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Cholinesterase inhibition and toxicokinetics in immature and adult rats after acute or repeated exposures to chlorpyrifos or chlorpyrifos–oxon

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ABSTRACT

The effect of age or dose regimen on cholinesterase inhibition (ChEI) from chlorpyrifos (CPF) or CPF–oxon (CPFO) was studied in Crl:CD(SD) rats. Rats were exposed to CPF by gavage in corn oil, rat milk (pups), or in the diet (adults) or to CPFO by gavage in corn oil. Blood CPF/CPFO levels were measured. With acute exposure, ChEI NOELs were 2 mg/kg CPF for brain and 0.5 mg/kg CPF for red blood cells (RBCs) in both age groups. In pups, ChEI and blood CPF levels were similar using either milk or corn oil vehicles. Compared to gavage, adults given dietary CPF (12 h exposure) had greater RBC ChEI, but lower brain ChEI at corresponding CPF doses, indicating an effect of dose rate. With repeated CPF exposures, ChEI NOELs were the same across ages (0.5 and 0.1 mg/kg/day for brain and RBCs, respectively). With CPFO dosing, the ChEI NOELs were 0.1 mg/kg (acute) and 0.01 mg/kg/day (repeated doses) for RBCs with no ChEI in brain at CPFO doses up to 0.5 (pup) or 10 mg/kg (adult) for acute dosing or 0.5 mg/kg/day for both ages with repeat dosing. Thus, there were no age-dependent differences in CPF ChEI via acute or repeated exposures. Pups had less ChEI than adults at comparable blood CPF levels. Oral CPFO resulted in substantial RBC ChEI, but no brain ChEI, indicating no CPFO systemic bioavailability to peripheral tissues.

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1. Introduction

Chlorpyrifos (CPF) is a broad spectrum organophosphate insecticide that inhibits acetylcholinesterase (AChE), which can result in accumulation of synaptic acetylcholine, neuronal overstimulation, and subsequently, cholinergic signs of toxicity (e.g., excessive salivation, lacrimation, urination, defecation, incoordination, tremors, etc.). Previous studies have demonstrated that young animals are more sensitive to lethality, some neurobehavioral effects and cholinesterase (ChE) inhibition following acute exposures to high doses of CPF (e.g., Pope et al., 1991). At 15–20 mg/kg, PND 17 rats exhibited the same magnitude of brain ChE inhibition as adult rats given 80 mg/kg CPF (Moser and Padilla, 1998; Moser et al., 1998). Functional neurobehavioral changes following acute CPF exposure have been correlated with ChE inhibition (Moser, 1995; Nostrandt et al., 1997). Generally, signs of cholinergic toxicity were seen when brain ChE was inhibited >60% (Nostrandt et al., 1997; Moser et al., 1998). ChE inhibition is considered the most sensitive endpoint for CPF toxicity based on previous dose–response studies and provides the most useful data for determining point of departure (US EPA, 2011). These differences in sensitivity are partially related to toxicokinetic differences between young animals and adults (i.e., young animals have a lower capacity to detoxify chlorpyrifos–oxon (CPFO), the active metabolite; Timchalk et al., 2006).

Investigators have demonstrated that age-related differences in sensitivity to CPF exist with acute, high-dose exposures and are proportional to the magnitude of ChE inhibition. Pope and Chakraborti (1992) demonstrated that there was a high correlation between brain and plasma ChE inhibitory potency (ED_{50} values) and sensitivity to acute toxicity at the maximum-tolerated dose across age groups with acute *in vivo* CPF exposure. Across three organophosphates (OPs), maximum-tolerated doses given to immature and adult rats produced similar degrees of brain ChE inhibition. With intermittent repeated exposures (i.e., 40 mg/kg CPF once every 4 days, total of four doses), adult rats were more sensitive to neurochemical changes, including brain ChE inhibition, than immature rats (PND 7–20) (Chakraborti et al., 1993). The paucity of data on age-related sensitivity at low dose levels was acknowledged by the US Environmental Protection Agency (US EPA) Scientific Advisory Panel (2008), which noted that there was uncertainty regarding the relative sensitivity of young animals to ChE inhibition after exposure to low doses of CPF.

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Aside from evaluating age-related sensitivity to CPF-induced ChE inhibition, there also were concerns for potential ChE inhibition due to exposures to CPFO. The US EPA's Environmental Fate and Effects Division has proposed that drinking water exposures to CPFO are possible. Estimated drinking water concentrations (EDWCs) of CPFO were based on Tier II surface water and Tier I groundwater model simulations for currently registered uses of CPF, because CPF is expected to transform to CPFO during drinking water treatment and there are limited environmental fate data available for CPFO, in part due to the short half-life of the oxon. Thus, due to the concern for potential drinking water exposures, acute and repeated-dose inhibition of ChE following CPFO exposure also was examined.

This study was derived from a standard US EPA Comparative Cholinesterase Study design to examine the relative sensitivity of adult rats and pups to ChE inhibition with the goal to better characterize age-dependent toxicity of CPF over lower portions of the dose–response curve. These data would establish whether previously identified age-related differences in ChE inhibition at high CPF doses apply at lower dose levels that are more relevant for human exposures. The study design also was expanded to examine the impact of dose vehicle, dose rate, and the effect of acute and repeated CPFO dosing on ChE inhibition with measurements of internal dose to provide context for these results. First, immature (postnatal day (PND) 11) and young adult rats were given acute (single bolus) or repeated (11 day) exposures to CPF or CPFO in corn oil. The impact of vehicle and dosing regimen was evaluated after bolus dosing in rat milk vehicle (pups) or 12 h dietary exposure (adults). The highest dose levels were selected to induce approximately 50–60% inhibition of brain ChE in the CPF studies and marked inhibition of RBC ChE in the CPFO studies. To examine behavioral neurotoxicity, clinical observations were included in the acute studies, whereas a functional observational battery (FOB) and motor activity were included after 10 daily exposures in the repeated dose study. Brain, RBC and plasma ChE inhibition were measured at the time-of-peak inhibition following both acute and repeated exposures to CPF or CPFO. Blood levels of CPF, CPFO, and/or trichlorpyridinol (TCP) were measured to examine the relationship between ChE activity and systemic exposure levels and to improve the existing physiologically-based pharmacokinetic/pharmacodynamic (PBPK/PD) model for CPF (Timchalk et al., 2002, 2006). Data from this study were modeled using benchmark dose analysis in the accompanying paper by Reiss et al. (2012).

2. Materials and methods

2.1. Materials and animal husbandry

Chlorpyrifos Technical (CPF; *O,O*-diethyl *O*-(3,5,6-trichloro-2-pyridinyl)ester phosphorothioic acid; 99.8% pure) and chlopyrifos-oxon (CPFO; diethyl 3,5,6-trichloro-2-pyridinyl ester phosphoric acid, 94.9% pure), a CPF metabolite that inhibits cholinesterase (ChE), were supplied by Dow AgroSciences LLC (Indianapolis, Indiana). Rat milk used as a vehicle in some PND 11 pup experiments was collected from untreated lactating dams or was purchased from Bioreclamation, Inc. (Hicksville, New York). Stability of CPF and CPFO in corn oil and milk was confirmed prior to the start of the pilot studies; stability of CPF in rodent diet was previously established for at least 30 days at concentrations spanning those used in this study. Concentration verification and homogeneity analyses were conducted for all dose solutions, and for the premix and all test diets.

Adult female Crl:CD(SD) rats were approximately 63 days of age at receipt. Females were selected for study, because adult females were either slightly more sensitive (e.g., Moser, 2000) or equally sensitive to adult males (Betancourt and Carr, 2004); in other studies, gender-related differences were not reported (Timchalk et al., 2006; Zheng et al., 2000). Male and female Crl:CD(SD) rat pups were 4 or 5 days of age at the time of arrival in the laboratory. Animals were allowed to acclimate under standard environmental conditions for approximately one week prior to study initiation.[1] Adults were singly housed and pups (8/litter) were housed with lactating dams. Unless otherwise stated, LabDiet Certified Rodent Diet #5002 (PMI Nutrition International, St. Louis, Missouri) and municipal tap water were provided *ad libitum* and pups were given free access to lactating dams. Adult female rats were assigned randomly to treatment groups using a computer program designed to increase the probability of uniform group mean weights and standard deviations at the start of the study. Within litters, pups were randomly assigned to treatment groups with a preference for using pups of intermediate body weights when possible (i.e., avoiding unusually large or small pups). For all studies, pups from the same litter were assigned to different treatment groups to control for litter effects at each time point. The study was approved by the Institutional Animal Care and Use Committee and complied with Good Laboratory Practice regulations.

2.2. Experimental design

The primary objective of this study was to provide data for a comparison of CPF- and CPFO-induced ChE inhibition in pre-weanling and young adult rats to determine whether age-related differences in sensitivity exist. Data were also collected to evaluate how differences in dose vehicle (corn oil vs. milk) or dose regimen (gavage vs. diet) affected the toxicokinetics and pharmacological effects of CPF. The study was conducted in four phases (Table 1): *phase 1*: a range-finding study; *phase 2*: an acute pilot; *phase 3*: a definitive acute study; and *phase 4*: a definitive repeat-dose study.

For each study phase, oral gavage doses were administered at a volume of 3 ml/kg based upon body weights collected just prior to dosing. Pups were removed from the dams approximately 1 h prior to dosing and returned to the dams after dose administration until the time of euthanasia. For gavage experiments, adult females were fasted overnight prior to dose administration, then were given *ad libitum* access to feed during the day. At termination, blood and brain samples were collected at each phase for the assessment of plasma, RBC and brain ChE levels.

2.2.1. Phase 1: range-finding study

A preliminary range-finding study was conducted to determine the approximate dose of CPF and CPFO necessary to produce marked (~50–60%) brain ChE inhibition in young adult female rats in order to anchor the dose–response curves with an effective dose. For this study, ~70-day-old female rats (3/dose group) were treated as shown in Table 1. There was a shared vehicle (corn oil) control group. All females were weighed and dosed by gavage in the morning and ChE samples were collected at 5 h following dose administration. Sample collection is described below. With CPFO, significant brain ChE inhibition was not seen, so RBC ChE inhibition was used for CPFO dose selection in subsequent study phases (Table 2).

2.2.2. Phase 2: acute pilot study

The pilot study was conducted to determine the time-of-peak ChE inhibition with the different dosing scenarios (Table 1). PND 11 rats (female) and young adult females (~70 days of age) were euthanized at numerous time points after administration of CPF (3 mg/kg in PND 11 pups; 10 mg/kg in adults) or CPFO (0.5 mg/kg in PND 11 pups; 0.3 mg/kg in adults) and ChE activity was

[1] Laboratory fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International).

Table 1
Chlorpyrifos comparative cholinesterase study design.

Age	Test compound	Vehicle	Dose level(s)[a] (mg/kg/day)	Duration	ChE sample collection time[b]	TK samples?
Phase 1: range-finding study						
Adult (~70 days of age)	CPF	Corn oil	0, 5, 10, 20	Single dose	5 h	N
	CPFO	Corn oil	0, 1, 5, 10	Single dose	5 h	N
Phase 2: time-of-peak inhibition study						
PND 11 (F)	CPF	Corn oil	0, 3	Single dose	2, 4, 6, 8, 12, 24 and 48 h	N
	CPF	Milk	0, 3	Single dose	2, 4, 6, 8, 12, 24 and 48 h	N
	CPFO	Corn oil	0, 0.5	Single dose	2, 4, 6, 8, 12 and 24 h	N
Adult (~70 days of age)	CPF	Corn oil	0, 10	Single dose	2, 4, 6, 8, 12 and 24 h	N
	CPF	Diet	0, 10	12-h Dietary exposure	2, 4, 6, 8, 12 and 24 h	N
	CPFO	Corn oil	0, 0.3	Single dose	2, 4, 6, 8, 12 and 24 h	N
Phase 3: acute dose–response study						
PND 11 (M and F)	CPF	Corn oil	0, 0.05, 0.1, 0.5, 2, 5	Single dose	6 h	Y
	CPF	Milk	0, 0.05, 0.1, 0.5, 2, 5	Single dose	8 h	Y
	CPFO	Corn oil	0, 0.005, 0.01, 0.05, 0.1, 0.5	Single dose	4 h	Y
Adult (~ 70 days of age)	CPF	Corn oil	0, 0.05, 0.1, 0.5, 2, 10	Single dose	8 h	Y
	CPF	Diet	0, 0.05, 0.1, 0.5, 2, 10	12-h Dietary exposure	8 h after removal of diet	Y
	CPFO	Corn oil	0, 0.01, 0.05, 0.1, 0.5, 1	Single dose	4 h	Y
Phase 4: repeat dose study						
PND 11 (M and F)	CPF	Corn oil	0, 0.05, 0.1, 0.5, 1, 3.5	11 doses; PND 11–21	6 h	Y
	CPFO	Corn oil	0, 0.01, 0.5	11 doses; PND 11–21	4 h	Y
Adult (~70 days of age)	CPF	Corn oil	0, 0.05, 0.1, 0.5, 1, 3.5	11 doses; ~70–80 days of age	8 h	Y
	CPFO	Corn oil	0, 0.01, 0.5	11 doses; ~70–80 days of age	4 h	Y

F, females; M, males.
[a] Whenever possible, a shared control group was used.
[b] Hours after administration of the last dose.

