding of age-related metabolic differences between adults and novices seemed reasonable. However, the Panel encouraged CAPHRA to reconsider the utility of the neurolemma studies, as the large uncertainties in the results seriously undermine the reliability of the results obtained, and the magnitude appear difficult to dissipate even with larger sample sizes.

Moving forward, the Panel urged CAPHRA to: (i) Continue pursuing *in vivo* studies, focus on developing new assays, and determine a clinical endpoint that could assess the clinical relevance of deltamethrin (DLM) presence in the brain; (ii) Examine an additional type II pyrethroid, cyhalothrin, in addition to the core pyrethroids; (iii) Complete the permethrin studies, relating ASR and DCO response to brain levels, as was done for deltamethrin.

On a more general level, the Panel recommended that CAPHRA re-evaluate the approach undertaken thus far to evaluate the effect of exposure to pyrethroids on the nervous system. More specifically, the Panel suggested a more transparent approach that would: 1) review all the potential risks of pyrethroid exposure on juveniles, 2) define the degree or frequency of impact that is acceptable, 3) examine indications of potential endpoints and outcomes of disease states/potential impacts, and 4) provide the most definitive evidence for such endpoints. As an

[3] Sheets, L.P., et al., *Age-Dependent Differences in the Susceptibility of Rats to Deltamethrin.* Toxicology and Applied Pharmacology, 1994. **126**(1): p. 186-190.

illustration, the Panel suggested potential endpoints to consider for the nervous system including activation and impacts on neuronal development, DNA damage, oxidative stress, reduction of neurogenesis, and loss of dopaminergic (DA) neurons. Finally, the Panel believed that while the human pharmacokinetic model is a useful tool for estimating the potential for exposure, the information provided by this model needs to be paired with an understanding of the dose-response relationship for an effect that implicates a specific outcome of a disease state.

c. *If you believe there is additional scientific value to conducting additional in vivo experiments (8b), please comment on the CAPHRA's proposed experiments. In the context of your response to Question 3, please include in your comments a discussion of dose levels and/or additional study design elements to improve existing preliminary evaluations.*

If *in vivo* neurotoxicity studies are continued, the Panel suggested that larger sample sizes be used and litter effects be considered, as proposed for further deltamethrin and permethrin studies. Relative to dose, the Panel recommended that 1 mg/kg dose be used in future deltamethrin studies, with unchanged time course and possible reduction in the number of trials per session to reduce habituation.

In regard to the use of corn oil as vehicle in previous experiments, the Panel suggested using the minimal volume necessary to enhance the rate of uptake and peak brain concentration in future experiments. Additional suggestions included: (i) performing additional experiments to monitor neuronal functions along with clinical observations of extracellular neurotransmitters release with brain microdialysis for better understanding age and dose dependent neurotoxicity of pyrethroids; (ii) considering ways to reduce tremor in rats as it might interfere with ASR; and (iii) examining how tissue concentration varies in the brain to provide better insight into what parts of the brain are more sensitive to pyrethroids.

Detailed Panel Recommendations

1. **High-throughput screening studies using human sodium channels expressed in mammalian cells with their regulatory Beta subunits (ChanTest Data):** Human voltage-gated sodium channels (Na_{Vs} 1.1, 1.2, 1.3, 1.6) were expressed in human embryonic kidney (HEK) cells and 9 pyrethroids were tested for their effects on channel conductance, as well as the effects of co-expressing the Beta 1 and 2 regulatory subunits.

a. Please comment on the ChanTest studies conducted for nine pyrethroids. Please include in your comments a consideration of their robustness (i.e. reproducibility, controls, statistics, background information, Na_V selection, etc).

The Panel acknowledged that heterologous gene expression systems have been an invaluable tool for gaining a better understanding of how protein structure relates to physiological function. The value of ion channel expression has been especially informative in our quest to understand how specific regions of sequence contribute to specific aspects of ion channel function. This includes identifying domains essential for the millisecond to millisecond characteristics of ion channel currents, including activation, inactivation, deactivation, and the selectivity filter. In addition, heterologous expression models have helped identify key determinants of protein-protein interactions, some of which mediate post-translational modifications and longer-term adaptation (integration) to cellular activity. With respect to Voltage Gated Sodium Channel (VGSC), interactions with beta subunits appear to be important for optimal targeting of the Na_V alpha subunit to the surface membranes and finer aspects of channel modulation. In this regard, Na_{Vs} subunits can interact with any one of 3 beta subunits, but the degree to which they do largely depends on cellular context.

Reproducibility

The Panel noted that comparative investigations based on expression of ion channel subunits in heterologous models must be tightly controlled in terms of the level of protein expression and targeting to the surface membrane. In the ChanTest case, the level of expression and targeting of each Na_V isoform is not determined by western blotting or immunofluorescence. The highly variable (>10X based on blot shown) transient expression of the beta2 subunit β2 (Fig 1) and an unknown level of expression of the beta1 subunit β1 (antibodies did not work), suggest a lack of precision in the current measurements made by ChanTest. Lack of precision is evidenced by the >200% (-0.7-1.6nA) variation in I_{peak} among $Na_V1.3$ cells. The variability in NaV1.3/β1β2 expression may explain why I_{peak} varies appreciably among cells (-1.4-2.7nA). Based on the information provided, and the degree of variability in the basic electrophysiological properties of each Na_V clone (with or without electroporation of β1β2), comparisons among isoforms would permit only moderate to large differences to be discerned with confidence, whereas more subtle differences may go unresolved. In this regard, summary statistics are not given in any of the Na_V summary tables (Tables 1A&B; 2A&B).

The Panel raised several statistical concerns. For instance in regards to sample size, how many data points in each group are being compared? The Panel noted that the sample sizes that were

presented were small, as low as n=3. When comparing the pyrethroids, there is uncertainty about the robustness of calculation for EC50s.

With regards to Controls

The Panel commented that inferences about the similarity or differences between human VGSC properties reported by ChanTest and those previously reported with the rat $Na_V1.3$ expressed in oocytes are neither warranted nor valid unless the homologous rat Na_V isoform lines (in the presence or absence of ß1ß2 electroporation) are recorded using the IonWorks Barracuda platform (at least NaV1.3 for validation). For valid cross species comparisons (human vs rat), it is necessary to make measurements under identical experimental conditions. The lack of such essential controls greatly weakens the usefulness of the ChanTest results with pyrethroids, especially when comparing results to other studies using completely different methodology.

For example, *"CAPHRA concludes that results from 7 pyrethroids tested in this first phase are consistent with and extended the Tan and Soderlund (2009) data on tefluthrin showing that human VGSC are much less sensitive than those in the rat. Comparison of homologous rat and human, Nav1.3 VGSC isoforms showed that the rat isoform was 4-fold more sensitive than the equivalent human sodium channel to the pyrethroid tefluthrin. This suggests that, in contrast to the situation with rats, the pyrethroids tested are poor modulators of the human sodium channel, which in turn suggests that the rat is likely an overly sensitive model to predict the neurotoxicity of pyrethroids."*

However, the results from HEK cell expressed human clones *vs.* rat channels expressed in *Xenopus* oocytes show large divergence in inactivation times, with rat $Na_V1.3$ channels exhibiting a 3-fold larger fraction of channels exhibiting the slow component of inactivation and ~3 fold slower kinetics at test pulse (V_t) of -15mV compared to human $Na_V1.3$ (Fig 1C&D; Tan & Soderlund 2009). It is uncertain if this fundamental difference is a result of model differences or if they would be observed in native neuronal models (e.g., primary embryonic neurons from rat *vs.* human). If this difference is artificial it may drive the difference in pyrethroid sensitivity. Key comparisons on inactivation parameters between human $Na_V1.3$ expressed in HEK (ChanTest) and those reported by Tan and Soderlund (2009) are not possible because the data are not included in the ChanTest report. Furthermore recent work has demonstrated that tefluthrin and deltamethrin exert different effects on Nav1.6 channels expressed in HEK293 cells (He and Soderlund, 2011) and on Nav1.6 channels expressed in *Xenopus* oocytes (Tan and Soderlund, 2010).

The Panel noted that the concentrations of pyrethroids used in the ChanTest experiments were a concern, especially with regard to the possible lack of specificity towards the intended Adverse Outcome Pathway (AOP) (i.e., selective alteration of VGSC kinetics). Tefluthrin concentrations extend to 100µM (>3 log units above its solubility; Fig 3). Tefluthrin shows a NOEL towards $Na_V1.3$ of 1µM, 20-fold above its aqueous solubility limit (ChemID). Allethrin, on the other hand is used within its solubility range (1-10µM). It is unclear if this fact explains the differential influence of the two pyrethroids on $Na_V1.3$ ß1ß2 or other isoforms?

There are several published papers that report that $Na_V1.3$ interacts with β1and/or β3, and at least one paper that indicates that β2 has no influence on $Na_V1.3$ singly or in combination with β1 or β3 (Meadows et al 2002). It is unclear what influence this has on the results reported by ChanTest.

There are some discrepancies in reporting the EC_{50} value for I_{tail} between Table 1A/Fig 4 and summary Table 2A (Allethrin 5.4 vs 7.8; Tefluthrin 2.0 vs 3.2). Again this reduces confidence in the general reproducibility of the measurements and raises questions about the validity of the conclusions extending comparisons of human Na_Vs to other studies that were done using very different expression models. Again, summary statistics are not given in any of the Na_V summary tables (Figures 1A&B; 2A&B).

The ChanTest does not take into consideration the occurrence of splice variants in individual sodium channel isoforms. For example, three splice variants of $Na_V1.3$ have been found in the human brain (Thimmapaya et al., 2005). While it is unclear the role that splice variants play in the toxicity of pyrethroids in humans, splice variants of sodium channels in insects have been demonstrated to produce pharmacologically distinct channels and are considered to be the basis for resistance.

Table 1 below summarizes quantitative measures of $Na_V1.3$ and $Na_V1.6$ channel function reported by ChanTest with addition of rat oral LD_{50} values and aqueous solubility for 6 pyrethroids included in the ChanTest report (LD_{50} and solubilities obtained from ChemID PLUS (http://chem.sis.nlm.nih.gov/chemidplus/). Evident from Table 1 is the lack of definable association between $Na_V1.3$ or $Na_V1.6$ channel parameters and acute toxicity. Collectively these data raise several uncertainties of whether the ChanTest experiments adequately address the key issues related to AOP and juvenile susceptibility.

