

# The Organophosphorus Pesticide Chlorpyrifos Induces Sex-Specific Airway Hyperreactivity in Adult Rats

Frances C. Shaffo,\* Ana Cristina Grodzki,\* Edward S. Schelegle,<sup>†</sup> and Pamela J. Lein\*<sup>1</sup>

<sup>\*</sup>Department of Molecular Biosciences; and <sup>†</sup>Department of Anatomy, Physiology, and Cell Biology, University of California, Davis, California 95616

<sup>1</sup>To whom correspondence should be addressed at Department of Molecular Biosciences, University of California, Davis, 1089 Veterinary Medicine Drive, Davis, CA 95616. Fax: (530) 752-7690. E-mail: pjlein@ucdavis.edu.

## ABSTRACT

Occupational and environmental exposures to organophosphorus pesticides (OPs) are associated with increased incidence of asthma and other pulmonary diseases. Although the canonical mechanism of OP neurotoxicity is inhibition of acetylcholinesterase (AChE), it was previously reported that the OP chlorpyrifos (CPF) causes airway hyperreactivity (AHR) in guinea pigs at levels that do not inhibit lung or brain AChE. The guinea pig is considered to have inherently hyperresponsive airways, thus, cross-species validation is needed to confirm relevance to humans. Additionally, sex differences in asthma incidence have been demonstrated in the human population, but whether OP-induced AHR is sex-dependent has not been systematically studied in a preclinical model. In this study, 8-week old male and female Sprague Dawley rats were administered CPF at doses causing comparable AChE inhibition in whole lung homogenate (30 mg/kg in males, 7 mg/kg in females, sc) prior to assessing pulmonary mechanics in response to electrical stimulation of the vagus nerves at 24 h, 48 h, 72 h, 7 d or 14 d post-exposure in males, and 24 h or 7 d post-exposure in females. CPF significantly potentiated vagally induced airway resistance and tissue elastance at 7 d post-exposure in males, and at 24 h and 7 d post-exposure in females. These effects occurred independent of significant AChE inhibition in cerebellum, blood, trachealis, or isolated airway, suggesting that AChE independent OP-induced airway hyperreactivity is a cross-species phenomenon. These findings have significant implications for assessing the risk posed by CPF, and potentially other OPs, to human health and safety.

**Key words:** airway hyperreactivity; asthma; chlorpyrifos; organophosphorus pesticides; rats.

Chronic exposure to organophosphorus pesticides (OPs) is a global human health concern. Epidemiological evidence links OP exposure to respiratory disease, including asthma (Amaral, 2014; Doust *et al.*, 2014; Hernández *et al.*, 2011; Mamane *et al.*, 2015; Ye *et al.*, 2013). Occupational and environmental OP exposures have been associated with asthma, wheeze, decreased lung function, increased risk of developing COPD, and an increase in general respiratory symptoms in both men and women in the United States and other countries (Chakraborty *et al.*, 2009; Fieten *et al.*, 2009; Hernández *et al.*, 2008; Hoppin *et al.*, 2002, 2008, 2009; Ndlovu *et al.*, 2014; Peiris-John *et al.*, 2005).

Experimental data support the epidemiologic evidence: OPs have been shown to induce bronchospasm in calves (Gustin

*et al.*, 1988) and increase lung resistance in guinea pigs (Segura *et al.*, 1999) at doses that inhibit acetylcholinesterase (AChE) but do not cause cholinergic crisis. Exposures to OPs at levels that do not inhibit AChE have also been causally linked to airway hyperreactivity (AHR), a hallmark symptom of asthma, in a guinea pig model (Fryer *et al.*, 2004; Lein and Fryer, 2005). Multiple OPs, including the widely used chlorpyrifos (CPF), were found to elicit AHR in adult male and female guinea pigs after a single sc injection in oil, which is used to mimic an occupationally relevant dermal exposure (Fenske *et al.*, 2012; Lein *et al.*, 2012). The observation that OPs induce AHR independent of AChE inhibition is of translational importance because many regulatory agencies, including the United States Environmental

Protection Agency (USEPA), set exposure limits for OPs based on inhibition of blood cholinesterase. The current USEPA exposure limits for CPF are 0.005 mg/kg/day for the general population and 0.00003 mg/kg/day for females of reproductive age (USEPA, [https://archive.epa.gov/scipoly/sap/meetings/web/pdf/hed\\_ra.pdf](https://archive.epa.gov/scipoly/sap/meetings/web/pdf/hed_ra.pdf); last accessed April 30, 2018).

Preclinical evidence of OP-induced AHR independent of AChE inhibition has yet to be validated in a second species. Cross-species validation is important for a weight-of-evidence determination in assessing human health risks. In choosing a preclinical model for further investigation of OP-induced AHR, it is important to consider the relevance of the model's pulmonary physiology, especially pulmonary innervation, and immune responses to the human condition. Rats have pulmonary innervation similar to that of humans in that their airway smooth muscle receives direct parasympathetic, but not sympathetic, innervation. In contrast, the guinea pig airway has substantial sympathetic innervation (Canning and Chou, 2008; Kummer et al., 1992). Moreover, guinea pigs are considered to have inherently hyperreactive airways compared with other species (Canning and Chou, 2008; Stengel et al., 1995); whereas the responsiveness of the rat airway is closer to that of humans. We also addressed the question of sex differences in OP-induced AHR, which is important because asthma is a sexually dimorphic disease in the human population. Finally, the time course of AHR development and resolution after a single OP exposure has not been previously described. To address these questions, we measured pulmonary function in adult male and female Sprague Dawley rats at multiple time points after a single sc exposure to CPF.

## MATERIALS AND METHODS

**Chemicals.** CPF (o, o-diethyl [o-3, 5, 6-trichloro-2-pyridinol] phosphorothionate; 99.5% purity) was purchased from Chem Service (West Chester, Pennsylvania) and used within 6 months of purchase with interim storage as recommended by the manufacturer. Solutions were made weekly in NEOBEE<sup>®</sup> M-5 oil vehicle (Spectrum Chemical, Gardena, California) at their final concentrations (5–30 mg/ml) and stored in a polypropylene container in the dark at room temperature.

**Animals and CPF Exposures.** Animals were maintained in facilities fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and all studies were performed with regard for alleviation of pain and suffering according to protocols approved by the UC Davis Institutional Animal Care and Use Committee. Adult male and female Sprague Dawley rats (8 weeks old; Charles River Laboratories, Hollister, California) were socially housed in groups of 2–3 animals under controlled environmental conditions (22 ± 2°C, 40%–50% humidity) with a 12 h light/dark cycle and *ad libitum* access to food and water. Male animals were administered 10 mg/kg CPF (10 mg/ml solution), 30 mg/kg CPF (30 mg/ml solution), or equal volume of vehicle (1 µl/g body weight) sc in the subscapular region. Female animals were administered 5, 7, 10, 20 or 30 mg/kg CPF sc, or equal volume of vehicle (1 µl/g body weight) also in the subscapular region.