Table 2
Phase 1: range-finding study – summary of CPF and CPFO ChE inhibition in adult female CD rats after single gavage exposure in corn oil (samples collected 5 h post-exposure).

Dose group (mg/kg)	RBC ChE (U/L) (100 μl sample)	RBC ChE (U/L) (50 μl sample)	Mean RBC ChE (U/L)	Mean plasma ChE (U/L)[a]	Brain ChE (U/L)	Mean brain ChE (U/L)
0	4296	4028	4247 ± 298	2377	48,198	50,400 ± 2812
	4650	4014			53,568	
					49,434	
5 chlorpyrifos	2614	2020	2293 ± 448	737	53,505	48,840 ± 6653 (3% decrease)
	2728	1808	(46% decrease)		51,794	
					41,222	
10 chlorpyrifos	636	324	434 ± 264	433	29,675	31,400 ± 4216 (38% decrease)
	112	664	(90% decrease)		28,319	
					36,205	
20 chlorpyrifos	UR	UR	UR	354	14,440	12,669 ± 1555 (75% decrease)
	UR	UR			12,038	
					11,529	
1 chlorpyrifos–oxon	396	UR	212 (95% decrease)	633	53,257	51,422 ± 3160
	28	UR			47,773	
					53,236	
5 chlorpyrifos–oxon	UR	UR	UR	295	52,353	51,839 ± 1221
	UR	UR			52,720	
					50,445	
10 chlorpyrifos–oxon	UR	UR	UR	362	50,331	52,183 ± 3574
	UR	UR			49,915	
					56,303	

U/L, international units/L; an international enzyme unit per liter (U/L) is defined as the activity of enzyme which converts 1 μmol/L of acetylthiocholine in one minute at standard conditions.
UR, under range (lowest range of standard curve = 20).
[a] Hemolysis was noted in some plasma samples (subsequent procedural change made to accommodate analytical sampling).

measured in various tissues (plasma, RBCs, brain). Time-of-peak ChE inhibition was determined experimentally using one dose level in the pilot study, then modeled across dose levels using the CPF PBPK/PD model (Timchalk et al., 2002, 2006; Supplemental data 1).

2.2.3. Phase 3: definitive acute dose–response study

The experimental design for the acute study examining the dose–response for ChE inhibition in PND 11 pups after acute CPF or CPFO administration is outlined in Table 1. Male and female pups (8/sex/dose; PND 11) and adult female rats (8/dose; ~70 days of age) were given a single gavage dose of 0, 0.05, 0.1, 0.5, 2, 5 (pups only) or 10 (adults only) mg/kg CPF in corn oil or 0, 0.005 (pups only), 0.01, 0.05, 0.1, 0.5, or 1 (adults only) mg/kg CPFO in corn oil. Prior to euthanasia, pups and adult female rats were given clinical observations to determine whether signs of cholinergic toxicity were present.

Samples (blood and brain) were collected at the time-of-peak ChE inhibition (CPF in corn oil: 6 h post-dosing in pups and 8 h post-dosing in adults; CPFO in corn oil: 4 h post-dosing in pups and adults).

A separate group of PND 11 male and female pups (8/sex/dose) were given a single gavage dose of 0, 0.05, 0.1, 0.5, 2, or 5 mg/kg

CPF in rat milk. This dosing scenario was designed to evaluate the effect of vehicle on ChE inhibition and blood levels of CPF, CPFO and TCP. Pups were terminated at the time-of-peak ChE inhibition (8 h after dosing as determined in the pilot study and by PBPK/PD modeling).

A separate group of females (8/dose; ~70 days of age) was exposed to CPF in the diet for 12 h at concentrations designed to achieve similar mg/kg doses to gavage CPF (0, 0.05, 0.1, 0.5, 2 or 10 mg/kg). This dosing scenario is more consistent with acute dietary exposures in humans and can be compared with adults exposed via CPF gavage in corn oil. Females in the dietary group were given CPF-supplemented diet at the start of the dark cycle for 12 h, and the amount of feed consumed was measured. Females were terminated at the time-of-peak ChE inhibition (8 h after removal of the CPF-containing diets as determined in the pilot study and by PBPK/PD modeling). Test material intake (mg/kg body weight/day) was calculated for the 12 h dietary exposures.

2.2.4. Phase 4: definitive repeat-dose study

ChE inhibition in plasma, RBCs and brain was examined in preweanling (male and female) and adult female rats after repeated gavage exposures (11 days) to CPF or CPFO in corn oil (Table 1). The experimental design used litters with all male or all female pups, and assigned pups within each litter to a CPF- or CPFO-exposure group. PND 11 male and female pups (8/sex/dose) and adult female rats (8/dose) were given daily gavage doses of CPF in corn oil at doses of 0, 0.05, 0.1, 0.5, 1, 3.5 mg/kg/day or CPFO in corn oil at doses of 0, 0.01 or 0.5 mg/kg/day for 11 days. CPF doses were selected to produce substantial brain ChE inhibition at the highest dose level. CPFO, which had only two treatment groups per age, was designed to include a high dose that caused RBC ChE inhibition, because brain inhibition was not seen with CPFO exposure. Pups and adults received daily clinical observations on PND 11–19 and 21 for pups and approximately 70–78 and 80 days of age for adults. A functional observational battery (FOB) was conducted at the approximate time-of-peak inhibition on PND 20 (preweanlings) or at ~79 days of age (adults) to determine whether signs of cholinergic toxicity were present. The FOB, which was conducted according to previously described procedures (Mattsson et al., 1986, 1997), included cage-side, hand-held and open-field observations, and measurements of body weight, rectal temperature, fore- and hindlimb grip performance, and motor activity. The FOB was conducted by an observer who was blind to the treatment status of the animal. The same observer was used for all rats. Adult and immature rats were euthanized at 80 and 21 days of age, respectively. Samples (blood and brain) were collected at the time-of-peak ChE inhibition determined in the acute study (6 h and 8 h after the last dose of CPF in pups and adults, respectively, and 4 h after the last dose of CPFO). In addition, an aliquot of the terminal blood sample was used to determine internal dosimetry for CPF, CPFO and/or TCP.

2.3. Sample collection

Rats were anesthetized with isoflurane. Blood samples were collected from the inferior vena cava (adults) or by heart nick with blood collection into capillary tubes (pups). Rats were euthanized by exsanguination followed by decapitation. An aliquot of blood (4/dose) was analyzed for CPF, CPFO, and TCP using analytical methods described in Brzak et al. (1998) and Mattsson et al. (2000). Remaining blood samples were placed into heparinized tubes and kept on ice. Brain tissue also was collected from each rat, weighed and quick frozen in liquid nitrogen. Blood was centrifuged to separate plasma and packed RBCs. RBCs were diluted in 1% Triton X-100. Samples were stored frozen (−80 °C) until shipped on dry ice to WIL Research Laboratories LLC (Ashland, OH) for analysis of ChE activity using a modified Ellman method (Ellman et al., 1961; Hunter et al., 1997). Frozen brain samples were diluted in 10 volumes of 1% Triton X-100 (based on brain weight), immediately homogenized, centrifuged, and analyzed for ChE activity.

2.4. Statistics

ChE activity was analyzed separately for adults, male and female pups to determine whether a significant, dose-related difference in ChE inhibition exists. These analyses involved a one-way analysis of variance (ANOVA) using dose as a factor. Descriptive statistics (mean and standard deviation) were reported for blood levels of CPF, CPFO and TCP. Statistical analyses were conducted on body weights (collected at FOB time points), grip performance, rectal temperature, motor activity and FOB observations, with analyses being conducted separately for each age, sex and test material. These analyses are described in Supplemental data 2.

3. Results

3.1. Phase 1: range-finding study

Adult females were exposed to 0, 5, 10 or 20 mg/kg CPF or 1, 5 or 10 mg/kg CPFO to identify dose levels that produced marked inhibition of brain ChE without maximum inhibition of RBC ChE activity. Samples were collected at 5 h post-dosing. The high dose level (20 mg/kg CPF) produced 75% brain ChE inhibition, with >99% inhibition of RBC ChE activity (Table 2). At 10 mg/kg CPF, brain and RBC ChE activity were decreased by 38% and 90%, respectively. The highest dose of CPFO (10 mg/kg) produced >99% RBC ChE inhibition, but did not alter brain ChE activity in adult female rats. The lowest CPFO dose (1 mg/kg) decreased RBC ChE activity by 95%. Based on these data, 0 and 10 mg/kg CPF and 0 and 0.3 mg/kg CPFO were selected for the time-of-peak inhibition studies in adults. Dose levels in the time-of-peak inhibition studies in PND 11 pups were adjusted to 0 and 3 mg/kg CPF and 0 and 0.5 mg/kg CPFO based on the magnitude of ChE inhibition seen in adult female rats.

3.2. Phase 2: time-of-peak inhibition studies

Studies were conducted to determine the time-of-peak inhibition for RBC, brain and plasma ChE in pups and adults using the different acute dosing scenarios. Time-of-peak inhibition for brain ChE was used for subsequent experiments. For PND 11 pups, the times-to-peak inhibition were 6 h post-dosing for CPF in corn oil, 4 h post-dosing for CPFO in corn oil and 8 h post-dosing for CPF in milk. For adult female rats, the times-to peak inhibition were 8 h post-dosing for CPF in corn oil, 4 h post-dosing for CPFO in corn oil, and 8 h post-exposure for CPF in diet (i.e., after conclusion of the 12-h dietary exposure to CPF-containing diet). Using the PBPK/PD model, the time-of-peak brain ChE inhibition was shown to be similar across dose levels within each age group; therefore, the same sample collection times could be used. The results of the time-of-peak inhibition studies and subsequent modeling appear in Supplemental data 1.

3.3. Phase 3: definitive acute dose–response study

The definitive acute comparative ChE study examined age-related differential sensitivity across multiple dose levels of CPF or CPFO after a single exposure. Samples were collected at the time-of-peak ChE inhibition. Blood levels of CPF, CPFO and TCP also were determined at these time points. There were no treatment-related clinical observations during any phase of the acute studies.

(a) CPF in corn oil: data for ChE activity measured in PND 11 male and female CD rat pups (8/sex/dose; 0.05–5 mg/kg sampled 6 h post-dosing) and adult female CD rats (8/dose; 0.05–10 mg/kg sampled 8 h post-dosing) exposed to a single gavage dose of CPF (corn oil vehicle) are shown in Table 3. At both ages, the highest dose level was designed to provide an anchoring point for the dose–response curve, causing consistent, measurable brain ChE inhibition. There were no discernable gender differences in ChE activity in male or female PND 11 pups in response to CPF treatment. At the highest dose of CPF (5 mg/kg in PND 11 pups and 10 mg/kg in adults), both pups and adults had significant and comparable decreases in brain ChE activity (42.5–49% of control brain ChE). Brain ChE was not inhibited in pups or adults at 2.0 mg/kg CPF. Both ages also showed significant RBC and plasma ChE inhibition at doses ⩾2 mg/kg. The 0.5 mg/kg CPF dose was considered a no-observed-effect-level (NOEL) for ChE inhibition across all tissues in both age groups with acute exposure.

Figs. 1A and 2A show blood levels of CPF and CPFO relative to ChE inhibition across tissues with samples collected at the time-of-peak ChE inhibition. Consistent with the ChE inhibition data, there were no apparent gender-related differences in blood levels of CPF or its metabolites in PND 11 pups; therefore, the figures show mean pup values. In PND 11 pups, CPF and TCP were detectable in blood at all doses of CPF, whereas CPFO was below the level of quantitation (LLQ) at ⩽0.1 mg/kg CPF and only had one value from four samples that was above the LLQ at 0.5 mg/kg CPF (Fig. 1A). Increases in blood CPF levels were approximately dose proportional at doses ⩽2 mg/kg, whereas at 5 mg/kg, CPF blood levels were 466× greater than blood levels at 0.05 mg/kg CPF with only a 100× increase in dose. This may suggest different kinetics at the high dose of 5 mg/kg than at lower dose levels. Furthermore, the [TCP]:[CPF] ratio was lower at 5 mg/kg than 0.05 mg/kg (21 vs. 300, respectively), suggesting that the relative amount of CPF metabolized to TCP is inversely related to CPF dose. This interpretation is consistent with predictions by the PBPK/PD model for CPF (Timchalk et al., 2002). In adult females, blood CPF was detectable at doses ⩾0.5 mg/kg and TCP was detectable at all dose levels in adult females; whereas CPFO was at or below the LLQ at all doses of CPF (Fig. 2A). It was difficult to fully evaluate dose proportionality (i.e., [TCP]:[CPF] ratio) in adult females with so few data points for CPF, but TCP levels were approximately dose proportional.

When comparing TK data with ChE inhibition data, it is important to note that samples were collected at 6 h post-dosing in PND 11 pups compared with 8 h post-dosing in adults. Furthermore, the time-of-peak ChE inhibition may not correspond with the time-of-peak blood levels of CPF or its metabolites, particularly given the slow recovery of ChE activity. The peak metabolite levels may occur earlier than the sampling times chosen for ChE measurements. However, these TK data were collected to aid in current understanding of metabolite ratios between dose levels, vehicle and age. These ratios provide useful information on relative metabolism of CPF between the experimental groups and are useful to refine PB/PK/PD models of chlorpyrifos in the adult and neonatal rat.

