Neurotoxicology and Teratology 52 (2015) 1–10

Contents lists available at ScienceDirect

Neurotoxicology and Teratology

journal homepage: www.elsevier.com/locate/neutera

Evidence for effects on thermoregulation after acute oral exposure to type I and type II pyrethroids in infant rats☆,☆☆

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ARTICLE INFO

Article history:
Received 19 May 2015
Received in revised form 4 September 2015
Accepted 9 September 2015
Available online 15 September 2015

Keywords:
Pyrethroids
Cumulative neurotoxicity
Infancy
Thermoregulation

ABSTRACT

Most pyrethroid (PYR) insecticides may be classified either as type-I compounds, which produce whole body tremors and hyperthermia, or type-II compounds, which produce salivation, choreoathetosis, and hypothermia (i.e., producing T and CS neurobehavioral syndromes, respectively). This classification is based on clinical observations in adult rats and mice after intracerebroventricular or intravascular administration of highly effective acute (bolus) doses. PYR neurotoxicity in infant animals is not characterized as much as in adult animals. Endpoints informing on vital determinants of mammal's maturation, such as body temperature may help recognizing age-related differences in susceptibility to PYRs. In this work, body temperature (T_b) was monitored at 30-min intervals after acute oral exposure to T-syndrome PYR bifenthrin (BIF), CS-syndrome PYR cypermethrin (CYPM), and a BIF–CYPM mixture in weanling rats by using a subcutaneous temperature monitoring system. In both single-compound assays, a time- and dose-related decline of T_b was the most evident impact on thermoregulation observed starting at ~2–3 h after dosing. Moreover, 15–18 mg/kg BIF induced a mild increase in T_b before the hypothermic action was apparent. The lowest effective dose for temperature perturbation was 15 mg/kg for BIF and 10 mg/kg for CYPM, and moderate neurobehavioral alterations were evident at 12 and 10 mg/kg, respectively. When low effective doses of BIF and CYPM were co-administered mild behavioral effects and a transient increase in T_b ($p = 0.02$) were observed at 1–2 h, and no T_b decline was apparent afterwards compared to control animals. Noteworthy, the hypothermic action of BIF in infant rats was quite different from the hyperthermia consistently reported in studies using mature animals. Our results suggest that body temperature monitoring may be useful as a complementary assessment to reveal qualitative age-specific pesticide effects in rats.

1. Introduction

Pyrethroids (PYRs) are synthetic insecticides, increasingly used in a wide range of indoor and outdoor pest control applications (CDC, 2003). A large body of data indicate that low-level exposure to multiple PYRs may occur in humans (CDC, 2005; FDA, 2008; Fortin et al., 2008; Heudorf et al., 2004; Jardim and Caldas, 2012; Li et al., 2014; Morgan et al., 2014; Tulve et al., 2006). A dual type-I/type-II toxicological classification for PYRs was established ~35 years ago based on their chemical structure (i.e., absence or presence of an α-cyano group, respectively) and acute effects in adult mice and rats administered high doses as a single-injection bolus via the intravascular or intracerebral routes. Type-I compounds produce marked whole body tremors (i.e., a T-syndrome), whereas type II compounds produce a syndrome characterized by choreoathetosis and salivation (i.e., a CS-syndrome) (Gammon, 1981; Lawrence and Casida, 1982; McDaniel and Moser, 1993; Verschoyle and Aldridge, 1980). Moreover, studies using acute oral exposures have shown opposite effects on thermoregulation depending on the PYR type, with type-I compounds producing hyperthermia and type-II compounds producing hypothermia (McDaniel and Moser, 1993; Soderlund et al., 2002). However, motor activity or neuromuscular strength assays have shown a similar outcome, a decline, for different PYRs regardless of the compound type (Wolansky and Harrill, 2008). In addition, no clinical report has yet been able to unequivocally link PYR exposures to the occurrence of the above-mentioned divergent type-I/type-II landmarks of neurotoxicity in humans (CDC, 2003; Miller and Menowsky, 2014; Pauluhn, 1998). Thus, a full understanding of the neurobehavioral effects of subconvulsive-doses of PYRs may require the use of a comprehensive battery of compound-specific endpoints (see ref. Wolansky and Tornero-Velez, 2013).

☆ *Competing interest declaration*: The authors have no competing interests.
☆☆ *Disclaimer*: Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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http://dx.doi.org/10.1016/j.ntt.2015.09.005
0892-0362/

The generation of accurate health risk estimates for PYRs is in progress (Shafer et al., 2005; Soderlund et al., 2002; Starr et al., 2012; Wolansky and Tornero-Velez, 2013). By using motor activity assays, Wolansky and co-workers previously tested the hypothesis that PYRs act in a dose-additive manner in adult rats (Wolansky et al., 2006, 2009). To this end, that research characterized the dose–effect functions for eleven PYRs and found that all PYRs produced dose-dependent reductions in activity, with more than 200-fold variation in potency. Moreover, a mixture of no-effect levels of these compounds produced a monotonic pattern of dose-related decline in activity, consistent with a dose-additive joint action. Thus, motor activity demonstrated to be a sensitive and reliable endpoint for the acute neurotoxicity of PYRs (Wolansky and Harrill, 2008). In addition, acoustic evoked startle response (ASR) assays have been efficient to describe sensorimotor reflex responsiveness to subconvulsive doses of PYRs, even though a limited capacity to detect age-specific susceptibility was apparent (Crofton and Reiter, 1984, 1988; Sheets et al., 1994; Sheets, 2000).

Vulnerability to pesticides is greater in children, especially in early life, than that in adults (Roberts et al., 2013). Developing individuals are not simply "small adults": during early life, there is a continuum of plastic changes in the microstructure and functional response of the nervous system, and the system of xenobiotic detoxifying enzymes is still immature (Rich and Boobis, 1997). Studies on age-related susceptibility to acute exposures in laboratory animals are available only for a few PYRs, including the CS-syndrome compounds deltamethrin (DLM) and cypermethrin (CYPM), and the T-syndrome ones permethrin and cismethrin (Shafer et al., 2005; Sheets, 2000). In these studies, several age-groups of rats were administered different oral doses, and toxicity was estimated using mortality and ASR assays. Results showed that the lethality of the PYRs examined decreased with age: two-week-old rat pups were +10-fold more sensitive whereas 3-week-old pups were 7.4-fold more sensitive than adults. Yet, such a degree of variation in the dose–response relationship was not observed between 3- and 9-week-old rats after exposure to DLM using ASR as an endpoint. Interestingly, pharmacokinetic data obtained in another study using developing rats exposed to DLM (Kim et al., 2010; Tornero-Velez et al., 2010) suggest that greater susceptibility in ASR assays would have been evident in infant rats if a longer testing time had been used in the aforementioned studies (Sheets et al., 1994). These studies underscore the need of a comprehensive selection of endpoints and testing times to complete the information on age-related vulnerability to PYRs.

