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CRITICAL CONSIDERATION OF THE MULTIPLICITY OF EXPERIMENTAL AND ORGANISMIC DETERMINANTS OF PYRETHROID NEUROTOXICITY: A PROOF OF CONCEPT

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Pyrethroids (PYR) are pesticides with high insecticidal activity that may disrupt neuronal excitability in target and nontarget species. The accumulated evidence consistently showed that this neurophysiologic action is followed by alterations in motor, sensorimotor, neuromuscular, and thermoregulatory responses. Nevertheless, there are some equivocal results regarding the potency of PYR in lab animals. The estimation of potency is an important step in pesticide chemical risk assessment. In order to identify the variables influencing neurobehavioral findings across PYR studies, evidence on experimental and organismic determinants of acute PYR-induced neurotoxicity was reviewed in rodents. A comprehensive analysis of these studies was conducted focusing on test material and dosing conditions, testing conditions, animal models, and other determinants such as testing room temperature. Variations in the severity of the neurotoxicity, under lab-controlled conditions, was explained based upon factors including influence of animal species and age, test material features such as chemical structure and stereochemistry, and dosing conditions such as vehicle, route of exposure, and dose volume. If not controlled, the interplay of these factors may lead to large variance in potency estimation. This review examined the scope of acute toxicological data required to determine the safety of pesticide products, and factors and covariates that need to be controlled in order to ensure that predictivity and precaution are balanced in a risk assessment process within a reasonable time-frame, using acute PYR-induced neurotoxicity in rodents as an exemplar.

The last decades have given rise to the marketing of hundreds of new pesticide products. The active ingredients of these products are chemicals with biological activity (e.g., repellence, attraction, knock-down, killing) against a wide repertoire of pests, from insects and molluscs to weeds and wild rodents. Once a natural or synthetic pesticide has undergone and successfully passed testing for efficacy and toxicity in a research and development (R&D) department, commercial product registrations require that a number of

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additional lab assays be conducted under specific guidelines to consider potential health risks in humans. Such guidelines are elaborated and enforced by authorities responsible for ensuring validity, adequacy, and sufficiency of lab data packet (Health Canada, 2009; U.S. Environmental Protection Agency [EPA], 2012a, 2013; Organization for Economic Cooperation and Development [OECD], 2012).

Systematic generation of lab animal data is a core step in considering chemical health risks in humans. In order to ascertain the potential harm that may be produced in humans once a new pesticide product is authorized to be marketed, several sources of information are incorporated into the decision making process. First, hazard information is collected following formal, systematic procedures demanded by legal mandates and regulatory authorities that allow for a comprehensive search on the potential target tissues and systems of the chemical, including teratology, neurotoxicity, carcinogenicity, and reproductive and developmental toxicity (European Food Safety Authority [EFSA], 2007; OECD, 2012; U.S. EPA, 2013). In addition, available lab animal and epidemiological studies may be reviewed to complete the body of evidence. Toxicological similarity is considered in terms of clinical signs of toxicity, mode of action (MOA), and structure–activity relationships (SAR), and the level of evidence supporting similarity is a core element toward a decision confirming or omitting the inclusion of the compound in a cumulative risk format for the insecticide class (U.S. EPA, 2002, 2012a). Second, lab animals are utilized for generation of time- and dose-response relationships, and the computing of potency estimates when applicable (U.S. EPA, 2002, 2012a). Several pesticide classes have already been subjected to cumulative risk assessment processes aimed to reconsider the health risks posed to humans by exposure to relevant mixtures of pesticides with a similar MOA. Recent U.S. EPA research efforts included generation of relative potency factors for a number of triazine herbicides, and organophosphate (OP), *N*-methyl carbamate, and pyrethroid (PYR) insecticides (U.S. EPA, 2012a).

Relevant doses from animal studies, such as the highest no-observed-adverse-effect level (NOAEL), the lowest dose producing an observed adverse effect (LOAEL), or a benchmark dose (BMD), are identified as a point of departure level (PoD) from which a reference dose (RfD) is established for humans (Izadi et al., 2012). An RfD is the estimated maximum amount of a chemical that may be taken daily without expecting any acute or chronic health impairment in humans (U.S. EPA, 1993; Solecki et al., 2005). Toxicokinetic information may be needed, particularly when parent compound and toxicologically active metabolites may accumulate in exposed organisms. Finally, regulatory agencies may require additional data before reaching a final decision on relative hazard and health risks. To date, effects data obtained using experimental animals have represented a critical component in formal estimations of risk (European Centre for Ecotoxicology and Toxicology of Chemicals [ECETOC], 2009; Patlewicz and Lander, 2013).

Derivation of an exposure limit for a pesticide chemical in humans such as RfD from an animal PoD requires considering several sources of uncertainty. In general, uncertainty is partitioned into an interspecies component (e.g., rodent to human extrapolation) and an intraspecies component (i.e., human variability). Additional factors may be incorporated into the risk estimation process when applicable (Kirman et al., 2005), including uncertainty factor in extrapolation from subchronic to chronic exposure (UF_S), uncertainty in use of a LOAEL (UF_L), and uncertainty for completeness of the database (UF_D). The 1996 Food Quality Protection Act (FQPA) requires that an additional 10-fold factor (UF_{FQPA}) be applied to protect infants and children unless evidence is presented to the contrary (FQPA, 1996).

$$RfD = \frac{PoD}{UF_{\text{interspecies}} \times UF_{\text{intraspecies}} \times UF_{other}}$$

The RfD estimate thus incorporates a number of precautionary uncertainty factors to the PoD. A combination of UF estimates make

up a global 100× to 1000× margin of safety (MOS) from the PoD considered the most adequate after thoughtful examination of the available evidence. In other words, given an MOS of 1000, a protective extrapolation process sets the human health risk standard (RfD) to 1/1000 of the acute PoD (animal based) to protect susceptible individuals in the population. This application of UFs is intended to provide a comfortable and conservative margin of safety. However, the uncertainty factors ("safety factors") do consider only a portion of the uncertainty inherent to the PoD estimate itself, that is, the UF_L (uncertainty due to a specific PoD, the LOAEL) and UF_D (sparse data to inform the PoD). This procedure to compute RfD estimates has been applied to all pesticide classes, including the PYR class reviewed in this investigation. Benchmark dose (BMD) methodology improves on a major pitfall of the NOAEL and LOAEL of sample size dependence, as the BMD is the dose that corresponds to a specified observable level of response. Provided the appropriate response model is specified, the lower confidence bound on the BMD captures uncertainties in the POD (Crump, 2002; Izadi et al., 2012). However, these uncertainties only correspond to the given experimental framework. Additional sources of PoD uncertainty may not be considered in the risk assessment. This review demonstrates a leading case for accumulated PYR research efforts, all of which make up a step forward toward a more comprehensive characterization of uncertainty.

Emerging evidence indicates that variations in pesticide dose-response relationships may occur in mammals as a function of biological factors and experimental dosing and testing conditions used in toxicological assays designed to support a chemical registration process. There were reports warning about the potential to observe large variations in acute toxicity and potency estimates as a function of the lab conditions selected for assay (McDaniel and Moser, 1997; Crofton et al., 1995; Karalliedde et al., 2003; Wolansky et al., 2007a). This issue is further compounded by the historical tendancy to explore only a few experimental conditions per compound in toxicological assays: that is, one age to consider effects "in adults," one set of physiological conditions as a model of toxic response in "healthy" animals, one vehicle to dissolve the test chemicals (i.e., when administration of a bolus dose dissolved in a vehicle is used in animal assays as required in dose-response studies of pesticides producing neurotoxicity), selection of a limited battery of measures to monitor clinical effects, one device to examine an entire functional domain, one testing time (or a very few) to identify and classify toxicity landmarks, and so on. As a result, for many currently marketed pesticide products, neurotoxicological information is often sparse; filling these data gaps for the numerous old and new pesticide products is a long-term challenge (U.S. National Research Council [NRC], 1984). Strategies to recognize the potential animal responses that are missing in the available knowledge base would help reduce the uncertainty intrinsic to the PoD selected for animal–human extrapolation. In recognition of the need to expedite chemical risk assessments and reduce uncertainty in species extrapolation deficiencies in current practices, the U.S. National Academy of Sciences released a report in 2007, "Toxicity Testing in the 21st Century: A Vision and a Strategy" (U.S. NRC, 2007; Krewski et al., 2010), that envisions a future in which virtually all routine toxicity testing would be conducted in human cells or cell lines in vitro by evaluating cellular responses in a suite of toxicity pathway assays using high-throughput tests (Rotroff et al., 2010; Judson et al., 2011; Leist et al., 2012). However, notwithstanding anticipated advances in this field, whole-animal data remain a critical component of human health risk assessment.

Pyrethroid (PYR) insecticides offer an adequate case to revisit the question of animal data sufficiency in a health risk assessment process. Synthetic PYR were first marketed about 50 years ago. Competitive insecticidal features were then observed in a series of newer compounds commercialized in the late 1970s and early 1980s (Katsuda, 1999), a period when increasing restrictions on agricultural and home

pest control applications got started for many organochlorine (OC) and OP insecticides due to safety concerns (Rogan and Chen, 2005; Lubick, 2010; U.S. EPA, 2012b). Pyrethroids usage has been estimated at 23–30% of the worldwide market of insecticides (Casida and Quistad, 1998; Katsuda, 1999; Centers for Disease Controll and Prevention [CDC], 2003; Sudakin, 2006; Freedonia, 2006). Emerging studies document a background exposure of humans to pyrethroids (CDC, 2005; Egeghy et al., 2011), consistent with recent figures showing environmental occurrence of multiple PYR residues (Department of Pesticide Control Service [DPCS], 2001; Moran, 2005; Woudneh and Oros, 2006; Tulve et al., 2006; Tornero-Velez et al., 2012a). Pyrethroid-induced neurotoxicity in mammals such as rats and mice has been characterized during the last 35 years. Clinical descriptions of PYR intoxications in humans have been reviewed by He (2000), Ray and Forshaw (2000), and Spencer and O´Malley (2006). In addition, evidence based on in vitro data and animal data supports a common mode of action (MOA), enabling a classification of PYR by structure and neurobehavioral syndrome, and the estimation of relative potencies (McDaniel and Moser, 1993; Soderlund et al., 2002; Shafer et al., 2005; Wolansky et al., 2006; Wolansky and Harrill, 2008; Soderlund, 2012). A comprehensive body of evidence is thus available to attempt identification of toxicity determinants, as well identification of experimental and biological factors that have apparently no influence on the clinical manifestation of PYR syndromes.

This review provides a compilation of data supporting the influence of a number of variables on functional measures of acute PYR-induced neurotoxicity. While most neurobehavioral data available in the open literature are derived from studies of toxic response in rats and mice after acute, single-bolus administration of PYR, there have been no attempts to use the same comprehensive approach to examine the global impact of study design conditions on BMD estimated after subchronic or chronic exposures. The reader should not expect that this analysis of determinants is exclusive to PYR insecticides. Indeed, a number of the determinants discussed herein are likewise responsible for variations in the syndrome severity observed after exposure to other pesticide classes such as OP (Karalliedde et al., 2003; Wolansky, personal communication) and OC (McDaniel and Moser, 1997) in mammals.

Toxicity determinants are here addressed separately as they pertain to the study design (test material chemistry, dose solution preparation, and dosing and testing conditions) or to the animal model (biological conditions such as age, species, strain, gender, and physiological status). A majority of the available studies reported in peer-reviewed literature on PYR have used young adult, male rats. Thus, this review is primarily based on articles reporting on male responses, with no inclusion of the larger body of data generated in guideline (i.e., confidential) studies using male and female animals regularly conducted in order to apply for a registration of a new pesticide product. Similarly, a consideration of the impact of PYR-mediated toxicity determinants in developing or aged animals is in most cases not possible due to lack of data. Data from rat and mice are therefore predominantly reviewed to discuss the potential interplay between experimental settings and their expected impact on the risk of neurotoxicity in humans. Using PYR as a case study, a systematic approach for reviewing lab data is presented and proposed as a tool to help identify critical data gaps and prioritize animal data needs in chemical risk assessment.