The TK data indicate that pups were exposed to higher blood concentrations of CPF than adults at high doses (e.g., at 5 mg/kg, pups had 3× higher blood CPF levels than adults given 10 mg/kg). In addition, pups had detectable levels of CPFO at these dose levels (5 vs. 10 mg/kg in adults), whereas pups had lower levels of TCP (67% of adult levels at these doses). At 2 mg/kg, PND 11 pups had 10× higher levels of blood CPF than adults; however, unlike the profile seen at the high dose, pups also had 2× higher blood TCP levels than adults, indicating that pups were metabolizing proportionately more CPF at this lower dose. There was no brain inhibition in either pups or adults at 2 mg/kg CPF.

Thus, the overall profile of effects was that pups were more sensitive to ChE inhibition at higher doses of CPF (e.g., 5 mg/kg); however, at lower CPF levels, pups had lower ChE inhibition than adults at comparable blood levels of CPF. There was no evidence of age-related differential sensitivity to acute CPF exposure over the lower portion of the dose–response curve.

(b) CPF in rat milk to PND 11 pups: data for ChE activity measured in PND 11 male and female CD rat pups (8/sex/dose; 0.05–5 mg/kg sampled 8 h post-dosing) exposed to a single gavage dose of CPF (rat milk vehicle) are shown in Table 4. Similar to the experiments using corn oil vehicle, there were no discernable gender differences in ChE activity in male or female PND 11 pups in response to CPF

Table 3
ChE inhibition in PND 11 pups and adult females following an acute dose of CPF in corn oil.

Dose (mg/kg)	RBC ChE (U/L)	RBC ChE (% control)	Brain ChE (U/L)	Brain ChE (% control)	Plasma ChE (U/L)	Plasma ChE (% control)
Adults – CPF in corn oil						
0	5409 ± 351	100.0	54,728 ± 1283	100.0	2143 ± 803	100.0
0.05	5146 ± 533	95.1	54,461 ± 2301	99.5	2317 ± 635	108.2
0.1	5494 ± 357	101.6	51,116 ± 2484	93.4	1944 ± 684	90.7
0.5	5975 ± 1150	110.4	52,243 ± 1586	95.5	1874 ± 262	87.5
2.0	**4360 ± 536***	80.6	52,194 ± 2580	95.4	**981 ± 284***	45.8
10.0	**851 ± 622***	15.7	**23,276 ± 11,567***	42.5	**283 ± 80***	13.2
PND 11 male pups – CPF in corn oil						
0	6891 ± 782	100.0	22,765 ± 3336	100.0	1407 ± 111[a]	100.0
0.05	7034 ± 923	102.1	25,891 ± 2664	113.7	1440 ± 221	102.3
0.1	7195 ± 861	104.4	24,339 ± 1508	106.9	1494 ± 177	106.2
0.5	6538 ± 544	94.9	25,202 ± 1847	110.7	1301 ± 89	92.4
2.0	**4434 ± 590***	64.3	22,346 ± 2910	98.2	**690 ± 63***	49.0
5.0	**804 ± 249***	11.7	**11,163 ± 2821***	49.0	**321 ± 54***	22.8
PND 11 female pups – CPF in corn oil						
0	6432 ± 776	100.0	24,629 ± 3768	100.0	1421 ± 106	100.0
0.05	6674 ± 634	103.8	25,125 ± 2987	102.0	1409 ± 150	99.1
0.1	6518 ± 677	101.3	24,910 ± 3080	101.1	1374 ± 200	96.7
0.5	6345 ± 1308	98.7	25,372 ± 2069	103.0	1283 ± 136	90.3
2.0	**4441 ± 392***	69.0	22,933 ± 3188	93.1	**752 ± 141*,a**	53.0
5.0	**873 ± 282***	13.6	**10,955 ± 1517***	44.5	**313 ± 69***	22.0

$n = 8$ pups/sex/dose level or 8 adult females/dose level unless otherwise indicated.
U/L, international units/L.
[a] $n = 7$ in the male pup plasma control group and the female pup plasma 2.0 mg/kg dose group.
* Significantly different from controls at alpha = 0.05 using Dunnett's test when raw ChE data were analyzed (Treatment-related effects in bold text).

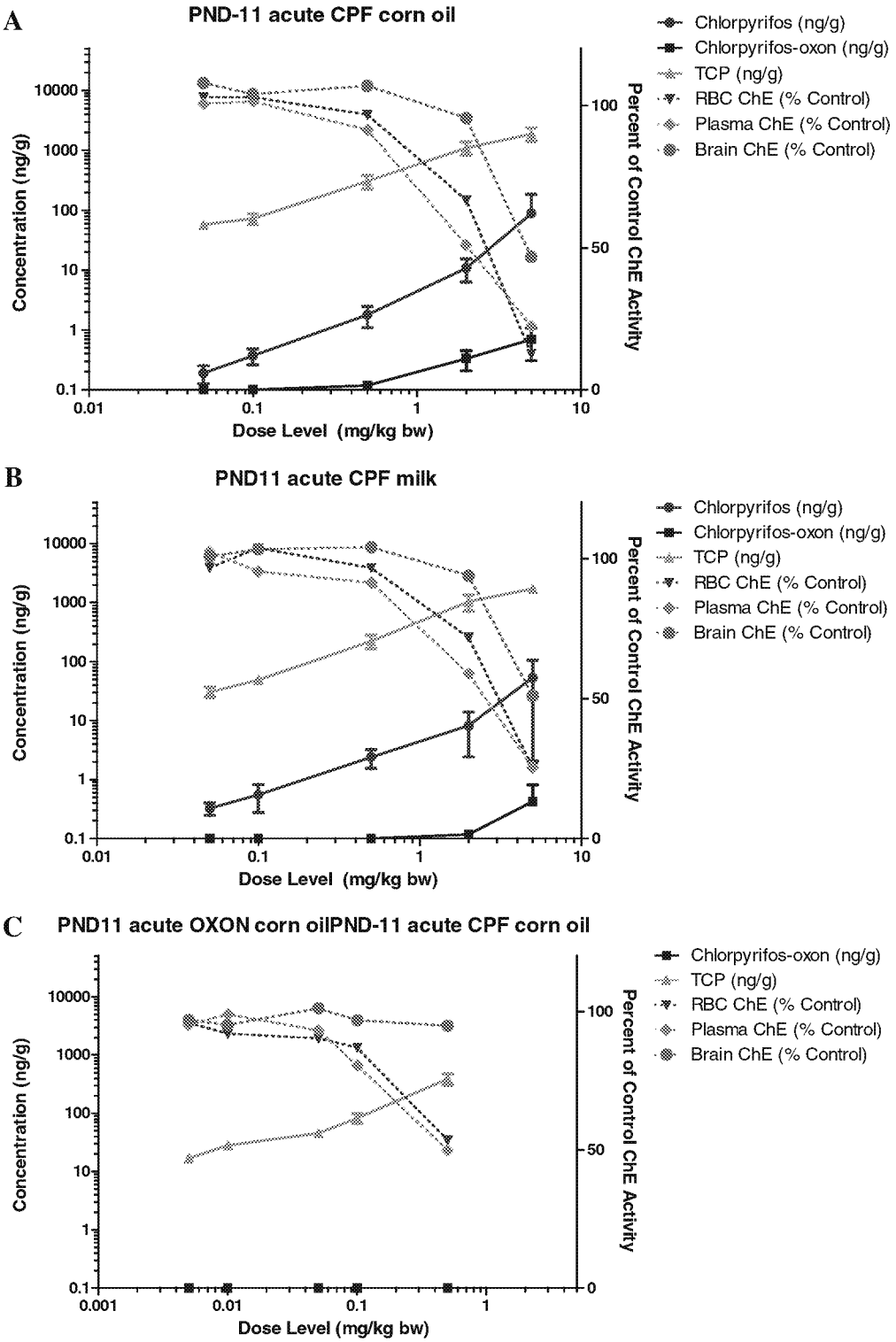

Fig. 1. Mean RBC, brain and plasma ChE inhibition relative to mean blood CPF, CPFO and TCP levels in PND 11 pups following acute gavage exposure at multiple dose levels to CPF in corn oil vehicle (A; sampled at 6 h post-dosing), milk vehicle (B; sampled at 8 h post-dosing), or CPFO in corn oil vehicle (C; sampled at 4 h post-dosing). Data show mean values of combined male and female pup data as there were no gender-related differences in either ChE inhibition or blood CPF/CPFO levels. (n = 7–8 pups/sex/dose level for ChE measurements; n = 4 pups/dose level for determination of blood CPF, CPFO and TCP levels).

treatment. ChE inhibition across tissues was comparable in PND 11 pups using either milk or corn oil vehicle. At 5 mg/kg CPF, brain ChE was inhibited to approximately 47% of control in corn oil compared with 51% of control in rat milk. As with corn oil, brain ChE was not inhibited at 2.0 mg/kg CPF in milk. Similarly, RBC and plasma ChE at 2.0 mg/kg CPF were inhibited to approximately 67% and 51% of control values using corn oil vehicle compared with 72% and 59% of control values using milk vehicle. There were no significant effects in ChE values at 0.5 mg/kg, which was the NOEL for ChE inhibition across all tissues regardless of vehicle.

Fig. 1B shows blood levels of CPF and CPFO relative to ChE inhibition across tissues with samples collected at the time-of-peak

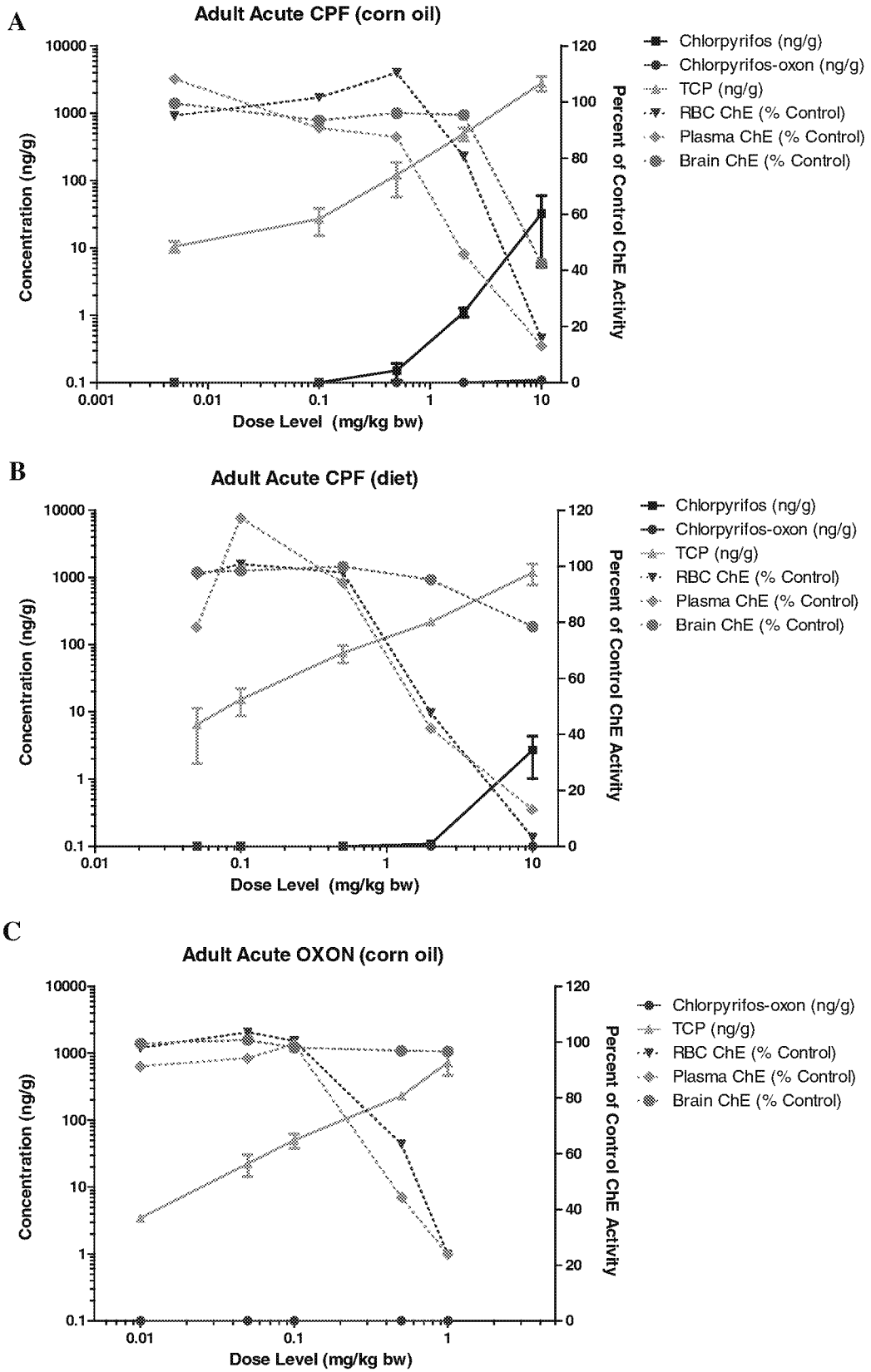

Fig. 2. Mean RBC, brain and plasma ChE inhibition relative to mean blood CPF, CPFO and TCP levels in adult female rats following acute exposure to CPF at multiple dose levels in corn oil vehicle (gavage) (A; sampled at 8 h post-dosing), 12-h dietary exposure (B; sampled 8 h after removal of test diet), or CPFO in corn oil vehicle (gavage) (C; sampled at 4 h post-dosing). ($n = 8$ rats/dose level for ChE measurements; $n = 4$ rats/dose level for determination of blood CPF, CPFO and TCP levels).

