EVIDENCE ON THE DEVELOPMENTAL AND REPRODUCTIVE TOXICITY OF

Chlorpyrifos

Draft

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AUTHORS AND REVIEWERS

The Office of Environmental Health Hazard Assessment's Reproductive and Cancer Hazard Assessment Branch was responsible for the preparation of this document.

Primary Authors

Poorni Iyer, DVM., Ph.D., D.A.B.T.
Staff Toxicologist
Farla Kaufman, Ph.D.
Staff Toxicologist
Reproductive and Cancer Hazard Assessment Branch

Contributing Author

K. Lily Wu, Ph.D.
Associate Toxicologist
Reproductive and Cancer Hazard Assessment Branch

Internal OEHHA Reviewers

George V. Alexeeff, Ph.D., D.A.B.T.
Deputy Director for Scientific Affairs

Lauren Zeise, Ph.D.
Chief, Reproductive and Cancer Hazard Assessment Branch

James M. Donald, Ph.D.
Chief, Reproductive Toxicology and Epidemiology Section
Reproductive and Cancer Hazard Assessment Branch

Technical Support

Heather Matwich
Reproductive and Cancer Hazard Assessment Branch

PREFACE

The Safe Drinking Water and Toxic Enforcement Act of 1986 (Proposition 65, California Health and Safety Code Section 25249.5 *et seq.*) requires that the Governor cause to be published a list of those chemicals "known to the state" to cause cancer or reproductive toxicity. The lead agency for implementing Proposition 65 is the Office of Environmental Health Hazard Assessment (OEHHA) of the California Environmental Protection Agency. This document by OEHHA addresses the reproductive toxicity of chlorpyrifos, and provides information on whether this compound should be identified as known to cause reproductive toxicity under Proposition 65

One of the mechanisms by which "a chemical is known to the state to cause cancer or reproductive toxicity [is] if in the opinion of the state's qualified experts the chemical has been clearly shown through scientifically valid testing according to generally accepted principles to cause cancer or reproductive toxicity" (Health and Safety Code Section 25249.8(b)). The "state's qualified experts" regarding findings of reproductive toxicity are identified as members of the Developmental and Reproductive Toxicant (DART) Identification Committee of OEHHA's Science Advisory Board (Title 27, California Code of Regulations, Section 25301) [1].

This draft document provides the DART Identification Committee with information relevant to the reproductive toxicity of chlorpyrifos. While this hazard identification document does not provide dose-response evaluation, exposure assessment, or determination of allowable or safe exposure levels, the document does provide information which may be useful in such appraisals.

A public meeting of the Committee will be held on November 20, 2008, in Sacramento, California. Following discussion and Committee deliberation, the Committee will determine whether chlorpyrifos "has been clearly shown through scientifically valid testing according to generally accepted principles" to cause reproductive toxicity.

[1] Formerly Title 22 California Code of Regulations, Section 12302

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A. Abstract

Chlorpyrifos (CPF) is one of the most widely used organophosphate (OP) pesticides in the United States and was one of the major insecticides used in residential settings until 2000, when nearly all residential uses were cancelled. It is used as an acaricide and miticide to control foliage and soil-borne insect pests on a variety of food and feed crops sites. Chlorpyrifos is bioactivated to chlorpyrifos oxon (CPO) in the liver through cytochrome P450-mediated desulfuration. Chlorpyrifos oxon can bind irreversibly with acetylcholinesterase (AChE) eliciting cholinergic hyperstimulation in the nervous system and in neuro-muscular junctions. The enzyme paraoxonase (PON1) plays an important role in the detoxification of CPF. Several polymorphisms of the PON1 gene have been shown to influence both the level of expression of the enzyme and its catalytic ability, thus determining the rates at which an individual will detoxify a specific pesticide Considerable variability has been seen in PON1 status across human populations, indicating varying sensitivity to CPF. In addition, PON1 levels have been shown to be low at birth, suggesting increased sensitivity of the young to exposure to CPF.

Epidemiologic studies examining developmental outcomes associated with CPF exposure included three prospective cohort studies, two conducted in New York City and one conducted in the Salinas Valley of California, as well as a study of agricultural exposure in India. Adverse outcomes reported in association with exposure to CPF included: lower birth weight; decreased birth length; decreased head circumference, associated with PON1 status; mental and motor delays; behavioral problems; as well as DNA damage. Study parameters differed and not all studies reported similar findings. Factors that differed across studies and which may account for some of the inconsistencies are discussed.

Several studies in laboratory animals have examined the potential developmental toxicity of gestational exposure to CPF. Some standard developmental toxicity studies have reported effects on survival, growth and maturation, although these findings are generally equivocal. In other studies that include endpoints not covered by standard developmental toxicity studies, neurochemical alterations after birth and resultant behavioral effects have been reported to occur after exposure to CPF during the gestational period. The underlying mechanism of neurotoxicity is not fully understood. Effects reported in a developmental neurotoxicity study include alterations in motor activity, auditory startle response, and brain structure (decreased measurements of the parietal cortex and hippocampal gyrus in the absence of significant brain weight deficits at 5 mg/kg-day). After exposure to 1 mg/kg-day, female offspring also exhibited significant dose- and treatment-related decreases in measurements of the parietal cortex at postnatal day (PND) 66, long after exposure to CPF had ended. This indicates an association of CPF with delayed alterations in brain development in offspring of exposed mothers who showed minimal plasma and erythrocyte cholinesterase inhibition. Oral exposure of dams to 3 mg/kg-day CPF was the lowest dosage that would result in significant (>10%) inhibition of brain ChE in the offspring on PND 1. In some studies, prenatal exposures to CPF that were nontoxic to the dam elicited deficits in cholinergic function in offspring that influence cognitive performance in adolescence and adulthood. In other studies, prenatal CPF exposure appears to elicit delayed-onset alterations, disrupting the program for the emergence of cholinergic activity.

Animal data both from guideline studies conducted to register the pesticide and from the peer-reviewed literature demonstrate effects in pups exposed during lactation to CPF in the milk. Reproductive effects included reduced pup weights and increased pup mortality in rats, at dose levels that induced parental toxicity as evidenced by inhibition of plasma, RBC and brain cholinesterase activities as well as histological lesions of the adrenal gland (vacuolation of cells of the zona fasciculata). Chlorpyrifos was detected in human breast milk in two studies, one of which determined, from the measured levels, that the intake of CPF in infants would be 0.041 mg/kg per day, which is 4.1 times greater than that recommended by the World Health Organization (WHO).

Male reproductive outcomes in humans were examined in a series of cross-sectional studies, five of which were based on the same sample population. Associations were reported between higher TCPY levels and increased DNA damage in sperm, lower reproductive hormone levels including testosterone and estradiol, higher TSH levels, and specific motion parameters in sperm.

No effects on the reproductive or fertility indices or on the histopathology of reproductive tissues were observed in animals at dose levels that resulted in significant cholinesterase inhibition. Severe testicular damage resulting in reduction in sperm count and fertility was noted in a study at higher dose levels.

B. Introduction

B.1. Chemical structure and main physical characteristics

Chlorpyrifos, O,O-diethyl-O-(3, 5, 6-trichloro-2-pyridinyl)-phosphorothioate (CAS Registry Number 2921-88-2; Molecular Weight 350.6, chemical formula $C_9H_{11}Cl_3NO_3PS$), is a broad spectrum organophosphate (OP) pesticide. Trade names of chlorpyrifos include Dursban®, Lorsban®, Empire 20®, Equity® and Whitmire PT270®. Technical chlorpyrifos (CPF) is a white crystalline solid with a melting point of 41.5–42.5ºC. Chlorpyrifos is stable in neutral and acidic aqueous solutions; however, stability decreases with increasing pH. Chlorpyrifos is practically insoluble in water, but is soluble in most organic solvents such as acetone, xylene and methylene chloride.

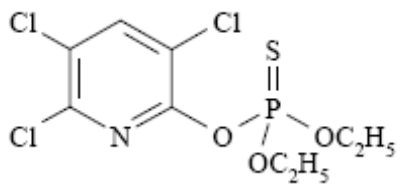


Figure B1. Chemical Structure of Chlorpyrifos

B.2. California use and exposure information

Chlorpyrifos was first introduced in 1965 and is one of the most widely used OP pesticides in the U.S. It is used as an acaricide and miticide to control foliage and soil-borne insect pests on a variety of food and feed crops sites such as cranberries, strawberries, citrus, apples, figs, pears, nectarines, cherries, peaches, plums, grapes, almonds, pecans, walnuts, nut trees, onions, peppers, kale, broccoli, brussel sprouts, cabbage, cauliflower, collards, cucurbits, asparagus, roots/tubers, corn, lentils, beans, peas, sorghum, tobacco, wheat, alfalfa, peanuts, soybeans, sunflower, cotton, sugar beets, mint, bananas, and pasture. It is also used in cattle ear tags, Christmas trees, and woodland. Other uses are on fire ant mounds, as a mosquito adulticide, on golf courses, shipholds, boxcars, industrial plants, and processed wood products.

Until 2000, CPF was commonly used as a residential pesticide for fire ants, cockroaches, and other household pests. The U.S. Environmental Protection Agency (EPA) cancelled most home, lawn and garden use products containing chlorpyrifos in mid-2000 based on human health risks. In addition, as of December, 2005, CPF products were no longer permitted for use in pre-construction termite control. Chlorpyrifos is still being widely used in agriculture. Data from the California Department of Pesticide Regulation's (CDPR) Pesticide Use Reports indicate a use of 1,919,625 lbs in 2006, the most recent year for which data are available (CDPR, 2007).

B.3. Pharmacokinetics and Metabolism

Absorption

Absorption of CPF varies with species. In humans, about 70% was absorbed after oral exposure of volunteers (Nolan et al., 1984). And for the metabolite, 3,5,6-trichloro-2-pyridinol (TCPY), the minimal dermal absorption was 1-3%. Additionally, it should be noted that chlorpyrifos is rapidly absorbed and transported to the brain with oral dosing (Mendrala and Brzak, 1998).

Distribution

The highest tissue concentrations were found in the liver and kidney, but chlorpyrifos did not bioconcentrate in tissue (Cochran et al., 1992). In a time course study, when pregnant rats were dosed with CPF at 7 mg/kg-day on GD 14–18, fetal brain TCPY concentration was twice as high as the maternal brain concentration, 5 hours after the last dose of CPF (Hunter et al., 1999).

Metabolism

The metabolic scheme for CPF is outlined in the figure below. Chlorpyrifos is bioactivated to CPO in the liver through cytochrome P450 mediated desulfuration. Chlorpyrifos oxon is subsequently hydrolyzed by A-esterase to diethylphosphate and 3,5,6-trichloro-2-pyridinol (TCPY), which is the major biological metabolite and environmental breakdown product of CPF (Figure B2). Chlorpyrifos oxon is the active metabolite of CPF, and undergoes further metabolism by both A- and B-esterase to form diethylphosphate (DEP) and TCPY, which can undergo phase II sulfation and glucuronidation (Timchalk et al., 2007). Chlorpyrifos oxon mediates the toxic effects of CPF by binding irreversibly with acetylcholinesterase (AChE) eliciting cholinergic hyperstimulation in the nervous system and in neuro-muscular junctions. A-esterase (e.g., chlorpyrifos oxonase and paraoxonase) and carboxylesterase are known to play an important role in the detoxification of CPF. Detoxification of CPF is not limited to carboxylesterase or A-esterase, bovine serum albumin has been shown to prohibit the effects of CPF on DNA synthesis in vitro (Qiao et al., 2001). In a study on human volunteers, the mean half-life ($t_{1/2}$) in the body was 26.9 hours (Nolan et al., 1984). The metabolic scheme for CPF is outlined in the figure below.

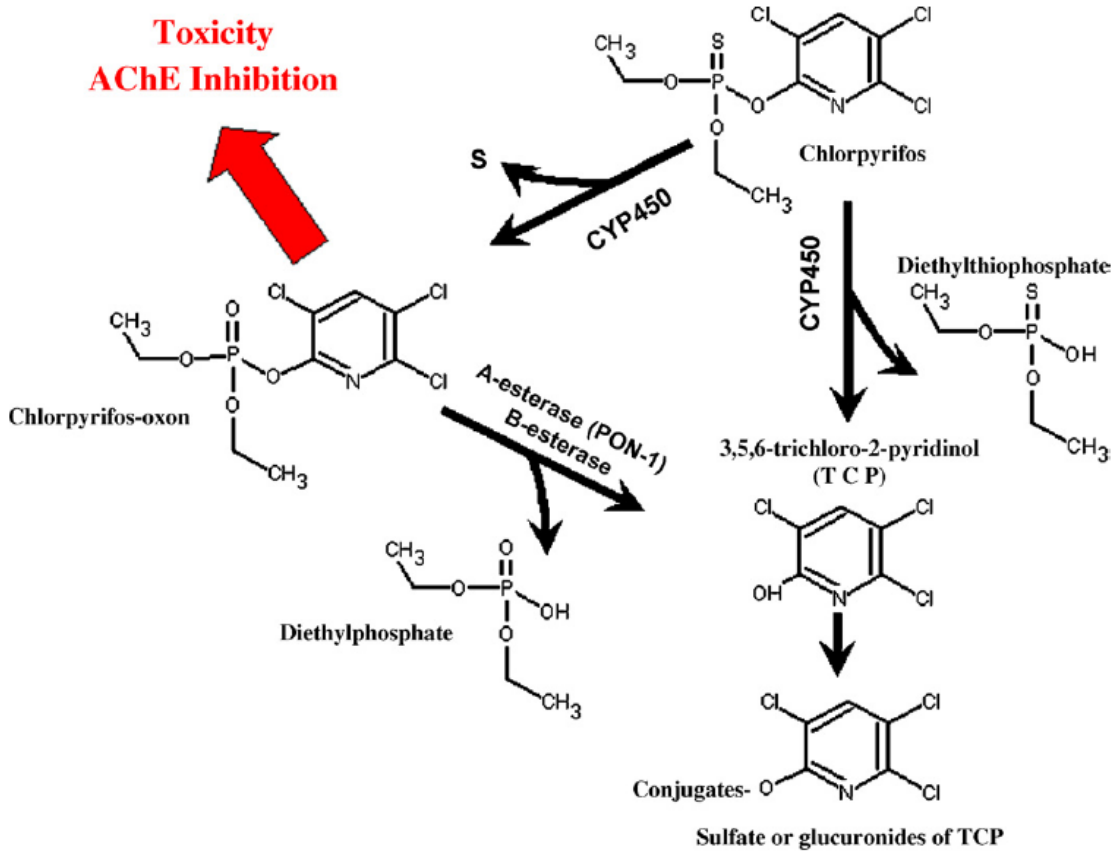


Figure B2. Metabolic scheme for chlorpyrifos (CPF) (*From Timchalk et al. (2007) Toxicology 237: 145-157*).

Paraoxonase

As noted above, OPs undergo metabolic activation to the corresponding oxons by a process of oxidative desulfuration catalyzed by cytochromes P450. Paraoxonase (PON1) is a high density lipoprotein liver and plasma enzyme that hydrolyzes the active metabolites (i.e., oxons) of specific OPs, including CPF, diazinon, and parathion. The oxon forms are much more potent inhibitors of cholinesterase than the parent compounds.

Several polymorphisms of the PON1 gene have been shown to influence both the level of expression and its catalytic ability, thus determining the rates at which an individual will detoxify a specific pesticide. In animal studies, transgenic mice expressing human PON1-R192 were much more resistant to cholinesterase inhibition by chlorpyrifos oxon exposure than PON1 deficient animals, with PON1-Q192 expressing animals demonstrating intermediate sensitivity, except at high doses, where the PON1-Q191 mice were essentially as sensitive as the PON1 deficient mice (Cole et al., 2005). In humans, the R192 alloform has a higher catalytic efficiency of hydrolysis than does the Q192 alloform for the oxon form of the pesticide and provides better protection against CPO exposures (Li et al., 2000), as seen in the table below. For diazoxon, both alloforms provide the same degree of protection, while for paraoxon, the substrate after which the enzyme was named, PON1 does not provide any protection due to an overall low catalytic efficiency of

PON1 toward this substrate (Costa, 2006). However, the antioxidant capacity of PON1 with respect to other non-OP substrates seems to be primarily associated with the Q192 alloform (Costa, 2006).

Table B1. In vivo protection of human PON192 alloforms and in vitro catalytic efficiency

OP	Degree of protection				Catalytic Efficiency[a]	
	Exogenous PON1[b]		hPON-TG[c]			
	Q192	R192	Q192	R192	Q192	R192
Chlorpyrifos oxon	1.5	4.2	1.5	4.0	152	256
Diazoxon	3.2	3.7	ND	ND	75	77
Paraoxon	0	0	ND	ND	0.7	6.3

Data are adapted from Li et al. (2000) and Cole et al. (2005) in Costa et al., (2006)

[a] V_{max}/km, measured in vitro under physiological NaCl conditions.

[b] Brain AChE activity (fold increase) following challenges with the OPs In PON1 knockout mice pretreated with equal amount of PON_{Q192} or PON_{R192} compared to untreated PON1 -/- mice.

[c] Brain AChE activity (fold increase) following challenge with CPO of transgenic mice expressing the human PON_{192R} or PON_{192Q} alloform over a knockout background, compared to PON1 knockout mice.

As the CPF oxon inhibits brain acetylcholinesterase at 1,000 times the rate of CPF, the rate of detoxification would be extremely important (Huff et al., 2001). In humans, considerable variability has been seen in PON1 status across populations (Sirivarasai et al., 2007). Approximately 50% of individuals of Northern European origin are homozygous for PON1-Q192 (Cole et al., 2005).

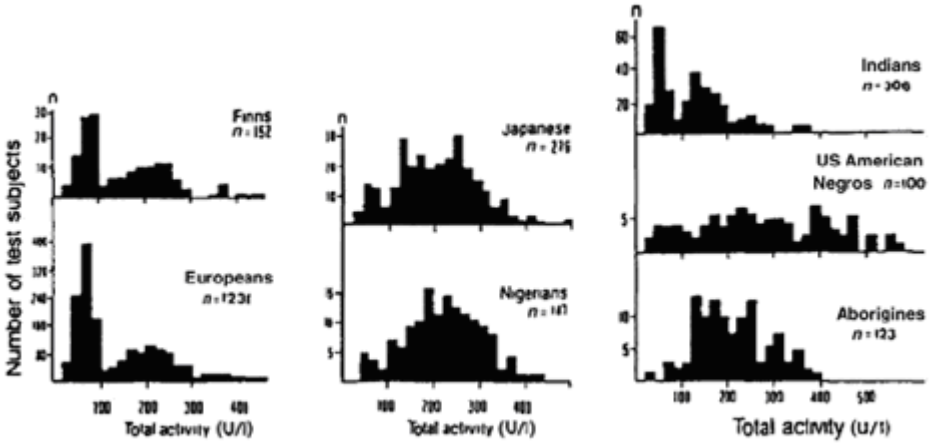


Figure B3. Histogram distributions of paraoxonase activity for different ethnic groups. Note the high frequency of low metabolizers in the Finns, Europeans, and Indians. The position of the antinode for low metabolizers allowed for the calculation of the gene frequency for low metabolizers in these populations, and in turn, calculation of the gene frequency of high metabolizers. Cited from Furlong (2007).

Genotyping alone does not provide the level of plasma PON1 activity, nor the phase of polymorphisms (i.e., which polymorphisms are on each of an individual's two chromosomes). A

functional determination of genomic analysis has been shown to be much more informative (Costa, 2006). PON1 status is the functional determination of PON1192 alloform(s) present in plasma, as well as the level of an individual's plasma PON1. PON1 status is determined with a high-throughput two-substrate assay and analysis where rates of diazoxonase hydrolysis are plotted against rates of paraoxonase hydrolysis using serum or non-EDTA preserved plasma samples.

PON1 levels have been shown to be low at birth, and plateau between 6 and 15 months of age, with high inter-individual variability (Cole et al., 2003). Furlong et al. (2006) found a 131 to 164 fold range in PON1 status in a Mexican-American population of women and their newborns, indicative of a wide range in sensitivity to CPO. The authors noted that many of the newborns and some of the mothers in their cohort would have increased sensitivity to the adverse effects of specific organophosphorus pesticide exposure.

Concerning adverse effects of exposure to CPF, much attention has been focused on the effects of the CPF oxon, the active metabolite, as the parent compound is a poor inhibitor of cholinesterases. However, recent research observed inhibition of cytochrome P450 isoform 3A4 (CYP3A4) during the bioactivation of CPF (Khawja et al., 2003). As CPY3A4 is an important enzyme in testosterone metabolism, this could have implications concerning CPF exposure during development (Dai et al., 2001).

Excretion

Chlorpyrifos is excreted primarily through the kidneys in the urine. Approximately 70% of an oral dose was excreted in the urine of humans, while 90% was excreted in rats (Cochran et al., 1992). The main route of excretion in humans, rats and goats was through the urine. Less than 0.1% of the dose administered to goats was found in the milk.

B.4. Non-DART effects

B.4.1. Acute Toxicity

Chlorpyrifos produces moderate acute toxicity in mammals, with a rat oral LD50 of 82–155 mg/kg (Gaines, 1969). The range of acute oral toxicity (LD50) for liquid formulations in male and female rats was 205–1414 mg/kg; for granular formulations it was 224–1630 mg/kg; for wettable powders it was 180–235 mg/kg; and for sprays it was 710–>5000 mg/kg (Cochran et al., 1992).

The acutely toxic effects of CPF and CPO are primarily through the persistent phosphorylation, by its metabolite CPO, of AChE at cholinergic synapses within the central and peripheral nervous systems. This persistent phosphorylation results in inhibition of AChE, and accumulation of the neurotransmitter acetylcholine (ACh) in the synapses, inducing hyperactivity of cholinergic pathways resulting from overstimulation of ACh receptors (Ecobichon, 1996). Acetylcholine is the neuro-chemical transmitter at endings of postganglionic parasympathetic nerve fibers, somatic motor nerves to skeletal muscle, preganglionic fibers of both parasympathetic and sympathetic nerves, and certain synapses in the central nervous system (CNS) (Murphy, 1986). The inhibition

of acetylcholinesterase (AChE) results in accumulation of endogenous acetylcholine in nerve tissue and effector organs and muscarinic, nicotinic and CNS receptors are stimulated with characteristic signs and symptoms occurring throughout the peripheral and central nervous systems (Lefkowitz et al., 1990; Ellenhorn and Barceloux, 1998) in acute toxic exposures to agents inhibiting AChE. The muscarinic effects can include increased intestinal motility, bronchioconstriction, increased bronchial secretions, bladder contraction, miosis, secretory gland stimulation and bradycardia. Nicotinic effects include muscle weakness, twitching, cramps and general fasciculations. The effects of severe poisoning with OP insecticides typically include slurred speech, tremors, ataxia, convulsions, depression of respiratory and circulatory centers followed by coma and death.

B.4.2. Subchronic and Chronic Toxicity

The non-DART subchronic toxicity of chlorpyrifos has been summarized as follows by the U.S. EPA in its Toxicology Chapter for the Reregistration Eligibility Decision (RED) for Chlorpyrifos (U.S. EPA, 1999).

> "Several subchronic studies are available for chlorpyrifos including two oral rat studies, one oral dog study, a 21 day dermal toxicity study in rats, and two inhalation studies in rats. The most sensitive toxicological endpoint following subchronic oral exposure is inhibition of plasma and red blood cell cholinesterase in dogs at 0.1 to 0.22 mg/kg/day (Barker 1989, McCollister et al. 1971, Kociba 1985) and plasma inhibition in rats at doses as low as 0.025 mg/kg/day (Crown et al. 1985). Rats exposed to higher doses exhibited hematological alterations at doses of 10 mg/kg/day (Crown et al. 1985) and increased brain and heart weight, adrenal gland effects and decreased body weight gain at 15 mg/kg/day (Szabo et al. 1988). No adverse effects were noted in rats exposed via inhalation to the highest attainable vapor concentration of 20.6 ppb (287 Fg/m3) (Corley et al. 1986a,b, Newton 1988). No adverse effects were observed in the 21-day dermal study in rats at doses as high as 5 mg/kg/day. However, in a 4-day dermal probe study, rats dermally exposed to doses of 0, 1, 10, 100, or 500 mg/kg/day exhibited reductions in plasma and red cell cholinesterase activities at doses of 10 to 500 mg/kg/day. The 21-day dermal NOAEL is 5 mg/kg/day based on a 45% and 16% inhibition of plasma and red blood cell cholinesterase, respectively in rats dermally exposed to 10 mg/kg/day for 4 days (MRID No. 40972801; Calhoun and Johnson, 1988)."

Similarly, the non-DART chronic toxicity of chlorpyrifos was summarized as follows by the U.S. EPA in its Toxicology Chapter for the Reregistration Eligibility Decision (RED) for Chlorpyrifos (U.S. EPA, 1999).

> "Chlorpyrifos was evaluated for chronic toxicity in dogs. In all animal species, the most sensitive effect is inhibition of plasma, red blood cell and brain cholinesterase that occurred at levels in the range of 0.03 to 3 mg/kg/day. Following chronic exposure, dogs appear to be the most sensitive species for cholinesterase inhibition and systemic effects, as noted by increased liver weights that could be an adaptive response in dogs exposed to 3 mg/kg/day. Rats exposed to 7-10 mg/kg/day had decreased body weight and decreased body weight gain, ocular effects, adrenal gland effects and altered clinical chemistry and hematological parameters. Mice appear to be the least sensitive to chronic chlorpyrifos exposure, as exposure to 45-48 mg/kg/day

resulted in decreased body 11 weight and an increased incidence of non-neoplastic lesions (i.e., keratitis, hepatocyte fatty vacuolation) (Gur 1992)."

B.4.3. Mutagenicity

Mutagenicity

Chlorpyrifos was not mutagenic in bacteria, or mammalian cells, but did cause slight genetic alterations in yeast and DNA damage to bacteria. Also, chlorpyrifos did not induce chromosome aberrations in vitro, was not clastogenic in the mouse micronucleus test in vivo, and failed to induce unscheduled DNA synthesis in isolated rat hepatocytes (U.S. EPA, 2000a). The results of numerous mutagenicity short-term assays, for genotoxicity obtained from Rahman et al. (2002) are provided in Table B2 below.

Table B2. Genetic and related effects of chloropyrifos

Short-term genotoxicity assays	Result
Mutation in *Escherichia coli*	-
Mutation in *Salmonella typhuimurium*	-
Mutation in *Bacillus subtilis*	+
DNA damage in prokaryotes	+
Mutation, gene conversion or mitotic crossing over in yeast	-
Unscheduled DNA synthesis in mammalian cells	-
Chromosomal aberrations in mammalians cells in vitro	+
Sister chromatid exchange in mammalian cells in vitro	-
Hypoxanthine guanine-phosphoribosyl transferase or TK mutations in mammalian cells	-
Mutation in *Drosophila melanogaster*	+
Mammalian micronucleus test in vivo	-
Mutations in higher plants	+

More recently DNA damage in lymphocytes of mice exposed to CPF at dose ranges of 0.28 – 8.96 mg/kg body weight was demonstrated using the comet assay (Rahman et al., 2002). Also, a dose-dependent increase in DNA damage in the liver and brain of rats following both acute and chronic exposure to CPF was noted at high doses of 50 and 100 mg/kg body weight (25% and 50% of rat LD50), via the intramuscular injection route (Mehta et al., 2008). DNA damage noted was thus only at high doses and not at clinically relevant doses. The methodology used in this study was also not entirely clear given that all organs typically used in the protocol were not evaluated. Also, given that the comet assay is a sensitive assay for DNA damage, typically additional positive responses in in vivo systems are required to be predictive for carcinogenicity in mammalian systems.

Cancer

The human health risk assessment by U.S. EPA (U.S. EPA, 2000b) concluded that there was no evidence of carcinogenicity.

C. Developmental Toxicity

Several studies have been identified in the toxicological evaluation of evidence on the effects of CPF on development. The studies in humans include epidemiologic data obtained from studies of exposure to CPF along with other pesticides. The individual studies have been decribed below. Animal studies include those conducted for regulatory purposes as reproduction studies per Federal Insecticide, Rodenticide and Fungicide Registration Act (FIFRA) guidelines as well as studies from the peer-reviewed literature. In addition to those involving gestational exposure, studies of exposure during the early postnatal period have been included since some periods of brain development that occur postnatally in animals are known to occur prenatally in humans.

C.1. Human studies

C.1.1. Individual studies

Among the epidemiologic data concerning CPF are studies from three separate ongoing prospective cohort studies, two based in New York City and one from the Salinas Valley in California. To orient the reader to these data, listed below are brief descriptions of the cohorts as well as the associated publications. An additional and recent cohort study by Samarawickrema et al. (2008) conducted in a rural farming community in Sri Lanka has also been reviewed below.

In addition to studies which assessed exposure specifically to CPF, related studies, in which exposure specifically to CPF was not assessed but which contain relevant information, are also presented and each is identified as a related study.

The prospective cohort study of pregnant women conducted by the Columbia Center for Children's Environmental Health was initiated in 1997. The sample population in this cohort included women 18–35 years of age, self identified as African American or Dominican, and residing in Washington Heights, Central Harlem, or the South Bronx of New York for > 1 year prior to pregnancy. The objectives of this study of inner city mothers and their newborns include the evaluation of the effects of indoor pollutants on birth outcomes, neurocognitive development, asthma, and procarcinogenic damage. The publications from this cohort include Perera et al., 2003, Whyatt et al., 2004, 2005; and Rauh et al., 2006. Since the study by Whyatt et al., 2005 is an update of the earlier research findings from Perera et al., 2003 and Whyatt et al., 2004, and contains the larger sample size, the discussion of the birth outcomes concerning fetal growth for this cohort will be centered around this study.

The Children's Environmental Health Study is a prospective cohort study of ethnically diverse, mostly Black or Dominican, mothers and infants enrolled at Mount Sinai Hospital in New York City during pregnancy from March 1998 to March 2002. The study was established to investigate the relationship between prenatal exposure to indoor pesticides and infant growth and development. The publications from this cohort include Berkowitz et al., 2004, Engel et al., 2007, and Wolff et al., 2007.

The Chamacos Study (which is an acronym for – Center for the Health Assessment of Mothers and Children of Salinas) is a prospective cohort study of the effects of exposure to pesticides and other environmental chemicals on the health of pregnant women and their children living in the Salinas Valley, in California. This population of mostly Mexican American mothers and their infants were enrolled in the study between 1999 and 2000 during pregnancy. The publications from this cohort include Eskenazi et al., 2004, Young et al., 2005, and Eskenazi et al., 2007.

In the discussion below all reported risk estimates are adjusted for relevant potential covariates or confounders as reported by the authors, unless otherwise noted.

Samarawickrema et al., 2008. Fetal effects of environmental exposure of pregnant women to organophosphorus compounds in a rural farming community in Sri Lanka.

In a cohort study, Sanarawickrema et al., 2008 examined the possible effects of low level, chronic exposure to OPs in pregnant women who presented for delivery at the Embilipitiya Base Hospital that serves a rural farming community in Southern Sri Lanka. Outcome measures included oxidative stress and oxidative tissue damage. Forty one women were recruited at the end of the July to August 2005 and January to February 2006 pesticide spray seasons, and 25 women were recruited before the commencement of the spray season in December 2005. Eligibility, as determined by a questionnaire administered in the hospital, included: 1) age between 20 and 30 years; and 2) living in a farming area for the duration of pregnancy but not directly handling or knowingly exposed to OPs. Questionnaire data also included the period of gestation at partus, approximate distance to residence from the closest site of spraying, approximate frequency of spraying at that site during the last four weeks of pregnancy, and dietary habits. Samples of plasma from maternal and cord blood were obtained at time of delivery; breast milk samples were obtained 24 hours post partum. Maternal blood sampling was not conducted during the first spray season, but the protocol was changed to allow sampling during the in-between spray season and the second spray season. Breast milk and plasma samples were analyzed for CPF, dimethoate, diazinon, and fenthion, the OPs identified as those predominantly used in the area. The OPs were analyzed in breast milk, and in maternal and cord blood using high pressure liquid chromatography (HPLC) - Tandem mass spectrometry with a level of detection of 0.5 μg/L. Chlorpyrifos and diazinon were also analyzed by gas chromatography with the levels of detection being 0.04 mg/L and 0.02 mg/L, respectively.

Plasma butyrylcholinesterase (BuChE) activity, superoxide dismutase (SOD), malondialdehyde (MDA) concentration and DNA damage were determined for all subjects. Assessment of DNA damage was done by an observer who had no knowledge of the subjects' exposure status. Butyryl cholinesterase levels were lower during both spray seasons than during the interspray season, and SOD and MDA levels were higher during the two spray seasons than during the interspray season. As there was consistency for these comparisons, which were made separately for each spray season with the interspray season, the authors pooled the data from the two spray seasons to increase the sample size and the power of the study. The subjects in the spray and in-between spray seasons were similar with respect to age, period of gestation, dietary habits, and approximate distance from closest site of spraying. Spraying activity at the closest spray site was, as expected, higher during the last 4 weeks of pregnancy of mothers in the spray season group.

Chlopyrifos was detected in only one maternal (0.564 μg/L) and cord blood sample (0.622 μg/L). No other pesticides were detected in any of the blood samples. Lower mean cord blood BuChE activity was seen during the spray season than in the between-spray season (p = 0.04) (Table C1). The BuChE activities of the newborn and maternal pairs, where data were available (n=46), showed no significant correlation (r = 0.1, p = 0.2). As shown in Table C1, there was no significant difference in the fetal SOD enzyme levels between the spray and in-between spray seasons. However, MDA production was significantly increased (p<0.001) in cord blood samples obtained during the spray season compared with those obtained during the in between season. DNA showed fragmentation in 20 of the 41 (48.8%) cord blood samples from the spray season compared with 2 (8%) of the 25 samples from the in between spray season.

Table C1. Comparison of results from 'spray' and 'in-between spray' seasons

Test	Group		
	'Spray' Season n = 41	'In-between spray' Season n = 25	P value¶
Fetal BuChE (Units/liter, U/L) – Mean (SD)	4984 (1137)	5557 (1047)	0.04
*Maternal BuChE (U/L) – Mean (SD)	5205 (1099)	5107 (1275)	0.7
Fetal SOD (U/g Hb) – Mean (SD)	3014 (1210)	2873 (688)	0.5
Fetal MDA (nmol/ml) – Mean (SD)	0.298 (0.017)	0.211 (0.045)	<0.001
Fetal DNA breakage – No. (%)	20 (48.8)	2 (8)	#0.001

*22 'spray' and 24 'in-between spray' samples.
¶ P values based on t-tests except # Chi-square test.

In comparing the groups with and without DNA damage (Table C2) there was significant inhibition of BuChE activity and increased MDA levels in cord blood samples that exhibited DNA damage. However, there was no significant correlation between BuChE activity, MDA levels and degree of DNA fragmentation. Although maternal BuChE activity was less in the samples with cord blood DNA damage, the difference between the groups was not significant.

Table C2. Comparison of cord blood samples with and without DNA breakage

Test	Samples: DNA Damage n = 22	Samples: No DNA Damage n = 44	P value¶
Fetal BuChE (Units/liter, U/L) – Mean (SD)	4778 (1003)	5410 (1144)	0.03
*Maternal BuChE (U/L) – Mean (SD)	4877 (975)	5288 (1263)	0.2
Fetal MDA (nmol/ml) – Mean (SD)	0.312 (0.126)	0.242 (0.072)	0.006

*15 with fetal 'DNA damage' and 31 with 'No fetal DNA damage'.
¶ P values based on t-test.

The authors state that these results suggest there is evidence of increased oxidative stress and high molecular weight fragmentation activity in fetal DNA and also lower BuChE activity during the spray season. This probably indicates some fetal exposure to organophosphorus compounds, although such exposure was not directly demonstrated. The lack of detection of OP metabolites in blood may be a result of the relatively short half-life of OPs since samples of blood and breast milk were taken following delivery in the hospital where, as noted by the authors, exposure to pesticide drift and direct spraying was likely to be lower than in the subjects' homes.

The many possible explanations for why low level exposure is associated with altered biomarkers in fetal blood but not in maternal blood include such differences between them as: 1) greater conversion to toxic metabolites or less metabolic detoxification capacity of the fetus; 2) distribution kinetics leading to trapping in the placental-fetal compartment; or 3) less effective antioxidant systems or less effective repair mechanisms in the fetus (e.g. slower BuChE synthesis or DNA repair). The authors cited literature that indicates toxic effects of chronic low-grade exposure to OPs may be mediated through AChE inhibition. However, they also note that objective signs of toxicity can occur in the absence of detectable AChE inhibition (Ray and Richards, 2001), and other mechanisms, such as oxidative stress, may mediate toxic effects of chronic low grade exposure (Kale et al., 1999; Bebe and Panemangalore, 2003; Vidyasagar et al., 2004).

The limitations of this study include the lack of control for possible confounding variables and the lack of clear distinction between the exposed and control groups. In addition, the assays' higher level of detection for CPF in blood and breast milk might have limited their ability to detect any CPF present. As mentioned by the authors, mothers in both groups would have experienced both seasons during their pregnancies. Women delivering at a time corresponding to in-between spray seasons would still have experienced increased pesticide exposures for at least 2 months, but just for half the time of those who delivered during the spray season, and not during the last two months of pregnancy. In addition, pesticides are used outside of the spray season, although at a much lower intensity. The exposed group, however, would have higher "spray season levels" of exposure closer to delivery than the "in between spray season" group.

Whyatt et al., 2005. Biomarkers in assessing residential insecticide exposures during pregnancy and effects on fetal growth.

This study conducted by the Columbia Center for Children's Environmental Health included women 18–35 years of age, self identified as African American or Dominican, and residing in Washington Heights, Central Harlem, or the South Bronx of New York for > 1 year prior to pregnancy. Nonsmoking women who were registered at the obstetrics and gynecology clinics at two area hospitals by the 20th week of pregnancy were enrolled in the study. Women were excluded from the study if they smoked cigarettes, or used other tobacco products during pregnancy, used illicit drugs, had diabetes, hypertension, or known HIV. Subjects were excluded if their cotinine levels in maternal blood samples or cord blood samples exceeded 15 ng/ml.

A total of 571 women were enrolled between January 1998 and January 2004. The retention rate was 82.72% at the three-year follow-up. Questionnaire data were collected during the 3rd trimester. Women wore a personal ambient air monitor during the daytime hours for two consecutive days and placed the monitor near the bed at night. Ten different pesticides were measured in these air samples including CPF. Up until publication of this study air samples were analyzed for 394 of the women. Umbilical cord blood was collected as close to delivery as possible. Samples of maternal blood were taken within 2 days postpartum. Blood samples were analyzed for 326 mothers and 341 newborns whose deliveries occurred between 1998 and 2002. In cases where infant cord blood CPF levels were not available, the value was derived from the regression of cord-blood levels on maternal blood levels. No mention was found in this paper of how many newborns' values were imputed; however, for the slightly smaller sample reported in Whyatt et al., 2004, of the 314 mother-newborn pairs cord blood CPF levels were available for 256 newborns and imputed for 31 newborns.

Regression analyses included examining the combined effects of CPF and diazinon which were assessed using methodology developed by U.S. EPA for cumulative risk assessment for organophosphates (U.S. EPA, 2001). Diazinon levels were put into CPF equivalents based on the ratio of CPF and diazinon relative potency factors calculated by the U.S. EPA (U.S. EPA, 2001).

The mean age of women in this sample population was 24.9 years, with 37% being African American, and 63% being Dominican. Seventy-three percent were single and 65% had at least a high school degree. A large percentage of women reported sighting pests in the home (85%), and 85% reported that pest control measures were used during pregnancy. Forty percent reported using only lower toxicity methods (such as traps and gels while 54% reported using one or more higher toxicity methods (such as spray by an exterminator, can sprays and pest bombs). The mean maternal and cord blood CPF levels were 3.9 ± 4.8 pg/g (range 0.3 – 35 pg/g) and 3.7 ± 5.7 pg/g (range 0.3 – 63 pg/g), respectively. Chlorpyrifos was detected in 99.7% of personal air samples. The correlation between CPF in maternal personal air and maternal blood samples was 0.21 ($p \leq 0.001$), and between maternal personal air and cord blood samples was 0.19 ($p \leq 0.001$). Maternal blood and cord blood samples were strongly correlated, 0.79 ($p \leq 0.001$). As shown in Figure C1, levels of CPF in maternal air samples decreased significantly between 1999 and 2002. Similarly, a significant decrease was seen in CPF levels in cord blood samples in infants born between 1998 and 2002.

Table C3. Insecticides in maternal personal air samples collected over 48 h during pregnancy and in blood samples collected from the mother and newborn at delivery

Pesticide	Personal air (ng/m^3) (*n* = 394)		Maternal blood (pg/g) (*n* = 326)		Cord blood (pg/g) (*n* = 341)	
	Percent > LOD[a]	Mean ± SD[b] (range)	Percent > LOD[a]	> Mean ± SD[b] (range)	Percent > LOD[a]	Mean ± SD[b] (range)
Chlorpyrifos	99.7	14.3 ± 30.7 (0.1–344.8)	70	3.9 ± 4.8 (0.3–35)	64	3.7 ± 5.7 (0.3–63)
Diazinon	100	99.5 ± 449.8 (1.0–6000)	45	1.3 ± 1.8 (0.3–25)	44	1.2 ± 1.4 (0.3–13)
Propoxur[b]	100	53.5 ± 124.5 (1.2–1420)	39	2.8 ± 2.7 (1.4–32.8)	40	3.0 ± 3.0 (1.4–26)

[a] LOD = limit of detection.
[b] 2-Isopropoxyphenol was measured in blood samples.

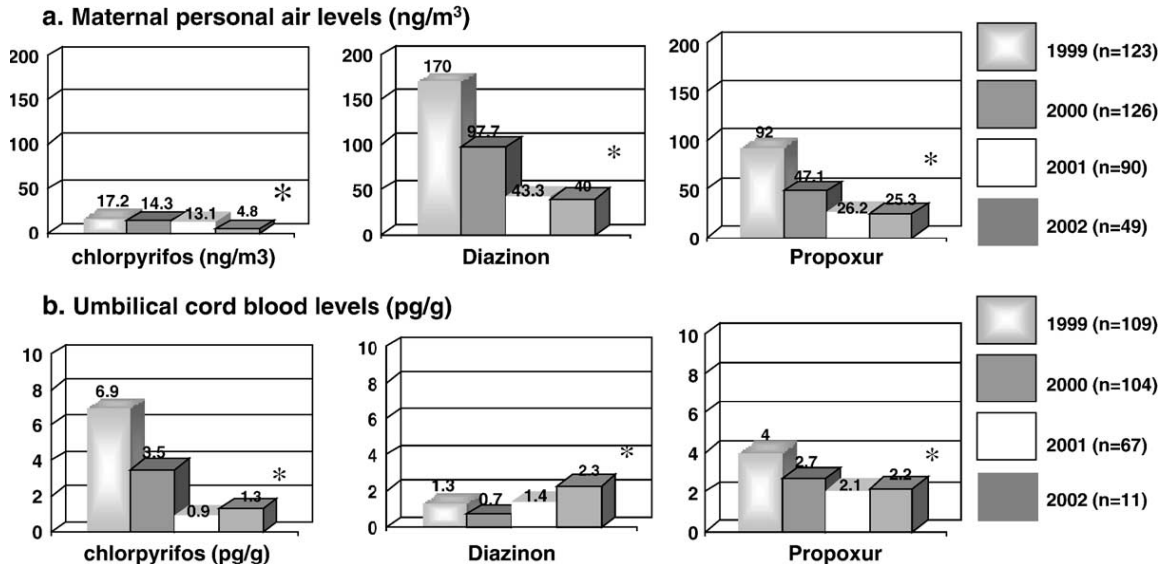


Figure C1. Mean insecticide levels in maternal personal air sample collected over 48 h during the 3rd trimester of pregnancy by year monitory (a) and in umbilical cord blood samples at delivery (b) by year of delivery.
* significant decrease between 1999 and 2002 ($p < 0.05$)

From the regression analysis an inverse statistically significant association was evident between log-transformed (ln)CPF levels and birth weight (B = -67.3 g/unit, 95% CI -116.6 to -17.8, p = 0.008) and birth length (B = -0.43 cm/unit, 95% CI -0.73 to -0.14, P = 0.004) in newborns born before 1/1/01 (n = 237) (Table C4). However, no significant association with birth weight was evident in infants born after 1/1/01, (B = 30.7 g/unit, 95% CI –108.6 to 169.9, p = 0.66). Of note is the smaller sample size and the wider confidence intervals. Similar results were observed when the combined levels of CPF and diazinon were examined (Table C4). There was a significant inverse association between CPF levels and birth weight in infants born prior to 1/1/01 (-72.5 g/unit, 95% CI -125.0 to -20.0, p = 0.0007), but not in those born after 1/1/01 (0.6 g/unit, 95% CI -144.7 to 145.9). Covariates included in these analyses were gestational age, maternal pre-pregnancy weight and weight gain during pregnancy, infant gender, parity, ethnicity, environmental tobacco smoke (ETS) in the home, and season of delivery. The authors

reported that in a model controlling for cord plasma 2-isopropoxyphenol (the chemical specific metabolite of propoxur), in addition to the other potential confounders, the associations between birth weight and birth length and cord plasma (ln)CPF, as well as the sum of cord plasma (ln)CPF and (ln)diazinon, remained significant ($p \leq 0.02$) and the effect size remained similar to that seen without 2-isopropoxyphenol in the model (data were not shown).

Table C4. Regression analyses[a] of birth weight and length and organophosphate levels in umbilical cord plasma samples for infants born before and after 1/1/01

	Birth weight (gm)		Birth length (cm)	
	β (95% CI)	*P* value	β (95% CI)	*P* value
Born before 1/1/01 (*n*=237)				
Chlorpyrifos	-67.3 (-116.6 – -17.8)	0.008	-0.43 (-0.73 – -0.14)	0.004
Sum chlorpyrifos and diazinon[b]	-72.5 (-125.0 – -20.0)	0.007	-0.46 (-0.77 – -0.14)	0.004
2-Isopropoxyphenol	-75.8 (-171.1 – 19.5)	0.12	-0.73 (-1.3 – -0.17)	0.01
Born after 1/1/01 (*n*=77)				
Chlorpyrifos	30.7 (-108.6 – 169.9)	0.66	0.07 (-0.65 – 0.79)	0.85
Sum chlorpyrifos and diazinon[b]	0.6 (-144.7 – 145.9)	0.99	-0.07 (-0.82 – 0.67)	0.84
2-Isopropoxyphenol	-107.3 (-298.7– 84.2)	0.27	-0.30 (-1.3 – 0.71)	0.56

[a] Each (ln) insecticide levels was entered as the independent variable into a parallel multiple linear regression model. Model covariates were gestational age of the newborn (in weeks), maternal pre-pregnancy weight and weight gain during pregnancy (in pounds), newborn gender (0 = male; 1 = female), parity (0 = nulliparous; 1 = at least one prior live birth), ethnicity (0 = Dominican; 1 = African American), ETS in the home (0 = no; 1 = yes), and season of delivery (dummy variable 1: 0 = summer; 1 = winter; dummy variable 2: 0 = summer; 1 = spring; dummy variable 3: 0 = summer; 1 = fall); models for head circumference included whether or not the delivery was by cesarean section (n = 0, 1 = yes).
[b] Sum of chlorpyrifos and diazinon in chlorpyrifos equivalents adjusted for relative potency.

Birth weight averaged 215.1 g less (95% CI -384.7 to -45.5, p = 0.01) among those with the highest versus the lowest combined cord plasma CPF and diazinon exposure levels (infants with levels in the highest third of detectable levels versus infants with no detectable levels of either pesticide).

This study's data collection spanned a period when CPF use had decreased. Some of the decrease may have been due to the phasing out of the pesticide by U.S. EPA for residential use (U.S. EPA, 2000b).

The results presented in this article are in agreement with an earlier article (Whyatt et al., 2004) based on this cohort but with a smaller sample size. No association was observed between CPF levels and head circumference in either study. CPF was detected in 99.7% of air samples and in 70% of maternal blood samples. There was a lack of a strong correlation between the levels of CPF in maternal blood and those measured in personal air samples. However, it may be that CPF in blood includes exposure from the dermal route of exposure (Whyatt et al., 2004), from ingestion (MacIntosh et al., 2001; Clayton et al., 2003), as well as from inhalation. The relatively short half-life of CPF in blood also may explain this lack of correlation as the personal air monitoring was conducted approximately one month earlier than the collection of blood samples, which were drawn within 2 days postpartum. In addition, it is likely that while in the hospital the pregnant women would have decreased exposure to CPF as compared to when she was at home. Therefore, depending on how much time elapsed between entering the hospital and

having the blood samples drawn within 2 days of delivery, the levels of CPF in blood may have been substantially lower. The half-life of CPF in blood is approximately 27 hours (Nolan et al., 1984).

Potential covariates were controlled for in the multivariate analyses in this study. In a previous paper, Perera et al. (2004) reported that a significant interaction between ETS exposure and exposure to polycyclic aromatic hydrocarbons (PAHs), as measured by benzo[a]pyrene-DNA adducts, had a significant effect on birth weight and head circumference. Although ETS exposure was included as a covariate in the current study it was not indicated that PAH exposure was also included. However, in order for exposure to PAHs to account for the effects on birth weight observed before and after the residential use ban on CPF, exposure to PAHs would also have to have changed over this same time period, which would seem to be highly unlikely.

Rauh et al., 2006. Impact of prenatal chlorpyrifos exposue on neurodevelopment in the first 3 years of life among inner-city children.

This is one of a series of reports on the ongoing prospective cohort of inner-city mothers and their newborn infants of the Columbia Center for Children's Environmental Health study. This study evaluated the effects of prenatal CPF exposure on behavior and child neurodevelopment during the first three years of life. Samples of umbilical cord blood were collected at the time of delivery. Maternal blood samples were collected within 2 days of delivery. The study sample included 536 participants (of the 648 consenting women) and 254 of their children who had reached the age of three years having: prenatal maternal interview data; biomarkers of CPF exposure levels in maternal and cord blood samples at delivery; postnatal observational data on the quality of the home caretaking environment; and a neurobehavioral outcome assessment at $\geq$ 1 yearly evaluation (12, 24 and 36 months). The retention rate was 82.72% at the three-year follow-up. The women lost to follow-up were similar to those retained in the study with respect to age, ethnicity, marital status, education, income and gestational age and birth weight of the newborn. Outcome assessments and other data at all three time points were available for 189 children.

A 45-minute questionnaire was administered to each woman during the third trimester of pregnancy. The Bayley Scales of Infant Development II (BSID-II) was used to assess cognitive and psychomotor development at 12, 24 and 36 months of age. Each scale provides a developmental quotient (raw score/chronological age), which generates a continuous Mental Development Index (MDI) and a corresponding Psychomotor Development Index (PDI) which are frequently used to diagnose developmental delay. The MDI is used to characterize a variety of cognitive abilities, and the PDI characterizes large muscle and fine motor coordination. The test is frequently used to diagnose developmental delay and is known to be sensitive to the effects of exposures such as low-level intrauterine lead (Bellinger et al., 1991). As noted by the authors, the BSID-II, administered at three years of age, has demonstrated only moderate predictive power for subsequent intelligence and school performance but is clinically useful for children performing in the subnormal range (Bayley, 1993; Burchinal et al., 1997; Sternberg et al.,

2001; Rauh et al., 2006). Children's status can be classified as normal or delayed (scores of ≤ 85) using a standardized cutoff of one standard deviation. Behavior problems were measured using the Child Behavior Checklist (CBCL) as reported by the mother. As explained in Eskenazi (2008), the CBCL assesses a range of behavior problems that produces scales consistent with the Diagnostic and Statistical Manaual of Mental Disorders diagnoses (APA, 2000). The Test of Nonverbal Intelligence was used to measure maternal nonverbal intelligence when the child was ~3 years of age. The Home Observation for Measurement of the Environment (HOME) instrument was used to measure the quality of the caretaking environment at 3 years. As described in this study, this instrument assesses physical and interactive home characteristics and has been used widely in studies of neurotoxicity (Bellinger et al., 1988; Rauh et al., 2006).

Breastfeeding was examined as a dichotomous variable and was found to have no relationship to any of the developmental outcomes or to CPF exposure. As described in Whyatt et al. (2005) the maternal and umbilical cord blood levels of CPF were strongly correlated ($r = 0.76$; $p < 0.001$). In the instances where umbilical cord blood was not collected (12% of subjects), the mother's values were used, based on the formula derived from the regression analyses described in the discussion above in Whyatt et al. (2005). All regression models included terms for prenatal ETS exposure, gender, ethnicity, gestational age at birth, quality of the home care-taking environment, maternal educational level (high school degree versus no high school degree), and maternal IQ.

Chlorpyrifos levels ranged from undetectable (LOD: 0.5 – 1 pg/g) to 63 pg/g. High exposure to CPF was classified as >6.17 pg/g which included 30.6% of the sample. The women in the high exposure group included 24.2% of black women and 14.9% of Dominican women. Women classified as being ETS exposed included 37% of the sample with cotinine levels ranging from 0.01 ng/mL to 8.79 ng/mL. Women who were highly exposed to CPF were significantly more likely to be exposed to ETS in the home during pregnancy (56%), compared with women with lower CPF exposure (31.9%).

The results of multivariate regression analyses found a marginally statistically significant ($p = 0.06$) adverse effect of prenatal CPF exposure on MDI scores (–3.3 points) at 36 months of age. At that age, other variables were statistically significantly related to lower MDI scores including mother's lack of a high school education, lower maternal IQ, and being male. Longer gestation was protective, as was having higher HOME scores. In addition, black children had higher scores than Dominican children. Children exposed to ETS during the prenatal period had a 3-point decrease in MDI scores at 24 months ($p = 0.06$), but this was not seen at 12 or 36 months. Interaction terms for the interaction of CPF levels with other exposure and sociodemographic variables were tested in the full model and none were found to be significant.