Table 1. *Summary of quantitative measures of $Na_V1.3$ and $Na_V1.6$ channel function reported by ChanTest with addition of rat oral LD50 values and aqueous solubility for 6 pyrethroids included in the ChanTest report (LD50 and solubilities obtained from ChemID PLUS (http://chem.sis.nlm.nih.gov/chemidplus/).*

	Pyrethrum	Prallethrin	Allethrin	Bifenthrin	Tefluthrin	Cypermethrin
Rat oral LD50 (mg/Kg)	200	460	685	54	22	57
I_{Tail}/I_{Peak} (Nav1.3&1.6@100μM)	0.25/0.27	0.62/0.70	0.25/0.26	ND (no effect)	0.078/0.084	ND
Aqueous Solubility (@20°C)	0.35-125mg/L	8mg/L	4mg/L	0.1mg/L	0.02mg/L	0.004mg/L
EC_{50} I_{Tail} (Nav1.3&1.6)	5.6/9.7μM	4/5μM	8/9μM	ND (no effect)	3/6μM	ND
EC_{50} Persistent late I (Nav1.3&1.6)						
TP1	2.4/>30μM	3.3/2.8μM	4.8/4.8μM	ND (no effect)	0.95/3.2μM	ND
TP20	2.4/>30μM	3.0/3.1μM	3.6/8.5μM	ND (no effect)	3.6 / 8.5μM	ND
Max Persistent late I (Nav1.3&1.6)						
TP1	7%/13%	13%/19%	6%/ 9%	ND (no effect)	4.5%/8.8%	ND
TP20	9%/15%	17%/22%	8%/11%	ND (no effect)	6.1%/9.4%	ND

b. *CAPHRA thinks that additional data from this line of evidence is unlikely to contribute useful information to characterize the potential for juvenile sensitivity. Thus, the CAPHRA has proposed NOT to continue this line of research. Please comment on this proposal and degree to which the ChanTest data inform the issue of evaluating the potential for juvenile sensitivity.*

The Panel agreed that the ChanTest approach is unlikely to contribute useful information to characterize the potential for juvenile sensitivity of pyrethroids. The approach is fundamentally limited by the heterologous expression system where the Na_V isoforms are over-expressed out of context and exposed to acute challenges to most pyrethroids above their solubility range.

A more defensible approach for high-throughput screening would be to implement primary neuronal networks in cultures isolated from juvenile rodent or human iPSC derived neuronal cells grown in culture and measure electrical network activity using FluoVolt or patterns of synchronous Ca^{2+} oscillations, which are tightly coupled to membrane electrical activity as an integral part of the AOP.

c. *The ChanTest experiments focus on human sodium channels and generally show weak response. In contrast, in vitro studies in rodents in the literature (e.g. Choi and Soderlund, 2006; Meacham et al., 2008; Tan and Soderlund, 2009[4]) show stronger responses to pyrethroids. Please comment on the extent to which the expressed human sodium channels can be used in combination with these sources to infer relative pharmacodynamic sensitivity between rats and humans.*

The Panel agrees that the ChanTest experiments as described do not sufficiently discriminate among the electrophysiological properties of expressed Na_V isoforms and do not discriminate differential influences of pyrethroids having large range of acute neurotoxicological potencies on Na_V isoforms. The data reporting stronger response of rat Na_V to pyrethroids from the above cited literature cannot be used in combination with the ChanTest data because those data were obtained from a non-mammalian expression system (*i.e.*, *Xenopus* oocytes) that could potentially have differences in post-translational modification of proteins, in membrane composition, or in phosphorylation status that could influence interactions between pyrethroids and Na_Vs (Meacham et al., 2008). The weak effects of pyrethroids in the ChanTest protocol and the lack of neuronal and developmental contexts are unlikely to add knowledge about isoform sensitivity, unless the rat and humans clones are tested under identical experimental conditions with a more robust sample size.

2. ***Transplantation of adult & juvenile rat brain synaptic membrane into Xenopus oocytes:*** *Purified neurolemma membranes from adult and juvenile rats were separately micro-injected into Xenopus oocytes. Patch clamp testing was performed on the oocyte membranes versus various doses of pyrethroids to determine their EC_{50} values for Na_V channel activation.*

Choi JS and Soderlund DM. 2006. Structure-activity relationships for the action of 11 pyrethroid insecticides on rat Na v 1.8 sodium channels expressed in Xenopus oocytes. Toxicol Appl Pharmacol. 2006 Mar 15;211(3):233-44. Epub 2005 Jul 26 Meacham, C.A., et al., *Developmentally-regulated sodium channel subunits are differentially sensitive to [alpha]-cyano containing pyrethroids.* Toxicology and Applied Pharmacology, 2008. **231**: p. 273-81. Tan, J. and D.M. Soderlund, *Human and rat Nav1.3 voltage-gated sodium channels differ in inactivation properties and sensitivity to the pyrethroid insecticide tefluthrin.* NeuroToxicology, 2009. **30**(1): p. 81-89

Inhibitors of competing channels were added to isolate the sodium channel conductance (i.e. chloride and calcium channels).

a. *Please comment on the synaptic membrane ("neurolemma") studies conducted for deltamethrin and permethrin. Please include in your comments a consideration of the robustness of data from the synaptic membrane transplantation into oocytes (reproducibility, control compounds, channel modulator reagents, statistics, use of Na blocker tricaine as an anesthetic, etc) and the degree to which these data inform the issue of evaluating the potential for juvenile sensitivity. Please comment on the confidence and uncertainties and associated findings in the oocyte experiments.*

The Panel recognized that the scientists performing the neurolemma experiments provided a detailed, thorough and comprehensive description of their procedures and results. A number of basic and preliminary experiments were conducted using appropriate channel modulator inhibitors reagents to assure reviewers that the model was a valid way of looking at rat sodium channels in a non-mammalian cellular system. The use of tricaine as an anesthetic for collection of *Xenopus* oocytes is well documented in the literature and deemed acceptable. Appropriate methodology was developed to look at sodium channels affected by pyrethroid insecticides. Concentration-related pyrethroid effects on brain neurolemma isolated from Postnatal Day 15 (PND15) and PND90 rats were measured, but no differences in potency (EC_{50} values between ages) were detected nor inactivation and deactivation tau values. Differences were observed in efficacy values (βmax) but this was suggested to be due to differences in the channel densities between the two ages rather than any sensitivity differences between the channels from rats of the different age groups.

The study does acknowledge the potential for incorrect orientation of the channel in the membrane and incorrect assemblage to the native state that are concerns with this methodology (Ivorra et al., 2002; Eusebi et al., 2009). The scientists indicated that they injected TTX into the oocyte to block any incorrectly oriented Na_V and the signal did not change. The scientists acknowledge that some yet to be characterized channels that are targets of some pyrethroids could be present in the preparation and that could influence the results obtained.

With respect to confidence in the findings of the oocyte experiments, the Panel raised the following concerns:

Sample size: Various statistical tests performed in the different stages of the experiments were based on a very small sample size. As an example, look at the effects of ion channel antagonists on outward ion currents measured during depolarization, one sample t-tests with n=3 were conducted (Figure 7, Appendix 2-2). Although the tests were significant, the very limited sample size raises concerns on the validity of the findings.

Fit of the response-concentration curve: Parametric curves were fit to the concentration-dependent response curves relative to NFA, KB-R7943 and TEA (see Figure 12, 14, 15 in Appendix 2-2 in the CAPHRA white paper). The fit in most cases were based on few sample points, with fits that in some cases looked appropriate (particularly, Figure 14) and fits that in other cases (especially TEA) looked more questionable. As IC50 are estimated from such curves, an increase in the sample size could remove some of the uncertainties related to these findings.

Large uncertainties: There are very large error bars (e.g. SEM) in the plots showing the effect of permethrin and deltamethrin concentrations on TTX-sensitive, inward current, particularly in the plots relative to NFA (see Figure 28, Appendix 2-2 in the CAPHRA white paper). The large uncertainties appeared to increase as concentrations increased (see Figure 28, Appendix 2-2) and they seriously undermine the confidence in the findings. Increasing the sample size and using more data, as the scientists themselves conclude (page 54 Appendix 2-2), could help clarify whether there is a different sensitivity to permethrin and deltamethrin in adult *vs* juvenile rats.

Robustness of the assay: The robustness of the assay is limited by the large variability of the primary data and by the requirement for multiple data transformations to obtain measurements that could be used for comparisons. The current assay also fails to take into account potential differences in the TTX sensitivity of the juvenile channel and how such differences can possibly impact data interpretation and differences between adults and juveniles with respect to pyrethroid sensitivity. The Panel recommended that additional validation studies are needed to clarify the relevance of this model.

b. In the context of your response to 2a, the CAPHRA has proposed to collect dose-response data in adult and juvenile rat synaptic membrane transplanted into oocytes for five more pyrethroids (including Type I, Type II, and mixed). CAPHRA's proposed path forward: If the additional five pyrethroids show similar patterns to deltamethrin and permethrin (i.e. no lifestage sensitivity observed), no additional pyrethroids will likely be tested in this system. Alternatively, if a different pattern is observed, additional pyrethroids are likely to be tested. Please comment on the CAPHRA's proposed path forward.

The Panel acknowledged that the system is capable of examining age-related differences in the sensitivity of rat brain sodium channels. However, the results with deltamethrin and permethrin did not demonstrate any age-related pharmacodynamic differences. It appears that the Na_V components of juvenile and adult P2-neurolemma are structurally identical only differing in the levels of $Na_V1.3$ and Na_V 1.6 present. Perhaps there is some value in testing additional pyrethroids, but the expectation that any age-related pharmacodynamic differences will be detected appears to be small unless a pyrethroid specifically targets $Na_V1.3$ or $Na_V1.6$. Therefore, it was the Panel's opinion that the overall usefulness of this system as the sole component for determining mechanisms associated with possible age-related differences in pyrethroid toxicities appears minimal.

3. ***Targeted in vivo studies in adult and juvenile rat: acoustic startle/detailed clinical observations:*** *Preliminary experiments that measure acoustic startle and detailed clinical observations have been conducted by the University of Cincinnati. In these experiments, juvenile and adult male Sprague-Dawley rats were treated with deltamethrin and permethrin as model Type II and Type I pyrethroids, respectively. In addition, brain and plasma concentrations were measured in PND 15 and 90 rats exposed to deltamethrin. The preliminary results suggest that juvenile rats are more sensitive to deltamethrin based on changes in detailed clinical signs and, to less extent, acoustic startle. However, there was no greater sensitivity in juvenile rats exposed to permethrin. Preliminary data from whole brain tissue samples with deltamethrin indicated that PND 15 rats had higher deltamethrin concentrations as compared to adult rats given the same dose. The brain concentration and toxicity data from the CAPHRA studies for deltamethrin are consistent to those previously published by the Bruckner lab (Kim et al., 2010) which showed that at a similar dose (2 mg/kg deltamethrin) PND 10 rats had increased severity of clinical signs and higher C_{max} of deltamethrin in the brain as compared to PND 21, PND 40, and PND 90 rats.*

a. *The in vivo behavior studies reported thus far are preliminary evaluations. Please comment on the study design in the preliminary in vivo studies for deltamethrin and permethrin conducted at the University of Cincinnati (Vorhees lab).*

In general, the Panel believed that these studies were carefully planned and that the study design included many facets to address scientific rigor and reproducibility. Preliminary studies were conducted to examine potential confounders and to validate the test system. In addition, the Panel commented that the study design also permitted rigorous statistical analysis. Along with these positive observations, the Panel also noted the following shortcomings associated with the studies.