ChE inhibition. Consistent with the ChE inhibition data, there were no apparent gender-related differences in blood levels of CPF or its metabolites in PND 11 pups; therefore, the figure shows mean pup values. At the time-of-peak inhibition, the blood CPF and CPFO

Table 4
ChE inhibition in PND 11 pups following an acute dose of CPF in rat milk.

Dose (mg/kg)	RBC ChE (U/L)	RBC ChE (% control)	Brain ChE (U/L)	Brain ChE (% control)	Plasma ChE (U/L)	Plasma ChE (% control)
PND 11 male pups – CPF in milk						
0	6305 ± 1460	100.0	23,911 ± 1873	100.0	1613 ± 185	100.0
0.05	6017 ± 1385	95.4	24,489 ± 3432	102.4	1629 ± 181	101.0
0.1	6591 ± 924	104.5	25,279 ± 2155	105.7	1573 ± 239	97.5
0.5	6320 ± 1028	100.2	25,325 ± 2815	105.9	1510 ± 142	93.6
2.0	4459 ± 1916	70.7	24,760 ± 2744	103.6	**985 ± 198***	61.1
5.0	**1805 ± 1706***	28.6	**13,918 ± 4867***	58.2	**472 ± 508***	29.2
PND 11 female pups – CPF in milk						
0	6324 ± 648	100.0	26,410 ± 3026	100.0	1586 ± 137	100.0
0.05	6197 ± 614	98.0	26,113 ± 2979	98.9	1646 ± 171	103.7
0.1	6501 ± 979	102.8	26,609 ± 2104	100.8	1476 ± 179	93.0
0.5	5877 ± 1505	92.9	26,969 ± 1413	102.1	1410 ± 160	88.9
2.0	**4600 ± 1759***	72.7	22,201 ± 4811	84.1	**896 ± 220***	56.5
5.0	**1375 ± 1634***	21.7	**11,511 ± 5332***	43.6	**341 ± 239***	21.5

$n = 8$ pups/sex/dose level.
U/L, international units/L.
* Significantly different from controls at alpha = 0.05 using Dunnett's test when raw ChE data were analyzed (treatment-related effects in bold text).

levels, as well as the magnitude of ChE inhibition across tissues, were similar with both milk and corn oil vehicles. As with CPF in corn oil, CPF and TCP were detectable in blood at all doses of CPF in rat milk, whereas CPFO was below the LLQ at ⩽0.5 mg/kg CPF (vs. the 0.1 mg/kg dose with CPF in corn oil) and only had one value from four samples that was above the LLQ at 2.0 mg/kg CPF. Increases in blood CPF levels were approximately dose proportional at doses ⩽2 mg/kg, whereas at 5 mg/kg, CPF blood levels were 165× greater than blood levels at 0.05 mg/kg CPF with only a 100× increase in dose. This value was not as great as the 466× differential seen with CPF in corn oil; however, there may have been an impact of collecting samples 2 h later with CPF in milk (8 h post-dosing vs. 6 h with corn oil). Again, these data suggest different kinetics at the high dose of 5 mg/kg than lower dose levels, regardless of milk or corn oil vehicle. Furthermore, the [TCP]/[CPF] ratio was lower at 5 mg/kg than 0.05 mg/kg (32 vs. 95, respectively), again suggesting that the relative amount of CPF metabolized to TCP was decreased when the CPF dose reached 5 mg/kg in milk. This interpretation is consistent with predictions by the PBPK/PD model for CPF.

At the time-of-peak inhibition, blood CPF and CPFO levels, as well as the magnitude of ChE inhibition across tissues, were similar with both milk and corn oil vehicles. Thus, PND 11 pups appeared to be equally sensitive to ChE inhibition following acute CPF exposure in either corn oil or rat milk vehicle.

(c) CPF in diet to adult females: data for ChE activity measured in adult female rats (8/sex/dose; 0.05–10 mg/kg) exposed for 12-h to dietary CPF are shown in Table 5. The dietary route was included as it was considered a more relevant dosing scenario. Based on body weights and feed consumption over the 12-h period, dietary test material intake for these females was 0.05, 0.10, 0.53, 2.06, or 9.59 mg/kg for the nominal doses of 0, 0.05, 0.1, 0.5, 2 or 10 mg/kg. Samples were collected at 8 h post-dosing as determined by a limited probe study (data in Supplemental data 1) and modeled using the existing PBPK/PD model. Results of these experiments are shown in Table 5. With a 12 h dietary exposure to CPF, adult females exhibited significant brain ChE inhibition at the same concentration as CPF given via gavage in corn oil (10 mg/kg); however, the magnitude of brain ChE inhibition was less by the dietary route (76.3% of control values compared with 42.5% of control in the oral gavage group). Similar to corn oil gavage, brain ChE was not inhibited at dietary doses ⩽2 mg/kg CPF. Conversely, RBC ChE activity appeared to be slightly more sensitive to dietary ChE with inhibition to 47.7% of control values at 2 mg/kg CPF (compared with 80.6% of control with gavage exposure at this dose). Again, RBC ChE was not inhibited at dietary doses ⩽0.5 mg/kg CPF as was seen with gavage dosing. Plasma ChE showed a similar response to RBC ChE activity with similar sensitivity via the dietary and gavage routes. As with corn oil gavage, 0.5 mg/kg CPF was the NOEL for ChE inhibition across all tissues.

Fig. 2B shows blood levels of CPF and CPFO relative to ChE inhibition across tissues with samples collected at the time-of-peak ChE inhibition. At the time-of-peak inhibition, the blood CPF and CPFO levels, as well as the magnitude of ChE inhibition across tissues, were different from the curves seen with corn oil vehicle. At 8 h post-exposure, blood CPF was >LLQ in all animals at 10 mg/kg and in one of four animals at 2 mg/kg and CPFO was below the LLQ in all samples, making the TK data somewhat limited for these analytes. However, TCP was detectable at all doses of CPF and increased in a dose-proportional manner. With only limited determinations, [TCP]:[CPF] ratio was similar to other dosing scenarios with a decrease in the proportion of CPF metabolized to

Table 5
ChE inhibition in adult females following an acute 12-h exposure to CPF in the diet.

Dose (mg/kg)	RBC ChE (U/L)	RBC ChE (% control)	Brain ChE (U/L)	Brain ChE (% control)	Plasma ChE (U/L)	Plasma ChE (% control)
Adults – CPF in diet						
0	5337 ± 305	100.0	53,888 ± 1242	100.0	1754 ± 395	100.0
0.05	5164 ± 575	96.8	52,700 ± 2265	97.8	1373 ± 249	78.3
0.1	5380 ± 525	100.8	53,020 ± 1698	98.4	2058 ± 458	117.3
0.5	5219 ± 575	97.8	53,844 ± 1737	99.9	1648 ± 364	94.0
2.0	**2548 ± 446***	47.7	51,294 ± 1639	95.2	**737 ± 190***	42.0
10.0	**167 ± 146***	3.1	**41,125 ± 7650***	76.3	**230 ± 82***	13.1

$n = 8$ adult females/dose level.
U/L = international units/L.
* Significantly different from controls at alpha = 0.05 using Dunnett's test when raw ChE data were analyzed (treatment-related effects in bold text).

TCP at the highest dose (10 mg/kg). Furthermore, blood CPF and TCP levels were 7.4× and 2.4× lower, respectively, by the dietary route than via corn oil gavage. This result suggests that dose rate impacts blood levels of CPF and its metabolites and subsequently, the magnitude of brain ChE inhibition.

While plasma ChE was similarly inhibited by both corn oil gavage and diet, RBC ChE was more inhibited by the dietary route at 2 mg/kg (47.7% compared with 80.6% by gavage), despite having approximately 10× and 2.3× lower blood levels of CPF and TCP, respectively. Thus, dose rate impacts blood levels of CPF and its metabolites and shifts the amount of ChE inhibition across tissues.

(d) CPFO in corn oil: data for ChE activity measured in PND 11 male and female CD rat pups (8/sex/dose; 0.005–1 mg/kg sampled 4 h post-dosing) and adult female CD rats (8/dose; 0.005–1 mg/kg sampled 4 h post-dosing) exposed to a single gavage dose of CPFO (corn oil vehicle) are shown in Table 6. At both ages, the highest dose level was designed to provide an anchoring point for the dose–response curve, causing consistent, measurable RBC ChE inhibition. There were no discernable gender differences in ChE activity in male or female PND 11 pups in response to CPFO treatment. There was no significant inhibition of brain ChE in either pups or adults at any dose level of CPFO. This result was consistent with results seen in the range-finding study with adult rats, in which no brain inhibition was seen at 10 mg/kg CPFO in corn oil, despite near complete inhibition of RBC ChE (Table 2). RBC ChE inhibition was seen at the same dose level (0.5 mg/kg) in PND 11 pups and adults (approximately 53% and 63% of control RBC ChE in pups and adults, respectively); however, PND 11 pups exhibited significant plasma ChE inhibition at a slightly lower dose than adults (0.1 mg/kg, where pup plasma ChE was approximately 80.5% of control compared with 99% in adult females).

Figs. 1C and 2C show blood levels of CPFO relative to ChE inhibition across tissues with samples collected at the time-of-peak ChE inhibition. Consistent with the ChE inhibition data, there were no apparent gender-related differences in blood levels of CPFO in PND 11 pups; therefore, the figures show mean pup values. Plasma ChE inhibition in pups at 0.1 mg/kg CPFO may be related to higher CPFO blood levels as suggested by the 1.6× higher blood TCP levels in pups at this dose level (CPFO was below the LLQ in pups and adults). In adults, CPFO was below the LLQ at all dose levels, whereas TCP was detectable at all doses of CPFO. In both pups and adults, blood TCP levels increased in a dose-related fashion, although the increase was lower than dose proportional in PND 11 pups at CPFO doses ⩾0.05 mg/kg (i.e., with a 100× increase in CPFO dose from 0.005 to 0.5 mg/kg, TCP levels increased only 23×) and at or slightly greater than dose proportional in adults.

With samples collected at the same time post-dosing (4 h), pups and adults had similar levels of ChE inhibition in RBCs and plasma at 0.5 mg/kg CPFO, although pups had 1.7× higher levels of blood TCP. At 0.1 mg/kg CPFO, neither pups nor adults had significant inhibition of RBC ChE, but pups had significant inhibition of plasma ChE. Pup blood TCP was again 1.6× higher than blood TCP levels in adults; thus, it was possible that higher CPFO dosimetry in pups explained this difference. At 0.05 mg/kg CPFO, pups had similar levels of TCP as adults at 0.1 mg/kg CPFO. At this internal dose, there was no inhibition of ChE in any tissue. Brain ChE was not inhibited at any dose of CPFO in either pups or adults when the highest oxon dose (0.5 mg/kg) yielded TCP levels of 387.5 ng/g and 724.8 ng/g, respectively.

Thus, the 0.1 mg/kg CPFO dose was considered a NOEL for ChE inhibition in RBC in PND 11 pups and a NOEL in both RBC and plasma for adult females with acute exposure. Due to plasma ChE inhibition at 0.1 mg/kg CPFO, 0.05 mg/kg CPFO was the NOEL across all tissues in PND 11 pups with acute CPFO exposure.

3.4. Phase 4: definitive repeat-dose study

The definitive repeat-dose study examined age-related differential sensitivity across multiple dose levels of CPF or CPFO after 11 daily doses administered by gavage. Blood levels of CPF, CPFO and TCP also were determined in terminal samples collected at the time-of-peak inhibition determined in the acute studies.

(a) CPF in corn oil: in the repeated dose study, there were no treatment-related clinical observations in male or female rat pups (8/sex/dose; dosed PND 11–21) or adult female rats (8/dose; dosed PND 70–80) following exposure to eleven daily gavage doses (corn oil vehicle) of 0, 0.05, 0.1, 0.5, 1, or 3.5 mg/kg/day CPF (data not shown). Neither pup nor adult female body weights were affected

Table 6
ChE inhibition in PND 11 pups and adult females following an acute dose of CPFO in corn oil.