The results of the multivariate regression model found a significant negative effect of high CPF levels on PDI scores at 36 months (Table C5), where PDI scores were 6.5 points lower compared with children with low CPF levels ($p = 0.003$). Other covariates which were statistically significant included race/ethnicity ($p = 0.034$) and gestational age ($p = 0.033$), while HOME score was of marginal significance ($p = 0.057$). Stratum specific analysis of race/ethnicity groups at 36 months found a significant adverse effect

of CPF on PDI scores for both black children (a deficit of ~ 7 points, p = 0.05), and Dominican children (a deficit of ~ 6 points, p = 0.05). A similar analysis of MDI scores found a significant effect for black children (a deficit of ~ 6 points, p = 0.05), while for Dominican children the deficit was not significant (slightly less than 2 points).

TABLE C5. Multivariate Linear Regression Models Testing Main and Interactive Effects of Prenatal Chlorpyrifos Exposure on 12-, 24-, and 36-Month PDI Scores, Adjusted for Race, Gender, Maternal Education, Maternal IQ, Gestational Age, and Prenatal ETS Exposure

Variable	Model 1:12 mo (*n*=228)		Model 2:24 mo (*n*=227)		Model 3:36 mo (*n*=228)	
	β,Mean ± SE	*P*	β,Mean ± SE	*P*	β,Mean ± SE	*P*
Constant	112.28 ± 34.64	.00	81.05 ± 32.45	.01	64.56 ± 36.64	.079
Prenatal chemical exposures						
ETS[a]	0.31 ± 1.76	.86	2.83 ± 1.63	.08	-0.14 ± 1.79	.940
Chlorpyrifos[b]	-3.30 ± 2.11	.12	1.17 ± 1.98	.56	-6.46 ± 2.18	.003
Covariates						
Race/ethnicity[c]	-2.00 ± 1.81	.27	2.15 ± 1.70	.21	3.88 ± 1.82	.034
Gender[d]	0.11 ± 1.64	.95	0.08 ± 1.54	.96	-2.95 ± 1.66	.077
Gestation age	-0.16 ± 0.56	.77	0.20 ± 0.53	.70	1.38 ± 0.64	.033
Maternal IQ[e]	-0.71 ± 6.26	.91	0.09 ± 5.56	.99	-5.78 ± 6.08	.343
Low maternal education[f]	-0.81 ± 1.77	.65	-1.26 ± 1.66	.45	1.69 ± 1.81	.350
HOME score	-0.08 ± 0.15	.61	0.09 ± 0.14	.53	0.30 ± 0.16	.057
R^2	0.024		0.035		0.107	

The sample included all children who had reached 3 years of age with data from the prenatal maternal interview, biomarkers of exposure, and complete data on all covariates. Sample sizes for age-specific models varied from 227 to 228 because of missed and/or unscorable assessments (<50). B indicates unstandardized regression coefficient.

[a] Prenatally exposed=1; not exposed=0.

[b] High exposure (>6.17 pg/g)=1; low exposure (≤6.17 pg/g)=0.

[c] Black=2; Dominican=1.

[d] Male=2; female=1.

[e] Measured with the Test of Nonverbal Intelligence, Second Edition,[42] natural logarithmically transformed.

[f] No high school degree=1; high school degree=0.

Logistic regression was used to estimate the adjusted risks of delays (MDI and PDI) as a function of prenatal CPF exposure. Covariates included in the analysis were gestational age, gender, ethnicity, maternal education, maternal intelligence, and home environment. No delays were observed in children up to 36 months of age. Highly exposed children had a higher odds of having mental delays at 36 months of age than children with lower exposure (OR = 2.37; 95% CI, 1.08 to 5.19) (Table C6). Similarly, but of greater magnitude, the OR for motor delays at 36 months was 4.52 (95% CI, 1.61 to 12.70) (Table C7).

TABLE C6. Logistic Regression Models Testing Effects of Chlorpyrifos on the Odds of Mental Delay at 12, 24, and 36 Months, Adjusted for Race, Gender, Gestational Age, Maternal Education, Maternal IQ, ETS Exposure, and Home Environment

Variable	Odds Ratio (95% CI)		
	Model 1:12 mo	Model 2:24 mo	Model 3:36 mo
Prenatal chemical exposures			
ETS[a]	0.58 (0.25–1.33)	1.26 (0.70–2.56)	1.29 (0.67–2.48)
Chlorpyrifos[b]	1.22 (0.48–3.06)	1.75 (0.86–3.60)	2.37 (1.08–5.19)
Covariates			
Race/ethnicity[c]	1.06 (0.48–2.38)	0.47 (0.26–0.87)	0.35 (0.17–0.72)
Gender[d]	1.66 (0.80–3.44)	1.68 (0.96–2.92)	1.90 (1.04–3.48)
Gestation age	1.00 (0.79–1.27)	0.89 (0.72–1.10)	0.81 (0.65–1.01)
Maternal IQ[e]	1.02 (0.98–1.05)	0.99 (0.96–1.01)	1.01 (0.99–1.04)
Low maternal education[f]	1.49 (0.69–3.22)	0.95 (0.52–1.73)	1.40 (0.74–2.67)
HOME score	0.96 (0.89–1.02)	0.96 (0.92–1.01)	0.90 (0.85–0.96)

Sample sizes for age-specific models ranged from 225 to 228 because of missed and/or unscorable assessments (<50).

[a] Prenatally exposed=1; not exposed=0.

[b] High exposure (>6.17 pg/g)=1; low exposure (≤6.17 pg/g)=0.

[c] Black=2; Dominican=1.

[d] Male=2; female=1.

[e] Measured with the Test of Nonverbal Intelligence, Second Edition,[42] natural logarithmically transformed.

[f] No high school degree=1; high school degree=0.

TABLE C7. Logistic Regression Models Testing Effects of Chlorpyrifos on the Odds of Psychomotor Delay at 12, 24, and 36 Months, Adjusted for Race, Gender, Gestational Age, Maternal Education, Maternal IQ, ETS Exposure, and Home Environment

Variable	Odds Ratio (95% CI)		
	Model 1:12 mo	Model 2:24 mo	Model 3:36 mo
Prenatal chemical exposures			
ETS[a]	0.95 (0.42–2.15)	0.77 (0.33–1.80)	1.80 (0.69–4.79)
Chlorpyrifos[b]	1.88 (0.78–4.53)	1.01 (0.37–2.76)	4.52 (1.61–12.70)
Covariates			
Race/ethnicity[c]	1.59 (0.69–3.67)	0.86 (0.36–2.09)	0.55 (0.19–1.54)
Gender[d]	0.91 (0.42–1.96)	0.74 (0.33–1.63)	1.89 (0.75–4.75)
Gestation age	1.00 (0.78–1.28)	0.88 (0.68–1.14)	0.97 (0.68–1.38)
Maternal IQ[e]	1.01 (0.98–1.04)	0.98 (0.95–1.02)	1.03 (0.99–1.06)
Low maternal education[f]	1.10 (0.49–2.50)	1.24 (0.54–2.81)	0.36 (0.12–1.10)
HOME score	0.99 (0.92–1.06)	1.01 (0.94–1.08)	0.94 (0.86–1.02)

Sample sizes for age-specific models varied from 227 to 228 because of missed and/or unscorable assessments (<50).
[a] Prenatally exposed = 1; not exposed = 0.
[b] High exposure (>6.17 pg/g) = 1; low exposure (≤6.17 pg/g) = 0.
[c] Black=2; Dominican = 1.
[d] Male=2; female = 1.
[e] Measured with the Test of Nonverbal Intelligence, Second Edition,[42] natural logarithmically transformed.
[f] No high school degree=1; high school degree = 0.

The results of logistic regression analyses examining the odds of behavioral disorders at 36 months of age are shown in Table C8. The adjusted analyses showed increases in the odds of having attention problems, attention deficit hyperactivity disorder (ADHD) problems and pervasive developmental disorder (PDD) problems with high exposure to CPF. Prenatal exposure to ETS was also significantly related to the odds of having ADHD problems. Although there was no evidence of interaction between ETS exposure and CPF exposure for these behavior outcomes, the authors note that the small number of subjects limited their ability to test these effects.

Table C8. Logistic Regression Models Testing Effects of Chlorpyrifos on the Odds of Behavioral Disorder at 36 Months, Adjusted for Race, Gender, Gestational Age, Maternal Education, Maternal IQ, ETS Exposure, and Home Environment (n = 228)

Variable	Odds Ratio (95% CI)		
	Attention Problems	ADHD Problems	PDD Problems
Prenatal exposures			
ETS[a]	2.81 (0.44–17.77)	8.10 (1.20–54.65)	0.75 (0.17–3.14)
Chlorpyrifos[b]	11.26 (1.79–70.99)	6.50 (1.09–38.69)	5.39 (1.21–24.11)
Covariates			
Race/ethnicity[c]	0.19 (0.02–1.61)	0.26 (0.04–1.82)	0.06 (0.01–0.65)
Gender[d]	2.84 (0.50–16.09)	1.05 (0.23–4.74)	2.40 (0.65–8.93)
Gestational age	0.62 (0.39–0.99)	0.62 (0.40–0.97)	0.81 (0.50–1.30)
Maternal IQ[e]	0.99 (0.94–1.06)	1.03 (0.97–1.09)	1.00 (0.95–1.05)
Maternal education[f]	0.22 (0.02–2.08)	0.35 (0.06–2.10)	1.75 (0.46–6.61)
HOME score	0.93 (0.80–1.08)	0.88 (0.77–1.01)	0.93 (0.82–1.05)

[a] Prenatally exposed = 1; not exposed = 0.
[b] High exposure (>6.17 pg/g) = 1; low exposure (≤6.17 pg/g) = 0.
[c] Black = 2; Dominican = 1.
[d] Male = 2; female = 1.
[e] Measured with the Test of Nonverbal Intelligence, Second Edition,[42] natural logarithmically transformed.
[f] No high school degree = 1; high school degree = 0.

Berkowitz et al., 2004. In utero pesticide exposure, maternal paraoxonase activity, and head circumference.

The study examined the effects of in utero pesticide exposure on fetal growth and neurodevelopment in a cohort of infants in New York City. The sample population included women who were primiparas with singleton births who had their first prenatal visit before 27 weeks gestation. Mothers and infants were excluded on a number of criteria including if the child was born at < 1,500 g or < 32 weeks of gestation. A total of 479 prenatal women were recruited. Of these, 75 were excluded due to a number of reasons (including medical complications, delivery of an infant with birth defects, inability to collect biologic specimens before birth, change of hospital, etc). The final sample size consisted of 404 births. During the third trimester a prenatal questionnaire was administered to the women to obtain information on pesticide and other environmental exposures, sociodemographics information, maternal health and lifestyle habits. Outcome information was acquired from the hospital database. During the third trimester maternal blood samples were taken and a urine sample was collected. Cord blood samples were obtained at birth. Urine samples were analyzed for TCPY, 3-phenoxybenzoic acid (PBA), a possible metabolite of several pyrethroid insecticides, and pentachlorophenol. The LOD for TCPY was 11.0 μg/L.

The proportion of TCPY values below the LOD was 57%. Urinary concentrations were corrected for creatinine levels. PON1 activity was categorized into low, medium or high based on its tertile distribution. The birth outcomes examined included birth weight, birth length, head circumference and gestational age.

The sociodemographic characteristics of these women are shown in Table C9. The predominant race/ethnicity was Hispanic, (49.8%), mostly Puerto Rican, the women were relatively young, and 46.8% were single. The median and interquartile range for TCPY were 7.6 ug/L, and 1.6 – 32.5, respectively (not corrected for creatinine). In the adjusted analyses, reported pesticide use was not associated with any of the birth outcomes. No significant associations were seen between any of the birth outcomes and TCPY, PBA, or PCP levels dichotimized above and below the LODs and adjusted for potential covariates.

Table C9. Distribution of maternal sociodemographic characteristics and pesticide use, Children's Environmental Health Study, Mount Sinai Hospital, 1998-2002 (total *n* = 404).

Characteristics	No. (%)
Maternal age (years)	
<20	143 (35.4)
20–24	132 (32.7)
25–29	44 (10.9)
30–34	63 (15.6)
≥35	22 (5.4)
Race/ethnicity	
White	85 (21.0)
African American	112 (27.7)
Hispanic	201 (49.8)
Other[a]	6 (1.5)
Marital status	
Married	116 (28.7)
Living with baby's father	99 (24.5)
Single/divorced/widowed/separated	189 (46.8)
Maternal education	
Lower/middle school	119 (29.5)
High school graduate	83 (20.6)
Some college	103 (25.6)
College graduate	98 (24.3)

Pesticide use by household member	186 (46.2)
Any reported indoor pesticide use	289 (71.5)

[a] Includes mixed race/ethnicity.

No significant associations were observed between the pesticide questionnaire data and the measured birth outcomes including birth weight, length, head circumference or gestational age. No significant associations were seen between TCPY levels above or below the LOD and adjusted birth weight or birth length. As seen in Table C10, there was a significant (p = 0.014) positive trend between maternal PON1 activity and head circumference in infants whose mothers had TCPY levels above the LOD. A similar trend was evident in the infants of mothers whose TCPY levels were below the LOD, and even though there were a larger number of subjects in this group and the measurements were similar, the trend was not statistically significant. The results were similar when maternal PON1 activity was examined in relation to head circumference, without consideration of TCPY levels. Mean levels were very close to those seen in Table C10 and the trend was significant (p = 0.004).

Table C10. Adjusted mean ± SD of fetal growth indices by tertiles of maternal paraoxonase activity and TCPY level, Children's Environmental Health Study, Mount Sinai Hospital, 1998–2002.

	Birth weight[a] (g)		Birth length[a] (cm)		Head circumference[a] (cm)	
	Mean ± SD	No.	Mean ± SD	No.	Mean ± SD	No.
TCPY < LOD						
Low PON	3,237 ± 456	76	50.3 ± 2.3	75	33.6 ± 1.8	76
Medium PON	3,255 ± 436	62	50.1 ± 2.2	62	33.7 ± 1.7	62
High PON	3,337 ± 444	71	50.3 ± 2.3	71	34.1 ± 1.7	70
TCPY > LOD						
Low PON	3,278 ± 395	47	50.9 ± 2.3	46	33.3 ± 1.5*	47
Medium PON	3,327 ± 406	57	51.0 ± 2.3	57	34.0 ± 1.5	57
High PON	3,270 ± 409	55	50.8 ± 2.4	55	34.1 ± 1.6	55

[a] Adjusted for race/ethnicity, infant sex, and gestational age. * p=0.014

(Related Study)

Wolff et al., 2007. Prenatal pesticide and PCB exposures and birth outcomes.

This study investigated the effects of in utero exposure to OPs and organochlorines on fetal growth in the prospective cohort of women enrolled at Mount Sinai Hospital during pregnancy. The details of this cohort were described under Berkowitz et al., (2004). This study examined birth outcomes including birth length, weight, ponderal index, gestational age, and head circumference in relation to exposure to the OPs, PCBs, DDE, and lead. However, unlike Berkowitz et al., (2004) where exposure was measured as urinary TCPY levels, in this study exposure to OPs was measured as total urinary

diethylphosphates (∑DEP), dimethylphosphates) and dialkylphophates (∑DAP). Maternal PON1, BuChE, and PON1Q192R gene variant were also determined. The sample size for this analysis was 404 infants. Urine samples with <20 mg/dL creatinine (n = 27) were excluded from the analyses. Models with DAPs included PON1 phenotype as tertiles or BuChE.

There was no difference in the levels of ∑DEP for any of the socio-demographic characteristics; however, race/ethnicity and marital status were associated with ∑DMP and ∑DAP, respectively. In examining fetal growth with exposure measures three inverse nonsignificant associations were seen: ∑DEP with birth weight, ∑DEP with ponderal index, and ∑DAP with head circumference. Similar to reported findings of Berkowitz et al., (2004), a significant association was observed between maternal PON1 activity and head circumference. Birth length was also found to be shorter by 0.68 ± 0.30 cm among mothers with PON192 RR than PON192QQ (p = 0.026). Based on the main effects observed with the DAPs, interactions between PON1 and the birth outcomes were examined. For birth weight the interaction terms PON1-∑DEP and PON192-∑DEP were not significant (Table C11). However, a 163 g deficit was seen in birth weight between the extremes of the interaction, i.e. fast activity-PON1 genotype/low ∑DEP and the slow-PON1/high ∑DEP (p = 0.042) (Table C11). A similar association was observed for the interaction of PON192 and ∑DEP (-199, p = 0.02). For birth length, there was an association with ∑DMP and birth length in mothers with the slowest PON1, whereby the mothers with the slowest PON1 and high ∑DMP had shorter babies (-0.9) than low ∑DMP (p = 0.032).

Table C11. Adjusted means ± SE of birth weight and length in relation to interactions between maternal urinary prenatal ∑DAP levels, PON192 genotype, and PON1 activity, Children's Environmental Health Study, Mount Sinai Hospital, 1998-2002

∑DEP and birth weight					
	Low maternal ∑DEP < median level		High maternal ∑DEP ≥ median level		
Maternal PON1 tertile*	*n*	Birth weight, g, mean ± SE	*n*	Birth weight, g, mean ± SE	*p* value for Δ-birth weight by ∑DEP within PON1
1st (slow)	60	3305 ± 53	53	3233 ± 56†	0.323
2nd	53	3348 ± 57	51	3282 ± 57	0.392
3rd (fast)	45	3396 ± 64†	56	3279 ± 54	0.138
	Low maternal ∑DEP < median level		High maternal ∑DEP ≥ median level		
Maternal *PON192***	*n*	Birth weight, g, mean ± SE	*n*	Birth weight, g, mean ± SE	*p* value for Δ-birth weight by ∑DEP within genotype
RR (slow)	39	3346 ± 69	55	3254 ± 63‡	0.291
RQ	84	3278 ± 46	66	3285 ± 50	0.907
QQ (fast)	33	3453 ± 60‡	42	3232 ± 52	0.005

∑DMP and birth length					
	Low maternal ∑DMP < median level		High maternal ∑DMP ≥ median level		
Maternal PON1 tertile§	*n*	Birth length, cm, mean ± SE	*n*	Birth length, cm, mean ± SE	*p* value for Δ length by ∑DMP within PON1
1st (slow)	61	51.1 ± 0.3	52	50.2 ± 0.3	0.032
2nd	55	50.3 ± 0.3	51	50.7 ± 0.3	0.258
3rd (fast)	49	50.4 ± 0.3‖	58	50.8 ± 0.3	0.418

∑DMP and birth length					
	Low maternal ∑DMP < median level		High maternal ∑DMP ≥ median level		
Maternal *PON192*¶	*n*	Birth length, cm, mean ± SE	*n*	Birth length, cm, mean ± SE	*p* value for Δ length by ∑DMP within genotype
RR (slow)	43	50.6 ± 0.4	53	49.9 ± 0.3‡‡	0.164
RQ	85	50.4 ± 0.3	70	50.7 ± 0.3	0.158
QQ (fast)	35	51.0 ± 0.3‡‡	41	50.8 ± 0.3	0.695

* *p* value for the interaction term in the model = 0.878.
† *p* value for extremes (third PON1/low ∑DEP *vs*. first PON1/high ∑DEP, 164g) = 0.042.
** *p* value for the interaction term in the model = 0.0755.
‡ *p* value for extremes (high *PONQQ*/low ∑DEP *vs*. low activity *PONRR*/high ∑DEP 199 g) = 0.020.
§ *p* value for the interaction term in the model = 0.036.
‖ *p* value for extremes (high PON1/low ∑DMP *vs*. low activity PON1/high ∑DMP, 0.3 cm) = 0.549.
¶ *p* value for the interaction term in the model = 0.230.
‡‡ *p* value for highlighted extremes (high *PONQQW*/low ∑DMP *vs*. low activity *PONRR*/high ∑DMP, 1.0 cm) = 0.019.
In the ∑DMP-greater-than-the-median group, length was shorter among the *RR* genotype than the *QR* ($p = 0.042$) or *QQ* ($p = 0.041$) genotypes.
The first tertile of PON1 and the *RR PON192* genotype are the slower activity groups.
Means adjusted for race, sex, gestational age, and creatinine level. Urinary metabolites included samples with creatinine >20 mg/dL.
Cutpoints for the *PON1* second tertile were >96 to <116.7 µg/mL/min.

(Related Study)

Engel et al., 2007. Prenatal organophosphate metabolite and organochlorine levels and performance on the Brazelton Neonatal Behavioral Assessment Scale in a multiethnic prenancy cohort.

This study was conducted in the Mount Sinai Children's Environmental Health Cohort to investigate the impact of pesticide and PCB exposure on child neurodevelopment. (Detailed methodology used in this cohort and demographics of this sample population were presented in Berkowitz et al. (2004)). Of 1,450 eligible women, 33% consented to participate (n = 479) and, after exclusions and loss to follow-up, etc., the final sample size was 404. However, for some of the analyses, specific BNBAS clusters, the sample size was as small as 144. Maternal blood and urine samples were obtained at a mean of 31.2 weeks gestation. Maternal urine samples were analyzed for six DAP metabolites and malathion dicarboxylic acid. Polychlorinated biphenyls and 1,1'-dichloro-2,2'-bis(4-chlorophenyl)ethylene (DDE) were measured in maternal blood. The BNBAS was administered before hospital discharge. The seven cluster method was used to score the BNBAS.

Women with prenatal total DEP levels above the median delivered infants who were 2.3 times more likely to have at least two abnormal reflexes (95% CI 1.1 to 5.0). Mothers with MDA levels above the median were also 3.6 times more likely to have at least two abnormal reflexes. Although no increased risk of abnormal reflexes were seen in association with DMP exposure, when PON1 levels were taken into consideration DMPs women in the lower tertile of PON1 expression had a significantly increased risk of abnormal reflexes. There were no adverse associations observed between PCBs and DDE exposure and any behavioral measures.

Eskenazi et al., 2004. Association of in utero organophosphate pesticide exposure and fetal growth and length of gestation in an agricultural population.

This longitudinal cohort study was conducted in an agricultural area in the central valley of California between October 1999 and October 2000. Pregnant women at least 18 years of age and less than 20 weeks gestation were enrolled at a county hospital or at one of five local clinics. The participation rate was 53.2% with women who declined to participate being similar to study subjects in age and parity but more likely to be English speaking and born in the United States. The final sample size was 488. Women were interviewed twice during pregnancy and again after delivery. The interviews occurred at a mean of 13 weeks gestation (range, 4–29) and 26 weeks gestation (18–39). Measures of exposure to pesticides were collected from mothers during pregnancy and at delivery and from the umbilical cord and included: six dialkyl phosphate metabolites, three DMP and three DEP which were summed to obtain total DAPs; seven different pesticide-specific metabolites measured in maternal urine during pregnancy (including MDA, TCPY, 4-nitrophenol (PNP); cholinesterase in whole blood; and butyryl cholinesterase in plasma). Outcome measures included length of gestation, birth weight, length, head

circumference, and ponderal index. The women in the study averaged 25 years of age, two thirds were multiparous, 80% were married, 84% were born in Mexico, and almost all the women were living within 200% of the poverty level. Few women reported smoking (6%), using drugs (2%), or consuming alcohol during pregnancy (1%). Some women were working in agricultural fields during pregnancy (~28%) with about another 14% working in agricultural-related jobs. Most women had agricultural workers living in their homes during their pregnancy (85%).

Pesticide measures, taken twice during pregnancy, were averaged for each woman. The LOD for TCPY was 0.26 ug/L with 76.3% of samples being over the LOD. The median was 3.3 ug/L (range 0.2–56.1). Linear and logistic analyses were conducted with all metabolites both adjusted and unadjusted for creatinine. For these analyses women were assigned to one of three groups with respect to TCPY measures: no detectable levels, detectable levels below the median of the detectable levels and detectable levels above the median.

No adverse association was found between maternal urinary levels of TCPY and parameters of fetal growth or gestational age (Table C12). Decreases in gestational duration were associated with two measures of *in utero* pesticide exposure: increased levels of metabolites of urinary DMP compounds were associated with a decrease of three days in gestational duration (B = -0.41 weeks per $\log_{10}$ unit increase, 95% CI -0.75 to -0.02; p = 0.02), and decreased levels of cholinesterase in umbilical cord blood was associated with shortened length of gestation by an average of 0.34 weeks (p = 0.001) (Table C12 and C13). The decrease in length of gestation was most clearly related to increasing exposure levels in the latter part of pregnancy. Increases in birth length and head circumference were observed in association with increases in average DAP concentration. After controlling metabolite levels for creatinine, the findings of increased birth length was no longer significant; however, increased head circumference and decreased gestational duration remained significant.

Table C12. Association of average urinary metabolites of organophosphate pesticides measured at two points during pregnancy[a] with length of gestation and fetal growth: CHAMACOS study, Salinas Valley, California, 2000-2001.

Metabolite	No.[b]	Length of gestation (weeks)[c]		Birth weight (g)[d]		Length (cm)[d]		Head circumference (cm)[d]		Ponderal index (g/cm^3)[d]	
		β (95% CI)	*p*-Value	β (95% CI)	*p*-Value	β (95% CI)	*p*-Value	β (95% CI)	*p*-Value	β (95% CI)	*p*-Value
Dialkyl phosphate metabolites (nmol/L, $\log_{10}$ scale)											
Dimethyl phosphates	485	-0.41 (-0.75–-0.07)	0.02**	41 (-40–122)	0.32	0.42 (-0.07–0.91)	0.09*	0.25 (-0.02–0.52)	0.07*	-0.03 (-0.10–0.04)	0.45
Diethyl phosphates	486	-0.16 (-0.53–0.22)	0.41	52 (-40–144)	0.26	0.40 (-0.15–0.94)	0.16	0.28 (-0.02–0.59)	0.07*	-0.01 (-0.09–0.07)	0.74
Total dialkyl phosphates	485	-0.20 (-0.55–0.15)	0.27	42 (-46–131)	0.35	0.52 (-0.01–1.05)	0.06*	0.32 (0.03–0.62)	0.03**	-0.04 (-0.12–0.04)	0.28
Pesticide-specific metabolites (µg/L)											
MDA											
No detectable levels	233	Referent		Referent		Referent		Referent		Referent	
Detectable levels < median	74	-0.13 (-0.55–0.30)	0.55	-45 (-154–63)	0.41	-0.53 (-1.18–0.11)	0.11	-0.16 (-0.52–0.19)	0.37	0.05 (-0.05–0.14)	0.33
Detectable levels ≥ median	75	-0.21 (-0.62–0.20)	0.32	56 (-49–161)	0.29	0.14 (-0.48–0.76)	0.66	0.11 (-0.24–0.46)	0.53	0.02 (-0.07–0.12)	0.60
TCPY											
No detectable levels	41	Referent		Referent		Referent		Referent		Referent	
Detectable levels < median	220	-0.17 (-0.74–0.40)	0.55	-6 (-138–126)	0.93	0.09 (-0.70–0.87)	0.83	0.06 (-0.37–0.49)	0.78	-0.01 (-0.12–0.11)	0.89
Detectable levels ≥ median	221	-0.06 (-0.63–0.20)	0.84	27 (-106–159)	0.69	0.44 (-0.35–1.22)	0.27	0.04 (-0.39–0.47)	0.85	-0.04 (-0.16–0.08)	0.50
PNP											
No detectable levels	124	Referent		Referent		Referent		Referent		Referent	
Detectable levels < median	179	-0.37 (-0.76–0.02)	0.06*	34 (-57–125)	0.46	0.60 (0.06–1.13)	0.03**	0.18 (-0.12–0.48)	0.23	-0.08 (-0.16–0.0)	0.06
Detectable levels ≥ median	179	0.18 (-0.21–0.57)	0.36	49 (-42–140)	0.29	0.41 (-0.13–94)	0.14	0.29 (-0.01–0.58)	0.06*	-0.03 (-0.11–0.05)	0.48

[a]Urinary metabolite levels are not adjusted for urinary creatinine concentration. [b]Numbers vary slightly for different outcomes due to missing data. [c]Models adjusted for timing of urine collection, timing of entry into prenatal care, maternal age, parity, country of birth, and poverty level. [d]Models adjusted for timing of urine collection, timing of entry into prenatal care, maternal age, parity, infant sex, country of birth, weight gain, BMI, poverty level, gestational age, and (gestational age)^2. *p<0.10; **p<0.05.

Table C13. Association of ChE and BuChE in maternal blood during pregnancy and at delivery and in umbilical cord blood with length of gestation and fetal growth: CHAMACOS study, Salinas Valley, California, 2000-2001.

		Length of gestation (weeks)[b]		Birth weight (g)[c]		Length (cm)[c]		Head circumference (cm)[c]		Ponderal index (g/cm^3)[c]	
Parameter	No.[a]	β (95% CI)	*p*-Value	β (95% CI)	*p*-Value	β (95% CI)	*p*-Value	β (95% CI)	*p*-Value	β (95% CI)	*p*-Value
ChE (µmol/min/mL)											
Maternal blood, pregnancy	340	0.01 (-0.15–0.17)	0.87	8 (-35–52)	0.71	0.05 (-0.20–0.29)	0.72	0.06 (-0.09–0.21)	0.45	0.00 (-0.03–0.03)	0.90
Maternal blood, delivery	357	0.09 (-0.04–0.23)	0.16	6 (-30–43)	0.73	0.05 (-0.17–0.27)	0.67	-0.07 (-0.19–0.05)	0.27	0.00 (-0.03–0.03)	0.95
Cord blood	292	0.34 (0.13–0.55)	0.001**	12 (-46–70)	0.68	-0.01 (-0.36–0.34)	0.95	-0.04 (-0.23–0.14)	0.65	0.02 (-0.03–0.07)	0.43
BuChE (µmol/min/mL)											
Maternal blood, pregnancy	340	-0.2 (-0.64–0.27)	0.42	56 (-67–179)	0.37	0.07 (-0.63–0.78)	0.83	0.12 (-0.31–0.56)	0.58	0.03 (-0.06–0.12)	0.51
Maternal blood, delivery	357	-0.1 (-0.48–0.36)	0.78	-90 (-206–25)	0.13	0.05 (-0.65–0.75)	0.89	-0.07 (-0.45–0.31)	0.73	-0.07 (-0.06–0.03)	0.16
Cord blood	292	-0.2 (-0.78–0.32)	0.41	111 (-35–257)	0.14	0.23 (-0.65–1.12)	0.6	-0.03 (-0.50–0.45)	0.91	0.05 (-0.07–0.17)	0.45

[a] Numbers vary slightly for different outcomes due to missing data. [b] Models adjusted for timing of urine collection, timing of entry into prenatal care, maternal age, parity, country of birth, and poverty level. [c] Models adjusted for timing of urine collection, timing of entry into prenatal care, maternal age, parity, infant sex, country of birth, weight gain, BMI, poverty level, gestational age, and (gestational age)^2. * *p<0.10;* ** p<*0.05.*

(Related Study)

Young et al., 2005. Association between in utero organophosphate pesticide exposure and abnormal reflexes in neonates.

As part of the CHAMACOS study this article examined the association between in utero and early postnatal OP exposure and neurobehavioral function in the cohort of women in the Salinas Valley of California. A description of the cohort was presented under Eskenazi et al., (2004). A total of 601 women were enrolled in the study between 1999 and 2000 with 528 being followed through delivery of a live born infant. A Brazelton Neonatal Behavioral Assessment Scale (BNBAS) was performed on 421 infants; however, the final sample size was 381 infants after the following exclusions: eight twins; 27 preterm infants and 5 infants whose BNBAS assessments were conducted more than 2 months after delivery. Administration of the BNBAS is appropriate up to the second month of life. Interviews during pregnancy were conducted at an average of 14 and 26 weeks gestation and again after delivery at an average of 7 days postpartum. The BNBAS was administered once to each infant.

Exposure was assessed by measurement of urinary DAP metabolites, including three dimethylphosphate metabolites (dimethylphosphate (DMP), dimethlyldithiophosphate (DMDTP), and dimethylthiophosphate (DMTP)), and three diethylphosphate metabolites (diethylphosphate (DEP), diethyldithiophosphate (DEDTP) and diethylthiophosphate (DETP)). Maternal urine specimens were generally collected at the time of the prenatal and postnatal interviews. The post delivery urines were collected within one week of delivery for 73% of the sample, with the remainder up to 176 days afterwards. The BNBAS consists of 28 behavioral items scored on a nine-point scale and 18 reflex items scored on a four-point scale. The scores were reduced to seven clusters. Higher scores on the behavioral clusters indicate more optimal functioning while higher scores on the reflex cluster indicate less optimal functioning.

Average median levels of total DAP, DMP and DEPs in maternal urine from the two pregnancy measurements were 132, 97 and 21 nmol/L, respectively, while the median post-delivery measurements were higher at 222, 160, and 27 nmol/L, respectively. The two measures of metabolite levels during pregnancy were not significantly correlated with each other or with the post-delivery measure. The median age at administration of the BNBAS was 3 days.

A significant association was seen between the average of urinary metabolite levels measured during pregnancy and the number of abnormal reflexes (expressed in nmol/L on a log scale) (total DAP (B = 0.23 (95% CI, 0.05 to 0.41), DMP (B = 0.18, 95% CI 0.02 to 0.34) and DEP (B = 0.22, 95% CI 0.04 to 0.40) (all associations were statistically significant at $p < 0.05$). No other significant associations were observed between these metabolite levels and BNBAS performances based on the clusters. In stratifying by age at assessment the same analysis found a significant association between DEP and the autonomic cluster in infants assessed within three days of delivery. This association,

however, was in the opposite direction from the hypothesis of a detrimental effect of exposure to OPs. No significant associations were observed for any other clusters. In infants assessed after three days of delivery the results were similar to those seen for the entire sample population where a detrimental association was observed between total DAP, DMP and DEPs metabolite measures and reflex functioning. No significant associations were observed for any other clusters.

In infants older than 3 days there was a significant trend of increasing proportion of more than three abnormal reflexes with increasing metabolite levels when metabolite levels were examined by quintiles. Using logistic regression, the odds of having more than three abnormal reflexes significantly increased with average urinary metabolite levels during pregnancy (OR = 4.9 for total DAP (95% CI, 1.5 to 16.1), OR = 3.2 for DMP (95% CI, 1.1. to 9.8), and OR = 2.4 for DEP (95% CI, 1.2 to 9.9) (all associations were statistically significant at $p < 0.05$)). This was based on a one unit change on the $\log_{10}$ scale, or a 10 fold increase such as from 10 to 100 nmol/L.

Eskenazi et al., 2007. Organophosphate pesticide exposure and neurodevelopment in young Mexican-American children.

This study investigated neurodevelopment and behavior in the CHAMACOS cohort by examining the association between maternal prenatal and child DAP levels with performance on the Bayley Scales through 2 years of age and with maternal reports of behavior using the CBCL at two years of age. Details of the methods for the CHAMACOS cohort were presented above under Eskenazi et al., (2004). A total of 531 women were followed to delivery of a live-born neonate. After exclusion based on a number of criteria or missing data the sample size varied from 372 to 396 infants for the Bayley analyses, with 356 mothers completing the CBCL at the 24 month visit.

Women were interviewed twice during pregnancy (at approximately 14 and 26 weeks gestation), shortly after delivery, and when the children were 6, 12, and 24 months old. Mothers completed the Peabody Picture Vocabulary Test to assess scholastic abilities at the six-month visit, as well as the Center for Epidemiologic Studies Depression Scale at the 12-month visit. The HOME instrument (as described under Rauh et al.) was completed at 24 months. The Bayley Scales of Infant Development was used to assess the developmental functioning of infants and young children. The CBCL was administered to mothers to assess 2-year-olds' emotional/behavioral problems and competencies. The following metabolites were measured in maternal urine: six nonspecific OP DAPs; three DMPs; three DEPs; MDA; and TCPY. Nonspecific DAPs were summed and transformed to the $\log_{10}$ scale. Multiple regression models were constructed for both MDI and PDI at 6, 12, and 24 months of age.

The demographics of the population are essentially the same as described in Eskenazi et al., 2004. Approximately 50% of mothers had symptoms of depression 1 year postpartum. Almost all the children were breast-fed (96.6%), with half breast-fed for $\geq$ 6 months. Ninety-one percent of mothers had detectable TCPY levels with an average

median level of 3.54 ug/L for the first and second pregnancy measurements. Levels of DAPs in children increased with age; the geometric mean levels at 6, 12, and 24 months were 45.5, 59.5, and 70.9 nmol/L, respectively. The Bayley PDI mean scores (± SD) at 6, 12, and 24 months were 96.4 ± 10.6, 106.0 ± 12.6 and 97.5 ± 10.6, respectively. The MDI scores at 6, 12, and 24 months were 95.7 ± 7.0, 100.6 ± 8.6 and 85.9 ± 11.8, respectively. At 24 months there was a large increase in the proportion of MDI scores <85, 50% at 24 months compared with 3–4% at 6 and 12 months. On the CBCL a similar proportion of children scored in the clinical range for attention problem syndrome and ADHD compared with the national reference sample (~2–4%). However, a much larger proportion of children scored in the clinical range on the DSM-oriented PDD, 14.4% compared with ≤ 3.3% in the national sample (p<0.0001). No associations were observed between TCPY levels and MDI or PDI scores, or with CBCL outcomes (Table C15). Children with higher prenatal and postnatal DAPs had a higher risk of PDD (prenatal, OR = 2.3; 95% CI, 1.0 to 5.2, p = 0.005) (Table C14). Elevated ORs were also observed between postnatal levels of DMP and DEP metabolites and PDD, while only DMP levels had significantly greater ORs for prenatal levels. Pregnancy DAP levels were generally negatively associated with MDI although not significantly; however, child measures were generally positively associated with MDI.

However, the authors caution that the negative associations between prenatal DAP levels and mental development should be interpreted with caution since the direction of postnatal associations was positive. One possible explanation for the postnatal findings is that the children with higher mental development scores were also more interactive with their enviroment and as a result came into contact with more pesticide residues (Eskenazi et al., 2008).

Table C14. Adjusted[a] ORs (95% CIs) for syndrome scores in the clinical (CL) or borderline clinical (BL) range on the CBCL at 24 months of age for DAPs urinary metabolites.

CBCL	Attention (BL)[a]	ADHD (BL)[a]	PDD (CL)[a]
Total DAPs			
Prenatal	0.77 (0.27–2.24)	1.34 (0.50–3.59)	2.25 (0.99–5.16)**
Child	1.41 (0.75–2.64)	1.11 (0.61–2.03)	1.71 (1.02–2.87)**
DMs			
Prenatal	0.78 (0.31–1.96)	1.27 (0.53–3.04)	2.19 (1.05–4.58)**
Child	1.54 (0.85–2.76)	1.10 (0.63–1.94)	1.52 (0.94–2.45)*
Des			
Prenatal	0.78 (0.26–2.31)	0.59 (0.21–1.68)	0.88 (0.37–2.07)
Child	1.02 (0.61–1.71)	1.18 (0.72–1.94)	1.72 (1.12–2.64)**

[a]Models adjusted for sex, exact age at assessment, breast-feeding duration, HOME score, household income above poverty threshold, parity, maternal PPVT, and maternal depression. *p≤0.10; **p≤0.05

Table C15. Adjusted[a] coefficients (β) (95% CIs) in points on the MDI and PDI of the Bayley Scales for prenatal urinary metabolites specific to malathion (MDA) and chlorpyrifos (TCPY)

Baylay Scales of Infant Development	MDI[a]	PDI[a]
MDA		
6 months	—	—
< LOD (reference)	0.98 (-0.85 to 2.81)	0.42 (-2.34 to 3.18)
< Median detected	-0.25 (-2.10 to 1.60)	-1.45 (-4.12 to 1.32)
≥ Median detected		
12 months		
< LOD (reference)	—	—
< Median detected	0.95 (-1.55 to 3.46)	-0.53 (-4.05 to 3.00)
≥ Median detected	2.40 (-0.13 to 4.94)	0.75 (-2.81 to 4.31)
24 months		
< LOD (reference)	—	—
< Median detected	-1.09 (-4.51 to 2.32)	-0.73 (-3.87 to 2.41)
≥ Median detected	0.24 (-3.03 to 3.52)	0.33 (-2.68 to 3.35)
TCPY		
6 months		
< LOD (reference)	—	—
< Median detected	0.24 (-2.12 to 2.60)	-0.56 (-4.03 to 2.91)
≥ Median detected	0.08 (-2.29 to 2.44)	-0.21 (-3.69 to 3.27)
12 months		
< LOD (reference)	—	—
< Median detected	-0.45 (-3.67 to 2.76)	-0.70 (-5.26 to 3.86)
≥ Median detected	-0.65 (-3.88 to 2.58)	-1.62 (-6.20 to 2.96)
24 months		
< LOD (reference)	—	—
< Median detected	-1.02 (-5.34 to 3.31)	-2.65 (-6.50 to 1.21)
≥ Median detected	-1.94 (-6.26 to 2.37)	-2.72 (- 6.57 to 1.12)

[a]Models adjusted for psychometrician, location, exact age at assessment, sex, breast-feeding duration, HOME score, household income above poverty threshold, parity, and maternal PPVT.

Rull et al., 2006. Neural tube defects and maternal residential proximity to agricultural pesticide applications.

This investigation pooled study populations from two case-control studies of neural tube defects (NTD) conducted in California. The studies combined pesticide-use report data and land-use data to determine pesticide exposure in these infants and fetuses born between 1987 and 1991. The study investigated whether maternal residential proximity to specific agricultural pesticide applications was associated with anencephaly and spina bifida.

Cases, including elective terminations, were confirmed diagnoses of anencephaly, spina bifida cystica, craniorrhachischisis, and iniencephaly. Unmatched controls were randomly sampled from all livebirths (with no structural congenital anomalies diagnosed before their first birthday). Information was collected on residential, medical, reproductive, occupational, nutritional, and family history, as well as on sociodemographic and lifestyle factors. Interviews over the phone were conducted on 265 mothers of NTD cases (84% of those eligible) and 481 mothers of controls (74% of those eligible) from the first study on average 3.8 years after the date of delivery. In-person interviews were conducted in the second study in 538 mothers of NTD cases (88% of those eligible) and 539 mothers of controls (88% of those eligible) at an average of five months after the date of delivery. A geographic metric was developed to evaluate potential exposures by linking land-use survey data of crops from the California Department of Water Resources and pesticide use data from the California Department of Pesticide Regulation. All the information, including geocoded residential addresses, were linked by geographic information system software and 500 and 1,000 meter circular buffers were drawn around each residence. Mothers were considered exposed to a pesticide if any crop type within the buffer was treated with the agent.

Odds ratios were computed for fifty-nine specific pesticides to which at least four cases and four controls were exposed using various logistic regression techniques. Using a single pesticide logistic regression model adjusted for covariates, elevated ORs were observed for CPF as well as several other pesticides including napropamide, benomyl, 1,3-dichloropropene, methomyl, dimethoate, disulfoton, naled, and fenbutatin-oxide. However, for conventional and hierarchical logistic regression using multiple pesticide models to adjust for multiple comparisons and correlated pesticide exposures, the ORs for CPF were not found to be elevated. Effect estimates remained elevated for benomyl and naled. In addition, elevated risks of NTDs were associated with exposure to combinations or categories of pesticides including OP pesticides.

Limitations of this study include the potential misclassification of exposure, and recall bias. Although some of the findings of this study suggest that pesticide mixtures applied in combination may increase the risk of NTD the low prevalence of exposure for any specific pesticide limited the ability to evaluate these potential synergistic effects.

Related Studies

Searles Nielsen et al., 2005. Risk of brain tumors in children and susceptibility to organophosphorus insecticides: the potential role of paraoxonase (PON1).

This study examined whether two common PON1 polymorphisms, C-108T and Q192R, are associated with the occurrence of childhood brain tumors (CBTs). The subjects for this population-based study of 66 cases and 236 controls were drawn from a previous case-control study of primary tumors of the brain, cranial nerves, or meninges. Cases were diagnosed at < 20 years of age in 1984–1991 while residing in Seattle-Puget Sound

area of Washington State covered by a population-based cancer incidence registry. Controls from the same area were identified by random digit dialing and were frequency matched to cases 2:1 by sex and age. A subset of this sample had been born after 1977, a time when dried blood spot cards were archived. Cards were located for 66 (94%) of the eligible cases and 137 (86%) of the eligible controls. An additional anonymous 100 controls that were randomly sampled for a pilot study were included to supplement the control group of this study. Approximately half of the cases had astroglial tumors, while the remainder included primitive neuroectodermal tumors (PNETs) and a heterogeneous group of other tumors. Most cases (71%) were diagnosed before the age of 5 years. Fewer mothers of cases (31%) than controls reported that the children's homes had ever been chemically treated for pests during pregnancy or childhood before the diagnosis date. Mothers were interviewed using a structured questionnaire that included questions about home pesticide treatment, defined as chemical treatment of the house for pests such as termites, fleas, ants, cockroaches or silverfish, during the index pregnancy and/or childhood up to the diagnosis date (cases) or similar reference date (controls).

Proportionally more cases (26%) than controls (17%) were homozygous for the inefficient PON1 promoter allele (PON1-108T). The risk of CBT was not significantly increased with increasing PON1-108T alleles (Table C16).

Table C16. Risk of CBT in relation to *PON1* C-108T and Q192R polymorphisms [*n* (%)].

	All subjects			Subjects with white parents[a]		
PON1 genotype	Cases (*n*=66)	Controls (*n*=236)	OR (95% CI)[b]	Cases (*n*=51)	Controls (*n*=125)	OR (95% CI)[b]
C-108T: PON1 promotion						
TT (inefficient)	17 (26)	39 (17)	2.1 (0.9–4.7)	14 (27)	22 (18)	2.6 (1.0–6.9)
CT (intermediate)	34 (52)	125 (53)	1.4 (1.0–2.2)	28 (55)	66 (53)	1.6 (1.0–2.6)
CC (efficient)	15 (23)	72 (31)	1.0 (reference)	9 (18)	37 (30)	1.0 (reference)
Q192R: $PON1_{R192}$isoform						
QQ (none)	32 (48)	100 (42)	1.5 (0.6–3.4)[c]	27 (53)	58 (46)	1.6 (0.5–4.6)
QR (some)	28 (42)	105 (44)	1.2 (0.8–1.9)[c]	21 (41)	57 (46)	1.3 (0.7–2.1)
RR (all)	6(9)	31 (13)	1.0 (reference)	3 (6)	10 (8)	1.0 (reference)

[a] Biologic mother and father both reportedly non-Hispanic and white; excludes 3 cases, 1 interviewed control, and 100 anonymous controls for whom father's race/ethnicity unknown. [b] For individual genotypes, with each polymorphism modeled linearly (0, 1, or 2 $PON1_{-108T}$ alleles; 0, 1, or 2 $PON1_{192Q}$ alleles) using logistic regression. [c] Adjusted for $PON1_{C-108T}$.

The strongest association, which was statistically significant, was seen in relation to the PNET histologic tumor type (for each additional PON1-108T allele: OR = 2.4; 95% CI, 1.1 to 5.4; p-value for trend = 0.03, based on 15 PNET cases, including 6 PON1-108TT homozygotes and 7 heterozygotes). No indication of an interaction between these two PON1 polymorphisms was seen in the logistic regression models (p = 0.75). Thus, these results suggest that having an inefficient PON1 promoter allele is associated with an increased risk of CBT. The limitations of this study include the small number of subjects and no direct measurement of exposure to CPF.

Case Reports

Two case reports regarding exposure to CPF and adverse developmental outcomes were also identified.

Sherman, 1996. Chlorpyrifos (Dursban) – associated birth defects: report of four cases.

In this case report multiple birth defects were noted in four children. All four children (two children were siblings) had ventricular, eye, and palate defects, as well as growth retardation. Three of the children had numerous defects including hydrocephaly, microcephaly, mental retardation, blindness as well as hypotonia, wide-spread nipples, and deformities of the teeth, external ears, and external genitalia. Three mothers were reportedly exposed to Dursban, a commercial product containing CPF, in their homes during pregnancy, with a fourth mother exposed at work. Some information concerning the timing of exposure during pregnancy was included in the report as during the first trimester. However, limited specific information was included concerning exposure.

Sebe et al. (2005). Organophosphate poisoning associated with fetal death: a case study.

This is a case report of a 23 year old woman who attempted suicide by ingesting "an excess amount" of Sarban2 dust (chlorpyrifos). She was taken to a rural hospital 2 hours after taking the chlorpyrifos because "she could not feel her baby's movements". She was hospitalized for 8 hours after gastric lavage and was diagnosed by fetal ultrasound with in utero fetal death at 19 weeks gestation. She was then transported to the emergency department at a university medical center. The fetus was delivered 12 hours after administration of 200 mg of misoprostol.

Chlorpyrifos was measured at 264 ppb in a fetal blood sample. The autopsy report noted that the fetus had traumatic lesions, tool wounds or morphological defects, but did not give any further details. The authors concluded that death was from CPF. The case study concluded that the report by the mother "of not feeling the baby's movements 2 hours after ingestion of the toxin, plus the autopsy finding of suppressed maternal serum pseudochloinesterase levels, high levels of CPF detection in fetal blood and lack of any macroscopic anomalies, lead us to conclude that CPF is fetotoxic and that it may have been the cause of fetal death in this case". In addition, the authors suggested that the mechanism of fetal death may have been fetal bradycardia and/or placental insufficiency because of maternal bradycardia.

C.2. Animal studies

C.2.1. Overview

Several studies of CPF developmental toxicity in animals were found in the literature. These included studies conducted to meet with regulatory requirements as well as studies in the published literature. Only those studies with exposure levels that contribute to hazard identification have been included. Studies with only high levels of exposure that caused excessive maternal toxicity have been omitted. Of the studies included, some reported reproductive or developmental toxicity, with endpoints such as resorptions, decreases in fetal weight and long-term effects on brain and behavior while others did not report reproductive or developmental toxicity. In addition, related articles were also identified and data from these are summarized below.

A glossary of some of the terms commonly used in studies evaluating neurobehavioral function in animals has been included at the end of the document in Appendix 1.

Chlorpyrifos affects the nervous system by reversibly inhibiting the activity of cholinesterase (ChE), an enzyme necessary for the proper functioning of the nervous system. Inhibition of ChE is the most sensitive effect in all animal species evaluated and in humans, regardless of exposure duration. In animals, significant inhibition of plasma and RBC ChE occur at doses below those that cause brain ChE inhibition. In animals, significant plasma and RBC ChE inhibition has been observed at oral doses as low as 0.025 to 0.3 mg/kg/day following exposure for two weeks to two years, while significant brain ChE inhibition has been observed at oral doses as low as 1 mg/kg-day following exposure for two weeks in pregnant rats (Hoberman, 1998). Female rats and especially pregnant rats appear to be more sensitive than adult male rats to cholinesterase inhibition (Hoberman, 1998; Moser et al., 1998a; Mattsson et al., 2000). Data from two human studies suggest that humans (adult males) are similarly sensitive and possibly more sensitive than rats and dogs following acute and short-term oral exposure and acute dermal exposure based on plasma ChE inhibition and/or possible clinical signs. It is likely that the human sensitivity for ChE inhibition relative to rats (but not dogs) is due to species differences in the constituents of plasma ChE between rats and humans. For example, in the toxicology chapter of the U.S. EPA Reregistration Eligibility Decision (RED) it is stated that in rats, plasma ChE consists of approximately a 60:40 ratio of acetyl cholinesterase (AChE) and butyryl cholinesterase (BuChE), while in most humans and dogs, plasma ChE is predominately as BuChE, which is more sensitive to inhibition than AChE (U.S. EPA 2000).

Given the predominant mode of action of OPs, studies examining the effects of CPF on AChE have been used in determining the relationship between ChE inhibition and developmental toxicity. From the work by Young and Grandjean 1988 (Table C17), exposure to 0.05 mg/kg-day in the diet of Fischer rats for twelve months demonstrated an inhibition of brain ChE that was significant in males (6%). In exposure of dams, 3 mg/kg-day CPF orally was the lowest dosage of CPF that would result in significant (>10%) inhibition of brain ChE in the offspring on PND 1 and 7mg/kg-day was the highest dosage that would not result in overt toxicity to either the dams or their offspring, defined as a significant decrease in body weight gain based on preliminary data and literature values (Lassiter et al., 1998; Mattsson et al., 2000).

Table C17. ChE Inhibition in the Rat

	LOEL (mg/kg-day)			NOEL (mg/kg-day)		
	PLASMA	RBC	BRAIN	PLASMA	RBC	BRAIN
SUB-CHRONIC - 13 weeks Szabo 1988	5 (95%)	5 (49%)		1	1	
CHRONIC - 2 years McCollister 1971		3 (76%)	3 (20%)	0.01	0.03	1
6 months Young and Grandjean 1988	1 (39%) M 1 (65%) F	1–24%M 10–41%F	0.05–6%M* 1–5%F*	0.1 M 0.1 F	0.1 M 1 F	NA –M 0.1 F

*12 month

It is noted that the State of California requires removal of workers from pesticide handling activities on the basis of plasma and erythrocyte activity ChE monitoring. The regulation states specifically: "If plasma cholinesterase falls to 60% or less of the baseline, or if red blood cell cholinesterase falls to 70% or less of baseline, the employee shall be removed from further exposure until cholinesterase values return to 80percent or more of their respective baseline values."

Also in a recent review of the California program (Fillmore and Lessenger, 1993), researchers found that plasma cholinesterase inhibition was predictive of pesticide-related illness. The researchers state this point as follows: "The relative risk of pesticide poisoning was increased in workers whose initial baseline plasma levels [of cholinesterase] were low, or if their levels had already dropped to 60–80% of their baseline previously in the season" (Fillmore and Lessenger, 1993). Accordingly, the developmental effects of the chemical can be evaluated in relationship to its effects on cholinesterase inhibition.

C.2.2. Developmental Toxicity Studies

Thompson et al., 1971
Three Generation Reproduction and Teratology Study in the Rat Following Prolonged Dietary Exposure to Dursban, O,O-Diethyl O-3,5,6-Trichloro-2-Pyridyl Phosphorothioate

Summary of the study was obtained from the California Department of Pesticide Regulation (CDPR). The purity and grade of CPF was not specified. The doses for the main portion of the reproduction study were 0, 0.1, 0.3, and 1.0 mg/kg-day in diet. The ChE inhibition NOEL was 0.3 mg/kg-day and general adult toxicity NOEL was 1.0 mg/kg-day at highest dose tested. The NOEL for reproductive system toxicity was 0.3 mg/kg-day based on slightly increased pup mortality in first 5 days post-partum.