In this work, we conducted single-compound time–dose–effect assays for the type-I-like compound bifenthrin (BIF), the type-II compound CYPM, and a mixture of low effective doses of both PYRs in weanling rat pups, using temperature monitoring assays. We were interested in exploring whether an evaluation of body temperature after acute exposure to PYRs may provide information similar to that previously reported in studies using motor activity and ASR assays. We selected BIF to model a potent tremorigenic compound, and CYPM to model a prototypical CS-syndrome compound. Moreover, BIF and CYPM rank at top positions in environmental studies and food pesticide residue surveys, what makes frequent oral exposure to these insecticides highly probable in general population (Melnyk et al., 2014; Tornero-Velez et al., 2012; Tulve et al., 2006; Weston et al., 2013). Our results extend prior evidence supporting the factors age, endpoint and testing conditions as major determinants of pesticide neurotoxicity in laboratory animals.

2. Materials and methods

2.1. Animal care

SPF-quality, experimentally naïve groups of animals were used for each experiment. Male Sprague–Dawley rats (School of Veterinary Sciences, University of Buenos Aires, UBA, Argentina) were obtained at 15–16 days of age, and housed 4–6 per cage in standard stainless steel cages (30 cm × 26 cm × 22 cm) containing heat-sterilized pine shavings at the time of arrival at the Animal Colony of the UBA School of Exact and Natural Sciences. All animals were maintained on a 12:12 h photoperiod (0600:1800), identical to that used at the animal colony. Colony and testing rooms were maintained at 22.5 ± 2.5 °C. Procedures recommended by the NRC's Guide for the Care and Use of Laboratory Animals, and the local Animal Colony Direction were followed to assure reducing animal suffering to the least possible. Food and tap water were provided ad libitum except when indicated. Beginning the third week of life rat pups start to increasingly use regular food pellets, spending less time in natural suckling behavior (Patel and Hiremagalur, 1992). In pilot work, we found that premature weaning pups (i.e. 15–16 days old) require a period of up to ~48–72 h to develop food-seeking and other appetitive behaviors. Infants were therefore confirmed to reach age-matched body weight before any experimental procedure was administered. This was controlled by offering Nestlé NIDO® (a dry milk powder designed for babies) dissolved in tap water twice a day, the first two days after maternal deprivation, as a supplementary diet. The age to begin premature weaning and the artificial milk formula (similar in protein/fat/carbohydrate concentrations to that reported in Sprague–Dawley female rat milk during late lactation) (Krinke, 2000; Pine et al., 1994) were carefully selected to assure that no major disruption of GIT development was present at the time of temperature monitoring assays (Boyle and Koldovsky, 1980).

2.2. Chemicals

Technical grade samples of BIF and CYPM were kindly supplied by FMC Corp (Philadelphia, PA). BIF (2-methylbiphenyl-3-ylmethyl (1RS,3RS)-3-[(Z)-2-chloro-3,3,3-trifluoroprop-1-enyl]-2,2-dimethylcyclopropanecarboxylate) and CYPM ((RS)-alpha-cyano-3-phenoxybenzyl-(1RS,3RS,1RS,3SR)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate) were 96.3% and 95% pure, respectively (see chemical structures of the examined compounds in Fig. 1).

Doses were calculated based on percent active ingredient. Eight doses of BIF, i.e., 0.1, 2, 6, 9, 12, 15, 18, and 22 mg/kg, and four doses of CYPM, i.e., 5, 10, 15, and 25 mg/kg were examined. Fresh dosing solutions were prepared before each experiment using corn oil as vehicle (Sigma Co., USA). Solutions were stirred and gently heated (40–50 °C) before dosing to assure full solubility.

2.3. Implant of transponders

An index measure of body temperature (T_b) was monitored using microchip technology based on implantable subcutaneous transponders (Bio Medic Data Systems, BMDS; Seaford, DE). Features of this BMDS system and its use in neurobehavioral studies have been already reported (Kort et al., 1998; Williams et al., 2007). In our work, implant procedures were conducted at 18–19 days old, i.e. ~72–96 h after the pups' arrival to our animal housing room. Briefly, antisepsis is carried out by cleansing the lowest portion of the interscapular region with ethanol 96%, and a sterile biocompatible glass-encapsulated thermistor (BMDS *Implantable Programmable Temperature Transponder* IPTT-300®; size: length, 14 mm; Ø = ~2 mm) is then subcutaneously injected in the rat back using a pre-loaded sterile syringe. Pups were allowed to recover for ~72 h to assure that no stress-related factor was influencing responsiveness to PYRs on the test day. Animals were tested at 21–22 days old.

2.4. Animal treatment

The dose–effect relationships for the individual actions of BIF and CYPM were examined in two independent experiments (Exps. 1 and 2, respectively). A third experiment (Exp. 3) was designed to assess the combined action of BIF and CYPM. These studies were divided into blocks, with N = 2–4 per treatment group at each block. Animals

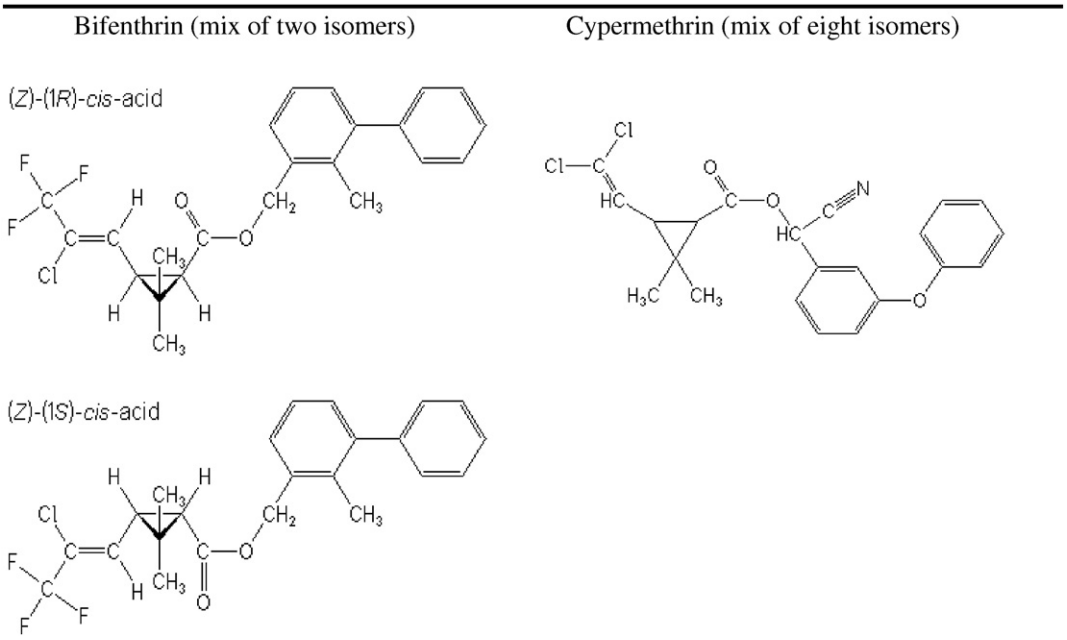

Fig. 1. Chemical structures of the pyrethroids examined (taken from Alan Wood's web site).

were transferred from the holding room to the adjacent testing room in translucent, polycarbonate cages (45 cm × 24 cm × 20 cm; one infant per cage) containing heat-sterilized pine shavings ~2 h before dosing. Dosing solutions were administered by oral route using a volume rate of 2 ml/kg. Dose groups were balanced for body weight and dosing run order. Control animals (administered corn oil vehicle only) were included in each block. The selection of dose schemes sought the inclusion of sub- and nearly effective levels, and at least two doses inducing evident changes in time–response patterns compared to control, leaving out nearly lethal exposures as possible. Administration was conducted using stainless steel 1.5″ intubation needles with round tips (Popper & Sons Inc., NY) attached to sterile 1-ml plastic syringes. Dosing procedures were carried out at ~09:30AM–11:00AM.