PYRETHROID STRUCTURE

Pyrethroids are neurotoxic insecticides developed in the last decades for optimizing insect killing, knockdown and repellent properties of a series of natural compounds called pyrethrins (Valentine, 1990). This insecticide class has expanded its structural variability, since the first PYR was marketed more than 50 years ago. Figure 1 illustrates the structural evolution of the pyrethrins/pyrethroids family of insecticides. Most PYR are esters, including

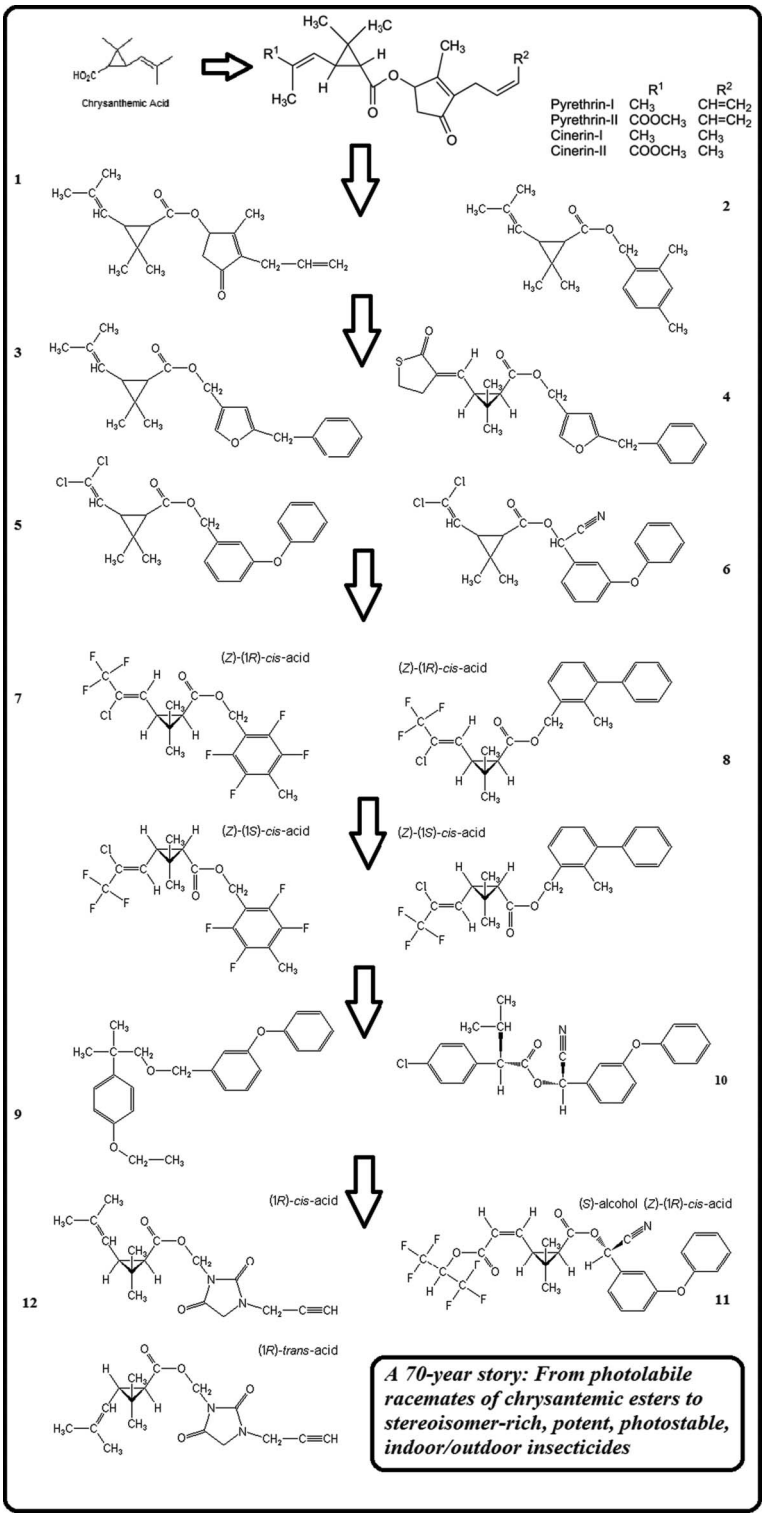

FIGURE 1. Structural diversification of pyrethroid insecticides. Early synthetic pyrethroids resulted from attempts to improve the photolability and relatively weak biocidal activity of the pyrethrins, a series of chrysanthemic acid esters naturally present in the flowers of chrysanthemum species. Two major historical landmarks in pyrethroids development are the incorporation of an α-cyano group (see permethrin and cypermethrin), and the commercialization of products containing pyrethroid materials enriched with the most potent stereoisomer as the active ingredient (see modern pyrethroids at bottom) (Katsuda, 1999; Wood, 2013; Wolansky and Harrill, 2008). Key for structures: 1, allethrin; 2, dimethrin; 3, resmethrin; 4, kadethrin; 5, permethrin; 6, cypermethrin; 7, tefluthrin; 8, bifenthrin; 9, etofenprox; 10, esfenvalerate; 11, imiprothrin; 12, acrinathrin.

an aromatic alcohol and a cyclopentanecarboxilate; a few ethers have been also developed (Katsuda, 1999). These are lipophilic chemicals with extremely low solubility in aqueous media, placed at the top in a ranking of pesticide $K_{o,w}$ coefficients (Finizio et al., 1997). More details on structure–activity relationships are provided in following sections.

PROPOSED MODE-OF-ACTION (MOA) FOR PYRETHROID NEUROTOXICITY

Pyrethroid insecticides act primarily on the nervous system in target and nontarget species. The commonly accepted primary MOA of PYR is the prolongation of the open state of voltage-gated sodium channels (VGSC) in targeted neurons (Soderlund et al., 2002; Soderlund, 2012). Pyrethroids produce a transient arrest of the VGSC system in the inactivation phase, prolonging inward Na^+ currents. This repolarization disruption results in repetitive neuronal firing or depolarizing block, depending on the duration the VGSC is kept open (Narahashi, 1996; Soderlund et al., 2002). The subsequent occurrence of a dosage-dependent elicitation of neurobehavioral signs to the observed syndrome suggests the existence of a relationship between dose(s) of parent compound at target tissue(s) and actual toxicity (Rickard and Brodie, 1985).

Other channel and receptor systems in neuronal tissues, including Ca^{2+} and K^+ channels and $GABA_A$ receptors, have been proposed to play a role in the generation of compound-specific effects (Crofton and Reiter, 1987; Hildebrand et al., 2004; Lawrence and Casida, 1982). Whole-cell and patch-clamp assays in cultured rat neurons suggest that T- and CS-syndrome PYR interact with VGSC binding sites by either competitive or allosteric actions (Song and Narahashi, 1996; Motomura and Narahashi, 2001). Moreover, structure-dependent interaction among PYR was proposed to occur in chloride channels of membrane patches from differentiated mouse neuroblastoma cells (Burr and Ray, 2004). As a preliminary conclusion, the action on the VGSC system of targeted neurons is presently considered the primary driver for neurotoxicity induced after acute exposure to individual or combined PYR insecticides, although one or more alternative sites in targeted neurons may likely account for type-specific neurological syndromes produced at high-effective doses in rats and mice (Soderlund et al., 2002; Burr and Ray, 2004; Cao et al., 2011; Johnstone et al., 2010; Soderlund, 2012). The regulatory matter of determining commonality among pesticides is outside the scope of this review. Specific literature is available to further explore this topic (Borgert et al., 2005), and to examine the knowledge base that was critically considered to propose dose-addition as a default hypothesis in the lab assays informing the cumulative risk assessment of PYR (Wolansky et al., 2009; U.S. EPA, 2011, 2012a).

PYRETHROID NEUROTOXICITY

Data consistently indicate that oral exposures of lab animals to PYR at doses well below those inducing lethality produce evident alterations in various neurobehavioral domains (McDaniel and Moser, 1993; Soderlund et al., 2002; Wolansky and Harrill, 2008). Acute exposures to PYR produced predominantly reversible alterations in the functionality of motor, sensorimotor, neuromuscular, thermoregulatory, and learning-related pathways in rodents. Exhaustive compilations of reports on the effects of PYR on neurobehavioral endpoints in rodents and a critical interpretation of them were published (Soderlund et al., 2002; Shafer et al., 2005; Wolansky & Harrill, 2008). Although primary mechanistic commonality is proposed for all PYR, observation of single-compound specific neurobehavioral profiles strongly suggests some toxicological heterogeneity among these insecticides (Crofton and Reiter, 1984; Peele and Crofton, 1987; McDaniel and Moser, 1993; Soderlund et al., 2002; Wolansky and Harrill, 2008; Soderlund, 2012). Further, neurotoxicological characterizations and classification of PYR in rats and mice were originally conducted by recording

cage-side or open-field observations carried out upon intravascular (iv) or intracerebral (ic) exposure to nearly lethal doses (Verschoyle and Aldridge, 1980; Lawrence and Casida, 1982; Gammon, 1985; Nishimura et al., 1984). These clinical descriptions of acute effects indicated the presence of three types of neurological syndromes: (1) those inducing whole-body tremors, (2) those inducing choreoathetosis and salivation, and (3) a smaller group producing a mixed syndrome including tremors and salivation. Accordingly, these have been termed *T-, CS-,* and *TS-syndromes*, respectively. In general, T-, CS-, and TS-syndrome compounds are also named "Type I," "Type II," and "Type I/II" PYR, respectively (Verschoyle and Aldridge, 1980; Lawrence and Casida, 1982; Gammon, 1985; McDaniel and Moser, 1993). A simpler, dual, Type I/Type II nomenclature is applicable to classify the patterns of neurotoxicity of high effective doses of most modern PYR (Soderlund et al., 2002; Wolansky and Harrill, 2008). Figure 2 shows the various clinical signs of PYR syndromes observed in rats as a function of the administered dose and time after dosing. In general, studies using single doses equivalent to 1/100–1/5 rat oral LD50 (LD50 range for PYRs = 22 to 10,000+ mg/kg) consistently showed a complete recovery of control performance in neurobehavioral assays after 12–24 h (Wolansky and Harrill, 2008). Indeed, the detoxification pathways for PYR in mammals are highly efficient (Soderlund et al., 2002), suggesting minimal accumulation of toxic levels in target tissues after low-level exposures such as those observed in recent environmental monitoring studies (CDC, 2005; Tulve et al., 2006; Starr et al., 2008). It is worthwhile noting that time elapsed between acute dosage and functional assessment, if not controlled, may lead to categorically different results in toxicological evaluations.

In addition, PYR-evoked neurobehavioral syndromes include a repertoire of adverse

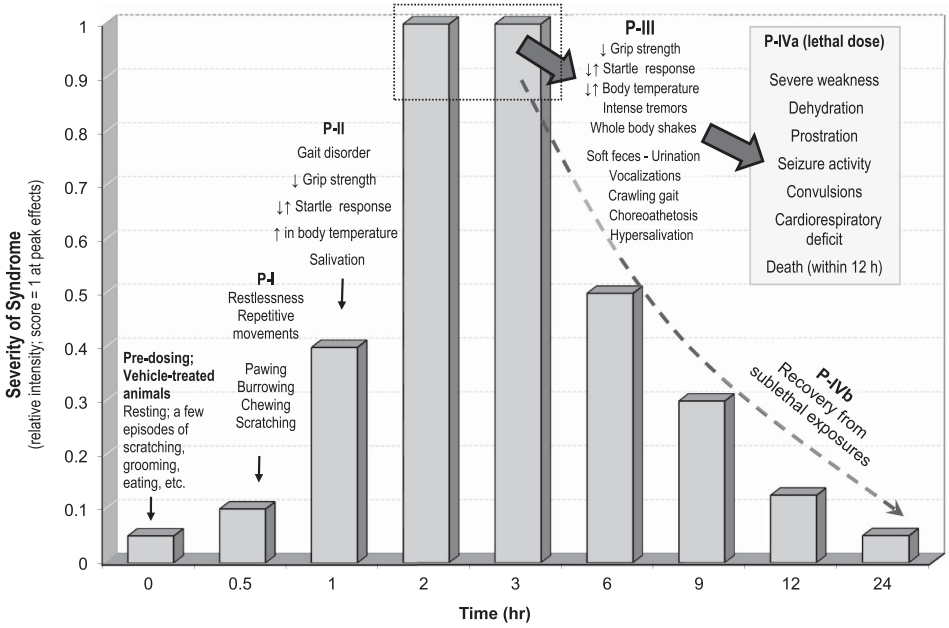

FIGURE 2. Neurobehavioral signs of pyrethroid toxicity. This scheme is mostly based on cage-side observations of male rats carried out during time- and dose-response assays for 11 pyrethroids dissolved in corn oil (dose volume = 1 ml/kg) (Wolansky et al., 2006, 2007; Crofton et al., 1995; Wolansky and Harrill, 2008; McDaniel and Moser, 1993; Soderlund et al., 2002). The *y*-axis shows a relative severity scale for the intensity of the endpoint alteration as observed at different time points after dosing, giving a score = 1 at the time of apparent peak toxicity, and the *x*-axis thus shows the time elapsed between single-bolus, oral administration of PYR and clinical observations, expressed in hours. The syndrome progression over time is illustratively divided in phases (namely P-I to P-IV), which are observed as a function of the administered dose from 1/100 to ~1/5 rat oral LD_{50} (see WHO, 2005; Wolansky et al., 2006; Wolansky and Harrill, 2008). The insert at right shows the progression of the syndrome when nearly lethal doses are administered (Phase P-IVa).

effects also observed after exposure to other insecticide classes primarily targeting neuronal excitability and neurotransmission. For instance, oral administration of the T-syndrome PYR bifenthrin was shown to produce increased startle response and hyperthermia among a battery of signs also present after acute oral exposure to the prototypic OC insecticide DDT (McDaniel and Moser, 1997; Wolansky et al., 2007a). Further, the neuromuscular weakness and tremorigenic actions produced by a number of PYR were similarly observed in OP studies (Ecobichon and Joy, 1993; Mileson et al., 1998; Pope, 1999; Wolansky and Harrill, 2008). Yet there is little information on the joint action of insecticides of different classes in rat studies using environmentally relevant exposure and quantitative neurobehavioral assays.