Table C18. Viability Index for the three generations of rats maintained on dietary levels of Dursban

	Viability Index					
Dursban	P1		P2		P3	
(mg/kg-day)	F1A	F1B	F2A	F2B	F3A	F3B*
0.00	94	90	71	87	91	--
0.03	97	96	--	--	--	--
0.10	96	92	77	92	95	--
0.30	91	94	74	91	90	--
1.00	--†	--	65	73	84	--

Viability index = Number of rats pups alive at PND 5 x 100 /Number of pups born alive
†Blank values indicate treatment groups not included in these segments of the study.
*F3B litters utilized on the teratogenic segment of the study.

Deacon et al., 1979
The Effects of Orally Administered Chlorpyrifos on Embryonal and Fetal Development in Mice.

Chlorpyrifos (presumed technical) was given to mice at 0, 0.1, 1, 10, and 25 mg/kg-day by gavage. Cholinergic effects such as salivation, tremors, etc. were noted at 10 mg/kg-day leading to a maternal functional NOEL of 1 mg/kg-day. However, based on significant inhibition of maternal plasma ChE at 1 mg/kg-day, the maternal NOEL was 0.1 mg/kg-day. Developmental toxicity as evidenced by decreased fetal length and weight and delayed ossification of skull and sternebrae at 25 mg/kg-day resulted in a developmental NOEL = 10 mg/kg-day.

Ouellette et al., 1983
Chlorpyrifos: Oral Teratology Study in Fischer 344 Rats.

In this study, CPF (96.6%) was administered to Fischer 344 rats at 0, 0.1, 3.0, and 15 mg/kg-day via gavage. This study was based on a pilot study where rats were given 0, 3, 10, and 30 mg/kg-day by gavage in cottonseed oil. In the pilot study 30 mg/kg-day was severely toxic to dams resulting in maternal deaths, typical cholinergic signs, and a high number of resorptions. Slightly matted haircoat and slight enlargement of adrenals were observed at 15 mg/kg-day. The developmental toxicity NOEL was 15 mg/kg-day, the high dose tested and the maternal NOEL was 0.1 mg/kg-day based on inhibition of plasma and RBC ChE inhibition at 3.0 mg/kg-day.

Rubin et al., 1987.
Pyrinex teratogenicity study in the rat. Makhteshim-Agan of North America Inc.,

The summary for this study was obtained from the CDPR. Three groups of 32 mated CD rats were dosed with Pyrinex in maize oil by oral (intragastric) intubation from Day 6 to Day 15 *post coitum* (p.c.), inclusive, at dosages of 0.5, 2.5, and 15 mg/kg-day, respectively. A fourth group served as a vehicle control. Ten rats from each group were bled at the end of the dosing period for plasma ChE determination and discarded. The remaining rats were sacrificed on Day 20 p.c. for examination of the uterine contents. Ten of these rats per group were bled prior to sacrifice for terminal plasma cholinesterase determination. Maternal toxic response to Pyrinex administration consisted of reduced body weight gain and a transient decrease in food consumption among animals dosed at 15 mg/kg-day, and dosage-related depression in plasma ChE activity in all treated groups. Terminal (day 20 p.c.) ChE activity returned to normal levels among low and intermediate dosage groups. Post-implantation fetal loss was more frequent among high dosage animals than among controls. This difference was statistically significant when arcsine transformed data were tested under parametric assumptions, but not when data were tested non-parametrically, by rank order methods. Fetuses in the high dosage group were significantly larger than controls, and showed enhanced skeletal development. The authors stated that the toxicological significance of these findings was not apparent. Fetal size has been shown to be inversely correlated with number of fetuses in the litter and may account for this occurrence. Pyrinex was concluded not to have shown a potential for teratogenicity and not to have caused fetal toxicity in the absence of maternal toxicity in the current test system.

Muto et al., 1992.
Embryotoxicity and neurotoxicity in rats associated with prenatal exposure to Dursban

Dursban, the widely-used OP insecticide has the active ingredient CPF. The teratogenic and neurotoxic potential of Dursban was evaluated in rats (strain unspecified) with about eight pregnant rats per dose for each treatment period. Embryos were exposed *in utero* on days 0–7 or days 7–21 of development to a formulation of 1% CPF, 6% xylene, and 93% water and diluted to an unspecified dosing volume with saline via intraperitoneal (ip) injection with the dose calculated to deliver 0.03, 0.1 or 0.3 mg CPF/kg. Dams were allowed to litter, then pups were evaluated for general viability, body weight and physical characteristics. Selected pups were evaluated for neurotoxicity on a rotorod on day 16, for motor behavior (by open field observation as a subjective measure of spontaneous motor activity per author's description) and for righting behavior on an inclined screen. An additional study evaluated the neurotoxicity and behavior following exposures of 0.1 or 0.3 mg (presumably ip, not clearly stated) as single doses on day 3, 10, or 12 postpartum, or as multiple doses on days 6–10 postpartum. All groups were evaluated on PND 16 for neurotoxicity or behavioral abnormalities. Increased embryo-lethality following dosing on gestation days 0–7 and gestation days 7–21 was noted with the highest embryo-lethality in the lowest dose group treated on gestation days 0–7 with 77% in the 0.03 mg group, 57% in the 0.1 mg and 68% in the 0.3 mg group. Also, incidences of physical abnormalities (decreased body weight, small hind and fore limbs, lack of spinal development, increased head circumference) were reportedly highest in 0.1 and 0.3 mg/kg-day groups (66 and 55%, respectively), among litters treated on gestation days 0–7. No corresponding control data

were presented other than the authors stating that "*all litters delivered by the saline-infused females were grossly normal in appearance and no lethalities were seen*." Rotorod performance was reported to be impaired in pups dosed at 0.3 mg/kg on PND 3, 10, and 12, and in offspring of dams dosed with 0.3 mg/kg on days 7–21 of gestation, and in offspring of dams dosed with 0.03, 0.1, or 0.3 mg/kg on days 0–7 of gestation. However, for all pups evaluated at PND 16 the control groups had a substantial difference in performance values making it hard to evaluate the data. The authors speculated that effects may be a result of delayed neurotoxicity consisting of demyelinization and neural degeneration progressing from distal extremities to central structures or due to the solvent (xylene) or potentiation by the solvent and/or the route of administration. The study report contained no numerical counts for the malformations noted and no control group data for lethality and defects. The study also contained serious misspellings and conceptual errors such as referring to a menstral (sic) rather than an estrous cycle in rats.

Chanda et al., 1995.
Comparative developmental and maternal neurotoxicity following acute gestational exposure to chlorpyrifos in rats

In this study, pregnant Sprague-Dawley rats were given CPF (200 mg/kg, subcutaneous (sc)) in peanut oil in an injection volume of 1ml/kg as a single dose on gestation day (GD) 12 and then sacrificed on GD 16, GD 20, or PND 3 for measurement of maternal and developmental indicators of toxicity and neurochemical changes. These included AChE inhibition and in vitro activity, muscarinic receptor binding and neurobehavioral testing. No overt signs were noted, but a subset (4/28) showed moderate to severe signs of "cholinergic" toxicity at 2-3 days after treatment, and these rats were omitted from further studies. Extensive AChE inhibition (82-88%) was noted in maternal brain at GD 16, GD 20, and PND 3. At GD 16 and GD 20, fetal brain AChE activity was inhibited 42-44%. While some degree of recovery in AChE activity was noted in pup brain by PND 3, AChE activity was still inhibited (30%) in treated pups cross-fostered to control dams. In vitro inhibition of maternal and fetal (GD 20) brain AChE activity by the active metabolite, CPO, suggested that the prenatal brain AChE activity was somewhat more sensitive. Maternal brain muscarinic receptor binding was more extensively reduced (30-32%) at GD 20 and PND 3 as compared to the developing brain at GD 20 (16%) and PND 3 (11%). A simple postnatal reflex test (righting reflex) was transiently altered by CPF with a significant increase in righting reflex time in pups at PND 1 followed by recovery by PND 3. Cliff avoidance behavior, however, was unaffected at all time points observed. The authors concluded that CPF exposure to dams during gestation at 200 mg/kg sc produces more signs of toxicity than in adult male rats at 250 mg/kg based on previous studies. Also, they noted extensive neurotoxicological effects in the dam relative to the developing fetus suggesting that the fetus may be relatively protected from neurotoxicity following exposure to CPF during gestation.

Nimphius, M. J. 1995.
(M.S. Dissertation)

A summary of this study was provided by the California Department of Pesticide Regulation (CDPR). The purpose of this study was to assess the potential of CPF (as the active ingredient in Dursban and Lorsban insecticides) and xylene to adversely affect embryonic and fetal development. This study also determined the distribution of the CPF and xylene throughout the dam and fetus and ChE levels in the maternal blood, brain, and fetus. Pregnant Sprague Dawley rats were given either CPF, xylene, or CPF with xylene. Doses were 0, 0.3, 3.0, and 10 mg/kg by sc injection on days 0 through 7 of gestation (typically 8 animals/dose/group). No evidence of maternal toxicity was noted. There were no deaths among the dams and no apparent resorbed litters in any of the treatment groups. The dams were sacrificed on days 19 and 20 of gestation. Maternal blood and brain tissue were collected for analysis. The fetuses were removed, weighed, and measured (crown to rump) and examined for external malformations. No obvious malformations were observed. A decrease in fetal body weights were noted in 95% of the CPF treated groups. A decrease in fetal body weights was seen in the higher xylene concentration dosage groups as well (data not available). The authors stated that the decrease in fetal weights indicate that CPF and xylene are potential developmental toxins. Maternal plasma cholinesterase inhibition was observed at the 10 mg/kg dose of CPF. Maternal erythrocyte ChE inhibition was observed at the 3.0 and 10 mg/kg CPF dose. Maternal brain tissue ChE was inhibited at the 10 mg/kg CPF and fetal tissue demonstrated inhibition at the 3.0 and 10 mg/kg CPF. None of the 0.3 mg/kg CPF or CPF/xylene treated groups demonstrated ChE inhibition in any of the tissues. Chlorpyrifos was detected in all of the tissues that exhibited ChE inhibition except in the 3.0 mg/kg CPF/xylene. Xylene did not attenuate the concentration of CPF or the enzyme inhibition.

Breslin et al., 1996.
Evaluation of the developmental and reproductive toxicity of chlorpyrifos in the rat

Chlorpyrifos was evaluated for its potential to produce developmental and reproductive toxicity in rats following oral exposure. Both these studies were conducted to meet with regulatory agency guidelines and the data were submitted to the CDPR around 1991. The findings were subsequently published in the open literature.

Developmental study

Pregnant Fischer 344 rats were given doses of 0 (corn oil vehicle), 0.1, 3.0, or 15 mg CPF/kg-day, by gavage, on GD 6 through 15. Maternal effects noted at the two higher dose levels included decreased ChE levels at 3.0 mg/kg-day and cholinergic signs (excessive salivation and tremors), decreased ChE levels, and decreased body weight gain at 15 mg/kg-day. No maternal effects were apparent at 0.1 mg/kg-day. The authors stated that the differential sensitivity between the dam and the fetus was likely to be both exposure and biologically based as other data have reported fetuses showing a delay to maximum total brain AChE inhibition, a decreased level of maximum brain AChE inhibition, and a faster rate of ChE recovery. Also, the rate and magnitude of ChE depression is likely exposure-related as the fetal brain represents a deep tissue

compartment and the rate of recovery may be biologically related to the rate of enzyme generation/regeneration. Hence, they proposed that the rapidly growing fetus would be expected to have an increased rate of brain growth, relative to the adult, resulting in an apparent increase in AChE recovery through the diluting of the inhibited enzyme with newly generated brain tissue/enzyme.

Table C19. Chlorpyrifos Developmental Toxicology Study: Maternal Parameters

		Chlorpyrifos (mg/kg-day)			
	Day	**0**	**0.1**	**3.0**	**15**
Number of dams		29	26	24	26
Body weights[a]	6	195 ± 10	191 ± 9	194 ± 7	192 ± 8
	9	199 ± 10	193 ± 9	199 ± 7	193 ± 10
	12	207 ± 10	204 ± 10	208 ± 7	198 ± 10*
	16	222 ± 11	219 ± 11	225 ± 7	213 ± 11*
	21	260 ± 15	254 ± 19	264 ± 11	250 ± 16
Body weight gain[a]	6-9	4 ± 3	2 ± 4	5 ± 3	2 ± 4
	9-12	9 ± 4	11 ± 6	10 ± 3	5 ± 4*
	12-16	15 ± 4	14 ± 3	17 ± 3	14 ± 4
	16-21	38 ± 8	36 ± 10	40 ± 8	37 ± 11
	6-21	65 ± 11	64 ± 14	71±12	58 ± 11
Liver weight					
Absolute[a]		9.60 ± 0.71	9.32 ± 1.20	9.88 ± 0.78	9.23 ± 1.15
Relative[b]		3.69 ± 0.23	3.66 ± 0.31	3.74 ± 0.26	3.70 ± 0.48
Plasma cholinesterase[c]		4.60 ± 0.59	4.28 ± 0.45	0.49 ± 0.03*	0.15 ± 0.02*
Erythrocyte cholinesterase[c]		1.12 ± 0.05	1.19 ± 0.04	0.31 ± 0.04*	0.25 ± 0.05*
Number of Dams		8	8	6	7

[a] Grams, mean ± SD.
[b] Grams, organ weight/100g body weight, mean ± SD.
[c] International unit/ml, mean ± SD. Determined on Gestation Day 15.
* Different from the control value by Dunnett's test, $\alpha = 0.05$.

Table C20. Chlorpyrifos Developmental Toxicity Study: Reproductive and Fetal Parameters Obtained at Necropsy

	Chlorpyrifos (mg/kg-day)			
	0	**0.1**	**3.0**	**15**
Number of females	31	32	33[a]	31
Number of pregnant	30	28	26	29
% Pregnant[b]	97 (30/31)	88 (28/32)	84 (26/31)	94 (29/31)
Pregnancies detected by stain	0/1	0/4	0/5	1/3
Dams removed, mistimed pregnant	1	2	3	2
Number of litters	29	26	24	26
Corpora lutea/dam[c]	11.4 ± 1.6	11.1 ± 17	11.3 ± 1.5	11.6 ± 1.4
Implantation sites/dam[c]	10.5 ± 1.8	10.0 ± 2.3	10.2 ± 2.4	10.0 ± 2.6
% Preimplantation loss[d]	8.1 ± 11.5	12.0 ± 16.2	11.1 ± 20.6	14.8 ± 19.6
Live fetuses/litter[c]	9.6 ± 2.5	9.1 ± 2.3	9.6 ± 2.5	9.4 ± 2.7
Resorptions/litter[c]	0.9 ± 1.3	0.8 ± 0.8	0.6 ± 0.8	0.6 ± 0.8
% Implantations resorbed	9 (26/304)	9 (22/259)	6 (15/245)	6 (15/259)
% Litters with resorptions	45 (13/29)	62 (16/26)	46 (11/24)	42 (11/26)
Litters totally resorbed	0	0	0	0
Dead fetuses	0	0	0	0
Sex ratio. M:F (%)	48:52	53:47	52:48	54:46
Fetal body weight (g)[e]	4.28 ± 0.15	4.37 ± 0.32	4.52 ± 0.14*	4.46 ± 0.40*
Fetal crown-rump length (mm)[e]	43.9 ± 2.7	43.9 ± 2.6	44.0 ± 2.1	43.6 ± 2.3

[a] Two animals were removed from the study during dosing (one exhibited glaucoma, one was inadvertently deprived of water overnight).
[b] Number of females detected as being pregnant by visual inspection of the uterus or by sodium sulfide stain/total number of females.
[c] Mean ± SD.
[d] Percentage per litter, mean ± SD.
[e] Mean of litter, means ± SD.
* Indicates statistical difference from control, $\alpha = 0.05$.

Table C21. Chlorpyrifos Developmental Toxicity Study: Incidence of Fetal Alterations among Litters of Pregnant Rats

		Chlorpyrifos (mg/kg-day)			
		0	**0.1**	**3.0**	**15**
		Number fetuses (number litters) examined			
External examination		278 (29)	237 (26)	230 (24)	244 (26)
Soft tissue examination		147 (29)	126 (26)	124 (24)	130 (26)
Skeletal examination		278 (29)	237 (26)	230 (24)	244 (26)
Bones of the skull		129 (29)	110 (25)	106 (22)	115 (25)
		Percentage affected (number affected)			
External observations					
Microphthalmia[a]	F[b]	0.4 (1)	0	0.4 (1)	0.8 (2)
	L	3 (1)	0	4 (1)	8 (2)
Anophthalmia[a]	F	0.4 (1)	0	0.4 (1)	0
	L	3 (1)	0	4 (1)	0
Soft tissue observations					
Cleft soft palate[a]	F	0	0	0	0.8 (1)
	L	0	0	0	4 (1)
Patent ductus arteriosus	F	0.7 (1)	0	0	0
	L	3 (1)	0	0	0
Hemorrhage in the liver	F	0	0.8 (1)	0	0
	L	0	4 (1)	0	0
Convoluted ureter	F	0	0.8 (1)	0	2 (2)
	L	0	4 (1)	0	8 (2)
Severely dilated renal pelvis[a]	F	0	0	0	2 (3)
	L	0	0	0	4 (1)
Skeletal observations					
Vertebrae					
Delayed ossification of centrum	F	6 (16)	6 (13)	1* (2)	5 (12)
	L	41 (12)	42 (11)	8 (2)	42 (11)
Ribs					
Fused[a]	F	0	0.4 (1)	0	0
	L	0	4 (1)	0	0
Spur	F	0.7 (2)	2 (4)	0	1 (2)
	L	3 (1)	15 (4)	0	8 (2)
Sternebrae					
Delayed ossification	F	41 (115)	44 (103)	37 (85)	34 (84)
	L	97 (28)	89 (23)	96 (23)	92 (24)
Extra site of ossification	F	0.4 (1)	0	0	0
	L	3 (1)	0	0	0

[a] Considered to be a malformation.
[b] F, fetuses; L, litters.
* Different from the control value by Wilcoxon test, $\alpha = 0.05$.

According to the authors, maternal toxicity was observed at these two higher exposure levels (3 and 15 mg/kg) and the fetal effects noted at these doses were not suggestive of developmental toxicity. However, as shown in Table C10, the only maternal parameters affected in the 3 mg/kg group were plasma and erythrocyte cholinesterase levels.

Reproduction study

In a two-generation reproduction study, groups of 30 male and 30 female first parental (PI) generation Sprague-Dawley rats were administered diets containing sufficient CPF to provide dose levels of 0, 0.1, 1.0, or 5.0 mg CPF/kg/day. The dose levels were selected on the basis of a previous study (unpublished data) in which rats given diets that provided a dose of 5.0 mg CPF/kg/day exhibited significant depressions in brain, plasma, and erythrocyte ChE. Significant depression of brain ChE is considered to be a toxic response and meets the criteria for a maximum-tolerated dose. The middle- and low-dose levels were selected to establish a dose response for ChE depression and a NOEL, respectively. Exposure to the treated diets was continuous throughout the duration of the study. The concentrations of CPF in the diets were adjusted weekly, based on weekly body weights and feed consumption data, to maintain targeted dose levels on a mg/kg-day basis. Concentrations of the test material in the diets ranged from 93 to 100% of the targeted concentrations and were stable in the diet for a minimum of 51 days. Treatment of the PI rats began at approximately 6 weeks of age. After approximately 10 weeks on test diets, PI rats were mated to produce the first filial generation (Fl) litters. Following weaning (3 weeks of age) of the Fl litters, 30 males and 30 females from each treatment group were randomly selected and assigned to the respective treatment group to become the second parental (P2) generation. After approximately 12 weeks of treatment following weaning of the last Fl litter, the P2 adults were bred to produce the second generation (F2) litters.

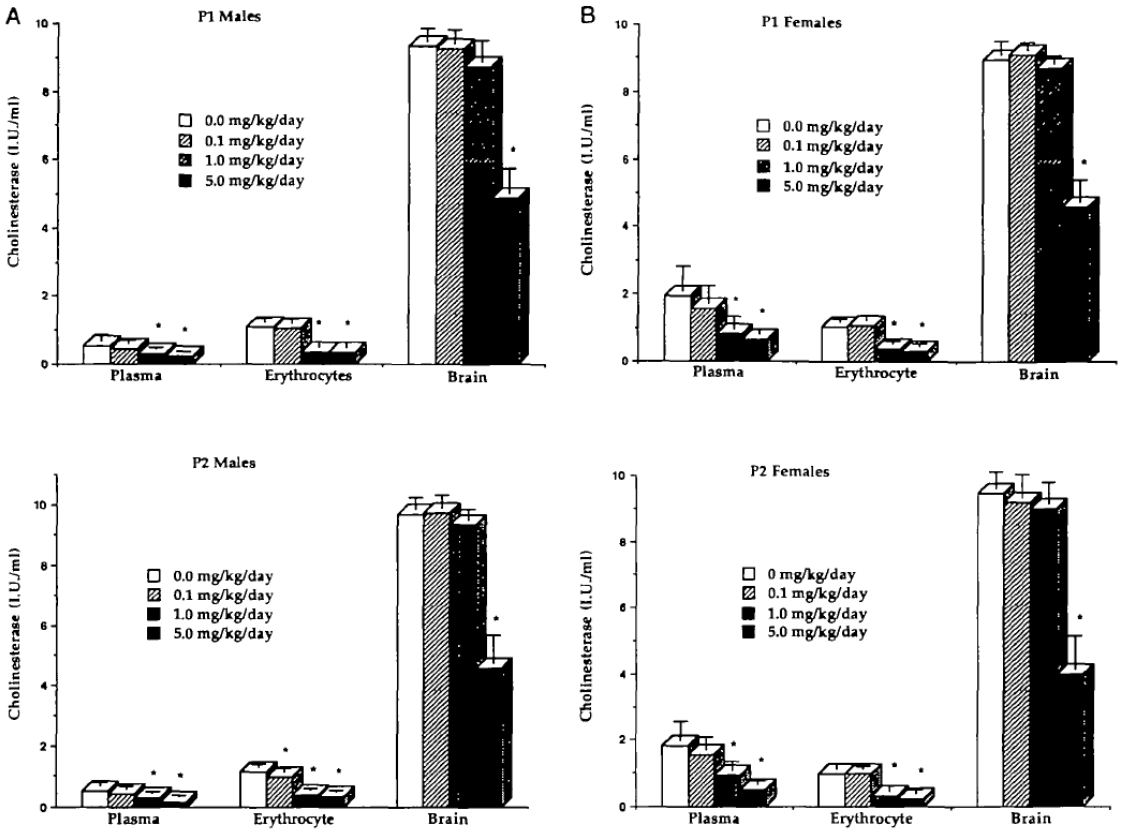


Figure C2. Cholinesterase levels, expressed as international units per milliliter (IU/ml), in plasma, erythrocytes, and brain of PI and P2 adult male and female rats. Cholinesterase determinations were made on PI and P2 adults following 19 and 21 weeks of exposure, respectively. The PI and P2 animals were 25 and 27 weeks of age, respectively, at the time of analyses. Error bars represent standard deviation. 'Statistically different from controls by the Dunnett's test, $\alpha = 0.05$.

Parental effects included (statistically significant from controls by the Dunnett's test, $\alpha = 0.05$) decreased plasma and erythrocyte ChE at 1.0 mg/kg-day and decreased plasma, erythrocyte, and brain ChE (Figure C2) and histopathological alterations of the adrenal zona fasciculata at 5.0 mg/kg-day. The histopathological alterations of the adrenal were characterized as very slight to slight vacuolation (consistent with fatty change) in males and very slight vacuolation and/or altered tinctorial properties in females.

No effects on reproductive or fertility indices or on the histopathology of reproductive tissues were observed at any dose level, and no neonatal effects were observed at 0.1 or 1.0 mg/kg-day in the Fl or F2 litters. Parental toxicity at the high dose was accompanied by decreased pup body weight and increased pup mortality in the Fl litters only. Also the neonatal effects observed in the F1 generation were not noted in the F2 generation.

Table C22. Chlorpyrifos Reproduction Study: Pup Body Weights

Day	Sex	Number of Litters	Dose (mg/kg-day) 0	0.1	1.0	5.0
			Pup body weight (g)—F1 litters			
1	Male	24–30	6.5 ± 0.6[a]	6.4 ± 0.7	6.4 ± 0.6	6.2 ± 0.5
	Female	24–30	6.2 ± 0.6	6.0 ± 0.7	6.1 ± 0.6	5.8 ± 0.5
4[b]	Male	23–30	8.5 ± 1.1	8.7 ± 1.0	8.4 ± 1.1	7.6 ± 1.2*
	Female	23–30	8.1 ± 1.1	8.2 ± 1.1	8.1 ± 1.3	7.2 ± 1.1*
21	Male	23–30	48.4 ± 4.0	49.1 ± 4.6	46.1 ± 4.3	43.0 ± 4.9*
	Female	23–30	46.1 ± 3.7	46.9 ± 5.5	44.7 ± 5.0	41.3 ± 4.8*
			Pup body weight (g)—F2 litters			
1	Male	22–26	6.8 ± 0.7[a]	6.6 ± 0.7	6.3 ± 0.6	6.3 ± 0.8
	Female	22–26	5.9 ± 0.7	6.1 ± 0.6	5.9 ± 0.6	5.9 ± 0.7
4	Male	18–25	8.6 ± 1.5	9.0 ± 1.4	8.6 ± 1.1	8.4 ± 1.9
	Female	18–25	8.2 ± 1.2	8.6 ± 1.2	8.2 ± 0.9	7.8 ± 1.7
21	Male	17–25	45.1 ± 6.1	47.6 ± 6.7	43.7 ± 6.7	43.4 ± 5.3
	Female	17–25	42.7 ± 5.1	45.7 ± 5.2	41.7 ± 4.3	41.0 ± 5.6

[a] Mean ± SD.
[b] After culling.
* Statistically different from control mean by Dunnett's test, α = 0.05.

Table C23a. Chlorpyrifos Reproduction Study: Assessment of Reproductive and Postnatal Parameters for P1 adults/F1 litters

	Dose (mg/kg-day)			
	0	0.1	1.0	5.0
	P1 adults/F1 litters			
Number of animals bred/sex	30	30	30	30
Female mating index[a]	100	100	100	100
Female conception index[b]	100	80.0*	93.3	93.3
Female fertility index[c]	100	80.0*	93.3	93.3
Male mating index[d]	96.7	90.0	96.7	100
Male conception index[e]	100	85.2	93.1	93.3
Male fertility index[f]	96.7	76.7	90.0	93.3
Gestation index[g]	100	100	100	100
Gestation survival index[h]	98.3	99.1	99.5	98.7
Gestation length (days)[i]	21.8 ± 0.4	21.6 ± 0.5	21.6 ± 0.5	21.6 ± 0.5
Time to mate (days)[j]	2.8 ± 2.1	3.6 ± 3.7	3.1 ± 2.8	2.3 ± 1.1
Live litter size at birth[k]	13.3 ± 2.2	13.6 ± 2.0	13.1 ± 2.1	13.9 ± 2.4
Litter size on Day 21	7.9 ± 0.3	7.6 ± 1.6	7.9 ± 0.3	7.5 ± 1.0
Day 1 survival index[l]	98.5	96.9	99.2	97.2
Day 4 survival index[l]	97.0	92.3	98.4	92.8
Day 7 survival index[m]	99.2	99.5	99.1	97.7
Day 14 survival index[m]	99.2	98.9	99.1	95.9*
Day 21 survival index[m]	99.2	98.9	99.1	95.5*
Pup sex ratio (male/female)	54:46	50:50	49:51	51:49

[a] Number of females with a sperm-positive vaginal smear or pregnant without additional evidence of mating/number of females cohoused with males (percentage).
[b] Number of females delivering a litter/number of sperm-positive females (percentage).
[c] Number of females delivering a litter/number of females cohoused with males (percentage).
[d] Number of males which mated resulting in a sperm-positive vaginal smear or pregnant female/number of males cohoused with females (percentage).
[e] Number of males which sired a litter/number of males resulting in a sperm-positive vaginal smear or pregnant female (percentage).
[f] Number of males which sired a litter/number of males cohoused with females (percentage).
[g] Number of females delivering a live litter/number of females delivering a litter (percentage).
[h] Percentage of newborn pups that were alive at birth.
[i] The duration of gestation in days; mean ± SD.
[j] The number of days the male and female were cohoused prior to mating; mean ± SD.
[k] Mean ± SD.
[l] Number of live pups on Day 1 or 4/number of pups born alive (percentage).
[m] Number of pups alive on Day 7, 14, or 21/number of pups alive on Day 4 (percentage).

Table C23b. Chlorpyrifos Reproduction Study: Assessment of Reproductive and Postnatal Parameters for P2 adults/F2 litters

	P2 adults/F2 litters			
Number of animals bred/sex	30	30[n]	30	30
Female mating index[a]	100	100	100	96.7
Female conception index[b]	80.0	76.7	86.7	75.9
Female fertility index[c]	80.0	76.7	86.7	73.3
Male mating index[d]	90.0	93.1	83.3	90.0
Male conception index[e]	81.5	81.5	88.0	77.8
Male fertility index[f]	73.3	75.9	73.3	70.0
Gestation index[g]	100	100	100	100
Gestation survival index[h]	96.9	97.4	99.4	99.6
Gestation length (days)[i]	21.7 ± 0.6	21.6 ± 0.5	21.8 ± 0.7	21.9 ± 0.4
Time to mate (days)[j]	3.3 ± 3.1	3.3 ± 3.0	4.3 ± 4.4	4.4 ± 4.2
Live litter size at birth[k]	11.5 ± 3.1	12.8 ± 3.7	12.4 ± 3.5	11.9 ± 3.7
Litter size on Day 21	6.8 ± 2.7	7.6 ± 1.4	7.2 ± 1.9	5.8 ± 3.3
Day 1 survival index[l]				
All[o]	98.6	99.0	97.2	95.4
Excl[p]	98.4	99.0	99.0	98.1
Day 4 survival index				
All	90.3	95.9	94.1	80.8
Excl	97.6	95.9	97.7	95.6
Day 7 survival index				
All	98.8	98.9	99.5	95.0
Excl	100	98.9	99.5	97.7
Day 14 survival index				
All	97.6	98.3	98.4	92.1
Excl	99.4	98.3	98.4	97.7
Day 21 survival index				
All	96.4	98.3	98.4	92.1
Excl	98.8	98.3	98.4	97.7
Pup sex ratio (male/female)	47:53	48:52	48:52	49:51

[a] Number of females with a sperm-positive vaginal smear or pregnant without additional evidence of mating/number of females cohoused with males (percentage).
[b] Number of females delivering a litter/number of sperm-positive females (percentage).
[c] Number of females delivering a litter/number of females cohoused with males (percentage).
[d] Number of males which mated resulting in a sperm-positive vaginal smear or pregnant female/number of males cohoused with females (percentage).
[e] Number of males which sired a litter/number of males resulting in a sperm-positive vaginal smear or pregnant female (percentage).
[f] Number of males which sired a litter/number of males cohoused with females (percentage).
[g] Number of females delivering a live litter/number of females delivering a litter (percentage).
[h] Percentage of newborn pups that were alive at birth.
[i] The duration of gestation in days; mean ± SD.
[j] The number of days the male and female were cohoused prior to mating; mean ± SD.
[k] Mean ± SD.
[l] Number of live pups on Day 1 or 4/number of pups born alive (percentage).
[m] Number of pups alive on Day 7, 14, or 21/number of pups alive on Day 4 (percentage).
[n] One male died prior to breeding resulting in 30 females and 29 males.
[o] All litters included in calculation of the index.
[p] Excluded from the index calculation all litters that died.

According to the authors, these data show that oral administration of CPF to rats at parentally toxic dose levels was not embryolethal, embryo/fetotoxic, or teratogenic and did not adversely affect fertility or the function or structure of the reproductive organs. Although effects on neonatal growth and survival were observed at a maternally toxic dose level in one generation, this effect was not observed in the subsequent generation. The authors also stated that the lack of effects on reproductive function and neonatal development observed in this study at dose levels as high as 1.0 mg/kg-day were consistent with the results of a previously conducted three-generation dietary reproduction and developmental toxicity study exposing rats to dose levels of 0, 0.1, 0.3, or 1.0 mg CPF/kg/day (Thompson et al., 1971) in which depressions in parental plasma and erythrocyte ChE were observed without effects on reproductive parameters, fetal development, or neonatal growth, survival, or histopathology, resulting in a reproductive and developmental NOEL of 1.0 mg/kg-day. However, in the study by Thompson et al., 1971 the viability index was decreased at the high dose level of 1 mg/kg-day (not statistically significant) and the Reproductive NOEL could be interpreted to be 0.3 mg/kg-day. No such decrease was noted at 1 mg/kg-day in this study.

Chanda and Pope, 1996
Neurochemical and neurobehavioral effects of repeated gestational exposure to chlorpyrifos in maternal and developing rats.

In this study, the relative neurotoxicity of repeated, lower-level CPF exposures during gestation in rats was examined. Pregnant Sprague-Dawley rats were exposed to CPF (6.25, 12.5, or 25 mg/kg-day, sc) in peanut oil from GD12-19. The dams and offspring were killed on GD 16 or 20 or PND 3. No clinical signs of maternal toxicity were observed at any dose level; maternal body weight gain values were similar to control for all treated groups. Fetal body weight was similar to control for all treated groups, but a significant decrease in fetal body weight was observed on postnatal day 1 in the 25 mg/kg/day dose group. A significant dose-related inhibition of AChE was observed following the three dosing regimens at GD 20. In each case, maternal brain AChE inhibition was greater than the fetal brain AChE inhibition with all three doses. AChE inhibition (83-90%) was noted in maternal brain at all three collection times following repeated exposures at 25 mg/kg-day. Higher AChE inhibition (58%) was noted in fetal brain at GD20 compared to 19-25% on PND3 in treated pups cross-fostered to control dams and in control pups cross-fostered to treated dams following repeated exposures (25 mg/kg-day). Specifically, in animals sampled on gestation day 16, 20 and PND 3 the brain AChE was inhibited in both dams and fetuses on gestation day 20 in a dose-related manner, with increased inhibition in dams. Brain AChE was also reduced significantly in control pups cross-fostered with treated dams and in treated pups cross-fostered to untreated dams on PND 3. A dose-related down-regulation of muscarinic receptors was also observed on GD 20 and also on PND 3. Although similar reductions in brain muscarinic receptor binding were observed at GD day 20 and PND 3 in dams and developing brain between acute (Chanda et al., 1995) and repeated dosing regimens, greater changes in [^{3}H]cis-methyl dioxolane and [^{3}H]cytisine binding were observed following repeated exposures in this study. A significant increase in righting reflex time and a decrease in percent cliff avoidance on PND 3 was noted in pups at the 25 mg/kg-day group whether cross-fostered with control dams or not. According to the authors, lower-level repeated exposures to CPF cause extensive neurochemical and neurobehavioral changes in developing rats in the absence of

maternal toxicity (signs of clinical toxicity and body weight gain data). Also repeated dosing of CPF during gestation resulted in AChE inhibition in both dams and fetuses at dose levels as low as 6.25 mg/kg-day sc, and that the maternal response, as measured by brain cholinesterase inhibition on GD 20, was more severe than the fetal response.

Hoberman, A. M. 1998.
Developmental neurotoxicity study of chlorpyrifos administered orally via gavage to Crl:CD7(SD)BR VAF/Plus 7 presumed pregnant rats

Chlorpyrifos (99.8%) in corn oil was administered orally via gavage to Crl:CD7(SD)BR VAF/Plus7 (Sprague-Dawley) presumed pregnant rats (25 dams/group), at Argus Research Laboratories, Inc., on GD 6 through lactation day 11 at 0, 0.3, 1, and 5 mg/kg-day. An additional 5 pregnant females/group were dosed at the same levels for ChE analyses on GD 20 on brain, plasma, and erythrocytes. Dams were examined for body weight, reproductive performance, number of viable pups and postpartum behavior. Offspring were examined for viability at birth, pup or litter survival, body weight, sex ratio and developmental landmarks such as eye opening, pinna detachment, vaginal opening and preputial separation as well as observed nursing behavior. On lactation day 5 the litters were standardized to 10 pups/litter (5 males and 5 females when possible), assigned to 4 subsets and observed as follows.
Subset 1: On PND 12, fixed brain weight measurements (10 pups/sex/dose) and neuropathological evaluation and morphometrics (6 pups/sex/dose) were performed with the remaining 10 pups/sex/dose necropsied for gross lesions.
Subset 2: On PND 23–25 and 62–92 learning and memory evaluations were performed (on 8 pups/sex/dose) and subsequently sacrificed on PNDs 97–101; with the remaining pups necropsied for gross lesions on PND 22.
Subset 3: On PND 14, 18, 22 and 61 pups were tested for motor activity and on PND 23 and 62 for auditory startle habituation, and subsequently sacrificed on PND 63 or 64.
Subset 4: On PND 66 fixed brain weight measurements (10 pups/sex/dose) and neuropathological evaluation and morphometrics (6 pups/sex/dose) were performed, with the remaining 10 pups/sex/dose necropsied for gross lesions on PND 66–77.

Table C24. Treatment Levels and Group Size

Dosage (mg/kg-day)	F0 generation females	F1 generation pups[1]	
		Males	Females
Control 0	25	80	78
0.3	25	80	79
1	25	80	80
5	25	64	69

[1]nominal number of pups to be assigned per group = 80

In the dams, brain ChE inhibition at 1 mg/kg-day (18%) and clinical signs of ChE inhibition were observed in 5 mg/kg-day leading to a maternal NOEL of 0.3 mg/kg-day. An increase in pup mortality, a transient decrease in pup body weight, brain size, brain layer thickness, transient changes in the startle response, delayed pinna detachment, vaginal opening and preputial

separation, all consistent with delayed maturation were seen in the presence of the maternal effects at 5 mg/kg-day. Cognitive functions such as learning, memory, and habituation were not impaired in the pups at any of the dose levels. According to the authors, the developmental NOEL of 1 mg/kg-day was based on the following observations at the high dose of 5 mg/kg-day - decreased neonatal survival; decreased pup growth with 11% reduction in body weight at 66 days postpartum in males; maturational delays of pinna unfolding, preputial separation in males, and vaginal patency in females; reduced morphometric dimensions of cerebellum and hippocampal gyrus at day 12 postpartum compared to concurrent and historical controls, reduced morphometric dimensions of parietal cortex and hippocampal gyrus at day 66 postpartum compared to concurrent and historical controls in high dose females, reduced motor activity at day 14 postpartum, reduced auditory startle habituation peak response and increased latency to response at day 23 postpartum.

Table C25. Maternal Clinical and Necropsy Observations [1]

Observation	Dose (mg/kg-day)			
	0	**0.3**	**1**	**5**
GESTATION				
Fasciculations	0	0	0	6/6*
LACTATION				
Fasciculations	0	0	0	30/16*
Hyperreactivity	3/2	7/7	3/2	27/17*
Hyperpnea	0	0	1/1	10/8*

[1] Total number of times finding was observed/number of animals with finding observed at least once during the designated period); * significant at p=0.001

Table C26. Mean (± S.E.) plasma, erythrocyte and brain cholinesterase activity[1]

Sample	Dosage (mg/kg-day)		
	0.3	**1**	**5**
Plasma	56.70 ± 2.69	31.13 ± 4.07	8.46 ± 1.19
Erythrocyte	58.74 ± 16.10	15.59 ± 6.80	0.13 ± 0.15
Brain	99.72 ± 1.48	82.11 ±2.80	10.18 ± 0.92

[1] as % of control group at GD20 in dams (5/group)

Evaluation of developmental landmarks (pinna unfolding, eye opening, preputial separation or vaginal opening) was also done. Time of preputial separation was not significantly delayed in high dose males, whereas time of vaginal patency was significantly delayed in high dose F1 females. Both parameters were considered by the investigators to be biologically significant, as indicated in Table C27 of demonstrated or indicated treatment effects.

Table C27. Survival and Body weights

Observation		Dose (mg/kg-day) 0	0.3	1	5
Surviving pups per litter	Day 1	12.3	13.3	13.0	12.7
	Day 5 pre-cull	12.2	13.1	12.7	8.9 [1]
	Day 5 post-cull	10.0	10.0	10.0	8.7 [1]
Mean pup weight (g)					
Males:	Day 1	6.6	6.7	6.4	6.1
	Day 5 post-cull	9.8	10.2	10.1	8.8 [1]
Females:	Day 1	6.3	6.2	6.1	5.6
	Day 5 post-cull	9.4	9.6	9.5	8.2 [1]
Pinna unfolding, % pups reached as of day:					
	2	7	3	1	0
	3	48	47	47	17 [2]
	4	94	99	91	71
	5	100	100	100	99
Found dead (total pups/litters per dose group)		1/25	2/24	2/24	50/23 [1]

[1] statistically significant; [2] not statistically significant, possibly treatment-related

Table C28. Sexual Maturation Parameters: Average Day of Age Achieved

Parameter Postpartum Study Day(s)	Dose (mg/kg-day) 0	0.3	1	5
Preputial Separation in Males	44.2±1.9	43.4±1.9	45.2±3.2	47.0±5.9
Vaginal Patency in Females	32.4±1.0	31.5±1.5	32.1±2.3	33.4±2.2*

* significantly different from control group, $p<0.02$

Neurobehavioral Evaluation:

Spatial delayed alternation studies at postpartum days 23–25 and 62–91, motor activity testing on postpartum days 14, 18, 22, and 61 and auditory startle testing on postpartum days 23 and 62 were undertaken. For the statistical analysis of spatial delayed alternation, the slope and intercept of regression lines fitted to delayed response data were compared for each group. Also submitted were validation studies for evaluation of spatial delayed alternation, which were based on temporal patterns of memory performance over sufficient duration to show a consistent linear change over time. There were no statistically significant differences between groups in average

acquisition and delayed response and no consistent pattern indicating a treatment effect for acquisition training data and delay training data. High dose males had 55% correct decisions after the 125 second delay, vs. 67% correct in controls. This was based on 8 rats/sex/group and 12 trials/rat. Thus there was considerable room for variability and little basis for discriminating minor effects. High dose females had virtually identical scores to corresponding female controls under the same circumstances. While delay training in high dose males at the later time interval (days 62 to 92 post partum) is suggestive of a treatment effect, overall there was a lack of treatment effect in either sex in other time periods of acquisition or delay training. There were no differences among groups for retention of information during PND 23–25 and 62–92. Motor activity (number of movements of each pup monitored by a passive infrared sensor and tabulated at 5-minute intervals for a 1 hour test session) was decreased in high dose male and female pups on PND 14 (56% decrease in males and 37% decrease in females) and increased in high dose females on PND 18 and PND 22 (51% increase at both time points). On PND 61, motor activity was increased in both males and females (16–17%). It was not possible to determine the statistical significance of these changes as insufficient statistical analyses were available i.e. Group x Sex x Time and Group x Sex x Time x Block interactions were not presented. Hence, while the authors considered any patterns observed to be spurious, it must be noted that the apparent reduction on PND 14 was accompanied by modest body weight decrements and was not dismissed by either U.S. EPA/OPP or Cal/EPA-CDPR reviewers. The latency to peak response during auditory startle habituation assessments was increased in high dose animals at PND 23 (16–25%) and at PND 62 (10–12%) compared to controls, while the peak response amplitudes were decreased (9–29%) in the high dose animals at these time points, although those changes were not statistically significant.

Table C29. Total Motor Activity recorded over 60 minutes

Time Interval Sex	Dose (mg/kg-day)			
	0	**0.3**	**1**	**5**
Postpartum day 14				
Male	246	182	168	109
Female	228	238	183	145
Postpartum day 18				
Male	373	328	390	319
Female	343	402	357	520
Postpartum day 22				
Male	314	249	299	302
Female	229	258	253	347
Postpartum day 61				
Male	585	612	616	681
Female	635	693	701	743

Table C30. Auditory Startle Habituation

Parameter **Study Day** **Sex**	**Dose (mg/kg-day)**			
	0	**0.3**	**1**	**5**
Mean Peak Response (g)				
Day 23 postpartum				
Males	57 ± 23	64 ± 30	57 ± 21	40 ± 10
Females	58 ± 18	58 ± 16	56 ± 17	49 ± 21
Day 60 postpartum				
Males	220 ± 100	156 ± 70	171 ± 92	168 ± 81
Females	147 ± 81	146 ± 89	97 ± 48	134 ± 82
Latency to Peak Response (msec)				
Day 23 postpartum				
Males	39 ± 7	39 ± 8	39 ± 9	49 ± 16
Females	37 ± 9	37 ± 7	38 ± 7	43 ± 8
Day 60 postpartum				
Males	37 ± 7	39 ± 9	38 ± 6	41 ± 12
Females	39 ± 9	41 ± 9	46 ± 11	43 ± 9

For pups, fixed brain weights and histopathology of brains after PND 12 sacrifice, brain weight evaluation during PND 66–71(10/sex/group), and neurohistopathology following *in situ* perfusion (6/sex/litter) were conducted. According to the authors, rats sacrificed on PND 12 did not present neurohistopathologic changes and the standard deviations of these data were often substantial so that large changes would be needed to distinguish treatment effects with the small sample sizes available. Brain morphometric data revealed that the cerebellar anterior/posterior dimension in 5 mg/kg-day male PND 12 pups was significantly below concurrent control dimension, and also outside the range of the available historical controls. Females did not suggest such a relationship at PND 12, and neither sex showed altered cerebellar anterior/posterior distance after 66 days. Thus, the data for high dose PND 12 male pups demonstrate significant decreases in the measurements of anterior to posterior cerebellum and cerebellum height. For the high dose PND 12 females, the caudate/putamen width measurement (significant at high dose) and cerebellum height was decreased. The decrease in thickness of the parietal cortex in the high dosage group males is smaller than the increase seen in the low and medium dosage group males.

Table C31. Brain Weights and Morphometry (at PND 12)

Observation	Dose (mg/kg-day)			
	0	**0.3**	**1**	**5**
Body & Brain Weights (g) n=10				
Body weight - males	23.5 ± 1.6	27.4 ± 2.4	25.9 ± 2.4	19.4 ± 4.3*
Brain weight - males	1.28 ± 0.04	1.41 ± 0.06	1.36 ± 0.08	1.17 ± 0.16*
Body weight - females	23.1 ± 2.3	23.2 ± 1.8	23.1 ± 2.8	18.8 ± 3.6*
Brain weight - females	1.28 ± 0.08	1.28 ± 0.04	1.27 ± 0.11	1.17 ± 0.13*
Morphometric data from F1 pups (units of µm, unless specified) n=6				
Males				
Brain weights - selected rats (g)	1.291	1.428	1.362	1.142*
Cerebellum (anterior to posterior - mm)	3.27 ± 0.31	3.45 ± 0.35	3.33 ± 0.19	2.47 ± 0.55*
Frontal cortex	1348	1360	1352	1272
Parietal cortex thickness	1336 ± 56.1	1448 ± 58.1	1448 ± 32.8	1256 ± 138*
Caudate putamen width	2240	2240	2312	2224
Hippocampal gyrus thickness	904 ± 93.2	1004 ± 114	972 ± 54.2	824 ± 65.6*
Cerebellum height	3504 ± 129	3456 ± 17.1	3416 ± 200	3008 ± 504*
External germinal layer, cerebellar cortex	37.2	38.3	40.0	37.7
Females				
Brain weights - selected rats (g)	1.292	1.278	1.261	1.179*
Cerebrum (anterior to posterior - mm)	12.40	12.70	12.80	12.15
Cerebellum (anterior to posterior - mm)	3.183 ± 0.22	3.033 ± 0.32	3.300 ± 0.17	3.000 ± 0.31
Parietal cortex thickness	1380 ± 54.2	1376 ± 19.6	1368 ± 80.3	1304 ± 72.3
Caudate putamen width	2384 ± 131	2224 ± 116	2288 ± 108	2152 ± 134*
Corpus callosum	307	286	304	274
Hippocampal gyrus thickness	936 ± 82	912 ± 50	932 ± 97	828 ± 79*
Cerebellum height	3512 ± 200	3176 ± 130	3120 ± 328	3208 ± 226*
External germinal layer, cerebellar cortex	38.7	36.3	41.2	40.8

* Statistically significant p<0.02; all morphometric data were analyzed by ANOVA. Both PND 12 and PND 66 findings for all groups were included in the statistical analysis.

At PND 66, the morphometry data were unremarkable in males. For female morphometry data, significant decreases in the dimensions of parietal cortex thickness at both mid and high dose and in the measurement for hippocampal gyrus at the high dose (5 mg/kg-day) were noted. The mean brain weights in high dose females at this time point were similar to controls though slightly reduced (0.3%). Brain weight was decreased less in the high dose group for PND 66 than PND 12, and brain size was decreased in the high dose group at PND 12 but not at PND 66. Data for male pups at PND 66 for the mid dose (1 mg/kg-day) were not provided.

Table C32. Brain Morphometry at PND 66

Dimensions in µm of Specified Cross-sections of F1 female pups (n=6)	Dose (mg/kg-day)		
	0	1	5
Parietal Cortex	1792 ± 36.1	1716 ± 36.4*	1700 ± 55.6*
Hippocampal Gyrus	1708 ± 57.6	1644 ± 129.5	1592 ± 86.8
Brain weight (g)	2.103 ± 0.071	2.127 ± 0.079	2.048 ± 0.050

*Statistically significant, p < 0.05 Dunnett's test

An overall examination of the brain morphometric data: brain weight and linear measurements expressed as percent of control at both time points is presented below.

Table C33. Overall Brain Morphometric Data (Female data in shaded columns)

	Dose (mg/kg-day)								
	PND 12						PND 66		
	Male			Female			Male	Female	
	0.3	1	5	0.3	1	5	5	1	5
Brain Weight	110.6	105.5	88.5*	98.9	97.6	91.3*	100.0	101.1	97.4
Ant/Post cerebrum	107.1	104.7	93.8	102.4	103.2	98.0	101.8	100.1	99.4
Ant/Post cerebellum	105.6	102.0	75.5*	95.3	103.7	94.3	99.7	99.7	97.6
Frontal cortex	100.9	100.3	94.4	100.9	98.5	99.4	98.7	100.2	98.9
Parietal cortex	108.4	108.4	94.0	99.7	99.1	94.5	102.1	95.8*	94.9*
Caudate-Putamen	100.0	103.2	99.3	93.3	96.0	90.3*	98.0	99.1	105.0
Corpus callosum	103.4	99.0	100.0	93.2	99.0	89.1	92.8	105.2	95.4
Hippocampal gyrus	111.1	107.5	91.2	97.4	99.6	88.5	98.3	96.3	93.2
Cerebellum height	98.6	97.5	85.8*	90.4	88.8*	91.3	99.1	97.4	99.0
Ext. Germinal layer	103.1	107.6	101.3	94.0	106.5	105.6	-	-	-

Statistical analyses were performed before conversion to percent of control.
*P<0.05, Dunnett's test; the findings for ANOVAs were also significant for A/P Cerebellum, parietal cortex and hippocampal gyrus for males at PND 12, in the absence of significant effects for individual comparisons.

The authors cited lack of a consistent dose-response trend in morphometry, an approximate 5-6% reduction over that of control animals for a few parameters, as well as small sample size and observations of substantial variability among controls as indications that the above findings were likely to have been incidental. The decrements at PND 66 were slightly higher

at 5 mg/kg-day than at 1 mg/kg-day, and the changes were consistent with the PND 12 sacrifice results. The high variability in the data, however, would more likely contribute to a lack of statistical significance in the face of true differences than a case of statistical significance in the absence of true differences given the small sample size. Also, the average coefficient of variation for the data at each of the time points (PND 12 and PND 66) as estimated by U.S. EPA scientists was below 8% overall and less than 3% for the parietal cortex thickness at PND 66. So, the effect was considered by them to be treatment-related, while the magnitude may not be dependent on dose resulting in the morphological alterations in the parietal cortex of female offspring at PND 66 being both statistically and biologically significant at the mid- and high-dose levels with a clear indication that the structure of the brain had been altered by treatment. Further, because of the absence of morphometric evaluation of the 0.3 mg/kg-day low dose group, they could not determine a NOAEL for this study. Their overall conclusion was "*It is not possible to definitively classify findings in the preweaning offspring as having originated with pre-or postnatal exposure, nor as resulting from developmental perturbation versus direct systemic-or neurotoxicity. However, adverse findings in the adult (PND 66) offspring, i.e., alterations in motor activity, auditory startle response, and brain structure (decreased measurements of the parietal cortex and hippocampal gyrus in the absence of significant brain weight deficits) observed at PND 66 in the offspring, long after exposure via the dam can be interpreted to represent long-term sequelae of developmental exposure*." (U.S. EPA, 2000a). Data on ChE inhibition were not presented for the offspring (fetuses and pups) in this study, so a correlation with the observed effects was not possible.

Maurissen et al., 2000
Lack of selective developmental neurotoxicity in rat pups from dams treated by gavage with chlorpyrifos

In this study, pregnant Sprague-Dawley rats were given CPF in corn oil by gavage from GD 6 through lactation day 10 at dosages of 0, 0.3, 1, or 5 mg/kg-day in a developmental neurotoxicity study that conformed to U.S. EPA (1991) guidelines. In the dams, toxicity was noted at the highest dosage level of 5 mg/kg-day with muscle fasciculation, hyperpnea, and hyperreactivity being observed. The authors reported a nonsignificant overall trend toward decreased weight gain and feed consumption in the dams at the high-dose group, with a statistically significant Group x Time interaction for reduced weight gain in the 5 mg/kg-day group near the end of gestation. Many developmental indices were normal, but pups from high-dose group had increased mortality soon after birth, gained weight more slowly than controls, and had several indications of slightly delayed maturation. The early deaths and delayed maturation were attributed to maternal toxicity, though a possible contributing role of direct pup toxicity in delayed development was also considered. In spite of the apparent delay in physical development, learning and memory as tested on T-maze spatial alternation tasks, motor activity and auditory startle were not affected in the high dose animals (pups) tested just after weaning. No overt effects were noted in either dams or pups at 1 or 0.3 mg/kg/day. On GD 20, brain AChE of the 1 mg/kg and 5 mg/kg groups were 82% and 10.2% of the controls. According to

the authors, CPF produced maternal and developmental toxicity in the 5 mg/kg-day group but did not cause selective developmental neurotoxicity.