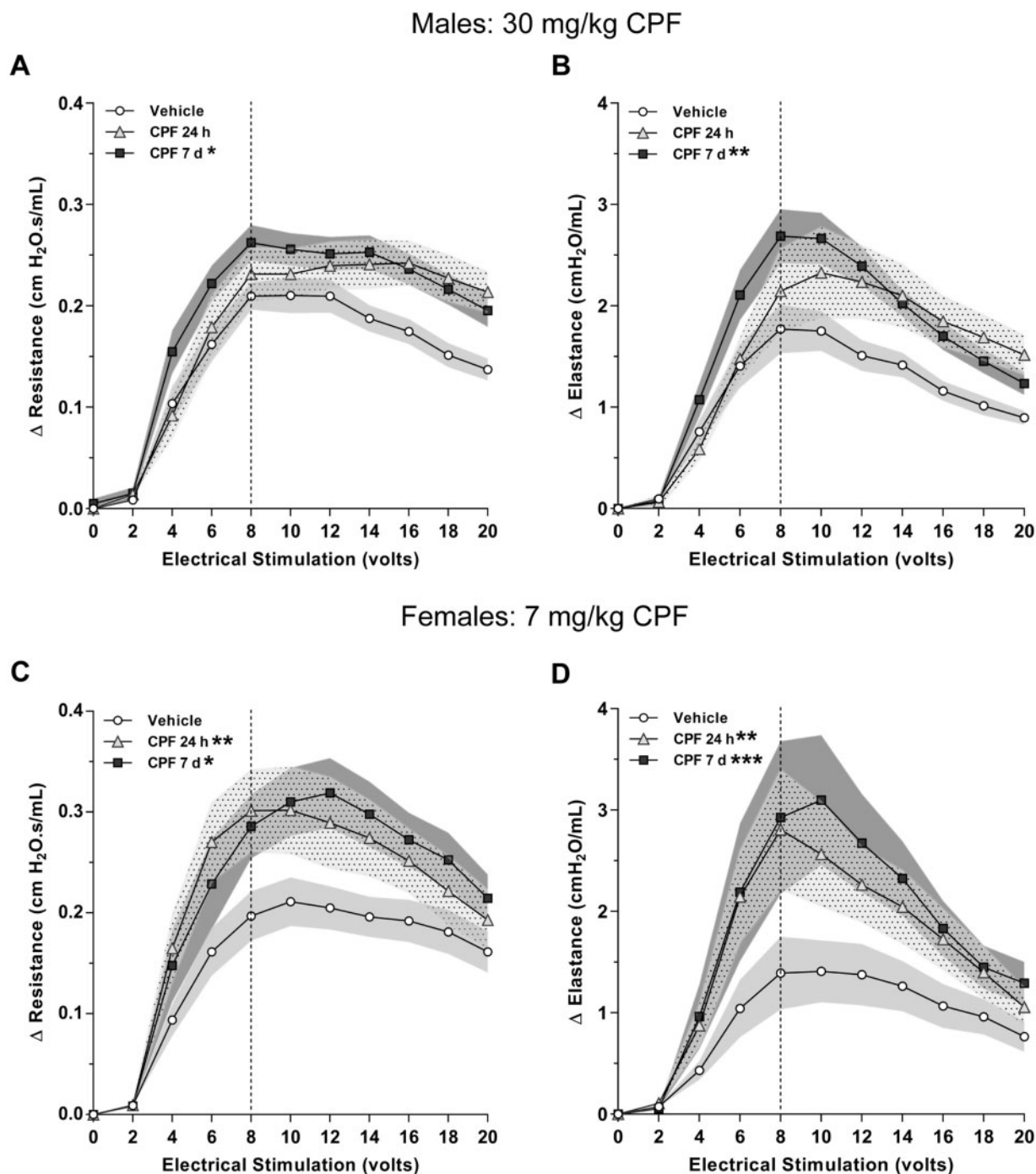
**Physiological Assessment of Pulmonary Function.** Animals were prepared for vagal nerve stimulation and measurement of pulmonary function at 24 h, 48 h, 72 h, 7 d or 14 d post-CPF injection. Animals were deeply anesthetized with a solution of 2% α-chloralose (Sigma-Aldrich, St. Louis, Missouri) and 12.5% urethane

(Sigma-Aldrich) in saline (0.50 ml/100 g, ip). A midline incision was made over the cervical trachea 10 min after administration of topical anesthetic (0.75% Bupivacaine in saline, Hospira, Lake Forest, Illinois) and the animal was intubated with an endotracheal tube. The endotracheal tube was connected to a constant-volume ventilator (FlexiVent4, SCIREQ Inc., Montreal, Canada) with a breathing frequency of 90 breaths/min and volume adjusted to 15 ml/kg. Once ventilated, animals were immobilized with succinylcholine (0.1 ml at 20 mg/ml, ip, Hospira) to eliminate spontaneous respiratory movement. Airway resistance (R) and tissue elastance (E) were recorded in real-time for each breath for the duration of each experiment.

During ventilation, the vagus nerves were isolated, stripped of their sheath, cut cranially and placed on a stimulating electrode (FHC, Bowdoin, Maine) connected to an electrical stimulator (Model S88K, Grass Technologies, West Warwick, Rhode Island). Vagus nerves were electrically stimulated for 10 s at 30 Hz and 0.5 ms pulse frequency every 2 min, 30 s after the animal received a total lung inflation maneuver to fully expand the lung. Stimulation voltage (V) ranged from 0 to 20 V at increments of 2 V. To confirm that response to vagal stimulation was mediated by release of acetylcholine (ACh) from parasympathetic nerves, a subset of animals were administered guanethidine (10 mg/kg, ip, Sigma-Aldrich) prior to ventilation to inhibit sympathetic nerve activity, or atropine (1 mg/kg, ip, Sigma-Aldrich) at the end of the stimulation protocol to inhibit muscarinic receptor activation. Animals were euthanized at the conclusion of physiological measurements by ip administration of greater than 2.5 ml/kg of a solution of pentobarbital sodium (31%) and phenytoin sodium (4.72%) (Euthasol, Virbac AH, Fort Worth, Texas).

**Methacholine (MeCh) and Acetylcholine (ACh) Challenge.** A separate cohort of animals that underwent the same surgical procedures as the vagal stimulation studies (including bilateral vagotomy) were used to determine airway resistance and tissue elastance responses to methacholine chloride (MeCh, MP Biomedicals, Burlingame, California), or acetylthiocholine iodide (ACh, Sigma-Aldrich) prepared in saline. During ventilation, animals were administered MeCh (6.25, 12.5, 25 µg/kg, iv) or ACh (4, 8, 16 mg/kg, iv) in sequentially increasing doses. Doses were spaced 4 min apart and were given 30 s after a total lung inflation maneuver. Animals were euthanized at the conclusion of physiological measurements with greater than 2.5 mL/kg Euthasol, ip.