Most PYR exert relatively low toxicity to mammals (Soderlund et al., 2002; Wolansky and Harrill, 2008; He, 2000; Power and Sudakin, 2007). Oral dose levels associated with dosage-dependent effects of individual PYR in animals (McDaniel and Moser, 1993; Wolansky and Harrill, 2008) are generally well above the emerging data on levels of human daily exposure to PYR (Food and Drug Administration [FDA], 2013; Tulve et al., 2006; Lu et al., 2006; Woudneh and Oros, 2006). Considering individual PYR separately, acute intoxications due to intake of PYR-residues contained in foods might not be attainable in humans. A relatively low incidence of registered cases of PYR-mediated intoxication (Power and Sudakin, 2007) is consistent with the proposed safety of PYR (Casida and Quistad, 1998; Katsuda, 1999).

The neurobehavioral database for PYR in mammals contains a classification of clinical syndromes that used nearly lethal, iv and intracerebroventricular (icv) exposures in rats and mice, and dose-response assays using a number of endpoints after sublethal, oral, and ip exposures (predominantly rats; Wolansky and Harrill, 2008). Most synthetic members of the PYR class (1) share a common structural ancestor (i.e., the basic structure of natural pyrethrins), (2) produce a similar action in the nervous system of target and nontarget species as a primary event required to elicit neurobehavioral toxicity (see next section), and (3) evoke qualitatively similar changes in various neurobehavioral endpoints. In general, PYR produce acute, mostly reversible decline in motor activity, motor coordination, and neuromuscular strength, and deficit in operant responses (Soderlund et al., 2002; Wolansky and Harrill, 2008). Increase in body temperature may occur at low effective doses (McDaniel and Moser, 1993; Wolansky et al., 2007a, 2007b; Pato et al., 2011). Contrary to this apparent commonality among pyrethroids, divergence of neurobehavioral profiles across PYR structures becomes evident as exposure levels increase, a matter addressed in the functional observational battery (FOB) study of 12 cyano and non-cyano PYR conducted by Weiner et al. (2009).

DETERMINANTS OF PYRETHROID NEUROTOXICITY

Pyrethroid-induced neurotoxicity is influenced by a variety of biological factors and experimental conditions. Next, a list of physical, chemical, and biological determinants of PYR-mediated neurotoxicity in rodent assays is proposed, organizing the available evidence as follows: test material, dosing solution, and dosing conditions (i.e., toxicokinetic factors), testing conditions, and animal models (i.e., toxicodynamic factors).

Pyrethroid Toxicokinetics-Related Factors

Test Material and Dosing Solution

Chemical structure Most PYR contain cyclopropane carboxylic acid moieties linked to aromatic alcohols through a central ester bond (Figure 1). Modifications to this basic structure may greatly influence activity for in vitro and in vivo models (Gammon, 1985; Yang et al., 1987; Valentine, 1990; Vijverberg and Van der Bercken, 1990; Naumann, 1998; Soderlund et al., 2002; Soderlund, 2012). Presence of an α-cyano group in the alcohol moiety confers an increase in potency (based on rat oral LD50 estimates) of approximately

one order of magnitude (Lawrence and Casida, 1982; Vijverberg and Van der Bercken, 1990; Valentine, 1990; Soderlund et al., 2002). Other structural modifications to the acid and alcohol moieties have been introduced, that is, variation in the identity and position of halogenated and hydrophobic chemical groups and in the stereochemical configuration (Naumann, 1998) (Figure 1, compounds 9–12). Rat oral LD_{50}s for old and modern PYR may have approximately 500-fold variation in toxicity based on compound structure according to the available acute oral toxicity classification documents for pesticides (CDC, 2003; WHO, 2005).

Pyrethroid stereochemistry The spatial conformation of a pesticide is often a major determinant of its toxicity (Glickman and Casida, 1982; Kurihara et al., 1997; Liu et al., 2005). Pyrethroids may include two to four chiral centers, and the resulting stereoisomers may greatly differ in potency in lab animals (Verschoyle and Aldridge, 1980; Glickman and Casida, 1982; Coats, 1990; Kurihara et al., 1997; Liu et al., 2005). For most PYR, preparations enriched in one or a few *cis*-isomers (e.g., *cis*-resmethrin, *cis*-permethrin, *cis*-bifenthrin, *cis*-cypermethrin) exhibit greater potency (ie, lower oral LD50) than preparations poor in *cis*-isomers (INCHEM, 1990a; WHO, 2005). Cismethrin, the 1R-*cis* isomer of resmethrin, is up to 50-fold more potent than resmethrin preparations enriched in the 1R-*trans* isomer in rats (Abernathy and Casida, 1973; White et al., 1976; Verschoyle and Barnes, 1972; Gray et al., 1980; Cremer and Seville, 1982; Crofton and Reiter, 1984; Wolansky et al., 2006). In lab rodents, potency variations in PYR samples differing in isomer ratios have been explained in terms of isomer-specific metabolism (Ueda et al., 1975). Tornero-Velez et al. (2012b) elaborated a physiologically based pharmacokinetic model of *cis*- and *trans*-permethrin disposition in rats and human. The faster clearance of the *trans* compounds was explained by rapid hydrolytic metabolism. In developing a quantitative structure–property relationship to estimate hydrolytic rates for pyrethroids, Chang et al. (2012) applied a three-dimensional (3D) pharmacophore approach to discern protein–ligand interaction features indicative of catalytically enabled ligand poses near the active site of carboxylesterase. The model successfully filtered out *cis* compounds with remarkable accuracy. In addition, pyrethroids with an α-cyano group may also assume different isomeric [αS] or [αR] configurations based on the spatial orientation of this group. The [αS] configuration has higher activity than the [αR] one in target and nontarget species (Elliott and Janes, 1978; Valentine, 1990; Soderlund et al., 2002). The difference in lethality is up to approximately three- to fourfold in rats when unresolved cypermethrin (CYPM) is compared to the most toxic "alpha-cypermethrin," a preparation containing approximately 50% (1R-*cis,* αS) and 50% (1S-*cis*, αR) (a higher proportion of the potent (1R-*cis* αS) isomer relative to cypermethrin) (Pronk et al., 1996; European Agency for the Evaluation of Medicinal Products [EMEA], 1998, 2003; McGregor, 1999; World Health Organization [WHO], 2005; Wood, 2012). Figure 3 illustrates the critical role of chiral centers for PYR-mediated toxicity in rats. Isomer-composition-dependent differences are evident for the three cyano (cyhalothrin, cyfluthrin, fenvalerate) and one non-cyano (permethrin, PRM) PYR. The maximum isomer ratio effect may be estimated from an approximately 7- to 50-fold factor, taking into account PRM and resmethrin studies, respectively.

In summary, the composition of stereoisomers in test materials of pyrethroids is a major determinant of PYR potency in susceptible organisms. Utilization of different lots of a PYR material (or dissimilar formulations) differing in the isomeric ratios may affect time- and dose-response assay reproducibility. Misleading interpretations across studies may thus arise by neglecting complete test material chemical information. Analysis of stereoisomers in a dosing solution aliquot and target tissue samples needs to be conducted when possible to confirm the nature of a PYR dose producing a certain neurotoxicity profile in mammals with greater accuracy.

Purity Little is known of the joint toxicity of PYR and other chemicals and the influence of purity on the toxicity of technical

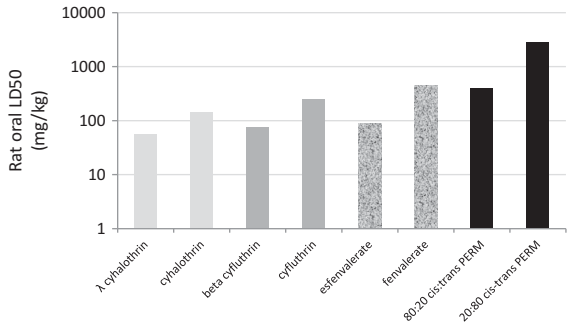

FIGURE 3. Influence of isomer ratios on pyrethroid toxicity. This chart illustrates the impact of individual isomers on the lethality of different preparations of pyrethroids. The *y*-axis shows rat oral LD_{50}s for the active-isomer rich preparations of one noncyano compound, *cis*-isomer rich permethrin (80:20 *cis:trans* PERM), and three cyano-compounds, λ-cyhalothrin, β-cyfluthrin, and esfenvalerate, compared to the least potent ratio of permethrin isomers (20:80 *cis:trans* PERM), and the corresponding parent cyano-compounds, cyhalothrin, cyfluthrin, and fenvalerate (LD_{50}s taken from WHO [2005] and INCHEM [1990a]).

and commercial preparations under experimental or environmental conditions in mammals. Even minor changes in purity were reported to markedly influence the toxicity of a number of insecticides such as the OP malathion (International Program on Chemical Safety [INCHEM], 1997a). Studies aimed to reconsider the safe levels of PYR were mostly conducted using technical-grade compounds of 88–99% purity (Soderlund et al., 2002; Breckenridge et al., 2009; Wolansky et al., 2006, 2007a, 2009). Yet since the first PYR was marketed in 1952 (INCHEM, 1989), hundreds of formulations containing one or more PYR and a variety of non-PYR ingredients have been registered (CDC, 2003; National Pesticide Information Retrieval System [NPIRS], 2011).

Coformulated chemicals may be able to modify the toxicological profile of PYR. In commercial products, PYR are often a minor fraction of the formulation (NPIRS, 2011). Pyrethroid-induced toxicity may be potentiated by non-PYR ingredients such as solvents (David, 1982; Grossman, 1995; Yang et al., 2002), metabolic inhibitors (Casida et al., 1976; Grossman, 1995), and other pesticides (Miyamoto, 1976; Audegond et al., 1989; Ortiz et al., 1995; Abou-Donia et al., 1996, 2001; Abu-Qare and Abou-Donia, 2003). The purity factor was studied in vitro by two labs using two different cell culture systems. First, environmentally relevant levels of two commercial formulations of bifenthrin produced greater toxicity than that of the technical material in a human adenocarcinoma cell line (Skandrani et al., 2006). In the other study, trade-secret, non-PYR ingredients of a commercial bifenthrin product produced changes in culture morphology in PC12 cells (Tran et al., 2006). In addition, a few studies reported on the influence of non-PYR ingredients of PYR commercial products with in vivo toxicological assays in rodents. An enhancement of PYR toxicity by co-formulated chemicals was demonstrated by testing different formulations of fenvalerate in mice (Williamson et al., 1989) and deltamethrin (DLM) in rats (Lepeshkin et al., 1992) after acute, single-dose exposure by oral route. In the former study, Swiss mice administered technical-grade preparations of PRM and fenvalerate or their commercial formulations ip (Ambush and Pydrin, respectively), were evaluated using a neurobehavioral endpoint. The commercial formulations showed greater toxicity than the corresponding active ingredients (Williamson et al., 1989). In contrast, a 40% pure PRM commercial formulation using petroleum distillates as a carrier (i.e., Hi-Yield Plus) produced attenuated toxicity (i.e., a later onset of clinical signs), using general motor activity as an endpoint, compared to a technical-grade, 92% pure sample of this Type I PYR in rats (Wolansky et al., personal communication). Internal doses accumulated after acute exposures to PYRs by dermal route may also be influenced by coformulated ingredients in PYR products. In general, percutaneous PYR absorption capacity in vivo (i.e., often considered $\leq$2% of the administered dose) is proposed to be well below 10% of that observed using oral administration schemes in rodents and humans. This greater efficacy as a natural biological filter for PYR suggests that skin exposures in humans would be more markedly relevant at occupational settings, spill accidents after handling concentrated formulations, and through use of permethrin-based shampoos to treat head and pubic lice infestations in children

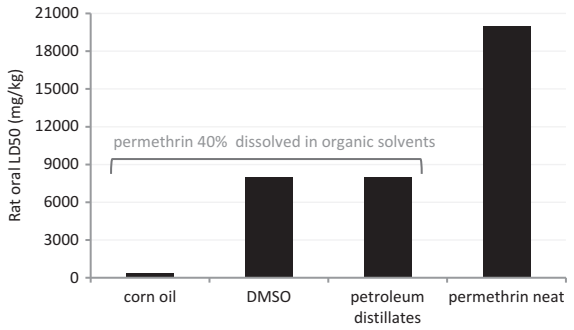

FIGURE 4. Influence of purity and carrier on pyrethroid toxicity. The intrinsic toxicity of a neat permethrin material (i.e., no carrier; LD_{50} > 20,000 mg/kg), expressed as oral LD_{50}, is compared to 40% w/v solutions prepared using petroleum, DMSO, or corn oil (LD_{50} = +8,000, +8,000, and 396 mg/kg, respectively). Formulation carrier may produce major changes in potency (INCHEM, 1990a).

and adults (CDC, 2003; Ross et al., 2011; Gunning et al., 2012). An extensive discussion of the potential influence of coformulated chemicals on transdermal penetration rates of commercial products based on permethrin was recently reported by Ross et al. (2011). These studies provide evidence of the potential role of impurities (i.e., other active or inactive ingredients) in increasing or decreasing PYR toxicity. Figure 4 illustrates the influence of purity and carrier composition on oral LD_{50} for PRM.