Qiao, et al., 2002.
Developmental neurotoxicity of chlorpyrifos: what is the vulnerable period?

Effects of CPF (sc injections in dimethyl sulfoxide-DMSO vehicle) were examined in nonpregnant female rats using 11 animals per treatment group. In addition, pregnant rats were given CPF at 0, 1, 2, or 5 mg/kg daily from GD 9-12, the period of neural tube formation, and then examined on GD 17 and GD 21 (n = 7-10). A second group received 0, 1, 2, 5, 10, 20, or 40 mg/kg daily on GD 17–20, a peak period of neurogenesis, and determinations were conducted on GD 21 (n = 7–9). This was based on previous exposure of neonatal rats to CPF that produced brain cell damage and loss, with resultant abnormalities of synaptic development.

Repeated administration of CPF to nonpregnant female rats had no effect on body weights at 10 mg/kg-day, but animals lost significant amounts of weight at doses of 20 mg/kg-day or higher. Pregnant rats, however, were more sensitive to CPF, with impaired weight gain seen when CPF was given on GD 9–12 at 5 mg/kg-day. The pregnant rats regained normal weights by GD 14. In the GD 17–20 group, reduction in maternal weight gain was seen at 5 mg/kg-day and above and there was no evidence of general fetotoxicity as assessed by the number of fetuses or fetal body and tissue weights both, at or below 5 mg/kg-day.

CPF treatment on GD 17-20. Chlorpyrifos (1 mg/kg-day) on GD 17–20 had no significant effect on ChE activity in fetal brain assessed on GD 21, 24 hr after the last dose. However, significant inhibition of ChE activity in the brain (15–20%) was seen at 2 mg/kg-day, rising to 80% inhibition at 40 mg/kg-day, with slightly greater effects on the forebrain compared with the brainstem. The authors state that differences in the rate of re-synthesis of ChE may also account for the smaller effects on enzyme activity in the brainstem compared with forebrain.

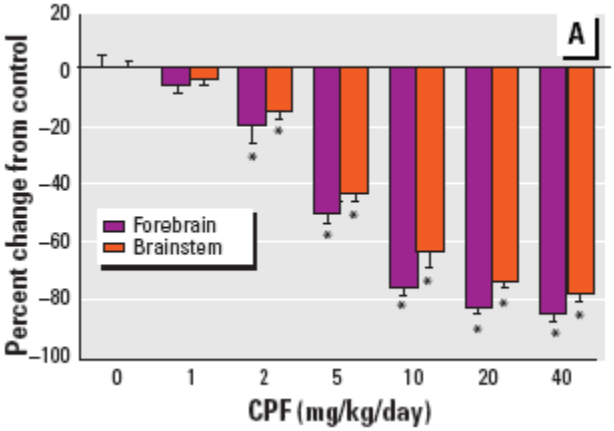


Figure C2. Effects of CPF (GD17-20) on cholinergic markers, assessed on GD 21, presented as the percentage change from corresponding control values. (*A*) Cholinesterase (ANOVA: treatment, $p < 0.0001$; treatment x tissue, $p < 0.0001$);

Chlorpyrifos had a smaller effect on choline acetyltransferase (ChAT) activity, a marker for development of presynaptic cholinergic nerve terminals. Reductions in ChAT were seen at all doses but did not achieve statistical significance until a threshold of 20 mg/kg-day, with the maximum deficit of 10%. Thus, indices of cholinergic synaptic development showed significant CPF-induced defects but only at doses above the threshold for ChE inhibition. Also "brain sparing", defined as reduced body weight with an increase in brain/body weight ratio, was noted above 5 mg/kg-day. Indices of cell packing density (DNA per gram of tissue) and cell number (DNA content) showed effects only on the liver; however, there were significant changes in the protein/DNA ratio, an index of cell size, in fetal brain regions at doses as low as 1 mg/kg, below the threshold for inhibition of fetal brain cholinesterase (2 mg/kg). At the two lowest doses cell size was enhanced in the brainstem and reduced in the forebrain, statistically significant overall by ANOVA as well as individually in *post hoc* tests.

CPF treatment on GD 9-12. With CPF treatment (GD 9–12), there was no evidence of general fetotoxicity or alterations of brain cell development at doses up to the threshold for maternal toxicity (5 mg/kg) assessed on GD 17 and GD 21; however, augmentation of cholinergic synaptic markers was detected at doses as low as 1 mg/kg. There was a consistent overall pattern ($p < 0.04$) of reduced cell size at low doses of CPF, with loss or reversal of the effect at the highest dose. Low doses of CPF given on GD 9–12 evoked significant elevations of ChAT, a constitutive marker for cholinergic nerve terminals in whole brain assessed on GD 17, with a loss or reversal of the effect at the higher dose of 5 mg/kg-day.

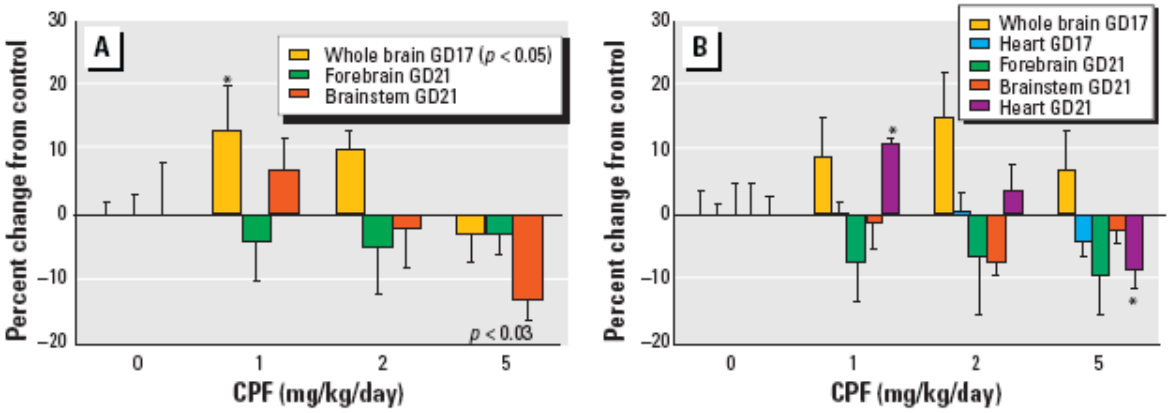


Figure C3. Effects of CPF (GD9-12) on cholinergic markers, assessed on GD 17 and GD 21, presented as the percentage change from corresponding control values. (*A*) ChAT (ANOVA: treatment, $p < 0.05$); (*B*) m2- AChRs (ANOVA: heart, $p < 0.005$).
*Individual values showing significant effects and main treatment effects across multiple tissues are shown the bar clusters.

According to the authors, compared with previous work on postnatal CPF exposure, the effects seen in this study required doses closer to the threshold for fetal weight loss implying a lower vulnerability in the fetal compared with the neonatal brain. The findings from this study suggest that the effects of CPF on fetal brain development are fundamentally different for exposure in early (GD 9–12) compared with late (GD 17–20) gestation. Exposure during the earlier period evoked an augmentation in cholinergic synaptic markers instead of the deficits seen with later treatment. Promotional effects were seen only at low doses (1 or 2 mg/kg-day), but were offset by a higher dose (5 mg/kg-day) that impaired maternal weight gain. During brain development, ACh appears to serve as a trophic factor, regulating the differentiation of target cells containing cholinergic receptors and its precursor and breakdown product, choline, also augments neural plasticity. During early gestation, low CPF exposures elicit promotional effects on neural cell differentiation that are offset when doses are raised to the point of cellular or general fetotoxicity. Overall these results point to a shifting spectrum of CPF effects on neurodevelopment, dependent both on the exposure window and on the dose. They concluded that although delayed neurotoxic effects of prenatal CPF may emerge subsequently in development, their results are consistent with the preferential targeting of late developmental events such as gliogenesis, axonogenesis, and synaptogenesis.

Levin et al., 2002.
Prenatal chlorpyrifos exposure in rats causes persistent behavioral alterations

Chlorpyrifos was administered to pregnant rats on GD 17—20, a peak period of neurogenesis, using doses of 1 or 5 mg/kg-day (in 1 ml/kg of DMSO via sc injection). Performance in the T-maze, Figure-8 apparatus and 16-arm radial maze, beginning in adolescence and continuing into adulthood, was evaluated. Chlorpyrifos elicited initial locomotor hyperactivity in the T-maze. Females showed slower habituation in the Figure-8 maze; no effects were seen in males (Figure

C4). In the radial16-arm radial maze, females showed impaired choice accuracy for both working and reference memory and again, males were unaffected (Figure C5). Despite the deficits, all animals eventually learned the maze with continued training. When challenged with the muscarinic antagonist, scopolamine, (to determine the dependence of behavioral performance on cholinergic function) control females showed impairment with scopolamine, as well as 5 mg/kg-day exposed females. However, 1 mg/kg-day CPF-exposed females did not, implying that the delayed acquisition of the task had been accomplished through alternative mechanisms (Figure C6). The differences were specific to muscarinic circuits, as control and CPF groups responded similarly to the nicotinic antagonist, mecamylamine. The adverse effects of CPF were greater in the group receiving 1 mg/kg as compared to 5 mg/kg and the authors suggest that promotional effects of ACh on cell differentiation may thus help to offset CPF-induced developmental damage that occurs through other noncholinergic mechanisms.

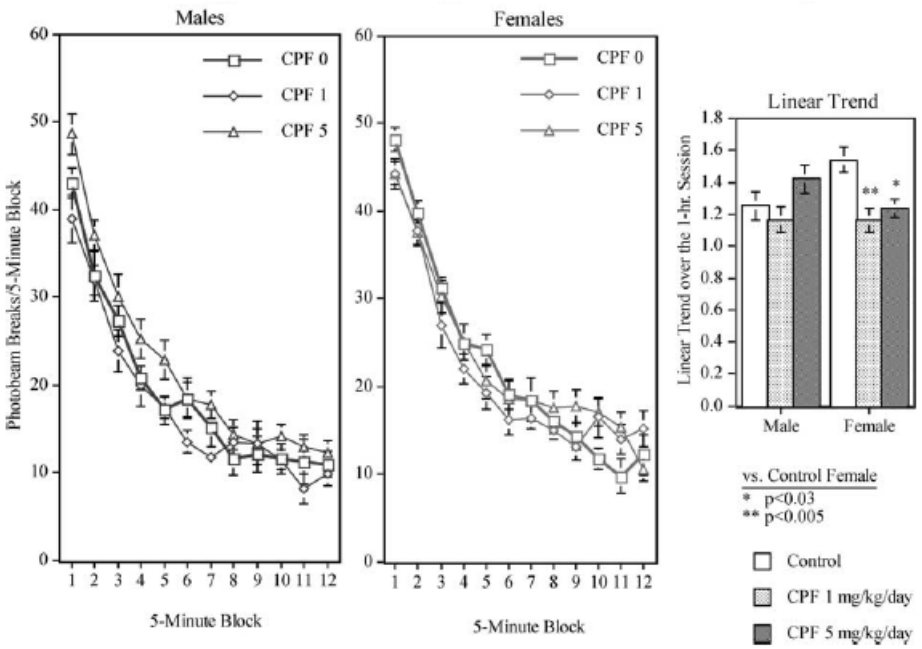


Figure C4. Effects of prenatal CPF exposure (0, 1 and 5 mg/kg-day on GD 17–20) on habituation in the Figure-8 apparatus during the first test session (mean ± S.E.M.).
Each time block was 5 min long. The linear trend (slope of decrease in activity over consecutive 5-min blocks) was significantly ($p<0.05$) lower in the CPF-exposed rats than controls.

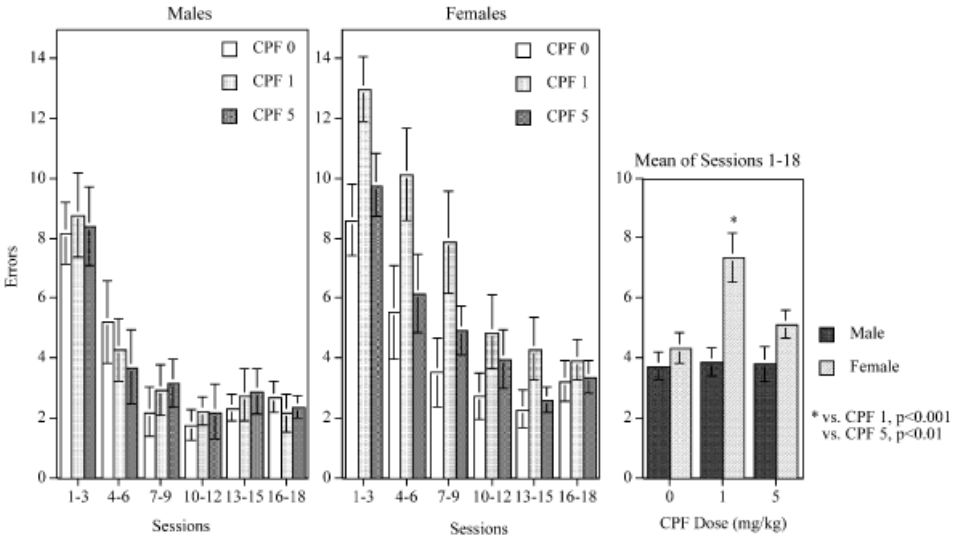


Figure C5. Effects of prenatal CPF exposure (0, 1 or 5 mg/kg-day on GD 17 – 20) on working memory performance during acquisition training (mean ± S.E.M.) in the 16-arm radial maze. Each session comprises one test on the maze.

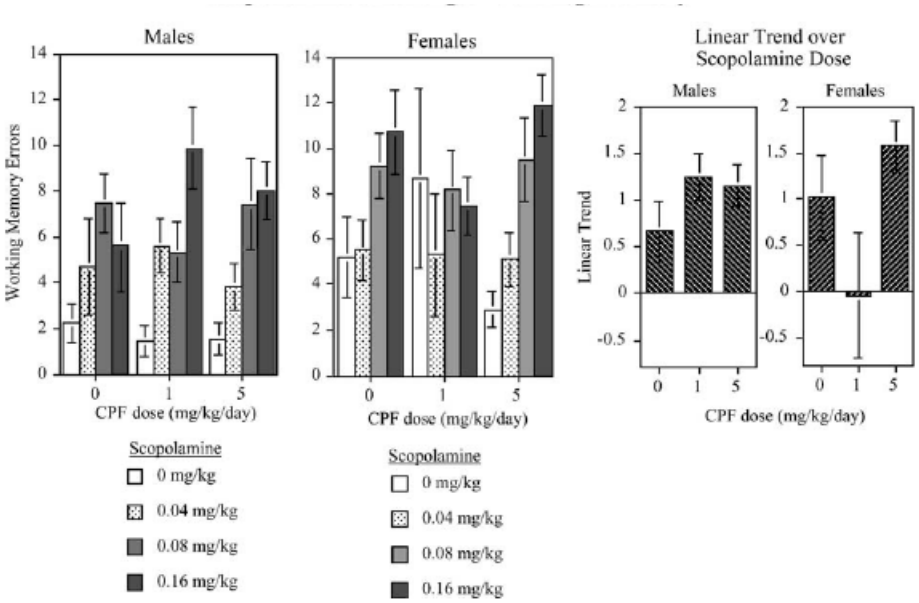


Figure C6. Prenatal CPF exposure (0, 1 or 5 mg/kg-day on GD 17–20) effects on the sensitivity of working memory performance to scopolamine (mean ± S.E.M.) in the 16-arm radial maze.

In this study, late prenatal exposure to CPF induced long-term changes in cognitive performance that are distinctly gender-selective. The strengths of using this study for risk assessment include the use of litter-based statistics. Also, a full report of the statistical analysis is provided and in conformity to practice standards, male and females were separated for analysis if sex effects or

sex treatment effects were found in the initial ANOVA. Repeated measures analysis of varianceANOVA was used for endpoints recorded sequentially in the same animals and a protected *post hoc* test was used. The sample size was 10 per sex per group and the groups were balanced for sex. In addition, the authors reported on general toxicity. For neurobehavior, error measures rather than latencies in learning and memory tests were considered; latency measures are more subject to interference by motor impairment. Also, knowing that the groups did not differ in spontaneous alternation supports a cognitive interpretation of the 16-arm radial arm maze data. The test for spontaneous motor activity confirms selective sensitivity of females as seen in the 16-arm radial maze task, and provides context for interpretation of radial 16-arm radial maze data in showing that locomotor activity was not grossly affected. The use of a linear trend across time to measure learning rather than comparing performance at any one time point between groups and the scopolamine challenge experiment support non- linear CPF dose response related to cholinergic brain systems. Issues that may be limitations of this study include the fact that the spontaneous alternation and activity tests took place during adolescence so they are not necessarily relevant to the maze test, which was in adults. Also, the study used sc injection, which is not the route of exposure for humans. However, this allows comparison of prenatal effects to previously determined postnatal effects. While no data on general toxicity are shown, the authors refer to other reports to support lack of general toxicity such as mortality and growth retardation.

Richardson and Chambers, 2003.
Effects of gestational exposure to chlorpyrifos on postnatal central and peripheral cholinergic neurochemistry

The effects of gestational exposure to CPF on postnatal neurochemistry (central and peripheral cholinergic) were investigated. Pregnant rats were orally dosed daily with CPF (0, 3, 5, or 7 mg/kg) in corn oil from GD 6 to 20. Dams were weighed daily, and treatments were administered on a vanilla wafer (Nabisco) at 0.5 ml/kg to reduce handling stress involved with oral intubation. Treated cookies were totally consumed within 10 min of administration. Pups were sacrificed on PND 1, 3, 6, 9, and 12 for the determination of brain, heart, lung, and serum cholinesterase, and brain choline acetyltransferase (ChAT) activities, along with liver carboxylesterase activity. Exposure to CPF did not produce signs of overt toxicity to the dams or developing offspring. Dams did not exhibit any signs of overt cholinergic crisis (i.e., lip smacking, lacrimation, diarrhea, or tremors) and no adverse effects on pup growth rate were noted. Cholinesterase activities were inhibited in a dose-related manner, with brain ChE inhibition of about 26%, 32%, and 45% on PND 1. Inhibition of brain cholinesterase persisted in all treatment groups until PND 6 and in the medium- and high-dosage groups through PND 9. By PND 12, activities of CPF-treated groups were not significantly different from control values. These results indicate that gestational exposure from GD 6 through GD 20 to CPF at 3, 5 and 7 mg/kg-day orally results in relatively persistent inhibition of brain ChE and a delayed depression of choline acetyltransferase (ChAT) at a time when brain ChE activity had returned to control levels in the high-dosage group. The authors also stated that while brain ChE inhibition may recover more quickly in the developing rat, there is still significant inhibition during the initial days of this rapid phase of brain development known as the brain “growth spurt” (Dobbing and Sands, 1979). Although it could be inferred that the persistence of the inhibition of brain ChE

was the result of lactational exposure, from the work of Mattson et al. (2000) the milk level of dams exposed to chlorpyrifos at 5mg/kg-day continually from GD6 was 3µg/ml on PND1, and estimated that pups would be exposed to only 0.126 mg/kg-day CPF which is similar to the threshold level for plasma ChE inhibition in adult rats of 0.1mg/kg-day as reported by Breslin et al., (1996). Also, transient ChE inhibition in serum and peripheral tissues of the offspring was noted at doses that did not elicit overt signs of toxicity in the dams.

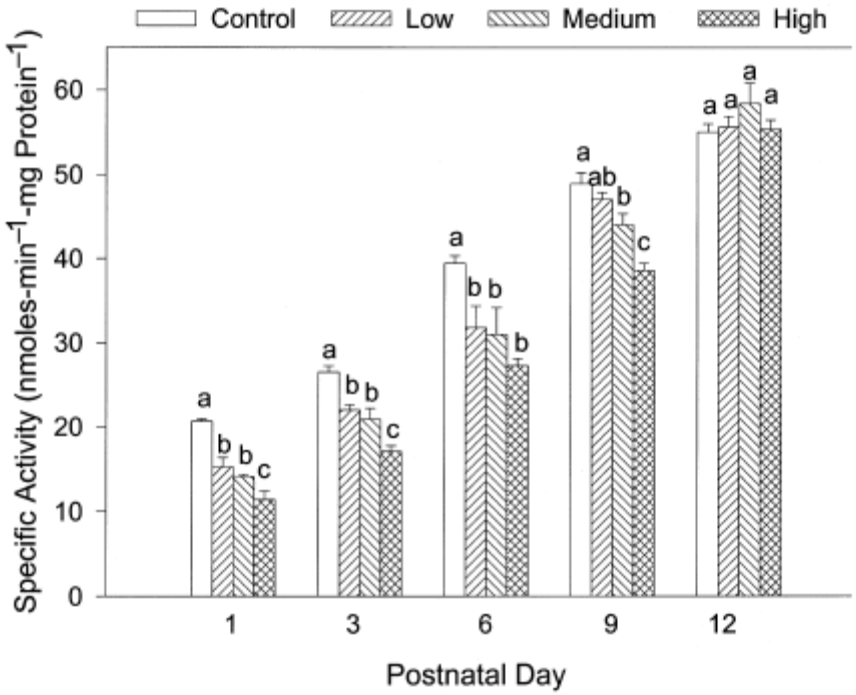


Figure C7. Specific activity of brain cholinesterase of rat pups exposed to 0 (control), 3 (low), 5 (medium), or 7 (high) mg/kg-day chlorpyrifos from gestation day 6 through 20. Bars labeled with the same letter on the same sampling day are not significantly different from each other ($p \leq .05$) by SNK post hoc test. Data are presented as the mean ± SEM (n = 3–4).

Liver carboxylesterase activity was also inhibited in a dose-related manner, with a recovery profile parallel to that of brain ChE. Choline acetyltransferase activity was decreased by about 13% in the high-dosage group on PND 9 and 12. The decrease in ChAT activity could reflect a developmental delay or a compensatory mechanism; however, the absence of significant ChE inhibition on PND12 argues against a compensatory mechanism. In this regard the authors speculated that the reduction in ChAT activity observed may be the result of insufficient availability of choline because of a decrease in high-affinity choline uptake (HACU) system which is the rate-limiting step in the synthesis of acetyl choline. Accordingly, they could not determine whether this was a transient or permanent effect.

Farag et al., 2003.
Developmental toxicity study of chlorpyrifos in rats

In this study, groups of 30 bred female Fischer 344 rats were given 0, 5, 15, and 25 mg/kg-day of CPF in corn oil by gavage on GD 6–15; the fetuses were evaluated on GD 21. Clinical signs of toxicity attributed to CPF were noted in dams receiving 15 and 25 mg/kg-day. Maternal effects in these groups also included depressed body weight and AChE activity. Tremors and salivation (signs of cholinergic toxicity) were noted in dams at 15 and 25 mg/kg-day. These effects appeared on day 10 of gestation (day 5 of treatment) and progressed throughout the period of treatment and were seen in 80% of dams at 25 mg/kg-day and in 60% of dams at 15 mg/kg-day. At 5 mg/kg-day, maternal and fetal brain ChE activities were 92 and 94% of control, respectively, and no effects were noted in the general appearance of animals at this dose level. While maternal brain ChE activity was markedly reduced at 15 and 25 mg/kg-day (69 and 51% of control, respectively), fetal brain AChE activity was not statistically altered at these doses (90 and 87% of control, respectively).

Table C34. Maternal parameters in rats after exposure to chlorpyrifos on gestation days 6-15 of gestation

	Days	Chlorpyrifos (mg/kg per day)			
		0	5	15	25
Number of dams		28	26	24	28
Body weight[a]	6	158.16 ± 13	160.90 ± 10	160.30 ± 10	161.93 ± 7
	9	162.34 ± 13	164.78 ± 10	166.66 ± 9	164.88 ± 7
	12	169.56 ± 10	170.80 ± 10	171.64 ± 9	168.84 ± 7
	16	184.13 ± 10	180.67 ± 11	171.44 ± 9*	170.25 ± 11*
	21	221.05 ± 11	218.73 ± 10	201.25 ± 10*	192.96 ± 10**
Liver weight					
Absolute[a]		7.62 ± 0.77	7.02 ± 0.23	7.98 ± 0.56	7.86 ± 0.41
Relative[b]		0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
Brain weight					
Absolute[a]		1.45 ± 0.22	1.27 ± 0.23	1.21 ± 0.14	1.18 ± 0.11
Relative[b]		0.01 ± 0	0.01 ± 0	0.01 ± 0	0.01 ± 0
Dam brain acetylcholineasterase (mg protein/min)		29.51 ± 1.14	27.05 ± 0.73	20.42 ± 1.41**	15.03 ± 0.76**
Fetal brain acetylcholineasterase (mg protein/min)		31.22 ± 1.45	29.29 ± 0.42	28.02 ± 0.77	27.36 ± 0.50

Data are presented as mean ± S.D.
[a] Body and organ weight in grams.
[b] Relative organ weight in grams (organ weight/body weight).
* Significantly different from control at $P < 0.05$.
** Significantly different from control at $P < 0.01$.

Maternal body weights were decreased significantly at 15 and 25 mg/kg-day. Fetal weight and viability were decreased, and fetal death and early resorption were increased at the 25 mg/kg-day maternal dose. Visceral, skeletal, and external variations were also increased in this group. Chlorpyrifos showed fetotoxic and teratogenic effects at a maternal dose of 25 mg/kg per day, along with maternal toxicity. No evidence of developmental toxicity was observed at 5 and 15

mg/kg-day. Since the developmental toxicity was noted only at the high dose of 25 mg/kg-day, along with severe maternal effects such as weight loss and cholinesterase inhibition, the data are not included in this document.

Tian and Yamauchi. 2003
Micronucleus formation in 3-day mouse embryos associated with maternal exposure to chlorpyrifos during the early preimplantation period

Chlorpyrifos was evaluated for its ability to induce cytogenetic damage in preimplantation embryos after maternal exposure. Pregnant female mice were administered a single ip dose of CPF (40 or 80 mg/kg) at 10:00 h on Day 0 of pregnancy. On day 3 of gestation, blastocysts were collected and evaluated for gross morphology, micronucleus (MN) frequency, and cell number. A significant increase in MN frequency indicating cytogenetic damage was observed in the treatment groups in comparison to control. The MN frequency revealed a clear dose-dependent increase. There was also a significant decrease in the embryo cell number in the 80 mg/kg treated group. This simultaneous decrease in the cell number and increase in MN frequency may reflect an embryonic developmental disadvantage resulting from maternal treatment with CPF. This same group of researchers also conducted a developmental toxicity study in mice described later in the document.

Qiao, et al., 2003.
Fetal chlorpyrifos exposure: adverse effects on brain cell development and cholinergic biomarkers emerge postnatally and continue into adolescence and adulthood

CPF (sc injections in DMSO) was administered to pregnant rats (Sprague-Dawley) on GD 17-20 at 0, 1, or 5 mg/kg and subsequent development of acetylcholine systems in forebrain regions involved in cognitive function were examined and compared with general biomarkers of cell development. On the day after birth, pups were randomized within treatment groups and redistributed to nursing dams with a litter size of 10 to maintain standardized nutrition. Randomization was repeated at several subsequent intervals, with dams rotated among litters to distribute any maternal caretaking differences randomly across litters and treatment groups. Animals were weaned on PND 21 and brain regions were examined in adolescence (PND 30) and young adulthood (PND 60). Tissues were frozen with liquid nitrogen and maintained at –45°C. At each age, each treatment group included 8–16 animals, evenly divided between males and females; the number of animals in each of the CPF groups was always matched to an equal number of controls, and determinations used no more than one male and one female from each litter. All assays were run such that all the animals for the control and both CPF groups were evaluated simultaneously to ensure that day-to-day variations in assays did not generate spurious treatment effects. Choline acetyltransferase, a constitutive marker for cholinergic nerve terminals, showed only minor CPF-induced changes during the period of rapid synaptogenesis. Hemicholinium-3 binding to the presynaptic choline transporter, which is responsive to nerve impulse activity, displayed marked suppression in the animals exposed to CPF; however, values returned to nearly normal by weaning. Deficits were again apparent in adolescence and

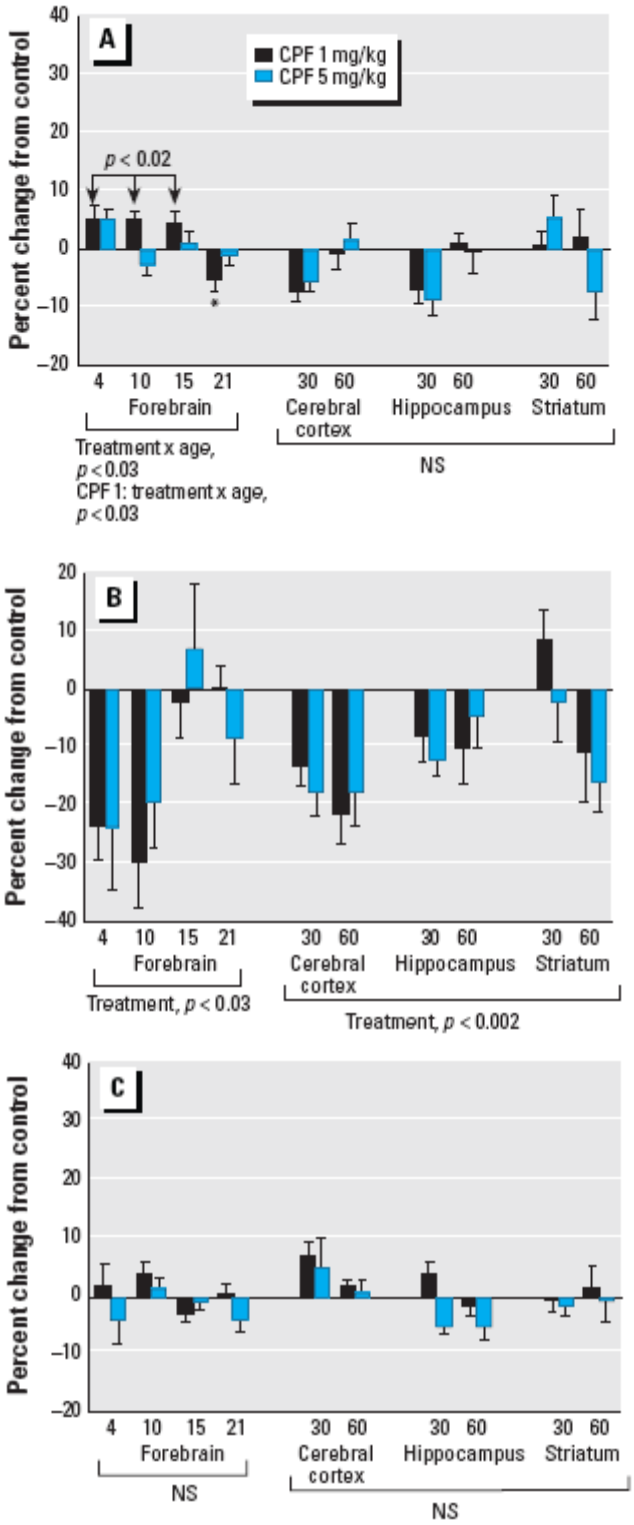


Figure C8. Effects of prenatal CPF exposure on postnatal development of cholinergic biomarkers: (*A*) ChAT activity; (*B*) HC-3 binding; and (*C*) m2AChR binding. NS = not significant. Data are presented as percentage change from control values at the postnatal ages (days). ANOVA results across ages and regions appear at the bottom of A–C.

adulthood and there was no compensatory up-regulation of cholinergic receptors, as m2-muscarinic cholinergic receptor binding was unchanged.

Until weaning, measurements were made in the whole forebrain; for determinations in adolescence and adulthood, the forebrain was divided into its constituent sub-regions: cerebral cortex, hippocampus, striatum. Tests of individual points where the CPF group differs from the corresponding control were carried out only where the global test indicated an interaction of treatment x age; this occurred only for the forebrain, which showed a main treatment effect across the first three age points in the low-dose group (arrows) and a decrease on PND 21 (asterisk). Testing of individual subregions on PND 30 and PND 60 was not conducted because of the absence of a treatment x region interaction. Results for males and females were combined because of the absence of a treatment x sex effect. A similar approach was used to evaluate postnatal development of cell protein biomarkers for relative cell size (total protein/DNA) and membrane surface area (membrane protein concentration, membrane/total protein) in Figure C9 below.

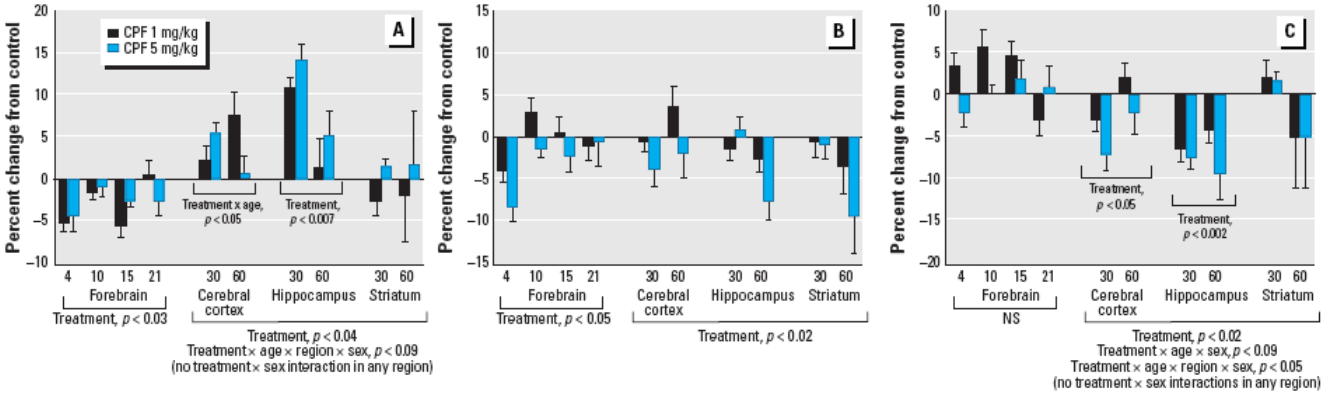


Figure C9. Effects of prenatal CPF exposure on postnatal development of cell protein biomarkers for (*A*) relative cell size (total protein/DNA) and membrane surface area ([*B*] membrane protein concentration, [*C*] membrane/total protein). NS = not significant. Data are presented as percentage change from control values, at the postnatal ages (days). ANOVA results across ages and regions appear at the bottom of A-C.

Chlorpyrifos also elicited delayed-onset alterations in biomarkers for general aspects of cell integrity, with reductions in cell packing density, increases in relative cell size, and contraction of neuritic extensions. According to the authors, neither the magnitude nor timing of these changes was predictive of the cholinergic defects, rather, the cellular effects may actually result from defective synaptic transmission.

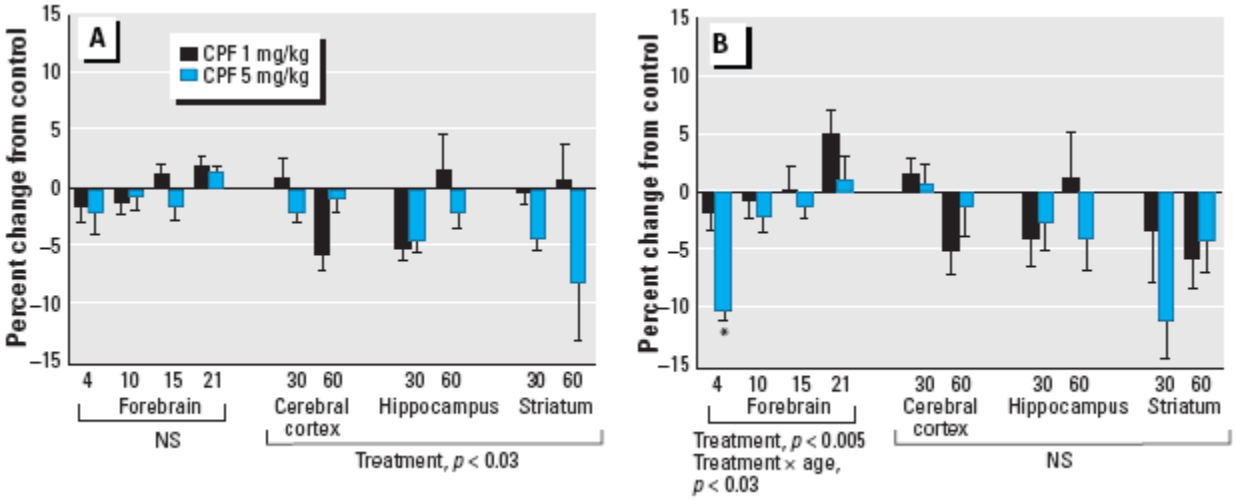


Figure C10. Effects of prenatal CPF exposure on postnatal development of biomarkers for (*A*) cell packing density (DNA concentration = DNA/g tissue) and (*B*) cell number (DNA content = DNA/region). NS = not significant. Data are presented as percentage change from control values at the postnatal ages (days) indicated.

The authors concluded that their findings indicate a wide window of vulnerability of cholinergic systems to CPF, extending from prenatal through postnatal periods, occurring independently of adverse effects on general cellular neurotoxicity. Further, the authors claim that the present results indicate that, despite the initial sparing, prenatal CPF exposure elicits marked alterations that emerge in the postnatal period identifying postnatal deficits in cholinergic activity that persisted into adulthood associated with, but not necessarily caused by, delayed, generalized effects on brain cell development. And, while CPF does concentrate in milk (Mattsson et al., 2000), its short biologic half-life (Lassiter et al., 1998; Hunter et al., 1999) makes it extremely unlikely that the observed effects of prenatal exposure, terminated 2 days before birth, reflect an indirect postnatal exposure from residual CPF. The authors therefore concluded that prenatal CPF exposure compromises the subsequent development of cholinergic synaptic function, characterized by deficits in an index of neural activity (decreased HC-3 binding) without substantial loss of nerve terminals (little or no change in ChAT). These changes persist into adolescence and adulthood and correspond to the long term defects in cholinergic components of working and reference memory (Levin et al., 2002).

Qiao., et al., 2004.
Chlorpyrifos exposure during neurulation: cholinergic synaptic dysfunction and cellular alterations in brain regions at adolescence and adulthood

Pregnant rats were given **0, 1 or 5 mg CPF/kg daily** (in 1 ml/kg of DMSO via sc injection) **on GD 9-12** with (7 dams/group), and subsequent development of acetylcholine systems was then examined and compared to those on general biomarkers of cell development. These included three different elements: ChAT and HC-3 binding (presynaptic components), and m2AChRs (mediate major cell signaling events evoked by acetylcholine release). On the day after birth,

pups were randomized within treatment groups and redistributed to nursing dams with a litter size of 10, to maintain standardized nutrition. Randomization was repeated at several subsequent intervals, with dams rotated among litters to distribute any maternal caretaking differences randomly across litters and treatment groups. Animals were weaned on PND 21 and brain regions were examined in adolescence (PND 30) and young adulthood (PND 60), focusing on the cerebral cortex, hippocampus and striatum, regions known to be potential targets for prenatal CPF exposure. Tissues were frozen with liquid nitrogen and maintained at -45 ºC. Each treatment group comprised 16 animals at each age point, evenly divided between males and females. Assays were run such that, for a given region and age point, all the animals for the control and both CPF groups were evaluated simultaneously to ensure that day-to-day variations in assays did not generate spurious treatment effects. Global analysis of variance (ANOVA) on data groupings corresponding to the three classes of measurements: weights, cholinergic biomarkers (ChAT, HC-3 binding, m2AChR binding) and indices of cell number, size and membrane surface area (DNA content and concentration, total protein/DNA, membrane protein/total protein) were conducted. Because each tissue homogenate contributed multiple assessments in each category, the various determinations were treated as repeated measures. The initial test indicated treatment effects differing among the different measures, so data were then examined separately for each measure, again using a multivariate ANOVA (treatment, region, age, sex). No signs of systemic toxicity were noted and neither was there any significant effect on maternal weights, litter sizes or neonatal viability. Across adolescence and adulthood, however, there were significant effects on body weight (main treatment effect, $p < 0.03$) that depended on sex (treatment x sex interaction, $p < 0.02$). Separate analyses of the body weights of males and females found a significant, albeit small, main effect only in the females ($p < 0.0008$, $p < 0.0002$ for control vs. CPF 1 mg/kg; $p < 0.05$ for control vs. CPF 5 mg/kg): PND 30, control 103 ± 2 g, CPF 1 mg/kg 95 ± 2 g, CPF 5 mg/kg 99 ± 2 g; PND 60, control 252 ± 8 g, CPF 1 mg/kg 225 ± 5 g, CPF 5 mg/kg 238 ± 6 g However, the sex-selective effects on body weight did not produce corresponding differences in brain region weights (data were not shown). Although there was a main treatment effect of CPF ($p < 0.03$), there was no sex-dependence (no interaction of treatment x sex), nor was there a consistent dose–effect relationship, as the small differences were confined to the group receiving the lower dose of CPF ($p < 0.02$). Re-examination of the tissue weights separately for each region found that the significant differences were confined to the hippocampus (main effect, $p < 0.005$), but again without sex-dependence and with the effect confined to the group receiving the lower dose of CPF ($p < 0.02$ compared to control). The differences were not significant when each age was considered separately. Across all three regions (cerebral cortex, hippocampus and striatum), both age points and both sexes, CPF had a significant effect on ChAT activity (main treatment effect, $p < 0.0001$) that reflected alterations for both the low and high dose groups ($p < 0.0001$ and $p < 0.0002$, respectively). In addition, the effect was regionally selective (treatment x region, $p < 0.01$), with increased ChAT in adolescence and adulthood, and statistically significant in the hippocampus and striatum.

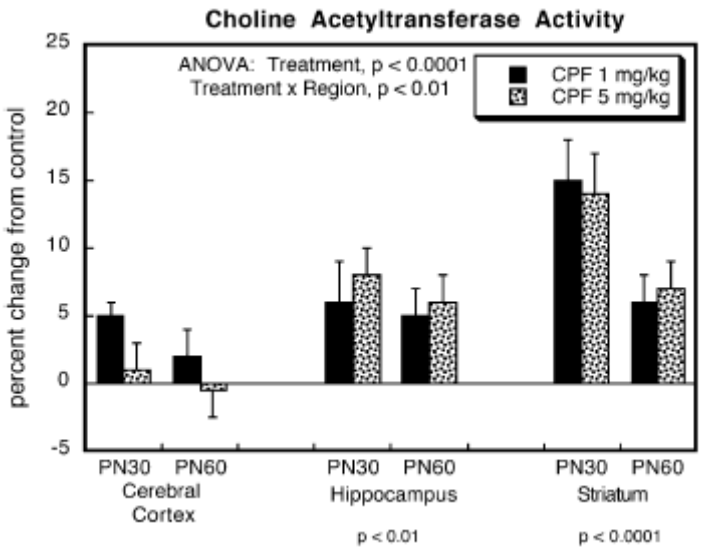


Figure C11. Effects of prenatal CPF exposure on postnatal development of choline acetyltransferase activity, presented as the percent change from control values. ANOVA across ages and regions appears at the top; tests for each region appears at the bottom. Testing of individual ages for each region not conducted because of the absence of treatment x age interactions.

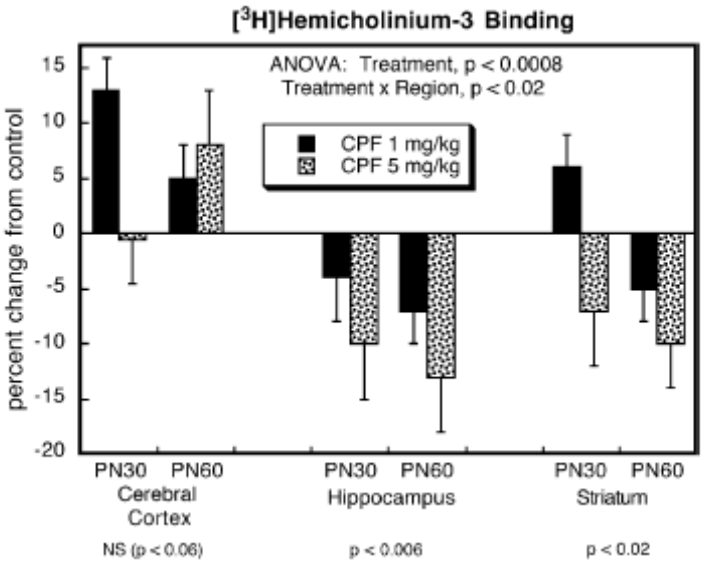


Figure C12. Effects of prenatal CPF exposure on postnatal development of the high affinity choline transporter, assessed by the binding of [3H]hemicholinium-3, presented as the percent change from control values. ANOVA across ages and regions appears at the top; tests for each region appears at the bottom. Testing of individual ages for each region was not conducted because of the absence of treatment x age interactions.

As illustrated in Figure C12, hemicholinium-3 (HC-3) binding to the presynaptic choline transporter, an index of nerve impulse activity, was markedly subnormal. In the cerebral cortex, prenatal CPF exposure elicited an overall increase in HC-3 binding that was at the margin of significance, whereas robust decreases were found in the hippocampus and striatum. These effects all persisted into adulthood.

The statistical pattern of CPF's effects on m2AChRs was different from that of the other two cholinergic markers with m2-muscarinic cholinergic receptor binding significantly reduced ($p < 0.02$), instead of showing the expected compensatory up-regulation for reduced neural input.

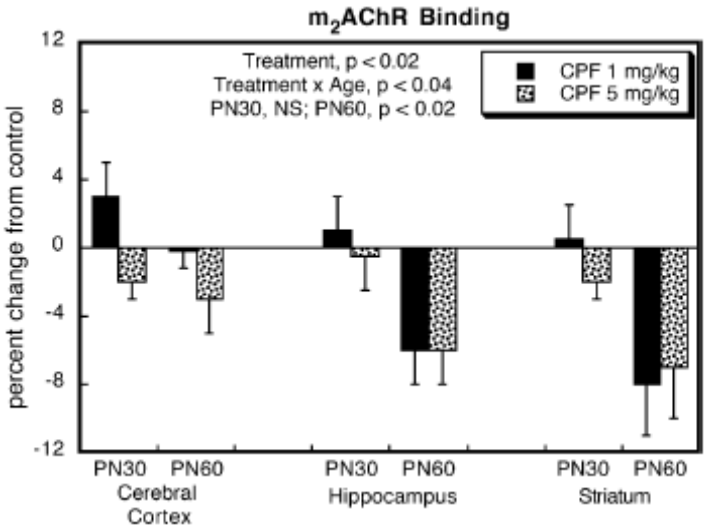


Figure C13. Effects of prenatal CPF exposure on postnatal development of m2AChR binding sites, presented as the percent change from control values. ANOVA across ages and regions appears at the top, along with separate tests for PN30 and PN60, as there was a significant treatment x age interaction. Testing of individual regions was not conducted because of the absence of a treatment x region interaction.

Chlorpyrifos also elicited delayed-onset alterations in biomarkers of cell packing density, cell number, cell size and neuritic projections, involving brain regions both with and without reductions in indices of cholinergic activity. According to the authors, the significant elevations in ChAT in the hippocampus and striatum were suggestive of increased density of cholinergic projections and if this were the only effect elicited by CPF exposure, then HC-3 binding would be augmented. While a marginal increase was seen in the cerebral cortex, that region had minimal changes in ChAT and the hippocampus and striatum, showed robust increases in ChAT with sustained decrements in HC-3 binding instead of the expected elevations. From previous work it appears that HC-3 binding, but not ChAT, is responsive to nerve impulse activity, hence the authors concluded the dichotomy between the two cholinergic markers indicated that cholinergic synaptic activity is markedly reduced by prenatal CPF exposure, with a distinct regional selectivity. Since m2AChR binding was impaired in the same two regions, this effect would serve to worsen any deficit in synaptic transmission and so the authors proposed that the promotional effect on ChAT may actually represent either nonfunctional cholinergic terminals,

or sprouting compensatory to a primary defect in cholinergic synaptic transmission. This conclusion appears to be in agreement with evidence for impaired cholinergic contributions to working memory in hippocampus-related behavioral tests. Overall the authors state that since the effects on hippocampus and striatum were not shared by the cerebral cortex, which showed nonsignificant changes in ChAT and a tendency toward increased HC-3 binding, this emphasizes that the effects of CPF were targeted toward specific brain regions and do not represent simply a global effect on the expression of cholinergically related proteins. Furthermore, presenting a compilation of data, gathered from this study and previous work, the authors postulated that the alterations actually emerge in adolescence and adulthood, rather than representing an initial deficit that simply continues into later life.

Table C35. Comparisons of persistent main treatment effects of chlorpyrifos when administered in different critical periods

Measure	GD 9-12	GD 17-20	PND 1-4	PND 11-14
ChAT	↑	(↓)	↓	↓
HC-3 binding	↓	↓↓	↓↓↓	↓
m_2AChRs	↓	±	ND	ND
DNA concentration	↓	↓	ND	ND
DNA content	↓	(↓)	ND	ND
Total protein/DNA	↑	↑↑	ND	ND
Membrane/total protein	↓	↓↓	ND	ND
Regional targets for cholinergic effects	hippocampus striatum	hippocampus cerebral cortex	hippocampus	hippocampus striatum
Regional targets for cellular effects	hippocampus striatum cerebral cortex	hippocampus cerebral cortex	ND	ND

Arrows indicate direction and comparative magnitudes of effects.
Parentheses denote effects with a consistent direction that did not achieve statistical significance
± indicates no consistent direction of change.
ND = no determination done.
Data for treatment regimens on GD 17-20, PND 1-4 and PND 11-14 were compiled from earlier studies.

According to the authors, these findings show that the hippocampus appeared to be adversely affected by CPF regardless of whether exposure occurs early or late in brain development, and defects emerged in adolescence or adulthood even in situations where normative values were initially restored in the immediate post-exposure period. Also, based on the disparate mechanisms by which CPF perturbs neuronal and glial cell development, CPF has an extraordinarily broad critical period for adverse effects on brain development, with a shifting target set of consequences dependent upon the period of exposure.

Icenogle et al., 2004.
Behavioral alterations in adolescent and adult rats caused by a brief subtoxic exposure to chlorpyrifos during neurulation

In this study, 0, 1 or 5 mg/kg-day of CPF was administered (in 1 ml/kg of DMSO via sc injection) to pregnant rats on GD 9–12**,** the embryonic phase spanning formation and closure of the neural tube. On the day after birth, pups were randomized within treatment groups and redistributed to nursing dams with a litter size of 10, to maintain standardized nutrition. Randomization was repeated at intervals of several days, with dams rotated among litters to distribute any maternal caretaking differences randomly across litters and treatment groups. Animals were weaned on PND 21 and behavioral evaluations were carried out on 10 males and 10 females from each treatment group, with no more than one male and one female from each litter. Tests included T-maze spontaneous alternation, Figure-8 maze locomotor activity, 16-arm radial maze, drug challenges in the radial-arm maze, startle reflex and prepulse inhibition and elevated plus-maze. After the completion of radial maze training, the rats were retested with drug challenges. Each rat received each test dose in a repeated-measures counterbalanced design, with at least 2 days between challenges. Scopolamine, a muscarinic acetylcholine antagonist and mecamylamine, a nicotinic receptor antagonist, were used to establish learning and memory dependence on cholinergic pathways. Scopolamine was administered at 0, 0.04, 0.08, and 0.16 mg/kg sc, and mecamylamine was administered at 0, 1.25, 2.5, and 5 mg/kg sc. The different doses of scopolamine and then mecamylamine were given in a counterbalanced order 20 min before the beginning of the test session in a volume of 1 ml/kg of isotonic saline vehicle: rats in each treatment group and of each sex received the challenge drug doses in all the different possible orders.

At the doses tested, CPF did not evoke any signs of systemic toxicity. There were no significant effects on litter size, neonatal viability, or postnatal body weight gain for either of the CPF treatments. No effects on growth or viability were noted, but behavioral abnormalities were observed when pups were tested in adolescence and adulthood in both males and females. In the CPF-exposed groups, locomotor hyperactivity was noted in early T-maze trials and in the elevated plus-maze; learning and memory in the 16-arm radial maze were adversely affected. The higher-dose group made more working and reference memory errors in early trials than did controls, whereas the lower-dose group was not significantly affected.

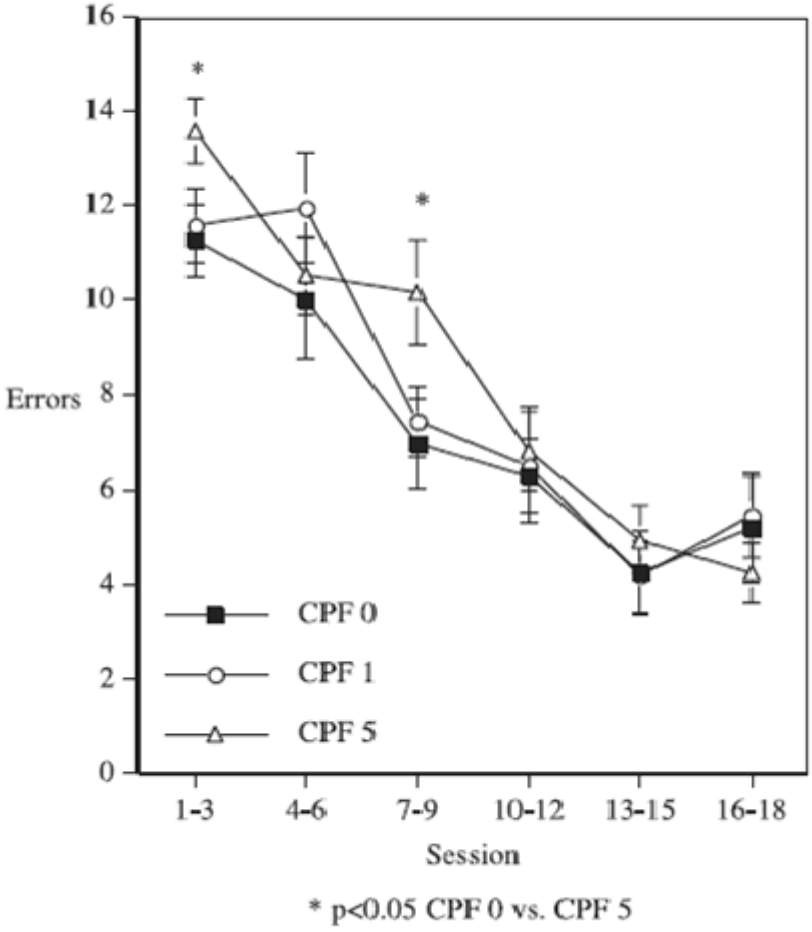


Figure C14. (a) Early prenatal chlorpyrifos (GD 9-12) radial-arm maze acquisition working memory errors.