2.5. Body temperature monitoring: single compounds (Experiments 1–2)

Body temperature has been successfully used in acute oral neurotoxicity studies of several carbamate, organophosphate, organochlorine and PYR insecticides (Gordon, 2005; McDaniel and Moser, 1993, 1997; Wolansky et al., 2007a, 2007b). Rat temperature signals emitted by subcutaneously implanted transponders were captured by a scanner-probe (BMDS model SP-6005) connected to a control unit (BMDS model DAS-6010 Mini Tower System), and transformed to °C units using the DAS-Host™ Windows based application software (BMDS; Seaford, DE). The implant procedures are simple and rapid (≤1 min). Puncturing, transponder discharge into the final destination in rat back, and injection device withdrawal is followed by immediate animal recovery. This system has already demonstrated an acceptable performance to inform on body surface temperature changes in rats and mice after exposure to pharmacological challenges (Williams et al., 2007) and infectious agents (Kort et al., 1998; Vlach et al., 2000). Moreover, subcutaneous temperature measures obtained using transponder-based systems or infrared thermometers have been reported to fairly correlate with core body temperature measures in small rodents (Hershey et al., 2014; Vlach et al., 2000). The implant location (i.e. subcutaneous), testing room conditions, and some experimenter–animal interaction required to take repeated scans may result in minor differences between data obtained using this microchip technology in contrast to systems using intraperitoneal transmitters. The latter is certainly considered the most reliable core temperature monitoring system in rats (Gordon et al., 2008).

Scans were conducted by sliding out cages from the cage rack, pulling off the cage cover, and placing the probe's tip at a few millimetre distance from the animal's back implant point. Temperature scans were first taken at 30 min prior to dosing time to obtain baseline (i.e., pre-dosing) T_b. Rats were administered the corresponding dose, and T_b monitoring and cage-side observations were conducted at 30-min intervals for an additional 3.5 h period. Collection of all scans took ~10–15 min per testing time. Datasets were further analyzed and modeled to estimate benchmark doses (see below). At the end of the monitoring period, all rats were euthanized by CO_2 asphyxiation.

2.6. Mixture study (Experiment 3)

Identical endpoint and testing procedures used in single compound assessments were used to examine a mixture of BIF and CYPM. This mixture simulated a worst case scenario of exposure to a simple combination of insecticides, including BIF, one of the most toxic PYRs (Julien et al., 2007; Melnyk et al., 2014; Tornero-Velez et al., 2012; Tulve et al., 2006; WHO, 2010). In addition, we sought to combine oral doses well below threshold levels for PYR-specific signs of neurotoxicity such as salivation, whole body tremors, and choreoathetosis, based on previous neurobehavioral studies in adult (Soderlund et al., 2002; Wolansky et al., 2006, 2007a; Wolansky and Harrill, 2008) and developing (Shafer et al., 2005; Sheets, 2000) rats, and a pilot work (data not shown). We analyzed data from single-compound assays by using a benchmark dose (BMD) modeling system (see below); co-administered doses of both PYRs had to be nearly these target BMD estimates. The time–dose–response study included a corn oil control group (2 ml/kg), and four PYR mixture doses: 1–X stock solution, and 0.1-, 0.2- and 0.5–X mixture dilutions.

2.7. Data modeling and statistical analyses

In time–dose–response studies, data were analyzed using linear mixed-effects models with differences in subcutaneous temperature from baseline ($\Delta T_i = T_{ti} - T_{t0}$; $t_0 = 30$ min before dosing) as the dependent variable, dose and time as fixed factors, and subjects as random factor. The models were fitted using the Restricted Maximum

Likelihood method. When data showed deviations from normality a square-root transformation was applied. F tests with type 3 hypotheses and Satterthwaite approximations for degrees of freedom were performed to build ANOVA tables for the REML mixed-effects models. These analyses were carried out in R, using the packages lme4, version 1.1-7, and lmerTest, v.2.0-2.0 (see details in: http://www.R-project.org). Student' t tests were carried out to assess whether ΔT_i was significantly different from 0 (no change from baseline), using times 1.5 h and 3.5 h as relevant testing points to analyze the temperature alterations over time. Bonferroni and Benjamini–Hochberg corrections of p-values were conducted as appropriate. In addition, we used the *Toxicodifussion Model* for repeated response measurements included in the USEPA Benchmark Dose Software package (v. 2.4) to estimate the BIF and CYPM doses producing mild alterations on thermoregulation which were then used in a binary mixture study (see tutored use in http://www.epa.gov/ncea/bmds/quickstart/rptresp.html). These benchmark dose (BMD) estimates may be considered nearly LOEL levels. In all cases, a default p-value of 0.05 was used to test the significance of main effects and post-hoc contrasts.

3. Results

3.1. Evaluation of single chemicals (Experiments 1–2)

3.1.1. Body temperature perturbation in control animals

Infant rats tolerated well all the procedures carried out in this work. In general, incision on the animal's back for transponder implantation produced no evident delay in body growth. On the implant day, pups unable to cope with early weaning were excluded. This criterion consisted in excluding rat pups unable to recover regular age-matched body weight gain trends (see more details in the Methods section). Historical body growth charts for Sprague–Dawley male rats from the animal colony provider and reports of regulatory toxicology studies conducted in this rat strain under GLP conditions (see NTP, 2010) were also considered. This exclusion occurred in only ~0–2 infants per block. Moreover, in the set-up assays, we found no relevant variation in body temperature trends after a mock intubation procedure or administration of corn oil using the same temperature monitoring system selected for the formal assays of the present work, except for transient mild increases in T_b at ~30–90 min after oral gavage intubation, which reached borderline significance when comparing intubated and implanted rats with no gavage procedure (data not shown).