Dose (mg/kg)	RBC ChE (U/L)	RBC ChE (% control)	Brain ChE (U/L)	Brain ChE (% control)	Plasma ChE (U/L)	Plasma ChE (% control)
Adults – CPFO in corn oil						
0	5630 ± 906	100.0	52,826 ± 2036	100.0	2106 ± 562	100.0
0.01	5510 ± 662	97.9	52,517 ± 1503	99.4	1922 ± 369	91.3
0.05	5830 ± 402	103.5	53,203 ± 2367	100.7	1984 ± 374	94.2
0.1	5655 ± 210	100.4	51,751 ± 991	98.0	2088 ± 736	99.2
0.5	**3572 ± 905***	63.4	51,169 ± 1299	96.9	**931 ± 369***	44.2
1.0	**1338 ± 497***	23.8	51,046 ± 1830	96.6	**502 ± 141***	23.8
PND 11 male pups – CPFO in corn oil						
0	6564 ± 513	100.0	25,539 ± 1711	100.0	1445 ± 118	100.0
0.005	6174 ± 828	94.1	24,558 ± 2114	96.2	1362 ± 115	94.2
0.01	5565 ± 1281	84.8	23,709 ± 2580	92.8	1466 ± 127	101.4
0.05	6159 ± 807	93.8	24,800 ± 3116	97.1	1378 ± 210	95.4
0.1	5506 ± 977	83.9	24,798 ± 2812	97.1	**1182 ± 190***	81.8
0.5	**3534 ± 489***	53.8	23,489 ± 3133	92.0	**738 ± 72***	51.1
PND 11 female pups – CPFO in corn oil						
0	6287 ± 856	100.0	22,994 ± 4168	100.0	1487 ± 210	100.0
0.005	6146 ± 1557	97.7	22,436 ± 1790	97.6	1428 ± 125	96.0
0.01	6227 ± 1234	99.0	22,358 ± 2620	97.2	1433 ± 172	96.4
0.05	5444 ± 1025	86.6	24,178 ± 1236	105.2	1352 ± 200	90.9
0.1	5651 ± 1274	89.9	22,202 ± 3687	96.6	**1179 ± 124***	79.3
0.5	**3329 ± 772***	52.9	22,462 ± 2964	97.7	**723 ± 62***	48.6

n = 8 pups/sex/dose level or 8 adult females/dose level.
U/L, international units/L.
* Significantly different from controls at alpha = 0.05 using Dunnett's test when raw ChE data were analyzed (treatment-related effects in bold text).

by repeated CPF exposure (data not shown except FOB body weights in Supplemental data 2). There were no treatment-related effects on neurobehavioral endpoints, which included a FOB and motor activity, evaluated at the time-of-peak ChE inhibition after the 10th dose of CPF (Supplemental data 2).

Data for ChE activity measured in PND 21 male and female rat pups and 80-day-old female rats exposed to various doses of CPF for eleven days are shown in Table 7. At both ages, the highest dose levels were designed to provide an anchoring point for the dose–response curves, causing consistent, measurable brain ChE inhibition. Samples for ChE inhibition were collected at the time-of-peak inhibition determined in the acute studies – 6 h post-dosing for the pups and 8 h post-dosing in the adults. At the high dose of CPF (3.5 mg/kg/day), pups had similar ChE inhibition to adults across all tissues. At 1 mg/kg/day, ChE was significantly decreased in both pups and adults across all tissues, although adult brain ChE activity was 91.1% of control, a change that was not considered biologically meaningful. At 0.5 mg/kg/day, there was no significant brain ChE inhibition in either adults or pups, but both age groups had significant decreases in RBC ChE activity and plasma ChE activity with no apparent difference in sensitivity. RBC ChE was significantly decreased in adults at 0.1 mg/kg/day, but this result was considered spurious because plasma ChE, which has similar or greater sensitivity to ChE inhibition by CPF (Lotti, 1995; Garabrant et al., 2009; UE EPA, 2008; Timchalk et al., 2006) was not inhibited at this dose. Thus, for a given CPF dose level, pups were equally sensitive to plasma ChE inhibition as adults. The 0.1 mg/kg/day CPF dose was considered a no-observed-effect-level (NOEL) for ChE inhibition across all tissues in both age groups with repeated exposure.

Fig. 3 shows blood levels of CPF and CPFO relative to ChE inhibition across tissues with samples collected at the time-of-peak ChE inhibition as determined in the acute CPF experiments. In PND 21 pups, CPF and TCP were detected in blood at all doses of CPF, whereas CPFO was below the LLQ (Fig. 3A). Overall, there were dose proportional increases in blood CPF and TCP levels; that is, as the doses increased from 0.05 to 3.5 (70× from low to high dose), blood CPF and TCP levels increased approximately 74× and 82×, respectively. There was no consistent pattern in the ratio of blood [TCP]:[CPF] across dose groups. For adults, CPF was detected in blood samples at doses ⩾0.5 mg/kg/day CPF, whereas CPFO was below the LLQ at all doses (Fig. 3B). TCP was detected in blood at all dose levels. There was a dose proportional increase in blood TCP levels; that is, as the doses increased from 0.05 to 3.5 (70× from low to high dose), blood TCP levels increased 78×. With limited data on CPF levels in blood, a pattern in the ratio of blood [TCP]:[CPF] could not be evaluated.

Blood levels of CPF and its metabolites facilitated interpretation of repeat-dose data. For example, at 0.5–1.0 mg/kg/day CPF, male pup RBC ChE was 63.2% and 38.7% of control compared with 81.8% and 56.0% of control in female pups at these dose levels. Based on blood values, there were no clear gender-related differences in CPF and TCP levels to support a differential sensitivity of male vs. female RBC ChE activity. Furthermore, plasma ChE inhibition was similar in both sexes at these doses. Therefore, it was concluded that this apparent gender-related difference in RBC ChE sensitivity was spurious. Gender-related differences in sensitivity were not seen in preweanling rats in other CPF studies (e.g., Moser and Padilla, 1998). As with the acute studies, pups had lower ChE inhibition across tissues at comparable CPF blood levels (e.g., approximately 27% RBC and plasma ChE inhibition and 2% brain ChE inhibition with CPF blood levels of 0.60 ng/g in pups compared with 71% RBC and plasma ChE inhibition and 9% brain ChE inhibition with 0.54 ng/g blood CPF in adults). The later sampling time in adults (8 h vs. 6 h in pups), coupled with a somewhat higher metabolic rate in adults, may have resulted in comparable blood levels. At 1 mg/kg/day, it appeared that RBC and plasma were less affected in pups, whereas brain was more affected; however, the magnitude of the brain ChE inhibition in pups was consistent with other brain ChE results for these blood CPF levels (i.e., mean pup blood CPF was 1.69 ng/g with 76% of control brain ChE activity; these values were between the blood CPF levels (0.54 and 2.21 ng/g, respectively) and brain ChE activity (91.1 and 31% of control, respectively) in adults given 1 or 3.5 mg/kg/day CPF, respectively). At 0.5 mg/kg/day, there was no significant brain ChE inhibition in either adults or

Table 7
ChE Inhibition in PND 21 pups and adult (~PND 80) females following repeated doses of CPF in corn oil.

Dose (mg/kg/day)	RBC ChE (U/L)	RBC ChE (% Control)	Brain ChE (U/L)	Brain ChE (% Control)	Plasma ChE (U/L)	Plasma ChE (% Control)
Adults – CPF in corn oil						
0	4903 ± 276	100.0	51,978 ± 1803	100.0	2254 ± 744	100.0
0.05	4655 ± 713[a]	94.9	51,929 ± 1031[a]	99.9	2892 ± 1052[a]	128.3
0.1	4120 ± 489*	84.0	51,990 ± 3291	100.0	2617 ± 770	116.1
0.5	**3946 ± 722***	80.5	51,694 ± 1862	99.5	**1220 ± 562***	54.1
1.0	**1335 ± 395***	27.2	**47,357 ± 1938***	91.1	**691 ± 316***	30.7
3.5	**135 ± 185***	2.7	**16,093 ± 2362***	31.0	**262 ± 73***	11.6
PND 21 male pups – CPF in corn oil						
0	6410 ± 1605	100.0	43,265 ± 1500	100.0	903 ± 111	100.0
0.05	5517 ± 1180	86.1	43,558 ± 1981	100.7	949 ± 148	104.9
0.1	5450 ± 1294	85.0	42,657 ± 1576	98.6	902 ± 136	99.6
0.5	**4054 ± 1112***	63.2	40,780 ± 1865	94.3	**645 ± 58***	71.3
1.0	**2478 ± 533***	38.7	**31,006 ± 4154***	71.7	**508 ± 70***	56.5
3.5	**540 ± 332***	8.4	**13,898 ± 988***	32.1	**187 ± 22***	20.8
PND 21 female pups – CPF in corn oil						
0	5951 ± 2082	100.0	42,289 ± 1616	100.0	928 ± 122	100.0
0.05	6388 ± 1021	107.3	44,726 ± 2598	105.8	921 ± 45	99.2
0.1	5882 ± 1282	98.8	43,499 ± 1409	102.9	864 ± 95	93.1
0.5	**4870 ± 829**	81.8	42,810 ± 1258	101.2	**713 ± 173***	76.8
1.0	**3333 ± 821***	56.0	**34,266 ± 2009***	81.0	**519 ± 64***	55.9
3.5	**723 ± 264***	12.1	**17,344 ± 2414***	41.0	**266 ± 51***	28.7

$n = 8$ pups/sex/dose level or 8 adult females/dose level unless otherwise indicated.
U/L, international units/L.
[a] $n = 7$ adult females in the 0.05 mg/kg/day dose group.
* Significantly different from controls at alpha = 0.05 using Dunnett's test when raw ChE data were analyzed (treatment-related effects in bold text).

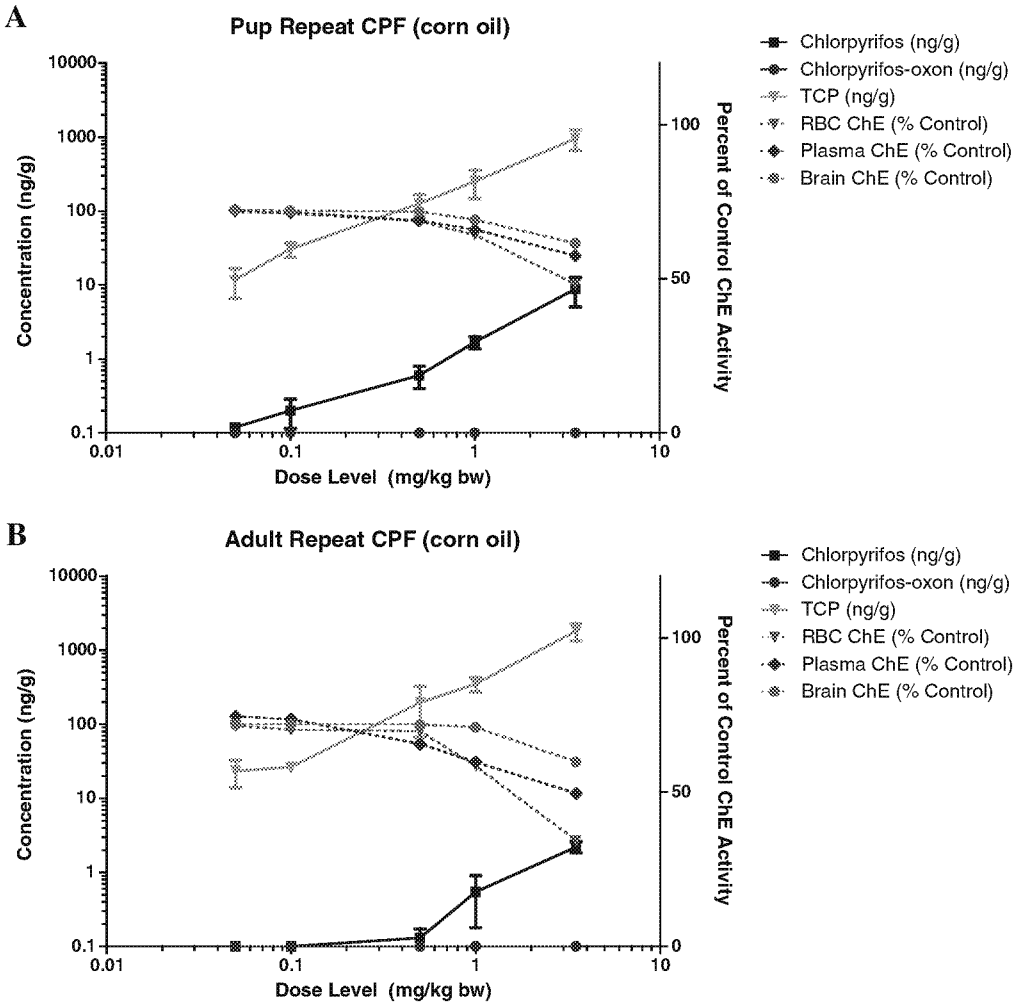

Fig. 3. Mean RBC, brain and plasma ChE inhibition relative to mean blood CPF, CPFO and TCP levels in PND 21 pups (A; sampled at 6 h after the last dose) or adult female rats (B; sampled at 8 h after the last dose) following daily gavage dosing with CPF in corn oil vehicle at multiple dose levels for 11 days. Data show mean values of combined male and female pup data as there were no gender-related differences in either ChE inhibition or blood CPF/CPFO levels. (n = 8 pups/sex/dose level or 7–8 adult females/dose level for ChE measurements; n = 4 pups or 4 adults in each dose group for determination of blood CPF, CPFO and TCP levels except for 3 pups in the control group and 3 adults in the 0.05 mg/kg/day group).

pups. Both age groups had significant decreases in RBC and plasma ChE activity with no apparent difference in sensitivity, despite a 4.6× higher blood CPF level in pups. Interestingly, the pup blood CPF level at 0.5 mg/kg/day was similar to blood levels in adults given 1 mg/kg/day CPF, a dose at which significant ChE inhibition was seen in all tissues in the adults. Thus, for a given blood CPF level, pups were equally or less sensitive to ChE inhibition as adults. As chlorpyrifos doses decrease below 1 mg/kg/day, there was no evidence of increased sensitivity in pups to CPF-induced ChE inhibition. At 0.1 mg/kg/day, CPF and CPFO were below the LLQ and TCP values were similar in adults and pups at 0.1 mg/kg/day, supporting the concept that pups would not be more sensitive to CPF at lower dose levels.