Each session represented one run on the maze (mean ± S.E.M.). The CPF 5 group showed significant ($P < 0.05$) impairments relative to control early in training.

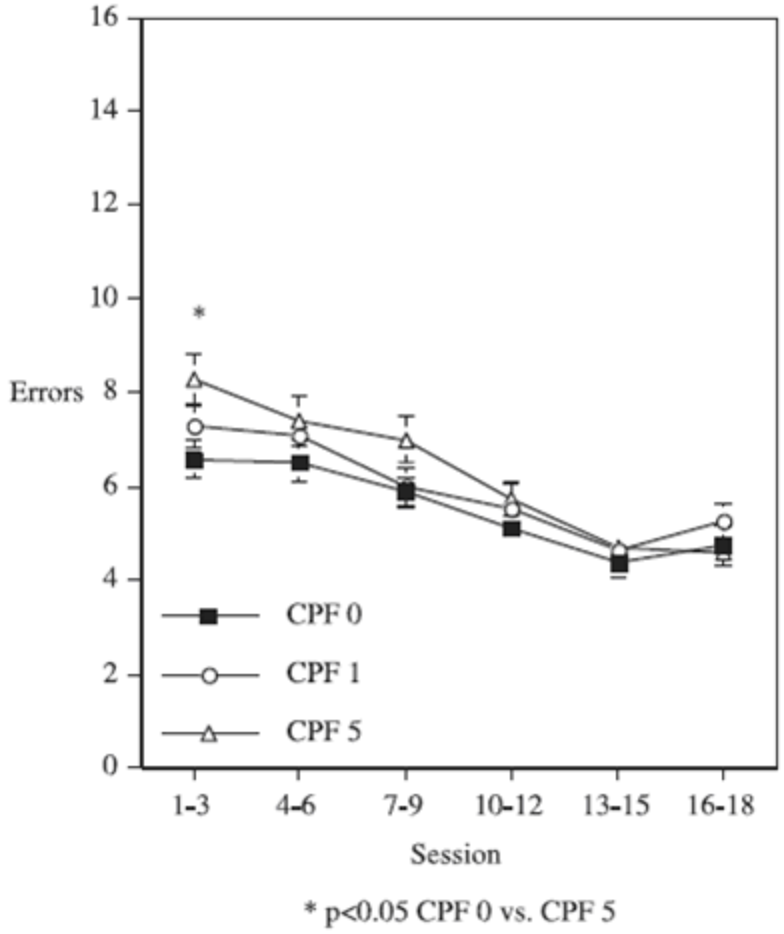


Figure C15. (b) Early prenatal chlorpyrifos (GD 9-12) radial-arm maze acquisition reference memory errors.

The effects displayed time-dependence, with errors in both of the memory parameters in the first test block and eventual resolution to control performance values in the later trials. Thus, while reference and working memory were impaired in the early training sessions, all CPF-exposed animals eventually learned the task although possibly through different mechanisms.

The characteristic amnesic effect of scopolamine, a muscarinic acetylcholine antagonist, was not seen in these tests suggesting that the behavioral anomalies observed selectively include impairment of cholinergic circuits used in learning and memory.

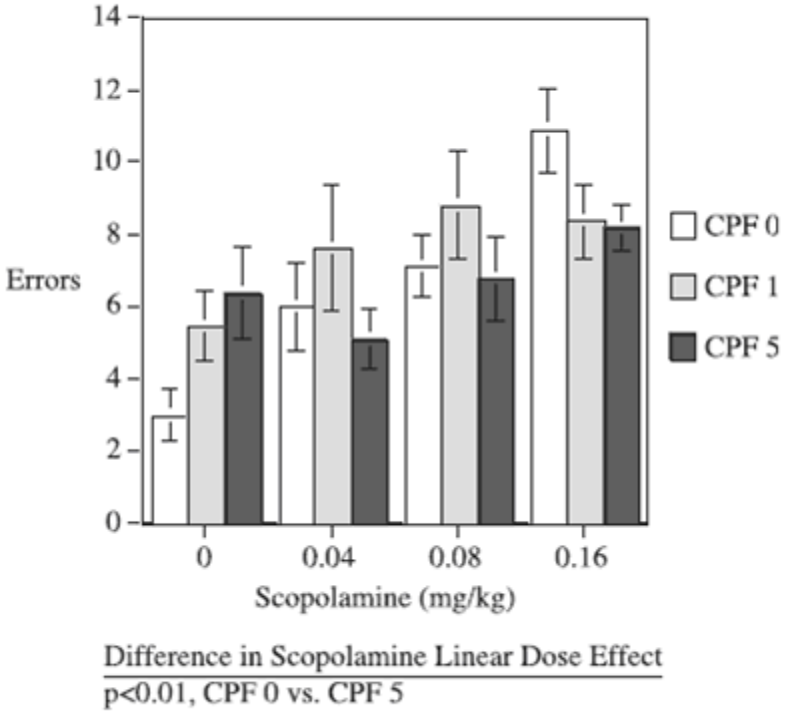


Figure C16. Early prenatal chlorpyrifos (GD 9-12) radial-arm maze scopolamine challenge working memory errors.

The CPF 5 group showed significantly less effect of scopolamine relative to control (difference in linear scopolamine dose– effect, $p < 0.01$). According to the authors, exposure to CPF during neurulation leads to cognitive impairment and changes in locomotor habituation and activity, with the former representing a specific defect in cholinergic synaptic function that is uncovered by pharmacologic challenge. Also, the results obtained here indicate that prenatal CPF exposure, as early as the neural tube stage, leads to locomotor abnormalities and impaired cognitive function in adolescence and adulthood. Pharmacologic challenges confirmed that, although the CPF-exposed animals exhibit competence for both reference and working memory (albeit acquiring these tasks at a slower rate than controls), they do so through abnormal mechanisms. The authors concluded that since these findings were similar to those noted after late gestational or neonatal CPF exposure, a prolonged window of vulnerability of brain development to CPF is indicated. One notable difference between the effects of CPF administered during neurulation and later treatments is that the effects on cognitive performance seen in this study were not sex-selective. In contrast, late gestational or early postnatal CPF treatment elicited cognitive deficits preferentially in females (Levin et al., 2002).

Aldridge et al., 2005.
Alterations in Central Nervous System Serotonergic and Dopaminergic Synaptic Activity in Adulthood after Prenatal or Neonatal Chlorpyrifos Exposure

This study tried to characterize the synaptic mechanisms underlying neuronal development of serotonin (5HT) and dopamine systems, and long-term alterations in behaviors related to 5HT function. Developing rats (Sprague-Dawley) were exposed to CPF at 1 and 5 mg/kg dissolved in DMSO injected subcutaneously, in three treatment windows: GD 17-20, PND 1-4, or PND 11-14. At birth all pups within each treatment group were pooled and randomly assigned to the mothers to maintain a litter size of 10 and subsequently rotated to avoid maternal caretaking differences. Animals were weaned on PND 21. On PND 60, one male and one female were selected from each litter and were decapitated and one pup/sex was used to analyze results presented. It was not clear how many rats were present in each group.

The cerebellum, including flocculi, was removed, and the forebrain was separated from the midbrain and brainstem by a cut made rostral to the thalamus. The forebrain was then further dissected into the regions containing major 5HT projections (cerebral cortex, hippocampus, striatum), and the midbrain and brainstem, which contain the preponderance of 5HT cell bodies, were separated from each other. The cerebellum, which is sparse in 5HT neurons or projections, was not evaluated. Tissues were frozen with liquid nitrogen and stored at –45°C.

In early adulthood (PND 60), basal neurotransmitter content and synaptic activity (turnover) in brain regions containing the major 5HT and dopamine projections were assessed. Across all brain regions, late gestational exposure to CPF elicited a significant net overall reduction (main treatment effect) in 5HT levels. However, the effect was seen only at the higher dose (5 mg/kg), which elicits maternal weight deficits and significant fetal brain ChE inhibition (Qiao et al., 2002; Garcia et al., 2003).

Chlorpyrifos exposure on GD 17–20 or PND 1–4 evoked long-term increases in 5HT turnover across multiple regions; the authors thought that the effects were not secondary to changes in neurotransmitter content, which was unaffected or even decreased. When the treatment window was shifted to PND 11–14, there were no long-term effects.

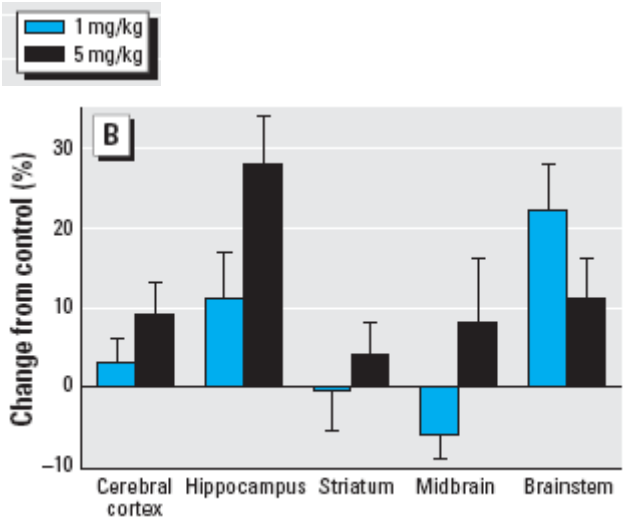


Figure C17. Effects of GD 17–20 CPF exposure on (B) turnover.
ANOVA across treatment, region, and sex: treatment, $p < 0.007$;
control versus 1 mg/kg, $p < 0.05$; control versus 5 mg/kg, $p < 0.002$.
Separate ANOVAs were not conducted for each region because of the absence of treatment × region interactions. Similarly, values are shown combined for males and females because of the lack of treatment × sex interactions.

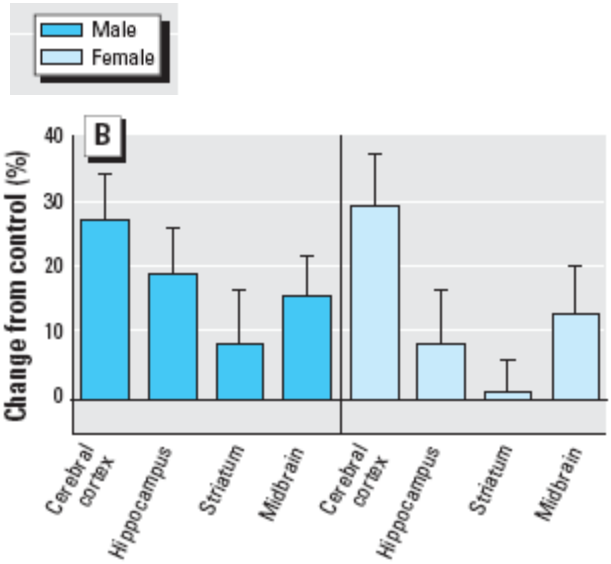


Figure C18. Effects of PND 1–4 CPF (1 mg/kg) exposure on 5HT turnover.
ANOVA across treatment, region, and sex: (B), treatment, $p < 0.0001$ (separate ANOVAs for males and females were not evaluated because of the lack of a treatment × sex interaction).
Separate ANOVAs were not conducted for each region because of the absence of treatment × region interactions.

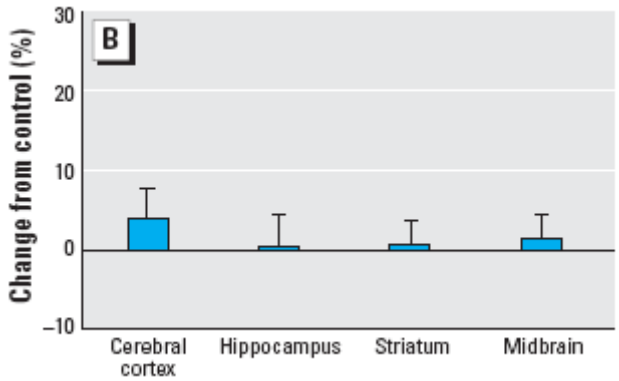


Figure C19. Effects of PND 11–14 CPF (5 mg/kg) exposure on 5HT turnover.
ANOVA across treatment, region, and sex: not significant.
Separate ANOVAs were not conducted for each region because of the absence of treatment × region interactions; similarly, values are shown combined for males and females because of the lack of treatment × sex interactions.

The pattern of effects on dopamine (DA) levels and turnover differed substantially from those seen for 5HT. Although DA content was unaffected in most brain regions, there were massive deficits in the hippocampus with either the low or high dose of CPF. Dopamine turnover was affected only at the higher dose, with significant elevations in cerebral cortex, striatum, and midbrain (data not shown here); DA turnover could not be assessed in the hippocampus because of the low levels of DA metabolites. Dopamine turnover also showed significant increases after CPF exposure on GD 17–20, but only when the dose was raised above the threshold for overt toxicity.

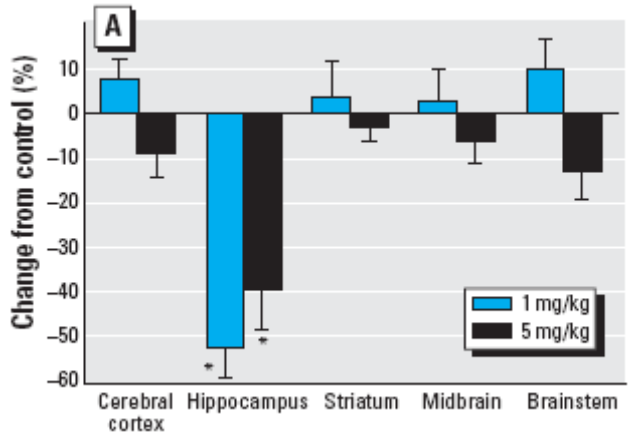


Figure C20. Effects of GD 17–20 CPF exposure on (A) DA content.
ANOVA across treatment, region, and sex: (A), treatment, $p < 0.04$; treatment × region, $p < 0.02$;
*Individual treatments and regions are significantly different; values are shown combined for males and females because of the lack of treatment × sex interactions.

Hippocampal dopamine content was profoundly subnormal after exposures below or above the acute, toxic threshold, suggesting outright neurotoxicity.

Also noted is the relative lack of sex selectivity to the effects on 5HT turnover, since effects on behavior and 5HT receptor expression show distinct sex differences (Aldridge et al., 2004; Aldridge et al., 2005a). Many of the functional consequences of CPF exposure represent the elimination of normal sex differences in synaptic function and behavior, partially masculinizing the patterns in females but feminizing males (Aldridge et al., 2004; Aldridge et al., 2005a); accordingly, CPF may interfere with sexual differentiation of the brain, which peaks during the vulnerable perinatal period found here (MacLusky and Naftolin, 1981; McCarthy, 1994). The authors therefore speculate that while CPF effects on males and females might be the same in regard to alterations of 5HT synaptic activity and/or miswiring of 5HT circuits, the outcome nevertheless can be expressed differently for males and females because of CPF's effects on sexual differentiation.

The authors suggest that these results indicate that apparently nontoxic exposures to CPF may produce lasting activation of 5HT systems in association with 5HT-associated behavioral anomalies, subsequent to exposure in a critical developmental period.

Tian et al., 2005.
Teratogenicity and developmental toxicity of chlorpyrifos. Maternal exposure during organogenesis in mice.

Chlorpyrifos was evaluated for potential teratogenicity and developmental toxicity in mice. Pregnant females were given a single ip injection (40 or 80 mg/kg) in olive oil on day 10 of gestation and fetuses were evaluated on GD 17. At 80 mg/kg, CPF treatment resulted in a significant reduction in numbers of live fetuses, and an increase in resorptions, versus control litters. There was no indication of maternal toxicity. External and skeletal malformations were observed at 80 mg/kg, but not 40 mg/kg. Rates of fetuses with cleft palate increased significantly ($p<0.05$) following 80 mg CPF/kg (5.97%) versus control litters (0.97%). Similarly, the absence of thoracic vertebrae was increased and the number of caudal vertebrae was significantly decreased.

Table C36. Effects of chlorpyrifos on reproductive and fetal parameters at day 17 of gestation (mean ± SE)

Group	Vehicle	Chlorpyrifos	
		40 mg/kg	80 mg/kg
No. of litters (fetuses) examined	16 (216)	15 (225)	19 (276)
No. of corpora lutea/litter	15.7 ± 0.8	17.3 ± 0.7	16.9 ± 0.7
No. of implantations sites/litter	13.5 ± 0.5	15.0 ± 0.6	14.8 ± 0.5
Body weight at day 10 of gestation	39.3 ± 1.0	38.1 ± 1.4	40.5 ± 1.1
Body weight at day 17 of gestation	58.4 ± 1.4	58.2 ± 1.7	56.9 ± 1.1
Maternal weight gain	19.2 ± 3.8	20.1 ± 0.9	16.4 ± 1.0
% of live fetuses	95.9 ± 2.1	93.3 ± 1.7	86.1 ± 2.9**
% of dead fetuses	0.8 ± 0.6	0.8 ± 0.8	2.3 ± 0.8
% of resorbed fetuses	3.3 ± 2.1	5.9 ± 1.7	11.6 ± 2.9*
Fetal body weight (g)/litter			
Male	1.17 ± 0.03	1.13 ± 0.03	1.09 ± 0.03
Females	1.13 ± 0.03	1.06 ± 0.02	1.05 ± 0.03
Ratio of males/total	0.41	0.49	0.49

* $p<0.05$ compared with controls by Mann-Whitney *U*-test.
** $p<0.01$ compared with controls by Mann-Whitney *U*-test.

Table C37. Incidence and types of external malformations in day 17 fetuses following maternal treatment with Chlorpyrifos at day 10 of gestation

Group	Vehicle	Chlorpyrifos	
		40 mg/kg	80 mg/kg
No. of litters (fetuses) examined	16 (206)	15 (209)	19 (237)
No. of litters with malformed fetuses (%)	2 (12.5)	2 (13.3)	11 (55.0)**
No. of malformed fetuses	2	3	15**
% of malformed fetuses/litters (mean and S.E.)[a]	0.83 ± 0.5	1.13 ± 0.9	6.62 ± 1.7
No. of fetuses with			
Cleft palate	2	0	12*
Open eyelids	0	3	3

[a] Mean ± standard error.
* $p<0.05$ compared with the control group by X^2-test.
** $p<0.01$ compared with the control group by X^2-test.

Administration with a single dose of 80 mg CPF/kg to pregnant mice on GD 10 produced embryotoxicity and teratogenicity as indicated by the decrease in number of live fetus and increases in resorptions, cleft palate, absent thoracic vertebrae and decreased caudal vertebrae (Tables C36, C37 and C38). These changes occurred without overt signs of maternal toxicity. It is suggested that CPF is teratogenic and embryotoxic in mice at doses below those that cause significant maternal toxicity.

Table C38. Effects of maternal treatment with chlorpyrifos on ossification in near-term fetuses

Group	Vehicle	Chlorpyrifos	
		40 mg/kg	80 mg/kg
No. of litters examined	16	15	19
No. of fetuses with absence of thoracic vertebrae/no. examined	4/206	5/209	22/237*
No. of caudal vertebrae/litter[a]	4.14 ± 0.19	3.83 ± 0.26	3.67 ± 0.16**
No. of extra ribs/litter[a]	4.74 ± 0.98	4.63 ± 2.3	4.11 ± 0.76
No. of metacarpals[a]	3.86 ± 0.06	3.87 ± 0.05	3.83 ± 0.06
No. of metatarsals[a]	4.78 ± 0.06	4.68 ± 0.06	4.74 ± 0.08

[a] Mean ± standard error.
* $p<0.05$ compared with the control group by X^2-tesst.
** $p<0.05$ compared with the control group by Mann-Whitney *U*-test.

The single ip injection of 80 mg CPF/kg used here was below doses that may cause significant inhibition of ChE activity, and such signs or symptoms were not observed in the exposed dams. It is not clear if the ChE levels in the brain were measured. The authors speculate CPF promoted cholinergic activity in the fetus to alter the timing or intensity of cholinergic receptors during palatogenesis and that higher sensitivity of the conceptus is due to weak detoxification systems such as A-esterases or carboxylesterases that are deficient in young rats (Padilla et al., 2000). At this time it is not known if this presumed overstimulation of cholinergic activity might alter the expression of genes controlling key morphogenetic processes (such as apoptosis) during palatogenesis.

Roy et al., 2005.
Quantitative morphological assessment reveals neuronal and glial deficits in hippocampus after a brief subtoxic exposure to chlorpyrifos in neonatal rats.

Chlorpyrifos was administered at **5** mg/kg sc daily on PND 11-14. Controls received equivalent injections of vehicle on the same schedule. Animals for each treatment were maintained in separate litters (12 litters per treatment). Male pups were selected 24 h (PND 15) and 6 days (PND 20) after the last CPF dose, utilizing no more than one from a given litter. Treatment groups comprised 4 controls on PND 15, 5 controls on PND 20, and 5 and 6 CPF-exposed pups at each respective age. Animals were euthanized under deep ketamine anesthesia and perfused transcardially with normal saline for 2 min, followed by perfusion for at least 20 min with freshly-prepared Karnovsky's fixative (4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, 4–10 ºC), after which the brains were dissected and preserved in fresh fixative overnight at 4 ºC. The slides were coded and the examiner was blinded to the animal number and treatment group and the validity of the morphometric measurements was confirmed by evaluating tissue shrinkage at the level of the anterior commissure. Morphometric measurements were carried out by a modification of the fractionator method using NIH Image software. On PND 15 and 20, quantitative morphologic examinations of neurons and glia in CA1, CA3, and dentate gyrus regions of the hippocampus were conducted. This was done to determine whether morphological alterations correspond or contribute to these adverse outcomes

expected at this exposure level. Although hippocampal morphology after CPF exposure was normal on gross observation, morphometric analysis revealed a significant overall reduction in the total number of neurons and glia. Superimposed on this basic effect, CPF elicited a delayed-onset increase in the neuron/glia ratio that emerged by PND 20, connoting selective gliotoxicity. The alterations in cell numbers were accompanied by significant perikaryal swelling and by enhanced development of astrocytic processes. Layer thickness also showed delayed-onset effects of CPF, with thinning of the CA1 and CA3 layers and enlargement of the dentate gyrus. The authors interpret their results to indicate that there are subtle morphological changes in the juvenile rat brain after neonatal CPF exposure that are detectable only with quantitative analysis and that correlate with regional and cell-specific targets identified earlier in neurochemical studies. Also, the intensification of these effects between PND 15 and PND 20, in the period after the termination of CPF exposure, is in keeping with earlier work showing delayed-onset cell loss, deterioration of cell signaling, shifts in neuronotypic and gliotypic protein expression, and alterations in neurons and glia in other brain regions. This simultaneous targeting of neurons and glia by CPF was considered by the authors to likely play an important role in its developmental neurotoxicant effects.

Akhtar et al., 2006.
Transplacental disposition and teratogenic effects of chlorpyrifos in rats.

Wistar rats were administered 9.6, 12 and 15 mg/kg-d in corn oil via gavage from GD 0-20 and examined for evidence of fetotoxicity and teratogenicity to evaluate the potential effects of technical CPF (97%). The number of implantations, number of corpora lutea and live fetuses/dam were not affected, but significant alterations were noted in percent increase in resorptions and fetal weight. No major malformations, but some minor anomalies such as reduced parietal ossification and absence of phalanges, were found at the high dose (significant) and were not considered as compound-related effects by the authors. The accumulation of CPF residue in dams was more in brain (0.0328 µg/g) than in liver (0.0071 µg/g) and the level of residue in fetuses was in the following order: liver (0.0531 µg/g) > brain (0.0364 µg /g) > placenta (0.040 µg/g) > amniotic fluid (0.0010 µg/g). It must be noted that at the low dose of 9.6 mg/kg, the residue in the brain tissue of the fetus (0.01 µg/g) was substantially higher than the dam (0.003 µg/g). While this trend was noted at the higher doses, the difference was not this marked.

Table C39. Residue levels* (µg/g) of Chlorpyrifos in different tissues of dams and fetuses.

	Organs	Dose (mg/kg-d)			Total organ Residue**
		9.6	12	15	
Dam	Brain	0.0030 ± 0.578[#]	0.035 ± 0.0051[#]	0.0603 ± 0.006[#]	0.0328[#] ± 0.333
	Liver	0.0025 ± 0.0005	0.00074 ± 0.0014	0.0115 ± 0.0003	0.0071 ± 0.001
	Amniotic fluid	0.002 ± 0.0001	0.0003 ± 0.00002	0.0003 ± 0.0001	0.0010 ± 0.0003
	Placenta	0.120 ± 0.011	0.0002 ± 0.0001	0.0006 ± 0.0001	0.040 ± 0.020
Fetus	Brain	0.0109 ± 0.011	0.0578 ± 0.0058[#]	0.0404 ± 0.0016[#]	0.0364 ± 0.333
	Liver	0.0147 ± 0.0004	0.0642 ± 0.0023[#]	0.0803 ± 0.0034[#a]	0.0531 ± 0.010
Dam	Brain+ liver	0.0028 ± 0.408	0.0212 ± 0.0066	0.0359 ± 0.011	0.0120[#] ± 0.009
Fetus	Brain+ liver	0.0128 ± 0.001	0.061 ± 0.0031	0.0603 ± 0.009	0.0447[#] ± 0.016

* mean ± SE of three samples each.
** mean ± SE of nine values each.
[#] Significant at the level of $p<0.05$ by three-way ANOVA.
[#a] $p<0.05$-0.01 by three-way ANOVA.
Note: samples of control animals did not show chlorpyrifos residue.

On the whole, the authors noted that although the total residue in the brain and liver was higher in fetuses (0.0447 µg/g) than in dams (0.0120 µg/g), the absence of abnormalities in fetal gross morphology, visceral and skeleton suggest that technical CPF at the tested dose levels is non-teratogenic in rats. The authors also contend that given these findings, the residue level alone may not produce morphological, visceral and skeletal abnormalities in rats

Venerosi et al., 2006.
A social recognition test for female mice reveals behavioral effects of developmental chlorpyrifos exposure.

CD-1 mice were exposed to CPF dissolved in peanut oil in a volume of 0.1 ml/kg both prenatally (GD 15-18; doses 0, 3 or 6 mg/kg) and postnatally (PND 11-14, doses 0, 1 or 3 mg/kg). Females at 4 months of age underwent a social recognition test in which ultrasound vocalizations (USVs) and social investigation behavior emitted by a resident female in the presence of a female partner were measured during two consecutive 3 min sessions (interval between the two sessions 45 min). Throughout the social recognition test a marked increase in USVs was found in females prenatally treated with the highest CPF dose; increase in USV was also paralleled by a selective increase in frequency but not in duration of social investigation.

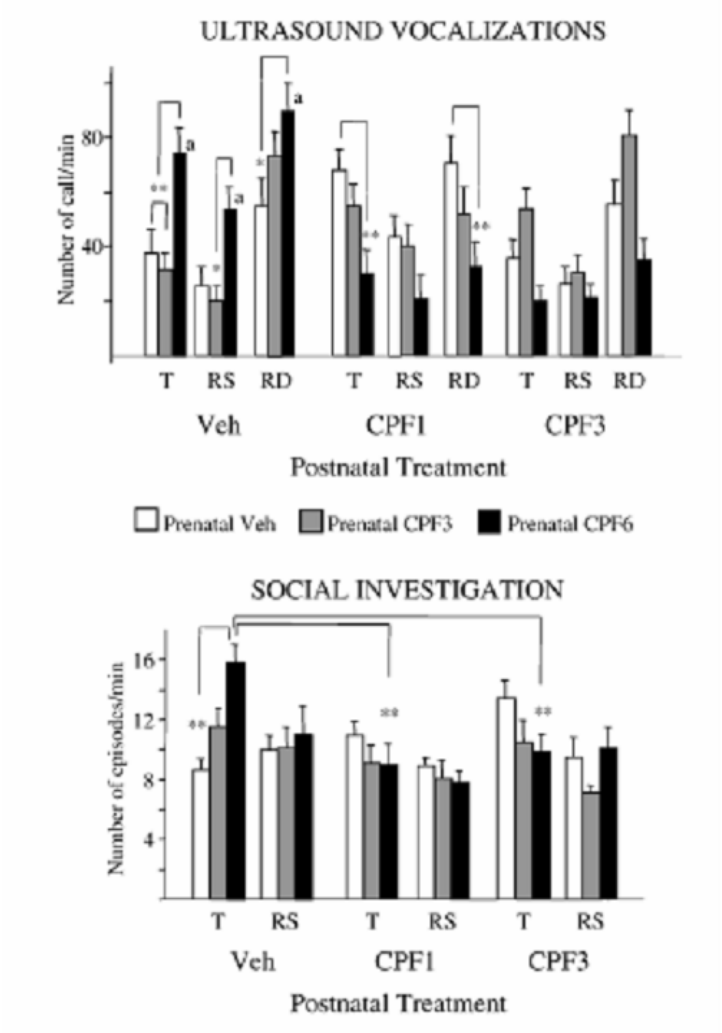


Figure C21. *Upper panel*: Effects of CPF exposure on frequency of USVs emitted by resident female during *Test* (T), *Retest Same* (RS) and *Retest Different* (RD) phases. Data are mean ± S.E.M.; n=10 in each group. *=p<0.05; **=p<0.01 after post-hoc comparison. (a)=significant difference after post-hoc cpmparisons (p<0.05) in CPF6-Veh vs. CPF6-CPF1 and CPF6-Veh vs. CPF6-CPF3. *Lower panel*: Effects of CPF exposure on frequency of social investigation displayed by resident females during T and RS phases. Data are mean ± S.E.M.; n=10 in each group. **=p<0.01 after post-hoc comparisons.

Prenatal exposure to the highest CPF dose increased USVs and frequency of social investigation during the first encounter in those females receiving only vehicle postnatally. Furthermore, such increased "baseline" levels did not appear to interfere with individual recognition of the other female, and was entirely counteracted by postnatal CPF exposure. These behavioral changes might be related to the well-documented effects of developmental CPF exposure on different neurotransmitter systems such as serotonergic, dopaminergic and cholinergic ones and may correlate with recent data of CPF effects upon serotonergic pathways (Aldridge et al., 2005), since serotonin modulation of anxiety levels in social contexts, and responsiveness to environmental and social cues has been also described in transgenic mice lacking the serotonin

1A receptor. Additionally, in all the three social recognition phases, the augmented response to the presence of an unfamiliar conspecific shown by CPF6–Veh females was totally blocked by subsequent exposure to CPF postnatally while the postnatal CPF exposure per se did not exert any significant effect on either social investigation or USVs. Since the last fetal week and the first postnatal ones are two different critical windows of susceptibility of CNS development in terms of neuronal differentiation, migration and synaptogenesis (Barone et al., 2000; Rice and Barone, 2000), the authors explain that the same agent administered at different developmental stages can produce different and even opposite effects on brain and behavior. They also caution that the "return" to control-like behavioral profiles induced by postnatal CPF in the social recognition test should not be generalized, since detrimental behavioral effects of the postnatal CPF exposure have been reported by other researchers in both mice (Ricceri et al., 2003) and rats (Dam et al., 2000; Aldridge et al., 2003). The overall conclusions by the authors were (i) gender selectivity of CPF behavioral effects is also dependent on time of administration; (ii) the social behavioral repertoire is a selective target of neurotoxic CPF effects. They also suggest that motivational rather than cognitive aspects of the mouse social behavior represent a sensitive marker for the CNS effects of developmental CPF exposure. The cognitive part of the social test was recognizing that the partner mouse was encountered before. Since the USVs go down from the Test to Retest Same and back up again from Retest Same to Retest Different phases and this was true of the CPF treated group, cognitive aspects are not apparently affected. However, under all three conditions the CPF mice had more USVs indicating that they were more responsive to the partner under all conditions. That would be considered the emotional or motivational part of the social response. Finally, the authors suggest that social recognition can be easily and rapidly assessed in the female mouse making it possible to evaluate, primarily by means of USV emission, even subtle alteration of social behavioral patterns dissociated from cognitive components of individual recognition.

Ricceri, et al., 2006.
Developmental neurotoxicity of organophosphorous pesticides: fetal and neonatal exposure to chlorpyrifos alters sex-specific behaviors at adulthood in mice.

Chlorpyrifos dissolved in peanut oil at 0,3 or 6 mg/kg-day (in a volume of 0.1 ml/kg) was given by oral gavage to pregnant females on GD 15-18 and offspring were treated sc (0, 1 or 3 mg/kg) on PND 11-14 resulting in nine treatment groups. Serum and brain AChE activity was evaluated at birth and 24 h from termination of postnatal treatments. On PND 70, male mice were assessed for spontaneous motor activity in an open-field test and in a socioagonistic encounter with an unfamiliar conspecific. Virgin females underwent a maternal induction test following presentation of foster pups. Both sexes were subjected to a plus-maze test to evaluate exploration and anxiety levels. At birth, brain AChE activity was unchanged by prenatal CPF treatment (both doses) but given the rapid rate of enzyme synthesis in the immature rodent, recovery from inhibition could have occurred by 24 hours.

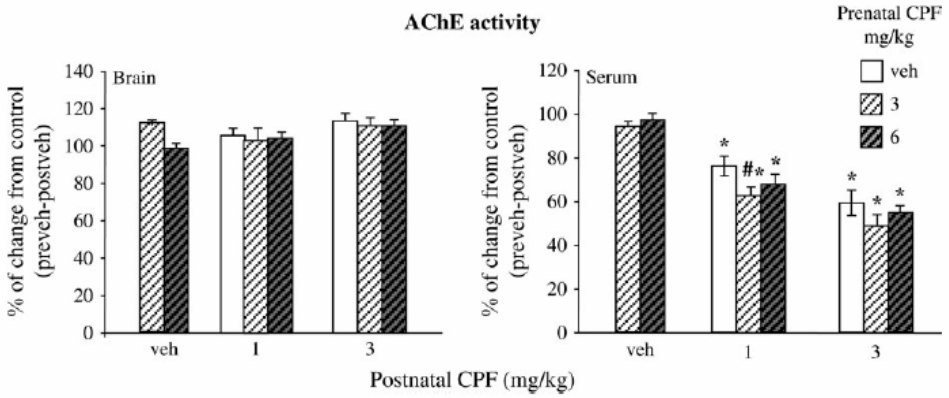


Figure C22. Cholinesterase (AChE) activity in the brain and serum of 15-day old mice, exposed to GDs 15-18 to Veh, CPF3, or CPF6 and on PNDs 11-14 to Veh, CPF1, or CPF3. * Indicates $p<0.05$ significantly different from control values (100%). # Indicates $p<0.05$ preCPF3-postCPF1 versus preVeh-postCPF1. AChE is expressed as the percent change from control (PreVeh-postVeh) values. Brain AChE control values were 6.39 ± 0.20 (mean ± SE; micromoles of AcThCh hydrolyzed per minute per gram of tissue) and serum AChE control values were 2.54 ± 0.11 (mean ± SE; micromoles of AcThCh hydrolyzed per minute per milliliter of blood).

The highest prenatal CPF dose (6 mg/kg) resulted in marked hyperactivity in adult male mice in the open-field test, an effect maintained also after re-exposure to 1 mg/kg CPF on PND 11–14. While postnatal exposure to CPF at 3 mg/kg-day also increased locomotor activity, the combined prenatal/postnatal exposure failed to enhance the hyperactivity shown by mice at CPF dose 6 mg/kg-day possibly, according to the authors, because of the ceiling effect produced by the highest prenatal CPF dose. Chlorpyrifos failed to affect habituation, as normal decrement of activity was observed throughout the open-field test. In addition, CPF-exposed mice were hyperactive neither during the socioagonistic encounters nor in the plus-maze test. The enhanced activity seemed to be specifically related to exploration of a novel environment, in line with previous experimental evidence in younger mice (Ricceri et al., 2003). Adult males exposed postnatally to 3 mg CPF/kg/day displayed increased frequency and duration of aggressive behaviors at the expense of defensive and submissive ones. Prenatal exposure also favored expression of offensive postures. The specificity of the pro-aggressive effect of CPF is supported by the lack of significant differences in responses related to general activity during the socioagonistic encounter.

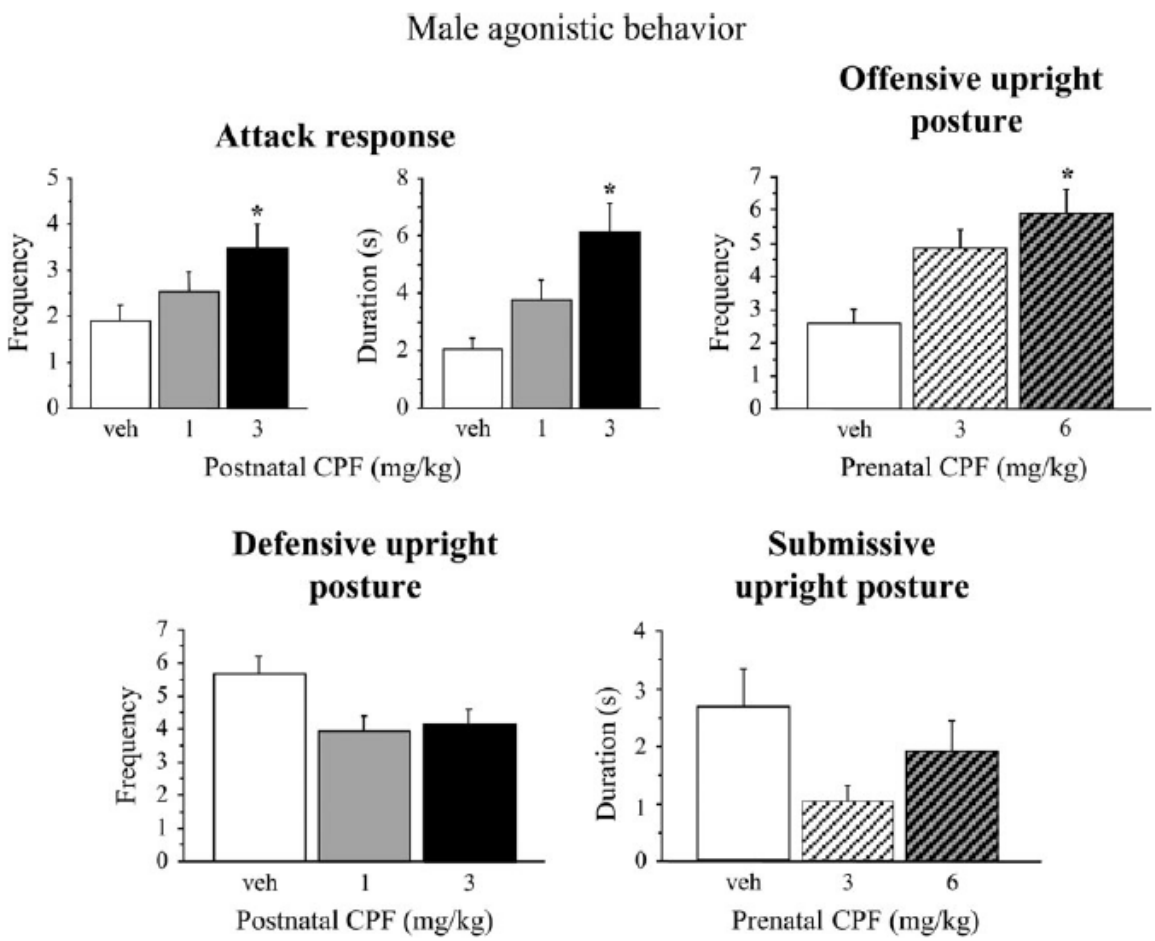


Figure C23. Effects of prenatal (GDs 15–18) and postnatal (PNDs 11–14) CPF treatment on different items of agonistic behavior recorded during a 20-min encounter of adult male mice with unfamiliar conspecifics. Data are mean frequency and duration on 5-min time units. * Indicates $p < 0.05$, post hoc comparisons with the corresponding control group

The authors also did not find any CPF-induced impairment in water maze acquisition in male mice treated with CPF. Data from virgin females show that postnatal CPF exposure enhanced responsiveness toward pups, favoring the expression of pup-directed behaviors. Neither a main effect of prenatal CPF nor an interaction between prenatal and postnatal treatment was found for pup-directed behaviors. Although data are not shown, the authors state that no effects of CPF were found on latency, frequency, and duration of nest building and pups' retrieving to the nest. In the Plus-Maze experiments, CPF appears to preferentially affect females' behavior, raising the open-arm time in females to the same levels as found in males as CPF appears to reduce anxiety levels blunting sex differences. Since female mice are more anxious than males when confronted with a novel environment (Augustsson et al., 2005), the observations in this study support previous data in the rat of CPF-induced anxiety reduction (Aldridge et al., 2005a) albeit in the opposite sex. From these results, the authors believe that CPF targets maturational CNS events that are not necessarily related to inhibition of brain ChE and behavioral changes are in fact observed following developmental exposure to doses inducing no or very limited brain

AChE inhibition. However, they do state that assessment of brain AChE activity shortly after treatment termination (1–4 h) would have revealed an effect of the repeated CPF treatment given that in their previous study, using the same mouse strain and the same CPF doses as in the present study, they found (20%) brain AChE inhibition 1 h after termination of PND 1–4 CPF treatment and no effect after treatment on PND 11–14 (Ricceri et al., 2003). The authors concluded that exposure to CPF enhances socioagonistic behavior in males and maternal responsiveness to pups in non-lactating females as well as reduced anxiety levels in both sexes, with females more affected than males, and induced hyperactivity in males. Additionally, the authors stated that CPF effects were for the most part due to the postnatal exposure, and the prenatal exposure did not increase the magnitude of such effects except for the induced hyperactivity in males. The enhanced activity seemed to be specifically related to exploration of a novel environment, and is in line with previous experimental evidence in younger mice (Ricceri et al., 2003). Alteration of activity levels might result from the interference of CPF with early development of cholinergic synaptic function as reported for late prenatal exposure periods (Qiao et al., 2003). The period of postnatal exposure spans through a phase of critical importance for development of basal forebrain cholinergic pathways, modulating arousal and inhibition (Berger-Sweeney and Hohmann, 1997) and effects observed can be interpreted accordingly. Late neonatal exposure on PND 11–14 was the most effective in causing behavioral changes. These findings support the hypothesis that developmental CPF may represent a risk factor for increased vulnerability to neurodevelopmental disorders in humans. While the authors claim that at these doses fetal and neonatal brain ChE is not inhibited the data supporting this were not provided. Altogether, developmental exposure to CPF at doses that do not cause substantial brain AChE inhibition appear to induce long-term alterations in sex-specific behavior patterns of the mouse species.

C.2.3. Relationship between developmental and maternal toxicity

As with many OP pesticides, developing animals appear more susceptible to the acute toxic effects of CPF than adults (Pope et al., 1991; Atterberry et al., 1997; Moser and Padilla, 1998b; Zheng et al., 2000), primarily because of lower levels of detoxifying enzymes such as carboxylesterases (CbxE), which stoichiometrically degrade CPO (Chambers et al., 1990), and A-esterases, which actively hydrolyze CPO (Mortensen et al., 1996; Atterberry et al., 1997; Moser et al., 1998a; Karanth and Pope, 2000). However, Lassiter et al. (1998) (study described below) have proposed that fetal brain ChE is simply able to recover more fully between each dose as compared to maternal brain ChE, giving the illusion that the fetal compartment is less affected than the maternal compartment.

Lassiter et al., 1998
Gestational Exposure to Chlorpyrifos: Apparent Protection of the Fetus?

To study selected toxicokinetic and toxicodynamic factors surrounding the toxicity of CPF in pregnant rats, dams were dosed daily (po) with CPF in corn oil (0 or 7 mg/kg) on GD 14 to 18. Animals were euthanized at 2 to 120 h after the last dose and tissues were collected for enzyme

analysis. The authors found that (1) the time of maximal ChE inhibition was the same (i.e., 5–10 h after dosing) for both maternal and fetal brain, (2) the degree of fetal brain ChE inhibition was 4.7 times less than maternal brain inhibition, and (3) the detoxification potential (i.e., CbxE and chlorpyrifos-oxonase) of the fetal tissues was very low compared to the maternal tissues. If pregnant dams received only **one** oral dose of 7 or 10 mg/kg CPF on GD 18, the degree of ChE inhibition in the fetal brain was comparable to the maternal brain ChE inhibition. Given the net increase (more than fourfold) in fetal brain ChE activity from GD 14 to 18 in control animals, the fact that maternal brain ChE was inhibited more than fetal brain ChE only in a repeated dosing regimen, led the authors to conclude that the fetus is not genuinely protected from the toxic effects of a given dose of CPF.

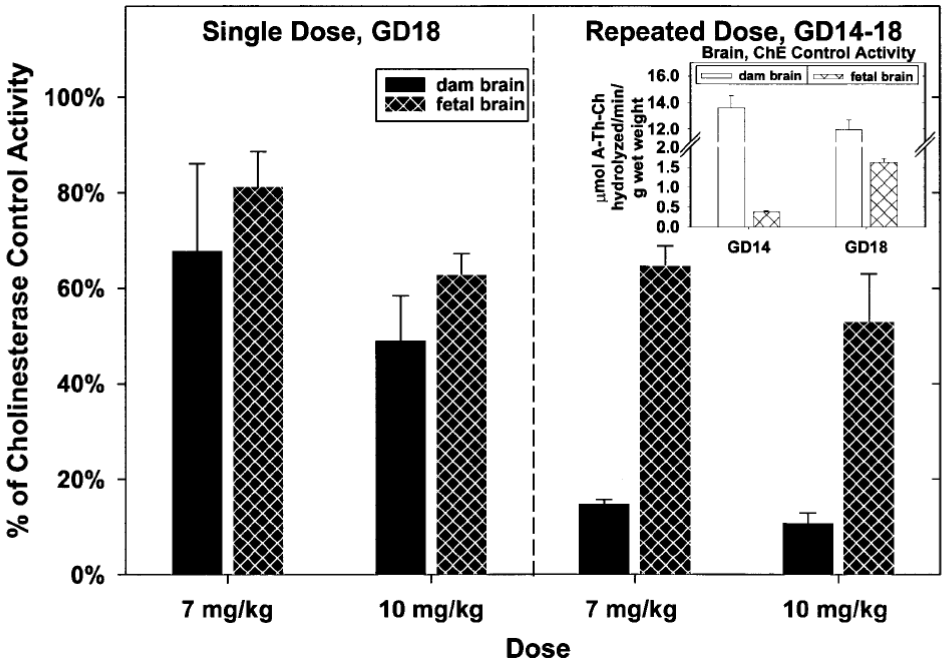


Figure C24. Cholinesterase activity in maternal and fetal brain following single or repeated exposure. (Inset) In controls, maternal brain cholinesterase activity does not change from GD14 to 18, but fetal brain cholinesterase activity increases 4.3-fold.

While the differential degree of ChE inhibition observed between fetal and maternal brain following repeated gestational CPF could be due to intrinsic differences in sensitivity of ChE molecules to inhibitors; differences in biotransformation and/or disposition of CPF; or protection of fetal brain ChEs by placental and/or fetal chlorpyrifos-oxonase and CbxE; it is the dynamics of recovery for fetal vs. maternal brain ChE that seem to be important. This exceptional recovery capacity of the fetal brain ChE may represent an alternative form of protection, but ultimately the pesticide appears to be still inhibiting ChE activity in the fetal brain. The authors thus concluded that moving beyond the traditional toxicological context of ChE inhibition, the inhibition of fetal brain ChE in itself may also be deleterious to the coordinated development of the brain.

Mattson et al., 2000
Lack of Differential Sensitivity to Cholinesterase Inhibition in Fetuses and Neonates Compared to Dams Treated Perinatally with Chlorpyrifos

Pregnant Sprague-Dawley rats were exposed to CPF by gavage (in corn oil) from GD 6 to PND 10. Dosages to the dams were 0, 0.3, 1.0 or 5.0 mg/kg-day. In dams and fetuses/pups, laboratory evaluations were made on GD 20, PND 1, 5, and 11: blood CPF, CPO, and TCPY; and ChE analyses of plasma, red blood cells, heart and brain. Chlorpyrifos and CPO analyses were conducted on milk from PND 1, 5 and 11. Cholinesterase analyses were conducted on dams and pups on PND 22, and as adults on PND 65. Separate groups of time-mated dams were used for each time period. The exposure regimen in this study was the same as the one used in a developmental neurotoxicity study (Hoberman, 1998) done according to previous U.S. EPA guidelines (U.S. EPA, 1991). The clinical examinations were conducted 3 to 4 h post-exposure in the developmental neurotoxicity study, but just before daily gavage in this study. On GD 20 (4 h post gavage), the blood CPF concentration in fetuses was about one-half the level found in their dams (high-dose fetuses 46 ng/g; high-dose dams 109 ng/g). Chlorpyrifos oxon was detected only once; high-dose fetuses had a blood level of about 1 ng/g. No blood CPF could be detected (limit of quantitation (LOQ) = 0.7 ng/g) in dams given 0.3 mg/kg/day; these dams had significant inhibition of plasma and red blood cell ChE. In contrast, fetuses of dams given 1 mg/kg-day had a blood CPF level of about 1.1 ng/g, but had no inhibition of ChE of any tissue. Thus, based on blood CPF levels, fetuses had less ChE inhibition than dams. Inhibition of ChE occurred at all dosage levels in dams, but only at the high-dose level in pups. Cholinesterase activity in dams, fetuses and pups (% of control activity) were presented. At the high dosage, ChE was inhibited in a pattern red blood cells ≈ plasma ≥ heart > brain (least inhibited). Inhibition was greater in dams than in fetuses or pups. Milk CPF concentrations were up to 200 times those in blood, and pup exposure via milk from dams given 5 mg/kg-day was estimated to be 0.12 mg/kg-day. The authors concluded that the no-observed-effect level for pups was 1 mg/kg-day of maternal exposure. The limitations were that the male and female fetal and pup data were combined and the clinical findings of the two studies cannot be directly compared because of the different timing of the examinations. However, clinical effects at 3 to 4 h post-exposure would be consistent with expected peak levels of blood CPF. While the blood CPF data presented above suggest that fetuses also have a lower exposure than their dams, since blood CPF levels were about twice as high in dams than fetuses, the associated standard deviations were very high (almost the same value as the mean value).

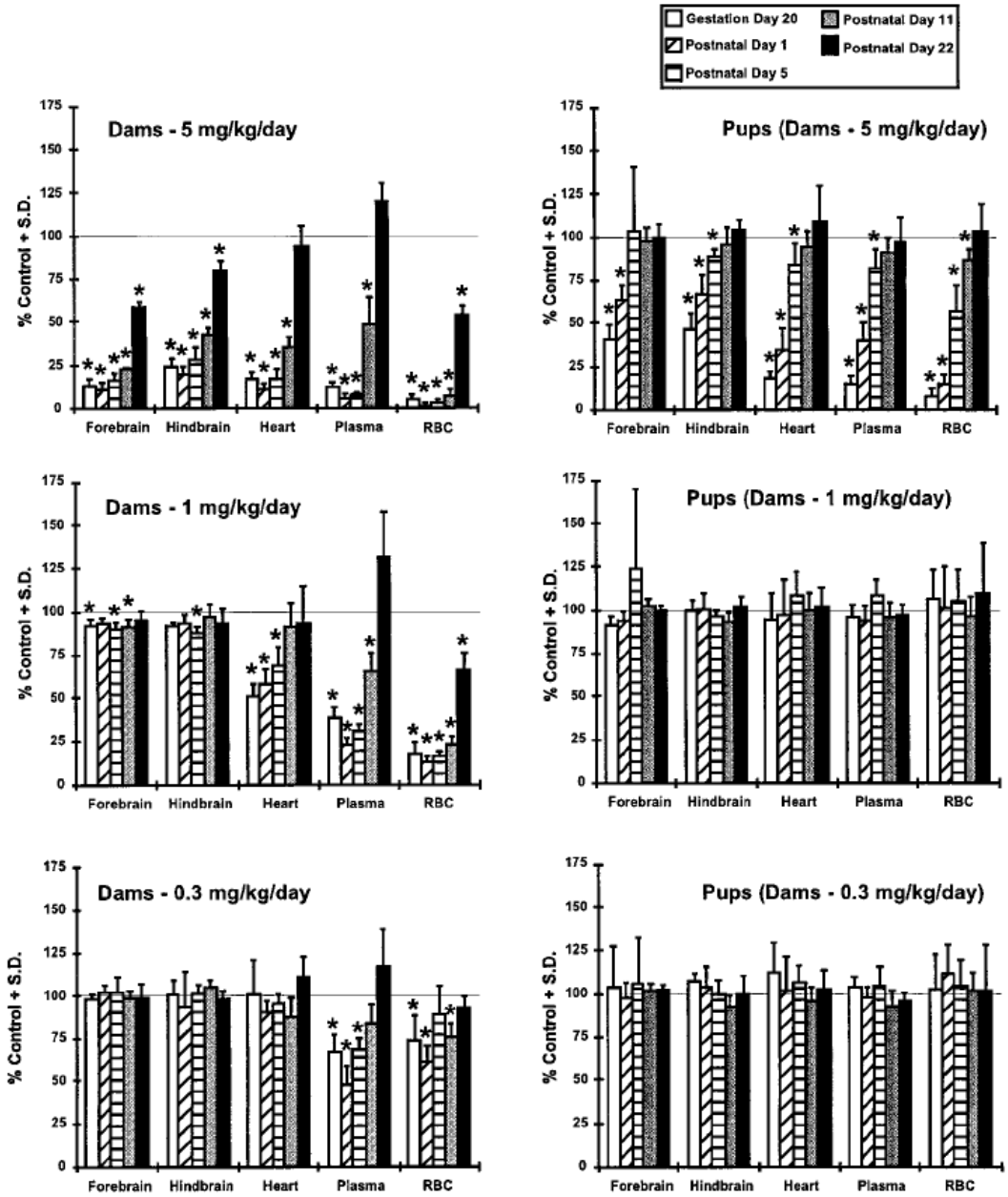


Figure C25. Cholinesterase activity in dams, fetuses, and pups (% of control activity). Male and female fetal and pup data were combined. *Indicates *p* values <0.02

Therefore, the dosage to nursing pups was much reduced compared to the dams exposure. In spite of exposure via milk, the ChE levels of all tissues of high-dosage pups rapidly returned to near control levels by PND 5. The authors used published equations from Sampson and Jansen (1984) and the rate of growth (6.1 g + 0.6 g/d) from the developmental neurotoxicity study of Hoberman (1998) to estimate that a newborn pup would drink about 1 ml of milk per day and concluded that at the high dose a nursing pup's exposure would be about 0.5 mg CPF/kg-day;

after further evaluation, they also estimated that high-dose nursing pups ingested about 0.1 mg/kg-day.