Further, evaluation of the formulation effect needs to include examination of potential interactions between active ingredients present in commercial PYR materials (Hodgson and Rose, 2008). There are commercial insecticide products formulated with more than one active ingredient, and some protocols for pest control recommend simultaneous or sequential use of more than one pesticide product. Pesticides such as DDT-like OC, OP, and PYR produce in rodents a number of common signs of neurotoxicity, such as tremors and alterations of neuromuscular and thermoregulatory responses (Herr et al., 1986; McDaniel and Moser, 1997; Soderlund et al., 2002; Ecobichon and Joy, 1993; Wolansky and Harrill, 2008; Krieger, 2010). To our knowledge there is no study available in vivo examining acute or chronic, cumulative effects produced by coexposure to environmentally relevant levels of pesticides with different MOA in rodents using endpoints informing on neurobehavioral toxicity. Yet there are a number of research articles (Gaughan et al., 1980; Audegond et al., 1989; Abou-Donia et al., 2001; Abu-Qare and Abou-Donia, 2003; Liu et al., 2006; Sexton et al., 2011) and reviews (Lydy et al., 2004; Hodgson and Rose, 2008; Hernández et al., 2012) indicating that synergy may occur between concurrent, suprathreshold exposures to pesticides in rodents and humans.

Finally, careful consideration of potential confounders needs to be conducted before any interaction between experimental factors is postulated. Interaction between intrinsic PYR-mediated toxicity, formulation, and route of exposure may explain the wide variation of PYR potency estimates that have been obtained under different dosing conditions (Chanh et al., 1984a, 1984b; Nishimura et al., 1984; Metker et al., 1977; Glomot, 1982; Crofton et al., 1995; Aboud-Donia et al., 2011; Abu-Qare and Abou-Donia, 2003).

Dosing Solution (Dosing Material Preparation Protocol) The protocol used for preparing dosing solutions and the storage of test materials may confound the apparent toxicity of PYR. Oral diet, accumulative exposure schemes using test materials of the older (i.e., photolabile) PYR such as allethrin, resmethrin, tetramethrin, or phenothrin dissolved in food pellets or in liquid solutions needs to be controlled for ultraviolet (UV)-induced photodegradation. Further, in studies using aqueous PYR dosing solutions, it is recommended to refer to solubility tables to prevent incomplete solubilization. The issue of solubility in most PYR studies using oral exposures may be resolved by dissolving the technical grade materials in oily vehicles and slightly warming up to 35–45°C prior to a dosing run. An alternative procedure is to prepare more diluted dosing solutions (i.e., using larger dosing volumes). Yet neurobehavioral studies for the OC DDT (McDaniel and Moser, 1997), the T-like PYR bifenthrin (Wolansky et al., 2007a; see later discussion), and the OP diazinon (Wolansky et al., unpublished data) indicate that a decrease in toxicity may occur

by increasing dosing volume using corn oil as a vehicle, regardless of the insecticide structure.

Route of Exposure and Vehicle

Route The route of exposure determines the organism's ADME profile (ADME is the acronym for absorption, distribution, metabolism, and excretion) and thus the portion of an administered dose reaching target tissues. Diet and hand-to-mouth behavior in children have greater relevance as PYR exposure pathways than inhalation in the general population (Soderlund et al., 2002; Tulve et al., 2006; Freeman et al., 2005; Lu et al., 2006; Starr et al., 2008). As mentioned earlier, skin absorption may have potential participation in intoxications occurring after chronic exposures (Gunning et al., 2012; Ross et al., 2011; Abou-Donia et al., 2011), for example, repeated handling of PYR products, a type of exposure scenario more likely observed in occupational settings (Hilton et al., 1994; Spencer and O´Malley, 2006).

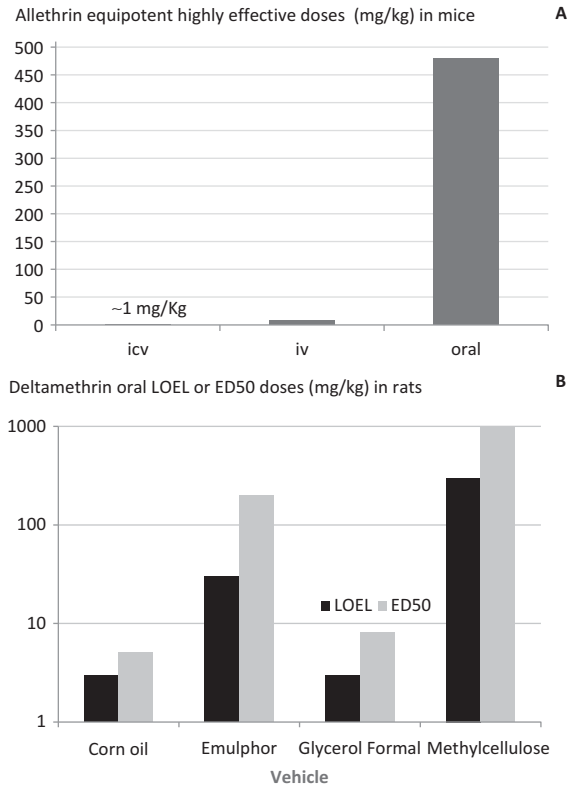

FIGURE 5. Influence of route of exposure and vehicle on pyrethroid toxicity. (A) The variation in potency observed by using different routes to administer allethrin in mice. Apparently equipotent, highly effective doses (expressed in mg/kg body weight [bw]) administered by each route are compared (Nishimura et al., 1984). (B) The evident influence of dosing vehicle on deltamethrin potency in rats. Deltamethrin dissolved in corn oil was observed to be up to ~200-fold more potent in producing a motor activity decline than other vehicles (i.e., ED_{50} from 5.1 to >1000 mg/kg). A similar trend is observed for LOEL estimates. Oral LOEL and ED_{50} doses are expressed in mg/kg (taken from Crofton et al., 1995).

For PYR, lethal doses and doses effective for producing evident changes in behavior in rodents vary across routes of exposure. A few studies tested the actions of PYR using multiple routes of exposure. Nishimura and coworkers (1984) characterized the progression of the T-syndrome induced by allethrin in mice using each of three routes of exposure in independent experiments. Apparently equitoxic (i.e., highly effective) single doses of allethrin were tested using each route as follows: 480 mg/kg (oral), 8 mg/kg (iv), and 0.3–0.9 mg/kg (icv). Almost a three-order variation in effective doses was evident across routes (Figure 5A), and differences in syndrome onset times and the variability of behavioral responses between animals were also dependent on route (Nishimura et al., 1984). Further, the influence of several vehicles on the toxicity of a single dose of the CS-syndrome compound DLM administered to rats by oral and ip routes was studied by Crofton and coworkers (1995) using motor activity as an endpoint. Regardless of the examined vehicles and routes, DLM induced decreases in motor activity. Yet with glycerol-formal (GF) as vehicle there was no marked difference between $ED50_{oral}$ and $ED50_{IP}$; for the other examined vehicles (i.e., corn oil, Emulphor, and methylcelulose) ED_{50} varied up to 28-fold for the same vehicle across routes of exposures (Crofton et al., 1995). Available data are thus sufficient to postulate a major influence of the selected route of exposure in the intensity and repertoire of functional observations in studies of PYR neurotoxicity.

Note that there may be interaction between route and vehicle factors: While PRM is less toxic to rats than cyfluthrin using an oral route and corn oil as a vehicle (Soderlund et al., 2002; Wolansky and Harrill, 2008), cyfluthrin

in PEG-400 by ip route is less potent than oral PRM in corn oil (INCHEM, 1990a, 1997b).

Vehicle This aspect was been introduced earlier as a determinant of PYR toxicity (Figure 4). The dosing solution vehicle per se has been observed to significantly influence PYR toxicity in rats. Pyrethroids are largely hydrophobic substances, with high affinity for lipid-rich biological or chemical substrates such as cell membrane phospholipids and lipophilic solvents (Coats, 1990; Laskowski, 2002). Moreover, most PYRs tend to aggregate and precipitate in aqueous carriers within the dosing solution concentration ranges needed to produce neurobehavioral effects. Indeed, λ-cyhalothrin present in an aqueous solution of the insecticide product ICON (oral NOAEL = 50 mg/kg; Ratnasooriya et al., 2002) was clearly less toxic to rats than a technical-grade λ-cyhalothrin dissolved in corn oil, when general motor activity was used as an endpoint (oral ED30 for λ-cyhalothrin = 1.3 mg/kg; Wolansky et al., 2006). Three studies have specifically addressed the issue of vehicle-dependent PYR-mediated toxicity in young adult rats. First, Crofton and coworkers (1995) tested the effect of four vehicles, GF, corn oil, Emulphor, and methylcelullose, on the motor activity alterations induced by DLM. Strikingly, there was a variation in potency (i.e., oral LOAEL and ED_{50} estimates for motor activity) of more than two orders of magnitude across vehicles. A quantitative comparison of this vehicle-effect in rats is shown in Figure 5B. Second, Kim and coworkers (2007) demonstrated that differences in gastrointestinal (GIT) absorption rates and bioavailability at target tissues may explain the differential toxicity of DLM administered in different vehicles. Doses of DLM in GF administered by the oral or iv route produced the expected salivation and slight tremors a few hours after exposure. DLM did not evoke any such effects when Alkamuls (Emulphor) was used as the vehicle. An approximate 10-fold higher blood C_{max} and area under the curve (AUC) were found in the GF animals than in the Alkamuls animals following oral administration of 10 mg/kg DLM. A microscopic analysis showed that Alkamuls did not achieve complete solubilization of the test PYR; DLM tended to aggregate, likely leading to a lower nervous system dose (Kim et al., 2007). Third, the comprehensive toxicological assessment of PRM conducted by Metker et al. (1977) exhibited up to an approximate 10-fold increase in toxicity when a neat PRM sample was diluted in corn oil before dosing. The mechanism by which oil and other fat vehicles enhance oral absorption of lipophilic compounds is not entirely known. Based on studies of DDT pharmacokinetics, Gershkovich and Hoffman (2007) proposed that following a high-fat meal, the lymphatic absorption and the plasma disposition levels of lipophilic compounds are enhanced by association with chylomicrons (structures bearing triglyceride-rich lipoproteins) formed in the absorptive cells of the small intestine. It is postulated that diets differing in the proportion of fatty foods might influence GIT absorption rates of ingested pyrethroid residues, although the impact in the human diet is presently unknown. In summary, the accumulated evidence indicates an influence of diluents and co-dissolved ingredients on critical toxicokinetic factors accounting for PYR potency in mammals.

Route of exposure and vehicle are critical determinants of PYR-mediated neurotoxicity as well as potential modifiers of the influence of other determinants such as species, age, and dosing solution. Studies in lab animals need to carefully control for aspiration during gavage procedures when the oral route is used. Administration route and vehicle may per se account for up to 5- to 28-fold (Figure 5A) and approximate 200-fold (Figure 5B) variation in PYR toxicity, respectively.

Organismic Factors

Animal Model (Organism Characteristics)

Species and strain A major source of biological variability across studies of neurobehavioral toxicity is the test animal. Data from lab rats and mice are presumed to provide relevant information of acceptable predictive value to protect human health from

toxic exposures to chemicals (Barlow et al., 2002). A few comparable PYR studies make possible ascertainment of the impact of the animal model in the susceptibility to PYR-induced neurotoxicity.

Regarding acute, single-dose exposure studies examining technical-grade test compounds, most neurobehavioral dose-response data for PYR were generated by employing Long-Evans (LE) rats, although a few investigations used Sprague-Dawley (SD) and Wistar strains (Wolansky and Harrill, 2008). There are four studies of deltamethrin (DLM) dose-effect relationship using similar oral corn oil dosing and testing conditions with figure-eight maze (F8M) activity as an endpoint. Three studies used the LE strain (Crofton and Reiter, 1984; Crofton et al., 1995; Wolansky et al., 2006), and one the SD strain (MacPhail et al., 1981). In all cases, dosage-related decreases in activity were observed. The ED_{30} were estimated to be within a 2.5–4 mg/kg range. In addition, LE and SD rat strains were used to examine λ-cyhalothrin effects in two labs under comparable experimental conditions (Hornychová et al., 1995; Wolansky et al., 2006). Both studies included general motor activity assays conducted a few hr after administration of a similar, single oral dose of λ-cyhalothrin. Equivalent motor changes were noted in both studies at 2–6 mg/kg. Thus, no strain-specific pattern of acute PYR toxicity in rats is apparent based on these described DLM and λ-cyhalothrin assays.