Maturation of metabolic and physiological mechanisms controlling circadian rhythms of core temperature and thermoregulatory responses to cold ambient temperatures and toxicological challenges is advanced at weanling, but may require 4–5 more weeks to be fully established and efficient in laboratory rats (Blatteis, 1998; Brody, 1945; Gordon, 2005, 1990). According to the mentioned knowledge base on thermoregulation in developing rats, no evident cold stress was a priori expected in the weanling rats of this work through the body temperature monitoring assay using a testing room temperature maintained at the rat thermoneutral zone (Gordon, 1990). Fig. 2 shows that these pups manifested only minor changes in T_b over time. Vehicle-injected control pups presented a T_b of 37.49 ± 0.34 °C before dosing, and group means showed individual responses either increasing or decreasing not more than ~0.4 °C after dosing compared to the corresponding pre-dosing (i.e., basal) values. As expected (see the set-up work synthesis above), apparent increases in T_b were observed at the initial 60–90 min after dosing. Subsequent scans indicated that T_b rapidly returned to basal values. We excluded testing times longer than 4 h in formal assays to preclude any potential impact of mild body temperature decrease due to single housing in the experimental animals. In addition, the highest doses of BIF produced exaggerated toxicity emerging at ~2–3.5 h after dosing, which supported a termination of temperature monitoring assays at 3.5 h as well.

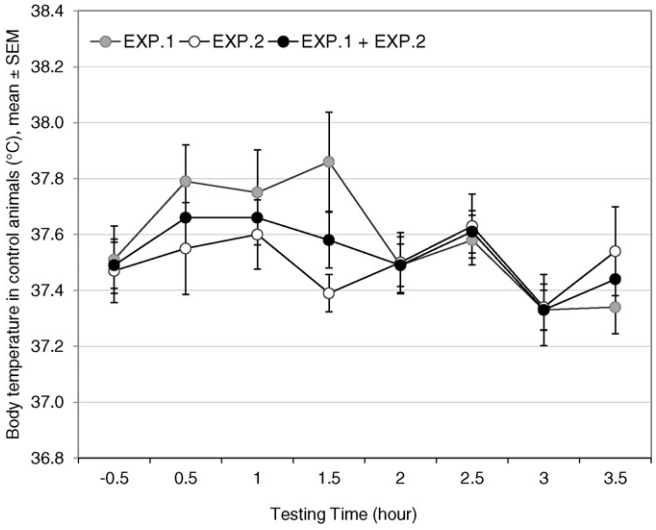

Fig. 2. Body temperature in control infant rats. This figure shows the ability of single-caged experimental infant rats to cope with the testing room temperature and other experimental conditions through the entire test period. Data from vehicle-control animals were collected at eight consecutive testing time points in Exp. 1 (BIF assessment; open circles) and Exp. 2 (CYPM assessment; light gray circles) (N = 8–10). A global trend for all control animals is also included ("Exp.1 + Exp.2"; dark gray circles) (N = 18). Results are expressed as group means ± SEM. Pre-dosing physiological T_b was recorded at 0.5 h before dosing (i.e., −0.5 h). Control pups were orally administered 2 ml/kg corn oil (i.e., "0-hour" time), and then T_b was measured at 30-min intervals. The RM-ANOVA computed significant differences for the within-subject factor *time* ($F_{7,112} = 2.29$, $p = 0.032$). For the main effects of the *experiment*, and the *experiment* ∗ *time* interaction, p-value > 0.1. These results are consistent with previous studies of circadian rhythms and changes in body temperature in response to handling in young rats (Gordon, 2005).

3.1.2. Body temperature perturbation in BIF and CYPM treated animals

In single-compound assays, BIF and CYPM produced alterations in behavior and T_b starting at 60–90 min after dosing. The most evident effect of both PYRs on body temperature was a dose-dependent hypothermia starting up at 1.5–2 h after dosing. Furthermore, a biphasic dose–response relationship was observed over time after exposure to BIF, which was not so apparent in CYPM assays.

3.1.2.1. Experiment 1. BIF effects on body temperature are shown in Fig. 3A. No PYR-specific sign of neurotoxicity was evident up to 9 mg/kg, and toxicity increased rapidly as the administered dose of BIF was greater than 12 mg/kg, which challenged the observation of a dose-related trend in animals expressing a sublethal syndrome. The ANOVA computed a weak effect of dose ($F_{7,43} = 1.94$, $p = 0.087$), and highly significant effects for testing time ($F_{6,251} = 13.66$, $p < 0.00001$) and the dose ∗ time interaction ($F_{42,251} = 6.75$, $p < 0.00001$). While a biphasic response was apparent over time, the monitoring period was split into two subsets of testing times to conduct a clinically relevant consideration of time–dose–effect data: we separately evaluated an early phase of subtremorigenic signs of toxicity from t_1 to t_4, featuring episodes of restlessness and an increase in T_b, and a subsequent phase of PYR-specific clinical alterations and hypothermia from t_4 to t_7 (see also ref. McDaniel and Moser, 1993; Pato et al., 2011; Wolansky et al., 2007a, 2007b). Moreover, we pooled the animals' data from the two highest dose groups based on the commonality of clinical observations (see Fig. 4), which optimized the fulfillment of the statistical model requirements. A dose of 12 mg/kg appeared to be an inflection point for the onset of a dose-dependent mild-to-moderate increase in T_b compared to the estimated (no-effect) baseline. This increase was observed at 1.5–2 h after exposure to 15–22 mg/kg ($p = 0.006$). Thereafter, a marked decline in T_b was observed at 18–22 mg/kg, with a peak hypothermic effect of BIF at ~3.5 h ($p = 0.006$).