C.3. Developmental toxicity: Other Relevant Data

Examining the literature for CPF, the role of the vehicle and route of exposure are issues that deserve attention. Since route, vehicle, dose, and frequency of administration can result in different systemic exposure to the test chemical and its metabolites, these issues need to be taken into consideration. Understanding the relationship between dosing regimes and body burden in pregnant rats, their fetuses, and neonatal rats is critical in order to understand internal dosimetry. The impact of different oral gavage vehicles (e.g., rat milk and corn oil), on pharmacokinetic (PK) parameters have been investigated. Accordingly, relevant articles in the literature are included below.

Marty et al., 2007.
The Effect of Route, Vehicle, and Divided Doses on the Pharmacokinetics of Chlorpyrifos and Its Metabolite Trichloropyridinol in Neonatal Sprague-Dawley Rats

In this study, Male CD (Sprague-Dawley derived) rats at PND 5 were dosed with CPF (1 mg/kg) using different routes of exposure, vehicles, and single versus divided doses. Divided doses were designed to model episodic CPF exposures. Blood concentrations of CPF and its primary metabolite, TCPY, were measured at multiple times through 24 h. Groups included were single gavage bolus versus divided gavage doses in corn oil (one vs. three times in 24h), single gavage bolus versus divided gavage doses in rat milk, and sc administration in DMSO. These data were compared with lactational exposure of PND 5 pups from dams exposed to CPF in the diet at 5 mg/kg-day for 4 weeks or published data from dams exposed to daily gavage with CPF at 5 mg/kg-day.

The experimental designs used in this study are outlined below. Postnatal day 5 pups were directly exposed to CPF (1 mg/kg-day) as a bolus oral dose in milk or corn oil or as a bolus dose via sc injection (A) or orally as a divided dose (0.33 mg/kg three times per day) in milk or corn oil (B). Pup blood was collected at 0.5, 1, 2, 4, 6, 8, 12, 18, and 24 h after the last dose. In the lactational experiments (C), PND 5 pups were exposed to CPF via lactating dams receiving 5 mg/kg-day CPF in the diet. Dams were at steady state, and dam blood and milk samples were collected at 6:00 A.M., 11:00 A.M., and 5:00 P.M. Pup blood was collected at 12:00 A.M., 7:00 A.M., and 1:00 P.M. Three to four pups were used per time point per dosing scenario.

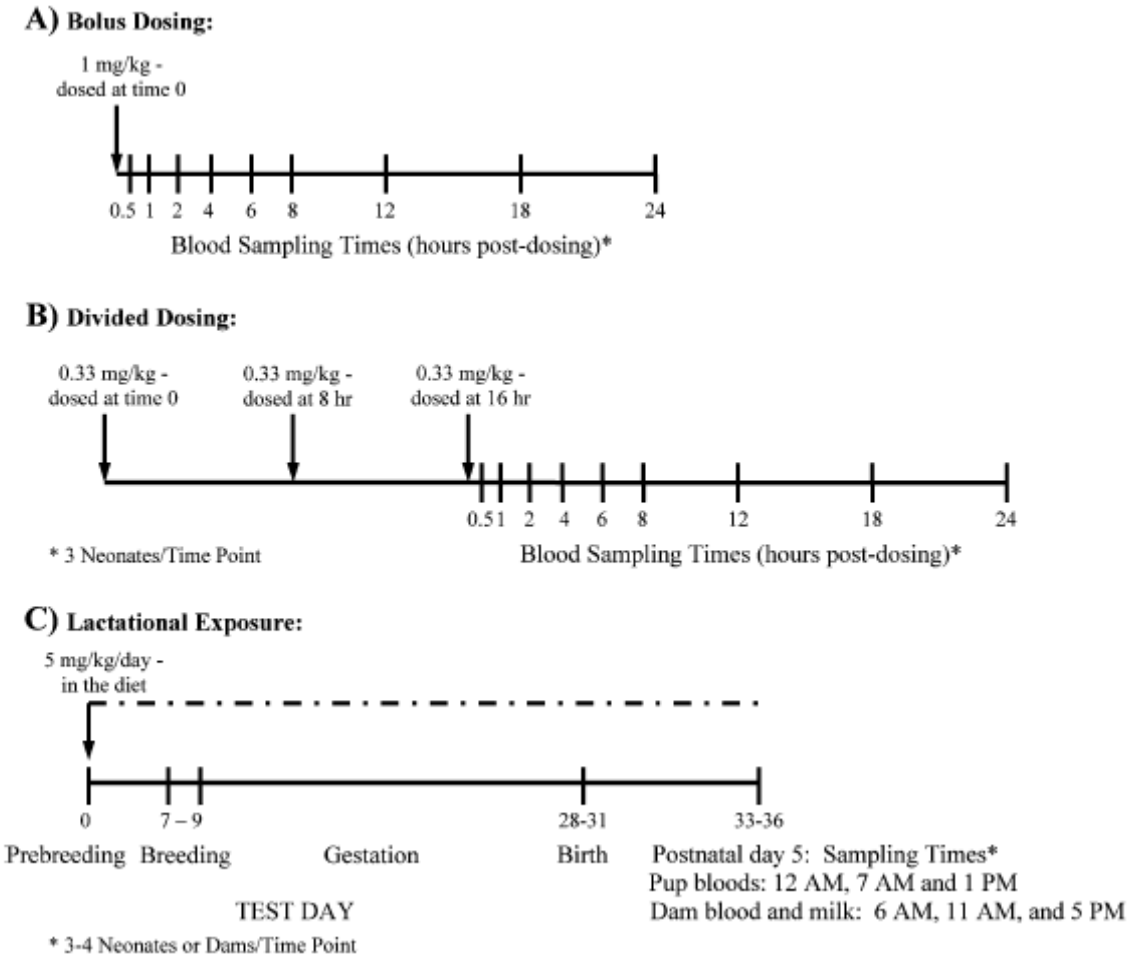


Figure C26. Diagram depicting the experimental designs used in the Marty et al., 2007 study.

In this study, neonatal body burdens following oral bolus gavage dosing (1 mg/kg, one time per day) or divided oral dosing (0.33 mg/kg, three times per day) on PND 5 were compared with exposure via nursing of milk from dams exposed to test material (5 mg/kg-day) in the diet. The designated injection site (dermal layers and a thin layer of underlying tissue), the carcass, and other tissues (liver, brain, and heart) were collected and analyzed for the presence of ^{3}H radioactivity. The injection site was analyzed to determine the qualitative rate at which the administered dose penetrated beyond the injection site.

Dietary exposure of Dams:

Chlorpyrifos and TCPY in the blood and milk of dams exposed to dietary CPF at 5 mg/kg-day for 6–7 weeks (during the prebreeding, breeding, gestation, and lactation periods and, therefore at steady-state levels) and pup blood levels for lactationally exposed offspring are noted below. Maternal blood levels of CPF were unquantifiable at all time points with dietary exposure (LOQ = 3.56–4.43 ng CPF/g blood). Milk CPF was fairly consistent at the three time points during 24 h, at about 118 ng/g milk, indicating that dietary exposure provided reasonably stable blood

levels of CPF. The TCPY levels of lactating dams from dietary CPF at 5 mg/kg-day varied four-fold over the course of the day.

Table C40. CPF and TCPY Concentrations in the Blood and Milk of Dams Exposed to 5 mg/kg-day CPF in the Diet and Pup Blood Levels Following Lactational Exposure (PND 5)

Sample time	Animal	CPF (ng/g blood)	TCPY (ng/g blood)	CPF (ng/g milk)	Estimated CPF (ng/g blood)[a]
12:00 A.M.	Neonates[b]	NQ[c]	48.63 ± 3.09	–	
6:00 A.M.	Dam[d]	NQ[e]	280.86	111.42	1.1
7:00 A.M.	Neonates	NQ	54.93 ± 2.10	–	
11:00 A.M.	Dam	NQ	1262.83	117.08	1.1
1:00 P.M.	Neonates	NQ	71.11 ± 8.00	–	
5:00 P.M.	Dam	NQ	566.40	127.28	1.2

[a]Estimated to be 1.1 ng CPF/g blood Assumes milk:blood partitioning of 104 from Mattsson *et al.* (2000).
[b]*n* = 3-4 neonates per group; mean ± SD for TCPY blood concentrations.
[c]Nonquantifiable; LOQ in neonatal blood ranged from 3.24 to 3.70 ng CPF/g blood.
[d]*n* = 1 dam/group.
[e]Nonquantifiable; LOQ in dam blood ranged from 3.56 to 4.43 ng CPF/g blood.

Neonatal blood levels of TCPY were more consistent across time points than diet-exposed dam TCPY levels, varying only by a factor of 1.5. Consistent with the dams, neonatal TCPY levels were highest at midday. At all time points, pup blood levels of TCPY were considerably lower than dam blood levels, and the PND 5 blood TCPY levels in pups were about 12 times lower than their dam's. The TCPY concentrations in the milk of dams exposed to CPF in the diet at 5 mg/kg were not measured, and the authors assumed that TCPY would partition into milk based on previous data and unpublished data available to the authors showing the transfer of TCPY through milk in other species.

Direct-dosing of pups:

Pharmacokinetic parameters for pup blood levels of CPF and TCPY following direct exposure to 1 mg/kg CPF via oral gavage and following sc administration of CPF as a single dose in DMSO are shown below. The area under the curve (AUC) values for bolus doses were calculated from time 0 (first dose) to 24 h after the dose was administered and in each of the direct oral dosing scenarios, the time to maximum blood concentrations (T_{max}) was the same, at 2 h post-dosing for CPF and 4 h post-dosing for TCPY. The maximum CPF and TCPY concentrations (C_{max}) were highest in pups given the single bolus dose of CPF compared with pups given split doses. Bolus dosing in corn oil yielded the highest total exposure based on both C_{max} and AUC values compared with the bolus milk group. According to the authors, absorption $t_{1/2}$ values for the two single oral dose groups were calculated from the TCPY blood data and were found to be comparable at 1.0 h for both the corn oil and the milk vehicle groups, but data were not shown. The approximately six-fold increase in plasma C_{max} via bolus dosing in corn oil versus milk was substantially higher than the 2.3-fold increase in plasma AUC for CPF and was also substantially higher than the 1.65-fold and 1.33-fold increases in TCPY C_{max} and AUC values seen for the corn oil versus milk vehicle groups. Hence, this specific increase in the C_{max} parameter for the

corn oil group suggests a decrease in the high rate of metabolism when CPF is administered in this dose vehicle. The ability of corn oil vehicle to markedly increase C_{max} and AUC values also appeared to be dose dependent because both CPF and TCPY C_{max} values were similar for pups receiving CPF divided doses of 0.33 mg/kg each in either vehicle (corn oil or milk). Thus, neonatal doses in excess of 0.33 mg/kg of CPF in corn oil vehicle appear to give a disproportionately higher C_{max} when compared to a milk vehicle. While the reason for the longer $t_{1/2}$ from milk vehicle compared with corn oil dose is unknown, the authors thought that it may be related to different distribution profiles for the parent compound administered in milk.

Following sc administration of CPF as a single dose in DMSO, the time to C_{max} of CPF in the blood was 2 h, the same time at which C_{max} occurred with direct oral exposures. The calculated AUC for parent CPF was 82 ng h/ml following sc administration, and the $t_{1/2}$ for parent CPF was 8.3 h. Thus, the CPF C_{max}, AUC, and $t_{½}$ values were similar to those seen with bolus oral exposure in a milk vehicle. However, the time to TCPY C_{max} was 6 h rather than 4 h as was seen with the other exposure routes. Also, the fate and disposition of CPF were not fully understood with sc administration in this study. The AUC for TCPY following the sc exposure was less than the TCPY AUC via the oral route suggesting that less test material was absorbed via the sc route. Overall with sc administration of 1 mg CPF/kg in DMSO, the AUC for the parent material was similar to that observed with the oral route using a milk vehicle and less than the AUC when using bolus corn oil gavage (as seen in Table C41 below). The plasma $t_{1/2}$ for CPF following sc injection in DMSO also was similar with the $t_{1/2}$ seen after bolus exposure in milk with the T_{max} for TCPY was 6 h compared with 4 h after exposure via the oral route. These data indicate that parental CPF may be available for a longer period (about 2 hours) from sc injection than from gavage to interact with target sites when administered under these dosing conditions.

Table C41. Effect of Dose Rate, Vehicle, and Exposure Route on PK Parameters in PND 5 CD Rat Pups Directly Exposed to 1 mg/kg-day CPF

			CPF					TCPY				
			T_{max}	C_{max}[b]		AUC[c]	$t_{1/2}$	T_{max}	C_{max}		AUC	$t_{1/2}$
Vehicle	Dose route	Dose frequency[a]	h	ng/ml	µmol/l	ng h/ml	h	h	ng/ml	µmol/l	ng h/ml	h
Corn oil	Oral	Single	2	48.7 ± 30.8	0.139	160	3.0	4	320 ± 112	1.53	2935	4.9
Rat milk	Oral	Single	2	8.6 ± 1.0	0.025	72	8.3	4	194 ± 120	0.93	2214	8.1
Corn oil	Oral	Split	2	6.9 ± 0.7	0.020	ND[d]	ND	4	112 ± NA[e]	0.54	ND	6.3
Rat milk	Oral	Split	2	5.6 ± 1.2	0.016	ND	ND	4	100 ± 33	0.48	ND	5.0
DMSO	sc	Single	2	9.5 ± 2.7	0.03	82	8.3	6	171 ± 90	0.82	1754	6.7

[a]A single dose of 1 mg CPF/kg or three doses of 0.33 mg CPF/kg administered 8 h apart.
[b]Mean concentration ± SD.
[c]Calculated from 0 to 24 h after last administered dose.
[d]Not determined.
[e]$n = 2$.

Overall maternal blood CPF levels were an order of magnitude lower from dietary exposure than gavage (1.1 vs. 14.8 ng/g), and blood CPF levels in PND 5 pups that nursed dietary-exposed or gavage-exposed dams were below the limit of detection. Single gavage doses of 1 mg/kg CPF in

corn oil vehicle in pups resulted in CPF blood levels of 49 ng/g and in milk vehicle about 9 ng/g. Divided doses led to lower peak CPF levels. While the authors claim that a bolus dose of 1 mg/kg CPF in DMSO administered sc appeared to have substantially altered the PK from orally administered CPF, from the data (see Table C41 above) the sc DMSO exposure is comparable to the exposure via rat milk (single dose more than split).

Table C42. PK Summaries from Various Studies Examining CPF and TCPY in Neonatal Rat Pups on PND 5

End point	CPF in maternal blood (LD 5)	TCPY in maternal blood	ChE inhibition[a] (dams on LD 5)	CPF in milk	Milk CPF ratio to 5 mg/kg gavage to dam	Estimated CPF exposures via milk	Pup exposure relative to dam	CPF in pup blood (C_{max})	CPF in pup blood (AUC)	TCPY in pup blood (C_{max})	TCPY in pup blood (AUC)	ChE inhibition[a] (pups on LD 5)
Gavage to dam: 0.3 mg/kg in corn oil	NQ at 4 h[b] (estimated 0.13 ng/g from milk data)	123 ng/g	Yes	13.5 ng/g[c]	0.009	0.003 mg/kg/day[d]	0.01	NQ[b]–2h[e]	–	NQ[f]–2 h[e]	–	No
Gavage to dam: 1.0 mg/kg in corn oil	NQ at h[b] (estimated 0.8 ng/g from milk data)	418 ng/g	Yes	81.8 ng/g[c]	0.05	0.017 mg/kg/day[d]	0.017	NQ[b]–2h[e]	–	NQ[f]–2 h[e]	–	No
Diet given dam: 5.0 mg/kg/day	NQ (estimated 1.1 ng/g from milk data	281-1263 ng/g	Yes[g]	111-127 ng/g	0.08	0.024 mg/kg/day[d]	0.005	NQ[h]	–	49-71 ng/g[i]	–	?[j]
Gavage to dam: 5 mg/kg/day in corn oil	14.8 ng/g at h	2048 ng/g	Yes	1534 ng/g[c]	1	0.1 mg/kg/day[k]	0.02	NQ[b]–2h[e]	–	49 ng/g–2 h[e]	–	Residual from *in utero* exposure
Neonatal sc injection: 1 mg/kg in DMSO	NA	NA	NA	NA	NA	NA	NA	9.5 ng/ml–2 h	82 ng h/ml[l]	171.3 ng/ml–6 h	1754 ng h/ml[l]	Yes[m]
Neonatal gavage (bolus): 1 mg/kg in rat milk	NA	NA	NA	NA	NA	NA	NA	8.4 ng/ml–2 h	90.1 ng h/ml[l]	194 ng/ml–4 h	2577 ng h/ml[l]	Not evaluated
Neonatal gavage (bolus): 1 mg/kg in corn oil	NA	NA	NA	NA	NA	NA	NA	47.5 ng/ml–2 h	125.5 ng h/ml[l]	320 ng/ml–4 h	3087 ng h/ml[l]	Yes[n]

Note: LD, lactation day.
[a]Plasma and/or red blood cell cholinesterase (ChE) inhibition at 4 h postgavage dosing of dams; Mattsson *et al.* (2000).
[b]Nonquantifiable with LOQ = 0.7 ng/g; Mattsson *et al.* (2000).
[c]Milk samples collected 4 h after dosing the dam via gavage, the time of peak blood concentrations in treated dams.
[d]Calculated based on concentration of CPF in milk X 2.35 ml milk consumed on PND 5 X 1/bw (~11.5 g).
[e]Peak blood levels for CPF were estimated to be at 4 h post-exposure in dams.
[f]Nonquantifiable with LOQ = 10 ng/g; Mattsson *et al.* (2000).
[g]Predicted response based on results from other studies cited in the table.
[h]Nonquantifiable with LOQ = 3.2–3.7 ng/g (current study).
[i]Peak time cannot be determined due to continuous exposure of pups via milk ingestion from CPF-exposed dams.
[j]ChE inhibition is uncertain; pups from dams given 5 mg/kg/day by gavage had ChE inhibition with lower TCPY values, but TCPY values were likely underestimated (samples were collected at 2 h; peak at 4 h).
[k]Mattsson *et al.* (2000).
[l]Calculated from zero to infinity.
[m]Song *et al.* (1997) with multiple doses.

[n]Timchalk *et al.* (2006).

Initial data from the sc DMSO dosing experiment indicated low recovery, either as CPF or as TCPY, of the total administered dose of CPF. But, in a follow-up study, wherein radio-labeled CPF was administered sc in DMSO and blood samples were taken soon after dosing until 2 h post-dosing to characterize the rapid absorption of CPF that was expected because of the use of DMSO vehicle, the majority of radiolabel remained at the injection site up to 2 h, coincident with peak blood CPF levels for this group. Figure C22 below illustrates the fate of radio-labeled CPF in rats injected with 1 mg/kg ^{3}H-CPF in DMSO and euthanized at 5, 10, 15, 30, 60, or 120 min after sc injection. Over the 2-h monitoring period, > 90% of administered dose of radiolabel remained at the injection site and in the carcass. The fate of this depot of radio-labeled material was not well understood by the authors.

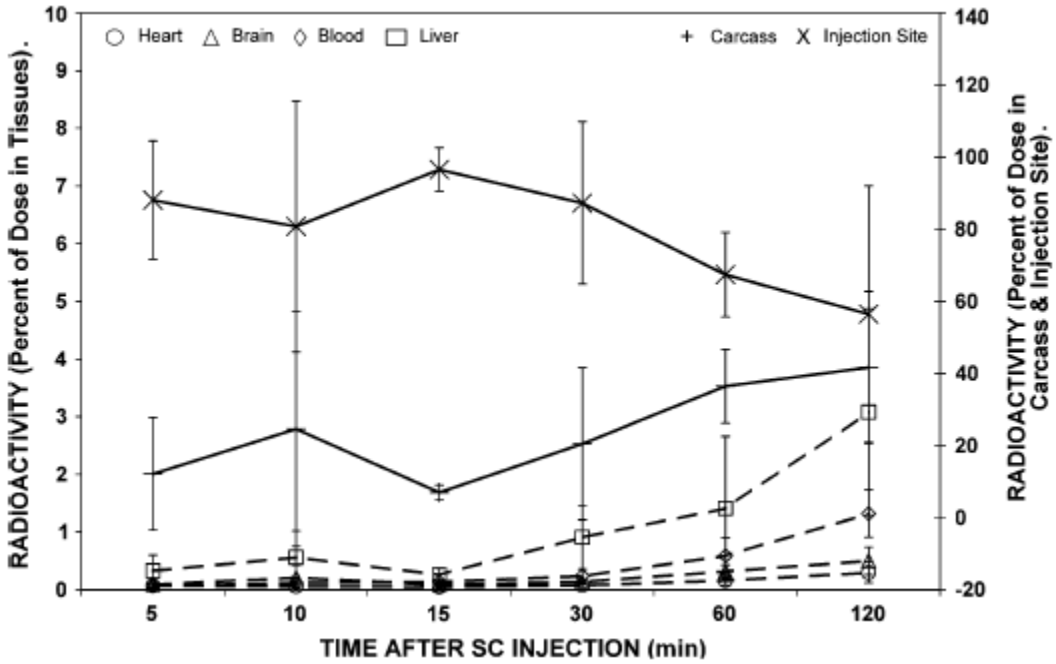


Figure C27. Fate of radio-labeled CPF in rats.

Since changes in the frequency of dosing and/or the vehicle may lead to significant differences in the disposition of a xenobiotic to target organs and a corresponding change in toxicological outcome, dose frequency and vehicle are important. The blood kinetic data for CPF administered sc fit poorly into the existing physiologically based pharmacokinetic (PBPK) model for juvenile animals (Timchalk et al., 2006). Thus, according to the authors, the longer TCPY T_{max}, the lower absorbed dose, the CPF depot at the injection site, and the poor PBPK model fit suggest that the PK via this route differ substantially from CPF administered orally. Accordingly, with the data presently available they were unable to determine whether the PK differences observed were due to the route of exposure (sc), vehicle (DMSO), or both. Although the authors state that the CPF doses selected were higher than environmentally relevant exposures but were typical

for doses commonly used in contemporary rodent studies, early on in the article they state that dietary CPF did not appear to cause systemic toxicity in either dams or pups as dams gained weight during gestation and lactation and there were no clinical signs of toxicity in either dams or pups.

The authors commented that the signs of discomfort (‘‘squirming’’) observed for several seconds to a minute or two following subcutaneous injection of 1 ml/kg BW DMSO, obligates exploration of alternative routes of administration that would result in less pain or discomfort. This obligation they felt was especially important for unusual routes of administration of test material in hazard evaluation studies, particularly when the utility of the resulting data for risk assessment of environmental exposures to chemicals is limited and because even transient pain can alter the chemistry of the developing brain, sometimes for long periods of time. Thus, given that CPF is known to be a mild irritant to the skin (WHO, 2007), in addition to the prompt signs of discomfort in the rats, the delay in absorption of sc CPF in DMSO raised questions of local irritation from this CPF depot.

It must be noted that the in vivo studies described earlier using macromolecular biomarkers conducted using the sc injection route of exposure and DMSO as the vehicle, had DMSO controls in all the studies. And though DMSO would result in a rapid uptake and full absorption of the compound, compounds administered via SC injection enter directly into the general circulation and bypass hepatic metabolism once, thus bypassing hepatic activation of chlorpyrifos to its active metabolite chlorpyrifos-oxon (U.S. EPA., 2000). While this is not a pathway of human exposure, these studies still provide important qualitative information on the potential for chlorpyrifos to affect neurodevelopmental processes.

These findings and other studies highlight another relevant issue. Given the relatively altricial state at which rat pups are born, exposure during PND 1–10 in the rat pup would be equivalent to a continuous *in utero* exposure in humans. The second most relevant exposure scenario would be lactational exposure following dietary exposure of the dams, as this scenario would provide an opportunity for continuous exposure as would occur *in utero* for humans. Lactational exposure may also be relevant for premature infants. To better understand the neurotoxic effects of diverse hazards on the developing human nervous system, researchers and clinicians rely on data collected from a number of model species that develop and mature at varying rates (Clancy et al., 2007). The findings from evolutionary and developmental biology show that the timing and sequence of early events in brain development are remarkably conserved across mammals (Finlay and Darlington, 1995) and form the basis for generalization across species. It is estimated that the rat brain at PND 1–10 equates to the third trimester in humans, or that rat neurodevelopment at PND 7 is equivalent to that of the human brain at birth (Dobbing and Sands, 1979; Andrews and Fitzgerald, 1997). A more recent study refines this time period, suggesting that PND 2–7 in rat corresponds to the human third trimester, and translating the human day of birth to rat PND 12–13 (Romijn et al., 1991). This is further confirmed by other conservative estimates which have placed the nervous system in a newborn human as equivalent to a rat pup on PND 10 or later (Marty et al., 2007).

Accordingly, exposure during the postnatal period in rodents appears to be relevant to human neurodevelopment. Thus, while studies where the exposure extends beyond the gestational period to early neonatal period have been described in the earlier section, studies with early postnatal exposure without prenatal exposure are included here.

Jett et al., 2001.
Cognitive function and cholinergic neurochemistry in weanling rats exposed to chlorpyrifos

In this study Long-Evans rats were injected sc with 0, 0.3 or 7.0 mg CPF/kg every 4 days before or after weaning and were tested with the Morris swim task from PND 24 through 28. The responses observed in this study appear to be classic effects on cognition as evidenced by a diminished spatial navigation ability in rats administered CPF at 7 mg/kg sc on PND 7, 11 and 15 (i.e., pre-weaning study) and in rats administered CPF at 0.3 mg/kg and 7 mg/kg sc on PND 22 and 26 (i.e., post-weaning study) and tested on PND 24 through 28. The lack of AChE activity may be a result of the timing when the animals were tested (3 hours, 24 hours or 5 days after sc injection). Other limitations of the study include lack of analysis of variance of group effect by time to evaluate rate of learning and fewer animals in the post-weaning study. Testing on the first day (day 24) revealed a significant difference between the controls and 0.3 mg/kg as well as the 7 mg/kg group in the pre-weaning study and it appears that in the post-weaning study, the day effect was not significant because both treatment groups had stable latencies higher than the controls throughout the study. Despite the variability associated with the test, it appears that even at 0.3 mg/kg the animals are demonstrating an impaired ability to learn the spatial navigation over the entire testing period. The probe test (built-in confirmatory test within the experimental protocol) conducted one day after the last day of testing demonstrated a significant difference between both treatment groups (7 mg/kg and 0.3 mg/kg) and control in the postweaning study and a significant difference only at the 7 mg/kg in the preweaning study. These findings indicate either a loss in memory which could be diminished recall or learning, either of which would constitute an effect on cognition. The authors concluded that there was cognitive impairment in weanling rats at low dose levels (despite the limitations noted) and the mechanisms for CPF-induced cognitive dysfunction were unknown.

Ricceri et al., 2003.
Developmental exposure to chlorpyrifos alters reactivity to environmental and social cues in adolescent mice.

Neonatal mice were treated daily on PND 1–4 or 11–14 with CPF, at doses of 1 or 3 mg/kg-day. Brain AChE activity was evaluated within 24 h of termination of treatments. Pups treated on PND 1–4 underwent ultrasonic vocalization tests (PND 5, 8, and 11) and a homing test (orientation to home nest material, PND 10). Pups in both the treatment schedules were then assessed for locomotor activity on PND 25, novelty-seeking

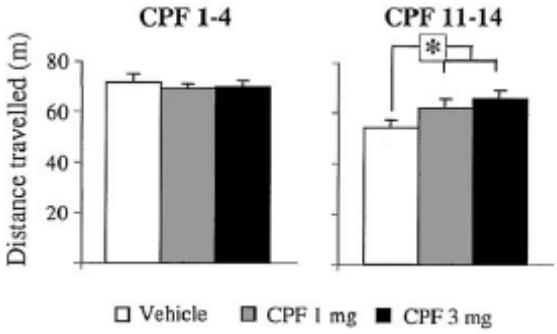
response on PND 35, social interactions with an unfamiliar conspecific on PND 45, and passive avoidance learning on PND 60.

Thus the aims were as follows:
1. To assess CPF effects at different developmental stages (neonatal, weaning, adolescent, and adult stage) and on different behavioral endpoints, ranging from those reportedly sensitive to CPF action in the rat (locomotor activity, habituation, and cognitive performances) to those not strictly cholinergically regulated, such as (i) ultrasound emission and orientation to home nest material in the neonates and (ii) social/affiliative behaviors and novelty-seeking behavior in adolescent mice;
2. To measure CPF anti-ChE effects on total, soluble, and membrane-bound AChE activities, providing more information on the CPF cholinotoxic effects during the developmental phase, and to analyze how the CPF inhibitory effects influence the G4 and G1 AChE isoforms in the soluble fractions, where G1 relative concentration is much higher in newborn than in young and adult animals;
3. To extend to the mouse species information on adverse CPF effects, up to now limited to the rat. The availability of genetically manipulated mice for OP detoxifying esterase (Shih et al., 1998) makes information on developmental CPF toxicity in the mouse relevant for future studies on individual susceptibility to pesticides' toxic effects.

The two CPF doses did not cause any overt signs of cholinergic intoxication at either age of treatment. The authors thought this was not surprising since depression of brain AChE activity in the order of 50% is usually associated with cholinergic signs (Bignami et al., 1975) and a correlation between depression of brain AChE activity and an appearance of cholinergic signs has been questioned (Allen, 1990).

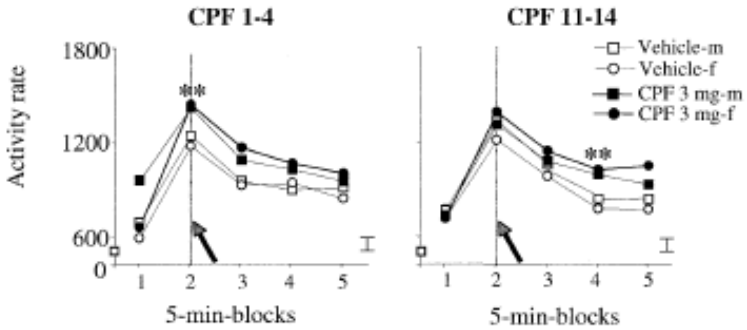
Acetylcholinesterase activity was reduced by 25% after exposure to CPF on PND 1–4, but not after PND 11–14. Also, CPF selectively affected only the G4 (tetramer) molecular isoform of AChE. The percentage of inhibition is comparable to that reported for rats at the same age and for similar CPF doses (Dam et al., 2000; Slotkin et al., 2002), but according to the authors the transience of the effects appears characteristic of the mouse species, and it is likely due to species-specific differences in AChE turnover and metabolism. Such transient inhibition was found in both the membrane-bound and the soluble AChE fractions, and rule out the hypothesis that adverse effects of CPF might be underestimated in developmental studies when measuring the total AChE fraction only. No significant inhibition was found following CPF treatment on PND 11–14 and on PND 32–35, thus indicating an apparent selective vulnerability of the PND 1–4 mouse CNS to the anti-ChE action of CPF. However, the rapid AChE activity increase by PND 5 could "mask" a persisting moderate inhibitory effect. Previous rat studies evidenced a significant AChE inhibition also after CPF injections on PND 11–14, but at a higher dose. The authors also noted that on PND 4 the anti-ChE action of CPF appears to target primarily the G4 form, the one also affected by diisopropyl fluorophosphate exposure both at adulthood and in the first postnatal week (Meneguz et al., 1989; Meneguz et al., 1992) indicating that developmental CPF exerts its anti-ChE action through the same mechanisms described for other OP in the adult rodent brain rather than by age-specific mechanisms.

Behavioral analysis showed that early CPF treatment failed to affect neonatal behaviors. Locomotor activity on PND 25 was increased in mice treated on PND 11–14 at both doses, and CPF-treated animals in both treatment schedules were more active when exposed to environmental novelty in the novelty-seeking test. All CPF-treated mice displayed more agonistic responses, and such effect was more marked in male mice exposed to the low CPF dose on PND 11–14. Passive avoidance learning was not affected by CPF.



Locomotor activity test on PND 25 (male and female data pooled)

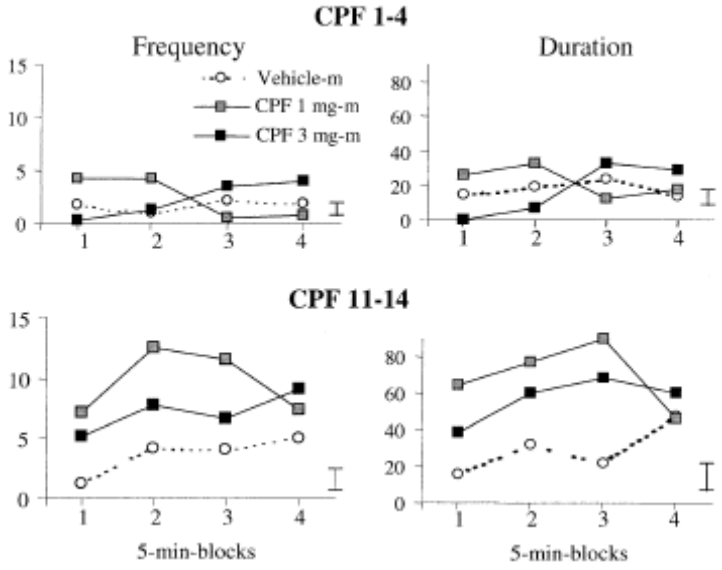
Figure C28. Data are mean distance traveled in a 20-min test (±SE) in CPF 1–4 and CPF 11–14 groups (CPF 1-4 = exposure to CPF on PND 1-4; CPF 11–14 = exposure to CPF on PND 11-14). *A significant increase in locomotion in CPF 11–14 treated mice ($P < 0.05$).



Novelty seeking test on PND 35

Figure C29. Data are mean activity rates before and after free access to the novel compartment. The gray arrow indicates time of partition opening. **A significant increase in locomotion in CPF 1–4 (exposure to CPF on PND 1-4) and CPF 11–14 (exposure to CPF on PND 11-14) treated mice ($P < 0.01$). Vertical bars to the right of each graph indicate pooled SE.

Examining the social interaction test on PND 45 (where the data are mean total aggressive responses displayed by 45-day-old mice during a 20-min social interaction test as seen below), the frequency of aggressive responses of CPF1 mice were significantly higher than vehicle in the first two 5-min blocks and the frequency of aggressive responses of CPF3 mice were significantly higher than vehicle in the last two 5-min blocks for PND 1–4. Both CPF1 and CPF3 mice displayed higher frequency and duration of aggressive responses throughout the 20-min test for CPF exposure on PND 11–14.



Social interaction test on PND 45

Figure C30. Data are mean total aggressive responses displayed by 45-day-old mice during a 20-min social interaction test. Vertical bars to the right of each graph indicate pooled SE.

The authors concluded that CPF appears to affect mouse neonatal brain development through multiple mechanisms, including interference with basal processes controlling cell replication and differentiation as well as specific effects on cholinergic system similar to effects in rats (Pope, 1999; Slotkin, 1999; Crumpton et al., 2000; Slotkin et al., 2002) and these data indicate that developmental exposure to CPF induces long-term behavioral alterations in the mouse species supporting the involvement of neural systems in addition to the cholinergic system in the delayed behavioral toxicity of CPF. Hence, the analysis of a wider range of behavioral and neurochemical endpoints than previously considered may help to elucidate the CNS targets for the delayed behavioral toxicity of CPF.

Mahjoubi-Samet et al., 2005.
Impact of chlorpyrifos on cerebrum and cerebellum maturity in suckling rats

The impact of CPF on cerebrum and cerebellum maturation in suckling rats was assessed in this study. Female Wistar rats were given 0.2 g CPF/L in drinking water, equivalent to40 mg/kg-day, from day zero until day ten after delivery. The standard diet contained 0.720±0.012 mg of iodine/g of diet. Food consumption was measured daily and the daily CPF intake ingested by lactating rats was calculated after measuring drinking water. The value of estimated CPF intake was 6.080±0.215mg/day-dam. In treated pups, plasma butyrylcholinesterase (BuChE) activity was inhibited by 70% and AChE activities in the cerebrum and cerebellum of the pups were reduced by 71 and 75%, respectively, compared with controls.

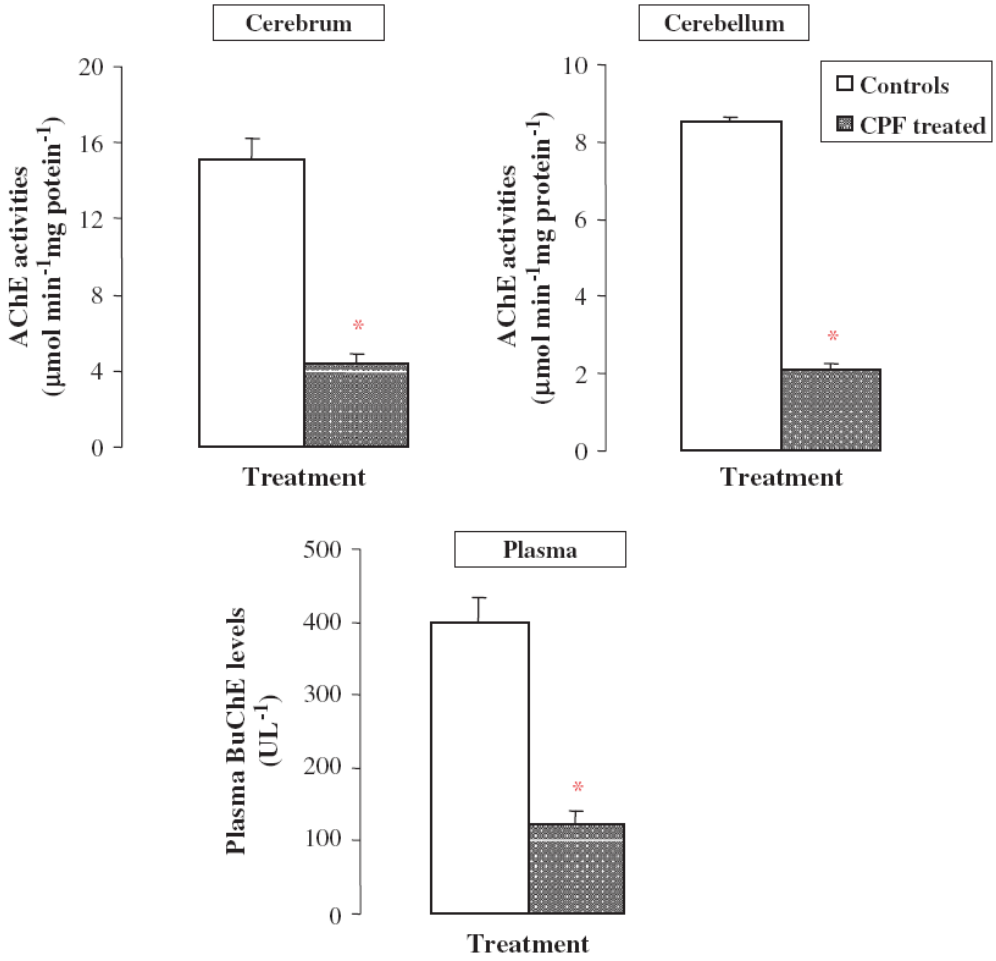


Figure C31. Plasma butyrylcholinesterase (BuChE) activity, cerebrum and cerebellum AChE activities in 10-day-old control and treated rats with chlorpyrifos (0.2 g/ L) administered in the drinking water of the mothers from day zero until day ten after delivery. n=6. Treated vs. Controls *: $p \leq 0.05$.

According to the authors, the results suggest that CPF was distributed in pup tissues through the milk of lactating mothers, resulting in inhibition of AChE activities and supporting previous work by Mattson et al. (2000). At the age of 10 days, the CPF-treated pups showed a 28% decrease in body weight, a 23 and 41% decrease in plasma free triiodothyronine (T_3) and thyroxine (T_4) levels and reduction in cerebral and cerebellar protein content by 36 and 38%, respectively.

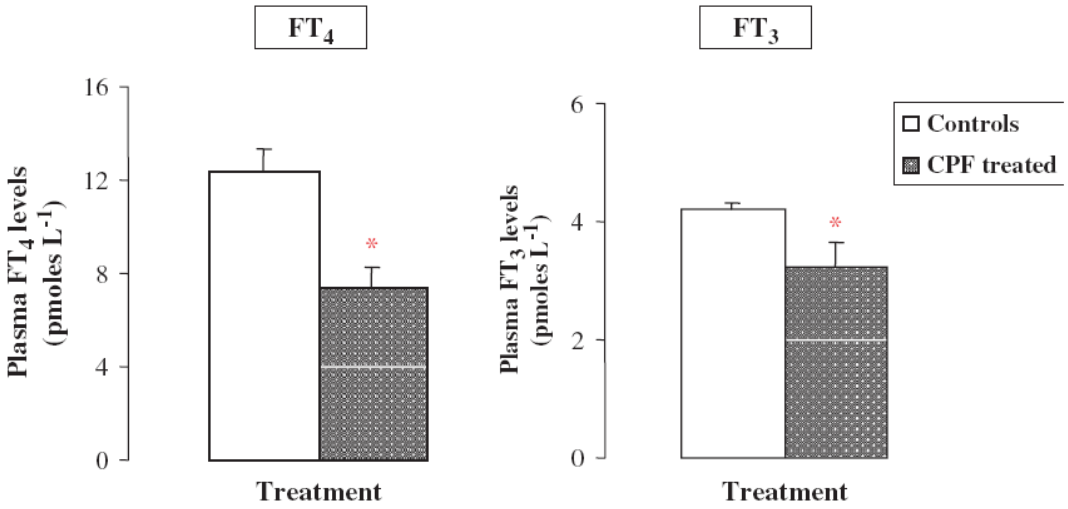


Figure C32. Plasma thyroxine (FT4) and triiodothyronine (FT3) levels of 10-day-old rats: controls and treated with CPF (0.2 g/L) administered in the drinking water of the mothers from day zero until day ten after delivery. n=6 Treated vs. Controls *: $p \leq 0.05$.

Thyroid hormones play an important role in the development and maturation of the central nervous system. Thyroid deficiency in early life has a marked influence on the functional development of the central nervous system, and is accompanied by significant effects on the structural and biochemical maturation of the cerebellum. The decrease in cerebrum and cerebellum protein content observed in this study is in agreement with previous studies using subcutaneous injection of CPF in DMSO to dams that indicated that acute CPF that exposure in young rats interferes with DNA and protein synthesis in different regions of the brain, despite the difference in route of exposure (Whitney et al., 1995; Dam et al., 1998).

Consistent histological changes were also found in the cerebellum of CPF treated pups, with the external granular layer being markedly developed, the Purkinje cell bodies being poorly differentiated and abnormally distributed into the internal granular layer. Figure C28, below, is a photomicrograph of histological cerebellum sections of 10-day-old rats.

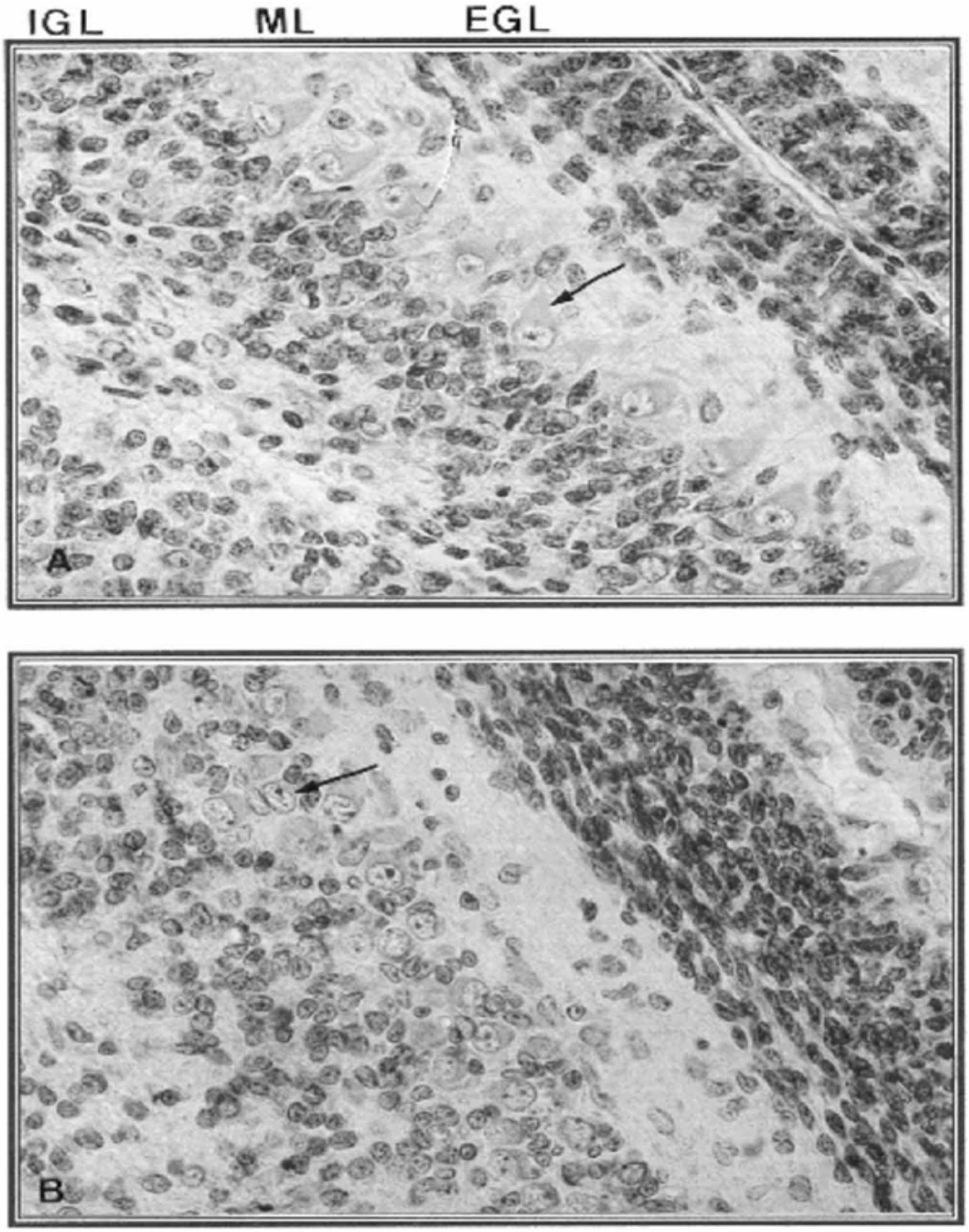


Figure C33. Histological cerebellum sections of 10-day-old rats: controls (A) and treated with CPF (0.2 g/L) (B) administered in the drinking water of the mothers from day zero until day ten after delivery with the arrows indicating Purkinje cells. (Magnification 400X; EGL: external granular layer, ML: molecular layer IGL: internal granular layer).

No information on clinical signs in dams or pups (which would be expected at this dose level) was provided. Similarly ChE levels in dams were also not measured. The overall conclusion was that CPF at this level of exposure (40 mg/kg-day) in the dams appears to interfere with thyroid function of the pups which plays a key role in cerebellum functional development.

C.3.1. Distribution and metabolism in pregnant females and conceptuses

Mattson et al. (2000) reported that the milk level of dams exposed to CPF at 5mg/kg-d continually from GD 6 was 3µg/ml on PND 1, and estimated that pups would be exposed to 0.126 mg CPF/kg-d. This value is similar to the threshold level for plasma ChE inhibition in adult rats of 0.1 mg/kg-d (Breslin et al., 1996). In addition, Tang et al. (1999) reported that a dosage of 200 mg CPF/kg given sc in corn oil to lactating dams on PND 1 resulted in about 26% inhibition of brain ChE in the offspring on PND 6. Given these findings, the neurobehavioral results on offspring from dams exposed during gestation to dose levels as low as 1 mg/kg-day subcutaneously (in DMSO) bears consideration. Several studies imply that the fetus is not protected from CPF when the dam is dosed orally and during late gestation the fetus is just as accessible, if not more accessible, as the dam, to orally administered CPF (Lassiter et al., 1998; Hunter et al., 1999). The higher levels of TCPY in the fetal brain than the maternal brain were not a result of increased accumulation within the fetal compartment, i.e., TCPY was created in and was unable to leave the fetal compartment. Because of the low levels of detoxification enzymes in the brain tissue, TCPY in the brain probably arose from the binding of the oxon to ChE or through TCPY in the circulation with some of the brain TCPY generated through ChE inhibition, and this is supported by the fact that both ChE inhibition and TCPY levels peak at the same time.

Studies have shown that immature organisms are more sensitive to CPF-induced ChE inhibition following acute high dose exposure, and the same degree of ChE inhibition can be achieved in PND 17 rats at a 5-fold lower dose compared to adults (Moser and Padilla, 1998b). The maximum tolerated dose (MTD) of CPF following sc injections was 45 mg/kg in neonatal rats at 7 days of age compared to 279 mg/kg in adult rats at 80-100 days of age (Pope et al., 1991). Pope and Chakraborti (1992) also studied the dose that would cause 50% inhibition of ChE activity (50% effective dose, ED50) following sc injections. The ED50 for brain ChE inhibition was 19.8 mg/kg in neonatal rats at 7 days of age compared to 44 mg/kg in adult rats at 3 months of age (Pope and Chakraborti, 1992). While young animals are more sensitive to the acute toxicity of CPF than adults, the difference is not evident following repeated exposure (Chakraborti et al., 1993; Liu et al., 1999; Zheng et al., 2000), and hence it is uncertain whether there is a similar age-related difference in sensitivity to chronic low dose exposure to CPF, which is more relevant to the environmental CPF exposure of the general human population.

C.3.2. Mechanism(s) of developmental toxicity.

While inhibition of ChE by its active metabolite CPO was once considered the lone mechanism of CPF neurotoxicity, recent studies have presented additional possibilities. There is evidence that CPF directly targets events that are specific to the developing brain and that are not necessarily related to the inhibition of ChE (Whitney et al., 1995; Song et al., 1997; Dam et al., 1998; Qiao et al., 2001; Qiao et al., 2003; Qiao et al., 2004; Qiao et al., 2005). Since immature animals actually recover more rapidly from ChE inhibition than do adults, measurements of ChE activity alone may not be sufficient for the assessment of adverse effects. Chlorpyrifos-induced neurochemical and neurobehavioral changes may in fact be unrelated to ChE inhibition and may be of equal concern for human health risk assessment. These include,

a) effects on the developing brain during cell division.
b) interference with RNA synthesis during differentiation.
c) interruption of cell signaling.
d) interference with important nuclear transcription factors involved in cell differentiation.
e) impairment of cholinergic synaptic function during development.
f) effects on the catecholamine system in the developing brain.
g) oxidative stress in the developing brain.
h) interference with gliogenesis and axonogenesis.

Such findings have led researchers to state that the vulnerable period for adverse effects of CPF is likely to extend into childhood or adolescence.

C.3.2.1. Biological mechanisms of action

Both AChE and ACh play important roles in brain development (Layer and Willbold, 1995; Buznikov et al., 1996; Brimijoin and Koenigsberger, 1999; Lauder and Schambra, 1999). In addition, the early appearance during neurogenesis of choline acetyltransferase (ChAT), the enzyme responsible for ACh synthesis and an indirect measure of functional cholinergic neurons, has also led to speculation on its involvement in neural development (Lauder and Schambra, 1999). These observations, along with the aforementioned greater sensitivity of juvenile animals to OP insecticides, suggest that any chemical that interferes with these components of cholinergic circuitry, such as CPF, may affect the proper development of the brain and possibly result in developmental abnormalities. It has been suggested that the cholinergic system of adult rats compensates for elevated levels of ACh resulting from the inhibition of ChE by decreasing ChAT activity (Russell and Overstreet, 1987). Choline acetyltransferase levels were not significantly affected by gestational CPF treatment until PND 12, and the absence of significant ChE inhibition at this time point argues against a compensatory mechanism (Richardson and Chambers, 2003). It has been established that either high-level acute exposure in adult rats (Liu and Pope, 1996; Liu and Pope, 1998) or repeated sc exposure to CPF in developing rats has resulted in reductions in both ChAT and high-affinity choline uptake (HACU) system with ChAT (Dam et al., 1999; Slotkin et al., 2001). Therefore, the reduction in ChAT activity observed by Richardson and Chambers (2003) may be the result of insufficient

availability of choline because of a decrease in HACU. However, it is not currently known whether the deficit noted was a transient or permanent effect and there are currently no data available on the effects of repeated gestational exposure to CPF on the development of the HACU system. Another explanation for the persistent inhibition of brain ChE in the offspring is that the enzyme is permanently inactivated or "aged" and can only recover through de novo synthesis of new enzyme (Wilson et al., 1992). However, there are currently no available data concerning the aging of brain ChE of offspring exposed *in utero* to CPF (Richardson and Chambers, 2003).