(b) CPFO in corn oil: in the repeat dose study, there were no treatment-related clinical observations in male or female rat pups (8/sex/dose; dosed PND 11–21) or adult female rats (8/dose; dosed PND 70–80) following exposure to eleven daily gavage doses of 0 (corn oil vehicle), 0.01 or 0.5 mg/kg/day CPFO (data not shown). There were no treatment-related effects on body weight and neither pups nor adult females exhibited effects on neurobehavioral endpoints (i.e., FOB and motor activity; Supplemental data 2) evaluated at the time-of-peak ChE inhibition after the 10th CPFO dose (i.e., 4 h post-dosing; Supplemental data 1).

Data for ChE activity measured in PND 21 male and female rat pups and 80-day-old female rats exposed to various doses of CPFO for eleven days are shown in Table 8. Once again, the high dose was selected to provide consistent, measurable RBC and plasma ChE inhibition. Samples for ChE inhibition were collected at the time-of-peak ChE inhibition determined in the acute studies. In response to repeated CPFO treatment, there was no discernable gender or age-related difference in ChE activity in male or female PND 21 pups or PND 80 adult females. There was no significant inhibition of brain ChE at any doses of CPFO (⩽0.5 mg/kg/day) in either pups or adults. The magnitude of RBC and plasma ChE in pups and adults was similar at 0.5 mg/kg/day CPFO, although adults had 2× the levels of TCP as pups. ChE was not inhibited in either of these tissues at 0.01 mg/kg/day in either adults or pups.

Fig. 4 shows blood levels of CPFO relative to ChE inhibition across tissues with samples collected at 4 h post-dosing. In both pups and adults, TCP was detectable in blood at all doses of CPFO (including two PND 21 control samples, which reflected some variability in detection around the LLQ; data not shown). TCP levels were increased in a dose-related fashion, although the increase was lower than dose proportional in pups (i.e., with a 50× increase in CPFO dose (0.01 to 0.5 mg/kg/day), TCP levels increased only 20×) vs. approximately dose proportional in adults (i.e., with a

Table 8
ChE inhibition in PND 21 pups and adult (~PND 80) females following repeated doses of CPFO in corn oil.

Dose (mg/kg/day)	RBC ChE (U/L)	RBC ChE (% control)	Brain ChE (U/L)	Brain ChE (% control)	Plasma ChE (U/L)	Plasma ChE (% control)
Adults – CPFO in corn oil						
0	4903 ± 276	100.0	51,978 ± 1803	100.0	2254 ± 744	100.0
0.01	5227 ± 1065	106.6	51,480 ± 1954	99.0	2814 ± 1122	124.8
0.5	**619 ± 228**[*]	12.6	51,161 ± 2204	98.4	**541 ± 160**[*]	24.0
PND 21 male pups – CPFO in corn oil						
0	6410 ± 1605	100.0	43,265 ± 1500	100.0	905 ± 111	100.0
0.01	5849 ± 1153	91.2	43,429 ± 1395	100.4	933 ± 136	103.9
0.5	**1013 ± 537**[*,a]	15.8	43,003 ± 1747[a]	99.4	**344 ± 51**[*,a]	38.3
PND 21 female pups – CPFO in corn oil						
0	5951 ± 2082	100.0	42,289 ± 1616	100.0	928 ± 122	100.0
0.01	5163 ± 1199	86.8	44,273 ± 1639	104.7	903 ± 95	97.2
0.5	**813 ± 304**[*]	13.7	38,762 ± 12,286	91.7	**362 ± 34**[*]	39.0

$n = 8$ pups/sex/dose level or 8 adult females/dose level unless otherwise indicated.
U/L, international units/L.
[a] $n = 7$ male pups in the 0.5 mg/kg/day dose group
[*] Significantly different from controls at alpha = 0.05 using Dunnett's test when raw ChE data were analyzed (treatment-related effects in bold text).

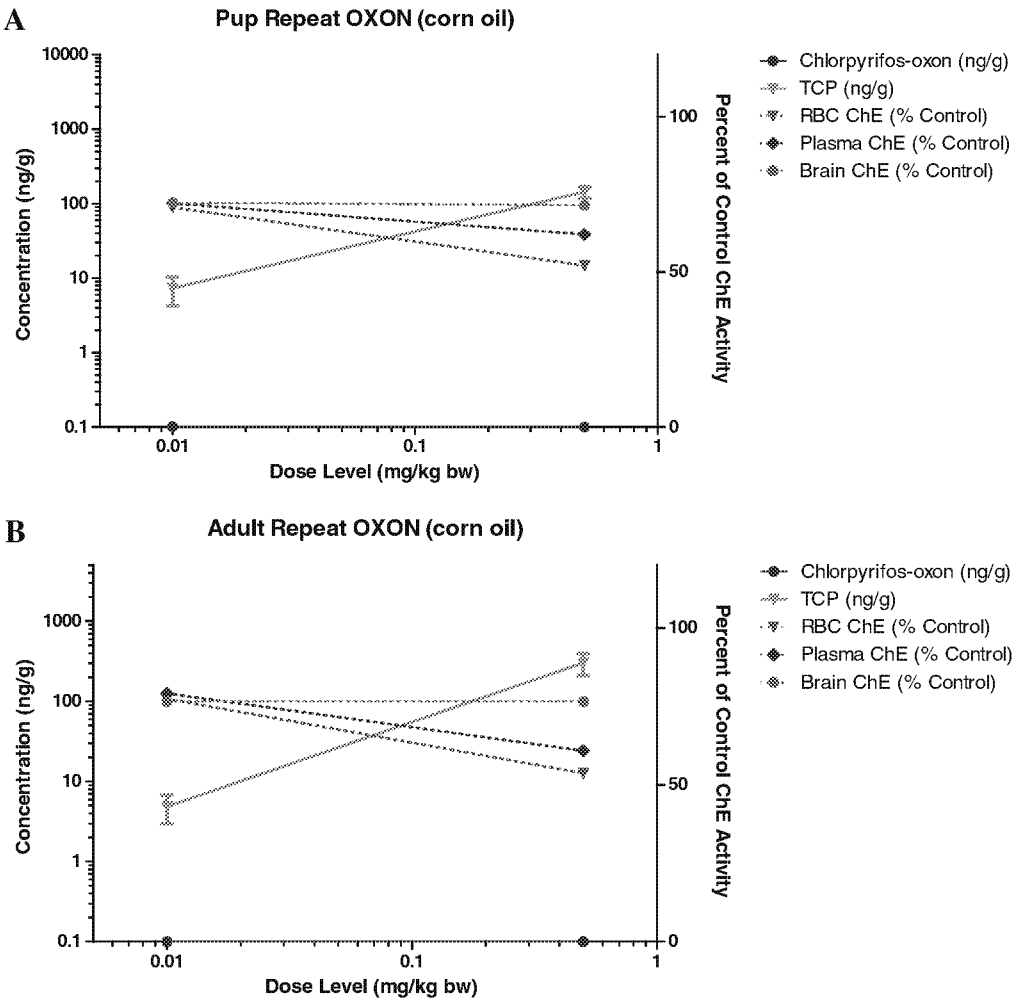

Fig. 4. Mean RBC, brain and plasma ChE inhibition relative to mean blood CPFO and TCP levels in PND 21 pups (A; sampled 4 h after the last dose) or adult female rats (B; sampled 4 h after the last dose) following daily gavage dosing with CPFO in corn oil vehicle at two dose levels for 11 days. Data show mean values of combined male and female pup data as there were no gender-related differences in either ChE inhibition or blood CPFO levels. ($n = 7$–8 pups/sex/dose level or 8 adult females/dose level for ChE measurements; $n = 4$ pups or 4 adults in each dose group for determination of blood CPFO and TCP levels except for 3 pups in the control group).

50× increase in CPFO dose, TCP levels increased 62×). Thus, adults had higher levels of TCP than pups at 0.5 mg/kg/day CPFO, whereas pups had 1.5× TCP blood levels compared with adults at the lower dose level of CPFO.

In conclusion, brain ChE was not altered at either dose of CPFO; thus, the 0.01 mg/kg/day CPFO dose was considered a no-observed-effect-level (NOEL) for ChE inhibition across all tissues in both age groups with repeated exposure.

4. Discussion

This comparative cholinesterase study was designed to examine whether there were age-related differences in sensitivity to ChE inhibition following CPF exposure. The effects of acute CPF exposure in PND 11 pups compared with adults showed that there were dose-dependent differences in sensitivity at high doses (i.e., 5 mg/kg in corn oil), where pups were more sensitive than adults. At lower dose levels, pups and adults showed significant plasma and RBC or brain ChE inhibition at the same dose levels. When using rat milk as an alternate vehicle to simulate lactational exposures, ChE inhibition in PND 11 pups was similar to levels achieved when administering CPF in corn oil. In adults, acute CPF exposure by gavage (corn oil vehicle) or using a alternate dosing scenario (i.e., 12-h dietary exposure in adult females) showed that dose rate affects the relative magnitude of ChE inhibition across tissues, because ChE inhibition was greater in RBC, but less in brain, with dietary exposures. With 11 daily exposures to CPF (corn oil vehicle), both pups and adults showed similar sensitivity to ChE inhibition across tissues. Brain ChE was not inhibited in either adults or pups at any dose of CPFO tested, despite similar sensitivity at ages to CPFO inhibition of RBC and plasma ChE. Together, these data indicate that young animals are not more sensitive than adults to CPF- or CPFO-induced ChE inhibition across the lower portion of the dose–response curves.

PND 11 pups were selected as the comparison group for adult females in this study based on EPA guidance for comparative cholinesterase studies. Across species, brain development follows predetermined developmental patterns; however, the timing of these developmental stages relative to birth varies. Consequently, the degree of functional maturity of the nervous system also varies at birth. There is ample data to support the concept that compared to humans, rats are altricial (i.e., born less mature) with respect to neurodevelopment. Using morphometric measurements and neurogenesis in different brain regions, Bayer et al. (1993) concluded that the full-term human brain at birth was approximately equal to a PND 14–21 rat brain. Similar conclusions also have been expressed by others (Vidair, 2004; Clancy et al., 2007). Data suggest that the blood–brain barrier (BBB) in humans is more developed at birth than the BBB in neonatal rats (Adinolfi and Haddad, 1977; Bonati et al., 1981). Thus, PND 11 rat pups represent an appropriately conservative model to examine potential effects in human infants.

When examining control animals from the acute phase of the comparative ChE study, adults had greater brain and plasma ChE activity, whereas pups had greater RBC ChE activity. In the repeat-dose study, when pups were euthanized on PND 21, brain ChE activity in the control group was approximately 80% higher than levels in PND 11 control pups. This was consistent with previous reports (Moser et al., 1998; Timchalk et al., 2006) that have demonstrated increases in brain ChE as rats mature. There was no apparent maturational pattern for RBC and plasma ChE activity, which were approximately similar at both PND 11 and 21. Timchalk et al. (2006) reported that RBC ChE enzyme activity increased between PND 5 and 12, then decreased slightly on PND 17, whereas plasma ChE activity was relatively stable across ages.

With acute exposures in corn oil, the relative sensitivity of adults compared with pups was dose dependent. At high dose levels, PND 11 pups were more sensitive to CPF-induced ChE inhibition because 5 mg/kg CPF induced similar levels of plasma, RBC and brain ChE inhibition as 10 mg/kg in adults. However, at lower doses of CPF, the dose–response curves for adults and immature rats intersected (see Figure S-21; Supplemental data 1), such that 2 mg/kg did not cause significant brain ChE inhibition in either adults or pups, but caused significant RBC and plasma ChE in both age groups. As reported previously (US EPA, 2011), significant RBC and plasma ChE inhibition occurred at lower dose levels than brain ChE inhibition in both PND 11 pups and adults. The NOEL for ChE inhibition across all tissues (0.5 mg/kg) was the same for both adults and pups. There was no significant difference in sensitivity to ChE inhibition between male and female PND 11 pups, consistent with other reports in preweanling animals (e.g., Moser and Padilla, 1998; Moser et al., 1998). Thus, at lower doses, adult female rats and PND 11 rat pups exhibited similar sensitivity across all tissues, although pups had higher blood levels of CPF at all dose levels.

The enhanced sensitivity of pups to acute CPF exposure at higher dose levels has been reported previously (e.g., Moser et al., 1998 at doses >5 mg/kg) and was partially attributed to the lower metabolic capacity in younger animals (Timchalk et al., 2006). There is evidence that pups metabolize high doses of CPF more slowly than adults. When examining [TCP]/[CPF] ratios across studies, which is an indicator of metabolic capacity of an animal, it appears that this ratio is ~50 in PND 5 pups at 6 h after dosing 1 mg/kg CPF in corn oil (Marty et al., 2007), 102–170 in PND 11 pups at 6 h after dosing with 0.5–2 mg/kg CPF in corn oil and ~449–810 in adult females at 8 h after dosing 0.5–2 mg/kg CPF in corn oil. These data show greater metabolic capacity was present in adults; however, TCP formation was favored in the older age groups as blood TCP concentrations exceeded parent CPF by a factor of 100-fold or greater by PND 11, consistent with the findings of Timchalk et al. (2006).