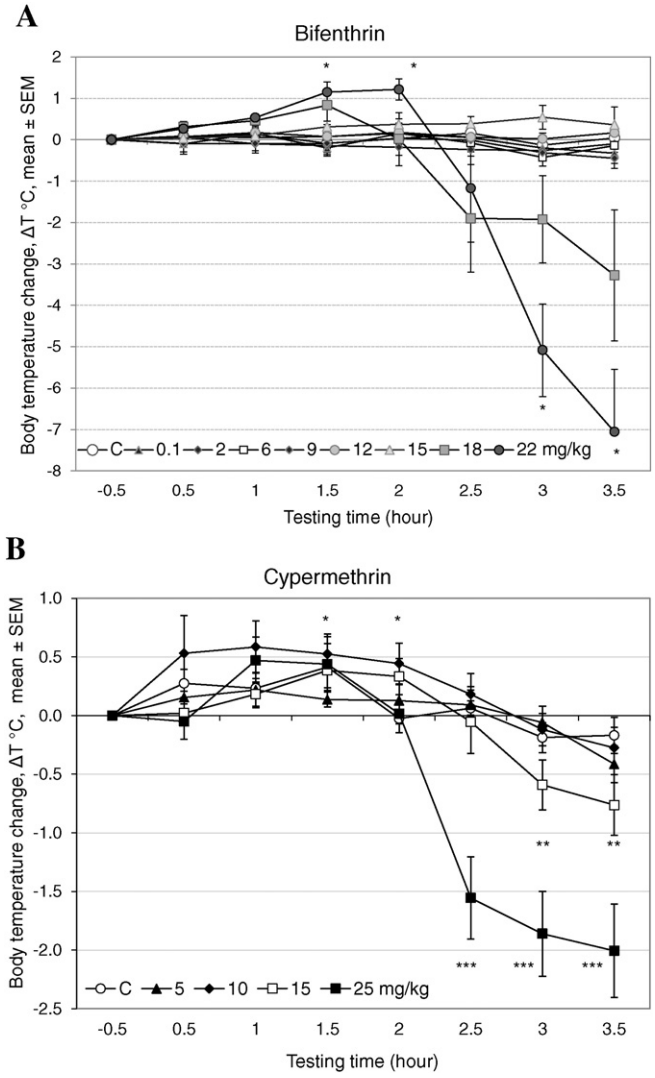

Fig. 3. Changes in body temperature after acute exposure to BIF and CYPM. Infant rats were tested after administration of BIF (Exp.1 — panel A) or CYPM (Exp.2 — panel B). Rat temperature (T_b) was monitored after dosing at 30-min intervals. T_b changes are expressed as variations (ΔT_b) at every testing time compared to each animal's baseline (i.e., pre-dosing T_b). Note that these insecticides do not belong to the same PYR type according to the canonical classification in adult animals (Soderlund et al., 2002; Wolansky and Harrill, 2008). Yet, in this work, the pattern of T_b changes observed in infant rats was fairly similar (BIF, N = 4–10; CYPM, N = 8–12). Panel A, *p = 0.006 at 15 and 18–22 mg/kg; **p = 0.006 at 18–22 mg/kg. Panel B, *p = 0.05 at 10–15 mg/kg, **p = 0.05 at 15 mg/kg; ***p = 0.01 at 25 mg/kg.

3.1.2.2. Experiment 2. Fig. 3B shows the time–dose–effect data obtained after exposure to CYPM. The ANOVA computed significant p values for both main factors (for dose, $F_{4,43} = 6.12$, $p = 0.00054$; for testing time, $F_{6,258} = 42.6$, $p < 0.00001$) and the dose * time interaction ($F_{24,258} = 6.66$, $p < 0.00001$). Moreover, a borderline significance was detected for a mild increase in ΔT_b at 10–15 mg/kg ($p = 0.05$) observed at 1–2 h after dosing. During the subsequent phase of the testing period, a dose-related decline of T_b was evident. Post-hoc Student's t tests computed borderline significant p values for mild-to-moderate decreases in ΔT_b at 5 and 15 mg/kg ($p = 0.05$), and a much greater decline in ΔT_b (i.e., −1.8 °C) was observed in the 25 mg/kg dose group compared to control ($p = 0.01$; see Fig. 3B).

3.1.3. Computing of benchmark doses of BIF and CYPM

Benchmark dose (BMD) modeling was used to estimate low effective doses for the hypothermic actions of BIF and CYPM. This analysis detected statistically significant effects of CYPM on the main factors *time*, *dose*, and *intercept* ($p < 0.05$); only dose and intercept significantly influenced T_b in infant rats exposed to BIF ($p < 0.05$) (see Table 1).

A BMD_{25} level of BIF and a BMD_{12} level of CYPM were selected as target doses to prepare the test mixture. The modeling program estimated that the time for BMD_{12} is 3.2 h for CYPM. This time may be assumed to indicate the time at which peak effects will be observed after acute exposure to a low effective dose of CYPM. BIF produced a slightly more delayed onset of hypothermia, and a low effective dose for its hypothermic effect was computed at >3.5 h, i.e., it fell off the monitoring period examined. A 1:1 mixing ratio was selected for an exploratory study of a CYPM–BIF mixture based on the molar rates of each compound, i.e., mixing up a BIF dose that was two times as potent for perturbing body temperature as the CYPM dose (see Table 1).

3.1.4. Neurobehavioral observations after single-chemical exposure

Fig. 4 shows cage-side, clinical observations collected during one block of the time–dose–response study of BIF. A qualitative, 5-point scale was designed to describe the severity of the syndrome as compared to vehicle controls. We did not observe PYR-related effects in BIF-treated animals compared to controls through the initial phase of the monitoring period. Moreover, while no PYR-related sign of neurotoxicity, such as whole body shakes, tremors, and increased reactivity to ambient stimuli (Wolansky et al., 2007a; Wolansky and Harrill, 2008) was evident at doses up to 12 mg/kg, only a dose range of 12–22 mg/kg is used to illustrate BIF syndrome progression over time. Some animals administered the highest doses suffered episodes of exaggerated toxicity, including intense body shakes and whole body tremors. As mentioned above, severe neurotoxicity, including a prolonged decline in T_b in most animals administered the highest doses, precluded conducting repeated T_b scans beyond 3.5 h due to ethical reasons.

As mentioned above, CYPM produced evident body temperature decline at 15–25 mg/kg, starting up at 1.5–2 h after dosing (see Fig. 3B). The most negative ΔT_b values for these doses (−0.75 to −2.0 °C, respectively) were observed at ~3.5 h. It should be noticed that a more marked hypothermia was observed at 15 and 25 mg/kg (i.e., −1.15 to −2.5 °C, respectively) if peak T_b during the initial phase of mild increase in temperature was considered as a reference point. Moreover, a few animals manifested body trembling at 2–3.5 h, followed by moderate weakness and gait disorder. No CYPM-treated infant rat presented salivation, i.e., a neurobehavioral hallmark of type-II syndromes in adult rats (McDaniel and Moser, 1993; Soderlund et al., 2002; Wolansky and Harrill, 2008), or signs of exaggerated toxicity.

3.2. Evaluation of a mixture of BIF and CYPM

3.2.1. Experiment 3

In order to ascertain whether additive effects may occur on body temperature after acute exposure to PYRs in developing rats, a mixture consisting of low effective doses of CYPM and BIF was examined under the same testing conditions used in the single-compound assays (see Table 1 and Fig. 5). Most animals at doses 0.5–1× started to show restlessness and exacerbated stereotyped behavior at ~1 h. Thereafter animals in these groups presented episodes of head and whole body shakes. Mild trembling and whole body tremors were apparent at ~2 h and ~3 h, respectively, but salivation, choreic locomotion or bizarre behaviors such as aggressiveness were not observed.

No evident trend for a hypothermic action was observed at any time. The 1× dose of the CYPM–BIF mixture produced mild changes in the time–response relationship consisting of increases in T_b during the initial 1.5 h of testing ($p = 0.06$), what was likewise apparent during the late phase of the monitoring. The ANOVA did not reveal any significant effect of the main factors dose ($F_{4,22} = 1.13$, $p = 0.37$) and time ($F_{6,132} = 1.43$, $p = 0.21$), but the interaction resulted significant ($F_{24,132} = 1.87$, $p = 0.01$). The differential influence of testing time on dose groups was confirmed using a Likelihood Ratio test ($\chi^2_{[df = 24]} =$