Use of DMSO as a vehicle for CPF administration in animal studies

Despite the many studies that have been conducted, the effects and the physiological and pharmacological properties of dimethyl sulfoxide (DMSO) are still incompletely understood. DMSO is thought to affect neurobehavioral function since 1 mg/kg of DMSO via i.p route retarded habituation of spontaneous exploratory activity, impaired acquisition of conditioned (auto-shaped) behavior and changed the dose-response relationship for d-amphetamine induced suppression of operant responding. Again, while the mechanisms of these actions are not certain, the toxic effects may be a consequence of DMSO's capacity to alter cell membranes so that target tissues become more vulnerable to other potentially toxic agents (Ali, 2001).

Also DMSO is thought to have antiChE activity (Sams, 1967; Sawada and Saito, 1975) These older studies have also stated that it enhances and may potentiate other anticholinesterases such as the organophosphates (Jacobs et al., 1982; Rubin, 1975), while another report demonstrated that DMSO reduced the toxicity of some irreversible antiChE (Kocsis et al., 1968). Studies in which CPF was administered orally by gavage at 5 mg/g-day during gestation day (GD) 6- postnatal day (PND) 10 (Mattsson et al., 2000) resulted in significant levels of ChE inhibition in the brain of fetuses at PND 5. Similarly, when given CPF orally on a vanilla wafer at 3 mg/kg orally during GD 6-20 (Richardson and Chambers, 2003), CPF caused inhibition of brain ChE in pups (26%) until PND 6. Thus, the concordance observed between studies that used DMSO as the vehicle and those that did not (Mattsson et al., 2000; Richardson and Chambers, 2003) underscores the effects of CPF at dose levels as low as 3 mg/kg in inhibiting brain ChE in pups subsequent to maternal exposure.

C.4. Integrative evaluation

C.4.1. Human data

A table (Table C43) summarizing developmental outcomes from the three cohorts is provided at the end of this section.

Studies examining the association between CPF exposure and fetal growth outcomes included the Columbia cohort (Whyatt et al., 2005; Rauh et al., 2006) which reported findings of decreased birth weight and birth length; the Mt. Sinai cohort (Berkowitz et al., 2004; Engel et al., 2007; Wolff et al., 2007) which reported smaller head circumference when considering paraoxonase activity; and the CHAMACOS cohort (Eskenazi et al., 2004; Young et al., 2005; Eskenazi et al., 2007) which reported no evidence of decreases in birth weight, birth length, or head circumference. Although Berkowitz et al., (2004), reported no findings of decreased birth weight, a subsequent related study from the same cohort (Wolff et al., 2007) did report deficits in birth weight in relation to ∑DEP and PON1, which were consistent with the findings of Whyatt et al., (2005), where a significant inverse association was seen between blood CPF levels and decreased birth weight.

The studies examining neurodevelopmental outcomes in relation to prenatal CPF exposure included the Columbia cohort (Rauh et al., 2006), the CHAMACOS study (Eskenazi et al., 2007; as well as the related study by Young et al., 2005) and the Mt. Sinai cohort (the related study by Engel et al., 2007). The study by Rauh et al. (2006), found greater proportions of children highly exposed to CPF, as measured in cord blood, had evidence of mental and motor delays, and were more likely to score in the clinical range for attention problems, attention-deficit/hyperactivity disorder problems and pervasive developmental disorder at 3 years of age, as compared with children with lower exposure. Eskenazi et al., (2007) found detrimental effects with maternal DAP and DMP levels, but not with DEP or TCPY, for mental development and pervasive developmental disorder. In the related studies of Engel et al., and Young et al., both studies were consistent in their findings of increased risk of abnormal reflexes associated with higher DEP levels.

These studies are difficult to compare as many factors differed between them including ethnic composition and genetic polymorphisms, age at developmental testing, as well as measures of exposure and levels of exposure. A number of these differences may explain some of the inconsistency in results. These factors are discussed below.

The ethnic composition of these three cohorts differed considerably. The Columbia cohort included African American or Dominican women, while the Mt. Sinai cohort was primarily Hispanic women from Puerto Rico, with African American and white women as well. In the CHAMACOS study most of the women were Mexican American. As presented earlier, there is a large amount of varibility in the polymorphisms of the PON1 enzyme between ethnic groups. The PON1 enzyme plays an important role in the detoxification of the CPF oxon, and the efficiency of the enzyme varies with different

polymophisms and the PON1 status. Only the Mt. Sinai cohort reported findings concerning PON1 genotype and phenotype in which low maternal PON1 activity and TCPY levels above the LOD were associated with decreased head circumference. If an effect modifier, such as the PON1 genotype, is not a confounder, the main effect, such as that between CPF exposure and fetal growth, would be an underestimate of the magnitude of the association among the most susceptible individuals and an overestimate of the magnitude among the less susceptible individuals (Bellinger, 2008). Thus, studies in which the PON1 status was not considered could be less likely to detect an effect where one was present.

The measure of exposure differed between the three cohorts. In the Columbia cohort, personal air sampling and blood CPF levels in the mothers and offspring were used. The CHAMACOS cohort and the Mt. Sinai cohort used urinary metabolite levels in the mothers and offspring. Cord blood CPF is perhaps the best biomarker of exposure as it is close to site of action and therefore may be more predictive of an effect (Needham, 2005). Urine levels of TCPY are easier to measure since there is a greater concentration of TCPY in urine than there is CPF in blood; however, urine levels of TCPY do not differentiate between exposure to CPF, to TCPY in the environment, or to chlorpyrifos-methyl (Needham et al., 2005). Since the Columbia study was the only one measuring blood CPF it is not possible to compare the levels of exposure in this study with those in the other two cohorts. The TCPY metabolite levels were higher in the Mt. Sinai cohort, in which a decrease in head circumference was observed, than in the CHAMACOS study which did not report any significant associations between TCPY levels and fetal growth outcomes.

Although exposure to CPF from dietary sources would be expected in all the participants in these studies, other exposures may differ substantially. In the studies of Eskenazi et al., (2004, 2007) and Young et al., (2005), the population was from an agricultural community where CPF is heavily used on crops grown in the Salinas Valley but very few of the home pesticides found in the homes of CHAMACOS participants contained CPF. In contrast, in New York City the environmental exposures in the Columbia and Mt. Sinai cohorts were primarily from the recurrent, spraying of CPF indoors with no exposure from agricultural use. Persistence of CPF is longer indoors due to diminished filtered sunlight, reduced moisture, air movement and surface area, as well as a lack of soil microorganisms (Krieger et al., 2001; Needham et al., 2005). Therefore, the ratio of the concentrations of parent pesticide to the less biologically active environmental degradates is likely to have been higher in indoor settings than in outdoor settings. In addition, it has been discussed that a higher percentage of cumulative exposure to CPF in the indoor settings was probably from inhalation, due to the longer duration in the homes and the amount of time spend indoors, thus women in the New York cohorts were probably more continuously exposed than those in the California cohort (Needham et al., 2005). The results of Whyatt et al., (2007) showed that pesticides were persistent in the home with little variability in the air concentrations over a two month period. Similar analyses for the CHAMACOS cohort was not found, although Eskenazi et al (2007) reported that preliminary results indicated that children's DAP measurements more than a few days apart were uncorrelated, suggesting considerable intraperson variability. In

addition, there may be a significant amount of exposure to the CPF oxon from the environment as the oxon has been measured in ambient air monitoring by the California Air resources Board (CARB, 1998). Exposure to the more potent oxon may have differed between these study populations.

An additional exposure factor considered in the Whyatt study (2005), was the association of exposure to CPF and birth outcomes in terms of before and after a ban on its residential use. An association between CPF exposure and birth weight and length that was observed before the ban was not seen after the ban. The study by Berkowitz et al., (2004) did not report conducting a similar analysis.

As estimated by Barr et al., (2005), CPF exposure at the 95th percentile would be about 0.06 ug/kg/day for children, which is within a factor of two of the chronic population-adjusted dose[2] (cPAD) for children (0.1 ug/kg body wt/day). However, as CPF levels in fetal brain in animals were found to be two to four times higher than levels in maternal liver (Hunter et al., 1999), exposure to the specific target tissues may be higher. In addition, since the levels of PON1 and the PON1 activity have been shown to be limited in the young, this population may be more sensitive to exposure (Furlong et al., 2005).

The strengths of these cohort studies include the prospective data collection during pregnancy, especially during the third trimester which would presumably include the critical window of exposure for outcomes such as birth weight and neurodevelopment. In addition, important covariates and potential confounders such as exposure to ETS, the home environment, and maternal intelligence for neurodevelopmental outcomes were measured and included in the analyses (Rauh et al., 2006; Eskenazi et al., 2007). The study by Whyatt et al. (2005) spanned the period before and after the ban of CPF for residential use, thus providing a unique opportunity for an epidemiologic study. Under these circumstances many of the variables that could be a source of bias in a study would have remained relatively constant in this cohort. Thus, the association seen between lower birth weight and birth length exposure to CPF before the ban but not after the ban is less likely to be the result of unmeasured covariates or confounding.

The period of most sensitivity or vulnerability in humans is likely to be the third trimester, during a spurt in brain growth. Another strength of these studies is that almost all urine and blood samples were collected during the third trimester, probably the critical window for the studied birth outcomes.

The limitations of prospective cohort studies include the lower participation rates and loss to follow-up. However, these studies were quite successful in retaining women in their study populations. In the neurodevelopmental studies, early childhood exposure to lead could be a confounder as it is a known neurotoxicant. Intrauterine lead levels were available for a subset of children (n = 89) in the Rauh study where no significant

[2] As explained in Barr et al., 2005, the PAD is defined by the U.S, EPA as the reference dose (RfD) adjusted to include the Food Quality Protection Act (FQPA) safety factor. For children and females 13-50 years, the acute and chronic CPF PAD are the RfDs divided by three. The general population PAD is equivalent to the RfD.

relationship was observed between prenatal lead levels and CPF levels (r = -0.08; p = 0.49). However, no childhood levels were reported. In addition, it is conceivable that a portion of the observed neurodevelopmental effects measured later in childhood in association with prenatal exposure to CPF could be a result of postnatal exposure to CPF. As mentioned earlier, the use of TCPY as a measure of exposure to CPF has its limitiations. Although Whyatt et al., (2005) did use cord blood CPF as a measure of exposure, these levels were not lipid-adjusted. Since the levels of CPF in blood depend somewhat on the equilibrium between its concentration in adipose tissue and blood, the blood concentrations are best evaluated both on a concentration basis and a lipid basis (Needham, 2005). Finally, exposure to more than one pesticide or to mixtures of pesticides can exacerbate, mitigate or mask the toxicity of the exposure of interest.

An additional study by Samarawickrema et al. (2008), found an association between CPF exposure, as determined by spray season, and DNA damage. Althought this was the only developmental study that examined this outcome, other studies of male reproductive outcomes reported DNA damage in sperm associated with CPF exposure.

The evidence of teratogenicity of CPF in humans includes a case-control study by Rull et al., (2006), which examined the association between CPF exposure, as assessed by maternal residential proximity to specific agricultural pesticide applications, and NTD such as anencephaly and spina bifida. Although CPF exposure was associated with NTD in initial analyses, this association was not signficant in subsequent analyses which controlled for exposures such as other pesticides. This study was limited in the precision of its exposure measure. A case report by Sherman (1996) described many birth defects in four children who were reportedly exposed to Dursban during pregnancy; however, very little specific exposure information was presented. Another case report (Sebe et al., 2005) described a fetal death following ingestion by the mother of an excess amount of CPF in a commercial product. The actual quantity of CPF ingested by the mother was not known but high levels of CPF were detected in the fetal blood. The difficulty concerning case reports include the lack of a comparison group and generally the lack of specific exposure information, frequently including exposure to the chemical as well as to other potential covariates. Lastly, in a related study by Nielsen et al., (2005), results suggested that having an inefficient PON1 promoter allele is associated with an increased risk of childhood brain tumors.

In summary, studies reviewed in this document found adverse outcomes associated with exposure to CPF, including lower birth weight, decreased birth length, decreased head circumference associated with PON1 status, mental and motor delays and behavioral problems, as well as DNA damage. Although the results for certain outcomes were not in agreement across some of the studies, factors discussed above may account for some of the inconsistencies.

Table C45. Epidemiologic Studies of Developmental Outcomes from the Three Cohorts

Study	Population	Exposures/ Outcomes	Results	Comments	Study Type
Studies reporting on fetal growth					
Berkowitz et al., 2004	Mount Sinai Children's Environmental Health Center, New York City N = 404	TCPY, classified above and below LOD Birth weight, length, head circumference and gestational age Considered PON1 activity	No association between questionnaire data or metabolite levels and any outcomes. With maternal PON1 levels (but not PON1 polymorphisms) there was a small but significant reduction in head circumference.	TCPY (uncorrected for creatinine) median = 7.6 μg/L (interquartile range = 1.6–32.5) Creatinine corrected median = 11.5 μg/g (interquartile range = 1.8–35.4) TCPY LOD = 11.0 μg/L (57% of values were below LOD) Urinary concentrations were corrected for creatinine and expressed as μg/g creatinine	Prospective Cohort
Whyatt et al., 2005 Previous publications from this cohort but with smaller sample size (Whyatt 2002, 2003, 2004, and Perera 2003)	Columbia Center for Children's Environmental Health, New York City N = 571	Air sampling – 2 consecutive days between 1998 and 2002 CPF in umbilical cord blood (drawn as close to delivery as possible) CPF in maternal blood (sampled within 2 days of delivery) Birth weight, birth length, and head circumference	Association between plasma CPF and decreased birth weight and birth length. Birth weight decreased by 67.3 g (95% CI -116.6 to -17.8 g; p=0.008) and birth length decreased by 0.43 cm (95% CI -0.73 to 0.14 cm; p=0.004) for each unit increase in log transformed cord plasma CPF. No association between birth weight and length and cord blood CPF among newborns born after 1/1/01 when CPF was banned for residential use.	CPF LOD = 1 pg/g (Barr, 2002) CPF in umbilical cord blood mean = 3.7 pg/g ± 5.7 (range 0.3–63) CPF in maternal blood mean = 3.9 pg/g ± 4.8 (range 0.3–35)	Prospective Cohort
Eskenazi et al., 2004	CHAMACOS Study (Center for the Health Assessment of Mothers and Children of Salinas) Salinas Valley, California N=488	TCPY, DAP metabolites in maternal urine during pregnancy Birth weight, birth length, ponderal index, head circumference, and length of gestation	No associations were observed between TCPY and any of the outcomes measured.	TCPY median = 3.3 μg/L (0.2–56.1) LOD = 0.26 μg/L	Prospective Cohort

Table C45 (Continued). Epidemiologic Studies of Developmental Outcomes from the Three Cohorts

Study	Population	Exposures/ Outcomes	Results	Comments	Study Type
Studies reporting on neurodevelopment					
Rauh et al., 2006	Same cohort as Whyatt et al., 2005 N=254	Bayley Scales of Infant Development II (provides a Mental Development Index and a Psychomotor Development Index) CBCL CPF in umbilical cord blood (drawn as close to delivery as possible) CPF in maternal blood (sampled within 2 days of delivery)	In highly exposed children there were statistically significant negative effects of CPF with mental delay 36 months (MDI) – OR = 2.37 (1.08 – 5.19) and psychomotor delay at 36 months – 4.52 (1.61–12.70) . Behavioral Disorder at 36 months – for attention problems 11.26 (1.70–70.99), ADHD 6.50 (1.09–38.69), PDD Problems 5.39 (1.21–24.11).	Although the PDI and MDI scores between the higher and lower exposed group differed by only 7.1 and 3.0 points, the proportion of delayed children in the highly exposed group was 5 times greater for the PDI and 2.4 times for the MDI.	Prospective Cohort
Eskenazi et al. 2007	Same cohort as Eskenazi et al., 2004 N=396	Bayley Scales of Infant Development II (provides a Mental Development Index and a Psychomotor Development Index) CBCL Maternal and child TCPY DAP	No associations were observed between TCPY levels and MDI or PDI scores, or with CBCL outcomes.	Associations were observed between DAP and neurodevelopmental outcomes	Prospective Cohort

C.4.2. Animal data

A summary table (Table C44) of data from animal studies of developmental toxicity is provided at the end of this section.

There have been several studies that have focused on the potential embryotoxicity and teratogenicity of gestational exposure to CPF (Deacon et al., 1980; Muscarella et al., 1984; Muto et al., 1992; Breslin et al., 1996). However, only recently has attention turned to the possibility that exposure to CPF during the gestational period may result in neurochemical alterations after birth and resultant behavioral effects. Hence, while the results on the teratogenicity studies are equivocal, given the proposed modes of action for this chemical, other end points of development may be relevant. Several recent studies suggest that CPF affects relatively late events in brain development, centered around the proliferation, differentiation, and functioning of glial cells (Garcia et al., 2001; Qiao et al., 2001), and the cells that provide metabolic support for neurons and those that guide axons to their proper targets within the developing central nervous system. These findings raise the issue of identifying the critical window for adverse effects of CPF on neurodevelopment. Typically the studies conducted per guidelines for regulatory agencies have focused on frank malformations and not on behavioral or other developmental milestones noted in rodents after birth. If late-occurring processes are involved, then in addition to correlating times of development in rodents with analogus periods in humans, vulnerability may extend into childhood, a period in which exposures may be particularly high (Fenske et al., 1990; Gurunathan et al., 1998; Landrigan et al., 1999).

Since the behavioral deficits noted in the studies by Levin (2002) and Icenogle (2004) were seen long after immediate cholinergic responses, the behavioral effects may be mediated via a non-cholinergic mode of action. Accordingly, prenatal CPF appears to elicit delayed-onset alterations, disrupting the "program" for the emergence of cholinergic activity. The functional significance of the later-occurring neurochemical anomalies is corroborated by behavioral deficits in cholinergic contributions to working and reference memory that emerge in adolescence and adulthood after fetal CPF exposure (Levin et al., 2002) and the same pattern is elicited by prenatal exposure to nicotine (Zahalka et al., 1992). Researchers therefore speculate that these long-term alterations reflect disruption consequent to elevated cholinergic activity during a critical period in fetal development. In addition to inhibiting ChE, CPF, like nicotine, interacts directly with nicotinic cholinergic receptors (Katz et al., 1997), such that exposures that do not cause significant ChE inhibition might still affect cholinergic signaling. Regardless of the underlying mechanism, prenatal exposures to CPF that are otherwise nontoxic elicit deficits in cholinergic function that influence cognitive performance in adolescence and adulthood.

Table C46. Data from animal developmental toxicity studies.

STUDY (SPECIES)	EXPOSURE DOSES	FINDINGS	NOEL
Teratology Range-finder (Ouellette et al., 1983) Fischer rats (Oral)	On days 6-15 of gestation 0, 3, 10, or 30 mg/kg/day (PartI) 0, 15 mg/kg/day (Part II)	Part I – 4 dams died at high dose. Cholinergic signs noted also at 15 mg/kg/day	2-day NOEL for cholinergic signs = 10 mg/kg/day
Teratology Fischer 344 Rats (Ouellette et al., 1983) (Oral)	On days 6-15 of gestation 0, 0.1,3, or 15 mg/kg/day	Cholinergic signs in dams at 15 mg/kg/day. No developmental toxic effects.	9-day maternal NOEL for decreased red blood cell ChE and plasma ChE activity = 0.1 mg/kg/day.
CD1-rats (Rubin et al., 1987) (Oral)	0, 0.5, 2.5, or 15 mg/kg/day on days 6-15 of gestation	Dose-related plasma ChE at all doses. Non dose-related slight increase in early resorptions at 15 mg/kg/day.	Decreased maternal body weight and cholinergic signs (tremors) at high dose of 15 mg/kg/day. Developmental NOEL = 2.5 mg/kg/day
CrlCD®(SD)BR VAF/Plus® rats (Hoberman, et al., 1998) (Oral)	0, 0.3, 1 and 5 mg/kg/day on gestation day 6 through lactation day 11	Clinical signs at 5 mg/kg/day, brain ChE inhibited at 1 mg/kg/day	Developmental LOEL = 1 mg/kg/day* based on morphometric effects noted decreased neonatal survival, decreased pup growth and delayed maturation (several endpoints) at 5 mg/kg/day
Sprague-Dawley Rats (Mattson et al., 1998) (Oral)	0, 0.3, 1.0, 5 mg/kg/day on gestation days 6- lactation day 10	On all PNDconcentration of CPF in milk was greater than in blood of dams.	Pup NOEL for brain ChE inhibition = 1 mg/kg/day
CF-1 Mice – Oral (Deacon et al., 1979)	0, 0.1, 1, 10, 25 mg/kg/day on days 6-15 of gestation	Cholinergic signs at 10 mg/kg/day Decrease in plasma ChE at 1 mg/kg/day Decrease in red blood cell ChE at 10 mg/kg/day	10 mg/kg/day (developmental) based on decrease in fetal length and weight, delayed ossification of skull and sternebrae at 25 mg/kg/day

* In the absence of data at the lower dose of 0.3 mg/kg/day

Table C46 (Continued). Data from animal developmental toxicity studies.

Sprague-Dawley Rats (Subcutaneous) (Chanda et al., 1995)	0 or 200 mg/kg/day single dose on day 12 of gestation	Terminated on day 16, or day 20, or PND 3. Maternal cholinergic signs detected 2-3 days after dosing and inhibition of maternal brain ChE noted at day 16, day 20, and PND 3 (2 weeks after dosing). Treated pups cross-fostered to untreated dams exhibited 30% inhibition of brain AChE activity. Untreated pups cross fostered to treated dams showed 23% reduction in brain AChE. Hence, it appears that CPF concentrates in milk and is taken up in the brain. Behavioral changes (no cliff avoidance, in treated pups on PND 3 and transient surface righting reflex on PND 1.
Sprague-Dawley Rats (Subcutaneous) (Chanda and Pope, 1996)	6.25, 12.5 or 25 mg/kg/day daily on days 12-19 of gestation	Animals sampled on gestation day 16, 20 and PND 1 and PND 3. Brain AChE was inhibited in both dams and fetuses on gestation day 20 in a dose-related manner, with increased inhibition in dams. Brain AChE was also reduced significantly in control pups cross-fostered with treated dams and in treated pups cross-fostered to untreated dams on PND 3. A dose-related down-regulation of muscarinic receptors was also observed on GD 20 and also on PND 3. A significant increase in righting reflex time and a decrease in percent cliff avoidance on PND 3 was noted in pups at the 25 mg/kg/day group whether cross-fostered with control dams or not.
Developmental Neurotoxicity in (SD) Rat Pups. Maurissen et al., 2000	Gestation day 6 – Lactation day 10 at doses of 0, 0.3, 1 or 5 mg/kg by oral gavage	In spite of apparent delay in physical development, learning and memory as tested on T-maze spatial alternation tasks, motor activity and auditory startle were not affected in the high dose animals. On GD 20, brain AChE of the 1 mg/kg and 5 mg/kg groups were 82% and 10.2% of the controls.
Cognitive function and cholinergic neurochemistry in weanling rats (Long Evans) Pre-weaning: (17-20 animals per group) Post-weaning: (7-8 animals per group) Jett et al., 2001	Preweaning: Exposed on postnatal days 7, 11, 15 and tested on postnatal days 24-28; Postweaning: Exposed on postnatal days 22 and 24 and tested on postnatal days 24-28 Exposure was via subcutaneous injection	Pre-weaning: Spatial navigation acquisition appears to be affected at 7 mg/kg on day 1 of testing and also during the rest of the testing days. Hence, pattern of learning may not be affected, but a diminished effect on day 1 of testing was statistically significant. Post-weaning: Spatial navigation acquisition was not significantly different from controls on day 1 of testing, but on day 5 of testing there was a significant difference between controls and both exposure groups (0.3 mg/kg and 7 mg/kg). However, group over time was not significant. But, the lack of day effect is because both the exposure groups did not vary over time and were consistently slow to learn the spatial navigation. Hence, while rate of learning per se may not be affected, the probe test is suggestive of either memory loss or impaired learning on day 5 (effects on cognition)

D. Female Reproductive Toxicity

Few human studies have examined the effects of CPF on the female reproductive system. These have primarily concentrated on the detection of CPF on different tissues/fluids in studies of exposure to CPF along with other pesticides in humans. No studies of human female reproductive function were identified.

Animal studies include those conducted for regulatory purposes as reproduction studies per FIFRA guidelines. Studies from the peer-reviewed literature include those involving lactational exposure and the effects observed from the presence of CPF in the milk.

D.1. Human Female Reproductive Studies

D.1.1. Individual studies

Wagner et al., 1990. Detection of phosphate ester pesticides and the triazine herbicide "Atrazine" in human milk, cervical mucus, follicular- and sperm fluid.

This investigation examined atrazine, CPF, and chlorfenvinphos in human milk, cervical mucus, follicular and sperm fluid in 41 specimens. It was not specified from what population the specimens were collected. Chlorpyrifos was detected in one of 11 samples of human milk (0.436 µg/kg), in three of 11 samples of cervical fluid (6.83 µg/kg, 1.80 ug/kg, and 0.62 µg/kg), and in one of 10 samples of sperm fluid (0.50 µg/kg). No CPF was detected in follicular fluid any of the 10 samples. The authors expressed surprise at finding pesticides in cervical mucus, which showed the highest levels, since it is more than 95% water with a high part of mucin-like proteins.

Sanghi et al. 2003. Organochlorine and organophosphorus pesticide residues in breast milk from Bhopal, Madhya Pradesh, India.

This study assessed status of human exposure to certain OP and organochlorine persticides by measuring their concentrations in breast milk. Samples of breast milk were collected from 12 women in Bhopal, India. Women were 19-45 years of age and of lower socioeconomic class. Among the pesticides measured in breast milk mean endosulfan concentrations were the highest (0.363 mg/L, range 0-0.763 mg/L) followed by CPF (0.230 mg/L, range 0.085-0.355 mg/L, CPF was detected in all 12 samples). Malathion, methyl-parathion and isomers of hexachlorocyclohexane were also detected in breast milk.

D.2. Animal Female Reproductive Toxicity Studies

D.2.1. Fertility

Dietz et al ,1983
Dursban Insecticide: Assessment of Neonatal Survival in a Two-Generation Reproduction Study in Rats.

The purpose of this study was to evaluate the potential effects of Dursban (a trademark of The Dow Chemical Company) insecticide on the reproductive capability, and neonatal growth and survival of rats fed the test material over two generations. Particular emphasis was placed upon evaluating effects of treatment on neonatal survival since equivocal results from a previous reproduction study (Thompson et al., 1971) have been interpreted as demonstrating a treatment-related increase in neonatal mortality. Male and female Sprague-Dawley rats, approximately 6 weeks of age, were fed diets providing 0, 0.5, 0.8, or 1.2 mg Dursban insecticide/kg-day for 135 days and then bred to produce F1 litters. On day 21 of lactation, pups for the following generation (F1) were randomly selected and the remaining pups were sacrificed. The F1 weanlings were dosed in a similar fashion for 120 days prior to their breeding to produce the F2 litters. These pups were also weaned on day 21 of lactation. All adult animals were sacrificed at the termination of each generation. No overt signs of toxicity were observed in any animals throughout the study. Treatment-related effects noted in the adult generations were limited to a decrease in body weights during the post-mating period for F1 males. These effects were generally limited to the 1.2 mg/kg-day F1 group, with slight effects observed at 0.8 mg/kg-day and no significant effects noted at 0.5 mg/kg-day. Adult food consumption was unaffected by the administration of Dursban insecticide at all three dose levels. No adverse effects on fertility, reproduction, or neonatal growth were observed among animals exposed to the test material for two generations. In addition, the dietary administration of up to 1.2 mg Dursban insecticide/kg-day did not adversely affect the survival of pups from F1 or F2 litters during the lactation period. In conclusion, these data demonstrate that the dietary ingestion of 0.5, 0.8, or 1.2 mg Dursban insecticide/kg/day by male and female rats for two successive generations did not result in any adverse effects on the ability of rats to mate, reproduce, or nurse their pups. Neonatal growth and survival were also unaffected by administration of the test material.

D.2.2. Reproductive Organ Toxicity

James et al., 1988.
The effect of Pyrinex (chlorpyrifos) on reproductive function of two generations in the rat.

Summary provided by the CDPR. Crl:CD7(SD)BR rats received diets containing 0, 2, 10, or 50 ppm CPF (95% purity) in diets over 2 generations (1 litter per generation).

Parental rats numbered 28/sex/group in the F0 generation, and 24/sex/group in the F1 generation. Protocol was that of a standard reproduction study, with a few preweaning developmental evaluations added (surface righting, air righting, and startle responses; and pupil reflex). There were no definitive treatment-related effects (report attributes 3 high dose deaths to treatment; however, there were deaths in other groups and no evident unique symptoms in high dose decedents).

Breslin, 1991.
Two-generation dietary reproduction study in Sprague-Dawley rats.

This study was conducted to meet with regulatory agency guidelines and the data were submitted to the CDPR around 1991. The findings were subsequently published in the open literature and have been discussed earlier in the document (Section C.2.2)

Chlorpyrifos (97.8% pure) was administered in the diet at dosages of 0, 0.1, 1.0 or 5.0 mg/kg-day to Sprague-Dawley rats (30 sex/group) 7 days/week for two generations. This study was submitted for regulatory purposes and was acceptable under FIFRA. The parental generation (F0) was exposed for 10 weeks prior to mating, and the F1 adults were dosed 12 weeks prior to mating. No adverse reproductive effects were observed, though F1 pup weights were significantly ($P<0.05$) reduced (10–11%) at the highest dosage. Parental effects included decreased plasma and erythrocyte ChE at 1.0 mg/kg-day, and decreased plasma, erythrocyte, and brain ChE and histopathologic alterations of the adrenal zona fasciculata at 5.0 mg/kg-day. The histopathologic alterations of the adrenal were characterized as very slight to slight vacuolation (consistent with fatty change) in males, and very slight vacuolation and/or altered tinctorial properties in females. No effects on the reproductive or fertility indices or on the histopathology of reproductive tissues were observed at any dose level, and no neonatal effects were observed at 0.1 or 1.0 mg/kg-day in the F1 or F2 litters. Parental toxicity at the high dose was accompanied by decreased pup body weight and increased pup mortality in the F1 litters only. These data show that oral administration of CPF to rats at parentally toxic dose levels was not embryolethal, embryo/fetotoxic, or teratogenic and did not adversely affect fertility or the function or structure of the reproductive organs. According to the authors, although effects on neonatal growth and survival were observed at a maternally toxic dose level in one generation, this effect was not observed in the subsequent generation and may not have been related to treatment.

D.3. Integrative Evaluation

According to generally accepted principles, changes in offspring growth or milk quantity and quality may constitute a female-specific endpoint of reproductive toxicity (U.S. EPA, 1996). Hence, the presence of CPF in the milk may be considered as affecting lactation, an essential component of female reproduction.

Of the two studies conducted on human samples, both found evidence of CPF in breast milk. The study by Wagner et al. detected CPF in only one sample of 10; however; since this study was conducted in 1990 it is possible that their limit of detection was higher than that of Sanghi et al. in which the stated LOD was 0.01 mg/kg. The latter study calculated the intake of CPF in infants weighing 2.9 kg and consuming 500 mL of milk daily would be 0.041 mg/kg per day, which is 4.1 times greater than that recommended by the Food and Agricultural Organization of the World Health Organization (WHO, 2006). On the whole, the animal data both from studies conducted for regulatory purposes per FIFRA guidelines as well as those from the peer-reviewed literature demonstrate lactational exposure and the effects observed from presence of CPF in the milk. Reproductive effects included reduced pup weights and increased pup mortality in rats, but only at dose levels that induced parental toxicity as evidenced by inhibition of plasma, RBC and brain cholinesterase activities as well as histological lesions of the adrenal gland (vacuolation of cells of the zona fasciculata).

E. Male Reproductive Toxicity

Several studies have examined the effects of CPF on the male reproductive system. These have primarily concentrated on the detection of effects of CPF on semen quality and male reproductive hormones in studies of exposure to CPF along with other pesticides in humans. Animal studies include those conducted for regulatory purposes as reproduction studies per FIFRA guidelines and from the peer-reviewed literature.

E.1. Human Male Reproductive Studies

E.1.1. Overview

Six epidemiologic studies were identified that examined CPF exposure and various outcome parameters of male reproductive toxicity, including one case control study (Swan et al., 2003) and five cross-sectional studies, all conducted in the same sample of men (Meeker et al., 2004a; Meeker et al., 2004b; Meeker et al., 2006a; Meeker et al., 2006b; Meeker et al., 2008). In addition to studies which assessed exposure specifically to CPF, two recent related studies are also presented (Perez-Herrera et al., 2008; Salazar-Arredondo et al., 2008). These two studies are an in vitro study and a study of exposure to multiple pesticides in which the interaction with PON1 data was also examined.

E.1.2. Individual studies

Swan et al., 2003.
Semen Quality in Relation to Biomarkers of Pesticide Exposure

In this case-control study the authors investigated the hypothesis that pesticides currently used in agriculture in the Midwest contributed to differences observed in semen quality between men from U.S. urban centers and a U.S. agrarian area. Subjects were chosen from men who had participated in the Study for Future Families, a multi-centered study of semen quality in fertile men. Men from the Missouri and Minnesota centers were chosen since, despite large difference in semen quality, they were comparable demographically and with respect to most risk factors for impaired semen quality. Cases were men for whom the average (abstinence-time adjusted) sperm concentration was below the population median, while controls included men for whom the average was above the median. Men were selected who had few or no risk factors for impaired semen quality; therefore, the sample included men 21–40 years of age, white, nonsmokers, with no history of infertility, sexually transmitted diseases (STDs), or fever in the three months preceding sample collection. As there were not enough men negative for these risk factors who met the criterion for low (or high) sperm concentration, 17 additional men with one or more risk factors were added. However, the authors note that as most men who were positive for one or more risk factors were controls, any bias should be in the direction of underestimating differences between cases and controls. Men whose sperm concentration was below the population median were selected as cases, while men whose sperm concentration was above the median were considered as controls.

In the initial analysis, the case group was formed by 30 men with the lowest sperm concentration while the control group included 30 men with the highest concentration. Of these 60 men, 24 were from Missouri and 36 were from Minnesota. To increase the power of the study to examine pesticide levels in relation to semen quality within Missouri, additional Missouri subjects were selected (4 cases and 22 controls) to achieve a total of 50 men (25 cases and 25 controls) within Missouri. A total of 36 men were from Minnesota (9 cases and 27 controls). Pesticide metabolite levels were measured in urine samples provided at the time of semen collection (including alachlor, atrazine, diazinon, 2,4-D, metolachlor, N,N-diethyl-m-toluamide (DEET), malathion, carbaryl, methyl parathion, para-aminophenol, acetachlor, CPF and carbofuran). Pesticide levels were categorized into low, medium, and high when at least 15 men had values above the LOD. Otherwise, two categories were used, low or high, defined as below the LOD, and greater than or equal to the LOD, respectively.

Recruitment rates were equal (27%) for the men in both Missouri and Minnesota. Alachlor, atrazine and diazinon were associated with decreased semen quality in Missouri subjects. The populations were similar on the basis of ethnicity, smoking, and several variables associated with decreased semen quality (history of infertility or STDs and fever in the 3 months before semen collection). Although the selection of cases was

initially based on sperm concentration, all semen parameters differed significantly between cases and controls, with the cases having poor semen quality and values for controls all being in the normal range (WHO, 1999). TCPY was found above the LOD (0.1 μg/g creatinine) for 86% of subjects in Minnesota and 50% of those in Missouri; however, the levels were unrelated to case-control status. The OR for low semen quality (sperm concentration, motility and morphology below the population median) associated with levels of TCPY $\geq$ 0.5 was elevated for men from Missouri, (OR = 6.4), however, the confidence interval encompassed one (95% CI, 0.5 to 86.3), while for the men from Minnesota the OR was 0.5 with the confidence intervals again encompassing one (95% CI, 0.04 to 8.3) (Table E1). Elevated ORs were observed for other pesticide levels, however, only those in the Missouri sample of men were significant (alachlor, IMPY, and atrazine). Further analyses were conducted on a number of pesticides; however, TCPY was not included.

Table E1. ORs for low semen quality for men exposed to elevated pesticide levels.

	Missouri				Minnesota			
Pesticide	Level (µg/g creatinine)	Cases	Controls	OR (95% CI)	Level (µg/g creatinine)	Cases	Controls	OR (95% CI)
Alachlor	<0.15	3	15	Reference	<0.15	6	21	Reference
	0.15–0.7	10	8	6.3 (1.3–29.4)	≥0.15	3	6	1.8 (0.3–9.2)
	>0.7	12	2	30.0 (4.3–210)				
IMPY	<0.1	6	20	Reference	<0.1	3	12	Reference
	0.1–3.0	9	3	10.0 (2.0–49.2)	0.1-3.0	3	9	1.3 (0.2–8.2)
	>3.0	10	2	16.7 (2.8–98.0)	>3.0	3	6	2.0 (0.3–13.1)
Atrazine	<0.1	17	24	Reference	<0.1	9	25	
	≥0.1	8	1	11.3 (1.3–98.9)	≥0.1	0	2	
Metolachlor	<0.15	5	11	Reference	<0.15	5	17	Reference
	0.15–0.3	11	8	3.0 (0.7–12.2)	≥0.15	4	10	1.4 (0.3–6.3)
	>0.3	9	6	3.3 (0.8–14.5)				
2,4-D	<0.1	20	19	Reference	<0.1	9	27	
	≥0.1	5	6	0.8 (0.2–3.0)	≥0.1	0	0	
1-Naphthol	<1.5	9	2	Reference	<2.0	3	8	Reference
	>1.5	12	1	2.7 (0.2–34.2)	2.0-4.0	2	4	1.3 (0.2–11.5)
					>4.0	4	15	0.7 (0.1–4.0)
3,5,6-Trichloropyridinol (TCPY)	<0.5	5	2	Reference	<1.25	1	3	Reference
	≥0.5	16	1	6.4 (0.5-86.3)	1.25–5.0	2	11	0.5 (0.04–8.3)
					>5.0	6	13	1.4 (0.1–16.2)
4-Nitrophenol	<0.1	20	3		<0.1	5	14	Reference
	≥0.1	1	0		≥0.1	4	13	0.9 (0.2–3.9)

The authors put forth that these data support the hypothesis that pesticides used commonly in mid-Missouri, but rarely in urban counties, contributed to the poor semen quality in men from Columbia relative to men from Minneapolis. Several pesticides were found to be associated with decreased semen quality, particularly alachlor, atrazine, and diazinon.

There were no actual values given for the concentrations of TCPY measured in the blood samples of subjects, therefore it is difficult to determine at what levels the subjects were exposed to the pesticide. In addition, TCPY, unlike alachlor, atrazine, and diazinon, was detected more often in subjects from Minnesota. Limitations of the study include the low participation rate (27%), although it is comparable to similar studies. The small number of subjects, especially the number of cases in Minnesota, limits the statistical power of the study. In addition, as ascertained from Table E1, small numbers of cases and controls were used to calculate the ORs for TCPY in subjects from Missouri (21 cases and 3 controls), even smaller than those used for alachlor, IMPY, and atrazine (25 cases and 25 controls).

Studies by Meeker et al.

The studies conducted by Meeker et al., (Meeker et al., 2004a; Meeker et al., 2004b; Meeker et al., 2006a; Meeker et al., 2006b; Meeker et al., 2008) are from the same sample of men and have many commonalities; thus, a brief description of the population and methodology that applies to all five studies will be presented first.

The five cross-sectional studies investigated various parameters of male reproductive outcomes and toxicity in association with exposure to CPF. Chlorpyrifos exposure was measured, as urinary TCPY, in men who were partners in sub-fertile couples seeking infertility diagnosis from an andrology clinic at Massachusetts General Hospital in Boston from January 2000 to April 2003. Approximately 65% of those approached to participate in the study consented to do so, with most men who declined citing lack of time on the day of their clinic visit as the reason. Men whose urine samples had specific gravity (SG) values outside the acceptable range ($1.01 < SG < 1.03$) were excluded from the primary analyses. However, sensitivity analyses were performed in which men with samples having SG outside the acceptable range were retained, and results were found to be similar in all instances. Specific gravity-adjusted and creatinine-adjusted concentrations of TCPY and the carbaryl metabolite 1N, also studied, were log-transformed. To improve interpretability, the regression coefficients were back transformed and expressed as a change in the dependent variable for an interquartile range (IQR) increase in insecticide levels. In addition, in all but one article, non-linear relationships between TCPY and 1N concentrations and the outcome measure of interest were assessed by regressing the latter on quintiles of insecticide concentrations.

Meeker et al., 2004a. Urinary levels of insecticide metabolites and DNA damage in human sperm.

This study examined the association between exposure to CPF and carbaryl and DNA damage in human sperm. A total of 368 men were recruited, and a semen sample was collected from each subject on the same day as a urine sample was collected for analysis of TCPY. Comet assay parameters and urinary insecticide metabolite concentrations were available for 260 men. The comet assay was not performed on 74 men for the following reasons: comet assay parameters were not available for the first 46 men recruited into the study because the study initially did not archive semen sample for comet assay analysis; 19 men were azoospermic (had no sperm cells available for the comet assay); and nine samples were lost in processing. In addition, insecticide metabolite levels were not available for 34 men.

The mean age of the remaining 260 men was 36.1 years, with 82% being white, 5% African-American, 6% Hispanic, and 6% Other. Nine% were current smokers. The geometric mean values for urinary TCPY were 2.03 μg/l for unadjusted, 2.21 μg/L for specific gravity-adjusted, and 1.71 μg/g for creatinine-adjusted, with the unadjusted and creatinine-adjusted distributions being very similar to the distributions from the National Health and Nutrition Examination Survey data, 1999-2000 (CDC, 2003). The LOD for TCPY was 0.25 μg/l; 92.7% of the samples were above the LOD. Forty-six men were excluded from the primary data analysis as their SG values were too concentrated (> 1.030) or too dilute (<1.010).

Sperm DNA integrity was assessed by neutral comet assay and reported as comet extent, percentage DNA in comet tail (Tail%), and tail distributed moment (TDM). As presented by the authors, "the comet assay relies on the electrophoretic movement of DNA out of single cells, in which low molecular fragments migrate away from the cell towards the positive electrode in a pattern resembling a comet tail". As presented in the paper "comet extent is a measure of total comet length from the beginning of the head to the last visible pixel in the tail. Tail% is a measurement of the proportion of total DNA that is present in the tail. TDM is an integrated value that takes into account both the distance and intensity of comet fragments." Cells with high DNA damage (CHD), which are cells too long to measure with VisComet software (>300 μm), were counted for each subject and used as an additional measure of DNA damage.

After adjusting for age and smoking status, the final multiple regression models showed that an IQR increase in urinary TCPY was associated with: 1) a significant increase in Tail% (2.8; 95% CI 0.9 to 4.6; p = 0.004); 2) a significant decrease in TDM (-2.5; 95% CI -4.7 to -0.2; p = 0.03); and 3) an insignificant decrease in comet extent (-2.7 μm; 95% CI -8.1 to 2.7) (Table E2). These coefficients represent the change in the specific comet parameter for an IQR change in insecticide metabolite. No significant association was observed between the number of arcsine-transformed CHD and TCPY. A borderline significant interaction was seen between smoking status and SG-adjusted ln(TCPY) (p=0.06). In the stratum-specific analyses for current smokers, SG-adjusted TCPY was inversely and significantly related to TDM (coefficient = -8.42 μm for an IQR increase in TCPY; p=0.01). For non-smokers the relationship was not statistically significant (coefficient = -1.64 μm for an IQR increase in TCPY; p=0.18). The interaction terms

between current smoking status and TCPY for comet extent and Tail% did not approach statistical significance ($p > 0.2$).

A negative correlation between Tail% and TDM was present only when stratified by comet extent. Therefore, it was suggested that these two parameters may be measuring different types of DNA damage within comet extent strata.

Table E2. Adjusted regression coefficients[a,b] for comet parameters associated with an interquartile range (IQR)[c] increase in SG-adjusted urinary insecticide metabolite concentrations, n=214[d]

	Percentage DNA tail (%)		Comet extent (μm)		Tail distributed moment (μm)	
	Estimate (95% CI)	P-value	Estimate (95% CI)	P-value	Estimate (95%CI)	P-value
TCPY	2.76 (0.89 to 4.62)	0.004	-2.72 (28.13 to 2.68)	0.3	-2.47 (-4.71 to -0.22)	0.03
1N	4.13 (1.92 to 6.32)	0.0003	-0.92 (27.39 to 5.55)	0.8	-2.18 (-4.88 to 0.50)	0.1

[a] Adjusted for age and smoking status.
[b] Regression coefficients represent the change in comet parameter for an IQR change in insecticide metabolite concentration (0 indicates no change in semen parameter for an IQR change in insecticide metabolite concentration, while a coefficient <0 indicates a decrease in comet parameter for an IQR change in insecticide metabolite concentration and a coefficient >0 indicates an increase in comet parameter for an IQR change in insecticide metabolite concentration).
[c] TCPY IQR = 1.28–4.74 μg/l; 1N IQR = 1.38–4.70 μg/l.
[d] Forty-six samples excluded with specific gravity >1.03 or <1.01.

Concentrations of 1N were significantly associated with increased Tail%, but also associated with a suggestive decrease in TDM.

The study found that increased urinary TCPY was associated with increased Tail% but also with decreased TDM. The significant interaction suggests that the inverse association between TDM and SG-adjusted TCPY concentration was stronger in current smokers than in current non-smokers. As noted by the authors, it is not clear which comet assay parameter is the most relevant measure of DNA damage in sperm; however, the percentage of DNA in the comet tail (Tail%) has been shown to be proportional to the frequency of DNA strand breaks (Olive et al., 1990). The authors also have hypothesized that the different comet assay parameters may reflect different mechanisms and types of DNA damage, such that double strand breaks "may lead to small DNA fragments that migrate large distances from the comet head but are of low intensity (contain a small amount of DNA). This in turn will have a larger influence on TDM, which is an integrative measure of distance multiplied by intensity, as compared with Tail % which is the proportions tail to head intensity."

The paper concludes that the significant associations found between urinary metabolites of these insecticides and Tail% suggest that environmental exposure to carbaryl and CPF may be related to increased DNA damage in human sperm. However, the need for future studies was noted to elucidate the inconsistent results between the different measures of DNA damage obtained by the neutral comet assay.

Meeker et al., 2004b. The relationship of urinary metabolites of carbaryl/naphthalene and CPF with human semen quality.

This study investigated the association between exposure to CPF and carbaryl and altered semen quality. The study included 272 of the subjects recruited from the infertility clinic. Semen quality was assessed as sperm concentration, percent motile sperm, and percent sperm with normal morphology, along with sperm motion parameters (straight-line velocity, curvilinear velocity, and linearity). Median urinary TCPY and 1N concentrations were 3.22 and 3.19 µg/L, respectively. These levels of TCPY were slightly higher than those seen in NHANES 1999-2000 (CDC, 2007).

Potential covariates considered include smoking status, race, body mass index, a previous exam for infertility, age, and abstinence time; with the last two being included in the regression models.

The authors reported "suggestive" associations between SG-adjusted TCPY with sperm concentration (adjusted OR = 1.0, 2.1, 2.4; p-value for trend = 0.09) and sperm motility (adjusted OR = 1.0, 1.6, 1.7; p-value for trend = 0.09) (Table E3). Compared with men in the lowest TCPY tertile, men in both the medium and high tertiles were more likely to have below-reference sperm concentration and sperm motility. The estimates, however, for the second and third tertiles suggest that the dose-response relationship was not monotonic. Sperm morphology was "weakly" associated with TCPY concentrations (OR = 1.0, 1.2, 1.9; p-value for trend = 0.10). Point estimates for the association between TCPY quintiles and below reference sperm concentration, motility and morphology were > 1.0; however, none of these were statistically significant.

Table E3. Adjusted ORs[a] (95% CIs) for SG-adjusted metabolite tertiles (n = 272)[b].

	Comparison subjects (n = 130)	Sperm concentration (<20 million/mL; n = 35)		Sperm motility (<50% motile; n = 119)		Sperm morphology (<4% normal; n = 59)	
		No.[c]	OR (95% CI)	No.[c]	OR (95% CI)	No.[c]	OR (95% CI)
1N[d]							
Low	53	5	1.0	27	1.0	20	1.0
Medium	39	14	4.2(1.4–13.0)*	45	2.5(1.3–4.7)*	17	1.4(0.6–3.0)
High	38	16	4.2(1.4–12.6)*	47	2.4(1.2–4.5)*	22	1.6(0.8–3.5)
p-Value for trend			0.01		0.01		0.20
TCPY[e]							
Low	52	8	1.0	33	1.0	17	1.0
Medium	39	12	2.1(0.8–5.6)	40	1.6(0.8–3.0)	16	1.2(0.5–2.7)
High	39	15	2.4(0.9–6.3)	46	1.7(0.9–3.2)	26	1.9(0.9–4.0)
p-Value for trend			0.09		0.09		0.10

[a]ORs adjusted for age and abstinence time. [b]Excluded 58 subjects with SG > 1.03 or < 1.01. [c]Number of subjects in each exposure tertile with below-reference semen parameters. The semen parameter categories were not mutually exclusive; a man could contribute data to one, two, or all three of the below-reference groups. [d]SG-adjusted 1N tertiles: low, < LOD to 2.36 µg/L; medium, 2.36-4.02 µg/L; high, 4.02-159.7 µg/L. [e]SG-adjusted TCPY tertiles: low, < LOD to 2.30 µg/L; medium, 2.30-4.42 µg/L; high, 4.42-40.7 µg/L. *p<0.05.

Multivariate linear regression analyses for computer-aided semen analysis (CASA) motion parameters showed significant inverse association between SG-adjusted TCPY and straight-line velocity (VSL) (-1.21 (95% CI -2.34 to -0.08, p<0.05) and linearity (LIN) -1.07 (95% CI -1.90 to -0.24, p<0.05)) (Table E4). The association with LIN became insignificant when examined by tertiles of TCPY while the association with VSL remained (0, -0.13, -2.79; p-value for trend = 0.05). Significant associations were seen between 1N exposure and below-reference sperm concentration, motility, and VSL.

Table E4. Adjusted regression coefficients[a,b] for a change in semen parameters and sperm motion parameters associated with an interquartile range (IRQ)[c] increase in SG-adjusted insecticide metabolite levels (*n* = 272)

	Coefficient (95% CI)	
	1N[d]	TCPY[d]
Semen parameters		
Concentration[e]	0.84 (0.71–1.01)	0.97 (0.83–1.12)
Motility (percent motile)	-3.87 (-7.28– -0.45)*	-2.16 (-5.05–0.73)
Morphology (percent normal)	-0.15 (-0.79–0.49)	0.15 (-0.39–0.68)
Motion parameters[f]		
VSL	-1.64 (-2.99– -0.27)*	-1.21 (-2.34– -0.08)*
VCL	-1.98 (-4.33–0.35)	-0.53 (-2.47–1.42)
LIN	-0.79 (-1.79–0.22)	-1.07 (-1.90– -0.24)*

[a] Regression coefficients were adjusted for age and abstinence time. [b] Regression coefficients for motility, morphology, and motion parameters represent the change in semen parameter for an IQR change in insecticide metabolite concentration (0, no change in semen parameter for an IQR change in insecticide metabolite concentration; < 0, a decrease in semen parameter for an IQR change in insecticide metabolite concentration; > 0, an increase in semen parameter for an IQR change in insecticide metabolite concentration). [c] 1N IQR = 1.80-5.02 µg/L; TCPY IQR = 1.76-5.01 µg/L. [d] 1N and TCPY were log transformed for regression analysis. [e] Sperm concentration was log transformed. The coefficient represents a multiplicative change in sperm concentration per IQR change in TCPY or 1N (1.0, no change in sperm concentration for an IQR change in insecticide metabolite concentration; < 1.0, a multiplicative decrease in sperm concentration for an IQR change in insecticide metabolite concentration; > 1.0, a multiplicative increase in sperm concentration for an IQR change in insecticide metabolite concentration). f VSL, VCL, and LIN analyses not performed on 9 azoospermic men; n = 263. TCPY IQR = 1.76–5.08 µg/L; 1N IQR = 1.77–5.02 µg/L. *p < 0.05.

Meeker et al., 2006a. Exposure to nonpersistent insecticides and male reproductive hormones.

This study was conducted to investigate one possible mechanism through which the pesticides CPF and carbaryl alter sperm parameters, that being disruption of the endocrine axis manifested as altered levels of reproductive hormones. Of the subjects participating in this study (n=322), 35% had a previous infertility examination. The mean concentration of serum testosterone was 422 ng/dL (SD = 147). Using multiple linear regression, an inverse association was observed between SG-adjusted TCPY and testosterone concentration (n=268). An increase in the IQR in TCPY was associated with a decrease in testosterone concentration (6.2% decrease, 95% CI -10.0% to -2.5%). An increase in the IQR in TCPY levels was also associated with a 7% decrease in free androgen index (-11% to -2%). In this study because both TCPY and 1N were inversely associated with testosterone, the authors tested for confounding and statistical interaction by including both metabolites as independent variables in regression models. There was essentially no change in any of the associations between the metabolites and the semen parameters and no evidence of interaction. An increase in the IQR for urinary 1N was also associated with a decrease in testosterone concentrations.

An increase in the IQR for TCPY was also associated with a decrease in LH by a multiplicative factor of 0.96 (95% CI 0.90 to 1.02), which represents a 6% decrease for the median value of LH. However, when ln-transformed LH was modeled with SG-adjusted TCPY quintiles, there was no evidence of a dose response pattern. Results for regressions models using unadjusted or creatinine-adjusted TCPY were comparable with those using the SG-adjusted levels.