The production of CPFO depends on the rate of hepatic activation of CPF and inactivation of CPFO by cytochrome P450 monooxygenases (Ma and Chambers, 1994; Sultatos, 1994; Sultatos et al., 1984). CPF is extensively metabolized into water soluble metabolites, which prevents the accumulation of CPF or its metabolites (US EPA, 2011). Other pathways involved in CPFO inactivation include interactions with esterases other than AChE (e.g., butyrylcholinesterases, which is hypothesized to scavenge CPFO to prevents its interaction with peripheral target sites; Maxwell 1992a,b; US EPA, 2011) or binding to B-esterases (e.g., carboxylesterases), both of which decrease the amount of CPFO available to interact with the target site (AChE). In addition, hydrolysis of the oxon by A-esterases (i.e., PON-1; CPF-oxonase; Behnke and Murphy, 1975; Costa et al., 1990) leads to the formation of TCP and diethylphosphate, which do not inhibit AChE. Data indicate that these detoxification pathways continue to mature postnatally in rats and the maturation of these systems parallels decreases in sensitivity to high-dose, acute CPF exposure (Mortensen et al., 1996; Atterberry et al., 1997; Maxwell, 1992a,b; Chand et al., 1997; Morgan et al., 1994). Moser et al. (1998) showed that preweanling rats have lower levels of both liver and plasma carboxylesterases and A-esterase activity than adults, which correlates with the gradual decrease in sensitivity as rats mature. However, in humans, available data indicate that liver carboxylesterase activity does not differ between infants and adults as activity appears to change relatively little during postnatal maturation (Pope et al., 2005). Furthermore, Smith et al. (2011) found no age-related differences in CPF metabolism *in vitro* using hepatic microsomes isolated from humans at 13 days to 75 years old, whereas age-dependent increases in CPFO esterase metabolism in human plasma (3 days to 46 years) were reported.

Levels of ChE inhibition following acute ChE exposure in the current study were generally consistent with previously published studies in immature animals, although this study included multiple dose levels at the lower portion of the dose response curve (i.e., <1 mg/kg). In the study by Timchalk et al. (2006), RBC and plasma inhibition were seen in PND 5 and 12 pups at 1 mg/kg

CPF in corn oil. This is consistent with the current study, where inhibition was seen in these tissues at 2 mg/kg, but was not seen at 0.5 mg/kg in PND 11 pups. Zheng et al. (2000) reported a decrease in plasma and RBC ChE at doses of 0.45 and 1.5 mg/kg CPF, respectively, in PND 7 pups. The reason for this difference in plasma ChE inhibition may be related to differences in study designs, sampling times, or ages of the pups from which ChE activity was measured. In the current study, there were no effects on brain ChE activity at 2 mg/kg on PND 11, which was consistent with Timchalk et al. (2006), who reported no effects on brain ChE activity at 1 mg/kg in PND 12 pups. Overall, when considering dose and pup age, the levels of ChE inhibition across tissues in this study were consistent with the existing scientific literature.

The variability in ChE measurements in the current study were consistent with variability reported in other studies, giving the current study a similar level of sensitivity to previous work. Coefficients of variation (CVs) for control ChE values appear in Supplemental data 3. In the acute dose–response studies ($n = 8$/sex/dose for pups or $n = 8$/dose for adult females), RBC CVs ranged from 6.5% to 23.2%, brain CVs ranged from 2.3% to 18.1% and plasma CVs ranged from 7.5% to 37.5%. In the repeat dose studies ($n = 8$/sex/dose for pups or $n = 8$/dose for adult females), RBC CVs ranged from 5.6% to 35.0%, brain CVs ranged from 3.5% to 3.8% and plasma CVs ranged from 12.3% to 33.0%. These results were consistent with expectations as brain ChE activity was the least variable of the tissues measured, whereas there was more variability in plasma ChE, which contains mixed activity (i.e., butyryl- and acetyl-cholinesterase). A CV comparison for treated animals was not included as variance was expected to be higher in treated animals due to inter-animal differences, including differences in absorption, distribution, metabolism, and excretion, individual differences in response to treatment (particularly in steeper portions of the dose–response curve), slight differences in dose delivered, etc. The similarity in CVs to other published studies shows that these assays were reasonably sensitive to detect changes in ChE activity when such changes were present.

When using rat milk as an alternative vehicle in PND 11 pups to simulate lactational exposures, ChE inhibition was similar to levels achieved when administering CPF in corn oil. At the time-of-peak inhibition, blood CPF and CPFO levels, as well as the magnitude of ChE inhibition across tissues, were similar with both milk and corn oil vehicles (Fig. 1A and B). This was unexpected as kinetic data for blood CPF and blood TCP in PND 5 pups (1 mg/kg CPF in corn oil or in milk) showed a similar time to maximal concentration (C_{max}) for both oil and milk vehicles with a notable increase in blood CPF C_{max} in pups dosed with corn oil (Marty et al., 2007). Based on the established PBPK/PD model for CPF in immature rat pups (Timchalk et al., 2002, 2006), the peak for pup blood levels of CPF after administration in rat milk was 5–7 h. Given the slow recovery of ChE activity, the ChE inhibition was comparable even with different time points examined (6 vs. 8 h post-dosing). In their recent assessment, the US EPA determined that RBC ChE inhibition in PND 11 pups exposed acutely to CPF in milk had the lowest oral point of departure in the CPF database (US EPA, 2011).

When using an acute 12-h dietary exposure in adults to simulate CPF exposures in the diet over a day, dose rate apparently impacted the relative magnitude of tissue ChE inhibition. The slower dose rate likely allowed more time for detoxification pathways, such that less CPF was available to interact with brain ChE. Data from these studies have shown that CPFO at <10 mg/kg did not inhibit brain ChE activity in adult females. Therefore, it is possible that the slower dose rate allowed greater opportunity for P450 metabolism and/or interaction of CPFO with carboxylesterases or other ChEs (e.g., butyrylcholinesterase) so that less CPFO was available to interact with brain ChE. Timchalk et al. (2006) reported that differences in tissue dosimetry (higher oxon AUC in blood relative to brain) contribute to enhanced sensitivity of blood relative to brain ChE. This seems plausible as RBC ChE showed greater inhibition with dietary CPF dosing, although plasma ChE inhibition was the same with both gavage and dietary treatment.

In the current study, there were no signs of cholinergic toxicity detected in either the acute study (clinical observations at ⩽5 mg/kg in pups or ⩽10 mg/kg in adults) or the repeat-dose study (clinical observations and FOB with motor activity at ⩽3.5 mg/kg/day in both age groups), despite significant brain ChE inhibition (~53–58% in the acute study and 59–69% in the repeat dose study). Moser (2000) reported that PND 17 female rats had a decrease in tail-pinch response at 6.5 h post-dosing with 4 mg/kg CPF, whereas males were not affected at this dose level. At a higher CPF dose (i.e.,10 mg/kg) than those given to pups in the current study, Moser (2000) observed alterations in numerous neurobehavioral endpoints in both male and female PND 17 pups including altered gait/ataxia, decreased arousal state, tail-pinch response (males), tremors, smacking (males) and lacrimation (females). In adults, decreased motor activity (total counts) was the most sensitive endpoint with decreases in males noted at 3.5 h post-dosing with 10 mg/kg, whereas both males and females were affected at 50 mg/kg, along with other cholinergic signs of toxicity (Moser, 2000). In a separate study (Moser et al., 1998), PND 17 female rats with a 50–60% decrease in brain ChE activity following acute exposure to 5 mg/kg CPF showed decreased open-field arousal, and in adult males, there was a correlation between a 40–50% decrease in brain ChE activity and decreased motor activity at 20 mg/kg CPF. Numerous factors may have contributed to differences in neurobehavioral effects in the previous studies by Moser et al. and the current study, including differences in dose levels (generally lower in the present study), developmental stage, neurobehavioral methods, or possibly receptor down regulation, which has been proposed to account for recovery of neurobehavioral performance (Bignami et al., 1975; Nostrandt et al., 1997; Moser and Padilla, 1998). Perhaps the gender-related differences in sensitivity to neurobehavioral effects in the Moser studies, in the presence of similar brain ChE inhibition in both sexes, indicate that these effects occurred near the threshold for neurobehavioral alterations, which could vary slightly across studies.

With repeated exposures, adults and PND 11 pups were similar in sensitivity to ChE inhibition by CPF. Significant brain ChE inhibition was seen in both adults and pups at 1.0 mg/kg/day CPF, whereas significant plasma and RBC inhibition occurred in both age groups at 0.5 mg/kg/day. In 2008, the US EPA Science Advisory Panel (2008) hypothesized that young animals might be less sensitive to repeated CPF exposure due to decreased levels of enzymes converting CPF to CPFO and/or a more rapid increase in AChE activity in tissues of young animals, likely due to increased rates of protein synthesis (e.g., Chakraborti et al., 1993; Liu et al., 1999). The current study verified that pups achieve higher blood levels of CPF for a given dose, presumably due to slower metabolism to TCP. Based on administered dose, these immature animals showed similar sensitivity to CPF-induced ChE inhibition as adults; however, based on blood levels, pups showed lower sensitivity to CPF-induced ChE inhibition. A recent physiologically based pharmacokinetic/pharmacodynamic (PBPK/PD) model using human CYP-specific kinetic parameters and age-based differences in hepatic CYP content predicted that 1 year-olds would be less sensitive than 19-year olds to CPF-induced butyryl- and acetyl-ChE inhibition, although age-related differences in PON-1 levels and microsomal liver content are needed to refine the model (Foxenberg et al., 2011).

In adult females, there was significant inhibition of RBC ChE activity at 0.1 mg/kg/day in the repeat-dose study, which was deemed incidental as plasma ChE was not significantly altered at this dose level. Plasma ChE has similar or greater sensitivity than

RBC to inhibition by CPF (Lotti, 1995; Garabrant et al., 2009; US EPA, 2008). Plasma ChE, which is comprised of half butyrylcholinesterase and half AChE (Timchalk et al., 2006), is more readily inhibited because butyrylcholinesterase is more sensitive to inhibition by CPFO than AChE (Amitai et al., 1998; Kousba et al., 2003; Timchalk et al., 2002). Across the current data sets, there were a few occurrences when samples collected at the same time showed RBC ChE inhibition that exceeded plasma ChE inhibition; however, this was in a minority of cases and when it occurred, RBC and plasma ChE samples were similar. Thus, the significant inhibition of RBC ChE in adult females at 0.1 mg/kg/day, which occurred in the absence of significant plasma ChE inhibition, was deemed spurious.

Overall, the dose–response for ChE inhibition with repeated CPF exposure in the current study was consistent with three previous studies examining CPF-induced ChE inhibition in rats. In a 90-day repeat-dose dietary CPF study with F344/DuCrl rats, Szabo et al. (1988) reported a significant decrease in RBC and brain cholinesterase activities at $\geqslant 1$ and $\geqslant 5$ mg/kg/day, respectively. In the adult 28-day dietary immunotoxicity study in CD rats (Boverhof, personal communication), significant RBC and brain cholinesterase inhibition were seen at similar dose levels ($\geqslant 0.4$ and $\geqslant 2$ mg/kg/day CPF, respectively) to the current study. RBC ChE inhibition at 0.4 mg/kg/day CPF was somewhat greater in the immunotoxicity study (53.7% of control compared with 80.5% at 0.5 mg/kg/day in the current study), which may have been related to the extended dosing period in the immunotoxicity study (11 days in the current study vs. 28 days in the immunotoxicity study) or the difference in exposure routes (oral gavage in corn oil in the current study vs. dietary in the immunotoxicity study). In a study by Carr and Nail (2008), ChE inhibition was measured in multiple areas of the brain in rat pups dosed daily by gavage from PND 10–16 with 5 mg/kg/day CPF in corn oil. Brain ChE inhibition ranged from 54% (cerebellum and medulla) to 64% (forebrain) with this dosing paradigm compared with whole brain ChE inhibition that ranged from 59% to 68% at 3.5 mg/kg/day from PND 11–21 in the current study. These results show comparable brain ChE inhibition despite slight differences in dose and exposure duration.

With CPFO exposure, brain ChE was not inhibited in either adults or pups at any dose level tested, despite similar sensitivity at both ages to CPFO inhibition of RBC and plasma ChE. These data indicate a lack of systemic bioavailability of CPFO to peripheral tissues (Bartels et al., 2011) and suggest that exposure to CPFO is less toxic to brain ChE than exposure to parent CPF. In preliminary studies, doses $\leqslant 10$ mg/kg CPFO did not inhibit brain ChE activity in adult female rats (Table 2). This finding differs from Betancourt and Carr (2004) who reported ~50–60% decreases in brain ChE in newborn rats (PND 1–6) exposed daily to 0.25 or 0.35 mg/kg/day CPFO via oral gavage. These results may differ from the current study because the pups were younger at the time of exposure; therefore, an incomplete blood–brain barrier and/or slower detoxification pathways for CPFO may have contributed to brain ChE inhibition. However, this PND 1–10 age range has generally been considered to be physiologically more consistent to human fetuses *in utero* (US EPA, 2011) and therefore, would not be relevant for evaluation of neonatal human exposures.