First, the Panel remarked that there is a need for more explanation on the selection of the Postnatal Day 15 (PND15) age in rats, especially as it relates to the development of the nervous system in a 1-3 year old human. This information is important since the Agency's overall interpretation is that "there was no greater sensitivity in juvenile rats exposed to permethrin". However, the rationale for the particular age chosen seems particularly important given that permethrin (PM) was shown to affect the ASR in PND17 and PND21 rats, but not PND15 rats. Thus, the presence of effects on PND17 and PND21 does not agree with the overall interpretation that "there was no greater sensitivity in juvenile rats exposed to permethrin" and leads to questions concerning the appropriateness of the selection of PND15 for testing. While it was indicated that the specific age chosen for juvenile testing was done in concurrence with EPA, the explanation behind this decision was not provided. However, it was revealed during the oral discussions at the public SAP meeting that the decision to use PND15 rats was based on the 2010 SAP where it was stated that the PND15 age was a compromise considering: 1) the neurobiological development required for the ASR tests, 2) brain development in a human child of the targeted age, and 3) age-related metabolic enzyme profiles in rats and humans. This rationale was not provided in the CAPHRA Overview Document and the rationale behind the use of only male rats in the CAPHRA *in vivo* studies was not discussed in the *in vivo* study reports. During the public meeting, it was stated that there were data demonstrating that there were no sex effects. The Panel noted that this data should have been discussed in the CAPHRA Overview Document.

Second, the Panel commented that the rationale for the dose selection in several studies was unclear. For example, there were higher permethrin doses in juveniles than adults. This led the Panel to question whether this range was expected to encompass doses that would cause no effect to doses that would cause a severe effect. However, the dose range finding studies do not appear to have demonstrated a dose that did not cause an effect.

Third, with regard to the pretesting of animals in order to improve performance, it is not clear how this was performed, and how this was used to match groups before the tests started. The potential for biases created by this design should be explained. For example, in Fig 1 of A2-4 the initial V_{max} of control animals varied significantly. This observation raised the question of whether or not this was related to the pretest grouping. It also introduced the question of if the pretesting approach had the potential for causing habituation, thus decreasing the ability to detect differences and reducing the dynamic range. Although this does not appear to be the case with the PM experiments in adults, it is not clear what effect this may have on the overall experimental design. Habituation in the ASR studies with adults after 2 hours could prevent direct comparison with juvenile studies that do not seem to exhibit this phenomenon. The Panel suggested that this issue should be addressed.

Fourth, the Panel noted was there was no clear explanation regarding why Sprague Dawley rats (SD) were selected for the studies instead of Long Evans (LE) rats. Although the data presented in the CAPHRA document certainly show that the SD rats are suitable for the studies, it appeared in the pilot studies the LE rats were giving a more robust response to permethrin.

Finally, the Panel expressed concern that even though both control pups and treated pups originated from the same litter, the litter should have been included in the statistical analysis. The basis for this was since the pups and dams were shipped to the investigators, the dams were required to care for the pups during transit and the shipping could have induced some level of stress in the dams. The magnitude of the response to that stress could have varied between dams and resulted in differential levels of care which could have impacted the behavioral outcomes of both treated and control pups in that litter. Litter could have been included to account for this possibility. During the public meeting, CAPHRA acknowledged that this was not the optimal method to obtain pups but was selected because of the short time frame required to obtain the preliminary data. CAPHRA also stated that in future studies, the pups would be obtained from a breeding colony onsite and not be subjected to the potential stress during shipping. The Panel also noted that there was no chemical characterization information provided for permethrin.

b. In 2010, FIFRA SAP commented on some challenges that were anticipated with the use of auditory startle to compare the relative sensitivity of juveniles and adults. In the studies conducted by the CAPHRA, both auditory startle and detailed clinical observations were evaluated. Please comment on the degree to which the auditory startle and detailed clinical observations provide useful data for evaluating the potential for juvenile sensitivity. Please include in your comments discussion of the dynamic range; the direction of the response varying between Type I and IIs; and the type of data obtained (continuous, ranked).

One study indicated that the tactile startle response (TSR) test provided a somewhat larger dynamic range; however, more studies may need to be done across ages, compounds and doses, to support the robustness of this conclusion. Given that they both appeared to be CNS-mediated, the Panel was uncertain whether switching to TSR could be justified based on the possibility that this would address the problem of non-specific peripheral effects.

The use of ordinal Detailed Clinical Observations (DCO) data is valuable as a secondary observation. However, these data are subjective, and it is not constitute a rigorous unbiased method for decisions related to hazard assessment across many compounds. Relative to DCO, the automated ASR (or TSR) approach is a better primary method for assessing *in vivo* toxicity. However, one panel member noted that the DCO observations, and tremors in particular, might better fit the "adverse" description as opposed to a compound effect.

Adult rats exposed to PM and tested in the ASR did not exhibit any clinical signs of toxicity, including tremors. The Panel suggested that perhaps these tremors did not likely affect the ASR since a proportional change (25%) did not occur in ASR. This suggestion may be true, but it is speculative without more data to support such a proportional relationship. For the PM pre-weaning results, there seems to be no clear relationship between the magnitude of the tremor response and the magnitude of the ASR response. It is unclear which is the more relevant indicator of toxicity. With respect to the ASR data for DLM, CAPHRA concluded that DLM did not induce statistically significant effects on ASR in PND90 rats (Figure 2-5a) while DLM induced statistically significant effects on ASR in PND15 pups (Figure 2-5b). These data suggest the presence of age differences in rats treated with DLM. However, the CAPHRA document states that "*This profile of age-dependent sensitivity to deltamethrin is consistent with published results, which show no difference in sensitivity of PND 21 and adult rats at low doses of deltamethrin, based on decreased ASR, and a marked increase in toxic response in juvenile rats as doses are escalated (Sheets et al., 1994).*" In the CAPHRA document, the dosage of 1 mg/kg did not result in statistical significance in PND15 pups but it was not tested in the PND90 animals. The lowest dosage used in both ages was 2 mg/kg that was statistically significant in PND15 rats but deemed to not be significant in PND90 rats at any time. However, the PND90 ASR data suggest the presence of a biologically relevant effect. For example, ASR was reduced in PND90 rats at 2 hrs. (Figure 2-5a. of the CAPHRA Overview Document) by 40-48% with all three dosages of DLM. The statistical model used by the scientists did not indicate any statistical significance. However, when the 95% confidence intervals are calculated using the means and standard error of the data, there is no crossing of intervals between the treated groups and the control group suggesting that they may be different. In fact, the only time treated and control confidence intervals crossed was at 4 hrs. in the 2 mg/kg DLM group. This issue decreased the confidence of the Panel concerning the absence of significant effects on ASR in PND90 rats exposed to DLM.

General Comment:

Historically the ASR study has been a measure of pyrethroid neurotoxicity and data generated by this study should be comparable to values in the literature. However, the relevance of the ASR to either observed or potential effects in humans has not been established. The question is whether there are other measures of functional neurotoxicity that could be dissociated from non-specific effects, and that have more relevance to observed neurotoxicity in humans. It is recognized that there may not be enough or any data in humans to design such a model, so tremors and/or writhing in rats may be more sensitive and relevant to the human condition. Another primary question is whether ASR is linked to any known mechanism of action for pyrethroids, including their effect on sodium channels, or other ion channels.

As an overall conclusion, the Panel concurs that it does appear that use of the ASR for juveniles is problematic because of the overlap with non-specific effects. Going forward, the Panel recommends further testing with the TSR, either alone or in combination with the ASR, to see if the test is sensitive enough to separate out the specific effects from the non-specific effects.

c. *Please provide comments comparing the temporal pattern and magnitude of the brain and plasma concentration data from the Vorhees & Bruckner labs and utility of such data to aid in the interpretation of the auditory startle and detailed clinical observation data.*

For DLM, the time of the rise in plasma and brain levels did not match the peak in ASR effects; however, the trends are consistent considering the slight delays. It is not expected that these should match perfectly since there are several unknown factors involved in the potential dose- and time-related mechanisms underlying the effect of DLM on ASR.

A plan to continue investigating the comparison in levels of neurotoxicity in young and adult rats with similar levels of exposure (concentrations) within the brain may be appropriate. If the data support the available findings to date, the Panel would concur that there would be no further *in vivo* work needed to resolve the pharmacodynamics question.

While it may be important to examine the effects of pyrethroids in young and old rats at a matched internal dose (e.g. brain/plasma concentration), the difficulties in obtaining a high enough dose in adults and the temporal variability in these doses may be prohibitive. This difficulty must be weighed against the added value of these data to the overall question of whether young rats/humans are more affected by a given external dose, regardless of intrinsic differences. The Agency will need to consider what information will be critical for the final hazard assessment and regulatory activity.

d. Please comment on the use of a 5 ml/kg dosing volume in the CAPHRA studies and how any impact on pyrethroid kinetics affects correlations with behavioral effects.

The Panel concluded that in the DLM experiments the dose volume did not significantly influence the timing of ASR effects. However it was clear that a large volume delayed the effect on ASR in other studies, and overall, it was not clear how the results of the experiments varying dose guided a final decision of using 5 ml/kg in future testing.

The Agency should consider the question: Is a slower release and delayed or extended period of the ASR desirable? For example, is it better to give a smaller volume and test at 2 hours, or larger volume and test at 4 or 6 hours? The answer may lie in the optimal study design for comparable results across compounds, as well as how best to design the model in order to simulate exposure in humans. The issue of habituation is relevant here as well since an extended period of exposure allows more time for habituation to occur. A particular concern is how differences in the apparent habituation responses of adult and juveniles contribute to understanding of physiological effects of pyrethroids.

Considering the Agency goal to modify toxicity testing not only to reduce animal use but also to test more human relevant doses, it might be worth a consideration going forward if it would really be necessary to push the doses of low potency compounds to such high levels. There does not yet appear to be a large database on this volume effect *in vivo,* but there could be a concern that for a potent compound an effect could be observed at a lower dose using the smaller volume, even with a later evaluation time for the larger dose volume. A positive control, such as DLM, could be included in testing of low or unknown toxicity compounds at the limit of solubility or if a higher dose volume was used.

The Panel questioned the justification of using 5 mL/kg in the CAPHRA studies given that previous studies in the literature used 1 mL/kg. CAPHRA stated that the methods were developed to allow testing of additional pyrethroids in the future. Many of these pyrethroids have very low toxicity and very high dosages would be required for testing.