**Analysis of Airway Resistance (R) and Tissue Elastance (E).** Resistance and tissue elastance were measured on a breath-by-breath basis using a single frequency forced oscillation technique that fits volume displacement, calculated air flow, and airway pressure obtained from the FlexiVent4 to a single compartment model (flexiWare software, version 7.6, SCIREQ Inc.). The maximum 3 s rolling average of R and E during the 30 s after each stimulation (vagal stimulation or iv MeCh/ACh) was recorded. These data were used to construct voltage-response or dose-response curves for each animal. General linear mixed-effects models were fit to test whether the effect of voltage (or ACh/MeCh dose) on R and E in OP-exposed animals differed from vehicle control. For the majority of experiments, drop-off in response after 8 V was observed, likely due to nerve depletion, so models were only fit up to 8 V. For voltage-response curves, there was a significant interaction between voltage and R, and voltage and E; therefore, these interactions were included in the model. For E-voltage response, Satterthwaite approximation was used in the model to account for unequal variances. For both ACh and MeCh dose-response curves, there was a



**Figure 1.** CPF exposure alters pulmonary physiology in male and female rats. Voltage-response curves of (A) airway resistance (R) and (B) tissue elastance (E) response to electrical vagal stimulation of adult male rats dosed with vehicle ( $n=12$ ) or 30 mg/kg CPF, sc, at 24 h ( $n=10$ ) or 7 d ( $n=14$ ) post-injection. Voltage-response curves of (C) R and (D) E of female animals exposed to vehicle ( $n=16$ ) or 7 mg/kg CPF, sc, at 24 h ( $n=8$ ) or 7 d ( $n=9$ ) post-injection. Data presented as mean response  $\pm$  SE (shaded regions). Voltage-response curves for male rats exposed to CPF at 10 mg/kg or 30 mg/kg at 48 h, 72 h, or 14 d post-injection are provided in Supplemental material. General linear mixed-effects models were fit up to 8 V to test whether the effect of voltage on R or E in CPF-exposed animals differed from vehicle control; \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$  relative to vehicle control.

significant interaction between dose and E, but not dose and R, and these interactions were included or excluded accordingly. All analyses were performed using SAS<sup>®</sup> (SAS Institute, Cary, North Carolina, version 9.4).

**Acetylcholinesterase (AChE) Activity.** AChE activity was assessed in animals exposed to CPF or vehicle. Animals were deeply

anesthetized with 4% isoflurane (Piramal Enterprise, Mumbai, India) in oxygen, blood was collected via cardiac puncture, and animals were then perfused transcardially with phosphate buffered saline (0.2M Na<sub>2</sub>HPO<sub>4</sub>, 0.2M NaH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, pH 7.2) using a Masterflex peristaltic pump (model 77200-50, Cole-Palmer Instrument Company, Vernon Hills, Illinois) at a rate of 15 ml/min until organs were cleared of blood. For CPF

**Table 1.** AChE Activity in Various Tissues of (A) Male and (B) Female Rats

(A) Males							
CPF dose	0 mg/kg	10 mg/kg	10 mg/kg	10 mg/kg	30 mg/kg	30 mg/kg	30 mg/kg
Time point		6 h	12 h	24 h	6 h	12 h	24 h
N	9	3	3	3	3	3	6
Blood	100 ± 20.5	115 ± 25.6	106 ± 14.0	126 ± 7.9	100 ± 31.0	117.3 ± 51.2	67 ± 26.7
Cerebellum	100 ± 15.3	103 ± 17.1	122 ± 12.8	122 ± 12.8	96 ± 15.7	104.3 ± 8.7	86 ± 10.7
Whole lung	100 ± 12.5	103 ± 20.2	110 ± 11.0	102 ± 28.3	117 ± 20.3	80.72 ± 6.4	<b>57 ± 10.2***</b>
(B) Females							
CPF dose	0 mg/kg	5 mg/kg	10 mg/kg	20 mg/kg	30 mg/kg		
Time point		24 h	24 h	24 h	24 h		
N	9	3	3	3	3		
Blood	100 ± 23.2	150 ± 11.1	135 ± 36.2	95 ± 51.6	130 ± 38.1		
Cerebellum	100 ± 22.0	83 ± 2.8	62 ± 16.4	88 ± 11.6	83 ± 14.7		
Whole lung	100 ± 7.5	<b>72 ± 9.5*</b>	<b>31 ± 8.4***</b>	<b>21 ± 5.9***</b>	<b>27 ± 7.3***</b>		

Data presented as the mean percent of vehicle control ± SD. Bold values are significantly different from vehicle group.

\* $p < .01$ . \*\*\* $p < .0001$ . Absolute AChE activity levels in the lung were approximately ten-fold lower than in the cerebellum or blood (data not shown).

dose-finding studies, a piece of distal left lung lobe and cerebellum were collected immediately after perfusion. For additional studies of AChE in airway-specific tissues, trachealis muscle and airway tissue were also harvested. For airway tissue, right lung lobes were inflated with agarose, and isolated bronchiolar airway was harvested from this preparation by microdissecting away the lung parenchyma as described previously (Van Winkle et al., 2017). AChE-specific activity was assessed in these tissues using Ellman's assay as described previously (Ellman et al., 1961) using acetylthiocholine iodide as the substrate and tetraisopropylpyrophosphoramidate (Sigma-Aldrich) to inhibit butyrylcholinesterase. Data were tested for normal distribution (Shapiro-Wilk normality test) and homogeneity of variance (Brown-Forsythe test) and analyzed using one-way ANOVA with  $\alpha$  set at 0.05 with Dunnett's correction for multiple comparisons (GraphPad Prism, Graphpad Software Inc., La Jolla, California, version 7).

## RESULTS

### *Chlorpyrifos (CPF) Causes Airway Hyperreactivity (AHR) in Both Male and Female Rats*

In adult male Sprague Dawley rats, exposure to CPF at 30 mg/kg, sc, significantly increased airway resistance (R) and tissue elastance (E) responses to vagal stimulation when measured at 7 d post injection, as evidenced by the upward shift of the voltage-response curve in CPF-exposed animals compared to vehicle controls (Figs. 1A and 1B). To characterize the time-to-onset and persistence of CPF-induced AHR, airway responses to vagal stimulation were tested in male animals at 24 h, 48 h, 72 h and 14 d after administration of CPF at 30 mg/kg. There were no significant effects on R or E responses at any of these other time points (Figs. 1A and 1B and Supplementary Figure 1). To identify a no-observed adverse effect level (NOAEL), vagal-induced airway responses were measured in male animals at 24 h and 7 d after exposure to CPF at 10 mg/kg, sc. At this lower dose, CPF had no significant effect on either R or E responses in males (Supplementary Figure 2).

To determine whether AChE was significantly inhibited at either of these doses, AChE activity was measured in the whole lung, blood, and cerebellum of a separate cohort of male rats exposed to CPF at 10 or 30 mg/kg, sc. Administration of CPF at 10 mg/kg sc, did not inhibit AChE activity at 6, 12, or 24 h post-injection in any of the three tissues examined. In contrast,

30 mg/kg CPF caused a progressive decrease in AChE activity in the lung over time, with significant inhibition (57% of sex-matched vehicle controls) occurring at 24 h after injection (Table 1A). However, 30 mg/kg CPF did not significantly inhibit AChE in the blood or cerebellum of males at any of the time points examined.