Further, a few comparable studies indicated a consistent trend for species-specific susceptibility for acute PYR-mediated toxicity in mammals. Mice are more susceptible than rats, and greater sensitivity is observed in rodents than dogs (Narahashi, 2000). An INCHEM document showed that male adult, oral LD_{50} for oily dosing solutions of DLM varied between 33 mg/kg for mice and 128 mg/kg for rats, well below $\geq$300 mg/kg (i.e., no mortality up to 300 mg/kg) observed using beagle dogs (INCHEM, 1990b). In addition, mice and rat oral LD_{50} estimates for CYPM (a 50:50 *cis:trans* sample, diluted in dimethyl sulfoxide [DMSO]) ranged from 138 to 303 mg/kg, respectively (INCHEM, 1990c). These potency differences are primarily based upon species-specific patterns of PYR metabolism (Ruzo et al., 1978, 1979; Ross et al., 2006). Recognizing that the parent pyrethroids are the proximate neurotoxic agents and that metabolism is detoxifying, variation in detoxifying metabolism within and between species may confound potency extrapolation to humans. For an advanced discussion of rat–human differences in metabolism the reader is encouraged to consult the studies of Ross et al. (2006), Crow et al. (2007), Godin et al. (2006, 2007), Scollon et al. (2009), and Knaak et al. (2012). More comprehensive comparisons of toxicity across species are hindered by confounding factors, such as variations in age, vehicle, dosing volume, and testing time. Species may thus account for two- to fourfold variability in susceptibility across small rodent models, according to the CYPM and DLM reports just discussed, respectively.

Age and body size The neurobehavioral toxicity attributed to PYR has been predominantly characterized using young adult animals (Wolansky and Harrill, 2008). Information on age-related factors influencing the neurotoxicity of environmental chemicals is increasingly relevant to informed regulatory decisions, particularly when projected figures for under-18 and over-65 years age groups are considered; these two age groups together make up 43.3% of the U.S. population in the 2050 projection (US Census Bureau, 2008). In general, exploration of potential age-related risks of neurotoxicity of insecticides has received little scientific consideration. For PYR, there are a few studies examining the vulnerability of developing rats and few data, if any, are available on the adverse neurological effects of PYR during aging of animals or humans.

Developing rats show greater vulnerability to PYR high-dose, acute neurotoxicity than adults**.** The influence of age on PYR toxicity was reviewed by Sheets (2000) and Shafer et al. (2005). Information on age-dependent susceptibility to neurotoxicity in rats and mice is only available for a few PYR: two PYR

of each type, that is, the CS-syndrome compounds DLM and CYPM, and the T-syndrome compounds PRM and cismethrin (Sheets et al., 1994; Sheets, 2000). In these studies, each of three age groups of rats was administered different dosages of PYR by the oral route, and toxicity was estimated using mortality and neurobehavioral (i.e., acoustic startle response) assays. The lethality of each of the PYR decreased with age. Rat pups were more than one order of magnitude more sensitive before two weeks of age than adults. Similarly, at 3 wk of age, pups were 7.4-fold more susceptible than adults. Interestingly, there was no evident variation in test performance between the 3- and 9-wk age groups using acoustic startle response (ASR) assays, when an oral, low-effective dose of DLM in corn oil was administered. Moreover, this investigation was the first study aimed to elucidate the relative weight of toxicokinetic and toxicodynamic factors on the apparent age-related susceptibility for the acute toxicity attributed to PYR. Target tissue (i.e., whole brain) determinations of DLM were carried out in weanling and adult groups of rats administered low-effective and lethal doses of DLM. When a dose well below lethality was used, target tissue level was higher in the weanling pups, and an opposite trend was observed when brain concentrations were determined after acute administration of a LD_{50} dose (Sheets et al., 1994). A recent study of age dependence in DLM pharmacokinetics by Kim et al. (2010) provides an additional perspective on these ASR results. Kim et al. (2010) showed that preweanling and weanling pups (a) displayed markedly elevated brain concentrations of DLM, which remained in this primary target tissue for longer periods of time than in adult rats (which quickly eliminated DLM), and (b) presented pronounced salivation, tremors, choreoathetosis, and eventual lethality. Tornero-Velez et al. (2010) developed an age-dependent, physiologically based pharmacokinetic model, simulating the data of Kim et al. (2010), by incorporating age-specific information for organ weights and metabolic rate contants. The model demonstrated minimal difference in brain concentrations between the 3- and 9-wk-old groups of rats at 2 h (the time of ASR assay), yet a large divergence at 6 h, with markedly lower levels in the older rats brought about by faster clearance kinetics. Thus, conducting an ASR assay at 6 instead of 2 h might demonstrate a greater sensitivity of younger rats. It is important to note that this may not be the case at low doses that do not exceed the limited metabolic detoxification capacity of immature rodents or humans (Anand et al., 2006; Kennedy, 2008). Another factor influencing the interpretation of age-related, [exposure]–[target tissue dose]–[effect] relationships would be the divergence of the time-course patterns for signs of neurotoxicity at either age group. The onset and recovery of clinical signs after acute oral PYR administration in rodents are typically rapid, predominantly occurring within 9 h (Figure 2). These studies underscore the need for evaluation of sensitive neurotoxicity endpoints in conjunction with pharmacokinetic profiles in order to reach decisions on age-dependent toxicity (Marino, 2012). This is not to suggest that the observed sensitivity in younger animals is solely a matter of pharmacokinetics. Immature animals may be more sensitive at the site of action. The *Xenopus* oocyte system is the primary model for acquiring information on the sensitivity of individual mammalian sodium channel isoforms (Soderlund, 2012). Using a *Xenopus* oocyte model to evaluate the influence of channel subunits on sodium channel currents, Meacham et al. (2008) observed that subunit combinations expressed in embryos were more sensitive to DLM and other cyano-bearing PYRs than subunit combinations which predominate in adults. This finding suggests that various toxicodynamic and toxicokinetic factors may concur to determine age-related differences in pyrethroid susceptibility.

A larger rat body size may a priori imply a delay and reduction in the distribution of an absorbed dose of PYR to target tissues. A greater amount of body fat in larger animals may facilitate extraction of PYR from the aqueous blood, so that less is available for central nervous system (CNS) uptake. Alternatively, as a fat-rich compartment, the CNS might act as a buffer

zone, partially attenuating the impact of the PYR burden present in blood. Narahashi (2000) proposed a body weight factor of twofold for size-related variations in susceptibility to PYR-induced toxicity across studies. In the comprehensive PRM study of Metker and coworkers (1977), no evident body-weight-related difference in LD_{50} was observed. In the case of studies comparing different developmental stages, the body size effect, if any, would be subsumed by age-related susceptibility (Sheets et al., 1994; Sheets, 2000; Shafer et al., 2005).

Concurrent aspects of PYR toxicology and the maturation of homeostatic mechanisms during early life may help explain a differential vulnerability through ontogenesis in mammals. The neurophysiological disruption that PYR produce in targeted neurons (Song and Narahashi, 1996), if prolonged in time, may be soon followed by altered neural output and neurobehavioral signs of toxicity (Brodie and Aldridge, 1982). Even a relatively low target tissue exposure to PYR at picomolar to nanomolar levels may be postulated to be effective in producing eventual signs of neurotoxicity (Ogata et al., 1988). In addition, the kinetics for a build up of PYR in target tissues greatly depend upon the detoxifying capacity of the organism. Pyrethroid metabolism is primarily carried out by liver cytochrome P-450 (OX) and carboxylesterase (CE) enzymes (Miyamoto, 1976; Soderlund et al., 2002; Godin et al., 2006). Differences in detoxifying capacity were proposed to play a major role in age-dependent vulnerability to PYR-induced neurotoxicity (Cantalamessa, 1993; Dybing and Søderlund, 1999; Sheets, 2000; Shafer et al., 2005). The age at which an adult-like neurobehavioral response to PYR is observed coincides with the period of final maturation of the xenobiotic metabolism (Anand et al., 2006).

In summary, susceptibility decreases as maturation progresses in high-dose, acute PYR exposure studies conducted in rodents. This is consistent with equivalent findings observed in insects (Bouvier et al., 2002), suggesting a potential role of age in PYR-mediated toxicity across target and nontarget species. This age effect, only emerging after oral exposure to high effective doses promoting it, would account for up to a one order of magnitude increase in toxicity in developing animals compared to young adults. A tentative, no-age-effect conclusion is proposed for environmentally relevant (i.e., low-level) exposures to PYR, although this conclusion awaits additional work to be confirmed. Limited evidence precludes a final conclusion on the body size as a factor, although it seems to contribute a marginal impact on potency estimates, if any, as compared to the age and other biological determinants of acute PYR toxicity in rodents.

Gender Little neurobehavioral evidence is available for PYR from acute exposure studies in rodents using females. Some subacute and chronic exposure studies are therefore reviewed below as an attempt to identify any suggestion of gender-specific susceptibility. Pyrethroid neurotoxicity would be expected to depend upon gender if gender-related differences occur in rates of absorption, distribution and excretion, capacity of detoxification, or in target tissue sensitivity. Soderlund et al (2002) reported blood and liver OX and CE enzymes are major determinants in PYR detoxification in mammals that may exhibit some gender-related differences (Hart et al., 2009). A few studies of male and female rats administered middle to high effective doses of PYR failed to show a consistent trend for gender-related susceptibility. In the FOB study conducted by McDaniel and Moser (1993), after acute oral exposure to PRM and CYPM, no gender-specific sensitivity was apparent. In conclusion, no clear evidence of a gender effect was found in neurobehavioral studies that used male and female animals subjected to similar schemes of acute, single-bolus, oral sublethal doses of PYR. No apparent comprehensive study of gender-related factors affecting neurobehavioral responses after environmentally relevant exposures to PYR was available in rodents.

Other Determinants of Pyrethroid Toxicity

Pyrethroid Metabolites Pyrethroids are efficiently metabolized in mammals; primary

and secondary metabolites are considered to be far less toxic than parent compounds (Gaughan et al., 1977; Roberts and Hutson, 1998; Soderlund et al., 2002). However, a few studies suggested a potential relevance of metabolic pathways for PYR-observed toxicity. Tralomethrin may transform to DLM by debromination within organisms; both PYR esters produce potent CS-like syndromes (rat oral LD_{50} < 100 mg/kg using an oily vehicle), and there is a 10-fold increase in half-life under aerobic conditions after this transformation (CDC, 2003; California EPA, 1996; WHO, 2005). A similar transformation and conservation of potency was observed in rats when tralocythrin is debrominated to form (1R,S)-*cis*-cypermethrin (Cole et al., 1982). Further, in rats, methyl-chrysanthemate, a common intermediate of the metabolizing pathways of a number of PYR, was reported to alter motor activity (Bères et al., 2000); changes in this endpoint also occur upon exposure to a number of the parent PYR regardless of their chemical structure (Wolansky and Harrill, 2008). In addition, acute oral (rats) or ip (mice) exposure to several metabolites common to a number of PYR metabolic pathways was found to evoke toxicity with LD_{50}s ranging from 371 mg/kg (i.e., 3-phenoxybenzyl alcohol, in mice) to 3600 mg/kg (i.e. 3-phenoxybenzaldehyde, in rats) (INCHEM, 1979). Nevertheless, emerging studies reported low-background exposure to PYR in humans (CDC, 2005; Tulve et al., 2006; Julien et al., 2007; FDA, 2013; Starr et al., 2008; Jardim and Caldas, 2012). Therefore, relatively high LD_{50} values and rapid biotransformation and clearence of PYR metabolites in mammals (Soderlund et al., 2002) preclude major clinical relevance of metabolites in health risk estimates for most, if not all, PYR. Yet taking into consideration tralomethrin → DLM, and tralocythrin → CYPM, cases of activation by metabolic transformation (Cole et al., 1982), the potential influence of the metabolic capacity to PYR-induced toxicity in rodents is assigned below a tentative (maximum) 1.5-fold factor, based on the ratio between tralomethrin and DLM oral LD_{50}s (WHO, 2005; U.S. EPA, 1997).