Several panel members were concerned that the 5 mL/kg dosage rate would be a large volume to administer especially to a PND15 pup. Other members were not as concerned given that a SD PND15 pup weighs on average 30 g and administering a 5 mL/kg solution would only be 0.15 mL which is only about 5% of the total stomach volume (2.8 mL). However, the Panel agreed that there is a concern since this amount was administered to a pup with a full stomach of milk. It was stated that 15 day old rat pups were not removed from the dam prior to dosing which did not allow gastric emptying. This is different from the studies with adults who were removed from food to allow gastric emptying prior to administration of the pyrethroids. It is possible that the milk in the stomach altered the absorption and pharmacokinetics of the pyrethroid. Given how a larger volume (5 mL/kg) of corn oil used to administer 120 mg/kg permethrin to adults delayed the onset of behavioral outcomes as compared to the same dosage administered in a lower volume (1 mL/kg) (Appendix A2-4), the presence of milk could have delayed the onset of behavioral outcomes in juveniles following DLM exposure.

4. ***Pharmacokinetic studies:*** *A number of pharmacokinetic studies using deltamethrin were performed by the CAPHRA in order to further refine and validate the developing rat PBPK model and construct a developing human PBPK model. For refinement of the rat PBPK model, tissue: plasma partition coefficients (in vivo measurements in PND 21 and adult rats), age dependent plasma protein binding (in vivo measurements in PND 10, 15, 21, and 90 rats), and cytochrome P450 (CYP) and carboxylesterase (CES) metabolism (in vitro measurements using rat liver and plasma preparations from PND 15, 21, and 90 rats) were evaluated. Further in vivo pharmacokinetic studies in rats were conducted with single IV (PND 90), single oral (PND 90), and multiple oral (PND 15, 21, and 90) doses to generate plasma and tissue data to validate the developing rat PBPK model. Additional experiments with deltamethrin were conducted for constructing the developing human PBPK model. Parameters evaluated in vitro included age dependent plasma protein binding derived in plasma from human donors aged from birth to adults, transport across the blood brain barrier using a human brain microvascular endothelial cell line, estimates of gastrointestinal absorption in caco-2 cells, and ontogeny data for CES, CYP1A2, and CYP2C8 determined in human liver tissue from donors (age 1-18 years old). Further in vitro experiments are currently underway by the CAPHRA to determine human adult and juvenile liver metabolism of deltamethrin by CYP and CES enzymes using human liver preparations or recombinant human enzymes*.

   a. *Please comment on the in vitro experiments to support the PBPK model development in the rat conducted thus far for deltamethrin.*

The *in vitro* work conducted so far by CAPHRA has generated important information for the development and validation of a PBPK model for deltamethrin in a developing human. Overall the Panel thought that the experiments that were either completed or underway were excellent in quality and appropriate for addressing the age-dependence of liver and plasma metabolism and protein binding. The age-dependence of these kinetic factors is particularly important to be captured in the model, and the experiments have quantitated important issues that are critical for the IVIVE approach such as looking at the age-related changes in the relative amounts of CES1 and 2 in microsomal and cytosolic fractions.

The Panel commented that the plasma binding study was thorough and produced high quality data, because it differentiated binding by age as well as by albumin, total protein, and lipoproteins; therefore Bmax and Kd values were useful for the model development.
The experiments clearly showed the age-dependence of plasma binding in rats with progressively increasing levels of albumin and total proteins. The extent of binding of deltamethrin at 250 nM was similar among ages except for the 1wk and 4wk age-groups, suggesting that total binding, at exposures equal to or lower than 250 nM may not have significant impact. This was noted to be true for total binding, protein binding, and albumin binding, but not lipoprotein binding.

For the vitro metabolism studies, the CAPHRA program has made considerable progress with proposed enzyme kinetic work on the metabolism of deltamethrin with rat hepatocytes, liver microsomal and cytosolic fractions, and plasma from male Sprague Dawley rats. Hepatic metabolism of deltamethrin in microsomal and cytosolic fractions was shown to be comparable to that by primary hepatocytes, which was an important confirmation on the use of microsomes

and cytosol as the surrogate for hepatocytes. The Panel thought this was critical information to support the IVIVE approach to be used for the development of the pyrethroid PBPK models. The Panel recommended that the CAPHRA program should complete the proposed metabolism work, in particular enzyme preparations from various age groups. The Panel also noted that the cell lines used for the blood-brain barrier penetration potential (hCMEC/D3) and for intestinal permeability and transport (Caco2) were cell lines that are widely used for the respective studies.

A few areas where the Panel identified deficiencies in the work included the lack of data or experiments addressing age-related differences in permeability and active transport at the blood brain barrier. This will be crucial information for reducing uncertainty in model predictions, particularly since permeability-surface-area cross product was an influential parameter governing brain concentrations of deltamethrin in the PBPK model.

In addition the Panel was not able to find or identify *in vitro* studies that addressed possible age-related changes in the absorption of deltamethrin. There was a lot of discussion during the meeting with regard to how absorption has been integrated into the model. The current PBPK model structure has absorption completely bypassing the liver and directly entering the lymphatic system. As these are critical model assumptions that affect model predictions of age-related changes in the brain Cmax, the Panel recommended conducting studies with appropriate in vitro systems to support the modeling work and parameter values.

For the metabolism studies, the Panel recommended additional studies including: 1) tissues from female rats to address sex-related differences in metabolism; and 2) extrahepatic tissues metabolism including the gastrointestinal tract and the brain. Information on the metabolic clearance of pyrethroids, including deltamethrin by intestinal tissue, could be important and may present challenges for modeling of any pyrethroid compounds that undergo significant biotransformation through intestinal CYPs and carboxylesterases. Some panel members recommended monitoring both the loss of deltamethrin and the formation of products to gain additional confidence on the results.

b. Please comment on the in vivo experiments to support the PBPK model development in the rat conducted thus far.

The CAPHRA program systematically carried out and collected high quality data for numerous toxicokinetic and tissue distribution studies, which had several strengths including: (1) use of various age-groups to determine age-related changes in toxicokinetics; (2) administration of deltamethrin by intravenous and oral dosing to investigate bioavailability (although some concern was expressed with regard to results); and (3) dosing over a range of deltamethrin concentrations doses to provide important information on the detoxification capacity (saturation). However, the Panel expressed some concerns about the study design and the data analysis. While single intravenous (IV) or oral dosing for pharmacokinetics studies are necessary for establishing kinetic parameters, it does not reflect true environmental exposure to deltamethrin or other pyrethroids. The charge question mentioned multi-dose studies as part of the suite of *in vivo* studies conducted, but Panel members were unable to locate the information in either the review documents or the presentations. Repeated administration with doses relevant to true environmental exposure should be considered. In addition the experiments also showed that

PND15 and PND90 but not PND21 groups had dose-dependent increases in Cmax values (Figure 3-2), but there was no explanation for this outcome, which should be addressed. The review documents indicated that male rats were used for the PND90 group, mixed sex was used for the PND21 group and unspecified sex was used for the PND15 group, but did not provide justification for the differences in sex selection.

One issue of concern to the Panel was the effect of delivery vehicle on absorption, in particular the absorption route to the systemic circulation (i.e. lymphatic system versus the portal system). There was also concern about the results and analysis of the kinetics from the IV versus oral exposure. As noted by one panel member, comparison of the Dose/AUC ratios would suggest less than 10% of the administered dose was bioavailable, but CAPHRA members stated that 30 to 40% of the administered dose is eliminated fecally. If first pass metabolism is not occurring due to absorption into the lymphatics, then CAPHRA needs to resolve the difference in measured fecal levels versus estimated bioavailability from the kinetic studies. This is important because the assumption of bypassing the liver in model may partly contribute to the modeling results, which predicted a higher brain Cmax in the adults versus children.

CAPHRA provided an explanation in their Overview Document about the limitations of *in vitro* or algorithm-derived partition coefficients for deltamethrin. Therefore, the Panel deemed the *in vivo* method to be appropriate for measuring the tissue plasma partition coefficients for various types of tissues. One area that was noted as needing explanation was the lack of age-related differences for permethrin as compared with deltamethrin. The lack of difference had been noted in the Overview Document, but no further information was provided. These differences in age-related effects among pyrethroids will be important to understand in order to reduce uncertainty in predictions for extrapolating the PBPK model across the family of pyrethroids. The Panel pointed out that although there are limitations and issues for the predictive ability of algorithms, these comparisons of the measured values with those derived empirically will be important as the modeling efforts advance to other pyrethroids that have not been proposed to have partition coefficients measured experimentally.

c. Please comment on the in vitro experiments in the human tissue conducted thus far. Please include in your comments a discussion of the ongoing in vitro experiments with recombinant enzymes for use in PBPK models and associated confidence and uncertainty with the use of such data.

The Panel noted the approach that CAPHRA was using for the IVIVE with recombinant enzymes seemed to be thorough and a logical method strategy to predict metabolism in the liver across age groups. The Panel noted several areas of confidence for the approach including: (1) Ontogeny data (expression) on human liver carboxylesterases (performed by Ronald Hines and colleagues) were consistent with data reported in the literature; (2) Ontogeny data (expression) on various CYP2C8 and CYP1A2 enzymes (performed by M Yoon and colleagues) in the liver were consistent with data reported in the literature as well; and (3) Enzyme kinetics will be determined with recombinant CYPs and CESs.

There were some aspects of the experiments that were of concern to the Panel and would generate uncertainty in the use of the data including: (a) lack of studies determining hepatic

clearance with primary human hepatocytes from various age populations (cryopreserved hepatocytes); and (b) determination of ontogeny expression of various enzymes by different laboratories. The Panel questioned why the same samples were not used for all types of enzymes to minimize variations raised from different laboratories. Although either purified recombinant enzymes or lysates from overexpression likely produce robust signals and the results from these preparations are informative, the recombinant enzymes may differ from native enzymes, for example with respect to posttranslational modifications and the cellular environment in which the enzyme resides. Therefore, some data from recombinant enzyme studies should be validated with native enzymes. Human liver enzyme preparations with different expression of an enzyme of interest are usually used for this purpose. In addition it would be interesting to have seen a proof of concept with using the scaling from recombinant to either a human hepatocyte or a combined microsomal/cytosolic incubation.