We next asked whether CPF induced AHR in adult female Sprague Dawley rats. We first determined whether the dose of CPF found to cause AHR in males (30 mg/kg) elicited a similar level of AChE inhibition in the whole lung of age-matched females. Interestingly, relative to males, AChE activity in the whole lung of females was more susceptible to inhibition by CPF (Table 1B). When measured 24 h after administration of 30 mg/kg of CPF, AChE activity in the female lung was approximately 27% of levels in sex-matched vehicle controls. To find a dose of CPF that inhibited AChE activity in the whole lung of females to approximately the same level observed in whole lung of males administered CPF at 30 mg/kg (57% of sex-matched vehicle controls), AChE activity was measured in female whole lung at 24 h after sc administration of 5, 10, or 20 mg/kg CPF. As observed in male, no significant AChE inhibition was observed in the blood or cerebellum of females at any of these doses. Pronounced AChE inhibition was observed in whole lung of females injected with CPF at 10 and 20 mg/kg (31% and 21% of vehicle, respectively). Moderate (72% of vehicle), but significant AChE inhibition was seen in the whole lung of females administered 5 mg/kg CPF. Because the percent inhibition of AChE activity in the whole lung of male rats exposed to CPF at 30 mg/kg fell midway between the percent inhibition of whole lung AChE in females administered 10 or 5 mg/kg, a CPF dose of 7 mg/kg was chosen for subsequent physiological experiments in female animals. Compared with sex-matched vehicle controls, R and E responses to vagal stimulation were significantly enhanced in females administered CPF at 7 mg/kg, sc, when tested at 7 d post-injection (Figs. 1C and 1D). In contrast to observations of males, exposure to CPF at 7 mg/kg, sc, also significantly enhanced R and E responses to vagal stimulation in females at 24 h post-injection (Figs. 1C and 1D).

### *CPF Effects on Pulmonary Physiology Responses to Vagal Stimulation Are Mediated at the Level of the Parasympathetic Nerves*

To determine whether CPF-induced AHR reflects changes in the presynaptic parasympathetic nerves or the postsynaptic airway



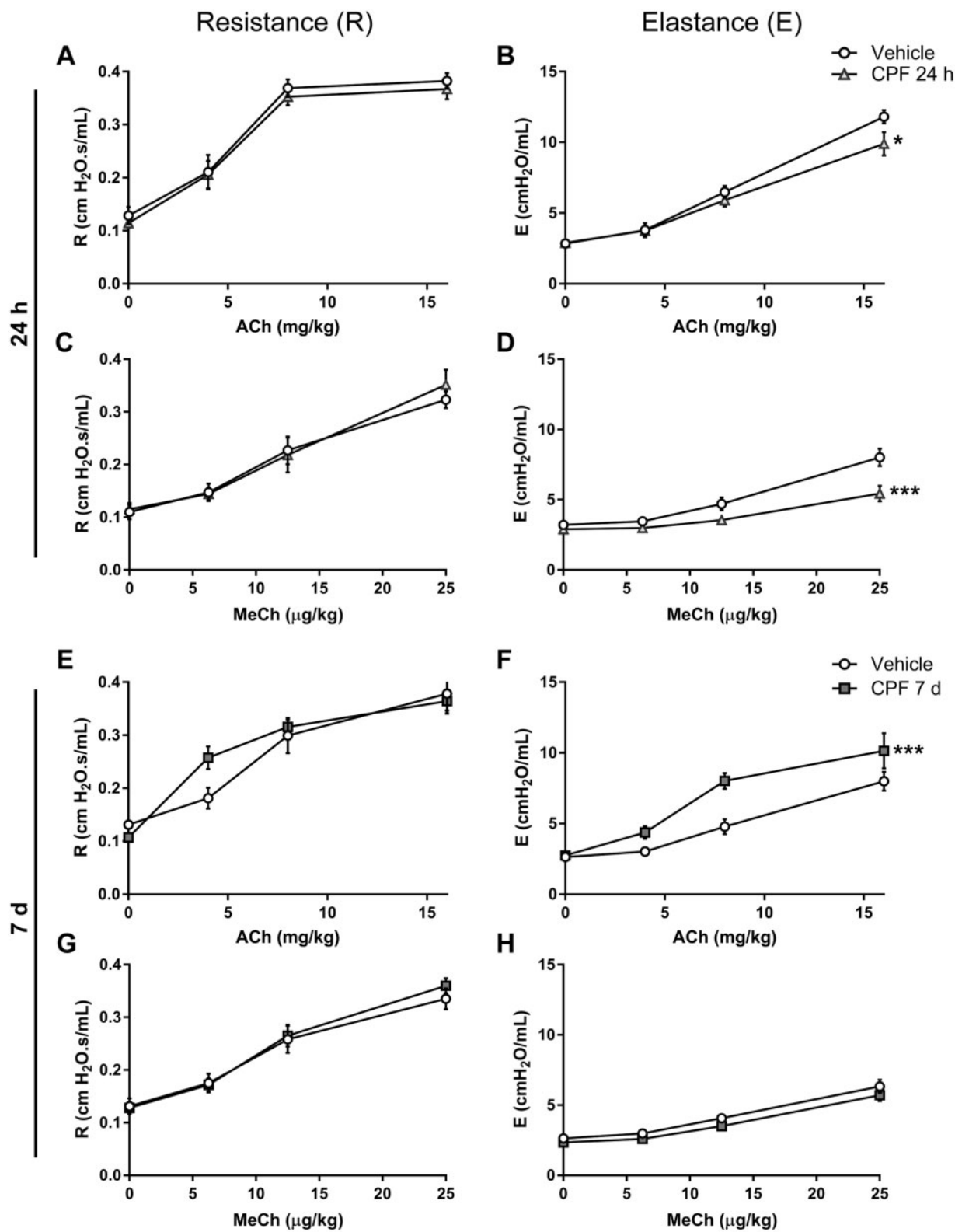
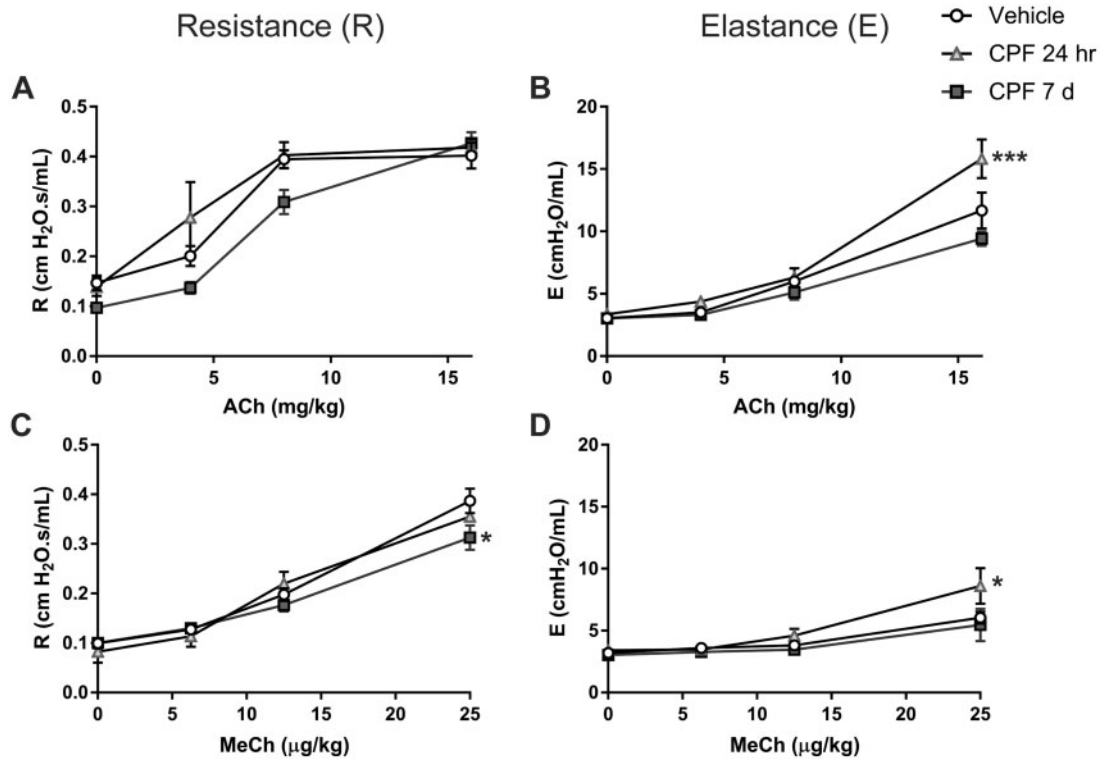