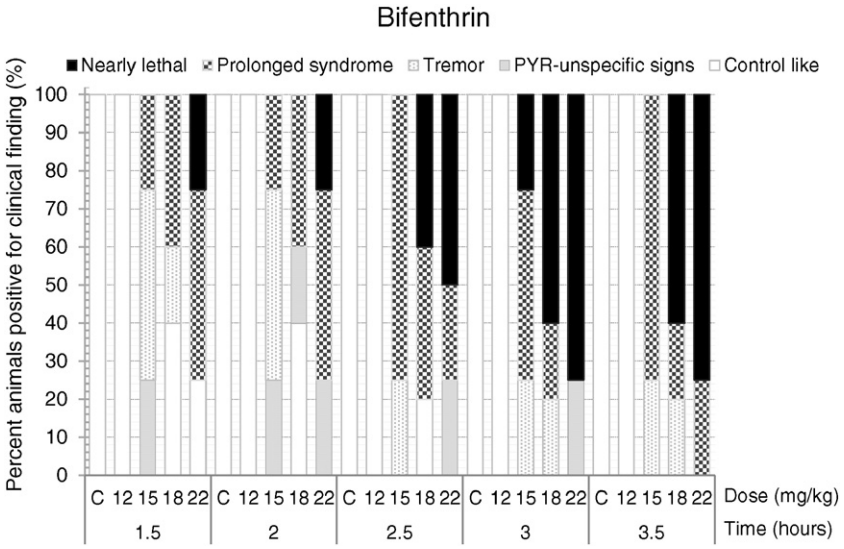

Fig. 4. Neurobehavioral alterations after acute oral exposure to BIF in infants. This chart shows a relative severity scale for the clinical signs of neurotoxicity observed at different testing times (in hours) after exposure to BIF 12, 15, 18 or 22 mg/kg (N = 3–5 per dose group; N = 2 for controls; total N = 18). Category "PYR-unspecific signs" stands for restlessness and exacerbation of stereotyped behaviors such as scratching and grooming. "Tremors" refers to muscular fasciculation and trembling progressing to whole body tremors. "Prolonged syndrome" stands for an extended duration of the specific and unspecific signs observed at less severe categories of clinical outcome. "Nearly-lethal" refers to animals suffering such a prolonged manifestation of intense signs of neurotoxicity as to expect that an ethical sacrifice might be required at 3.5 h after dosing. Note that one out of four animals administered the highest dose presented exaggerated toxicity signs emerging at 1.5 h after dosing. Furthermore, one out of four animals administered 15 mg/kg was classified as nearly-lethal at t = 3 h, although recovery started to be apparent in the subsequent testing time, suggesting that 15 mg/kg would be a nearly threshold dose for BIF lethality.

47.32, p = 0.003), thus suggesting that mild effects of dose were present. This interaction was mostly related to the action of the 1× and 0.5× doses at 1.5 h and 3 h, respectively (t test, p = 0.02 in both cases). Panels B and C in Fig. 5 show separate analyses of area-under-the-curve (AUC) values for the early and late parts of the dose–effect relationships over time to further explore the global actions of dose on thermoregulation. Minor differences of borderline statistical significance were only detected at the 1× mixture dose.

4. Discussion

Our results show that BIF and CYPM may alter body temperature control in infant rats, extending previous findings in studies using adult rats (Gordon, 2005; Soderlund et al., 2002; Wolansky et al., 2007a; Wolansky and Harrill, 2008; Wolansky et al., 2007b). The most evident action of CYPM was a dose-related hypothermia. Noteworthy, the disruption of normothermia caused by BIF was qualitatively dissimilar to that previously reported in the literature: hypothermia was observed in this work instead of the characteristic hyperthermic action observed after acute oral exposure to noncyano PYRs in adult rats. Furthermore, coadministration of nearly LOAEL doses of CYPM and BIF produced exacerbation of locomotor and nonlocomotor behaviours, and a mild increase in T_b through a few hours after dosing. The various experimental and biological factors accounting for the susceptibility of weanling infants to BIF and CYPM are discussed below.

PYRs act on the nervous system in target and nontarget species (Soderlund et al., 2002). A proposed primary mechanism-of-action for all PYRs is the prolongation of the open state of neuronal voltage-dependent sodium channels. This action results in altered neuronal excitability (Narahashi, 2000). The mechanistic pathways responsible for a divergence between neurobehavioral syndromes produced by type-I, type-II and mixed type-I/II compounds in rats and mice are not well understood (Soderlund et al., 2002). CYPM has been classified as a type-II compound, whereas BIF has been reported to present many features of typical T-syndrome compounds that have been classified as type-I in canonical studies (McDaniel and Moser, 1993; Soderlund et al., 2002; Wolansky et al., 2007a; Wolansky and Harrill, 2008).

In adult rats, ≥6 mg/kg BIF dissolved in corn oil produces a dose-related increase in core temperature using a dose volume rate of 1 ml/kg (Wolansky et al., 2007a, 2007b, in preparation). Interestingly, the dose volume rate may influence PYR neurotoxicity. ED30 estimates for BIF effects on motor activity are 3–6 mg/kg and 11–13 mg/kg using 1 or 5 ml/kg dose volume rates, respectively (Wolansky et al., 2006, 2007a, 2007b). Likewise, dosing volume-related changes in body temperature disruption were noticed using colorectal probes (Wolansky et al., 2007a) or intraperitoneal transmitters (Wolansky et al., unpublished) to monitor thermoregulation. In contrast, no alteration in body temperature was observed in adult rats in a Functional Observational Battery (FOB) study of BIF using a dose scheme ranging from 10 to 75 mg/kg (Soderlund et al., 2002), which may have been most likely accounted by the use of a large dose volume rate (Wolansky et al., 2007a). A recent review has concluded that threshold doses for PYRs are greatly influenced by multiple biological and experimental factors, such as test compound structure and isomer composition, administered dose, vehicle, dose volume rate, testing time, and endpoint (Wolansky and Tornero-Velez, 2013).

In the present study, hypothermia was evident at ≥15 mg/kg BIF in infant rats using a dosing rate of 2 ml/kg (Fig. 3A). No previous BIF

Table 1
Estimation of benchmark doses. Time–dose–response data obtained in single-compound assays were modeled using the benchmark dose (BMD) modeling system to estimate low effective doses of each compound. BMD_{12} and BMD_{25} are dose levels producing 12 and 25% declines in T_b compared to the corresponding no-effect baseline. A mixing ratio of ~1 was used in the mixture study.