5. ***Physiologically-based pharmacokinetic model in rat:*** *Using data described in Question 4, the CAPHRA has developed a PBPK model for deltamethrin using age-specific metabolism parameters in rats to simulate plasma and brain internal exposures in young and adult rats. This PBPK model relies on in vitro to in vivo extrapolation (IVIVE) to use age-specific metabolic data collected in vitro to estimate hepatic metabolic clearance in vivo. The deltamethrin brain or plasma concentrations estimated by the model in rats are then compared to measured concentrations from in vivo rat studies to verify the model. Non-chemical specific physiological parameters for rats were obtained from the published literature, including body weight, cardiac output, hematocrit levels, tissue volumes, and tissue blood flows. As discussed in Question 4, recently generated data by the CAPHRA and published data were used for compound-dependent parameters, including partition coefficients, metabolic rate constants, absorption rates, protein binding, compartments and tissue permeability.*

a. *Please comment on the robustness of the rat PBPK model for simulating internal exposures in the developing rat. In your response, please include evaluation of the structure and parameters used to build the model, as well as its ability to accommodate different oral absorption scenarios (i.e. different vehicles used for in vivo studies) and discussion of confidence, accuracy and uncertainties associated with the deltamethrin developing rat model. Please also comment on the sensitivity analyses of parameters CAPHRA has completed thus far.*

Comments concerning the model were based on CAPHRA's presentations during the public meeting and the information given in the overview. Panel members did have some concern about commenting on the confidence, accuracy and uncertainty of a model that did not appear to be in the final form. However, the Panel recognized the fluidity of the modeling process and commended the researchers and modelers on the iterative nature of the work with the experiments informing the model and vice versa.

The model-predicted concentrations of deltamethrin in the brain and plasma of PND10, PND15 and PND90 rats have been simulated with a PBPK model structure, which built upon the model by Tornero-Velez et al. (2010) and expanded it to account for: (1) vehicle-specific absorption of deltamethrin; (2) plasma metabolism; (3) age-specific plasma protein binding; and (4) the metabolic contribution of CES to pyrethroid hydrolysis. As shown in Tables 3-1 and 3-2 of the

overview document, parameters for the rat PBPK model were derived from a variety of sources, including both values reported in the literature and CAPHRA-generated values. The Panel felt the incorporation of this additional information increased the confidence and accuracy of the model.

With regard to CAPRHA's sensitivity analyses conducted thus far, the only local sensitivity analysis (LSA) that was available in either the presentation or the overview was for the human, and not the rat. The Panels' comments relating to the results of that analysis were included in Charge question 6a as it related to confidence and uncertainty in certain model parameters with regard to the human. As a general statement, the Panel considered conducting sensitivity analyses as an important component during model development and parameter calibration, but one that has to be interpreted in light of the existing model structure and parameter values, since it is a local sensitivity analysis. For example the sensitivity coefficients (SC) may have changed based on the dose or within the range of plausible values for the parameters. Therefore a global sensitivity analysis was recommended for the modeling work. In addition with regard to the LSA, the SC will change over time, therefore inclusion of other times may be important to gain confidence over the entire time profile of pyrethroid concentrations in the brain.

Model Structure:

There were two places where the comparison between the model simulations and the data raised concern among the Panel about either the adequacy of the model structure or the parameters used for the model. Slides 40 to 44 presented the model comparisons with literature and newly generated data for different age rats. There was deviation of the model fit from the data both with regard to prediction of Cmax and Tmax and with comparisons during the terminal regions of the concentration time profiles, particularly for the brain concentrations in the older rats. The altered model structure of a two-compartment absorption system was an appropriate method to accommodate delayed absorption when administered orally and sought to address the difference in vehicle-related kinetics CAPHRA noted in the rats. However the parameters appear to need to be calibrated to provide better correlation of predicted versus observed plasma concentrations.

There was some concern among panel members about the model structure switch from uptake to the portal system to completely bypassing the liver via the lymphatic system. Not enough evidence was presented to support this model structure assumption in humans, nor were modeling comparisons given to allow the panel to judge the effect on model predictions in humans. Therefore, there was low confidence in model predictions because of the uncertainty in the validity of this structure. As demonstrated during the CAPHRA slide presentations, lymphatic absorption would bypass the liver and the pyrethroid would not be subjected to first pass metabolic degradation by hepatic CES and CYP, which have age-related differences. Therefore, a model with only lymphatic absorption eliminates the potential for age-related differences in the systemic concentrations of the pyrethroids due to first pass metabolism, and the Panel was concerned that could lead to errors in comparing age-dependent levels of pyrethroid reaching the brain. However, when one panel member briefly played with the model and the parameters, there appeared at least for the PND 90 rat to not be a large difference in assuming gut uptake vs lymphatic uptake on brain concentrations. The Panel was unable to successfully load the human model, which may be different from the rat.

Model Parameters:
In addition, there was some uncertainty with regard to how the absorption parameters were determined for the various vehicles. The documentation seemed to only show the values for corn oil. In the model there were three parameters that could be changed to affect the rate of absorption either to the gut or through the lymphatics. The Panel was uncertain from the documentation whether better fits could be obtained for the early time point data for the different vehicles. There should be a quantitative versus visual assessment conducted to determine whether the parameter values are vehicle-specific. The lack of a quantitative assessment of the adequacy of fit for the absorption parameters decreased the confidence in the model predictions.

With regard to the discrepancy in model predictions at the terminal region of the brain concentration versus time curve, one parameter that could have influenced the predicted brain concentrations was the permeability-surface-area cross product. The model assumed there was no age-dependent change in the blood-brain barrier permeability, however it was curious to note that the model provided better predictions in the younger rats (PND10 and 15) than in PND 90. Upon working briefly with the rat model code, one panel member found that decreasing the PABRC (the permeability-surface-area cross product for the brain) resulted in prolonged retention of deltamethrin in the brain. Therefore there was a lack of confidence in the assumption of no age-dependent changes for the permeability-surface-area cross products, which affects the assumptions regarding humans. This appeared to be a parameter that was highly uncertain and the Panel recommended the penetration of the blood-brain barrier as a function of age should be investigated and incorporated in the model.

In briefly looking through the rat model code, the majority of the code looked appropriately described. One minor note from the Panel: In the model code, the percentage of brain attributed to blood volume in the tissue was slightly higher than that reported in Brown et al. 1997. Also the volume in Brown et al. (1997) is for total blood, therefore the volume in the model needs to be adjusted to represent the plasma volume, since the models used tissue: plasma partition coefficients. The cardiac output and tissue flows were adjusted to represent plasma flow to the tissues.

In addition, as one panel member noted, the robustness of the rat PBPK model could have been evaluated by comparing the predicted concentration curve of deltamethrin in plasma and brain in PND10, PND15 and PND90 rats with both data reported in published studies and data collected by CAPHRA-supported studies (the new CAPHRA data).) As noted in the document, the modality of administration used was different in the two sets of studies, the first used glycerol as the oral gavage vehicle at a volume of 1ml/kg body weight, while the new CAPHRA studies used corn oil at 5 ml/kg body weight. Also the dosage used in the two sets of studies was different: 2 and 10 mg/kg in the published studies for PND90 and 0.4, 2 and 10 mg/kg in for PND10.

b. In the context of your response to 5a, please comment on the extent to which additional data are/are not needed to refine the developing rat model.

The Panel recommended several studies that were needed to better validate the predictions of the current model and provide further refinement,particularly with respect to predicted brain concentration of deltamethrin in older versus younger rats. In charge question 4, there was mention of multi-dose studies, but the Panel could not locate the study results in the main document, presentations, or appendices. The model has been compared only with single oral doses; however, data from repeated dosing of pyrethroids are important to look at the potential accumulation in the brain and as a source to validate model predictions. The Overview Document stated there was no expected accumulation of deltamethrin, but it did not present supporting data. Since the neurological effect of pyrethroids are correlated to Cmax, this information is important to document, as well as model accuracy. In addition repeated exposure may result in induction of enzymes, which will affect the toxicokinetic profile. The induction also would need to be performed with hepatocytes from different ages to assess possible age-related changes.

The model structure with the absorption directly into the lymphatic system was regarded by the Panel as plausible but not well supported by the current experimental data or by model predictions of the data. The Panel thought that information on the impact of the lymphatic absorption in the model would be useful if it is to be incorporated into the final structure, and *in vivo* measurement of the lymphatic transport as supporting evidence would be crucial. Another aspect of the bioavailability that was not incorporated into the model was metabolism in the GI tissues. Extrahepatic metabolism, and in particular potential age-related differences, will be important to address, since it will affect plasma and brain concentrations. The model currently assumes 100 percent bioavailability as a worst case scenario, but is also predicting higher brain concentrations in the adult versus infant. Portal system absorption with greater metabolism in the adult versus infant, may result in model predictions of greater concentrations of pyrethroids in the brains of infants versus adults.

Data are also needed to evaluate the possibility of age-dependent differences in the blood-brain barrier. Based on the discussions of the Panel and the demonstrated influence of the model's brain permeability-surface-area cross product on predicted brain concentrations, this will be important data to provide confidence in the use of the PBPK model for risk assessment for children.

6. Physiologically -based pharmacokinetic model for human: *Similar to the rat PBPK model, the human PBPK model integrates non-chemical specific physiological parameters for humans from the literature. Compound-dependent parameters, such as partition coefficients and oral absorption parameters, were adapted from the rat PBPK model. Recently generated data by the CAPHRA were used for the remaining compound-dependent parameters, such as metabolic rate constants, protein binding and tissue permeability. With respect to metabolic constants, CYP and CES enzymes involved in metabolism of a given pyrethroid will be identified and the in vitro metabolic constants for those enzymes will be determined for integration into the PBPK model. Intrinsic clearance for each active enzyme will be scaled to in vivo using scaling factor data collected by the CAPHRA and the SIMCYP database. The*

ontogeny of enzyme expression (also from the CAPHRA data and the SIMCYP database) will be incorporated into the process of obtaining distributions of age-specific intrinsic clearance for each enzyme. Preliminary simulations have been conducted for deltamethrin to demonstrate the process used with the PBPK model.

a. *Within the context of understanding potential juvenile sensitivity, characterize the robustness of the PBPK model for extrapolating age-specific internal tissue exposures for humans. In your response, please comment on the structure and parameters used to build the model and include discussion of confidence, reliability, and uncertainties associated with the deltamethrin human model. Please include in your comments a discussion of the data from the McCarver/Hines Laboratory (submitted as part of the CAPHRA package) for providing ontogeny of CES enzymes.*

The Panel appreciated the effort of CAPHRA to develop a human PBPK model to extrapolate internal tissue exposure to pyrethroids (specifically deltamethrin) in humans, with the ultimate goal to assess whether juveniles experience an increased sensitivity to pyrethroid neurotoxicity. Following on the same construct of the rat PBPK model, the human PBPK model for deltamethrin estimated/predicted tissue exposure to deltamethrin by modeling age-related physiological and metabolic processes that affect the chemical kinetics. Several age-specific parameters were used to represent the various physiological processes: body weights, tissue volume, tissue body weight fraction, partition coefficients, tissue permeability, etc. Values for these parameters were derived from various sources including among others the published literature, CAPHRA-supported studies, and rat models. In some cases, as for example, for the metabolic rate constants, the values refer to adults and not juveniles. The Panel expressed the following concerns regarding some of these model parameters:

1- The Panel noted some discrepancies between some of the model parameters/model structure reported in CAPHRA's presentation during the public meeting and the parameter values/model structure reported in the Overview Documents (specifically, with respect to the GI uptake and the permeability-surface-area cross products). The Panel recognizes that a possible justification for the discrepancy could be the continuous efforts of CAPHRA to update and improve the model. The Panel also encountered difficulty in accessing the model files from the provided Dropbox folder because some of the files, in particular the executable for the human model in the software acs1X, did not load properly.