Figure 2. CPF alters pulmonary responses to iv acetylcholine (ACh) and methacholine (MeCh) in male rats. Dose-related response of airway resistance (R) and tissue elastance (E) at 24 h (A–D) and 7 d (E–H) post-CPF injection to ACh (A, C, E, G) and MeCh (B, D, F, H). Data presented as mean  $\pm$  SE ( $n = 5$ –7 animals per group); \* $p < .05$ , \*\*\* $p < .001$  relative to vehicle control.

smooth muscle, physiological responses to iv administration of acetylcholine (ACh) or methacholine (MeCh) were tested at 24 h and 7 d post-CPF injection. In males exposed to CPF at 30 mg/kg,

sc, R responses to varying doses of ACh or MeCh were not altered relative to sex-matched vehicle controls at 24 h (Figs. 2A and 2C) or 7 d (Figs. 2E and 2G) post-injection. The effects of CPF



**Figure 3.** CPF alters physiological response to iv acetylcholine (ACh) and methacholine (MeCh) in female rats. Dose-related responses to ACh (A, B) and MeCh (C, D) in female rats at 24 h and 7 d post-CPF injection on airway resistance (R) and tissue elastance (E). Data presented as mean  $\pm$  SE ( $n=4-6$  animals per group); \* $p < .05$ , \*\*\* $p < .001$  relative to vehicle control.

on E responses to ACh and MeCh were more complex. At 24 h post-exposure, the E response to either ACh or MeCh was significantly decreased in CPF-exposed males relative to sex-matched vehicle controls (Figs. 2B and 2D). However, at 7 d post-exposure, the E response to ACh was increased in CPF-exposed males relative to vehicle control males (Figure 2F), but the E response to MeCh was not altered by CPF exposure (Figure 2H).

In females exposed to CPF at 7 mg/kg, sc, the R response to ACh was not significantly altered by CPF at either 24 h or 7 d post-exposure (Figure 3A). Although the R response to MeCh in CPF-exposed females relative to sex-matched vehicle controls was no different at 24 h, it was significantly decreased at 7 d post-exposure (Figure 3C). The ACh and MeCh dose-response curves for E were shifted upwards at 24 h but unchanged at 7 d relative to sex-matched vehicle controls (Figs. 3B and 3D).

To confirm that R and E responses to vagal stimulation are mediated by parasympathetic neurotransmission, a subset of vehicle animals (male and female) were administered guanethidine (10 mg/kg, ip) to block signaling by the sympathetic nervous system, or atropine (1 mg/kg, ip) to antagonize muscarinic receptors. As seen in representative voltage-response traces (Figure 4), guanethidine had no effect on airway responses to vagal stimulation (Figure 4A), whereas atropine abolished all airway responses to vagal stimulation (Figure 4B). Atropine also abolished all vagally induced responses in males exposed to CPF at 30 mg/kg and females exposed to CPF at 7 mg/kg at 24 h and 7 d post-CPF injection (data not shown).

#### CPF Has No Effect on AChE Activity in Specific Lung Compartments at Doses That Alter Pulmonary Physiology

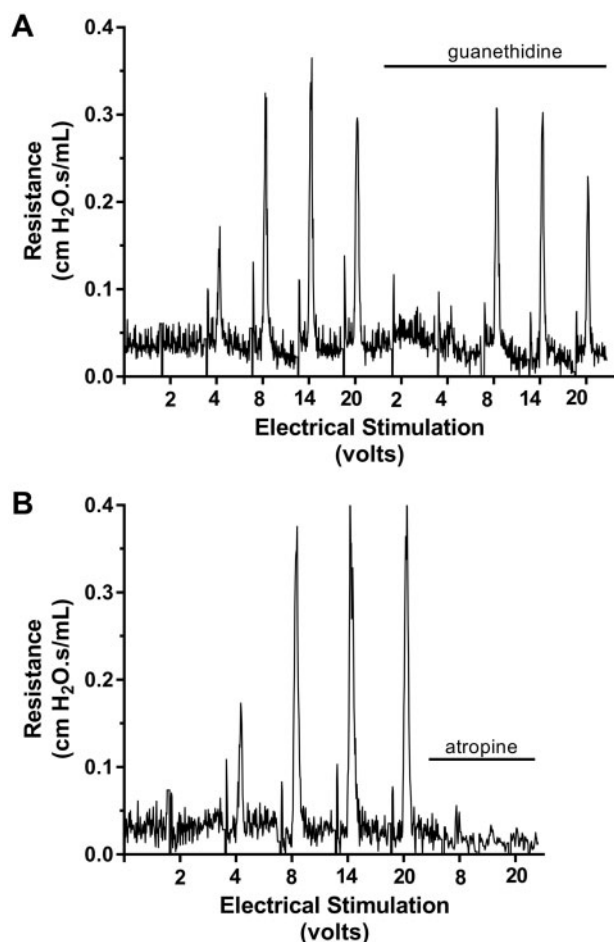
AChE activity was measured in airway tissue and trachealis isolated from lungs of vehicle and CPF-exposed animals of both

sexes (males, 30 mg/kg; females, 7 mg/kg) at 24 h, 48 h, 72 h and 7 d post-CPF injection. No significant inhibition of AChE activity was observed in isolated airway (Figure 5A), trachealis (Figure 5B), blood (Figure 5C), or cerebellum (Figure 5D) at any time point in CPF-exposed male or female animals relative to sex-matched vehicle controls.

## DISCUSSION

This study describes the first evidence of OP-induced AHR in the rat, providing cross-species validation of the toxicologic effect originally described in guinea pigs (Fryer et al., 2004; Lein and Fryer, 2005). This study extends the earlier studies by identifying specific lung compartments impacted by CPF. The guinea pig studies used methods to assess airway physiology that do not separate pulmonary resistance into its primary components of airway resistance (R) and tissue elastance (E). R primarily measures resistance in the large conducting airways, whereas E is impacted by changes in the more distal airways (Grinnan and Truwit, 2005). E is the pressure required to inflate the lung, thus an increase in pulmonary elastance means the lung is getting stiffer. The primary determinant of airway resistance is the caliber of the conducting airways, which is how bronchoconstriction leads to an increase in R; E on the other hand is driven by other factors, such as proliferation of myofibroblasts, collagen content and caliber of small distal airways (Faffe et al., 2001; Rocco et al., 2001). The observation that both R and E responses to vagal stimulation are increased by CPF exposure in males and females suggests that both the large proximal airways and small distal airways are impacted by CPF.