Physiological Status (Circadian Rhythms) As mentioned earlier, molecular targets of pyrethroids include voltage-gated channels, which are involved in maintaining organism stability of normal transmission of electrochemical signals and generation of action potentials in neurons. Predosing activity levels and extraneous sensorimotor stimuli may therefore influence nervous system susceptibility and insecticide potency estimates in experimental animals (Ray, 1997). In addition, rats and mice are nocturnal animals that present circadian rhythms of general activity, eating, drinking, and internal temperature (Zucker, 1971; Weinert and Waterhouse, 1998; Gordon, 2005). Piercy and coworkers (unpublished WHO document, 1976) observed greater rat susceptibility in rats if a high dose of PRM was administered after a 24-h starvation period, compared to toxicity produced in animals fed ad libitum (INCHEM, 1990a). Intraday variations in adverse responses were also observed in rats after exposures to other insecticides such as OP using thermoregulatory response as an endpoint (Kupferberg et al., 2000; Gordon, 2005). In light of the few data available for rodents on the interaction between physiological status and PYR-induced neurotoxicity, maintaining similar dosing and testing times across neurobehavioral studies of PYR seems to be the rule of thumb to avoid potential inconsistencies within and across labs.

Morbid Conditions (Health Status) It is presently unknown how comorbid conditions in humans may influence the major biological determinants of PYR-induced neurotoxicity, such as target tissue uptake and the PYR-specific actions on neuronal excitability. All PYR studies in rodents involving nervous-system function were conducted using illness-free animals (Soderlund et al., 2002; Wolansky and Harrill, 2008). The specific-pathogen-free ("SPF") certification and optimal housing protocols are mandatory requirements for animals selected for regulatory studies. This precludes learning of the potential interaction between toxicogenic pathways of chemical hazards and the most prevalent morbid conditions observed

in human populations. Regarding the accumulated knowledge base for diabetes, epilepsy, obesity, chronic pain, elder-related neurodegeneration and cerebrovascular diseases, and other pathological alterations of human health, it is reasonable to postulate that comorbid conditions might modify the toxicokinetics and toxicodynamics of pesticides in lab animals as well as vulnerability of humans (Janssen et al., 2012). Pyrethroids disrupt neuronal excitability as a primary MOA by targeting VGSC channels in the nervous system (Soderlund et al., 2002; Soderlund, 2012). An example of this potential interaction between PYR-mediated toxicity and chronic pathologies may be apparent by analyzing VGSC system integrity and diabetes-related neuropathic pain. Alterations in VGSC expression and function were proposed to produce clinically relevant changes in pain-related signaling pathways of nociception, including changes in pain thresholds observed in diabetic neuropathy patients (Hong and Wiley, 2006; Cummings et al., 2007). In addition, an old PYR compound (i.e., a tetramethrin-like structure) was shown to alter the action of insulin-like growth factors (IGF; i.e., IGF-I and IGF-II) on calcium homeostasis in BALB/*c* 3T3 cells; most relevant, some concentration-dependent effects were observed at the low nanomolar range (Kojima et al., 1988). There are limited epidemiological or animal data, if any, on susceptibility to PYR toxicity in diseased individuals. Lack of data creates uncertainty on whether an additional vulnerability factor is present in individuals suffering the early or late stages of chronic diseases such as diabetes. This lack of systematic exploration of the morbidity factor in the regulatory pesticide toxicology arena is surprising, taking into account that there have been values available for more than three decades consistently indicating that people illness free for their entire life constitute a minority of the general population worldwide (CDC, 2010).

Testing Conditions (Endpoint and Testing Device) Threshold doses and potency estimates vary depending on the neurobehavioral endpoint. The FOB study of McDaniel and Moser (1993) tested PRM and CYPM effects on 14 endpoints examined under identical dosing conditions. For most endpoints, at least 60 mg/kg CYPM was needed to induce evident functional alterations. Lowest effective doses were lower (i.e., 20–40 mg/kg) in motor activity, sensorimotor performance (ASR), reactivity (touch and click response), and body temperature assays, showing apparent greater sensitivity of these endpoints for CYPM. Overall, an approximate threefold variation in the lowest levels of the effective dose range was evident across assays. In addition, a number of equivalent FOB assays were used to characterize the influence of dose-volume (i.e., 1 vs. 5 ml/kg) on neurotoxicity induced by acute exposure to bifenthrin (Wolansky et al., 2007a). Some endpoints (e.g., internal temperature, tremorigenic activity, motor activity) exhibited evident dosage-related effects at low doses; moreover, syndrome severity and onset time were clearly influenced by dose volume. However, when sensitivity to handling and tail-pinch response were assayed, no evident effect trend was evident at bifenthrin doses below the threshold dose for lethality, and a dependency of functional performance on dose volume was unclear. Figure 6 summarizes dose-response data from the nine assays included in this bifenthrin study (see panels A and B). It is apparent that a threshold-dose estimate for bifenthrin-induced neurotoxicity is dependent on assay endpoint, spanning 6–20 mg/kg from the most to the least sensitive testing protocol.

For any test chemical, its observed neurobehavioral toxicity may be influenced by testing apparatus and testing-room features (Tapp et al., 1968; Crofton et al., 1991). Thus, it follows that potency estimates may also vary as a function of these factors. Apparent contradictory results were observed in two independent studies evaluating the effect of CYPM on motor activity: An increase in activity was observed using Motron, cage-like stations, whereas a reduction in activity was found using F8M mazes. Dissimilar results were mostly attributed to the spatial distribution of photocells in these motility meter systems (Reiter et al., 1981; Crofton and MacPhail, 1996). In other studies, PRM and DLM produced

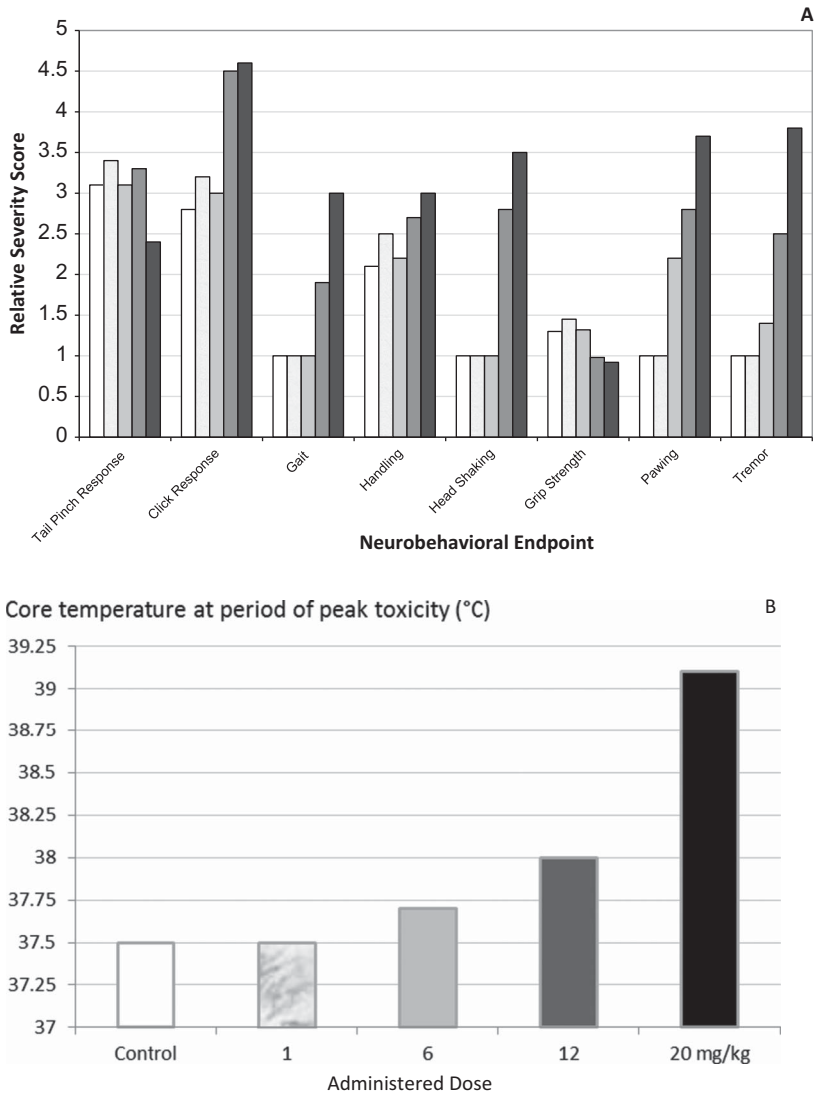

FIGURE 6. Influence of the selected endpoint on potency estimates for bifenthrin neurotoxicity. Dose-response patterns observed in rats at ~4 h after oral exposure to bifenthrin in corn oil using FOB neurobehavioral assays (Wolansky et al., 2007a). In all assays (save internal temperature changes), authors used a scoring scale from 1 (control-like performance) to 4 (severe impairment) (see panel A) for the effects of 1, 6, 12, and 20 mg/kg bifenthrin (ascending doses denoted as increasingly darker figure bars). Note the variability of lowest effective doses across neurobehavioral domains. Handling and tail-pinch response were mostly unaffected, though a low-effective dose for tremor, pawing (A), and body temperature (B) was observed at ~6 mg/kg, consistent with a low-effective, ED_{30} dose of 3.2 mg/kg (95% confidence interval: 2.6–3.8 mg/kg) for alteration of motor activity as estimated in a prior bifenthrin study where similar dosing and testing conditions were used (Wolansky et al., 2006).

dosage-related decreases in F8M activity assays (Crofton and Reiter, 1984, 1988; Wolansky et al., 2006); however, the opposite outcome, a dose-related rise, was noted using an identical animal model, the same test chemicals, and similar dosing conditions when general motor activity was monitored using a cage-based system by radiotelemetry (Wolansky et al., 2007b). The design of the testing system and the actual manner in which raw data are collected and interpreted may thus critically demonstrate inconsistent findings across PYR studies.

Classification of PYR in subgroups based on neurobehavioral findings is endpoint specific. As mentioned earlier, all PYR (save the

Wolansky et al. 2007b study), tested as single compounds or mixtures, produced dose-related reduction in motor activity and other functional responses (MacPhail et al., 1981; Crofton and Reiter, 1984, 1988; McDaniel and Moser, 1993; Hornychova et al., 1995; Wolansky et al., 2006a, 2009; Wolansky and Harrill, 2008). However, compound structure-dependent effects were found when other endpoints were evaluated. Full effective doses of the Type-II PYR CYPM and DLM produced hypothermia, and Type-I-like PRM and bifenthrin produced hyperthermia (Soderlund et al., 2002; Wolansky et al., 2007a; Wolansky and Harrill, 2008). Yet, at a low effective dose range all four PYR produced moderate increases in body temperature, and this dose-related, biphasic pattern of these Type-II compounds was visible only when a second endpoint, that is, thermoregulatory response, was tested in the same lab using dosing conditions comparable to those used in motor activity assays (McDaniel and Moser, 1993; Wolansky et al., 2006, 2007a, 2007b; Pato et al., 2011).

In addition, the available FOB studies of PRM, CYPM and bifenthrin suggest that various relative potency estimates may be obtained for PYR by selecting different neurobehavioral assays. While a ratio of ED_{30} for DLM and CYPM in F8M motor assays (i.e., $ED30_{DLM}/ED30_{CYPM}$) is approximately 0.1–0.24 (Crofton and Reiter, 1984, 1988; McDaniel and Moser, 1993; Crofton et al., 1995; Wolansky et al., 2006), this ratio declines to approximately 0.05 if effects data from ASR assays are used for ED_{30} estimation (Crofton and Reiter, 1984, 1988). In addition, alternative use of Motron and F8M devices in independent studies of motor activity reflected device-specific findings for PYR-mediated neurotoxicity (Reiter et al., 1981; Crofton and MacPhail, 1996). In conclusion, endpoint- and device-related sensitivity in estimating PYR potency may produce variations in pesticide potency across assays. A threefold endpoint effect based on dose-effect trends observed in the FOB studies of PRM, CYPM, and bifenthrin may thus be assumed (McDaniel and Moser, 1993; Wolansky et al., 2007a).

Ambient Temperature Ambient temperature is another experimental variable that determines the potency of PYR insecticides with in vitro assays (i.e., disruption of neuronal excitability and signal transmission in cell cultures), and in vivo in rodents (Narahashi, 2000; Krieger, 2010). A fall in sample temperature enhances the toxicity of PYR by increasing the decay time of tail currents of Na^+ through VGSC system in vitro (Ginsburg and Narahashi, 1999; Narahashi, 2000). Similarly, low ambient temperature within the testing lab enhances PYR potency in vivo: Acute oral LD_{50} values for cismethrin in rats decrease from >1000 mg/kg at 30°C to 157 mg/kg at 4°C, a ≥6-fold room-temperature effect (White et al., 1976). In addition, PYR produced changes in body temperature (Soderlund et al., 2002; Wolansky et al., 2007b; Wolansky and Harrill, 2008) that may last 24–48 h (McDaniel and Moser, 1993). Excessive metabolic activity associated with tremors was proposed as a tentative explanation for the transient hyperthermia observed in rats upon intoxication with the tremorigenic compounds PRM and cismethrin (Cremer and Seville, 1982; McDaniel and Moser, 1993). However, there is no certainty on how the CS-syndrome landmark hypothermia (McDaniel and Moser, 1993; Soderlund et al., 2002) is generated above middle-effective dose levels of most cyano-pyrethroids. According to the already mentioned data on PYR-induced alterations of thermoregulation in rats and interaction between adverse response and ambient temperature (White et al., 1976; Gordon, 2005), neurobehavioral assessments of PYR-mediated toxicity need to control for ambient temperature consistency within and across studies. Lab studies are typically carried out at 22–25°C (Gordon, 2005); however, human exposures in the general population rarely occur in such a narrow ambient temperature range. Based on the cismethrin study of White et al. (1976), ambient temperature might be expected to produce up to a sixfold variation in PYR potency in lab rodents.