2- The Panel noted a difference in the values for the permeability-surface-area cross products listed in the overview document and the parameters presented by CAPHRA during the public meeting. The Panel could not understand the reason for a discrepancy in the values. The values in the presentation did not match the values listed in the m files for the model, which the Panel noted as fitted. As noted in the CAPHRA's overview document, brain permeability was an influential parameter in the model, since accurately predicting brain kinetics will be necessary to correctly predict toxicity.

The Panel had the following concerns regarding the model structure specifically:

1- The Panel raised concerns regarding the fact that the model switched from uptake to the portal system to bypassing the liver completely via the lymphatic system.
In addition, the Panel was concerned about whether there were differences between rats and humans in the role of transporters in gut absorption.

2- The Panel expressed concerns regarding how the structure of the brain was parameterized in the model and how that would impact Cmax predictions. CAPHRA appears to have conducted experiments to address this aspect of deltamethrin pharmacokinetics and the summary data provided appears to not support significant involvement of transporters. However the low brain: plasma concentration ratio from the steady state experiments seemed to suggest that there is more than just a permeability limitation for the distribution.

3- The Panel recognized as a major challenge the difference among rats and humans in the metabolism of deltamethrin. While hydrolysis seems to be more important than oxidation in humans, the opposite is true in rats. In light of this observation, the Panel raised concerns about whether the hydrolytic clearance was over-estimated in humans, and they advised CAPHRA to investigate whether the estimated clearance supported by human hepatocytes varies with age.

4- The Panel highlighted the fact that the available human data showcased large variability among individuals, presumably and at least in part due to induction.

5- The Panel found it unclear why 1-year olds were used since the significant differences were found for 1 month-old. Additionally, the Panel was unclear on how the Monte Carlo approach was implemented (distributions used, number of simulations, etc).

The Panel recognized that the model does appear to incorporate reliable sources for the average age-specific physiological parameter values. One parameter that the Panel encourages CAPHRA to look at more closely is the percentage body fat: a fairly narrow range is given for children compared with adults. The Panel believed that there should be more variability in body fat percentage especially if sex is taken into account: the common approach in the literature on percentage body fat algorithms is to account for both age- and sex-specific variation.

The Panel suggested that CAPHRA consider the following suggestions in the next modeling endeavors:

1. Change the definition of clearance: The use of average clearance for an age group with large individual variations tends to exaggerate the capacity of clearance and under-estimate the risk for those whose clearance capacity is at the bottom end. Thus, the Panel suggested replacing average clearance with the 10^{th} percentile of clearance in the model.

2. Change the age grouping when modeling the liver function: During the first year of life, the liver undergoes a functional switch: from a blood-production organ to a metabolic organ. Based on the expression of carboxylesterases, it is not appropriate to group children altogether. Rather, the Panel believed it is reasonable to have several age subgroups: one month, 2-3 month, 3-6 month and 7-12 months.

3. A void using western blotting: Western blotting can detect a relatively narrow range of variations (struggling with even 10-fold). In comparison, the activity can detect a large dynamic range of variation. In particular, for a given group of samples, the range of variation detected by Western blotting tends to be much smaller than the range of variation detected by activity. Therefore, a western-blotting based approach tends to exaggerate the capacity of clearance.

Given the issues mentioned above, and the fact that the Panel was not provided with sufficiently updated information, neither in the background material nor in the presentations, the Panel found it very hard to comment on the confidence, reliability and uncertainty of the predictions of age-related increase in Cmax in the brain.

One aspect of the modeling effort that the Panel expressed confidence in was the expertise of the individuals involved in the work: the investigators involved in the modeling have extensive expertise and knowledge in the field and are experienced in extrapolating among species and with IVIVE.

6b. Please comment on the proposed use of SIMCYP for providing enzyme ontogeny patterns and deriving population distributions for metabolic parameters in humans. Please include in your comments whether or not other tools with similar capacity to SIMCYP are available.

The Panel recognized that there is a general acceptance of the SIMCYP Simulator by the modeling community as a tool to provide enzyme ontogeny patterns and derive population distributions for metabolic parameters in humans. This Simulator has a pediatric module that integrates developmental physiology and age-related drug elimination pathways. As for the elimination pathways, the SIMCYP Simulator is equipped with well-characterized enzyme systems such as cytochrome P450s and UDP-glucuronosyltransferases as well as some transporter systems such as P-glycoprotein. Generally, using SIMCYP Simulator, an adult model is built and then scaled down to children. This approach has been used to build models for drugs and some of them are quite predicative.

The Panel believed that pediatric modeling with the SIMCYP Simulator was robust particularly for drugs that are 1) primarily excreted through the kidney, 2) undergo minimal metabolism or are metabolized by a single enzyme system, 3) show some but not extensive protein-binding and 4) have limited involvement of active transport. The Panel, at the same time, pointed out potential challenges for the pediatric modeling of pyrethroids. These challenges are raised from two perspectives. First, pyrethroids, as a class of chemicals, exhibit the unique modeling-challenging characteristics including high-levels of protein binding, extensive metabolism by multiple enzyme systems and potential involvement of active transport as suggested by CAPHRA for uptake. Second, there are several knowledge gaps regarding the metabolism of pyrethroids in humans.

1. Both hydrolysis and oxidation are involved in the metabolism of pyrethroids such as deltamethrin. It is not clear whether and to which extent these two elimination pathways differentially contribute to the overall clearance of a pyrethroid.
2. Both hydrolysis and oxidation are present in the liver and extrahepatic tissues, probably varying depending on an organ. There is no information on organ-specific clearance of a pyrethroid as well as age-dependency in this regard.

3. Pyrethroids are activators of pregnane X receptor that supports differential induction of cytochrome P450s over carboxylesterases in humans. It is not clear how the differential induction leads to changes of the contribution of respective elimination pathways. The complication is that induction of drug-metabolizing enzymes is inversely related to age, meaning the pediatric induction has a greater impact on the changes of drug clearance.

Throughout the public SAP meeting, particularly during the pharmacokinetic discussion, the Panel made some suggestions that will likely enhance the confidence and performance of the pediatric model for this class of insecticides.

1. *Incorporation of organ-specific clearance ($CL_{int,\ app}$) into the modeling (liver, brain and intestinal mucosa)*: The clearance rates should be estimated based on the metabolism of a pyrethroid with subcellular fractions or intact cells if possible such as pediatric and adult human hepatocytes.
2. *Specification of differentially regulated expression of hydrolysis and oxidation pathways by pyrethroids*: If possible, induction studies such as in hepatocytes should be performed to ascertain how the hydrolytic and oxidative elimination pathways are differentially impacted by pyrethroids.
3. *Subgrouping of children at 1 year of age or younger*: The first year of life undergoes tremendous changes in metabolic capacity. This age group should be sub-grouped further to reflect these changes to minimize the impact of large variations.
4. *Alternative modeling platform*: In addition to the SIMCYP Simulator, an alternative modeling platform should be used for comparison. There are other softwares for modeling and simulation as reported by Jamei M et al, 2009 and by Khalil and Läer, 2014.

7. Physiologically -based pharmacokinetic models for humans with other pyrethroids*: Thus far, the CAPHRA has focused its human PBPK efforts on deltamethrin and, to a lesser extent, permethrin. Soon laboratory efforts will turn to other pyrethroids. CAPHRA's proposal is to conduct fewer studies and in lieu of such data, use read across and computational approaches.*

a. Please comment on the appropriateness of the current human PBPK model to be used for other Type I, Type II, or mixed type pyrethroids. Please include in your response evaluation of the path forward provided by the CAPHRA regarding in vitro and in vivo studies for other Type I, Type II, or mixed type pyrethroids.

The Adverse Outcome Pathway is a combination of pharmacokinetics and pharmacodynamics. Both have to be adequately understood to read across (i.e. extrapolate) to other compounds. The Panels comments on this charge question were focused only on extrapolating the pharmacokinetics across the family of pyrethroids. The Panel pointed out that it was difficult to make statements on a model that appeared to still be in development. The model structure and results differed between material provided in the overview document and the presentation of the work during the meeting. The Panel felt that they were being asked to make comments and recommendations on a concept that had not yet been thoroughly vetted.

The Panel noted that in theory, if the issues raised with regard to the rat and human model were addressed to decrease uncertainty in the model and increase accuracy of model predictions compared with data, then a read across with chemical-specific parameters would be appropriate. The Panel did not anticipate that the pharmacokinetic model structure would need to be different for the different pyrethroids, but the parameter values would differ. As pointed out by one panel member, one challenge of modeling these compounds is variability in metabolism. For example, bioallethrin (type I) and deltamethrin (type II) are both preferably hydrolyzed by CES1. Bioallethrin, compared with deltamethrin, is a better substrate of CYP3A4. Adding to the complication is the fact that hydrolysis varies depending on the isoform. For example, cis-permethrin is hydrolyzed comparably by CES1 and CES2. However, trans-permethrin is predominately hydrolyzed by CES2. Metabolic clearance for individual pyrethroid compounds must be determined to allow models to be developed with better predictive ability:

Based on the presentation of the path forward that was presented by CAPHRA, the Panel made the following recommendations:

1. The proposed path for quantitation of the partition coefficients would be appropriate if QSAR or predictive algorithms are demonstrated to be useful for predicting measured values from the early stage pyrethroids. In addition it is not just partition coefficients that need to be assessed among the pyrethroids but also the permeabilities that are influential in determining the tissue concentrations. These permeabilities, particularly in the brain, need to be determined.

2. The *in vitro* kinetics were recommended to be determined for the juvenile microsomal and cytosolic fractions for at least permethrin because of its isomeric forms. This may affect the proposed IVIVE approach with recombinant enzymes. Deltamethrin does not have isomeric forms, therefore, permethrin may serve as a prototype to guide modeling of other pyrethroids.

3. The Panel recommended developing the PBPK models in adult rats for all pyrethroids if the data is generated. This would provide further support for the approach for comparison of the adult with the juvenile rats. The data would be available and the modeling would not require extensive effort once the approach is refined with deltamethrin and permethrin.