Further, this study identifies sex-specific responses to CPF in the rat lung. A major sex difference is the effective toxicological dose of CPF. AHR was observed in males exposed to CPF at



**Figure 4.** Vagally mediated bronchoconstriction is driven by parasympathetic innervation. Representative traces of airway resistance (R) response to electrical vagal stimulation in vehicle control males before and after administration of 10 mg/kg guanethidine (A) or 1 mg/kg atropine (B).

30 mg/kg, but not 10 mg/kg, whereas, in females, 7 mg/kg CPF triggered AHR. Moreover, the magnitude of airway hyperresponsiveness in females at 24 h and at 7 d post-exposure was greater than that observed in CPF-exposed males at 7 d. This was especially evident in the maximum tissue elastance response, which was more than doubled in CPF-exposed females at both time points relative to sex-matched vehicle controls, whereas the maximum elastance response in males at 7 d post-exposure was approximately 50% greater than that of sex-matched vehicle controls. These findings suggest that relative to males, females are more susceptible to the pulmonary effects of CPF.

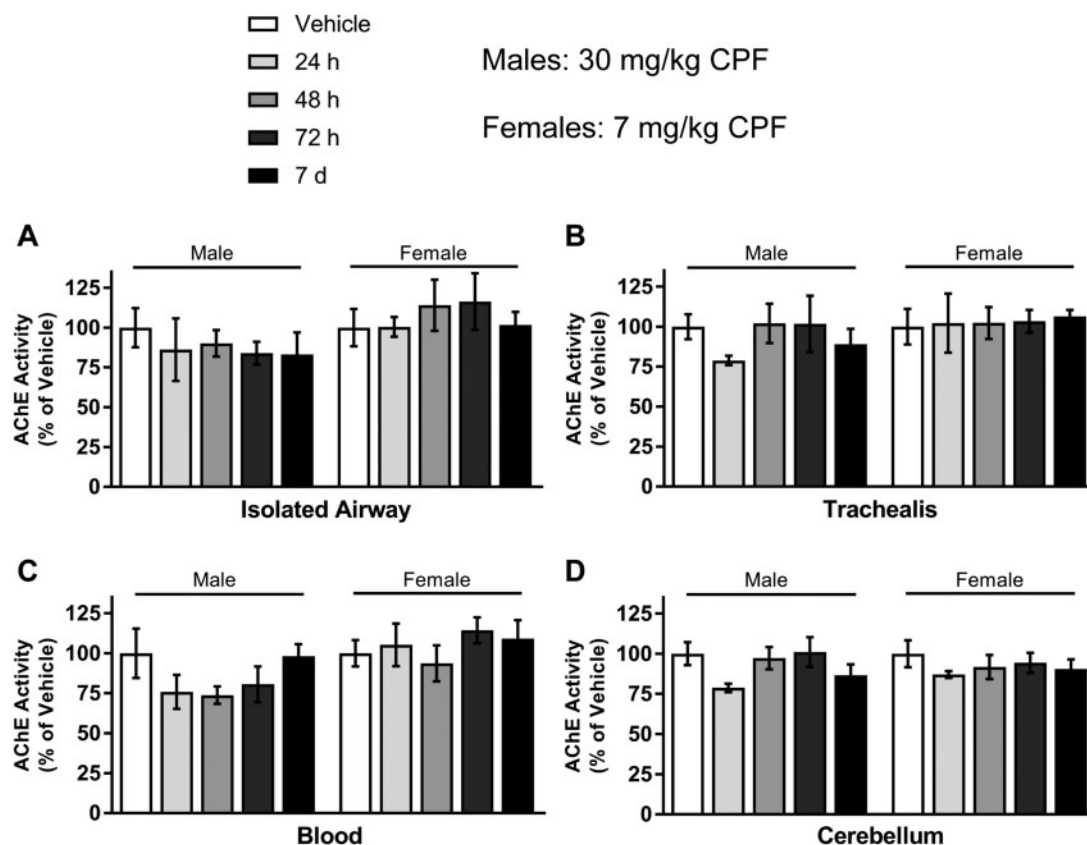
A third major sex difference is the time course of AHR development. AHR is not observed in male rats until 7 d post-CPF exposure. These findings suggest that in males, CPF-induced AHR is a delayed response, which is resolved by 14 d post-exposure. In contrast, females develop AHR as early as 24 h post-exposure, and their airways are still hyperreactive at 7 d post-exposure. The marked sex difference in the time to onset of CPF-induced AHR raises questions regarding mechanisms that mediate the effects of CPF on airway responsiveness. One possible explanation is that the mechanism of CPF-induced AHR differs between males and females. Alternatively, it is possible that the mechanism of CPF-induced AHR changes over time, and the early mechanism does not manifest in males. The mechanism of AHR has been demonstrated to change over time in other

models of non-allergic AHR. For instance, in ozone-induced AHR, the mechanism changes from one mediated by muscarinic receptor dysfunction at 1 d post-exposure to one mediated by neurogenic inflammation at 3 d post-exposure (Verhein et al., 2008, 2011). Because the mechanism identified in guinea pigs at 24 h post OP exposure is dependent on an inflammatory response, specifically TNF $\alpha$  release from macrophages (Proskocil et al., 2013) it is plausible the initial insult could set up an inflammatory cascade resulting in persistent or delayed AHR.

The observed sex differences in this study are not surprising as asthma is known to be a sexually dimorphic disease in the human population. Relative to men, adult women have higher rates of asthma, especially severe, refractory, nonatopic (nonallergic) asthma (Kenyon and Jarjour, 2003). Although the reason for this sex difference in the human population is unknown, it has been postulated that hormones play a role (Bonds and Midoro-Horiuti, 2013). Sex-dependent susceptibility to CPF-induced AHR may also reflect significant sex differences in metabolizing enzymes, including the cytochrome p450 (CYP) enzymes responsible for the bioactivation and detoxification of CPF. In humans, bioactivation of CPF to its active metabolite CPF-oxon is primarily catalyzed by CYP2B6, whereas detoxification primarily occurs via CYP2C19 and PON1 activity (Foxenberg et al., 2011, 2007). It has been suggested that CYP2B6 may have higher expression in human females compared with males (Yang et al., 2010). CYP2B6 is also one of the most highly polymorphic CYPs with 9 major variants identified in the Caucasian population alone, which may also explain differences in individual susceptibility to CPF intoxication (Crane et al., 2012). The rat homolog of CYP2B6 is CYP2B1, which is highly expressed in type II alveolar cells (Låg et al., 1996), which could explain why the whole lung tissue, which is largely comprised of alveoli, was the only tissue in which significant inhibition of AChE activity by CPF was observed.

AChE inhibition does not, however, appear to play a major role in CPF-induced AHR in the rat model. The most direct evidence in support of this conclusion is that doses of CPF that triggered AHR in male and female rats, did not significantly inhibit AChE in airway specific tissue. Although the dose of CPF that produced AHR in males inhibited AChE in the whole lung to 50% of vehicle control levels, it did not inhibit AChE in the trachealis or isolated airway, where AChE inhibition would have an effect on airway smooth muscle contraction. This would suggest the observed AChE inhibition in whole lung homogenate is occurring in the lung parenchyma. Further evidence that AChE inhibition is not mediating AHR in this model is the lack of a hyperreactive resistance response to iv ACh challenge in CPF-exposed animals. Vagally induced bronchoconstriction is mediated by binding of ACh released from prejunctional parasympathetic nerves onto M3 muscarinic receptors expressed by the airway smooth muscle. Therefore, if AChE inhibition were the primary mechanism driving CPF-induced AHR, iv administration of ACh would be predicted to trigger a hyperreactive response.