Pyrethroid	Benchmark dose, in mg/kg (hypothermic action phase, $t_{2\ h}$ to $t_{3.5\ h}$)	Relative toxicity in BMD estimates	Dose in test mixture, in mg/kg	Mixing ratio in test mixture: CYPM = 1
BIF	BMD_{25} = 14.8	Assumed to be low effective dose levels for thermoregulation	15	1.05
CYPM	BMD_{12} = 14.3		14.3	1.00

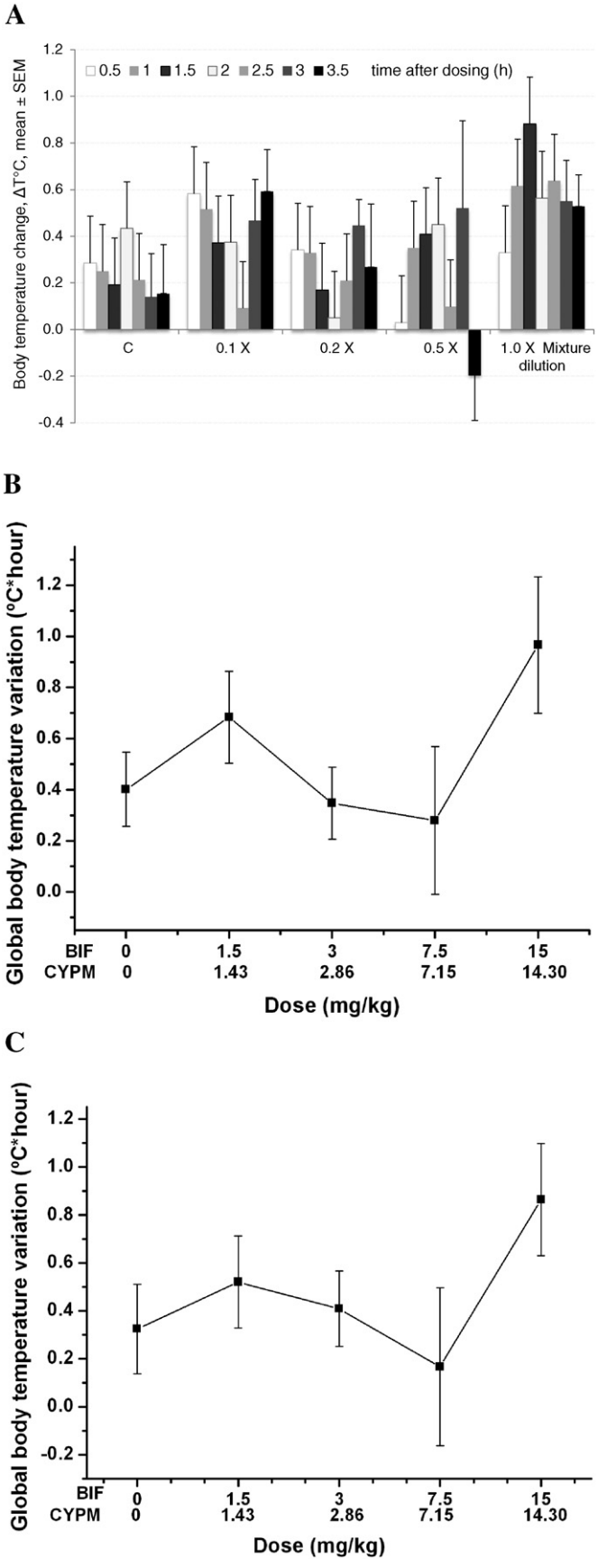

Fig. 5. Changes in body temperature after acute exposure to a mixture of BIF and CYPM. Low-effective doses of BIF and CYPM for the hypothermic action (i.e., ~BMD_{25} and BMD_{12}, respectively) were coadministered to weanling rats. The mixture comprised 14.3 mg/kg CYPM and 15 mg/kg BIF (i.e., 1× dose). Greater-than-additive effects were not evident. Moreover, a trend for an increase in AUC (i.e., up to +0.9 °C * h) was observed in animals exposed to the undiluted mixture compared to vehicle controls (see panel A). These results were consistent with the dose–response relationships for single compounds (see Fig. 3). Panels B–C show AUC analyses of the early (0.5 to 2 h) and late (2 to 3.5 h) phases of the monitoring period, respectively. Global trends for a mild increase in T_b over time at mixture dose 1× were apparent. Results are expressed as mean ± SEM.

study has used this dose volume rate in rats. In an attempt to determine whether age-related changes in BIF toxicity may occur, we compare our present results with a recent study of BIF effects on young adult rats conducted in our laboratory applying identical subcutaneous transponders to monitor body temperature but using a lower dose volume rate of 1 ml/kg (Mosquera Ortega et al., 2013; Pato and Sosa Holt, 2011). Fig. 6 shows the time–dose–response curves for those adult animals. A period of peak effects for hyperthermia was observed at 2.5–3 h after dosing, i.e., slightly earlier than the time of peak effects for hypothermia evident in Fig. 3 of this work. Moreover, the estimated BMD_{25} dose of BIF in infant rats was 14.8 mg/kg ($CI_{95\%}$ lower limit, BMD_{25}-L = 12.6 mg/kg), and 3 mg/kg was a nearly LOAEL dose in the adult rats (see Fig. 6). No greater susceptibility in infants should be thus proposed based on comparisons of our present work with previous neurobehavioral studies using motor activity and body temperature as endpoints. However, according to the neurobehavioral observations in this (Fig. 4) and previous studies of BIF (Mosquera Ortega et al., 2013; Pato and Sosa Holt, 2011; Scollon et al., 2011; Soderlund et al., 2002; Wolansky et al., 2006, 2007a), nearly lethal syndromes appear to be evident at ~15–18 mg/kg (2 ml/kg) and ~16–20 mg/kg (1 ml/kg) in infant and adult rats, respectively. Regarding the dose volume factor influencing acute toxicity (Wolansky and Tornero-Velez, 2013), our results suggest that threshold doses for BIF lethality are lower in infants than those in adults, but this age-related difference seems to faint when low-dose effects are examined, as previously proposed by Sheets et al. (1994) testing different age-groups in sensorimotor response assays after exposure to subconvulsive doses of deltamethrin. More interestingly, the attenuated manifestation of the hyperthermic action and the strong hypothermic effects observed in the infant animals of our work (compare results in Figs. 3A and 6) strongly suggest that qualitative differences may occur in the susceptibility to acute PYR exposures in early infancy.

The infant rats were responsive to CYPM as well: 5 mg/kg was a no-effect level, restlessness and mild increase in T_b were apparent at 10 mg/kg, and dose-dependent declines in T_b were evident at 15–25 mg/kg (Fig. 3B). High oral doses of CYPM have been reported to produce hypothermia, a hallmark in neurobehavioral descriptions of type-II syndromes in adult rats (McDaniel and Moser, 1993; Soderlund et al., 2002); however, when a low-effective dose of CYPM is administered, an increase in body temperature shows up through the first 2 h after oral dosing (McDaniel and Moser, 1993; Wolansky et al., 2007b). In addition, a low-effective acute oral dose of CYPM to decrease motor activity (i.e., an ED30 level) is ~11–27 mg/kg (Wolansky and Harrill, 2008), and a mild increase in temperature is observed after administration of 20 mg/kg CYPM in corn oil (McDaniel and Moser, 1993) using 1 ml/kg as the dosing volume rate. We used a similar CYPM sample but a higher dosing volume rate than that in the FOB study of McDaniel and Moser (1993). Hence, weanling pups were apparently slightly more susceptible to acute oral exposure to CYPM than adults, a finding mostly consistent with studies on PYR actions in developing rats reported to date (Cantalamessa, 1993; Shafer et al., 2005; Sheets, 2000; Wolansky and Tornero-Velez, 2013).