8. Integration of lines of evidence:

a. The tissue dosimetry data from the rat suggest higher brain levels in juveniles compared to adults. In contrast, the preliminary PBPK modelling for human predicts slightly lower brain concentrations in young children compared to adults. Please comment on these differences, including comments on the key inputs that lead to this difference, human variability associated with the key parameters, and the confidence and uncertainties associated with the difference between the rat and human internal dosimetry.

The Panel recognized that, through both the overview material and the background information provided for each project, CAPHRA has generated a significant volume of information on pyrethroid toxicity in addition to having compiled, discussed, and presented historical data and current research by others. The data generated constitutes different lines of evidence that may point in different directions with respect to the ultimate goal of evaluating juvenile sensitivity to

pyrethroids. Tissue dosimetry from the rat model suggest that there is an expectation of higher brain levels of pyrethroid concentrations in juvenile compared to adults, while the PBPK model for humans predicts lower concentrations in young children vs. adults' brain.

Combining these two lines of evidence to form a conclusion using a weight of evidence approach requires a better understanding of the uncertainty, confidence, and reliability of each line of evidence since weight of evidence is an approach that, by means of qualitative or quantitative methods, integrates individual lines of evidence to form a conclusion (Linkov et al., 2009). A fundamental issue in the weight of evidence approach is a critical understanding of the reliability, robustness, and relevance of all the evidence.

The Panel has already addressed issues regarding the uncertainty and confidence on the individual lines of evidence in charge questions 2, 3 and 6.

In particular, with respect to the human PBPK model, while the Panel recognized that the model has the advantage of predicting internal tissue concentration for humans, the Panel also expressed skepticism with regard to the PBPK modeling results that showed increasing Cmax with age in humans, thus implying that children potentially would be less susceptible to adverse neurological responses from exposure to deltamethrin than adults. The Panel hypothesized that the PBPK model-predicted Cmax in the brain was dependent on the model structure and parameter values. In particular, at the exposure conditions simulated, the brain concentration is mainly influenced by 1) the brain permeability surface area cross product, the parameter controlling diffusion across the blood brain barrier; and 2) the blood flow to the liver, as systemic clearance is predicted to be blood flow limited, and not metabolically limited. Since children have a higher blood flow per gram of liver, they would clear deltamethrin from the plasma more rapidly than adults if the protein binding is assumed to be the same for the ages simulated. Therefore, the brain permeability surface area cross product and the blood flow to the liver are key inputs to the model. The Panel, as stated in responses to other charge questions, has concerns about the values of these parameters and the model structure, and therefore, is not confident in the conclusions that CAPHRA has made thus far with respect to the differences in the rat and human internal dosimetry.

The Panel recognized that tissue dosimetry was a measurable endpoint, but it can only be measured in rats and the concordance between rats and humans may be questionable. In particular, as humans have fewer genes than rats, members of the Panel believe that a rat model cannot be a good model for humans. For future studies, members of the Panel have recommended the following two alternatives for internal dosimetry experiments: 1) to use guinea pigs as they might provide a better model for humans than rats or 2) use ES1 plasma carboxylesterase knockout mice, now available. The latter have the advantage of being physiologically more relevant to predict neurotoxic effect of pyrethroid to humans. Although this type of mouse lacks the ES1 gene, they have normal levels of carboxyleterase in liver, intestine, lung, and other organs.

Finally, as discussed in other charge questions (charge question 3 and 6), another major obstacle to integrating the two lines of evidence is the significant uncertainty associated with both the PBPK model and the experimental data. In light of the above points, the Panel does not believe

that there is enough understanding or confidence in the results obtained from the two lines of evidence – e.g. the experimental data and the human PBPK - to utilize a weight of evidence approach and draw a conclusion regarding internal tissue dosimetry.

The Panel recommended considering quantitative WOE approaches being formalized by the Organization for Economic Co-operation and Development (OECD) for developing Adverse Outcome Pathways (AOPs) (OECD, 2014, Becker et al., 2015). The Bradford-Hill (BH) considerations (Hill, 1965), originally developed for the evaluation of causality of associations observed in epidemiological studies, provide a useful approach for evaluating the extent of support for hypothesized AOPs. A formalized framework would have to be applied in order to compare the confidence and uncertainty associated with the differences in the rat and human dosimetry. Following Becker et al. (2015), it is critical to evaluate the reliability, robustness, and relevance of all the evidence using a formal weight of evidence (WoE) approach. The purpose of a WoE evaluation is to document certainty in inferring responses beyond interpolation within the range of empirical observations in a transparent manner. Confidence in inference is underpinned by the degree of certainty that the lines of evidence support the hypothesized inference.

b. CAPHRA has proposed to continue in vivo behavioral testing for deltamethrin and permethrin in definitive dose-response evaluations. Pyrethroids have been studied for decades and thus there is a large body of evidence for these pesticides. Given your response to 8a along with 1) the extensive body of scientific literature on pyrethroid toxicity syndromes and high dose studies in juvenile rats (e.g., Sheets et al, 1994[5]); 2) neurolemma studies supported by the CAPHRA (Question 2) along with additional in vitro studies (e.g. Meacham et al., 2008); and 3) recent in vivo studies from the Vorhees & Bruckner labs, please comment on the additional scientific value that would be provided in conducting further in vivo rat experiments to assess potential for PD sensitivity of human infants and children.

The CAPHRA summary document and presentations clearly indicated that CAPHRA's proposed focus moving forward would be on pharmacokinetics and on the *in vitro* neurolemma system, with minimal if any further behavioral testing (limited to completing definitive studies on permethrin and deltamethrin). The conclusions presented in the documents were that there was no evidence for any differences in pharmacodynamics in juvenile versus adult rats, but there was clear evidence from the deltamethrin studies that brain levels in juvenile rats were higher than those in adults at a given dose. The current human model predicts the opposite, with equivalent or higher brain levels predicted for the adult. In discussions about the model, CAPHRA pointed out that the model prediction of lower brain levels in humans was only for deltamethrin, and it will be interesting to see modeled concentrations for other planned test pyrethroids.

Panel discussions highlighted the great uncertainty surrounding the neurolemma and ion channel studies (charge question 2) as well as the need for further work on the PBPK model (charge question 6). In particular, the great levels of uncertainty surrounding the neurolemma and ion channel studies raised concerns among panel members on the usefulness of (more) neurolemma and ion channel studies. A large majority of the Panel believes that the uncertainty in the results of the neurolemma studies would not be dissipated even with a very large sample size.

Although the Panel, in general, agreed with CAPHRA's plan to focus on the pharmacokinetics and expand the information on age-related metabolism differences in juveniles and adults, the Panel also recognized that *in vivo* studies should not be abandoned. In particular, the Panel suggested that CAPHRA explores new assays that would facilitate an understanding of the clinical relevance of the presence of DLM (deltamethrin) in the brain. The Panel emphasized that it is fundamental that a clinical endpoint that measures the level of DLM in the brain and its clinical relevance be investigated by CAPHRA. Clearly, data that would increase confidence in and expand to other pyrethroids the prediction of the equivalent or lower levels of pyrethroids in the brains of young children at a given exposure level is the most critical. This would also impact the judgment of how relevant the rat studies are to assessing the relative risks of pyrethroid exposure in children and adults.

The Panel suggested that in addition to core pyrethroids, CAPHRA should also examine another type II pyrethroid, cyhalothrin, whose toxicity is not associated with the same symptoms associated with deltamethrin. For example, deltamethrin produces choreoasthetosis, while

[5] Sheets, L.P., et al., *Age-Dependent Differences in the Susceptibility of Rats to Deltamethrin.* Toxicology and Applied Pharmacology, 1994. **126**(1): p. 186-190.

cyhalothrin does not. Several references on the differential actions of cyhalothrin on neurotransmission are available (Hossain et al., 2004, 2006, 2006, 2013).

.

As that database is expanded, the need for further behavioral testing of additional pyrethroids can be reconsidered. It was stated that the blood and tissue level work conducted in parallel with the permethrin rat study is nearly completed, and the data from that study should be evaluated in a manner similar to that used for the deltamethrin data, relating ASR and DCO responses to brain levels, before deciding if and how to proceed with the definitive studies with permethrin and deltamethrin.

The Panel recommended framing the question about the value of additional studies that can be conducted to reduce uncertainties in the knowledge related to pyrethroid risks, using a formal Value of Information (VoI) framework (Keisler et al., 2014). The Panel found that the approach taken in the CAPHRA report suffered from a limited focus and from an evidence-driven line of inquiry and did not discuss contribution of individual lines of evidence to the overall goal. Specifically, although the Adverse Outcome Pathway to detect an effect on nerves is critical for understanding the mechanism and is useful in predicting a response to exposure along that specific pathway, it is only one piece of information that should be considered in the decision process and collecting additional information should be brought into the perspective of the decisions at hand.

A more transparent process for pyrethroid risk assessment would be to enumerate all the potential consequences of exposure and the level of effect that is tolerable given the benefits of exposure to these compounds. This decision-focused approach ensures that tolerable or intolerable endpoints are agreed to before the evidence of the exposure and consequences are reviewed. For example, the increased startle response in juvenile rats may indicate risks in children of neurosensitivity, attention disorders, autism, or other long-term impacts. The relationship between startle repose and these other endpoints should be researched. Once these connections are understood or hypothesized, the potential risk can be calculated for each endpoint. Where the evidence is unavailable for specific endpoints or exposure levels, those studies can be designed to inform the decision- making process based on an understanding of the limitations of specific models or approaches. When these data are available to inform the process, their role in the decision process can be clearly identified and the studies reviewed for relevance and quality.