LABORATORY-CONTROLLED DOSING SCHEME VERSUS REALISTIC EXPOSURES

Most published studies designed to establish absolute and relative potencies of pesticides in mammals have used single-chemical exposure schemes (Janssen et al., 2012). Real scenarios of human exposure to PYR (and to other pesticides) suggest that daily exposures to residues of multiple pesticides are more a rule than an exception in the general population (Chun and Kang, 2003; Tulve et al., 2006; Lu et al., 2006; FDA, 2013; CDC, 2005; CDPR, 2007; Riederer et al., 2008; Jardim and Caldas, 2012). Studies evaluating the combined neurobehavioral actions of environmentally relevant levels of pesticides with different MOA in mammals are apparently not available. There is some evidence on the combined effects of low dose mixtures after acute, single-bolus dose exposure to a number of PYR in rodents. A dose-response study examined the simultaneous action of subthreshold levels of 11 pyrethroids administered by the oral route to young adult rats (Wolansky et al., 2009). The test mixture included cyano and non-cyano compounds previously classified as T-, CS-, or TS-syndrome-inducing PYR (Soderlund et al., 2002; Wolansky and Harrill, 2008). General motor activity was used as a neurobehavioral endpoint. Although the individual chemical constituents were present at subthreshold levels, the mixture was effective in producing dose-dependent declines in activity. Use of rigorous mathematical and statistical procedures to test a default hypothesis of additivity indicated that the cumulative effect of the 11 compounds was consistent with the joint action proposed by the dose-addition theory (Wolansky et al., 2009). While the test mixture was not based on observed proportions in the environment (i.e., equipotent doses of 11 chemicals were used), the subthreshold nature of the co-administered doses of the examined chemicals certainly carries environmental relevance. Additivity was also determined in a follow up study using a five-chemical mixture. The test compounds and mixing ratios in this second study were selected based on the proportions of PYR detected in the floor residues of 168 child care centers across the United States, a nationally representative sample (Starr et al., 2012; Tornero-Velez et al., 2012 a; Marshall et al., 2013).

Although an efficient PYR metabolism in humans would be expected to completely eliminate trace amounts ingested with food daily, or by their residential use, a low but persistent burden of PYR seems to be the rule rather than the exception for many populations globally, as evidenced by various studies of urine samples in humans (Schettgen et al., 2002; Heudorf et al., 2004; Riederer et al., 2008; Whyatt et al., 2002; Wielgomas et al., 2013; Wu et al., 2013). For this reason, recent mixture studies emphasize the relevance of experimentally assessing the joint action of multiple exposures to insecticides in dose levels individually considered subtoxic in order to fully characterize the risk of neurotoxicity posed by realistic exposures to PYR co-occurring in the environment and food. In addition, there are a few reports on the joint toxicity of environmentally relevant, binary mixtures of PYR and OP insecticides in non-neurobehavioral endpoints, such as markers of endocrine disruption in rats (Liu et al., 2006), which are beyond the focus of this review.

A series of studies with the non-cyano PYR allethrin suggests a complex interaction between the accumulated pattern of chemical pesticide exposures during early development, route of exposure, and vulnerability to realistic PYR exposures at any age (Eriksson and Talts, 2000; Tsuji et al., 2002). A low oral, repeated exposure to bioallethrin in 10 day-old mice at 0.7 mg/kg/d for 7 d was subeffective in producing any evident clinical sign of toxicity in developing or adult mice, but otherwise effective in facilitating enduring neurochemical and neurobehavioral alterations when treated pups were reexposed to the same low bioallethrin dose as adults at 5 mo of age (Eriksson and Talts, 2000). In addition, another study using a full range of *d*-allethrin doses administered by inhalation, including the already-mentioned 0.7-mg/kg dose, a similar period of early postnatal exposure, and an equivalent battery of endpoints of neurotoxicity in adulthood, was

unable to detect any long-term neurochemical or behavioral alterations (Tsuji et al., 2002). These and other studies examining the developmental neurotoxicology of PYR were reviewed by Shafer et al. (2005). The limited evidence available calls for research efforts to ascertain whether persistent neurobehavioral effects of environmentally relevant exposures to PYR are possible in individuals who experienced subclinical, nearly threshold exposures to pesticides during early life.

INTERPLAY OF DETERMINANTS OF PYRETHROID NEUROTOXICITY

This review shows the multiplicity of experimental and organismic factors that may significantly influence the severity of the PYR syndromes, as manifested in lab rodents under controlled conditions. These determinants are classified in Table 1 according to the toxicological domain mostly responsible for the outcome variation and the actual maximal influence expected upon manipulation of each determinant.

There is a body of evidence that establishes that test material composition, dosing solution preparation, and testing-room features may account for major variations in PYR potency**.** The most relevant physicochemical and toxicokinetic determinants of PYR syndrome severity include structure, isomer composition (i.e., ratios of active and nonactive isomers), route of exposure, vehicle, and dosing volume. The digestion of food in the GIT of experimental animals would be assumed to be a potential modifier of PYR absorption and metabolism. There is certainly limited evidence that fasted animals may suffer slightly greater toxic responses from oral exposures. This seems to be consistent with a similar trend observed with decreasing vehicle volume (from 5 to 1 ml/kg). The smaller dose volume (1 ml/kg) enables a greater rate of GIT absorption (Wolansky et al., 2007a).

Species is perhaps the principal organismic factor, contributing up to a fourfold effect on PYR potency. Time of the day when dosing and testing are conducted (i.e., circadian rhythms), and testing conditions such as endpoint and test device also affect dose-effect relationships and potency estimation. All factors result in up to two- to fourfold variation in PYR potency. The size of the animal (Narahashi, 2000) and testing device design (Crofton and MacPhail, 1996) need to be taken into consideration when interpreting PYR actions on rodents, especially when results from different animal lots or sensitivities of alternative testing systems are compared. Lastly, testing-room ambient temperature needs to be maintained within a small range, ideally the same used in the animal colony (i.e., typically 22–25°C), whether a comparative analysis within or across studies is required. Other organismic aspects of the study design, including age, strain, and gender, exert little or no impact on neurobehavioral measures of PYR-induced acute neurotoxicity when nearly threshold or low-effective dose regimens are used.

The influence of experimental and organismic factors on PYR-induced toxicity in mammals confirms the importance of study design on toxicological assays. The already-mentioned determinants of PYR toxicity are not exclusively relevant for this insecticide class. The toxicological literature is replete with examples of chemicals with a plethora of factors affecting, to a lesser or greater degree, insecticide toxicity in small rodents. Many of the determinants of PYR-mediated toxicity alluded to in this review were also found to be relevant for the toxicogenic pathways of OP (Karalliedde et al., 2003). Chemical structure is also a major determinant of acute toxicity for OC and OP, demonstrating an approximate 500-fold variability in rat oral LD_{50}, a span of potency across compounds similar to that observed for PYR (Patnaik, 2007; WHO, 2005). In addition, many OC, PYR, and OP were previously found to display enantiomer-specific activity responsible for at least 10-fold variation in LC_{50} estimates in invertebrate models used in ecotoxicology (Liu et al., 2005). The neurotoxicity evoked by OC in rats was reported to depend upon dose volume (McDaniel and Moser, 1997), in

TABLE 1. Impact of Experimental and Organismic Factors on Pyrethroid Neurotoxicity

Toxicological aspect	Toxicity determinant	Maximum factor effect in single-compound assays (pyrethroids case)	Is consideration of the impact of these study design aspects a standard procedure in risk assessment for pesticides?
Toxicokinetics	Chemical structure	++++++ (500-fold)	Yes
	Isomer composition	+++ (25-fold)	Yes
	Formulation/purity	+++ (50-fold)	No
	Vehicle	+++++ (200-fold)	No
	Route of exposure	+++ (28-fold)	Yes
	Dose volume	+ (3-fold)	No
	Dosing solution	+ (≤2-fold)	No
	Metabolic activation	+ (≤1.5-fold)	No
	Age (detoxification system maturation)	++ (10-fold)*	Yes
Toxicodynamics	Species (rodent)	++ (4-fold)	Yes
	Age (nervous system development)	No effect?*	Yes*
	Endpoint	+ (3-fold)	No**
	Physiological status	+ (2-fold)	No
	Morbidity	No data available	No
Testing room	Ambient temperature	++ (6-fold)	No
History of pesticide exposure episodes (from gestation to adulthood)	Realistic sequential exposures vs. laboratory-controlled acute exposure	Few data available	No

Note. The table classifies each determinant of toxicity with respect to pyrethroid toxicokinetics (TK) or toxicodynamics (TD) in laboratory animals. A relative score for the impact of each factor on potency estimation is also provided (maximum factor-effect between brackets in third column). Lack of information on the potential impact of these determinants on single-chemical risk assessments is marked in the fourth column when applicable. Data on cumulative risk of neurotoxicity of pesticides have recently started to be generated. For a part of the listed TK and TD factors, the influence of the experimental and organismic determinants on pyrethoid potency has not been incorporated to animal–human extrapolation procedures yet.

*Little evidence suggests no evident toxicodynamics-related age effect for acute oral exposures to low-effective doses of individual pyrethroids in small rodents. Moreover, no information is available for the influence of aging on pyrethroid vulnerability.

**The PoD used in risk assessment is regularly derived from the most sensitive endpoint assay; there is no analysis available of endpoint sensitivity for a representative number of Type I, Type II, and mixed Type I/II pyrethroids using a full dose range scheme of administered doses.

a magnitude comparable to that estimated for bifenthrin (Wolansky et al., 2007a). Overall, these findings reveal a strong relationship between study design and the estimated insecticide potencies.

The myriad of factors that may greatly influence the observed patterns of neurotoxicity raise a question of the animal model, test material, and dosing and testing conditions that need to be selected in order to be as realistic and protective as possible in health risk assessments of PYR or other insecticides. For many insecticides in use, there is no apparent information on how several of the already-mentioned experimental and organismic factors influence toxicity in mammals. Moreover, to our knowledge, there is no there is no apparent information on how interaction of two or more determinants of toxicity may produce unexpected attenuation or exacerbation of PYR-induced toxicity. Indeed, it was mentioned earlier that inconsistent results were obtained by a lab that tested different formulations of PRM but did not use the same vehicle to prepare dosing solutions in each case (Williamson et al., 1989). Should one expect intakes of a 10-μg PYR dose diluted in an oily food (e.g., at 0.01 mg/ml concentration) or in a mostly aqueous, more concentrated beverage (e.g., 0.1 mg pyrethroid/ml drink) to be equitoxic in mammals including humans? There is a lack of empirical data to respond to this type of question, and current interpolation and

extrapolation procedures do not consider these potential scenarios of exposure in terms of their impact on estimates of health risk. An experienced reader may expect that a great portion of the uncertainty in the preceding example is already recognized, acknowledged, and considered when toxicokinetic (TK) studies are conducted and used in risk assessment processes. Yet an advanced TK knowledge base is not always generated during risk characterization at industry R&D departments and government labs. Thus, the answer to this question is unknown at present. A major utility of identifying and characterizing the determinants of PYR-mediated neurotoxicity in rodents becomes apparent: Lab animal data are used in decision-making processes to protect humans from the unwanted impact of low-level, daily exposures to pesticide residues present in foods and pest control products used indoors and outdoors.

It may be impractical to generate information on all determinants of toxicity for all old and new insecticide compounds. Therefore, a simple format for analyzing the adequacy of the available information needs to be designed for use in the early steps of a decision making process on health risks. While many determinants of neurotoxicity are not exclusive for a particular insecticide class, the potential maximal influence of each experimental factor in potency estimates seems to be of the same order of magnitude across insecticides (as suggested for the reported chemical structure, isomer composition, vehicle, purity, and dose volume effects on ED_{50}s and LD_{50}s); the PYR case apparently illustrates a situation applicable to other insecticide classes. In the following, an exploratory system applicable as a decision rule for animal data sufficiency is proposed for PYR.

ESTABLISHING SUFFICIENCY IN ANIMAL TEST DATA

It was noted that manifestation of PYR-induced toxicity in experimental in vivo assays and PYR potency estimations in animals greatly depend on lab-controlled conditions used to examine these insecticides. Research efforts aimed to estimate the cumulative risk of neurotoxicity posed to humans after exposure to environmentally relevant levels of PYR (Tulve et al., 2006; Wolansky et al., 2006, 2009; Mirfazaelian et al., 2006; Starr et al., 2008; Scollon et al., 2009, 2011; Jardim and Caldas, 2012) thus require a knowledge of the exposure scheme dependence and testing protocol dependence of PYR potency (Crofton et al., 1995; Wolansky et al., 2007a, 2009). Hence, it is opportune to identify how much data are sufficient before using experimental information in well-informed health risk decisions.