In conclusion, both the acute and repeated-dose data indicate that young animals are not more sensitive than adults to CPF or CPFO over the lower portion of the dose response curves. This conclusion has been confirmed subsequently using Benchmark Dose Modeling (Reiss et al., 2012). Thus, with low-level, environmentally relevant exposures, higher sensitivity of young animals to ChE inhibition would be unlikely. Furthermore, there is no indication of altered brain ChE activity in either pups or adults following 11 daily exposures to <0.5 mg/kg/day CPFO, the proximate toxicant with CPF exposure. These data suggest that there should be little, if any, concern for CPFO-mediated brain ChE inhibition at environmentally relevant exposure levels.

Conflict of interest

The authors of this article, with the exception of M.J. Beck, are employed by The Dow Chemical Company or Dow AgroSciences, LLC, which produce chlorpyrifos and funded this study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.yrtph.2012.03.015.

References

Adinolfi, M., Haddad, S.A., 1977. Levels of plasma proteins in human and rat fetal CSF and the development of the blood–CSF barrier. Neuropadiatrie 8, 345–353.

Amitai, G., Moorad, D., Adani, R., Doctor, B.P., 1998. Inhibition of acetylcholinesterase and butyrylcholinesterase by chlorpyrifos–oxon. Biochem. Pharmacol. 56, 293–299.

Atterberry, T.T., Burnett, W.T., Chambers, J.E., 1997. Age-related differences in parathion and chlorpyrifos toxicity in male rats: target and nontarget esterase sensitivity and cytochrome P450-mediated metabolism. Toxicol. Appl. Pharmacol. 147, 411–418.

Bartels, M., Marty, M.S., Hotchkiss, J.A., Juberg, D.R., 2011. Imparct of non-linear pharmacokinetics and metabolism of chlorpyrifos on biological response in the rat. Abstract # 2279. 2011 Itinerary Planner. Society of Toxicology, Washington, DC.

Bayer, S.A., Altman, J., Russo, R.J., Zhang, X., 1993. Timetables of neurogenesis in the human brain based on experimentally determined patterns in the rat. Neurotoxicology 14, 83–144.

Behnke, G.M., Murphy, S.D., 1975. The influence of age on the toxicity and metabolism of methyl parathion and parathion in male and female rats. Toxicol. Appl. Pharmacol. 31, 254–269.

Betancourt, A.M., Carr, R.L., 2004. The effect of chlorpyrifos and chlorpyrifos–oxon on brain cholinesterase, muscarinic receptor binding, and neurotrophin levels in rats following early postnatal exposure. *Toxicol.* Sci. 77, 63–71.

Bignami, G., Rosic, N., Michalek, H., Milosevic, M., Gatti, G.L., 1975. Behavioral toxicity of anticholinesterase agents: methodological, neurochemical, and neuropsychological aspects. In: Weiss, B., Laties, V.G. (Eds.), Behavioral Toxicology. Plenum, New York, pp. 155–216.

Bonati, M., Latini, R., Marra, G., Assael, B.M., Parini, R., 1981. Theophylline distribution in the premature neonate. Dev. Pharmacol. Ther. 3, 65–73.

Brzak, K.A., Harms, D.W., Bartels, M.J., Nolan, R.J., 1998. Determination of chlorpyrifos, chlorpyrifos oxon, and 3,5,6-trichloro-2-pyridinol in rat and human blood. J. Anal. Toxicol. 22, 203–210.

Carr, R.L., Nail, C.A., 2008. Effect of different administration paradigms on cholinesterase inhibition following repeated chlorpyrifos exposures in late prewenling rats. Toxicol. Sci. 106, 186–192.

Chakraborti, T.K., Farrar, J.D., Pope, C.N., 1993. Comparative neurochemical and neurobehavioral effects of repeated chlorpyrifos exposures in young and adult rats. Pharmacol. Biochem. Behav. 46, 219–224.

Chand, S.M., Mortensen, S.R., Moser, V.C., Padilla, S., 1997. Tissue-specific effects of chlorpyrifos on carboxylesterase and cholinesterase activity in adult rats: an *in vitro* and *in vivo* comparison. Fundam. Appl. Toxicol. 38, 148–157.

Clancy, B., Kersh, B., Hyde, J., Darlington, R.B., Anand, K.J.S., Finlay, B.L., 2007. Web-based method for translating neurodevelopment from laboratory species to humans. Neuroinformatics 5, 79–94.

Costa, L.G., McDonald, B.E., Murphy, S.D., Omenn, G.S., Richter, R.J., Motulsky, S.G., Furlong, C.E., 1990. Serum paraoxonase and its influence on paraoxon and chlorpyrifos–oxon toxicity in rats. Toxicol. Appl. Pharmacol. 103, 66–76.

Ellman, G.L., Courtney, K.D., Andres Jr., V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.

Foxenberg, R.J., Ellison, C.A., Knaak, J.B., Ma, C., Olson, J.R., 2011. Cytochrome P450-specific human PBPK/PD models for the organophosphorus pesticides: chlorpyrifos and parathion. Toxicology 285, 57–66.

Garabrant, D.H., Aylward, L.L., Betent, S., Chen, Q., Timchalk, C., Burns, C.J., Hays, S.M., Albers, J.W., 2009. Cholinesterase inhibition in chlorpyrifos workers: characterization of biomarkers of exposure and response in relation to urinary TCPγ. J. Expo. Sci. Environ. Epidemiol. 19, 634–642.

Hunter, P.L., Marshal, R.S., Padilla, S., 1997. Automated instrument analysis of cholinesterase activity in tissues from carbamate-treated animals: a cautionary note. Toxicol. Meth. 7, 43–53.

Kousba, A.A., Poet, T.S., Timchalk, C., 2003. Characterization of the *in vitro* kinetic interaction of chlorpyrifos–oxon with rat salivary cholinesterase: a potential biomonitoring matrix. Toxicology 188, 219–232.

Liu, J., Olivier, K., Pope, C., 1999. Comparative neurochemical effects of repeated methyl parathion or chlorpyrifos exposures in neonatal and adult rats. Toxicol. Appl. Pharmacol. 158, 186–196.

Lotti, M., 1995. Cholinesterase inhibition: complexities of interpretation. Clin. Chem. 41 (12 part 2), 1814–1818.

Ma, T., Chambers, J.E., 1994. Kinetic parameters of desulfuration and dearylation of parathion and chlorpyrifos by rat liver microsomes. Food Chem. Toxicol. 32, 763–767.

Marty, M.S., Domoradzki, J.Y., Hansen, S.C., Timchalk, C., Bartels, M.J., Mattsson, J.L., 2007. The effect of route, vehicle, and divided doses on the pharmacokinetics of chlorpyrifos and its metabolite trichlorpyridinol in neonatal Sprague–Dawley rats. Toxicol. Sci. 100, 360–373.

Mattsson, J.L., Johnson, K.A., Albee, R.R., 1986. Lack of neuropathologic consequences of repeated dermal exposure to 2,4-dichlorophenoxyacetic acid in rats. Fund. Appl. Toxicol. 6, 175–181.

Mattsson, J.L., Charles, J.M., Yano, B.L., Cunny, H.C., Wilson, R.D., Bus, J.S., 1997. Single-dose and chronic dietary neurotoxicity screening studies on 2,4-dichlrophenoxyacetic acid in rats. Fundam. Appl. Toxicol. 40, 111–119.

Mattsson, J.L., Maurissen, J.P., Nolan, R.J., Brzak, K.A., 2000. Lack of differential sensitivity to chloinesterase inhibition in fetuses and neonates compared to dams treated perinatally with chlorpyrifos. Toxicol. Sci. 53, 438–446.

Maxwell, D.M., 1992a. The specificity of carboxylesterase protection against the toxicity of organophosphorus compounds. Toxicol. Appl. Pharmacol. 114, 306–312.

Maxwell, D.M., 1992b. Detoxification of organophosphorus compounds by carboxylesterases. In: Chambers, J.E., Levi, P.E. (Eds.), Organophosphates Chemistry, Fate and Effects. Academic Press, New York, pp. 183–199.

Morgan, E.W., Yan, B., Greenway, D., Parkinson, A., 1994. Regulation of two rat liver microsomal carboxylesterase isozymes: species differences, tissue distribution, and the effects of age, sex, and xenobiotic treatment of rats. Arch. Biochem. Biophys. 315, 513–526.

Mortensen, S.R., Chanda, S.M., Hooper, M.J., Padilla, S., 1996. Maturational differences in chlorpyrifos–oxonase activity may contribute to age-related sensitivity to chlorpyrifos. J. Biochem. Toxicol. 11, 279–287.

Moser, V.C., 1995. Comparisons of the acute effects of cholinesterase inhibitors using a neurobehavioral screening battery in rats. Neurotox. Teratol. 17, 617–625.

Moser, V.C., Padilla, S., 1998. Age- and gender-related differences in the time course of behavioral and biochemical effects produced by oral chlorpyrifos in rats. Toxicol. Appl. Pharmacol. 149, 107–119.

Moser, V.C., Chanda, S.M., Mortensen, S.R., Padilla, S., 1998. Age- and gender-related differences in sensitivity to chlorpyrifos in the rat reflect developmental profiles of esterase activities. Toxicol. Sci. 46, 211–222.

Moser, V.C., 2000. Dose–response and time-course of neurobehavioral changes following oral chlorpyrifos in rats of different ages. Neurotoxicol. Teratol. 22, 713–723.

Nostrandt, A.C., Padilla, S., Moser, V.C., 1997. The relationship of oral chlorpyrifos effects on behavior, cholinesterase inhibition, and muscarinic receptor density in rat. Pharmacol. Biochem. Behav. 58, 15–23.

Pope, C.N., Chakraborti, T.K., 1992. Dose-related inhibition of brain and plasma cholinesterase in neonatal and adult rats following sublethal organophosphate exposures. Toxicology 73, 35–43.

Pope, C.N., Chakraborti, T.K., Chapman, M.L., Farrar, J.D., Arthun, D., 1991. Comparison of in vivo cholinesterase inhibition in neonatal and adult rats by three organophosphorothioate insecticides. Toxicology 68, 51–61.

Pope, C.N., Karanth, S., Liu, J., Yan, B., 2005. Comparative carboxylesterase activities in infant and adult liver and their sensitivity to chlorpyrifos oxon. Regul. Toxicol. Pharmacol. 42, 64–69.

Reiss, R., Neal, B., Lamb, J., 2012. Acetylcholinesterase Inhibition Dose–Response Modeling for Chlorpyrifos and Chlorpyrifos–oxon. Regul. Toxicol. Pharmacol. 63, 124–131.

Smith, J.N., Timchalk, C., Bartels, M.J., Poet, T.S., 2011. In vitro age-dependent enzymatic metabolism of chlorpyrifos and chlorpyrifos–oxon in human hepatic microsomes and chlorpyrifos–oxon in plasma. Drug Metab. Dispos. 39, 1353–1362.

Sultatos, L.G., 1994. Mammalian toxicology of organophosphorus pesticides. J. Toxicol. Environ. Health 43, 271–289.

Sultatos, L.G., Shao, M., Murphy, S.D., 1984. The role of hepatic biotransformation in mediating the acute toxicity of the phosphorothionate insecticide chlorpyrifos. Toxicol. Appl. Pharmacol. 73, 60–68.

Szabo, J.R., Young, J.T., Grandjean, M., 1988. Chlorpyrifos: 13-week dietary study in Fischer 344 rats. Summarized in: chlorpyrifos (Dursban®, Lorsban®) dietary exposure assessment. Health Assessment Section, Medical Toxicology Branch. Department of Pesticide Regulation. California Environmental Protection Agency. May 8, 1992.

Timchalk, C., Nolan, R.J., Mendrala, A.L., Dittenber, D.A., Brzak, K.A., Mattsson, J.L., 2002. A physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model for the organophosphate insecticide chlorpyrifos in rats and humans. Toxicol. Sci. 66, 34–53.

Timchalk, C., Poet, T.S., Kousba, A.A., 2006. Age-dependent pharmacokinetic and pharmacodynamic response in preweanling rats following oral exposure to the organophosphate insecticide chlorpyrifos. Toxicology 220, 13–25.

US EPA, 2008. United States Environmental Protection Agency. Chlorpyrifos Reassessment. Appendix B – Mode of Action: Inhibition of Acetylcholinesterase (AChE). Health Effects Division, Office of Pesticide Programs, August 27, 2008.

US EPA, 2011. United States Environmental Protection Agency. Chlorpyrifos preliminary human health risk assessment. DP No. D388070. Office of Chemical Safety and Polution Prevention. June 30, 2011. Available from: <http://www.epa.gov/oppsrrd1/registration_review/chlorpyrifos/EPA-HQ-OPP-2008-0850-DRAFT-0024%5B1%5D.pdf> (accessed on 12.02.12).

US EPA Scientific Advisory Panel, 2008. A set of scientific issues being considered by the Environmental Protection Agency regarding: the Agency's evaluation of the toxicity profile of chlorpyrifos. Arlington, VA; September 16–18, 2008, SAP minutes no. 2008-04. Available from: <http://www.epa.gov/hsrb/files/1e2-sap-meeting-minutes-121708.pdf> (accessed on 12.02.2012).

Vidair, C.A., 2004. Age dependence of organophosphate and carbamate neurotoxicity in the postnatal rat: extrapolation to the human. Toxicol. Appl. Pharmacol. 196, 287–302.

Zheng, Q., Olivier, K., Won, Y.K., Pope, C.N., 2000. Comparative cholinergic neurotoxicity of oral chlorpyrifos exposures in preweanling and adult rats. Toxicol. Sci. 55, 124–132.