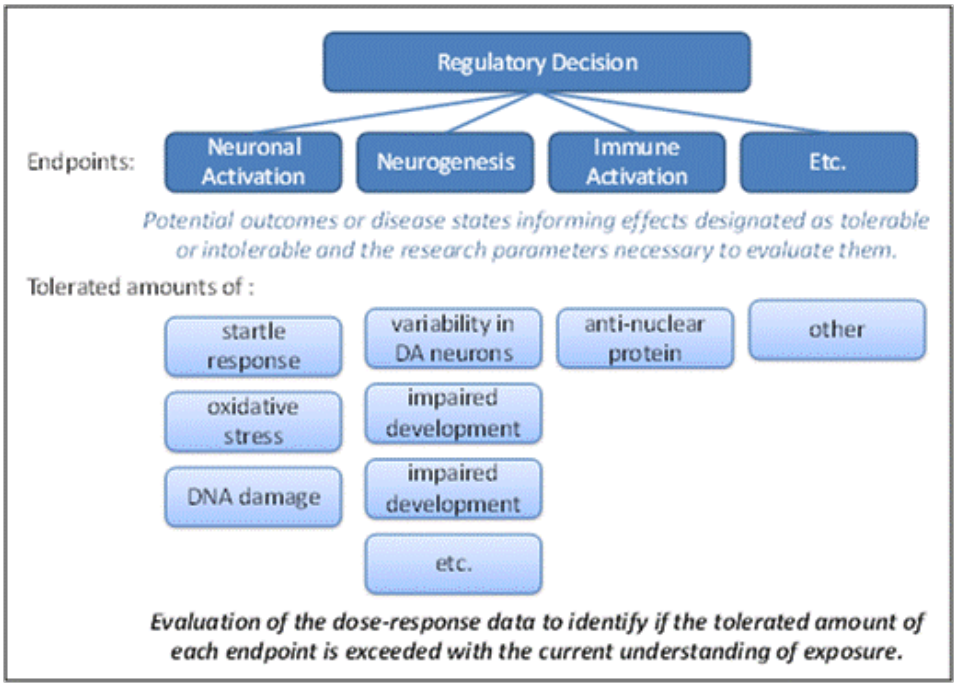

Figure 1: *A schematic diagram for a decision-focused approach for evaluating the existing research on the effect of exposure to pyrethroids on children with the ultimate goal of evaluation and regulation of pyrethroids.*

The first step in the process would be to review all the potential risks of pyrethroid exposure to juveniles and agree to the degree or frequency of impact that is acceptable. Based on the outcomes of such a review, there should be a discussion on what is the most definitive evidence for that impact/endpoint (i.e. discussion on what is/is not a good model for that endpoint, and what supplementary information is deemed important). In this context, the relevance of the high throughput screening or Xenopus oocyte models should be considered (Questions 1 & 2). Does the binding and activation of the sodium receptor provide adequate evidence of the effects that are indicative of increased risk? In addition to the issue of juvenile susceptibility to neurotoxicity, there are several other endpoints of concern according to EPA's TEACH technical summary for Permethrin and Resmethrin (2007). Endpoints to consider in the nervous system include activation and impacts on neuronal development, DNA damage and oxidative stress, depression of neurogenesis and loss of dopaminergic (DA) neurons. Other endpoints that should be considered include skin irritation, respiratory irritation, and immune system activation. The mechanism(s) to consider for these endpoints may be outside the scope of the Adverse Outcome Pathway for sodium channel activation.

With each of these endpoints, the tolerable level of impact should be designated. It may be important to consider the understanding of the long-term impact of small differences in juveniles, such as the relationship between DA neuron and Parkinson's disease. When these decisions are set in advance, then the application of research or the identification of the necessary research to make a clear decision becomes transparent. With the designation of the tolerable levels of risk associated with each endpoint, there should be a discussion of the research/data necessary to evaluate that endpoint. Specifically, what exposure levels are common, by what means, and in what models.

The human pharmacokinetic model is a useful tool for estimating the potential for exposure. However, the information provided by the PBPK model needs to be paired with an understanding of the dose-response relationship for an effect that implicates a specific outcome of a disease state. The AOP for juvenile nervous system activation is a piece of evidence that informs the endpoint(s) of interest associated with neuronal activation. If the AOP does not predict the level of activation designated as not indicative of an outcome/disease state requiring a change in regulation, then the activation endpoint is considered "safe" given the current understanding of exposure levels and consequences. The rest of the available literature should be reviewed for relevance to the evaluation of the endpoint and the risk of research in the designated level of effect. Gaps in the literature can be clearly identified, as well as the necessary assumptions for making a decision given the currently available information. One benefit of this approach is that it should facilitate subsequent review of future research and be able to identify those aspects of understanding that would change the decision to alter the regulation of these chemicals.

c. *If you believe there is additional scientific value to conducting additional in vivo experiments (8b), please comment on the CAPHRA's proposed experiments. In the context of your response to Question 3, please include in your comments a discussion of dose levels and/or additional study design elements to improve existing preliminary evaluations.*

If *in vivo* neurotoxicity studies are continued, the Panel suggested that the proposed study designs for the permethrin and deltamethrin definitive studies discussed by CAPHRA are pursued. CAPHRA has discussed in the public presentation some strategies to enhance the study design, including larger sample sizes and consideration of litter effects. (The basis of the determination of the optimal sample size should be presented with the study report.) The doses selected appeared reasonable. It would not appear critical to expand the time course. In fact, given the considerable discussions concerning the effect of the corn oil vehicle, it may be worth, in future experiments, to consider using the minimal volume necessary to enhance the rate of uptake and peak brain concentration. Reducing the number of trials per session to reduce habituation, as presented, may improve the ability to measure the response.

If further *in vivo* studies with deltamethrin are continued, the inclusion of a 1 mg/kg dose group (and maybe lower) in the PND15 rats should be considered. According to the overview document, one study was done with PND15 rats with 0, 1, 2, 4 mg/kg dose levels. Although not significant at 1 mg/kg, the authors pointed out that the 1 mg/kg group showed a trend of decreasing ASR at 8 hrs. (Figure 10, Appendix A2-4) when data from two studies with PND 15 rats were pooled. A larger number of rats should be used to detect any effects. Note that some mortality was observed at 3 mg/kg with mortality increasing at 5, 6, and 8 mg/kg in PND 15 rats. At 2 mg/kg in PND15 rats, mobility was mildly affected and rats had mild tremor. The observation of DCO at the dose of 1 mg/kg and possibly lower (not previously done) could also provide information on adverse neurological effects.

As neurotoxicity of pyrethroid insecticides is reflected in the alteration of neuronal functions, the Panel suggested that CAPHRA conduct additional experiments to monitor neuronal functions along with clinical observations of extracellular neurotransmitters release with brain microdialysis for better understanding age and dose dependent neurotoxicity of pyrethroids.

CAPHRA stated that tremor might reduce ASR since it overlaps with ASR. Further, a suggestion for future *in vivo* studies would be to counter dose it with zonisamide (no specific sodium and calcium channeler blocker) to prevent the tremor by blocking of Na_v.

Finally, as a general comment, the Panel emphasized that Questions b) and c) asked about the value of additional studies that can be conducted to reduce uncertainties in our knowledge related to pyrethroid risks. Generally, additional evidence is only material if it will reduce the uncertainty in a way that changes the decision at hand. A formalized "value of information" (VoI) approach can be used to determine the value of an additional study, specifically, what would be needed to ensure that information is definitive (statistical power, timing, verification of exposure over what time course and with what sensitivity, etc.)? Also the amount of evidence needs to be considered. Specifically, what kind of replication of any new study is critical (different labs, rat strains, behavioral assays)? To reduce uncertainties regarding the effects of pyrethroid exposure, a more transparent process in the context of pyrethroid risk assessment should be undertaken.

Literature Cited

Eusebi, F., Palma, E., Amici, M., and Miledi, R.. (2009) Microtransplantation of ligand-gated receptor-channels from fresh or frozen nervous tissue into Xenopus oocytes: A potent tool for expanding functional information. *Progress in neurobiology* 88 (1), 32-40.

He, B. and Soderlund, D.M. (2011). Differential state-dependent modification of rat $Na_v1.6$ sodium channels expressed in human embryonic kidney (HEK293) cells by the pyrethroid insecticides tefluthrin and deltamethrin. *Toxicology. Applied. Pharmacology*. 257(3):377-387. PMID: 21983428.

Hossain, M.M., Suzuki, T., Unno, T., Komori, S., Kobayashi, H. (2008). Differential presynaptic actions of pyrethriod insecticides on glutamatergic and GABAergic neurons in the hippocampus. *Toxicology*. 243:155-163.

Hossain, M.M., Suzuki, T., Sato, N., Sato, I., Takewaki, T., Suzuki, K., Tachikawa, E., Kobayashi, H. (2006). Differential effects of pyrethroid insecticides on extracellular dopamine in the striatum of freely moving rats. *Toxicology. Applied. Pharmacology*.217: 25-34.

Hossain, M.M., Suzuki, T., Sato, I., Takewaki, T., Suzuki, K., Kobayashi, H. (2004). The modulatory effect of pyrethroids on acetylcholine release in the hippocampus of freely moving rats. *NeuroToxicology* 25(5):825-833.

Hossain, M.M., Suzuki, T., Kobayashi, H., Richardson, J.R. 2013. Acute effects of pyrethroids on serotonin neurotransmission in the striatum of awake rats: in vivo microdialysis study. J. Biochem. Mol. Toxic. 27(2):150-156.

Ivorra, I., Henriquez, M., Lax, P., Riquelme, G., and Morales, A. (2002). "Functional transplantation of chloride channels from the human syncytiotrophoblast microvillous membrane to Xenopus oocytes." *Pflügers Archiv* 444 (6): 685-691.

Jamei, M, Marciniak, S, Feng, K. et al (2009) The Simcyp population-based ADME simulator. Expert Opin Drug Metabolism Toxicology, 5, 211-223.

Keisler, J. M., Collier, Z. A., Chu, E., Sinatra, N., & Linkov, I. (2014). Value of information analysis: the state of application. *Environment Systems and Decisions*, *34*(1), 3-23.

Khalil & Läer (2014) Physiologically Based Pharmacokinetic Models in the Prediction of Oral Drug Exposure Over the Entire Pediatric Age Range—Sotalol as a Model Drug *American Association of Pharmaceutical Sciences Journal* Mar; 16(2), 226–239.

Linkov, I., Loney, D., Cormier, S., Satterstrom, F. K., & Bridges, T. (2009). Weight-of-evidence evaluation in environmental assessment: review of qualitative and quantitative approaches. *Science of the Total Environment*, *407*(19), 5199-5205.

Meadows L.S., Chen Y.H., Powell A.J., Clare J.J., & Ragsdale D.S. (2002) Functional modulation of human brain Nav1.3 sodium channels, expressed in mammalian cells, by auxiliary beta 1, beta 2 and beta 3 subunits. *Neuroscience*. 114(3)745-753.

Meacham, C.A., Brodfuehrer, P.D., Watkins, J.A., Shafer, T.J. (2008). Developmentally-regulated sodium channel subunits are differentially sensitive to α-cyano containing pyrethroids. *Toxicology. Applied. Pharmacology* 231(3)273-281. PMID: 18538810.

Organization for Economic Co-operation and Development (OECD), (2014) Adverse Outcome Pathways, Molecular Screening and Toxicogenomics. Available online at: http://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm.

Tan, J. and Soderlund, D.M. (2010). Divergent actions of the pyrethroid insecticides S-bioallethrin, tefluthrin, and deltamethrin on rat $Na_V1.6$ sodium channels. Toxicology Applied Pharmacology 247(3):229-237. PMID: 20624410.

Thimmapaya, R., Neelands, T., Niforatos, W., Davis-Taber, R.A., Choi, W., Putman, C.B., Kroeger, P.E., Packer, J., Gopalakrishnan, M., Faltynek, C.R., Surowy, C.S., Scott, V.E. 2005. Distribution and functional characterization of human $Na_V1.3$ splice variants. Eur. J Neurosci. 22(1):1-9. PMID: 16029190.