For any pesticide class, a number of determinants of toxicity are expected to coparticipate in defining the ultimate clinical consequences of actual exposures in both lab animals and humans. Table 1 provides a summary of the most relevant organismic and experimental factors influencing PYR-induced toxicity and relative (theoretical) impact of each factor on estimated risks, as established based on the available information that was compiled in this review. An indication if each individual factor is considered in current risk assessment protocols and risk uncertainty estimations for individual and combined PYR compounds is also provided. Since all relevant test materials, and dosing- and testing-related factors may individually produce moderate-to-strong variations in PYR-mediated toxicity in rats and mice (i.e., 3- to 500-fold changes in potency measures; see Table 1), interplay between the modifying impacts of these factors may theoretically yield large variations in risk measures. The interplay between dose volume and vehicle may result in up to 600-fold variation in toxic potency in animals (i.e., computing the product of the individual maximal impacts of these toxicokinetic factors; see Table 1). Similarly, potentiated or attenuated syndromes may result from the combination of particular dosing and testing conditions. Various factors included in Table 1 are regularly examined by research labs in industry and government as currently mandated by local and national authorities during procedures for hazard characterization and health risk assessment. Animal species, animal age, and route

of exposure are duly noted in virtually all toxicological assessments, with good reason, as they might contribute an estimated global uncertainty to risk estimates of approximately 10^3 ($4 \times 28 \times 10$). However, these factors need to be considered in the context of isomer composition, formulation/purity, and vehicle, which contribute an estimated global uncertainty of approximately 10^5 ($25 \times 50 \times 200$). Although a global impact of 10^5 is unlikely to be realized, it serves as a caution in study design where such factors may combine multiplicatively. Moreover, although recent research efforts in industry and government (Wolansky et al., 2006, 2007, 2009; Starr et al., 2012; Weiner et al., 2009; Breckenridge et al., 2009) demonstrate exceptional rigor in study design, the acute PYR toxicity case may still serve as a proof of concept on how to scrutinize sufficiency of data from animal assays in regulatory processes.

Figure 7 synthesizes all the information on toxicity determinants mentioned in this review for PYR. For any chemical class, a graphical representation like this would be helpful to determine animal data sufficiency for risk assessment, if it allows a sensible and rapid evaluation of three questions, as follows: (1) Have all relevant organismic and experimental factors been evaluated using low, realistic exposure levels? (2) Have all major determinants of toxicity been appropriately examined? For instance, if ratios of enantiomers are critical for toxicity, has the maximal isomer effect been estimated for a few prototypical members of each toxicological class? (Note: For PYRs, this would include at least one Type-I, one Type-II, and one Type-I/II compound.) (3) Are any major determinants of toxicity assigned a factor effect score of relevance for realistic exposures in humans? The graphical presentation in Figure 7 enables testing most of these questions after a quick visual analysis of the determinant factors, ordered from greater to lower impact on toxicity.

This illustration makes clear that most of the uncertainty related to the animal model and experimental design used for PoD derivation has been already informed in the PYR case. This might be not the case for other pesticide chemical classes. In a quasi-optimal situation similar to the one described here for PYR, environmental data would be considered to incorporate to PoD computing only the variability related to the most relevant determinants of toxicity, that is, not the determinants exerting greater impact but those producing any variation in health risk in realistic situations of exposure.

In summary, this review compiled animal data relevant to the acute neurobehavioral characterization of PYR. It is our expectation that confirming that comprehensive lab data have been collected, and accounting for the aforementioned experimental and biological factors may reduce uncertainty in risk assessments pertinent to subclinical or mild neurotoxicity. Further this approach will provide the necessary thinking to consider risks associated with other endpoints. Indeed, the knowledge base on other health risks such as endocrine disruption or immune response deficiency may contain less available data (Van Balen et al., 2012), and it is also unknown how Figure 7 would appear if chronic PYR exposure studies were considered instead of the acute exposure effects data that are predominantly available at present. Yet it is clear that a similar comprehensive consideration of the determinants may help to reach greater accuracy in risk estimates for those endpoints (Janssen et al., 2012).

All research sectors (i.e., industry, government, academia) contributing to generate exposure, toxicokinetic, mechanistic, and effects information may achieve their ultimate goals in optimizing chemical safety faster and more accurately by reaching a consensus on the minimal toxicological information that needs to be generated before establishing estimates of absolute and relative potencies for individual compounds representing a toxicological class. This, in turn, would ensure building up environmentally realistic, risk assessment projects using more sensible, predictive, and protective designs. Accordingly, the generation of an advanced neurobehavioral toxicity knowledge base and recent efforts considering the acute

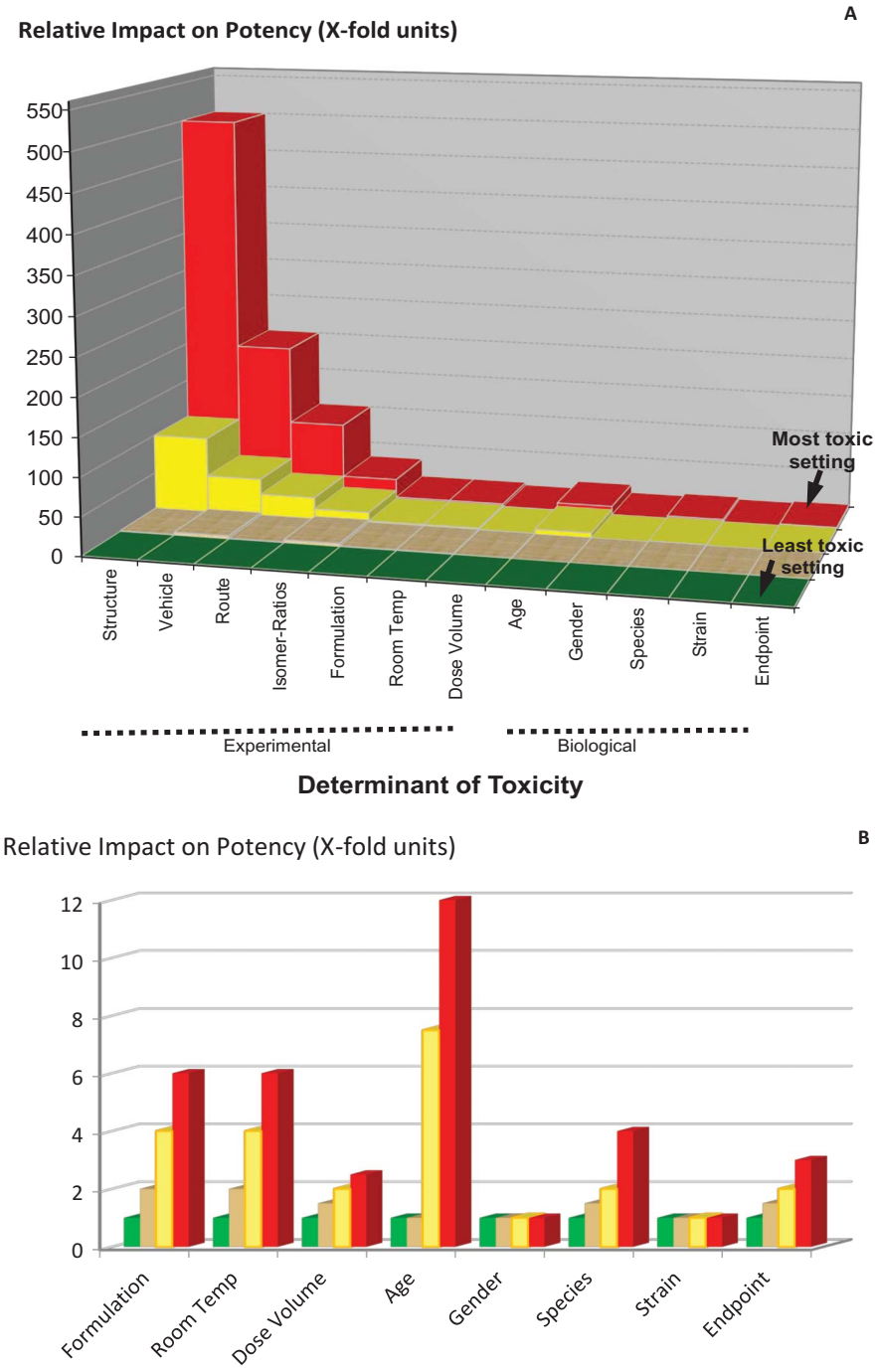

FIGURE 7. (A, B) Influence of 12 determinants on pyrethroid potency in laboratory animals. This figure shows the influence that might be expected on potency estimations by using different experimental and biological conditions. As reviewed studies present more than one assay condition for each determinant examined (e.g., as occurred in the assessment of the influence of four different vehicles on DLM's ED_{30} for motor activity in the study by Crofton et al. [1995]), this graphical approach shows the expected relative impacts on potency estimates, from the least to the most sensitive study conditions. These determinants of toxicity are ordered from those producing the greatest impact at left to those at right from which only minor variations in potency would be expected across studies differing in design. Panel B allows for distinguishing differences among factors having 1-12-fold impact on potency.

Note: For factor "Age," the alluded maximal effect would be only possible at oral exposure levels equivalent to $\geq 1/20$ LD_{50} (see oral LD_{50} for permethrin in rat pups and adults, in http://www.epa.gov/teach/chem_summ/pyrethroids_summary.pdf). Data to construct this figure were taken from: *Structure*. Rat oral LD50s observed across pyrethroid insecticides, from the least toxic compound (LD_{50} > 10 g/Kg) to the most potent compound (LD_{50} = 22 mg/kg) (WHO, 2005; Wolansky and Harrill, 2008). *Isomer ratio*. Studies of the differential acute

FIGURE 7. (*Continued*) oral lethality of several preparations of permethrin differing in *cis*- and *trans*-isomer ratios in rats (taken from INCHEM, 1990a). *Formulation*. Three studies were considered: a comparison of the lethality of two formulations of deltamethrin (Lepeshkin et al., 1992), a comparison of the neurotoxicity of two formulations of permethrin and fenvalerate in mice (Williamson et al., 1989), and a study of the comparative effects of two permethrin test materials (a technical-grade preparation and a 40% pure, commercial formulation) on motor activity in time-course and dose-response assays conducted in rats (Wolansky, unpublished data). *Dose volume*. Study of the toxicity observed in rats after oral administration of bifenthrin in corn oil. The two dosing conditions examined in this study are compared, 1 vs. 5 ml/kg (Wolansky et al., 2007). *Route*. Studies of the toxic action of deltamethrin in rats (Crofton et al., 1995) and allethrin in mice (Nishimura et al. 1995) using different routes of single-dose, acute administration. Monitoring of motor activity in mazes was conducted in the former study, and observation of toxic signs and tremorigenic activity scoring was used in the latter. *Vehicle*. Study of the influence of four vehicles used to dissolve the test compound on the neurobehavioral toxicity of deltamethrin in rats, using motor activity as an endpoint (Crofton et al., 1995). *Species*. A mild trend for a higher vulnerability in animals of smaller body size is apparent (Narahashi, 2000). According to a few studies of deltamethrin and cypermethrin, mice appear to be two- to fourfold more susceptible than rats (taken from INCHEM, 1990b; INCHEM, 1990c). *Gender–strain*. Limited evidence suggests no relevant influence on pyrethroid neurotoxicity. *Age*. Based on the lethal toxicity and the acoustic-evoked startle response assays conducted in infant, weanling and adult rats after a single, oral dose of DLM in corn oil (Sheets et al., 1994; Sheets, 2000). *Metabolites*. Limited information is available suggesting no or very low neurotoxicity potential of pyrethroid metabolites in small rodents (Soderlund et al., 2002; see also difference in LD_{50} for deltamethrin and tralomethrin in section "Pyrethroid Metabolites"). *Morbid condition*. No information is available. *Endpoint*. Two FOB studies examining permethrin, cypermethrin (McDaniel and Moser, 1993), and bifenthrin (Wolansky et al., 2007a) actions on young adult male rats under identical dosing conditions were taken into account. The figure shows a variation in minimum effective doses across endpoints in the bifenthrin study. *Room temperature*. Study of the differential oral lethality of resmethrin in adult rats tested at three ambient temperatures, 4°C, 20°C, and 30°C (White et al., 1976). *Realistic vs. laboratory-controlled exposures*: Available data (Eriksson and Talts, 2000; Tsuji et al., 2002) are still insufficient to draw conclusions on possible pyrethroid sensitization manifesting from early-life exposures to low doses of pyrethroids (color figure available online).

risks PYR insecticides demonstrates a successful experience that may guide and inform the decision-making processes of other pesticide classes.

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