The observed AHR in males and females also does not appear to be mediated by changes in airway smooth muscle contractility, as no increase in R response to iv MeCh was observed at 24 h or 7 d. MeCh is a nonhydrolyzable analog of the ACh, which binds directly to post-junctional M3 muscarinic receptors on the airway smooth muscle to cause contraction irrespective of prejunctional ACh release. Thus, the lack of airway hyperresponsiveness to iv MeCh in CPF-exposed animals suggests that CPF did not alter contractility of the airway smooth muscle. These observations are consistent with findings in guinea pigs (Fryer et al., 2004; Lein and Fryer, 2005). Based on data from the guinea pig model, it has been proposed that OP-induced AHR is



**Figure 5.** Doses of CPF that alter pulmonary physiology have no effect on AChE activity. AChE activity as a percent of sex-matched vehicle controls in isolated airway (A), trachealis (B), blood (C), and cerebellum (D) of CPF exposed male (30 mg/kg) and female (7 mg/kg) rats at 24 h, 48 h, 72 h, and 7 d post-CPF injection. No significant effects of CPF exposure were identified by one-way ANOVA ( $\alpha = 0.05$ ). Data presented as the mean  $\pm$  SE ( $n = 5-6$  animals per group).

mediated by dysfunction of autoinhibitory M2 muscarinic receptors on prejunctional parasympathetic nerves, which enhances release of ACh in response to vagal stimulation (Fryer *et al.*, 2004; Lein and Fryer, 2005). Whether OPs cause M2 receptor dysfunction in rats has yet to be established, but the findings reported herein are consistent with this model.

Tissue elastance was effected in males and females at 24 h, although in different directions; E response was decreased in males but increased in females. ACh receptors exist on many other cell types besides neurons, and nonneuronal cholinergic signaling plays many important roles in the lung, including immune modulation, collagen synthesis, and cellular proliferation (Kummer *et al.*, 2008; Verhein *et al.*, 2009). Many of these nonneuronal cholinergic processes could impact tissue elastance, which could explain the unexpected effects of ACh and MeCh on E. Although it is unclear why there are also sex differences in this effect, it is not surprising given the difference in vagal response 24 h post-CPF in males and females, and further highlights the importance of studying sex differences in susceptibility.

These studies provide further proof-of-concept evidence that environmentally relevant levels of OPs, such as might be encountered in occupational or residential settings, could feasibly contribute to asthma pathogenesis or exacerbation of asthmatic symptoms. The evidence supporting an AChE independent mechanism of OP toxicity is highly relevant to human health and safety as AChE inhibition is used as a benchmark by regulatory agencies, including the United States Environmental Protection Agency and Occupational Safety and Health Agency, for determining exposure limits for many OPs.

These studies also raise a number of questions about sex-specific respiratory effects of OPs. Future studies should further elucidate mechanisms of CPF-induced AHR in the rat, including whether AHR at 24 h and 7 d post-CPF in females is mediated by the same or distinct mechanisms. This information will be important to inform regulatory decisions about exposure limits to OPs for susceptible groups. The possibility that women may be more susceptible to respiratory dysfunction following OP exposure should be considered, as many women are occupationally exposed to these compounds in the agricultural sector, although little epidemiological data has been collected on this specific group to date. The fact that AHR is seen in both sexes 7 d after a single CPF exposure raises questions about long-term respiratory impacts of OP exposure, especially because environmental exposures in the human population are ubiquitous.

## SUPPLEMENTARY DATA

Supplementary data are available at Toxicological Sciences online.

## ACKNOWLEDGMENT

The authors acknowledge Mabelle Wilson, PhD, for providing statistical consultation.

## FUNDING

This work was supported by the United States National Institutes of Health [grant numbers R01 ES017592 to P.J.L.,



T32 HL07013 to F.C.S., U54 HD079125 to P.J.L. and A.C.G., P30 ES023513 pilot project funding to A.C.G., and UL1 TR001860]; and the J.B. Johnson Foundation [unrestricted gift to P.J.L.]. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH. Further, the granting agencies do not endorse the purchase of any commercial products or services mentioned in the publication.