Table E5. Adjusted* Coefficients (95% Confidence Intervals) for a Change in Serum Hormone Levels Associated With an IQR Increase in Insecticide Metabolite Levels†

	TCPY			IN		
	SG-Adjusted (n = 268)	Creatinine-Adjusted (n = 301)	Unadjusted (n = 322)	SG-Adjusted (n = 268)	Creatinine-Adjusted (n = 301)	Unadjusted (n = 322)
FSH‡	0.99 (0.91 to 1.06)	0.97 (0.90 to 1.04)	0.99 (0.93 to 1.05)	1.01 (0.92 to 1.10)	0.98 (0.91 to 1.06)	1.02 (0.93 to 1.11)
LH‡	0.96 (0.90 to 1.02)	0.94 (0.89 to 1.01)	0.96 (0.91 to 1.01)	0.99 (0.91 to 1.07)	0.98 (0.92 to 1.05)	1.00 (0.92 to 1.07)
Inhibin B§	2.17 (-6.65 to 11.0)	0.20 (-8.85 to 9.24)	-2.76 (-10.1 to 4.54)	-3.71 (-14.8 to 7.34)	-2.54 (-12.7 to 7.60)	-7.21 (-17.3 to 2.81)
Testosterone §	-25.2 (-40.4 to -10.1)	-25.7 (-41.1 to -10.3)	-18.4 (-30.8 to -5.8)	-24.3 (-43.3 to -5.21)	-17.5 (-35.0 to 0.04)	-21.8 (-39.1 to -4.48)
SHBG‡	1.02 (0.97 to 1.07)	1.01 (0.95 to 1.06)	1.00 (0.96 to 1.05)	1.00 (0.94 to 1.07)	1.00 (0.94 to 1.06)	1.01 (0.96 to 1.07)
Free androgen index‡	0.93 (0.89 to 0.98)	0.93 (0.89 to 0.98)	0.96 (0.91 to 0.99)	0.95 (0.90 to 1.01)	0.96 (0.92 to 1.03)	0.90 (0.90 to 1.00)

*Adjusted for age, BMI, smoking (current vs former or never), and time of day that blood was collected for hormone analysis. The testosterone models were also adjusted for (ln) SHBG.

†Ln-transformationations of LH, FSH, SHBG, and free androgen index. Testosterone and inhibin B concentrations were untransformed. In all models, ln-transformations of insecticide metabolite concentrations.

‡Coefficient represents a multiplicative change in hormone level for an IQR change in insecticide metabolite concentration after backtransformation of both hormone and insecticide metabolite concentrations. For an IQR change in insecticide metabolite concentration, a coefficient equal to 1.0 indicates no change in hormone level, a coefficient <1.0 indicates a multiplicative decrease in hormone level, and a coefficient >1.0 indicates a multiplicative increase in hormone level.

§Coefficient represents the change in hormone level for an IQR change in insecticide metabolite concentration after backtransformation of the insecticide metabolite concentrations. For an IQR change in insecticide metabolite concentration, a coefficient equal to zero indicates no change in hormone level, a coefficient <0 indicates a decrease in hormone levl, and a coefficient >0 indicates an increase in hormone level.

When exposure was divided into quintiles the inverse association between TCPY exposure and testosterone levels appeared to be dose-dependent, with the highest TCPY quintile being associated with a decrease in testosterone of 83 ng/dL (-128 to -39) when compared with the lowest quintile.

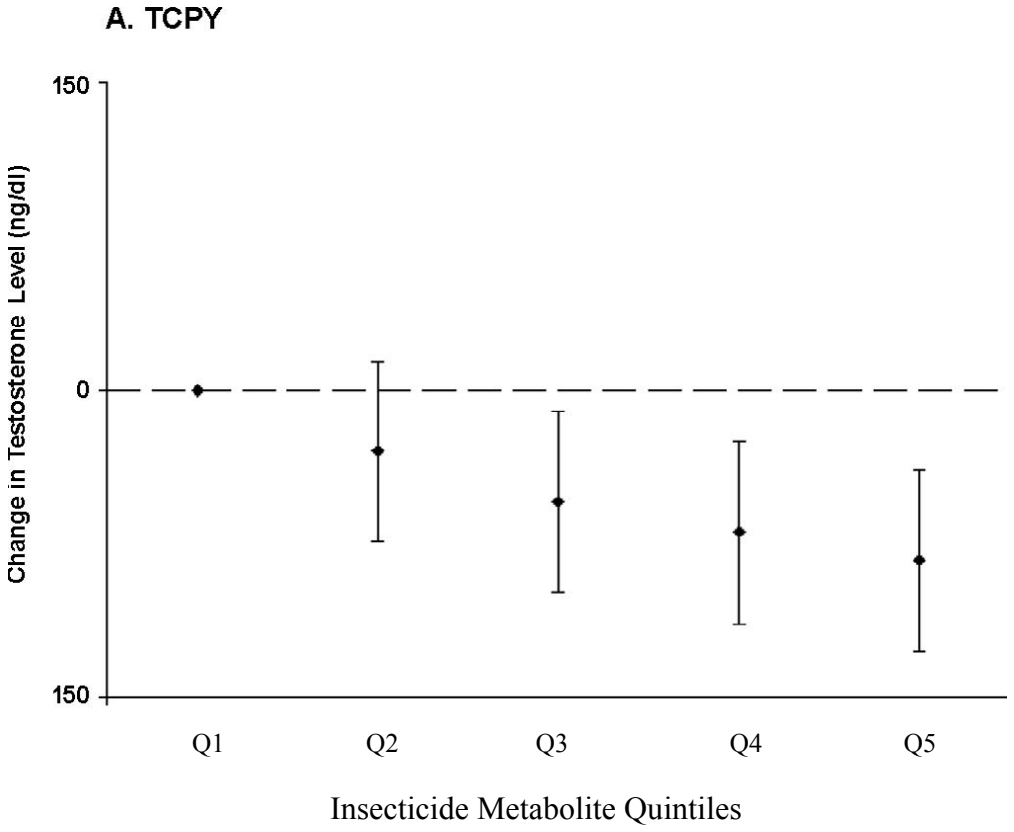


Figure E1. Regression coefficients (diamonds) and associated 95% confidence intervals for a change in testosterone level associated with increasing quintiles of specific gravity-adjusted insecticide metabolite TCPY, adjusted for age, BMI, ln-transformed SHBG, smoking (current vs. former or never), and time of day that blood was collected for hormone analysis (n=268).

The authors cite the results from this study, as well as their related studies, to suggest that the strong association between TCPY and testosterone suggests that CPF may be associated with altered endocrine function of the male hypothalamic-pituitary-gonadal axis. In contrast, the strong associations of 1N with sperm motility and DNA damage suggests that carbaryl or naphthalene may be associated with direct damage to developing or mature sperm.

Meeker et al., 2006b. Thyroid hormones in relation to urinary metabolites of non-persistent insecticides in men of reproductive age.

This study investigated the association between exposure to CPF and carbaryl and levels of the thyroid hormones thyroxine (T4), triiodothyronine (T3), and thyrotrophin (TSH). A total of 268 subjects were included in the multiple regression analysis using SG-adjusted TCPY concentrations. All models were adjusted for age, BMI, current smoking and season although none of these variables were very strongly associated with urinary TCPY concentrations. Samples collected in winter had T4 concentrations which were slightly lower than those collected during the other seasons.

There was an inverse association between TCPY concentration and serum T4 levels. An increase in the IQR of TCPY was associated with a 0.03 ng/dL decrease in T4 (95% CI, -0.06 to 0.0003, p =0.05). (Table E6), which represents a 2.5% decrease at the median level of T4 (1.26 ng/dL). In contrast, there was a positive association between SG-adjusted TCPY and TSH. An IQR increase in TCPY was associated with a 1.09 fold (9%) increase in TSH (95% CI, 1.00 to 1.18), which represents a 0.13 μIU/mL increase in the median TSH level (1.44 μIU/mL).

Table E6. Adjusted[a] coefficients for a change in serum thyroid hormone and TSH levels[b] associated with IQR increase in insecticide metabolite levels[c]

	n	Free T_4[d] (ng/dL)	Total T_3[d] (μIU/mL)	TSH[e]
SG-adjusted				
TCPY	268	-0.031 (-0.063, 0.0003)	-0.018 (-0.041, 0.0005)	1.09 (1.00, 1.18)
1N	268	-0.033 (-0.071, 0.005)	-0.007 (-0.034, 0.021)	1.05 (0.94, 1.16)
Creatinine-adjusted				
TCPY	301	-0.034 (-0.067, -0.003)	-0.017 (-0.040, 0.006)	1.10 (1.01, 1.19)
1N	301	-0.025 (-0.062, 0.012)	-0.004 (-0.030, 0.021)	1.05 (0.95, 1.15)

[a] Adjusted for age and BMI. The model for 1N and TSH was also adjusted for smoking (current vs. former or never) and season (winter vs. spring, summer, or fall).

[b] log-transformations of TSH concentration were used. Free T_4 and total T_3 concentrations were modeled untransformed.

[c] In all models log-transformations of insecticide metabolite concentrations were used.

[d] Coefficient represents the change in hormone level for an IQR change in insecticide metabolite concentration after back-transformation of the insecticide metabolite concentrations. For an IQR change in insecticide metabolite concentration, a coefficient equal to 0 indicates no change in hormone level, a coefficient <0 indicates a decrease in hormone level, and a coefficient >0 indicates an increase in hormone level.

[e] Coefficient represents a multiplicative change in hormone level for an IQR change in insecticide metabolite concentration after back-transformation of both hormone and insecticide metabolite concentrations. For an IQR change in insecticide metabolite concentration, a coefficient equal to 1.0 indicates no change in hormone level, a coefficient <1.0 indicates a

multiplicative decrease in hormone level, and a coefficient >1.0 indicates a multiplicative increase in hormone level.

When categorized into quintiles, a statistically significant inverse trend was seen for TCPY and T4 (p =0.03), while a positive trend was observed for TCPY and TSH (p =0.01) (Figure E2). However, these trends were not monotonic. The authors stated that the associations between TCPY and T4 and TSH suggest altered T4 production or secretion by the thyroid gland, as opposed to effects on the hypothalamus or anterior pituitary.

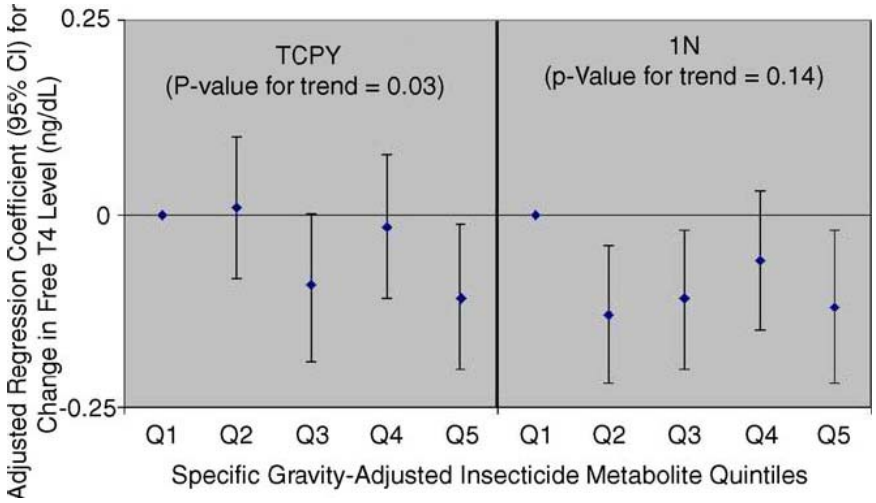


Figure E2. Regression coefficients (diamonds) and 95% confidence intervals for a change in T4 associated with increasing quintiles of SG-adjusted TCPY or 1N. Adjusted for age and BMI.

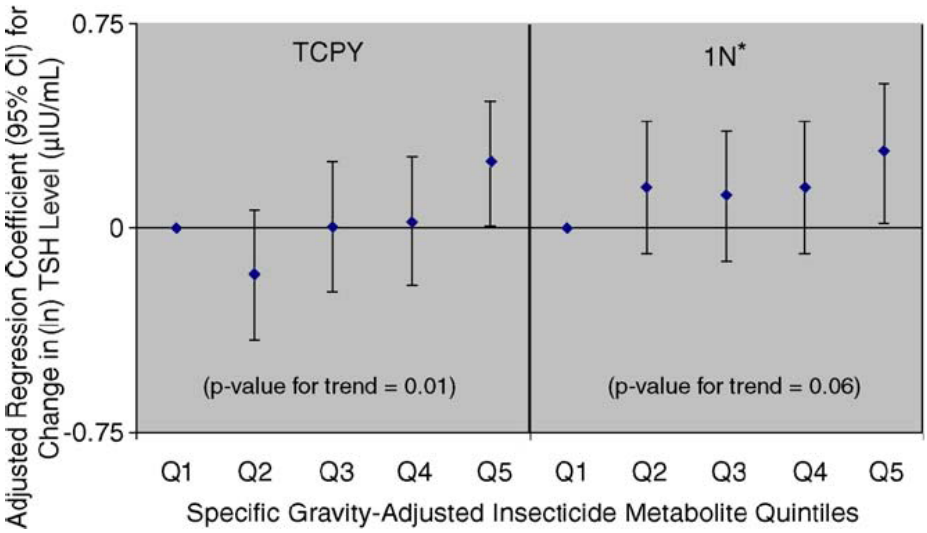


Figure E3. Regression coefficients (diamonds) and 95% confidence intervals for a change in ln-transformed TSH associated with increasing quintiles of SG-adjusted TCPY or 1N. Adjusted for age and BMI. (*) The model for 1N and TSH was also adjusted for smoking and season.

Potentials mechanisms for reduced T4 may be increased thyroid hormone metabolism whereby increased hepatic metabolism of thyroid hormones results in decreased T4 and increased TSH. The authors also explored the plausibility that exposure to low concentrations of CPF may alter thyroid function, leading to a decline in circulating testosterone, as seen in the previous study (Meeker et al., 2006b) and potentially a decrease in sperm concentration and/or motility (Meeker et al., 2004b). In synthesizing the results of this study with their earlier studies to examine the relationships between T4, testosterone, and semen quality, the results show that while TCPY was associated with a decrement in both T4 and testosterone, the relation to semen quality remains unclear.

Meeker et al., 2008. Circulating estradiol in men is inversely related to urinary metabolites of nonpersistent insecticides.

As evidence in the literature suggests that nonpersistent pesticides may adversely impact estradiol levels (Jeong et al., 2006; Usmani et al., 2006), and that estradiol plays an important role in male reproductive health (Eddy et al., 1996; Hess et al., 1997), the authors examined the association between CPF and carbaryl and circulating estradiol levels in this population of men. The geometric mean estradiol level for the 322 subjects was 29.3 pg/mL. Estradiol was inversely associated with age and positively associated with BMI. Multivariate regression analyses were adjusted for SHBG, age, BMI and season.

In the multiple regression analysis TCPY concentrations were inversely associated with estradiol, with a 4.6% decrease in median estradiol level (95% CI -8.8 to -0.5%, p = 0.03) for an IQR increase in TCPY. No significant association was observed between TCPY

concentrations and prolactin concentrations. When 55 men were excluded due to their SG being out of the accepted range, the results remained largely unchanged though the point estimate became slightly smaller.

When serum hormone levels were regressed on quintiles of TCPY a decreasing (though not monotonic) trend in estradiol was observed (p=0.03) (Figure E4).

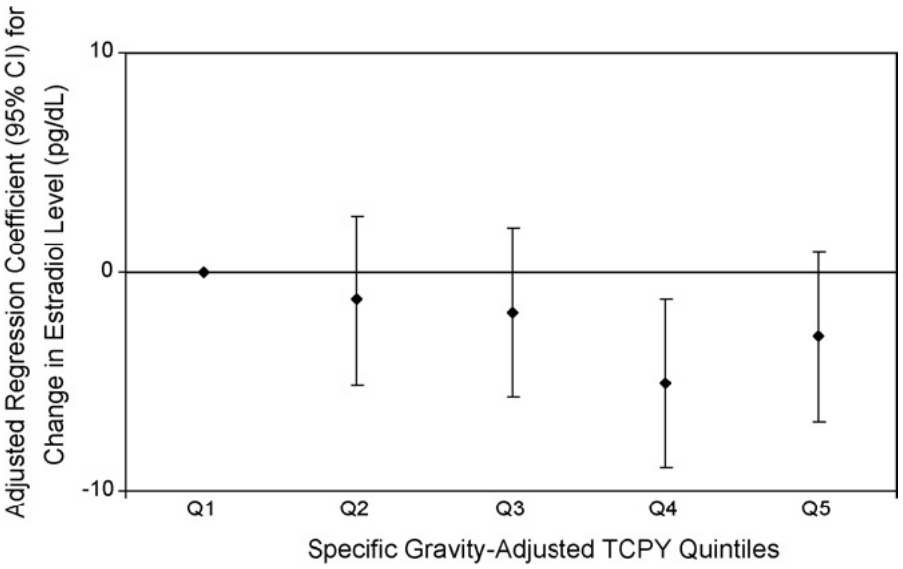


Figure E4. Adjusted (adjusted for ln-transformed SHBG, age, BMI, and season) regression coefficients for a change in estradiol level associated with increasing quintiles of SG-adjusted TCPY (*n*= 322). *p*-value for trend = 0.03.

In a similar analysis with prolactin, the highest quintile of TCPY was associated with significantly higher prolactin levels compared with the lowest quintile (Figure E5).

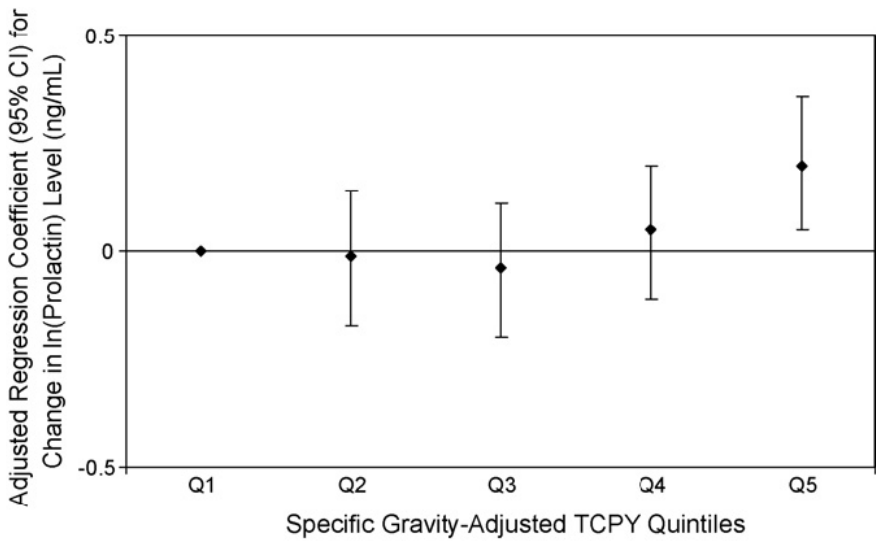


Figure E5. Adjusted (adjusted for age and time of day blood sample was collected) regression coefficients for a change in ln-adjusted prolactin level associated with increasing quintiles of SG-adjusted TCPY (N = 322). p-Value for trend = 0.01.

As a proportion of men (10.8%) had estradiol concentrations below the detection limit, multivariate logistic regression was conducted in which estradiol levels were divided into quintiles. Figure E6 shows the resulting dose-dependent increase in odds ratios for the lowest quintile versus increasing quintiles of TCPY.

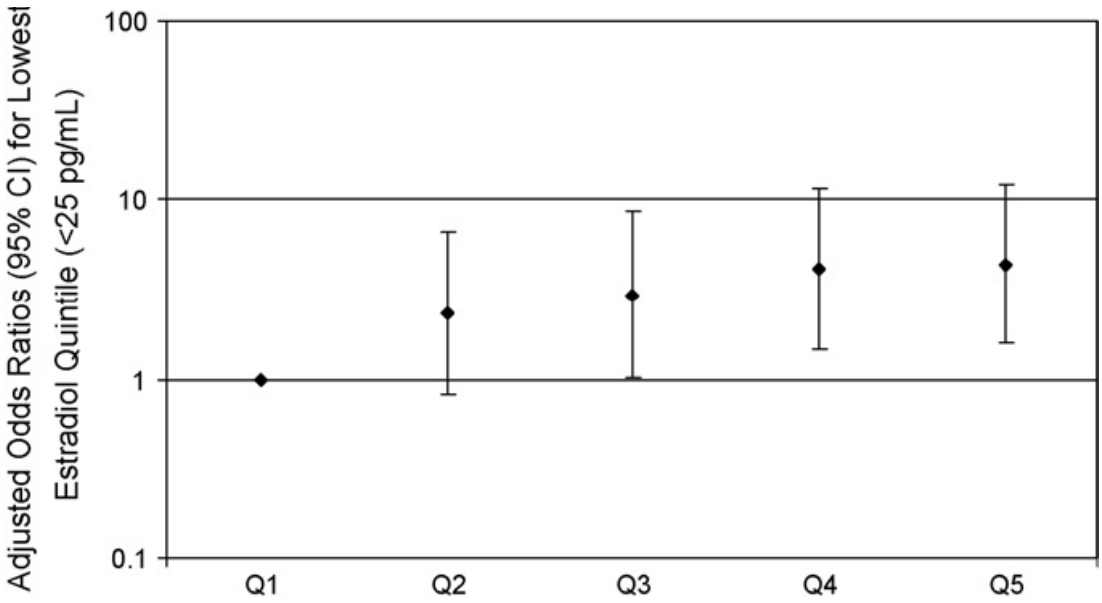


Figure E6. Adjusted (adjusted for ln-transformed SHBG, age, BMI, and season) odds ratios for having estradiol level in the lowest quintile (<25 pg/mL) with increasing SG-adjusted TCPY quintiles (*n* = 322). *p*-value for trend = 0.002.

The limitations associated with the above studies by Meeker et al., include the use of a single sample to measure hormone levels and a one day estimate of CPF exposure. However, as reported in Meeker et al. (2005), the use of a single urine specimen is predictive of a 3–month average exposure when classifying exposure into highest or lowest exposure tertiles. In addition, as there were a number of different outcomes examined in the same population in the different studies there may be a greater probability of finding spurious associations.

The studies included both fertile and infertile men. Although the subjects were recruited from an infertility clinic, the authors cite a recent study of a cohort of men overlapping with this one where no differences were reported when semen characteristics were compared between participants and nonparticipants. Although the participation rate was 65%, it is a higher participation rate than other male reproductive health study designs and this design may reduce the potential for selection bias.

Related studies

Salazar-Arredondo et al., (2008). Sperm chromatin alteration and DNA damage by methyl-parathion, CPF and diazinon and their oxon metabolites in human spermatoza.

In this recent study, Salazar-Arredondo et al., examined damage to sperm DNA from in vitro exposure to organophosphate compounds and their oxons. Human ejaculated spermatozoa from healthy volunteers with normal semen quality parameters (according to the WHO, 2001) were incubated with different concentrations of three OP insecticides

(methyl-parathion, CPF, and diazinon) and their corresponding oxon metabolites. Concentrations (50-750 μM) of the six different tested compounds were not cytotoxic to mature sperm cells except for 750 μM methyl-paraoxon. The percentage of cells with DNA damage is represented by % DFI (DNA fragmentation index) (as assessed by the sperm chromatin structure assay).

All OP compounds caused DNA damage in comparison to controls; however, the degree varied. Methyl parathion and its oxon were the most toxic showing significant effects at 300 μM. CPF and its oxon showed increases in % DFI at 500 and 750 μM, with significant increases of 33% and 45% of the control value, respectively, with the oxon metabolite showing increases from 7% to 47% at 300 μM and above (Figure E7).

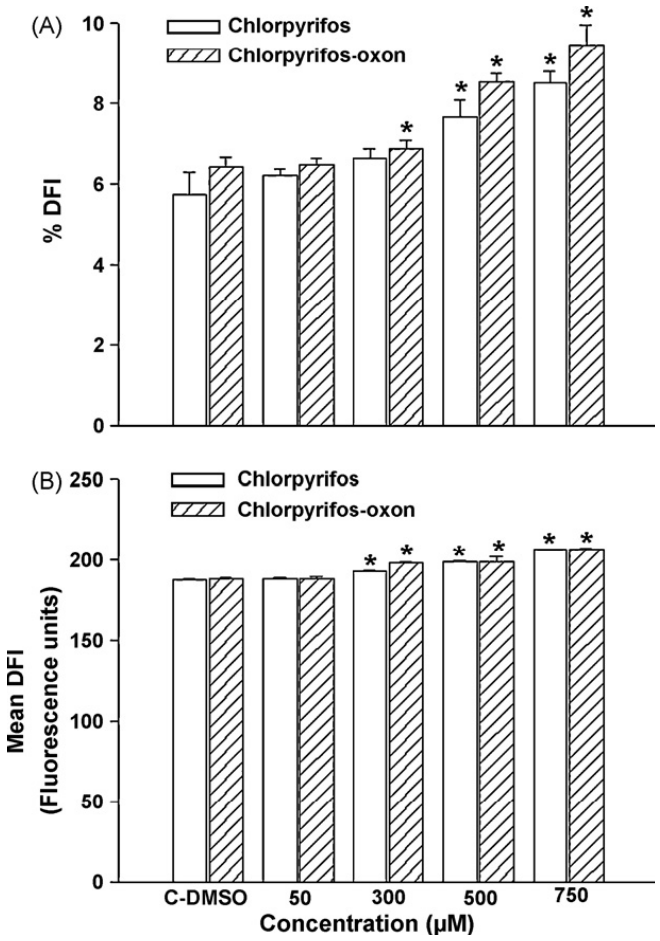


Figure E7. Effects of chorpyrifos and chlorpyrifoxon on sperm chromatin structure. (A) % DFI and (B) mean DFI. Bars represent mean ± S.D. (*n* = 3 separate experiments made by sextupling from each donor). *p < 0.05 vs. the control group, according to the analysis of variance (ANOVA) and the Bonferroni multiple comparison test. C-DMSO= control group added with DMSO.

All the OPs and their metabolites showed dose-response effects in sperm DNA damage with correlation values ranging from 0.7852 to 0.9731; CPF and its oxon correlations

were 0.8889 and 0.8992 ($p<0.001$), respectively. Linear regression analysis was used to estimate the concentration of OPs that may cause sperm DNA damage at (DFI) $\geq$ 30%, a widely used value at which fertility is compromised. The concentrations (mM) at which CPF and the CPF-oxon were found to cause sperm DNA damage were 4.302 and 3.549, respectively, while the concentrations for methyl-paraoxon and diazoxon were 0.332 and 10.251, respectively. Thus, methyl-paraoxon was most toxic and diazoxon was least toxic.

Table E7. Estimation of OP concentrations that may cause sperm DNA damage (DFI)≥30%

Compound	Concentration[a] (mM)
Methyl-paraoxon	0.332
Methyl-parathion	3.435
Chlorpyrifos-oxon	3.549
Chlorpyrifos	4.302
Diazinon	6.238
Diazoxon	10.251

[a] Concentration of OP compounds that cause a DFI $\geq$ 30% (value that compromises fertility in humans) according to a simple linear regression analysis.

The authors conclude that their results strongly suggest that the oxon metabolites of methyl parathion, diazinon, and CPF have the ability to damage mature sperm chromatin, particularly the DNA, being more toxic than their parent compounds, and that OP metabolism may be involved in this genotoxicity, possibly through the induction of oxidative stress.

Perez-Herrera et al., 2008. PON1Q192R genetic polymorphism modifies organophosphorous pesticide effects on semen quality and DNA integrity in agricultural workers from southern Mexico.

This cross-sectional study was conducted in a cohort of traditional agricultural workers from the Yucatan State in southeastern Mexico in 2005 to investigate the role of PON1Q192R polymorphism on the susceptibility of semen quality and DNA integrity to OP toxicity. Eligible subjects were healthy agriculture workers, aged 18-55 years old. Men with a vasectomy were not included as well as one subject with azoospermia. Fifty-four workers agreed to participate (participation rate = 26%). Semen quality was evaluated according to WHO guidelines and included morphology, volume, motility, viability, concentration, and NT positive cells (the percent of cells with endogenous nicks, the nick translation assay). Questionnaire data was collected on factors including sociodemographic characteristics, lifestyle habits, and pesticides use history. An

involved exposure assessment included information from two exposure indexes, which were constructed for all pesticides and for the group of OPs; one at the month of sampling (representing recent exposure, of spermatids and mature spermatozoa), and another during the preceding three months before sampling (representing exposure of cells during a complete spermatogenic cycle). The two exposure indexes were estimated from the following factors: 1) frequency of exposure to all pesticides, 2) frequency of exposure to OPs, 3) use of personal protection, and 4) habits that may modify the exposure. A protection factor was created considering personal protection used during handling pesticides according to absorption rates of potential exposures such as wearing waterproof gloves (15%), mask (60%), mouth cover (10%), etc. The habits (frequency) variable included washing the spraying equipment (which increased the exposure by 30%), storing pesticides in the home (60%), eating while preparing the pesticide mixture (30%), etc. The exposure index was then calculated for each worker by multiplying, first, the frequency of OP or all pesticide use by the protection factor, and second, by the habits factor, then adding these two values together.

The mean age of the subjects was 39.2 years (SD $\pm$10.2). Forty-three% of subjects were current smokers and 48% had Mayan ancestry. Pesticides used by these workers consisted of 29 different active ingredients, including CPF as well as diazinon, malathion and methamidophos. PON1Q192R genotype frequencies for 192R and 192Q alleles were 0.54 and 0.46, respectively, and the frequencies for 192RR, 192QR and 192QQ genotypes were 0.28, 0.51 and 0.21, respectively. A wide variation in PON1 activities was observed among participants and a good agreement was seen between genotype and phenotype using paraoxon and phenylacetate as substrates. Homozygote subjects for the 192R allele had the highest levels of PON1 activity to both substrates, paraoxon and phenylacetate. No significant differences were observed in the general characteristics of subjects and OP exposure among the PON1Q192R genotypes. Semen quality analysis showed morphology and DNA integrity (as evaluated by the percentage of NT-positive cells) were the only parameters that showed mean values outside the reference ranges (Sakkas et al,. 1996, WHO, 2002). However, a varied percentage of subjects showed abnormal values of some of the semen quality parameters; all participants had abnormal morphology, 46% had low ejaculate volume, 30% showed low sperm motility and 13% had low sperm viability. Alteration of sperm DNA integrity was seen in 88% of subjects, higher than that reported in fertile men (< 10%, Sakkas et al., 1996), and higher than in individuals living in the same area but not occupationally exposed to pesticides who showed a mean value of 6.3% (data not shown). No associations were observed between sperm DNA integrity and semen quality parameters; however, a borderline association was seen with sperm viability ($p = 0.087$) (data not shown). As seen in Table E8, using regression analysis, no associations were seen between semen quality parameters and OP exposure.

Table E8. Simple and multiple linear regression analysis of semen quality and DNA integrity modeling OP exposure

Parameter	At the month of sampling			During 3 months before sampling		
	Coefficient	r^2	p^a	Coefficient	r^2	p^a
Morphology (%)[b]	0.001	0.032	0.467	0.001	-0.039	0.750
Motility (%)	0.005	-0.0282	0.812	-0.001	-0.015	0.918
Viability (%)[c]	237.780	0.155	0.528	-10.283	0.094	0.849
Concentration (10^6 cells/ml)	0.038	0.035	0.621	0.004	0.037	0.695
Volume (ml)[d]	-0.001	0.065	0.227	-0.001	0.052	0.778
NT-positive cells (%)[d]	-0.001	-0.113	0.508	0.001	-0.104	0.772

[a] Adjusted by cigarette smoking, alcohol drinking, caffeine intake and exposure to all pesticides at the month and during 3 months before collecting the samples.
[b] Values are transformed by square root.
[c] Values are transformed by cubic root.
[d] Values are transformed by logarithm.

When subjects were stratified by genotype, the multiple linear regression analysis for OP exposure at the month of sampling showed a significant association with DNA integrity (NT-positive cells) only in homozygote subjects for the 192R allele (p = 0.025) (Table E9). No other associations were observed except for a non-statistically significant decrease in sperm viability in homozygote subjects for the 192R allele (p = 0.086).

In examining the same stratified analysis as in Table E10, except for OP exposure during the three months before sampling, associations were seen in the homozygote subjects for the 192R allele with DNA integrity (p = 0.059), with motility (p = 0.043), and with viability (p = 0.008). In addition, an association with observed with ejaculate volume (p = 0.001) in homozygote subjects for the 192Q allele.

The authors concluded that the effects on sperm DNA integrity and semen quality parameters (viability, motility, and morphology) were modified by PON1Q192R polymorphism, with subjects featuring the 192RR genotype being more susceptible to developing adverse effects from exposure to the OP mixtures used by this population. Additionally, it is suggested that cells at the last stages of their maturation, as represented by the month of sampling and probably at earlier stages of spermatogenesis, are sensitive to OP toxicity. The authors also point out that the lack of association seen between sperm DNA damage and conventional semen quality parameters (except for viability) has been reported previously (Sanchez-Pena et al., 2004); Spano et al., 1998; Xia et al., 2005) and is consistent with the concept that the sperm chromatin or DNA integrity are independent parameters of semen quality and may show early stages of damage.

The limitations of this study include the low participation rate and the use of questionnaire data to assess exposure. However, the questionnaire data was extensive and very detailed and may provide a more representative assessment of exposure over time. Participants were exposed to a mixture of pesticides, mostly OP pesticides: methamidophos, CPF, methyl parathion, malathion and diazinon, in that order, which

would make it difficult to determine the effects of a single pesticide. However, since the PON1 polymorphisms preferentially metabolize different OP pesticides the observed interaction between the polymorphisms and pesticide exposure in relation to the outcomes suggests effects of specific pesticides.

Table E9. Multiple linear regression modeling OP exposure at the month of sampling according to PON1Q192R genotype

Genotype									
Parameter	QQ			QR			RR		
	Coefficient	r^2	p^a	Coefficient	r^2	p^a	Coefficient	r^2	p^a
Morphology (%)[b]	0.001	-0.016	0.951	0.001	-0.1335	0.650	-0.029	-0.0026	0.372
Motility (%)	-0.140	0.870	0.214	0.052	-0.022	0.121	-0.140	0.870	0.214
Viability (%)[c]	-2143.942	0.626	0.270	224.370	0.0308	0.709	-13,106.55	0.1755	0.086
Concentration (10^6 cells/ml)	-0.695	0.709	0.102	0.131	0.009	0.149	0.882	0.0332	0.547
Volume (ml)[d]	0.018	0.848	0.003	-0.001	0.023	0.534	-0.014	-0.075	0.384
NT-positive cells (%)[d]	-0.003	-0.129	0.448	-0.001	-0.072	0.598	0.013	0.279	0.025

[a] Adjusted by cigarette smoking, alcohol drinking, caffeine intake and exposure to all pesticides at the month and during 3 months before collecting the samples.
[b] Values are transformed by square root.
[c] Values are transformed by cubic root.
[d] Values are transformed by logarithm.

Table E10. Multiple linear regression modeling OP exposure during 3 months before sampling according to PON1Q192R genotypes

Genotype									
Parameter	QQ			QR			RR		
	Coefficient	r^2	p^a	Coefficient	r^2	p^a	Coefficient	r^2	p^a
Morphology (%)[b]	-0.001	-0.064	0.878	-0.001	-0.258	0.585	-0.003	0.148	0.098
Motility (%)	-0.018	0.845	0.434	0.019	0.204	0.158	-0.053	0.385	0.043
Viability (%)[c]	-425.410	0.625	0.313	-126.286	-0.049	0.624	-1361.658	0.419	0.008
Concentration (10^6 cells/ml)	-0.114	0.797	0.115	0.051	0.004	0.191	-0.141	0.265	0.102
Volume (ml)[d]	0.003	0.909	0.001	-0.001	0.035	0.640	-0.001	-0.064	0.358
NT-positive cells (%)[d]	-0.001	-0.901	0.696	-0.006	-0.114	0.366	0.003	0.202	0.059

[a] Adjusted by cigarette smoking, alcohol drinking, caffeine intake and exposure to all pesticides at the month and during 3
months before collecting the samples.
[b] Values are transformed by square root.
[c] Values are transformed by cubic root.
[d] Values are transformed by logarithm

E.2. Animal male reproductive toxicity studies

Everett. 1982
Effect of Dursban 44 on semen output of Holstein bulls.

Dursban 44, an insecticide for lice control, was applied to 185 Holstein bulls 9 to 52 months of age. These sires were in various stages of progeny testing at an artificial insemination center. Application of this product killed seven bulls, and the remaining bulls exhibited varying severity of illness with six classified as very sick. This study evaluated the effect of this illness on semen production. Semen output on 40,950 ejaculates from 583 Holstein bulls collected from July 1, 1975, through March 31, 1981, was analyzed to establish normal semen production and to estimate the effect of illness caused by Dursban 44 treatment. Ejaculate number, days between collections by previous number of ejaculates, calendar months, years, and ages of bulls affected the semen output characteristics, original volume, sperm concentration, percent motile sperm, total sperm per ejaculate, percent pre-freeze discards, percent post-thaw sperm motility, and percent post-thaw discards. Ejaculate volume, motility, total percent pre-freeze discards, and percent post-thaw discards were influenced negatively on the 6 very sick bulls. Percent post-thaw discards were higher on all bulls treated with Dursban 44 for up to 6 months post-treatment. The actual amount of CPF exposure was not available.

Dietz. et al., 1983
Dursban Insecticide: Assessment of Neonatal Survival in a Two-Generation Reproduction Study in Rats.

The purpose of this study was to evaluate the potential effects of Dursban insecticide on the reproductive capability, and neonatal growth and survival of rats fed the test material over two generations. Particular emphasis was placed upon evaluating effects of treatment on neonatal survival since equivocal results from a previous reproduction study (Thompson et al., 1971) have been interpreted as demonstrating a treatment-related increase in neonatal mortality. Male and female Sprague-Dawley rats, approximately 6 weeks of age, were fed diets providing 0, 0.5, 0.8, or 1.2 mg Dursban insecticide/kg/day for 135 days and then bred to produce F1 litters. On day 21 of lactation, pups to be bred for the following generation (F1) were randomly selected and the remaining pups were sacrificed. The F1 weanlings were dosed in a similar fashion for 120 days prior to their breeding to produce the F2 litters. These pups were also weaned on day 21 of lactation. All adult animals were sacrificed at the termination of each generation. No overt signs of toxicity were observed in any animals throughout the study. Treatment-related effects noted in the adult generations were limited to a decrease in body weights during the post-mating period for F1 males. These effects were generally limited to the 1.2 mg/kg-day F1 group, with slight effects observed at 0.8 mg/kg-day and no significant effects noted at 0.5 mg/kg-day. Adult food consumption was unaffected by the administration of Dursban insecticide at all three dose levels. No adverse effects on fertility, reproduction, or neonatal growth were observed among animals exposed to the test material for two generations. In addition, the dietary administration of up to 1.2 mg Dursban

insecticide/kg/day did not adversely affect the survival of pups from F1 or F2 litters during the lactation period. In conclusion, these data demonstrate that the dietary ingestion of 0.5, 0.8, or 1.2 mg Dursban insecticide/kg/day by male and female rats for two successive generations did not result in any adverse effects on the ability of rats to mate, reproduce, or nurse their pups. Neonatal growth and survival were also unaffected by administration of the test material

Jameset al.. 1988
The effect of Pyrinex (chlorpyrifos) on reproductive function of two generations in the rat

Summary provided by the CDPR. Crl:CD7(SD)BR rats received diets containing 0, 2, 10, or 50 ppm CPF (95% purity) in diets over 2 generations (1 litter per generation). Parental rats numbered 28/sex/group in the F0 generation, and 24/sex/group in the F1 generation. Protocol was that of a standard reproduction study, with a few pre-weaning developmental evaluations added (surface righting, air righting, and startle responses; and pupil reflex). There were no definitive treatment-related effects (report attributes 3 high dose deaths to treatment, however there were deaths in other groups and no evident unique symptoms in high dose decedents).

E.2.1. Multi-generation Studies

Breslin 1991
Two-generation dietary reproduction study in Sprague-Dawley rats.

This study was conducted to meet with regulatory agency guidelines and the data were submitted to the CDPR around 1991. The findings were subsequently published in the open literature and have been discussed earlier in the document (Section C.2.2)

Chlorpyrifos (97.8% pure) was administered in the diet at dosages of 0, 0.1, 1.0 or 5.0 mg/kg/day to Sprague-Dawley rats (30 sex/group) 7 days/week for two generations. This study was submitted for regulatory purposes and was acceptable under FIFRA. The parental generation (F0) was exposed for 10 weeks prior to mating, and the F1 adults were dosed 12 weeks prior to mating. No adverse reproductive effects were observed, though F1 pup weights were significantly ($P<0.05$) reduced (10-11%) at the highest dosage. Parental effects included decreased plasma and erythrocyte ChE at 1.0 mg/kg-day, and decreased plasma, erythrocyte, and brain ChE and histopathologic alterations of the adrenal zona fasciculata at 5.0 mg/kg-day. The histopathologic alterations of the adrenal were characterized as very slight to slight vacuolation (consistent with fatty change) in males, and very slight vacuolation and/or altered tinctorial properties in females. No effects on reproductive or fertility indices or on the histopathology of reproductive tissues were observed at any dose level, and no neonatal effects were observed at 0.1 or 1.0 mg/kg-day in the F1 or F2 litters. Parental toxicity at the high dose was accompanied by decreased pup body weight and increased pup mortality in the F1

litters only. These data show that oral administration of CPF to rats at parentally toxic dose levels was not embryolethal, embryo/fetotoxic, or teratogenic and did not adversely affect fertility or the function or structure of the reproductive organs. According to the authors, although effects on neonatal growth and survival were observed at a maternally toxic dose level in one generation, this effect was not observed in the subsequent generation and may not have been related to treatment.

Joshi et al 2007
Testicular toxicity of chlorpyrifos (an organophosphate pesticide) in albino rat.

Chlorpyrifos at the dose levels of 7.5, 12.5 and 17.5 mg/kg body weight/day in olive oil was administered orally by gavage to male Wistar rats (4 groups of 6 animals each) for 30 days to evaluate the toxic alterations in testicular histology, biochemistry, sperm dynamics and testosterone levels. Control group animals were administered the vehicle (olive oil). The body weight of animals did not show any significant change; however, a significant reduction in testes weight was observed. Marked reduction in epididymal and testicular sperm counts in exposed males and a decrease in serum testosterone concentration were noted. Histopathological examination of testes showed mild to severe degenerative changes in seminiferous tubules at various dose levels. Testes from the low-dose CPF group showed a slight change from the normal histological features with decrease number of sperm and irregular germinal epithelium. Testicular sections from the high-dose groups showed severe degenerative changes marked with damaged epithelium, irregular and incomplete spermatogenesis, lumen filled with cellular debris and was devoid of sperm.

Fertility testing (method not specified in the article) showed 85% negative results for the high dose group. However, mating data were not included. A significant reduction in the sialic acid content of testes and testicular glycogen was also noticed, whereas the protein and cholesterol content was raised significantly. All these toxic effects were moderate at low doses and became severe at higher dose levels. From the results of the present study the authors concluded that CPF induces severe testicular damage and results in reduction in sperm count and thus affects fertility. The low dose in this study (7.5 mg/kg-day) was higher than the high dose in the previous study by Breslin et al., 1996 (5 mg/kg-day) and the data observed appear to match the expected dose-response pattern.

E.3. Integrative evaluation

Human studies

A summary table (Table E11) of the studies of human male reproductive outcomes is provided at the end of this section.

Two epidemiologic studies of male reproductive toxicity examined semen quality in association with pesticide exposure (Swan et al., 2003; Meeker et al., 2004b). In the case-control study by Swan et al. (2003), associations were observed between certain pesticides and reduced semen quality, but the OR for TCPY, although elevated, was not significant. This study could be limited by the very small number of subjects used in the analysis involving TCPY. No distribution of TCPY values was given in the article by Swan et al. (2003). The categories used for the logistic regression analysis were <1.25 μg/g, 1.25–5.0 μg/g and >5.0 μg/g. However, it is not clear whether exposure as measured by these TCPY levels was comparable or lower in this population than exposure reported for males in the U.S. (unadjusted 50th and 95th percentile = 2.6 μg/L and 14.9 μg/L, respectively and creatinine adjusted 50th and 95th percentile = 1.8 μg/g and 10.3μg/g, respectively) (Meeker et al., 2004a; CDC, 2005). The TCPY levels in the subjects from the Meeker et al. studies were comparable to those levels reported for the male U.S. population (creatinine adjusted 50th and 95th percentile = 1.9 and 7.2 μg/g, respectively) (CDC, 2005). In addition, the TCPY values in the study by Swan et al. (2003) were creatinine adjusted. It has been suggested that adjusting urine insecticide metabolite concentrations using specific gravity (SG) may be more appropriate (Meeker et al., 2004a).

In the cross-sectional study by Meeker et al. (2004b), associations between TCPY and semen parameters (concentration and motility), while suggestive, were not statistically significant. However, significant associations were observed between TCPY levels and the CASA motion parameters VSL and LIN. The study by Swan et al. (2003) reported on semen quality, but there is no indication that CASA motions parameters were also examined. The related cross-sectional study by Perez-Herrera et al. (2008) also reported an association between OP pesticide exposure and semen quality in agricultural workers; however, only in those men with the PON1Q192RR genotype. In these susceptible men an inverse association was seen between reported OP exposure and semen quality parameters (viability and motility). Earlier in this document, PON192RR polymorphism was observed to be protective against adverse developmental outcomes unlike what has been reported here. It is unclear whether these reflect conflicting results or if a protective effect in this respect is operating through another mechanism. For instance, as reviewed in the PON1 overview, an antioxidant capacity of PON1 toward oxidized lipids appears to be primarily associated with the Q192 form (Costa, 2006).

The other cross-sectional studies by Meeker et al., (2004a, 2006a, 2006b, 2008), based on the same sample population, reported associations between higher TCPY levels and

increased DNA damage in sperm, lower reproductive hormone levels including testosterone and estradiol, and higher TSH levels. Two related studies reported similar associations between pesticide exposure and DNA integrity (Perez-Herrera et al., 2008; Salazar-Arredondo et al., 2008). The study by Perez-Herrera et al., (2008), as mentioned above, reported an association between exposure to OP and the percent NT-positive cells ($p = 0.059$), a measure of DNA integrity in the men with the PON1 192RR allele. The in vitro study by Salazar-Arrendondo et al. (2008) found genotoxic effects of the oxon metabolites, as well as the parent compounds of several OPs, at levels that were not cytotoxic. The results of this study suggest the ability of the oxon metabolites to damage mature sperm DNA, at even a higher degree than the parent compounds. These studies found associations between OP exposures and DNA damage using a variety of methods, such as the comet assay and sperm chromatin specific assay.

As mentioned in the integrative summary for developmental toxicity, studies show that within the population there are sub-groups with differing PON1 genotypes and varying PON1 activity levels. The related studies by Perez-Herrera et al. (2008) and Salazar et al. (2008), suggest that associations between exposure to OP and adverse effects on semen quality and DNA integrity differ according to one's PON1 status. As mentioned earlier, if an effect modifier, such as the PON1 genotype, is not a confounder, the main effect, such as that between CPF exposure and fetal growth, would be an underestimate of the magnitude of the association among the most susceptible individuals and an overestimate of the magnitude among the less susceptible individuals (Bellinger, 2008). The epidemiologic studies of Swan et al. and Meeker et al. did not examine the PON1 status in their sample populations. Thus, if there was genetic variability in these populations with respect to the PON1, it could be less likely that an association would be detected if one existed, or an effect would appear be less significant than it actually is.

Table E11. Epidemiologic Studies of Male Reproductive Outcomes

Study	Population	Exposures/ Outcomes	Results	Comments	Study Type
Swan et al., 2003	Partners of pregnant women who were recruited at prenatal clinics N = 122	TCPY in urine Semen quality – sperm concentration, motility, morphology	No association was observed between TCPY and any of the parameters.	No levels of TCPY were reported.	Case-control
Meeker et al., 2004a	Males attending Massachusetts fertility clinic between January 2000 and April 2003 N = 260	TCPY in urine Sperm DNA	Significant increase in Tail% associated with an interquartile range increase in TCPY (2.8; 0.9 to 4.6)	Geometric mean TCPY = 2.03 μg/L LOD = 0.25 μg/L	Cross-sectional
Meeker et al., 2004b	Same population as above N = 330	TCPY in urine Semen quality	Suggestive, borderline-significant associations for TCPY with sperm concentration 0.97 (0.83 – 1.12) and motility -2.16 (-5.05 – 0.73). A statistically nonsignificant association was observed with sperm morphology.	Mean TCPY = 3.19 μg/L	Cross-sectional
Meeker et al., 2006a	Same population as above N = 268	TCPY in urine Male reproductive hormones in blood	Inverse association between TCPY and testosterone. Decrease in testosterone appeared dose dependent when examined as quintiles. Highest TCPY quintile associated with decreased testosterone of 83 ng/dL as compared with lowest quintile.		Cross-sectional
Meeker et al., 2006b	Same population as above N = 322	TCPY in urine Altered thyroid function	Interquartile increase in TCPY associated with a 9% increase in TSH.		Cross-sectional
Meeker et al., 2008	Same population as above N = 322	TCPY in urine Estradiol levels in blood	An IQR increase in TCPY was associated with a 4.6% decrease in estradiol. For quintiles – dose dependent increase in OR of lower estradiol with higher TCPY.	Geometric mean TCPY = 2.59 μg/L Estradiol = 29.3 pg/mL	Cross-sectional

Animal data

No effects on the reproductive or fertility indices or on the histopathology of reproductive tissues were observed at dose levels that resulted in significant cholinesterase inhibition in a study in rats conducted by Breslin et al., (1991) per FIFRA guidelines. Severe testicular damage resulting in reduction in sperm count and fertility were noted in another study at much higher dose levels (Joshi et al., 2007). Since the low dose in the Joshi et al., 2007 study (7.6 mg/kg-day) was higher than the high dose in the Breslin 1991 (5 mg/kg-day) study, the effects observed seem appropriate for the dose levels exposed. Typically, studies in laboratory animals focus on fertility or sperm abnormalities and do not examine DNA damage *per se*. The findings of DNA damage noted in few of the studies conducted for evaluating genotoxicity may support the findings in the human studies; however, similar endpoints were not examined in the animal studies.

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Appendix 1

Glossary of terms for evaluating neurobehavior

Agonistic combative

Alternation training consists of the food or water reinforcer being located at alternating arms on successive trials.

Altricial requiring excessive care when born

Avoidance tasks have been used to screen chemicals affecting cognitive enhancement
Passive avoidance
This tests the memory of the animal to remember a negative experience (such as a foot shock by measuring the latency to for the animal to enter the otherwise preferred dark chamber.

Active avoidance
The animal has to leave the dark chamber to escape the foot shock

Auditory startle test
This provides a good measure of gross hearing ability and auditory threshold based on the principle that a suddenloud noise will elicit a startle reflex in the animal.

Conspecific of the same species; unfamiliar conspecific amouse not encountered before i.e., a stranger

Fasciculations a small contraction of muscles visible through the skin, representing a spontaneous discharge of a number of fibers innervated by a single motor nerve filament.

Habituation is an example of non-associative learning in which there is a progressive diminution of behavioral response probability with repetition of a stimulus and thus habituation is a simple form of learning.

Morris Maze swim test
Th is a frequently used paradigm to evaluate learning and memory abilities. This is a spatial navigation task in which the animal swims to find a hidden platform using visual cues to locate the platform, with escape from water being the positive reinforcement. The task was originally used to investigate anatomical brain structures required for spatial learning and memory in rats. Impaired acquisition of the Morris water task have been observed with lesions of the hippocampus, medial septum/diagonal band, entorhinal/perirhinal cortex and also after pharmacological treatments with cholinergic and glutamate receptor antagonists. Aged animals also show impairments in performance of this task

Spontaneous alternation

When it is given consecutive trials in a *T* maze, the rat will typically enter one arm of the maze on the initial trial and the opposite arm on the second. This phenomenon has been termed "spontaneous alternation". Lesions to the hippocampus or the administration of cholinergic blocking drugs, processes which have been found to interfere with learning and remembering, also impair spontaneous alternation. This suggests that the rate at which an animal spontaneously alternates could be a function of its ability to acquire and retain information.

Working memory

Delay training introduces a time delay in the range of 30 seconds to 5 minutes between successive trials, so the subject must remember which arm was reinforced last time to make the correct choice on the next trials. This is a test for working memory.

Appendix 2

Acronyms and Abbreviations

5HT – serotonin
ACh – acetylcholine
AChE – acetylcholinesterase
ADHD – attention deficit hyperactivity disorder
ANOVA – analysis of variance
AUC – area under the curve
BNBAS – Brazelton Neonatal Behavioral Assessment Scale
BuChE – butyrylcholinesterase
CBCL – child behavior checklist
CBT – childhood brain tumors
CbxE – carboxylesterases
CDPR – California Department of Pesticide Regulation
ChAT – choline aceyltransferase
ChE – cholinesterase
C_{max} – maximum concentration
CNS – central nervous system
CPF – chlorpyrifos
CPO – chlorpyrifos oxon
DA – dopamine
DAP – dialkylphosphate
DDE – 1,1'-dichloro-2,2'-bis(4-chlorophenyl)ethylene
DETP – diethylthiophosphate
DEP – diethylphosphate
DMP – dimethylphosphate
DMSO – dimethyl sulfoxide
ED_{50} – 50% effective dose
ETS – environmental tobacco smoke
FIFRA – Federal Insecticide, Fungicide, and Rodenticide Act
GD – gestation day(s)
HACU – high-affinity choline uptake
HOME – Home Observation for Measurement of the Environment
HPLC – high performance liquid chromatography
IQR – interquartile range
LD50 – Lethal Dose, 50%
LOD – level or limit of detection
LOEL – lowest observed effect level
LOQ – limit of quantitation
MDA – malondialdehyde
MTD – maximum tolerated dose
NOEL – no observed effect level
NTD – neural tube defect
NTE – neurotoxic esterase

NTP – National Toxicology Program
OP – organophosphate pesticide
PAH – polycyclic aromatic hydrocarbon
PBA – 3-phenoxybenzoic acid
PDD – pervasive developmental disorder
PK – pharmacokinetic
PND – postnatal day(s)
PNET – primitive neuroectodermal tumor
PON1 – paraoxonase
RBC – red blood cell
SG – specific gravity
SOD – superoxide dismutase
$t_{1/2}$ – half-life
TCPY – 3, 5, 6-trichloro-2-pyridinol
T_{max} – time to maximum blood concentration
U.S. EPA – United States Environmental Protection Agency
WHO – World Health Organization