The reported variations in the acute toxicity of PYRs along postnatal development may greatly rely on several toxicokinetic factors (Casida et al., 1975/1976). Biotransformation rates of PYRs by liver enzymes are dependent on isomer composition and animal age (Anand et al., 2006; Kim et al., 2010). Rats reach nearly full maturity in liver enzymes

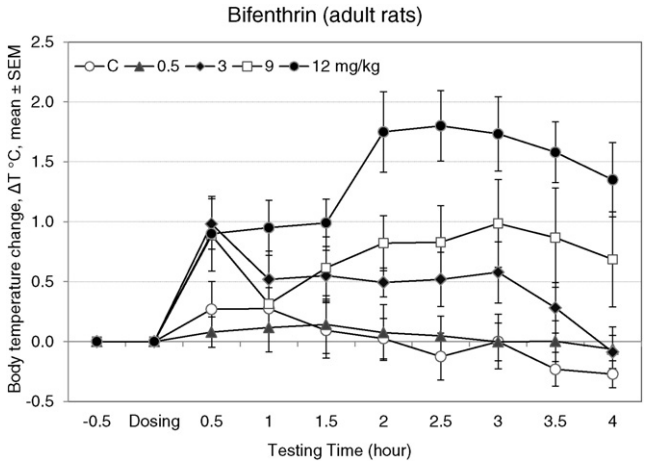

Fig. 6. Changes in body temperature after acute oral exposure to BIF in adult rats. BIF has been recently evaluated in our laboratory in young adult animals under identical exposure and subcutaneous temperature monitoring conditions save for the 1 ml/kg dose volume rate used in that study (Mosquera Ortega et al., 2013). Here we show the hyperthermic effects of 0.5–12 mg/kg BIF (N = 6–8 per group). Note that BIF produced a dose-related increase in body temperature, with an apparent LOAEL at 3 mg/kg, and no trend for a decline in T_b was observed at any testing time.

responsible for PYR detoxification at ~40 days of age, although some enzyme specificity is observed along infancy and adolescence (Tornero-Velez et al., 2010). CYPM is a mix of eight isomers greatly differing in acute toxicity in mammals (EFSA, 2011), whereas BIF consists of only a few highly toxic isomers (ECHA, 2011). Additionally, expression of multidrug-resistance protein P-glycoprotein, an efflux transporter localized on the apical membrane of brain capillary endothelial cells (Jette et al., 1995), reaches adult levels of expression at ~28 days of age (Matsuoka et al., 1999). Thus, the finding of a greater susceptibility in infants compared to adult rats after exposure to CYPM may be a consequence of maturing enzymes with age-related, differential detoxifying activity towards its isomers, which may in turn determine larger peak tissue concentrations of the most toxic CYPM isomers in the infant than in the adult nervous system, as proposed by Anand et al. (2006) and Cantalamessa (1993).

Thermoregulation is a vital physiological feature in mammals (Brody, 1945), and a major biological determinant of the neurobehavioral responses observed in studies of pesticides disrupting body temperature control mechanisms (Gordon, 2005). During the early postnatal life, rat pups develop both behavioral and non-behavioral mechanisms to control body temperature. This allows neonates, which are mostly unable to regulate body temperature, to evolve into homeothermic organisms able to resist cold stress at ~4–6 weeks (Adolph, 1957; Blumberg, 2001; Gordon, 1990). Equivalent maturational changes occur during the first year of life in humans; suckling babies may have severe clinical sequelae after disruption of thermoregulation (Kumar et al., 2009; Leon, 1986). Interestingly, using telemetered rats and thermoregulation as an endpoint, Mack and Gordon (2007) also found differential trends of susceptibility to anticholinesterase insecticides in 17-day-old pups compared to adult animals. The results of our present work confirm the utility of body temperature monitoring assays to characterize the vulnerability of early development to pesticides interfering thermoregulation (Gordon, 2005).

Evaluation of a mixture of CYPM and BIF consisting of doses producing mild changes in the subcutaneous index measure of body temperature used in this work provided no evidence of greater-than-additive effects in 21-day-old rat pups. In adult rats, the joint administration of subthreshold doses has been found to act on motor activity as predicted by the dose-addition theory (Wolansky et al., 2009). Moreover, as doses of coadministered PYRs increase to suprathreshold levels, mixture effects may be more accurately predicted using a response-summation hypothesis, as it was apparent in a study of high-effective doses of permethrin and deltamethrin in young adult rats using core temperature as an endpoint (Wolansky et al., 2007b). Based on the single compound dose–response curves (Fig. 3), the low-effective nature of the combined doses (Table 1), and the common primary mode of action proposed for PYRs (Narahashi, 2000; Soderlund et al., 2002) our default hypothesis was that no evident hypothermic action would be produced by the test mixture. The experimental results confirmed this prediction (Fig. 5). Nevertheless, most animals administered the two highest mixture doses (0.5–1×) presented mild-to-moderate signs of neurotoxicity, including neuromuscular fasciculation suggestive of mild tremors. More interestingly, the mixture also produced a mild increase in T_b, a finding consistent with the mild increases in T_b independently produced by the combined doses of CYPM and BIF in the single compound assays (compare responses at testing times $t_{1\ h}$–$t_{2\ h}$ in Fig. 3 with mixture actions on T_b at the same time interval in Fig. 5). Hence, the results are suggestive of an algebraic summation of single compound effects. Last, this and previous studies (Marshall et al., 2013; Sheets et al., 1994; Starr et al., 2012; Wolansky et al., 2009, 2007b) suggest that additivity as a default hypothesis would be predicting or slightly over-predicting the cumulative toxicity produced in adult and infant rats after acute exposure to PYRs depending on the nature (i.e., sub- or supra-threshold level) of the single compound doses contributing to the combined effects. This suggestion would be confirmed by designing extensive dose–effect assays and appropriate statistical models to test additivity in developing rats such as those recently used to characterize PYR mixture effects in adult rats (Marshall et al., 2013; Wolansky et al., 2009).

5. Conclusions

In conclusion, by using body temperature as an endpoint to assess PYR neurotoxicity, we were able to expand the knowledge base on PYR effects in developing rats. More importantly, our results strongly suggest age-related qualitative changes in response after acute exposure to BIF, which would not have been predicted if only knowledge based on the available studies in adult animals had been considered.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgements

The authors thank FMC Química do Brasil Ltda. and FMC Latin America for the help in obtaining the bifenthrin sample. MW wants to express his gratitude to W. Kobel, S. Allen, C. Breckenridge, T. Pastoor, and F. Suarez for a donation of scientific equipment used in this study. This work was funded by grant PICT-2007-1340, National Agency for Promotion of Science and Technology (ANPCyT), Argentina Ministry of Science and Technology. MJW is an Argentina National Research Council Scientist and a University of Buenos Aires Adjunct Professor. CSSH and AMP were advanced UBA students during their participation in this work. The Universidad Nacional Autónoma de México through its office of doctoral programs funded the international trainee position of UB at the University of Buenos Aires, by fellowship No. 228586 from Consejo Nacional de Ciencia y Tecnología de México. SIN is a research assistant on data modeling and biostatistics at the IQUIBICEN Research Institute (CONICET).

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