## REFERENCES

- Amaral, A. F. S. (2014). Pesticides and asthma: Challenges for epidemiology. *Front. Public Health* 2, 1–3.
- Bonds, R. S., and Midoro-Horiuti, T. (2013). Estrogen effects in allergy and asthma. *Curr. Opin. Allergy Clin. Immunol.* 13, 92–99.
- Canning, B. J., and Chou, Y. (2008). Using guinea pigs in studies relevant to asthma and COPD. *Pulm. Pharmacol. Ther.* 21, 702–720.
- Chakraborty, S., Mukherjee, S., Roychoudhury, S., Siddique, S., Lahiri, T., and Ray, M. R. (2009). Chronic exposures to cholinesterase-inhibiting pesticides adversely affect respiratory health of agricultural workers in India. *J. Occup. Health* 51(6), 488–497.
- Crane, A. L., Klein, K., and Olson, J. R. (2012). Bioactivation of chlorpyrifos by CYP2B6 variants. *Xenobiotica* 42, 1255–1262.
- Doust, E., Ayres, J. G., Devereux, G., Dick, F., Crawford, J. O., Cowie, H., and Dixon, K. (2014). Is pesticide exposure a cause of obstructive airways disease? *Eur. Respir. Rev.* 23, 180–192.
- Ellman, G. L., Courtney, K. D., Andres, V., Jr., and Feather-Stone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* 7, 88–95.
- Faffe, D. S., Silva, G. H., Kurtz, P. M., Negri, E. M., Capelozzi, V. L., Rocco, P. R., and Zin, W. A. (2001). Lung tissue mechanics and extracellular matrix composition in a murine model of silicosis. *J. Appl. Physiol.* (1985) 90, 1400–1406.
- Fenske, R. A., Farahat, F. M., Galvin, K., Fenske, E. K., and Olson, J. R. (2012). Contributions of inhalation and dermal exposure to chlorpyrifos dose in Egyptian cotton field workers. *Int. J. Occup. Environ. Health* 18, 198–209.
- Fieten, K. B., Kromhout, H., Heederik, D., and Joode, B. v. W. d. (2009). Pesticide Exposure and Respiratory Health of Indigenous Women in Costa Rica. *Am. J. Epidemiol.* 169(12), 1500–1506.
- Foxenberg, R. J., Ellison, C. A., Knaak, J. B., Ma, C., and Olson, J. R. (2011). Cytochrome P450-specific human PBPK/PD models for the organophosphorus pesticides: chlorpyrifos and parathion. *Toxicology* 285, 57–66.
- Foxenberg, R. J., McGarrigle, B. P., Knaak, J. B., Kostyniak, P. J., and Olson, J. R. (2007). Human hepatic cytochrome p450-specific metabolism of parathion and chlorpyrifos. *Drug Metab. Dispos.* 35, 189–193.
- Fryer, A. D., Lein, P. J., Howard, A. S., Yost, B. L., Beckles, R. A., and Jett, D. A. (2004). Mechanisms of organophosphate insecticide-induced airway hyperreactivity. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 286, L963–L969.
- Grinnan, D. C., and Truwit, J. D. (2005). Clinical review: respiratory mechanics in spontaneous and assisted ventilation. *Crit. Care* 9, 472–484.
- Gustin, P., Dharm, A. R., Lomba, F., Lekeux, P., Van de Woestijne, K. P., and Landser, F. J. (1988). Measurement of total respiratory impedance in calves by the forced oscillation technique. *J. Appl. Physiol.* (1985) 64, 1786–1791.
- Hernández, A. F., Casado, I., Pena, G., Gil, F., Villanueva, E., and Pla, A. (2008). Low Level of Exposure to Pesticides Leads to Lung Dysfunction in Occupationally Exposed Subjects. *Inhalation Toxicology* 20(9), 839–849.
- Hernández, A. F., Parrón, T., and Alarcón, R. (2011). Pesticides and asthma. *Curr. Opin. Allergy Clin. Immunol.* 11, 90–96.
- Hoppin, J. A., Umbach, D. M., London, S. J., Alavanja, M. C. R., and Sandler, D. P. (2002). Chemical Predictors of Wheeze among Farmer Pesticide Applicators in the Agricultural Health Study. *Am J Respir Crit Care Med* 165(5), 683–689.
- Hoppin, J. A., Umbach, D. M., London, S. J., Henneberger, P. K., Kullman, G. J., Alavanja, M. C. R., and Sandler, D. P. (2008). Pesticides and Atopic and Nonatopic Asthma among Farm Women in the Agricultural Health Study. *Am J Respir Crit Care Med* 177(1), 11–18.
- Hoppin, J. A., Umbach, D. M., London, S. J., Henneberger, P. K., Kullman, G. J., Coble, J., Alavanja, M. C. R., Beane Freeman, L. E., and Sandler, D. P. (2009). Pesticide use and adult-onset asthma among male farmers in the Agricultural Health Study. *Eur Respir J* 34(6), 1296.
- Kenyon, N. J., and Jarjour, N. N. (2003). Severe asthma. *Clin. Rev. Allerg. Immunol.* 25, 131–149.
- Kummer, W., Fischer, A., Kurkowski, R., and Heym, C. (1992). The sensory and sympathetic innervation of guinea-pig lung and trachea as studied by retrograde neuronal tracing and double-labelling immunohistochemistry. *Neuroscience* 49, 715–737.
- Kummer, W., Lips, K. S., and Pfeil, U. (2008). The epithelial cholinergic system of the airways. *Histochem. Cell Biol.* 130, 219–234.
- Låg, M., Becher, R., Samuelsen, J. T., Wiger, R., Refsnes, M., Huitfeldt, H. S., and Schwarze, P. E. (1996). Expression of CYP2B1 in freshly isolated and proliferating cultures of epithelial rat lung cells. *Exp. Lung Res.* 22, 627–649.
- Lein, P. J., and Fryer, A. D. (2005). Organophosphorus insecticides induce airway hyperreactivity by decreasing neuronal M2 muscarinic receptor function independent of acetylcholinesterase inhibition. *Toxicol. Sci.* 83, 166–176.
- Lein, P. J., Bonner, M. R., Farahat, F. M., Olson, J. R., Rohlman, D. S., Fenske, R. A., Lattal, K. M., Lasarev, M. R., Galvin, K., Farahat, T. M., et al. (2012). Experimental strategy for translational studies of organophosphorus pesticide neurotoxicity based on real-world occupational exposures to chlorpyrifos. *Neurotoxicology* 33, 660–668.
- Mamane, A., Raheison, C., Tessier, J.-F., Baldi, I., and Bouvier, G. (2015). Environmental exposure to pesticides and respiratory health. *Eur. Respir. Rev.* 24, 462–473.
- Ndlovu, V., Dalvie, M. A., and Jeebhay, M. F. (2014). Asthma associated with pesticide exposure among women in rural Western Cape of South Africa. *American Journal of Industrial Medicine* 57(12), 1331–1343.
- Peiris-John, R. J., Ruberu, D. K., Wickremasinghe, A. R., and van der-Hoek, W. (2005). Low-level exposure to organophosphate pesticides leads to restrictive lung dysfunction. *Respiratory Medicine* 99(10), 1319–1324.
- Proskocil, B. J., Bruun, D. A., Jacoby, D. B., Rooijen, N., Lein, P. J., and Fryer, A. D. (2013). Macrophage TNF- $\alpha$  mediates parathion-induced airway hyperreactivity in guinea pigs. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 304, L519–L529.
- Rocco, P. R., Negri, E. M., Kurtz, P. M., Vasconcellos, F. P., Silva, G. H., Capelozzi, V. L., Romero, P. V., and Zin, W. A. (2001). Lung tissue mechanics and extracellular matrix remodeling in acute lung injury. *Am. J. Respir. Crit. Care Med.* 164, 1067–1071.

- Segura, P., Chávez, J., Montaña, L. M., Vargas, M. H., Delaunoy, A., Carbajal, V., and Gustin, P. (1999). Identification of mechanisms involved in the acute airway toxicity induced by parathion. *Naunyn Schmiedebergs Arch. Pharmacol.* **360**, 699–710.
- Stengel, P. W., Yiamouyiannis, C. A., Obenchain, R. L., Cockerham, S. L., and Silbaugh, S. A. (1995). Methacholine-induced pulmonary gas trapping in guinea pigs, hamsters, mice, and rats. *J. Appl. Physiol.* (1985) **79**, 2148–2153.
- Van Winkle, L. S., Kelty, J. S., and Plopper, C. G. (2017). Preparation of specific compartments of the lungs for pathologic and biochemical analysis of toxicologic responses. *Curr. Protoc. Toxicol.* **71**, 24.5.1–24.5.26.
- Verhein, K. C., Fryer, A. D., and Jacoby, D. B. (2009). Neural control of airway inflammation. *Curr. Allergy Asthma Rep.* **9**, 484–490.
- Verhein, K. C., Hazari, M. S., Moulton, B. C., Jacoby, I. W., Jacoby, D. B., and Fryer, A. D. (2011). Three days after a single exposure to ozone, the mechanism of airway hyperreactivity is dependent on substance P and nerve growth factor. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **300**, L176–L184.
- Verhein, K. C., Jacoby, D. B., and Fryer, A. D. (2008). IL-1 receptors mediate persistent, but not acute, airway hyperreactivity to ozone in guinea pigs. *Am. J. Respir. Cell. Mol. Biol.* **39**, 730–738.
- Yang, X., Zhang, B., Molony, C., Chudin, E., Hao, K., Zhu, J., Gaedigk, A., Suver, C., Zhong, H., Leeder, J. S., et al. (2010). Systematic genetic and genomic analysis of cytochrome P450 enzyme activities in human liver. *Genome Res.* **20**, 1020–1036.
- Ye, M., Beach, J., Martin, J. W., and Senthilselvan, A. (2013). Occupational pesticide exposures and respiratory health. *Int. J. Environ. Res. Public Health* **10**, 6442–6471.